Staphylococcal Responses to Antimicrobial Skin Lipids

Josephine Moran

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

September 2014

Abstract

Staphylococcal Responses to Antimicrobial Skin Lipids

Josephine Moran

Antimicrobial lipids on skin are proposed to form a barrier against microbial colonisation. Skin lipids, such as unsaturated fatty acids and sphingosines, cause membrane permeabilisation and/or proton motive force disruption . These lipids may be crucial in determining the diversity and degree of staphylococcal skin colonisation. Specifically, antimicrobial lipids may inhibit skin colonisation by *Staphylococcus aureus* while permitting the growth of *Staphylococcus epidermidis*. Here it was shown that skin fatty acids sapienic acid and linoleic acid are more active against *S. aureus* than *S. epidermidis*. This supports a role for fatty acids in the prevention of *S. aureus* skin colonisation. The most anti-staphylococcal skin lipid tested was D-sphingosine; no differences in resistance levels between *S. aureus* and *S. epidermidis* to D-sphingosine were observed.

The genetic response and basis for resistance to skin antimicrobial lipids of *S. epidermidis* and *S. aureus* was investigated using next generation sequencing. The transcriptomic response of both species to sapienic acid was determined using RNA-Seq. Additionally, *S. epidermidis* and *S. aureus* were passaged in sapienic acid or D-sphingosine. Isolates with increased lipid resistance after passaging were genome sequenced, and mutations associated with increased resistance were characterised. From these approaches, several genes and pathways potentially involved in the responses of both species to skin lipids became apparent. These components included cell wall biosynthesis, transport and production of small molecules, ammonia production, albumin binding proteins and putative lipid efflux pumps. Cellular components identified as specifically involved in *S. aureus* resistance to sapienic acid included capsule and staphyloxanthin biosynthesis. Cellular components involved specifically in *S. epidermidis* resistance to sapienic acid were also speculatively identified, though the functions of these components were not resolved.

This study has increased our understanding of staphylococcal molecular interactions with host antimicrobial lipids, which could lead to applications in the design of novel antimicrobial compounds.

Acknowledgements

I'd like to thank Mal Horsburgh, for his advice and support throughout my PhD studies, I couldn't have asked for a better supervisor.

I'd like to thank Jennifer Kelly and Rosanna Coates for training me in the world of bioinformatics.

I'd like to thank all those who've helped me in the lab, but especially Dave Rooks, Marta Veses Garcia and James Houghton, who were always around to steer me in the right direction.

I'd like to thank all my friends and family, particularly Thomas Seddon, for listening even when they didn't understand what I was saying and for generally keeping me sane.

Contents

List of Tables

List of Figures

Chapter 1 Introduction

1.1 Staphylococci

Staphylococci are dominant colonisers of the skin and mucosa of mammals and birds (Cogen *et al*., 2008; Costello *et al*., 2009; Taylor *et al*., 2003; Nagase *et al*., 2002). Though there are more than forty recognised species of staphylococci, only a selection are regular colonisers of human skin. These include the coagulase-negative staphylococci species: *Staphylococcus epidermidis*, *Staphylococcus capitus*, *Staphylococcus cohnii*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus saprophyticus*, *Staphylococcus simulans, Staphylococcus warneri* and *Staphylococcus xylosus* (Kloos and Schleifer, 1975; Kloos and Musselwhite, 1975; Coates *et al*., 2014). *S. epidermidis* is the best characterised and most frequently isolated, having been found to colonise up to 100 % of healthy individuals (Foster, 2009; Roth and James, 1988). In contrast, the coagulase-positive *S. aureus* is a transient skin coloniser, though it colonises human anterior nares relatively frequently (Kluytmans and Wertheim, 2005; Moss and Squire, 1948; Cho *et al*., 2010).

S. aureus, *S. epidermidis* and several other staphylococci cause nosocomial infections in immuno-compromised and long-term catheterised patients, however, those of *S. aureus* tend to be more severe (Vuong and Otto, 2002). *S. aureus* also causes infections that other staphylococci usually do not, such as toxic shock syndrome, pneumonia, meningitis, scalded skin syndrome, impetigo and abscesses (Cogen *et al*., 2008). This greater pathogenicity has been widely studied and is attributed to *S. aureus*' greater repertoire of virulence determinants (Massey *et al*., 2006; Christensen and Bruggemann, 2014).

1.2 The structure of human skin

Skin is a barrier, preventing excessive water loss and the entry of microbes into sterile areas of the body. The skin consists of layers of cells that become progressively differentiated as they migrate towards the outermost layer (Fig. 1.1). In order of least to most differentiated, these layers are classified as the

basal layer (stratum basale), the spinous layer (stratum spinosum), the granular layer (stratum granulosum) and the stratified layer (stratum corneum) (Candi *et al.*, 2005; Proksch *et al.*, 2008). In the basal layer are the proliferative keratinocyte cells. The rate of proliferation in these cells is matched by the rate of desquamation, with complete turnover occurring every 14 days (Candi *et al.*, 2005; Roth and James, 1988).

Keratinocytes in the granular layer are typified by the presence of lamellar granules, or Odland bodies. These small organelles contain stacks of lipid lamellae consisting of phospholipids, cholesterol, glucosylceramides and various enzymes such as acid hydrolases (Candi *et al.*, 2005).

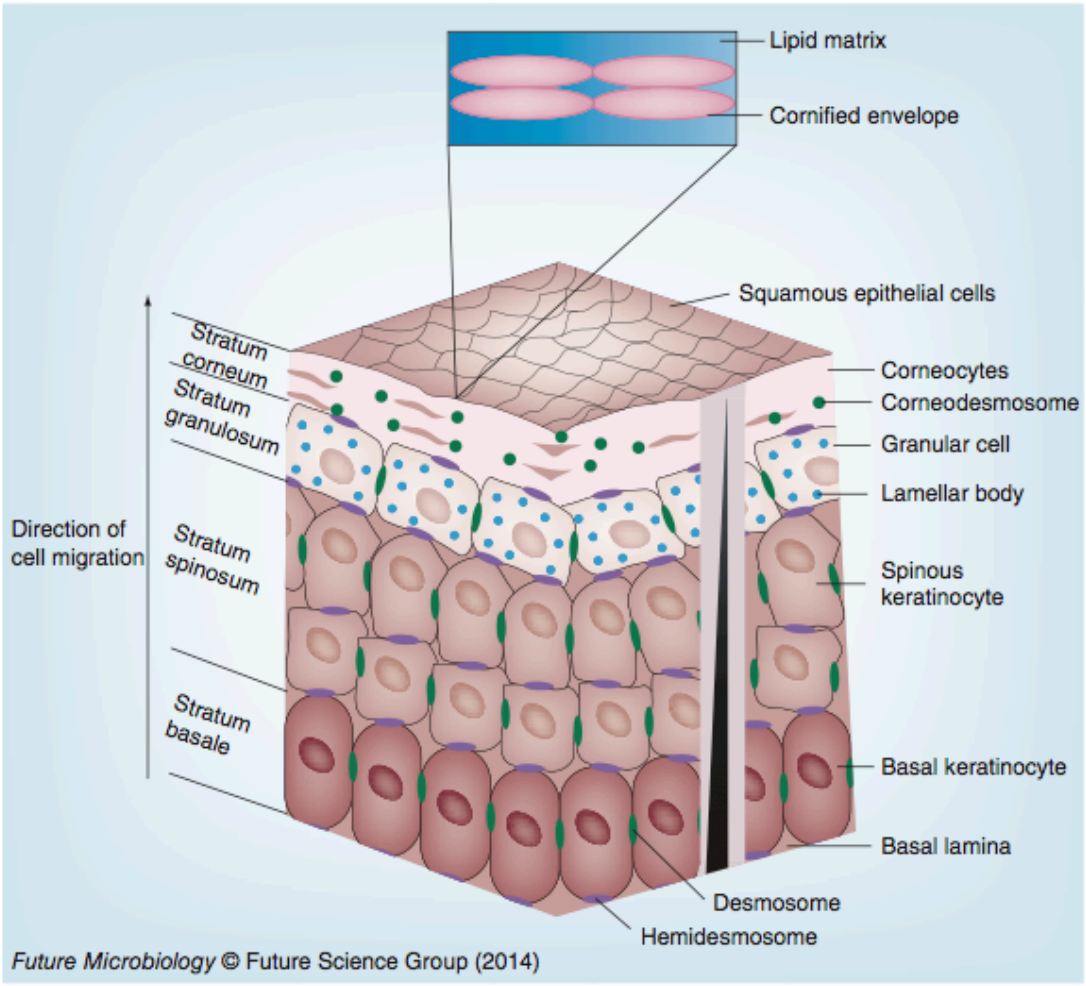


Figure 1.1 Structure of the skin

(Coates *et al.*, 2014)

The majority of changes resulting in terminally differentiated keratinocytes occur between the granular layer and the stratum corneum (Candi *et al*., 2005). The lamellar granules are extruded during the transition between the granular layer and the stratum corneum. Many of the phospholipids and glucosylceramides released from the lamellar granules are hydrolysed to ceramides and free fatty acids (Madison, 2003; Drake *et al*., 2008). Keratin is bound by fillagrin into tight bundles, resulting in flattening of the keratinocytes (Candi *et al*., 2005). Beneath the plasma membrane the cornified envelope forms; the cornified envelope is a protein layer cross-linked by isopeptide bonds making it extremely resistant to common proteolytic enzymes (Proksch *et al*., 2008). A lipid envelope, consisting of ω-hydroxyceramides, is covalently attached to involucrin, envoplakin and periplakin of the cornified envelope by ester linkage (Proksch *et al*., 2008; Candi *et al*., 2005; Madison, 2003). The plasma membrane disintergrates as it is replaced by the lipid envelope and the cells organelles are degraded as they undergo apoptosis (Candi *et al*., 2005). These terminally differentiated cells are known as cornified cells or corneocytes (Candi *et al*., 2005).

Corneocytes are embedded within a lipid matrix of intercellular lamellae formed from lipids released from the lamellar granules (Proksch *et al*., 2008; Madison, 2003). The ω-hydroxyceramides of the lipid envelope non-covalently interact with intercellular lamellae to retain the lipid matrix around the corneocytes (Proksch *et al*., 2008). The lipid matrix consists of ceramides (45-50 %), cholesterol (25 %), free fatty acids (10-15 %) and other lipids (< 5 %) (Madison, 2003). It is thought this lipid matrix forms both gel phase domains, which are highly ordered and relatively impermeable, and liquid crystalline phase domains, which are more fluid and provide flexibility (Madison, 2003).

Corneocytes are bound together by corneodesmosomes, which differ from desmosomes found in lower layers of the epidermis due to additional structural proteins such as desmoglein-1 (Candi *et al*., 2005). Proteolytic degradation of corneodesmosome proteins results in shedding of the stratum corneum (Candi *et al*., 2005).

1.3 The human skin microbiome

There are between 10^2 and 10^7 microorganisms per cm^2 of human skin (Schroder and Harder, 2006). Numbers of microorganisms vary between areas of the skin and between individuals, probably because of variations in environmental conditions on the skin. For example, higher numbers of microbes are normally found in areas that are naturally occluded, such as the axilla. Occluding the forearm by wrapping increases the number of microbes dramatically; humidity, temperature and pH also increase, indicating that these factors are responsible for increased growth in occluded areas (Roth and James, 1988).

On the skin there are transient flora, that are found sporadically on the skin after being transferred by exogenous sources, and there are resident flora, that are found consistently on the skin in reasonably stable numbers (Roth and James, 1988). More recently, research arising from the Human Microbiome Project has led to members of microbiomes being classified as "core" (taxa commonly shared between individuals) or "minor" (taxa infrequently shared between individuals) (Li *et al*., 2013).

The microbiome of the skin has been studied by both culturing and 16S rRNA gene sequencing techniques. Propionibacteria have been found to be the dominant anaerobe by both culturing and 16S rRNA techniques (Cogen *et al*., 2008; Costello *et al*., 2009; Grice *et al*., 2009). Conversely, whilst coagulase-negative staphylococci are found to be the dominant bacteria capable of aerobic growth on the skin by culturing techniques (Cogen *et al*., 2008), both corynebacteria and staphylococci can dominate by 16S rRNA gene sequencing techniques dependent on body location and individual factors including gender (Taylor *et al*., 2003; Costello *et al*., 2009; Callewaert *et al*., 2013; Grice *et al*., 2009). Since only approximately 14 % of the skin microbiota can be cultured (Gao *et al*., 2007), 16S rRNA gene sequencing results are likely to give a more realistic impression of the skin microbiome. Only staphylococci, propionibacteria and corynebacteria are considered members of the core skin bacterial microbiome (Li *et al*., 2013), however, other common skin colonisers

include *Micrococcus* spp., *Brevibacterium* spp. and *Malassezia* spp. (Callewaert *et al*., 2013; Kong, 2011; Cogen *et al*., 2008).

The bacterial skin flora are not typically pathogenic, though many of them are opportunistic pathogens. *S. epidermidis* is one of the most common causes of indwelling medical device infections (Cogen *et al*., 2008; Vuong and Otto, 2002), and causes 5% of native valve endocarditis (Roth and James, 1988). *Corynebacterium jeikeium* can cause sepsis or endocarditis in immunocompromised patients (Cogen *et al*., 2008). *Corynebacterium diptheriae* can cause diptheria, septicaemia, endocarditis and osteomyelitis (Cogen *et al*., 2008). *Propionibacterium acnes* is frequently associated with acnes vulgaris, but can cause more serious infections such as infections of the eye that often lead to blindness and occasional systemic infections in the immuno-compromised (Cogen *et al*., 2008).

Overall, the interaction of the skin microbiome with their human host is considered beneficial. To colonise the skin, bacteria must adhere through interactions with receptors on the skin; resident flora bound to these receptors are theorised to block exogenous, potentially pathogenic bacteria from binding. Some resident flora also produce antimicrobial peptides that can act synergistically with peptides of the innate immune system, and boost the innate immune defences (Cogen *et al*., 2010a).

1.4 Staphylococcal skin survival

Conditions on the skin surface are inhospitable, with various host factors challenging bacterial skin colonisation. The low moisture, acidic pH and high salt conditions of the skin ensure microbial growth is inhibited or impeded (Roth and James, 1988; Coates *et al*., 2014). To colonise the skin, microbes must first adhere to the skin, a process that is hindered by the complete shedding of the skin every 14 days (Roth and James, 1988). To persist on the skin, microbes must be able to evade components of the innate immune system such as antimicrobial peptides (AMPs) and antimicrobial lipids (Coates *et al*., 2014). Further, microbes must also be able to outcompete other microbes; many skin

bacteria produce their own antimicrobial peptides to target competitors (Cogen *et al*., 2008; Cogen *et al*., 2010a; 2010b).

The ability of *S. epidermidis* to colonise skin whilst *S. aureus* cannot has been linked to differences in their ability to overcome the challenges described above (McEwan *et al*., 2006; Cho *et al*., 2001; Melnik, 2006; Ong *et al*., 2002; Arikawa *et al*., 2002; Takigawa *et al*., 2005; Ishikawa *et al*., 2010). Several recent reviews have focussed on determinants that enable staphylococci to overcome these challenges, however, little is known about what determinants account for the differences in skin colonisation observed between *S. aureus* and *S. epidermidis* (Coates *et al*., 2014; Johannessen *et al*., 2012; Sollid *et al*., 2014; Ryu *et al*., 2014).

1.4.1 Adhesion

To thrive on the skin bacteria must be able to adhere so they are not pulled away be sheer forces, but must also be able to re-attach to the new layers before the cells are lost through desquamation. Adhesins specific for receptors on the skin mediate strong attachments to prevent loss. To date it has not been studied if adhesion to the uppermost layer of the skin is sufficient for long-term skin survival or if penetration and adhesion to deeper layers of the skin is necessary, though bacteria are found at both the upper and lower layers of the stratum corneum (Brooker and Fuller, 1984; Zeeuwen *et al*., 2012; Grice *et al*., 2009).

Staphylococcal adhesins can be classified as one of three groups; MSCRAMMs (microbial surface components recognising adhesive matrix molecules), SERAMs (secretable expanded repertoire adhesive molecules) or non-proteinaceous adhesins (Heilmann, 2011). MSCRAMMs are covalently anchored to the staphylococcal cell wall and bind one or more host factor. SERAMs are non-covalently bound to the staphylococcal surface and frequently have enzymatic functions as well as roles in adhesion (Heilmann *et al*., 2003; Bowden *et al*., 2002). Non-proteinaceous adhesins are usually embedded in the membrane lipids and to date have been found to act as adhesins through non-covalent bonds (Heilmann, 2011). Staphylococcal adhesins known to adhere to

the human epidermis and their receptors (where known) are summarised in Table 1.1.

Table 1.1 Adhesins of staphylococci for skin and their receptors.

Host receptor	Adhesins	Species	Reference
Undetermined receptor on desquamated epithelial cell	Aap	*S. epidermidis*	(Macintosh *et al*., 2009)
Undetermined receptor on desquamated nasal cells	SasG, ClfB, SdrC, SdrD, IsdA	*S. aureus*	(Corrigan *et al*., 2007; 2009)
Undetermined receptor on keratinocyte cell line	Spa, Coagulase, FnbpA/FnbA, FnbpB/FnbB, ClfA	*S. aureus*	(Mempel *et al*., 1998)
Collagen type I	SdrF, GehD	*S. epidermidis*	(Arrecubieta *et al*., 2007; Bowden *et al*., 2002)
	SdrI	*S. saprophyticus*	(Sakinc *et al*., 2006)
	Can, Eap/Map, Emp	*S. aureus*	(Snodgrass *et al*., 1999; Hansen *et al*., 2006; Hussain *et al*., 2001)
Fibrinogen	Aaa, ClfB, FnbpA/FnbA, IsdA, Atl, Eap/Map, Emp, FnbpB/FnbB, ClfA	*S. aureus*	(Heilmann *et al*., 2005; Ni Eidhin *et al*., 1998; Wann *et al*., 2000; Clarke *et al*., 2004; Hirschhausen *et al*., 2010; Hussain *et al*., 2001; Burke *et al*., 2011; McDevitt *et al*., 1994)
	Fbe/SdrG	*S. epidermidis*	(Hartford *et al*., 2001)
	UafB	*S. saprophyticus*	(King *et al*., 2011)
	Fbl	*S. lugdunensis*	(Nilsson *et al*., 2004)
Fibronectin	Aaa, FnbpA/FnbA, FnbpB/FnbB, IsdA, Atl, Eap/Map, Emp	*S. aureus*	(Heilmann *et al*., 2005; Flock *et al*., 1987; Jonsson *et al*., 1991; Clarke *et al*., 2004; Hirschhausen *et al*., 2010; Hussain *et al*., 2001; Hussain *et al*., 2001)
	Embp, Fbe/SdrG, Aae	*S. epidermidis*	(Williams *et al*., 2002; Nilsson *et al*., 1998; Heilmann *et al*., 2003)
	Aas, UafB, SdrI	*S. saprophyticus*	(Hell *et al*., 1998; King *et al*., 2011; Sakinc *et al*., 2009)
	AtlC	*S.caprae*	(Allignet *et al*., 2002)
Involucrin	IsdA	*S. aureus*	(Clarke *et al*., 2009)
Loricrin	IsdA, ClfB	*S. aureus*	(Clarke *et al*., 2009; Mulcahy *et al*., 2012)
Cytokeratin-10	IsdA, ClfB	*S. aureus*	(Clarke *et al*., 2009; O'Brien *et al*., 2002)
Keratinocyte lipids/glycolipids	Pls	*S. aureus*	(Huesca *et al*., 2002)

Since adhesins are relatively specific it is likely that the repertoire of adhesins a strain expresses could specify which hosts and areas of the host the bacterium can adhere to. Demonstration of host specificity provided by adhesins comes from studies investigating the ability of staphylococcal strains from one mammalian host (such as canine) to adhere to keratinocytes of other mammalian hosts (such as feline) (Woolley *et al*., 2008; Lu and McEwan, 2007). These studies found differences in adhesion dependent on both host type and strain origin (Woolley *et al*., 2008; Lu and McEwan, 2007).

Demonstration of niche specificity provided by adhesins comes from studies of the adhesin accumulation associated protein (Aap). Aap, found in many strains of *S. epidermidis*, binds buccal epithelial cells and desquamated terminally-differentiated keratinocytes (Macintosh *et al*., 2009). In contrast SasG, a *S. aureus* adhesin and Aap homologue, binds desquamated nasal cells but not buccal epithelial cells or cells from a keratinocyte cell line (Roche *et al*., 2003).

Staphylococcal adhesion may be facilitated by different proteins at separate stages of colonisation. It is known that *S. aureus* adheres to highly keratinised squamous cells both on the skin and in the nose. It is suggested that wall teichoic acid (WTA) is important in the initial stages of colonization of the nose (Weidenmaier *et al*., 2008; 2004). Whereas clumping factor B (ClfB) is indicated to be important in long-term colonisation of the nose, since a *S. aureus* ClfB mutant, though capable of initial colonisation, was eliminated more quickly than the wild type (Wertheim *et al*., 2008). However, it is still unclear how the methods of adherence change as colonisation progresses.

1.4.2 Osmotic stress

The risk of osmotic stress has long been considered a limiting factor for bacterial growth and survival on skin. Osmotic stress can be divided into the categories: low relative humidity (RH), matric water stress (desiccation) and ionic stress (salts or ions) (Potts, 1994). All three affect colonisation of the skin environment. On skin, RH is often low and ionic stress high, whereas matric water stress tolerance is known to be important for staphylococcal

transmission but may also affect survival on skin if the RH falls below the limits at which staphylococci could grow. Skin bacteria also face the added stress caused by sudden increases in water availability brought on by increased sweating or washing.

1.4.3 Osmotic stress resistance

It was shown that *S. aureus* and *S. epidermidis* can grow at lower RH levels than other bacteria. Whilst *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas fluorescens* required 92-94.5% RH to grow, *S. aureus* could grow at 87% RH and *S. epidermidis* 81-84% (de Goffau *et al*., 2009). This could be significant for exclusion on the skin, since at high RH, *S. epidermidis* growth was inhibited by *Sphingomonas paucimobilis*, but at lower RH *S. epidermidis* had the competitive advantage (de Goffau *et al*., 2009). As *S. epidermidis* is able to grow at lower RH than *S. aureus*, it is plausible that this factor could influence the enhanced survival of *S. epidermidis* over *S. aureus* on the skin.

When *S. aureus* or *S. epidermidis* are grown at RH levels approaching their RH growth limit they become larger, form thicker cell walls and form cuboidal packs of eight cells rather than their typical grape-like clusters (de Goffau *et al*., 2009; 2011). Similar observations have been made for staphylococcal autolysin mutants, suggesting this morphism is autolysin controlled (de Goffau *et al*., 2011). Alteration in morphology could offer protection from osmotic stress through a reduction in surface area to volume ratio and subsequent reduction of water loss and maintenance of turgor pressure (de Goffau *et al*., 2011). Further work of de Goffau has indicated that staphylococci grown at low RH are more hydrophilic, which would aid in water acquisition (de Goffau *et al*., 2011).

RP62a was the most resistant *S. epidermidis* strain tested by de Goffau; it was speculated that this could be linked to the ability to form biofilms, as biofilm structure would further reduce surface area to volume ratio (de Goffau *et al*., 2009). However, it may also be linked to the ability of Rp62a to produce the exopolysaccharide (EPS) polysaccharide intracellular adhesin (PIA) (McKenney *et al*., 1998), as work on *Pseudomonas* has shown that EPS acts like a buffer

against desiccation (Roberson and Firestone, 1992). EPS offers protection from desiccation *in vitro* ostensibly by slowing the drying of the bacteria and providing more time for the bacteria to adjust; EPS could offer similar protection upon wetting preventing too rapid a rehydration (Roberson and Firestone, 1992). Theoretically, PIA could play a similar role in staphylococci on the skin to that of EPS in *Pseudomonas* in the soil. However, PIA has been indicated to cause a fitness cost for human skin colonisation (Rogers *et al*., 2008).

A factor observed to protect *S. epidermidis* against high NaCl concentration (2 M) is poly-γ-glutamic acid (PGA) biosynthesis (Kocianova *et al*., 2005). PGA forms a capsule around *S. epidermidis* ubiquitously in both clinical and commensal isolates tested (Kocianova *et al*., 2005). PGA biosynthesis genes (*pgsBCAD*/*capBCAD*) were also found to be present in other coagulase negative staphylococci including *S. capitis*, *S. caprae*, *S. haemolyticus*, *S. warneri* and *S. hominis*, but are not present in *S. aureus* (Kocianova *et al*., 2005).

ClpX and YjbH are proteases found to be important in ionic and matric stress in *S. aureus* possibly through global regulation of other factors, and were demonstrated to be important in osmotic stress survival of *B. subtilis* (Chaibenjawong and Foster, 2011).

Cardiolipin (CL) is known to play a role in osmotic stress responses in various bacteria. The amount of membrane CL and other zwitterionic phospholipids decreases with increasing salinity in both Gram-positive and Gram-negative bacteria. CL synthase levels were shown to increase in *E. coli* in response to ionic stress (Romantsov *et al*., 2009), CL synthase catalyses the conversion of peptidoglycan into CL and glycerol, however levels of CL were not seen to significantly increase in *S. aureus* in response to NaCl (Tsai *et al*., 2011). In *S. aureus* there are two CL synthase genes, one is used for housekeeping and the other in response to stress. Mutation of both genes resulted in reduced long-term survival in high salinity media (Tsai *et al*., 2011). CL was found to provide membrane stiffening activity (Nagamachi *et al*., 1992), thus it may reduce membrane permeability and loss or gain of small solutes. Alternatively, resistance may be provided by CL interaction and regulation of the

mechanosensitive channel MscL and the osmosensory transporter ProP (Romantsov *et al*., 2009).

Extracellular matrix (ECM)-binding protein homologue (Ebh) is a cell wall and membrane-associated protein found in *S. aureus* and *S. epidermidis* but absent from *S. haemolyticus* or *S. saprophyticus*. Ebh mutants exposed to high salt conditions were observed to have invaginated vacuoles along their septum within 30 minutes, suggesting that this protein is important in initial ionic stress resistance (Kuroda *et al*., 2008).

A common resistance mechanism in bacteria to ionic stress is the import of solutes known as osmoprotectants (Potts, 1994). Glycine betaine, proline betaine and L-proline all offered protection of *S. aureus* to 1 M NaCl (Amin *et al*., 1995). Proline betaine was the most effective and L-proline was the least effective in the majority of *S. aureus* strains tested. Similarly in *S. saprophyticus* glycine betaine and proline betaine offered maximal protection whereas L-proline offered limited protection (Amin *et al*., 1995). Proline betaine was also the most effective in the *S. epidermidis* strain tested, however in this strain L-proline was a more effective osmoprotectant than glycine betaine (Amin *et al*., 1995).

1.4.4 Acid

The surface of the skin has been described as having an "acid mantle" that contributes to innate defence against microbial colonisation, particularly by Gram-negative bacteria (Matousek and Campbell, 2002). Across the skin layers there is an acidic gradient, increasing throughout the stratum corneum from the pH of 7.4 in the stratum granulosum to pH 4-5 at the skin surface (Rippke *et al*., 2002; Matousek and Campbell, 2002; Schmid-Wendtner and Korting, 2006). The human skin surface is more acidic than most other mammals, which may act to separate the flora of human and animal skin (Matousek and Campbell, 2002).

As well as the direct effect of acid against microbes, causing damage to proteins and DNA via denaturation, acid can increase the activity of cationic AMPs (CAMPs) and antimicrobial fatty acids (AFAs) (Walkenhorst *et al*., 2013; Cartron

et al., 2014). Converesely, *S. aureus* was found to have increased CAMP resistance at more acidic pH (Walkenhorst *et al*., 2013).

Beyond its antimicrobial role, the acid gradient also enables the activity of skin enzymes such as β-glucocerebrosidase and serine proteases to be regulated dependent on their location within the stratum corneum (Rippke *et al*., 2002; Schmid-Wendtner and Korting, 2006). The regulation of these enzymes by pH ensures timely differentiation and desquamation of keratinocytes (Matousek and Campbell, 2002; Schmid-Wendtner and Korting, 2006). As such, altered pH is indicated as a factor in skin diseases associated with irregular skin differentiation and desquamation, such as atopic dermatitis and ichthyosis (Rippke *et al*., 2002; Matousek and Campbell, 2002; Schmid-Wendtner and Korting, 2006).

This acidic gradient is provided by a number of components including lactic acid and free amino acids from sweat, free fatty acids from sebum, the filaggrin degradation products urocanic acid and pyrrolidone carboxylic acid, and cholesterol sulphate (Rippke *et al*., 2002; Matousek and Campbell, 2002). The majority of these components are present in higher levels towards the skin surface, whilst cholesterol sulphate is present in higher levels towards the stratum granulosum (Rippke *et al*., 2002). Molecules that raise the pH of the skin, such as ammonia, carbon dioxide and bicarbonate, are also produced in the skin and act to buffer the pH (Matousek and Campbell, 2002).

1.4.5 Acid resistance

Acid resistance in Gram-positive bacteria is mediated by mechanisms that: increase the internal pH; repair DNA and protein damage caused by the acid; or change the cell envelope architecture (Cotter and Hill, 2003).

F_1F_0-ATPase is a well-studied proton pump that mediates acid resistance (Cotter and Hill, 2003). F_1F_0-ATPase is capable of generating a proton motive force (PMF) that increases intracellular pH as protons are extruded in a process fuelled by ATP (Cotter and Hill, 2003). Glutamate decarboxylases can also increase acid resistance by increasing the internal pH (Cotter and Hill, 2003).

These decarboxylases combine glutamate with a proton then export the product out of the cell (Cotter and Hill, 2003). In *S. aureus*, F_1F_0-ATPase is downregulated in response to acid, but NADH dehydrogenase is upregulated indicating that the latter is more important in *S. aureus* for acid resistance (Bore *et al*., 2007). NADH dehydrogenase moves $2H^+$ out of the cell whilst converting NADH to NAD^+ (Bore *et al*., 2007).

D-alanylation of WTA is encoded by the *dlt* operon and was shown to reduce proton permeability in *Streptococcus mutans* (Cotter and Hill, 2003). The effect of this operon on *S. aureus* acid resistance is unstudied, though it was not differentially expressed after acid shock (Bore *et al*., 2007).

Ammonia produced by Gram-positive bacteria binds hydrogen ions to form NH_4^+ and thus increase the internal pH (Cotter and Hill, 2003). Ammonia production is facilitated by ureases, which convert urea to ammonia and carbon dioxide, and the arginine deiminase pathway, which converts arginine to ornithine, ammonia and carbon dioxide (Cotter and Hill, 2003). Urease genes, but not arginine deiminase pathway genes, were observed to be upregulated in *S. aureus* following acid shock (Bore *et al*., 2007). Production of acetoin may similarly reduce the internal pH (Bore *et al*., 2007).

The staphylococcal duplication of the *cls* gene, encoding CL synthase, is also important in resistance to acids. Whilst *cls2* produces CL under normal conditions, it was not capable of producing CL under acute acid stress, under these conditions *cls1* produces CL (Ohniwa *et al*., 2013). CL is important for osmotic stress resistance (see section 1.4.3), and plays a role in cell membrane structure, particularly in stationary phase (Ohniwa *et al*., 2013). Osmoprotectant transporters were upregulated following acid stress in *S. aureus*, suggesting a link between osmotic stress resistance and acid stress resistance (Bore *et al*., 2007).

1.4.6 Competition

The dominance of either corynebacteria or staphylococci on skin indicates there may be competitive exclusion between these two genera. Indeed, inhibition of staphylococci by corynebacteria has been observed in the nasal cavity (Frank *et*

al., 2010; Libberton *et al*., 2014). Other species associated with competitive exclusion of *S. aureus* from the nasal cavity include *S. epidermidis*, *S. haemolyticus*, *Finegoldia magna*, *Corynebacterium accolens*, *S. hominis* and *Micrococcus* spp. (Libberton *et al*., 2014; Wos-Oxley *et al*., 2010; Iwase *et al*., 2010).

Another group of potential competitors on the skin are bacteriophages. The presence of anti-staphylococcal phage on mammalian skin was suggested by Slanetz and Jawetz (1941) who observed the presence of lytic phages in bovine milk. Phages capable of infecting *S. epidermidis* have since been shown to be present at low levels in the anterior nares (Aswani *et al*., 2011).

1.4.7 Outcompeting the competition

Resistance to competition by bacteria and yeasts can be mediated by several mechanisms: through blocking colonisation via competitive interference by adhering to a range of receptors (competition interference); through resistance to competitor bacteriocins (similar to AMP resistance described in section 1.4.9); and through production of compounds that inhibit growth of competitors.

Staphylococci produce a range of bacteriocins (or staphylococcins) active against other staphylococci, group A *Streptococcus* (GAS) spp., group B *Streptococcus* spp., *Enterococcus faecalis*, *Lactobacillus* spp., *Micrococcus luteus* and *Corynebacterium fimi* amongst others (Cogen *et al*., 2010a; Gamon *et al*., 1999; Potter *et al*., 2014). The mechanisms of action of staphylococcins remain uncharacterised, but are potentially as variable as human AMPs (see section 1.4.8).

Phenol-soluble modulins (PSMs) δ and γ of *S. epidermidis* were also shown to have antimicrobial activity against *S. aureus*. Phenol soluble modulins are a family of amphipathic, α-helical staphylococcal proteins, whose functions range from haemolysins (δ-toxin) to biofilm dissemination (Periasamy *et al*., 2012). The antimicrobial activity of PSMs is thought to arise from their ability to insert into membranes, resulting in lipid vesicle leakage (Cogen *et al*., 2010b).

Secreted products other than bacteriocins can also act to inhibit competition. For example, under anaerobic conditions, *S. epidermidis* ferments glycerol into succinic acid, which is capable of inhibiting *P. acnes* growth *in vitro* and reducing *P. acnes* lesions in mouse models (Wang *et al*., 2014).

S. epidermidis is also capable of stimulating production of human β-defensins 2 and 3 (hBD2 and hBD3), antimicrobial peptides of the innate immune system that are active against *S. aureus*, but not *S. epidermidis* (Lai *et al*., 2010; Iwase *et al*., 2010). Esp is a serine protease, that unaided can disrupt *S. aureus* biofilm formation, and acts synergistically with hBD2 against *S. aureus* (Iwase *et al*., 2010; Park *et al*., 2011).

Staphylococci on the skin may also face competition from temperate bacteriophage. Resistance to bacteriophages can be mediated by Clustered Regularly Interspaced Palindromic Repeats (CRISPRs). CRISPRs and their associated Cas proteins provide phage resistance in many bacteria and archaea and have been described in some staphylococci (van der Oost *et al*., 2009; Vale and Little, 2010; Marraffini and Sontheimer, 2008; Ramia *et al*., 2014). The Cas proteins process phage derived-DNA and store them as short repeat regions within CRISPR regions in the genome (Vale and Little, 2010). These repeat regions in association with Cas proteins then lead to sequence-specific interference and degradation of phage genetic material in subsequent infections with a similar phage or reactivation of a prophage within the cell (Vale and Little, 2010).

1.4.8 Antimicrobial peptides

There are various AMPs found constitutively on the skin and upregulated when skin is wounded or exposed to pathogens (Cho *et al*., 2010). Skin AMPs include cathelicidin, hBD2, hBD3, psoriasin and dermcidin 1, whilst RNase 7 and lysozyme also contribute to innate antimicrobial activity of the skin, as previously reviewed (Niyonsaba and Ogawa, 2005).

Cathelicidin LL-37 is expressed in the eccrine glands and stored in lamellar granules; it is active against Gram-positive bacteria, Gram-negative bacteria and

the yeast *Candida albicans* (Schroder and Harder, 2006). It is an α-helical AMP observed to cause loss of turgor pressure at higher concentrations and inhibit cell growth, possibly through interference with peptidoglycan synthesis, at lower concentrations in *Bacillus subtilis* (Barns and Weisshaar, 2013).

The antimicrobials hBD2 and hBD3 are active against GAS, *S. aureus* and certain viruses (Cho *et al*., 2010). Where hBD2 is cationic and found in lamellar bodies, hBD3 is highly basic and is found in tissues throughout the body (Schroder and Harder, 2006).

Psoriasin is highly bacteriocidal to *E. coli*, and also promotes host cell growth, which may be important for wound healing (Glaser *et al*., 2005; Shubbar *et al*., 2012; Anderson *et al*., 2009). Psoriasin overproduction has been associated with psoriasis and growth of certain cancers (Shubbar *et al*., 2012; Anderson *et al*., 2009).

RNase 7 has activity against *S. aureus, E. coli* and enterococci (Simanski *et al*., 2012; Schroder and Harder, 2006). Though RNase 7 has ribonuclease activity, the antimicrobial activity of this cationic peptide is suggested to be caused by its ability to induce membrane permeabilisation (Huang *et al*., 2007).

Dermcidin 1 is produced by the eccrine glands and is active against *S. aureus*, *E. faecalis*, *E. coli* and *C. albicans* (Schroder and Harder, 2006). Dermcidin 1 inhibits RNA and protein synthesis, possibly through ion channel formation (Song *et al*., 2013a; Senyurek *et al*., 2009; Rieg *et al*., 2005).

Lysozyme is a peptidoglycan N-acetylmuramoylhydrolase (murimidase) that is active against Gram-positive cell walls, resulting in cell lysis (Niyonsaba and Ogawa, 2005). It is present on the skin due to secretion from apocrine glands (Niyonsaba and Ogawa, 2005).

1.4.9 AMP resistance

Staphylococcal resistance to CAMPs, such as cathelicidins and defensins, can be mediated by the *dlt* operon products and MprF. Dlt proteins mediate addition of D-alanine to wall teichoic acid (Peschel *et al*., 1999). MprF is a flippase that

modifies anionic phospholipids with L-lysine or L-alanine and also translocates lysyl-phosphatidylglycerol to the outer leaflet of the membrane (Ernst and Peschel, 2011; Peschel *et al*., 2001). These modifications result in a more positive cell surface that repels the CAMPs.

PGA biosynthesis is another factor that may prevent interaction of AMPs with the cell membrane. PGA biosynthesis mutants had increased susceptibility to cathelicidin LL-37 and hBD3 (Kocianova *et al*., 2005).

GraRS is a two-component sensor-regulator activated by CAMPs (Yang *et al*., 2012). GraRS regulates over 200 genes, activating defence mechanisms against CAMPs including the *dlt* operon (Herbert *et al*., 2007). Mutation of GraRS increased staphylococcal sensitivity to CAMPs and attenuated *S. aureus* virulence in a mouse infection model (Kraus *et al*., 2008; Herbert *et al*., 2007; Yang *et al*., 2012). The gene *vraG*, encoding an ABC efflux pump, is contranscribed with *graR*; VraG mutants also show increased susceptibility to cationic peptides indicating VraG also plays a role in AMP resistance (Yang *et al*., 2012). It is likely that other regulatory components also contribute to AMP resistance.

Proteases are an important means of defence against AMPs (Lai *et al*., 2007). In *S. epidermidis*, and presumably other staphylococci, AMPs induce expression of proteases (Lai *et al*., 2007). Though proteases are active against all human AMPs (to the authors knowledge), SepA of *S. epidermidis* is particularly efficient in mediating dermcidin 1 resistance (Lai *et al*., 2007).

The majority of staphylococci are lysozyme resistant. This trait has primarily been attributed to OatA, which O-acetylates muramic acid residues in peptidoglycan and sterically hinders interaction of lysozyme with peptidoglycan (Bera *et al*., 2005). The presence of WTA at lysozyme binding sites on peptidoglycan and the high degree of cross-linking by transpeptidases in staphylococcal cell walls adds another layer of resistance against lysozyme (Bera *et al*., 2007). Mutation of genes known to mediate CAMP resistance, such as *dltA* and *gra*RS, further increased susceptibility of staphylococci to lysozyme in OatA mutants, but not wild type (Herbert *et al*., 2007).

1.4.10 Antimicrobial skin lipids

Antimicrobial lipids of the skin include sphingoid bases (sphingosines) and fatty acids. Fatty acids and sphingosines are liberated from ceramides, phospholipids and glucosylceramides released from lamellar granules (Drake *et al*., 2008; van Smeden *et al*., 2014). Fatty acids are also released from the hydrolysis of triglycerides and biglycerides, some of which are released from lamellar granules, but the majority of which come from the sebum (Kohler *et al*., 2009; Madison, 2003; Drake *et al*., 2008).

AFAs are active against staphylococcal and micrococcal species, whereas sphingosines are widely active against bacteria and fungi (Drake *et al*., 2008; Desbois and Smith, 2010). Beyond their antimicrobial role on skin, sphingosines, fatty acids, and their precursors contribute to barrier formation. This barrier prevents excessive water loss as well as entry of the majority of substances (Pullmanová *et al*., 2014; van Smeden *et al*., 2014). Fatty acids help maintain skin homeostasis by reducing inflammation and promoting wound healing (Brogden *et al*., 2012; Huang *et al*., 2014; Lai *et al*., 2009). As described above, fatty acids are also believed to contribute to the acid mantle of the skin (Fluhr *et al*., 2001).

Not all free fatty acids on skin are antimicrobial. Those that are include sapienic acid (C16:1Δ6), linoleic acid (C18:2Δ9Δ12) and oleic acid (C18:1Δ9) (Drake *et al*., 2008). AFAs tend to be unsaturated and between 12 and 20 carbons in length (Desbois and Smith, 2010; Zhang *et al*., 2012). It is possible to predict the antimicrobial activity of AFAs based upon their carbon chain length, degree of unsaturation, location of unsaturated bonds, presence of hydroxide radicals, degree of esterification and presence of other functional groups, though this has only been confirmed in one strain of *S. aureus* to date (Zhang *et al*., 2012).

The antimicrobial activity of sphingosine is inhibited at deeper layers of the skin by cholesterol sulphate ($ChSO_4$) with which it forms an undissociated salt (Payne *et al*., 1995).

1.4.11 Resistance to antimicrobial lipids

Staphylococcal resistance mechanisms to AFAs have been investigated, however, there has been very little investigation into sphingosine resistance mechanisms. Some mechanisms that protect against AFAs are likely to also protect against sphingosines. For example, factors such as WTA and IsdA that decrease cell surface hydrophobicity, should decrease the capability of antimicrobial lipids to interact with the cell (Kohler *et al*., 2009). Cell wall thickening would further decrease the overall cell surface hydrophobicity, and gene expression data indicates this could occur in response to AFA challenge (Kenny *et al*., 2009).

Capsule biosynthesis is another factor that may protect against sphingosines and AFAs by preventing their interaction with the cell. Capsule biosynthesis genes were up-regulated in response to AFAs in *S. aureus* (Kenny *et al*., 2009).

Further AFA resistance mechanisms are unlikely to offer resistance to sphingosines. Carotenoid production, for example, has been postulated as a mechanism for AFA resistance in *S. aureus*. Carotenoid acts as a membrane "stiffener", and could potentially counteract AFA induced increases in membrane fluidity (Chamberlain *et al*., 1991). *S. aureus* strains with higher levels of carotenoid have greater resistance to AFAs, and carotenoids are up-regulated in response to AFAs (Chamberlain *et al*., 1991; Kenny *et al*., 2009). Despite this, the majority of staphylococci do not have carotenoids. In these staphylococci other factors that can act as membrane stiffeners, including cardiolipin and host-derived cholesterol (Romantsov *et al*., 2009), could offer protection against AFAs, though this has not been investigated to date.

Fatty acid modifying enzyme (FAME) provides resistance to AFAs in staphylococci. FAME is an exoprotein, known to be produced by *S. aureus* and *S. epidermidis*, that esterifies lipids with cholesterol or primary alcohols, seemingly inhibiting the mechanism of toxicity (Chamberlain and Brueggemann, 1997; Kapral *et al*., 1992). FAME is inhibited by triglycerides and diglycerides, which may explain why there is strong correlation between FAME and lipase production amongst staphylococci (Long *et al*., 1992; Lu *et al*., 2012). Lipases

hydrolyse triglycerides and diglycerides releasing free fatty acids that FAME can act upon.

Recently it was proposed that some of the myosin-cross reactive antigen (McrA) family of cell wall anchored proteins in staphylococci provide protection against AFAs, including linoleic acid. These proteins include the *S. aureus* surface protein F (SasF) and the *S. saprophyticus* surface protein F (SssF), with homologues of these proteins present in all sequenced staphylococci (King *et al*., 2012). An *S. aureus* SasF mutant was shown to have reduced survival to linoleic acid, which could be complemented by SasF and also by SssF (Kenny *et al*., 2009; King *et al*., 2012). Clinical isolates of *S. saprophyticus* with the SssF gene were shown to be more resistant to linoleic acid than those without (King *et al*., 2012). It was speculated that these proteins detoxify the AFAs by acting as fatty acid hydratases (King *et al*., 2012), as demonstrated in McrA family proteins of *Streptococcus pyogenes* and *Bifidobacterium breve* (Volkov *et al*., 2010). This activity was seen previously in *S. aureus* strains that could cause the saturation of the fatty acid linoleic acid to form its less-toxic derivatives oleic acid, stearic acid and mysteric acid, though this activity was not linked to any specific protein at the time (Campbell *et al*., 1983).

Kenny *et al*. (2009) found genes encoding arginine deaminase pathway enzymes (such as the *arcABDC* operon) were upregulated in response to AFAs and that an *arcA* mutant showed increased sensitivity to AFAs. ACME (Arginine catabolic mobile element) was predicted to improve survival of *S. aureus* on the skin in community acquired MRSA isolates (Diep *et al*., 2006). Moreover, it was suggested that the arginine deaminase genes on ACME could account for increased survival by providing protection from acid by producing ammonia (Foster, 2009), potentially neutralising any acidic pH caused by AFAs.

Mutants of *S. aureus* VraE (an ABC transporter permease) and SAR2632 (a putative efflux pump proposed to be involved in lipid transport) showed decreased survival when grown on linoleic acid containing agar compared to the wild type (Kenny *et al*., 2009). Increased transport might be important for AFA survival, this may be because AFAs interfere with cellular transport, and the cell may attempt to restore membrane polarisation or increase export of

AFAs from the cell. Specific fatty acid efflux pumps have been shown to confer AFA resistance in meningococci (Schielke *et al*., 2010), and are indicated to play a role in staphylococcal AFA resistance (Truong-Bolduc *et al*., 2014).

1.5 Atopic dermatitis and staphylococci

Atopic dermatitis (AD) is a disease characterised by dry, flaky skin lesions that can become infected and form abscesses (Bieber, 2008). AD patients have unusually high levels of *S. aureus* colonisation, with up to 80 % of their skin microflora being comprised of *S. aureus*, even on non-lesional skin (Roth and James, 1988; Takigawa *et al*., 2005; Kong *et al*., 2012; Higaki *et al*., 1999). AFAs, sphingosines and AMPs are all reduced in AD patients, and therefore have been linked to *S. aureus* skin exclusion (Cho *et al*., 2010; Schafer and Kragballe, 1991; Arikawa *et al*., 2002). Other factors associated with AD but not to *S. aureus* skin exclusion to date include decreased filaggrin production and increased skin pH (Rippke *et al*., 2004; Irvine and McLean, 2006).

The AMPs hBD2 and hBD3 are reduced in AD patients and have therefore been linked with susceptibility to *S. aureus* colonisation (Cho *et al*., 2010). Levels of *S. epidermidis* also increases on AD skin (Soares *et al*., 2013; Kong *et al*., 2012), as *S. epidermidis* is not susceptible to hBD2 or hBD3 (Cho *et al*., 2010), it seems unlikely that the reduction of these AMPs fully accounts for the increase of staphylococci on AD skin. Expression of hBD2 and hBD3 is reduced in response to elevated Th2 cytokines. Th2 cytokines are elevated in response to tissue damage and inflammation, which occurs in healthy skin when there is an overgrowth of *S. aureus* or an *S. aureus* infection (Cho *et al*., 2010); hence the reduced hBD2 and hBD3 could be a direct consequence of high *S. aureus* colonisation.

More recently, reduced skin barrier function as assessed by transepidermal water loss (TEWL) was correlated to *S. epidermidis* colonisation in AD (Jinnestal *et al*., 2014). Though this could be related to mutations in the filaggrin gene previously linked to AD, barrier function and TEWL are mediated in part by skin lipids (Irvine and McLean, 2006; Pullmanová *et al*., 2014; van Smeden *et al*., 2014). The link between TEWL, *S. aureus* colonisation and skin lipids agrees

with previous findings that the degree of *S. aureus* colonisation is inversely proportional to the levels of AFAs and sphingosines on the skin of AD patients (Takigawa *et al*., 2005). *S. epidermidis* is indicated to be more resistant to the AFA sapienic acid than *S. aureus*, however, there has been no systematic analysis comparing more than one strain of each species (Takigawa *et al*., 2005).

1.6 Mechanism of action of unsaturated fatty acids

The effects of AFAs upon staphylococci are multifold. One of the most tendered mechanisms of action is their ability to insert into bacterial membranes. This mechanism has been used to explain observations of increased membrane fluidity and permeabilisation, altered intracellular pH and leakage of low molecular weight (< 20 kDa) compounds from the cell, including ATP and amino acids (Greenway and Dyke, 1979; Cartron *et al*., 2014; Parsons *et al*., 2012). In some microorganisms AFAs dissolve the plasma membrane, however this has not been observed in staphylococci (Bergsson *et al*., 2001).

Unsaturated AFAs tend to be more potent than saturated AFAs, if AFAs do insert into membranes this could be explained by the kinked structure of unsaturated AFAs (Fig. 1.2), which could result in inferior packing of phospholipids in the cell membrane, thereby increasing fluidity and disorder (Desbois and Smith, 2010). Unlike some other antimicrobials like vancomycin, sapienic acid has not been found to localise to any particular point in the membrane, however, like vancomycin, it does interfere with proper cell division (Cartron *et al*., 2014).

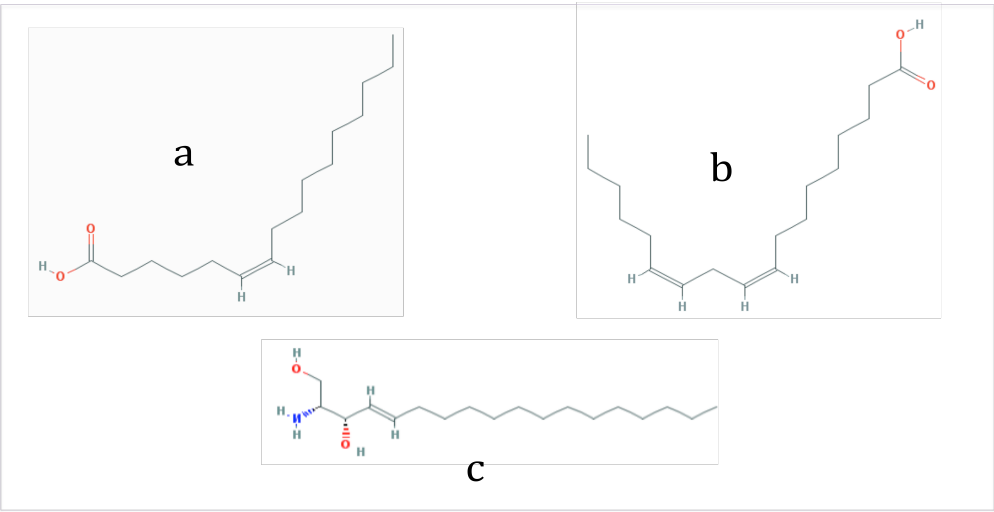


Figure 1.2 Chemical structures of antimicrobial skin lipids

a) linoleic acid, b) sapienic acid and c) D-sphingosine.

Whilst there is evidence to support membrane insertion by AFAs, membrane insertion was not essential to inhibit growth (Greenway and Dyke, 1979; Cartron *et al*., 2014). Indeed, AFA membrane insertion has been suggested as a bacterial resistance mechanism to reduce toxicity of fatty acids below a concentration threshold (Cartron *et al*., 2014). Studies using ^{14}C labelled fatty acids showed that non-toxic fatty acids were metabolised, whist toxic fatty acids could not be; this indicates the normal mechanism to cope with fatty acids is uptake and metabolism (Parsons *et al*., 2012). Since insertion is not essential for growth inhibition, it is unlikely that membrane permeabilisation is the primary mechanism of action for AFAs.

Other reported activities of the AFAs include inhibition of respiration, disruption of the electron transport chain and collapse of the proton gradient (Cartron *et al*., 2014; Parsons *et al*., 2012). These effects of AFAs are likely a result of the ability of AFAs to act as a protonophores and thus as uncouplers of oxidative phosphorylation (Cartron *et al*., 2014; Parsons *et al*., 2012; Jezek *et al*., 1998). The *S. aureus* MIC of sapienic acid increased six fold when grown anaerobically; the authors suggested that this was because *S. aureus* is not dependent on a proton motive force during anaerobic growth (Cartron *et al*., 2014). The fact that *S. aureus* was still inhibited by sapienic acid under anaerobic conditions, albeit at a higher concentration, indicates a secondary

mechanism of action, this is likely to be membrane permeabilisation. It has been argued that the greater damage to cell metabolism caused by AFAs than typical protonophores indicates that AFAs main mechanism of action is through pore formation which would lead to membrane depolarisation rather than protonophore action (Parsons *et al*., 2012). However, with the data that AFAs can rapidly inhibit growth without inserting into the membrane it is likely that their protonophore activity is the primary mechanism of action, with membrane permeabilisation acting as a secondary mechanism that compliments their protonophore activity.

An AFA mechanism of action resulting in uncoupling of oxidative phosphorylation would explain why so many energy dependent bacterial processes, such as DNA, RNA, protein, cell wall and membrane synthesis, are reported as being inhibited by AFAs (Parsons *et al*., 2012; Greenway and Dyke, 1979). It would also explain why, though membrane insertion is not essential to inhibit growth, reduced membrane access can act as a resistance mechanism.

1.7 Mechanism of action of sphingoid bases

The mechanisms of action of sphingosines are less studied than AFAs, though it has been suggested that the mechanisms of action against *S. aureus* are the same (Parsons *et al*., 2012). There is some evidence to support sphingosines and AFAs having the same mechanism of action; D-sphingosine was also seen to cause release of low molecular weight components into the media (Parsons *et al*., 2012). However, it was revealed that AFAs carboxyl group is essential for their toxicity (Zheng *et al*., 2005), but sphingosines do not have carboxyl groups. Sphingosines have also been shown to be more potent than AFAs against *S. aureus*, and are active against Gram negatives *E. coli* and *Fusobacterium nucleatum* as well as *Corynebacterium* spp., none of which are strongly inhibited by AFAs (Fischer *et al*., 2012). Taken together, these data make it unlikely that AFAs and sphingosines have identical mechanisms of action.

Unlike AFA-treated *S. aureus* cells, which have no significant morphological changes (Parsons *et al*., 2012), sphingosines cause profound morphological changes (Fischer *et al*., 2013). Morphological changes of *S. aureus* treated with

D-sphingosine or dihydrosphingosine included a decrease in cell size, ruggate appearance, more pronounced septal grooves, loss of cell wall, flocculated cytoplasm, electron dense intracellular inclusion bodies and visible cell lysis (Fischer *et al.*, 2013). Phytosphingosine caused the same morphological changes with the exception that it did not reduce cell size; this corresponded with a considerably higher MIC for phytosphingosine than D-sphingosine and dihydrosphingosine (Fischer *et al.*, 2013; 2012).

Although it was observed that *S. aureus* incorporated sphingosines, it is uncertain whether they become localised in the membrane, as with AFAs, or if they are taken into the internal environment of the cell (Fischer *et al.*, 2013). Sphingosines are evidently more efficient than AFAs at causing membrane disruption as they lead to visible effects and cell lysis. It seems likely that the sphingosine mechanism of action against *S. aureus* is, at least in part, due to their ability to permealise the cell membrane. It remains possible that they have a secondary mechanism of action; sphingosines are known to interact with a multiple proteins to cause signal transduction within mammalian cells (Spiegel and Milstien, 2000), and they could have a similar effect in bacteria.

1.8 Thesis aims

S. aureus and *S. epidermidis* show large differences in their ability to colonise human skin. Studies of individuals with atopic dermatitis has led to discovery of multiple host factors that could affect *S. aureus* survival on skin. However, few studies have investigated the microbial basis for the impact of these host factors on *S. aureus* and *S. epidermidis*. This study investigates the effects of host antimicrobial cutaneous lipids upon *S. aureus* and *S. epidermidis*, with a focus on discovering microbial components that may affect antimicrobial lipid resistance.

Chapter three aims to assess the hypothesis that host antimicrobial cutaneous lipids permit colonisation of skin by *S. epidermidis* but not *S. aureus*. If true, it would be expected that *S. epidermidis* would have higher resistance to these lipids than *S. aureus*.

Chapter four will aim to characterise and compare the transcriptional response of *S. aureus* and *S. epidermidis* to an antimicrobial skin lipid. Chapter five aims to characterise and compare the evolutionary response of *S. aureus* and *S. epidermidis* to antimicrobial skin lipids. Differences in responses of *S. aureus* and *S. epidermidis* may help explain their differential survival on skin. Both of these techniques may implicate specific genes as important for skin survival and as resistance determinants to antimicrobial skin lipids.

Through the comparison of *S. aureus* with *S. epidermidis* it is hoped that a better understanding of the genetic determinants accounting for their differential survival on skin will be found. Understanding the genetic basis for skin survival in these two species may facilitate greater understanding of skin colonisation. Understanding the genetic basis for differential skin colonisation between *S. aureus* and *S. epidermidis* may elucidate new targets for antimicrobials that specifically target detrimental members of the microbiome. Alternatively, such knowledge could be used to improve microbial replacement therapies for skin disorders caused by microbiome disbiosis.

Chapter 2 Methods and Materials

2.1 Bacterial strains, growth conditions and plasmids

The strains used in this study and their metadata are shown in Table 2.1. All strains were tested for resistance to antimicrobial lipids. Strains Newman and Tü3298 were selected for RNA-seq analysis. Strain Rp62a was selected for experimental evolution work, clones of which were sequenced along with previously evolved SH1000 (Campbell-Lee, 2012).

All overnight cultures were grown for 18 h at 37˚C with shaking unless stated otherwise. Todd Hewitt broth (THB) or agar (THA) was used as the medium for all work unless otherwise stated.

Table 2.1 Bacterial strain data.

A summary of strains used in this study.

Species	Strain identifier	Isolation site	Reference
S. epidermidis	Rp62a	Intravascular catheter	(Christensen *et al*., 1982; 1985)
S. epidermidis	Tü3298	Unspecified	(Allgaier *et al*., 1986)
S. epidermidis	NCTC 1457	Intravenous catheter	(Mack *et al*., 1992)
S. epidermidis	A19	Skin (forearm)	(Kelly, 2013)
S. epidermidis	B19	Skin (forearm)	(Kelly, 2013)
S. epidermidis	O16	Skin (forearm)	(Kelly, 2013)
S. aureus	Newman	Osteomyelitis	(Duthie and Lorenz, 1952)
S. aureus	SH1000	Lab strain (8325 derivative)	(Horsburgh *et al*., 2002)
S. aureus	MSSA476	Osteomyelitis	(Holden *et al*., 2004)
S. aureus	MRSA252	Fatal bacteraemia	(Holden *et al*., 2004)
S. aureus	BL169	Nasal	(Libberton, 2011)
S. aureus	BL137	Nasal	(Libberton, 2011)

2.2 Minimum inhibitory concentration assay

The MIC method was based upon the method by Tatham (2011). The assay was executed in a 96 well plate containing linoleic acid, sapienic acid or D-sphingosine at a range of concentrations as shown as a percentage of the stock solution in Table 2.2. The range was produced by diluting a stock solution 1 in 2 in broth across the plate, then half diluting again with the addition of the bacterial suspension. Linoleic acid stock was 400 mM, sapienic acid stock was 400 μg ml^{-1} (1.57 mM) or 1000 μg ml^{-1} (3.93 mM) if a higher concentration was required and D-sphingosine stock was 500 μg ml^{-1} (1.67 mM). Linoleic acid and sapienic acid stocks were made in 100% (v/v) ethanol, D-sphingosine stock was made at 1 mg ml^{-1} in 100% ethanol (v/v) and half diluted with THB immediately before use, all stock solutions were stored at -20˚C and allowed to reach ambient temperature before use. Control plates were made using ethanol adjusted to the appropriate concentration with THB. Overnight cultures were diluted to OD_{600} 0.2 ± 0.005 using broth, and 100 μl of diluted culture was added to each well, making a total volume of 200 μl per well.

As the fatty acids emulsified in media it was not possible to assess growth by optical density; instead 10 concentrations were chosen that represented the range of the plate and these were serially diluted 10 fold up to $1x10^{-6}$ and plated by the Miles and Misra method. The lowest concentration with CFU ml^{-1} lower than or equal to the initial CFU ml^{-1} was deemed to be the minimum inhibitory concentration (MIC). The lowest concentration that reduced viability of the inoculum by more than 2 log was deemed to be the minimum bacteriocidal concentration (MBC).

Table 2.2 Concentrations per well used in minimum inhibitory concentration assay.

Concentrations are shown as percentages of stock solutions and shown in the locations they would appear in the 96 well plates used for the experiment. Wells E-H were repeats of wells A-D. The – or + represent a negative or positive control respectively.

	1	2	3	4	5	6	7	8	9	10	11	12
A	25	12.5	6.25	3.13	1.56	0.78	0.39	0.2	0.1	0.05	0.02	0.01
B	21.7	10.84	5.42	2.71	1.36	0.68	0.34	0.17	0.09	0.04	0.02	0.01
C	18.3	9.16	4.58	2.29	1.15	0.57	0.29	0.14	0.07	0.04	0.02	-
D	15	7.5	3.75	1.88	0.94	0.47	0.23	0.12	0.06	0.03	0.02	+

2.3 Agar plate-based linoleic acid survival assay

Linoleic acid was added to molten THA to produce plates with 1, 2.5, 5, 7.5 or 10 mM linoleic acid. Cultures were grown overnight either under normal conditions or with 0.01 mM linoleic acid in the media, a concentration that was previously determined as sub-inhibitory (Kenny *et al*., 2009). Overnight cultures were 10-fold serially diluted up to 1 x 10^{-6}; at least duplicate 20 μl spots were plated per dilution per linoleic acid concentration. Controls were plated onto THB agar plates with no linoleic acid. The counts were converted to CFU ml^{-1} and expressed as a percent survival of controls. Growth was assessed at 24 h for *S. epidermidis* strains and SH1000, and at 48 h for all other *S. aureus* strains.

2.4 Growth curves

Overnight cultures were adjusted to an OD_{600} of 0.5 using THB; 2 ml of the diluted culture was used to inoculate 50 ml of media in 250 ml flasks. Flasks were incubated in a water bath with 250 rpm linear shaking at 37 ˚C. Samples were taken hourly between 0 and 7 h and diluted ten-fold in phosphate buffered saline (PBS) before recording the OD_{600}.

This work aimed to determine the concentration of sapienic acid to be used for challenge conditions that would be used in future RNA seq work. The ideal concentration should be the lowest concentration at which sapienic acid could be added to the bacteria during the exponential phase (at OD_{600} 0.5) and be seen

to reduce growth rate 20 min post challenge when results were displayed graphically. An 8 mg ml^{-1} stock of sapienic acid was used to make all concentrations during this experiment. Initial concentrations used were $^1/_{10}$ to $^1/_4$ of the MIC value; adjustments were then made empirically based on previous results.

2.5 DNA and RNA quality control

2.5.1 Agarose gel electrophoresis

Agarose gels were made to a concentration of 1 % or 2 % (w/v) agarose in TAE buffer. The 1 % gels were used for gDNA integrity analysis whilst 2 % gels were used for analysis of PCR products and small plasmids. Molten agarose was stored at 50 °C until required. Ethidium bromide or Midori green (Nippon Genetics) were mixed into molten agarose at 4 μl or 2 μl per 100 ml respectively. When necessary, a loading dye was added to samples before loading samples into the gel. Samples were run in TAE buffer at 80-100 v for between 45 min and 2 h.

2.5.2 Qubit analysis

Qubit analysis was performed using the Qubit dsDNA BR or Qubit RNA BR assay kit (Life Technologies) according to the manufacturer's instructions.

2.5.3 Nanodrop analysis

Nanodrop (Thermoscientific) analysis was done according to the manufacturer's instructions for DNA or RNA samples.

2.6 Experimental evolution

2.6.1 Evolution passaging

Passages were set up with the appropriate concentration of sapienic acid or D-sphingosine in ethanol and were made up to a total concentration of 0.5 % (v/v)

ethanol in THB. Overnight cultures were used to inoculate serial passage cultures to OD_{600} 0.02 in a final volume of 5 ml. For the first passages concentrations of a tenth, quarter and half MIC were tested; of the cultures that grew, those with the highest concentration of sapienic acid or D-sphingosine were used for the subsequent serial passages. For each strain, a control was also passaged through THB with 0.5 % ethanol.

The concentrations of sapienic acid and D-sphingosine were increased in the subsequent passage if after overnight growth the culture reached an optical density consistent with control growth. In these cases, a range of increases were tested, values tested were empirical. The highest concentration that supported culture growth was selected for subsequent passages. Samples were taken and frozen at -80 °C in 15 % (v/v) glycerol prior to increases in concentration of sapienic acid or D-sphingosine.

2.6.2 SNP analysis

Sequence reads were processed by the Centre for Genome Research, University of Liverpool (CGR) to remove library adapter sequences and poor quality reads. Briefly, this meant trimming with cutadapt version 1.2.1 (Martin, 2011) to remove adapter sequences from reads followed by a further trim with Sickle version 1.200 to remove bases with lower than 20 base quality score. Reads shorter than 10 bp after trimming were filtered from the data files, if the paired read was not filtered out in this process it was moved to another file designated "R0". After this initial processing by the CGR, read quality was assessed using the NGS QC toolkit v2.3 (Patel and Jain, 2012).

If filtered reads were assessed to be of good quality, reads were aligned to their reference genome using the Burrows-Wheeler aligner (BWA) aln and sampe packages (Li and Durbin, 2009; 2010) version 0.5.9-r16. Samtools (Li *et al*., 2009) version 0.1.18-r580 was used to process SAM files (sequence alignment/map) to create a BCF file (binary variant call format) for SNP calling.

SNPs were called using a bespoke Perl script (mpileup_SNPs_v2.pl) that utilises the SNPEFF package (Cingolani *et al*., 2012) version 3.4e (build 2013-12-21) to

determine the effect of the SNP (e.g. Synonymous, non-synonymous or truncation). Another bespoke Perl script was used to filter out from the data SNPs found in the evolved strains and parent or ethanol control strains (unique_SNPs_bwa.pl). Non-synonymous SNPs and SNPs in potential promotor regions (<200bp upstream of a gene) were deemed of import to this study.

2.6.3 Protein modelling

HHpred (Remmert *et al*., 2011; Soding, 2005; Soding *et al*., 2005) was used to align gene sequences to the protein data bank (PDB) and conserved domains database (CDD) to determine potential protein domains. Providing the protein did not have predominantly coiled coil domains, a model protein structure for the gene was constructed using HHpred automatic template selection with multiple templates. The model was visualised using the Pymol Molecular Graphics System v 1.5.0.4 (Schrödinger, LLC).

2.7 RNA sequencing

2.7.1 Notes

All work with RNA was done in RNase free plastic-ware using RNase free filter tips. Benches and pipettes were cleaned with RNase Zap (Ambion). Any water used was incubated at 37 °C with 0.1 % (v/v) diethypyrocarbonate overnight prior to sterilisation by autoclaving (DEPC-treated water).

2.7.2 Growth and preparation of cells for RNA extraction

Cultures were set up as described previously (section 2.4). When bacteria reached an OD_{600} of 0.5, sapienic acid in ethanol (test) or the equivalent volume of ethanol (control) was added. The final concentration of sapienic acid for *S. epidermidis* Tü3298 was 15 μg ml^{-1}, for *S. aureus* Newman it was 11.25 μg ml^{-1}.

At 20 minutes after challenge with sapienic acid or ethanol cells were harvested by pelleting for 5 min at 4,000 RCF and 4 °C. Pellets were resuspended in 2 volumes of RNAlater (Qiagen) and incubated overnight at 4 °C.

2.7.3 Lysis of cells for RNA extraction

Lysis was performed as adapted from Tatham (2011). Briefly, the protocol was as follows. Per extraction, 3 ml of the *S. epidermidis* preparation or 1 ml of the *S. aureus* preparation was used. The bacteria were pelleted at 6,000 RCF for 5 min at 4 °C, and resuspended in 100 μl TE containing 6 mg ml^{-1} lysostaphin and 400 U ml^{-1} mutanolysin.

This lysis mix was incubated at 37 °C for 15 min for *S. aureus* and 30 min for *S. epidermidis*, mixing every 5 min. Bacteria were incubated for a further 30 min at 37 °C after the addition of 25 μl of Proteinase K (Qiagen).

2.7.4 RNA extraction

RNA was extracted using the RNeasy kit (Qiagen), with slight alterations to the manufacturer's protocol. Briefly the method was as follows: 350 μl buffer RLT containing 1 % (v/v) β-mercaptoethanol was added to the lysed cells and mixed before the addition of 250 ml ethanol. This suspension was centrifuged through the RNeasy column for 15 s at 10,000 RCF. The column was then washed twice with 700 μl buffer RW1 for 15 s at 14,000 RCF. The column was then washed thrice with 500 μl buffer RPE for 15 s at 14,000 RCF. Collection tubes were changed between buffers. The column was centrifuged in a clean collection tube for 1 min with the column lid off. The column was then air dried for 2-5 min. RNA was eluted twice with 30 μl water prewarmed to 45 °C.

RNasin (Promega) was added according to the manufacturer's instructions. A 2 μl aliquot of the sample was used for total RNA quantification using Qubit RNA assay kit (Invitrogen) according to manufacturer's protocol.

2.7.5 DNase treatment of RNA

Samples with > 3 μg total RNA were DNase treated using turbo DNase (Ambion) according to manufacturer's instructions. DNase was removed using the RNeasy MinElute clean up kit (Qiagen) according to manufacturer's instructions, with

the addition of 1 % beta-mercaptoethanol to the buffer RLT and elution was in 20 μl water. An aliquot of 4 μl was taken for quality control analysis whilst the rest was frozen at -80 °C.

2.7.6 RNA quality control

Quality control analysis was conducted using a Qubit (Invitrogen) for quantification, 2100 Bioanalyser (Agilent technologies) to assess degradation levels and Nanodrop (Thermoscientific) to assess protein or solvent contamination (see section 2.5). Tests were carried out according to manufacturer's instructions for bacterial RNA. Samples with a paired control and test condition sample with Qubit read indicating ≥ 3 μg RNA, Bioanalyser RIN ≥ 7.0, Bioanalyser trace indicating good integrity, Nanodrop 260/280 and 260/230 ≥ 1.8 were suitable for sequencing.

2.7.7 RNA library preparation

Library preparation was performed by the CGR. Total RNA samples were rRNA depleted using Ribo-Zero magnetic kit for Gram-positive bacteria (Epicentre); this was repeated for samples with poor initial rRNA removal. The concentration of RNA was normalised, then libraries were then prepared using strand specific ScriptSeq kits (Epicentre). Samples were sequenced using paired-end sequencing on the HiSeq platform (Illumina).

2.7.8 RNA sequencing differential expression analysis.

Bowtie (Langmead *et al*., 2009) and Edge R (Robinson *et al*., 2010; Robinson and Oshlack, 2010) were used to map reads and determine the differentially expressed (DE) genes respectively. Genes with mapped transcripts that had a false discovery rate > 0.05, as determined by Benjamin and Hochberg analysis, were filtered out of the dataset. The remaining gene set was considered differentially expressed between control and test conditions. This analysis was produced by the Centre for Genomic Research (CGR), Liverpool.

Changes in gene expression in biosynthetic pathways were assessed using DE gene sets with KEGG mapper- search and color (Kanehisa *et al*., 2012; Kanehisa and Goto, 2000). Gene ontology (GO) terms were attached to DE genes using Uniprot (UniProt, 2014).

2.7.9 COG analysis

The sequences for all genes within the genomes of *S. aureus* Newman and *S. epidermidis* Tü3298 were extracted into a fasta file using the Galaxy tool "Extract genomic DNA" (Goecks *et al*., 2010; Giardine *et al*., 2005). A bespoke perl script (DNA_fasta_to_protein_fasta.pl) was then used to convert these gene sequences into protein sequences. These protein sequences were then submitted to WebMGA function annotation (COG) (Wu *et al*., 2011), which assigns a COG ID to each gene.

Another bespoke Perl script was used to convert the names that had been assigned to the genes by the Galaxy extract genomic DNA tool back to their true gene names (replace_names.pl). The gene lists of DE genes were labelled with their assigned COGs using a bespoke Perl script (label_cogs.pl). The numbers of genes in each COG class and the percentage of the genome accounted for by each COG class was then calculated using a further bespoke Perl script (counting_cogs.pl). Pearson's Chi^2 test was used to assess if the number of genes DE in each COG class was significantly more or less than expected.

2.7.10 SAMMD analysis

Prior to SAMMD analysis, the gene names in the DE gene list were converted to their homologue in *S. aureus* N315 using a bespoke Perl script (convert_to_SA_homologs.pl) which utilises the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990). The top hit from BLAST was selected using Excel (Microsoft), this list was used to create lists of homologous up or down regulated genes using a bespoke Perl script (lists_for_SAMMD.pl). The DE homologue gene list was then submitted to SAMMD (Nagarajan and Elasri, 2007) as an advanced search and the resulting data was turned into Venn

diagrams using Inkscape. The genes that were up- and down-regulated in both sapienic acid and linoleic acid challenge in both *S. aureus* Newman and *S. epidermidis* Tü3298 were determined using a bespoke Perl script (sammd_compare.pl).

2.8 Genome sequencing

2.8.1 DNA extraction

For DNA extractions, 1.5 ml of overnight cultures of staphylococci were pelleted at 4,000 RCF for 2 min in sterile Eppendorf tubes. The DNeasy Blood and tissue kit (Qiagen) was then used according to the manufacturer's instructions for Gram-positive bacteria, with the addition of lysostaphin at a final concentration of 25 $\mu g\ ml^{-1}$.

2.8.2 DNA Quality Control

Quality control analysis was conducted using the Qubit (Life Technologies) for quantification, agarose gel electrophoresis to assess DNA fragmentation and Nanodrop to assess protein or solvent contamination (as described in section 2.5). Samples used for sequencing with TruSeq DNA sample prep kit (Illumina) had Qubit reads indicating ≥ 1 $\mu g\ ml^{-1}$ DNA in ≤ 55 μl, an agarose gel result indicating low fragmentation of DNA and Nanodrop 260/230 and 260/280 ≥ 1.8. Samples for sequencing with Nextera DNA sample prep kit (Illumina) had the same requirements except that DNA concentration had to be ≥ 0.1 $\mu g\ ml^{-1}$.

2.8.3 Pooled DNA samples

For samples that were pooled prior to sequencing, DNA was extracted from individual clones and the DNA was pooled. DNA was pooled in equimolar concentrations based upon Qubit readings made using the same mastermix or using two internal control to normalise results when using a different mastermix.

2.8.4 DNA library preparation

DNA libraries for samples to be used as reference genomes for RNA sequencing were prepared using a Nextera DNA sample prep kit (Illumina) according to the manufacturer's instructions. DNA libraries for experimental evolution clones were prepared using TruSeq DNA sample prep kit (Illumina) by the CGR, Liverpool. All samples were sequenced on the MiSeq platform (Illumina) by the CGR, Liverpool.

2.8.5 Genome assembly

The NGS QC toolkit v2.3 (Patel and Jain, 2012) was used to filter out reads with less than 70 % of the read with quality scores over Q20 and to trim 3' ends with less than Q20 quality scores. VelvetOptimiser version 2.2.5 (Victorian Bioinformatics consortium), which utilises Velvet version 1.2.08 (Zerbino and Birney, 2008), was used to assemble genomes using k-mer lengths between 19 and 99 bp. This produced contigs that could be used as a reference for RNA-Seq data.

2.9 qPCR

2.9.1 Primer design

Primers were taken from the literature where possible or designed using primer-BLAST (Ye *et al.*, 2012). Primers with a length between 100 and 200 bp, predicted to have only one product, a Tm of 60 ±2 °C, low level of single base repeats and a GC clamp towards the 3' end were selected where possible.

Primers were confirmed to amplify a product of the predicted length without any secondary products using standard PCR with gDNA as a template. The PCR mix was made using BioMix Red (Bioline) and ACCUZYME DNA polymerase (Bioline) according to the manufacturer's instructions.

Primer efficiency was then confirmed to be within 90-100 % using a dilution curve with gDNA as described previously (Nolan *et al.*, 2006). Efficiency testing was done using the same conditions as for qPCR reactions (described below).

The reaction mix in a total volume of 20 μl was set up with 0.5 μM of each primer, 10 μl SensiFAST and a dilution range of gDNA between $1x10^{0}$ – $1x10^{-4}$, with a starting concentration of 10 million copies. Negative controls without template were also implemented. Efficiency values were generated by the qPCR machine software, and an average of at least three resulting efficiency values was taken.

2.9.2 cDNA generation

The tetro cDNA synthesis kit (Bioline) was used for cDNA synthesis according to the manufacturer's instructions using random hexamer primers and approximately 2 μg RNA per reaction. Only RNA samples determined to have high integrity were used for these reactions.

2.9.3 qPCR conditions

All qPCR reactions were done using SensiFAST SYBR Hi-ROX kit (Bioline) with the ABI StepOnePlus (Life Technologies). The reaction mix contained 10 μl Sensifast, 0.5 μM of each primer, 80 μg cDNA and DEPC-treated water up to a total reaction size of 20 μl. The run cycle was 95 °C for 5 min, then 40 cycles of 95 °C for 10 s, 62 °C for 30 s.

Data analysis was done using the ABI StepOnePlus software. At least two technical replicates and three biological replicates were used to determine fold change in gene expression between samples.

Chapter 3 Antimicrobial skin lipid resistance of *Staphylococcus aureus* and *Staphylococcus epidermidis*

3.1 Introduction

3.1.1 Measuring antimicrobial activity of lipids

This Chapter is primarily concerned with the antimicrobial activity of skin lipids upon *S. aureus* and *S. epidermidis*. As such, it is important to consider methods for antimicrobial activity testing, particularly those that have been used for testing the susceptibility of bacteria to lipids.

Methods for analysing antimicrobial resistance of bacteria vary depending on the organism type, antimicrobial or researcher preference. Antimicrobials routinely used in clinical settings often have guidelines for best practise available from organisations such as the clinical and laboratory standards institute (CLSI) or the European committee for antimicrobial susceptibility testing (EUCAST). As antimicrobial lipids are not used routinely in clinical settings, best practise guidelines are limited to generalised options, resulting in variations in methodologies. Here the different methodologies used for testing bacterial susceptibilities to lipids, their advantages and disadvantages are discussed.

Broth microdilution methods to determine minimum inhibitory concentrations (MICs) or minimum bactericidal concentration (MBC) are common in antimicrobial lipid studies (Desbois and Lawlor, 2013; Clarke *et al*., 2007; Chamberlain *et al*., 1991; Kenny *et al*., 2013; Fischer *et al*., 2012). For the broth microdilution method, first, a range of concentrations of the antimicrobial in broth is created by two-fold serial dilution from a stock solution. Bacteria are inoculated into each concentration and incubated for between 1 and 24 h (Desbois and Lawlor, 2013; Clarke *et al*., 2007; Chamberlain *et al*., 1991; Kenny *et al*., 2013; Fischer *et al*., 2012). Guidelines recommend an inoculum of 1-5 x 10^5 CFU ml^{-1} and an incubation time of 18-20 h (Jorgensen and Ferraro, 2009).

The lowest concentration with no growth, usually assessed by changes in optical density, is considered the MIC (Jorgensen and Ferraro, 2009; Clarke *et al*., 2007; Desbois and Lawlor, 2013). For fatty acids MIC assessment by changes in CFU ml^{-1} would be advisable as at high concentrations they can affect the turbidity of the media (Fischer *et al*., 2012). The lowest concentration required to kill 99.9 % of the inoculum, as assessed by CFU ml^{-1}, is the MBC (Desbois and Lawlor, 2013). There are some disagreements in MIC and MBC definitions; Fischer *et al.* (2012) consider the MIC to be the lowest concentration at which growth is inhibited by 50 % compared to control growth, and the MBC is the lowest concentration with no growth. An alternative measure of antimicrobial activity is the median lethal dose (LD_{50}), the lowest concentration required to kill 50 % of the inoculum, assessed by CFU ml^{-1} (Chamberlain *et al*., 1991).

The advantage of broth microdilution methods is that they quantitatively assess antimicrobial activity, allowing direct comparison of inhibitory concentrations between antimicrobial agents (Jorgensen and Ferraro, 2009). The disadvantage is that inoculum size, media, culture growth conditions, strain choice and even culture growth conditions for the inoculum can all affect the MIC and MBC values as is clearly seen in Table 3.1 and discussed below. This is compounded by the disagreement of what constitutes an MIC or MBC.

Table 3.1 *S. aureus* MICs and LD_{50}s for fatty acids and sphingosines from literature.

All concentrations were converted to Molar to aid comparisons.

*The MIC using the common definition as the lowest concentration that inhibits growth, though these are referred to as the MBC by the author. Abbreviations: MHB – Mueller-Hinton broth; TB – 1 % tryptone broth; CL – CL medium; CT-TSB – iron limited tryptic soy broth; TSB – trypticase soy broth.

Lipid	MIC (μM)	LD_{50} (μM)	Media	Strain	Reference
D-sphingosine	4.3	-	MHB	ATCC 29213	(Fischer *et al.*, 2012)
	7.8	-	TB	RN4220	(Parsons *et al.*, 2012)
Phytosphingosine	24.6*	-	MHB	ATCC 29213	(Fischer *et al.*, 2012)
Dihydrosphingosine	15.6 *	-	MHB	ATCC 29213	(Fischer *et al.*, 2012)
Lauric acid (12:0)	1248.1*	-	MHB	ATCC 29213	(Fischer *et al.*, 2012)
	99.8	-	CL	SH1000 & Newman	(Clarke *et al.*, 2007)
	250	-	TB	RN4220	(Parsons *et al.*, 2012)
Sapienic acid (16:1Δ6)	245.7*	-	MHB	ATCC 29213	(Fischer *et al.*, 2012)
	196.5	-	CT-TSB	SH1000 & Newman	(Clarke *et al.*, 2007)
	39.3	-	CT-TSB	SH1000 & Newman	(Clarke *et al.*, 2007)
	31	-	TB	RN4220	(Parsons *et al.*, 2012)
Capric acid (10:0)	58.1	-	CL	SH1000	(Clarke *et al.*, 2007)
	232.2	-	CL	Newman	(Clarke *et al.*, 2007)
	>500	-	TB	RN4220	(Parsons *et al.*, 2012)
Oleic acid (18:1Δ9)	-	8.6	TSB	18Z	(Chamberlain *et al.*, 1991)
	>500	-	TB	RN4220	(Parsons *et al.*, 2012)
Linoleic acid (18:2Δ9,12)	71.3	-	CL	Newman & SH1000	(Clarke *et al.*, 2007)
	250	-	TB	RN4220	(Parsons *et al.*, 2012)
Palmitoleic acid (16:1Δ9)	78.6	-	CL	Newman & SH1000	(Clarke *et al.*, 2007)
	31	-	TB	RN4220	(Parsons *et al.*, 2012)

To avoid MIC and MBC differences caused by media choice, Mueller-Hinton broth is recommended for MIC and MBC studies (EUCAST and ESCMID, 2000). However, many studies do not use Mueller-Hinton broth. It has been indicated that the broth used in broth microdilution MIC determination can have dramatic effects on MIC and MBC values, probably because certain resistance mechanisms require particular components to be effective, as well as pH playing a significant part (Reimer *et al*., 1981; Li *et al*., 2014). Media components have been shown to affect lipid MICs too, for example sapienic acid was shown to be more potent in chemically defined media than rich media (Table 3.1) (Clarke *et al*., 2007). Other studies have already assessed the effects of additional factors on fatty acid activity against bacteria. Addition of factors including metal ions Ba^{2+}, Sr^{2+}, Ca^{2+} and Mg^{2+} offered protection to bacteria against linoleic acid and lauric acid in a dose dependent manner, possibly by acting to precipitate the fatty acids out of solution (Galbraith and Miller, 1973). Conversely, Fe^{2+} and Fe^{3+} have been shown to increase the potency of sapienic acid and linoleic respectively against *S. aureus*; this was suggested to be due to the increase in IsdA (iron-regulated surface determinant protein A) production, and therefore hydrophobicity, under iron-starvation conditions (Galbraith and Miller, 1973; Clarke *et al*., 2007). The pH also has a considerable effect on potency of lipids; both linoleic and lauric acid were found to be more bactericidal at acidic pH (Galbraith and Miller, 1973). Increase in bactericidal activity at lower pH correlated with increased uptake of fatty acid with decreasing pH; this was postulated to be related to the increase in oil/water partition coefficient with decreasing pH (Galbraith and Miller, 1973). Such effects are important considerations when studying MIC and MBC values.

Many studies have used killing assays to determine the effects of lipids on bacteria. These killing assays involve resuspending washed overnight cultures in a buffer containing the lipid of interest, and assessing decreases in viability over time (Cartron *et al*., 2014; Clarke *et al*., 2007; Takigawa *et al*., 2005; Galbraith and Miller, 1973; Bibel *et al*., 1992). The advantage of this method is that it can easily be used to assess both the rate of killing and the effects of reaction conditions including pH or salt concentration (Takigawa *et al*., 2005; Cartron *et al*., 2014; Galbraith and Miller, 1973). The disadvantages of this

method are that it is labour, media and space intensive compared to microdilution based methods.

Both microdilution and killing assay methods usually use solvents such as ethanol or DMSO to dissolve the lipids before addition to broth or buffer. The disadvantage of using solvents is that they have been proposed to act synergistically with lipids (Drake *et al*., 2008), potentially exaggerating lipid antimicrobial activity. This is particularly likely if the mechanism of action involves enhancing permeability of the bacterial membrane, as ethanol and DMSO are both known permeability enhancers (Drake *et al*., 2008). It is possible to avoid using solvents with lipids and still determine MIC or MBC values as has been done recently (Fischer *et al*., 2012). Fischer *et al*. (2012) dissolved the lipid in solvent, dried the lipid under nitrogen in a suitable container then resuspended the lipid by sonication in 0.14 M NaCl solution. The MICs are higher in the Fischer *et al*. (2012) study (Table 3.1); these differences may be a result of ethanol use, but could also be attributed to strain variation, media variation or subtle differences in methodologies. A direct comparison would be necessary to uncover the extent that solvents influence the antimicrobial activity of lipids.

Another method used primarily for screening linoleic acid resistance is an agar plate-based assay. This method uses between 0.5 and 1 mM linoleic acid in agar with up to 1 % ethanol, overnight cultures are serially diluted and between 2.5 ul and 20 ul are plated. After incubation, reduction in growth relative to growth on control plates is calculated (Kenny *et al*., 2009; Kenny *et al*., 2013; King *et al*., 2012). The advantage of this method is that it tests resistance during sessile growth, which is more representative of growth on skin where these bacteria would encounter these antimicrobial lipids. This method could also be used without solvents to enable calculation of MIC, MBC or LD_{50} values. The main disadvantages are that the assay is media and space intensive compared to a microdilution based method.

Greenway and Dyke (1979) assessed growth inhibition by linoleic acid by calculating the difference in mean generation time between treated and control cultures. The advantages of this method are the relatively low concentrations of

lipid used and the potential to give better assessment of how lipids interact with bacteria during growth. The disadvantages of this method are that it is relatively space, media and labour intensive compared to microdilution methods, and is susceptible to variability introduced through media and strain choices.

Bibel *et al.* (2002; 2005) used an *in vivo* assay to test the antimicrobial activity of lipids. This method involved applying the lipid in solvent or solvent control to the forearm of healthy volunteers. Once the solvent had evaporated, 10^5 CFU of *S. aureus* was overlaid onto the lipid or control patch. Following a 3 h incubation, a skin-scrubbing technique was used to collect *S. aureus* from the lipid or control patches for viability counting (Bibel *et al*., 1992; Bibel *et al*., 1995). This method is the only one that assesses the effect of lipids *in vivo*, however there are disadvantages. Firstly, it is not possible to alter any factors to see how lipids and bacteria interact with separate components of the skin, but it is likely that unknown interactions are occurring. Secondly, tests involving human subjects requires ethical consent and willing volunteers, which may not be easily accessible. As such, this method should not be used alone, but is a useful complementary method for comparative assessment of the antimicrobial activity of skin antimicrobials.

3.1.2 Antimicrobial activity of skin lipids on staphylococci

When investigating antimicrobial activity, it is important to know what has been determined previously. This helps to identify what experiments would add to what is already known. Further, it will provide a benchmark for what is expected within results.

Numerous studies have reported the effects of fatty acids and sphingosines upon staphylococci (Arikawa *et al*., 2002; Bibel *et al*., 1992; Bibel *et al*., 1995; Cartron *et al*., 2014; Chamberlain *et al*., 1991; Clarke *et al*., 2007; Fischer *et al*., 2012; Fischer *et al*., 2013; Greenway and Dyke, 1979; Galbraith and Miller, 1973; Kenny *et al*., 2009; Kenny *et al*., 2013; King *et al*., 2012; Parsons *et al*., 2012; Takigawa *et al*., 2005). The interest in these compounds is due to the absence of toxicity on skin and their wide spectrum of antimicrobial activity against skin bacteria despite constant exposure of skin bacteria to these lipids,

indicating a low probability of resistance emerging. This makes these lipids ideal candidates for use as antibacterial skin treatments. This interest has increased following reports that decreased levels of sapienic acid and sphingosines on skin, as described in patients with atopic dermatitis, directly correlates with increased levels of *S. aureus* on the skin (Arikawa *et al*., 2002; Takigawa *et al*., 2005). This relationship indicates that these components could be used to directly control the levels of *S. aureus* on skin.

Previous studies of antimicrobial skin lipids primarily focus on *S. aureus*, though the effects of fatty acids on single strains of *S. saprophyticus* and *S. epidermidis* have been investigated (King *et al*., 2012; Takigawa *et al*., 2005). Some studies investigating antimicrobial lipids have determined the *S. aureus* MIC for fatty acids and sphingosines, their findings are summarised in Table 3.1.

Most reports indicate that fatty acids kill or inhibit growth of *S. aureus* within 1 h at MIC levels (Desbois and Lawlor, 2013; Parsons *et al*., 2012; Cartron *et al*., 2014), however, a slower kill rate of 24 h has also been described at ten times MIC (Fischer *et al*., 2012). Sphingosines have been reported to kill within 1 h at ten times MIC (Fischer *et al*., 2012).

To conclude, there have been multiple studies investigating the activity of skin lipids against *S. aureus* using a limited numbers of strains. Results between investigators are not easily comparable and are sometimes conflicting due to differences in methodologies. The method that was least labour and media intensive and gave quantitative results was the broth microdilution MIC assay.

3.2 Aims

Skin colonisation by *S. aureus* only occurs on skin with low levels of skin lipids, however other staphylococci, such as *S. epidermidis* are ubiquitous skin colonisers regardless of skin lipid levels. As discussed, many studies have examined the antimicrobial effects of skin lipids on *S. aureus*, showing that *S. aureus* is susceptible to various antimicrobial skin lipids. However, little is known about the effects of antimicrobial skin lipids on *S. epidermidis*. Here, it was hypothesised that *S. epidermidis* has a greater resistance to skin antimicrobial lipids than *S. aureus*.

The main aim of this Chapter was to determine if there was a significant difference in *S. aureus* and *S. epidermidis* resistance to antimicrobial skin lipids. A previous study compared the resistance of *S. aureus* and *S. epidermidis* to sapienic acid in a non-quantitative assay, and found that *S. epidermidis* was more resistant, though this was assessed using only one strain of each which may not be representative (Takigawa *et al*., 2005). This Chapter reports the use of a quantitative broth microdilution MIC assay to compare resistance levels to sapienic acid, linoleic acid and D-sphingosine. Multiple strains from different sources were be tested to better represent the resistance levels across the species. Further, tests based on growth rate assays and agar plate based assays were used to assess resistance of *S. aureus* and *S. epidermidis* to these antimicrobials as described previously (Greenway and Dyke, 1979; Kenny *et al*., 2009). The final aim was to determine resistance levels and growth conditions that can be used in subsequent experiments that further compare *S. aureus* and *S. epidermidis* responses to antimicrobial lipids.

3.3 Results

3.3.1 Comparison of minimum inhibitory concentrations (MIC) of skin lipid matrix components of *S. aureus* and *S. epidermidis*.

Whist it has previously been suggested that sapienic acid may be significant in prohibiting *S. aureus* growth on the skin without having an effect on *S. epidermidis* (Takigawa *et al*., 2005), it has not been comprehensively tested. It has also been inferred that sphingosines play a role in suppressing *S. aureus* growth on skin (Arikawa *et al*., 2002). The minimum concentration required to inhibit growth (MICs) or kill 99.9% of the inoculum (MBC) of *S. aureus* and *S. epidermidis* strains by two important skin fatty acids, sapienic acid and linoleic acid, and a skin sphingosine, D-sphingosine were determined. The *S. aureus* and *S. epidermidis* MICs for these lipids were then compared to assess if either species had greater resistance to any of the lipids tested.

The assay to determine MIC and MBCs used a broth microdilution method as described in section 2.2. Briefly, stock solutions of antimicrobial lipids in ethanol were serially two-fold diluted in Todd Hewitt broth in 96 well microtitre plates before inoculating with overnight cultures of bacteria at a final OD_{600} 0.1. Assay plates were incubated at 37 °C for 24 h, then bacterial viability counts were measured using the Miles and Misra technique.

The MIC values of the fatty acids reveal a clear trend, with *S. epidermidis* strains having higher MICs than *S. aureus* for linoleic acid and sapienic acid. There is no difference between *S. aureus* and *S. epidermidis* MICs for D-sphingosine (Table 3.2). The MBCs results also follow this trend, though this is less apparent for sapienic acid.

There is a good correlation between linoleic acid and sapienic acid MIC values, which is not evident between D-sphingosine and either linoleic acid or sapienic acid MIC values.

Table 3.2 MICs and MBCs for sapienic acid, linoleic acid and D-sphingosine for strains of *S. aureus* and *S. epidermidis.*

MICs and MBCs were determined using the broth microdilution method. MICs are the lowest concentration found to inhibit growth, MBCs were the lowest concentration that reduced CFU ml^{-1} by ≥ 3 log.

Species	Strain	Linoleic acid (mM)		Sapienic acid (μM)		D-sphingosine (μM)	
		MIC	MBC	MIC	MBC	MIC	MBC
S. epidermidis	Tu3298	37	37	144	171	38	38
	NCTC 1457	37	43	197	394	8	8
	Rp62a	25	25	98	144	38	38
	A19	25	50	144	171	38	38
	B19	5	37	144	197	8	8
	O16	5	15	98	144	8	8
S. aureus	Newman	5	11	59	98	8	38
	SH1000	5	37	144	>394	8	8
	MRSA 252	0.6	5	98	197	38	38
	MSSA 476	0.6	0.6	72	197	38	38
	BL137	5	5	72	72	38	38
	BL169	5	5	72	98	38	38

3.3.2 Effects of sapienic acid on growth of *S. epidermidis* and *S. aureus*

MIC values indicated that *S. epidermidis* is more resistant to fatty acids than *S. aureus*. Microdilution MIC values are the standard format to test bacterial resistance levels to antimicrobials as they give quantitative data, however, these results did not inform as to the effects of fatty acids on growth. To assess this, representative lab strains of *S. epidermidis* and *S .aureus* were grown with sub-MIC levels of sapienic acid. Strains Newman, NCTC 1457 and Tü3298 were selected based upon MIC results. Sapienic acid was selected over linoleic acid as it did not affect turbidity of the media at low concentrations, allowing assessment of growth using optical density. This work was also designed to determine optimal conditions for RNA-Seq experiments; sapienic acid was favoured for RNA-Seq work as it is the most abundant fatty acid in sebum, is

directly linked to *S. aureus* inhibition on skin and had not been used in transcriptomics analysis at the time of selection.

As these experiments would be used to decide the optimal conditions for RNA-Seq experiments, a similar experimental design was used to one previously used for fatty acid transcriptional response analysis (Kenny *et al*., 2009). This study used two growth conditions. The first method was to grow strains to mid-log growth then to challenge them with a concentration of fatty acid that affected growth, but would not cause cell lysis (challenge conditions). In the second method, bacteria were grown with sapienic acid from 0 h (exposure conditions). Here we tested a range of concentrations of sapienic acid, based upon fractions of sapienic acid MIC values, to produce growth reduction.

When challenged with 11.25 μM (*S. aureus* Newman and *S. epidermidis* NCTC 1457) or 15 μM (*S. epidermidis* Tü3298) sapienic acid during mid-exponential growth (Fig. 3.1, A, C & E), bacteria showed a growth inhibition for approximately 1 h followed by growth at a rate reduced by 27 % in *S. epidermidis* Tü3298, 37 % in *S. epidermidis* NCTC 1457 and 64 % for *S. aureus* Newman. At 15 μM, NCTC 1457 (Fig. 3.1, C) decreases in optical density for a 2 h period after challenge, indicating cell lysis, followed by growth rate dramatically reduced by 72 %.

When exposed to sapienic acid from the time of inoculation (Fig. 3.1, b, d & f), growth effects varied depending on the sapienic acid concentration. At concentrations below 3.75 μM for *S. epidermidis* strains and 1.5 μM for Newman, there was no significant difference in growth. For *S. aureus* Newman (Fig. 3.1 b) at 7.5 μM sapienic acid, growth was inhibited after 1 h, followed by growth at a rate reduced to 38 % from 3 h. NCTC 1457 and Tü3298 (Fig. 3.1 d & f) at 15 μM sapienic acid, showed an increased growth rate for the first hour (by 33 % and 21 % respectively), followed by growth inhibition. *S. epidermidis* strains NCTC 1457 and Tü3298 at 37.5 μM showed no growth for the first 2 h followed by decreased growth rate, though this was higher for Tü3298 than NCTC 1457.

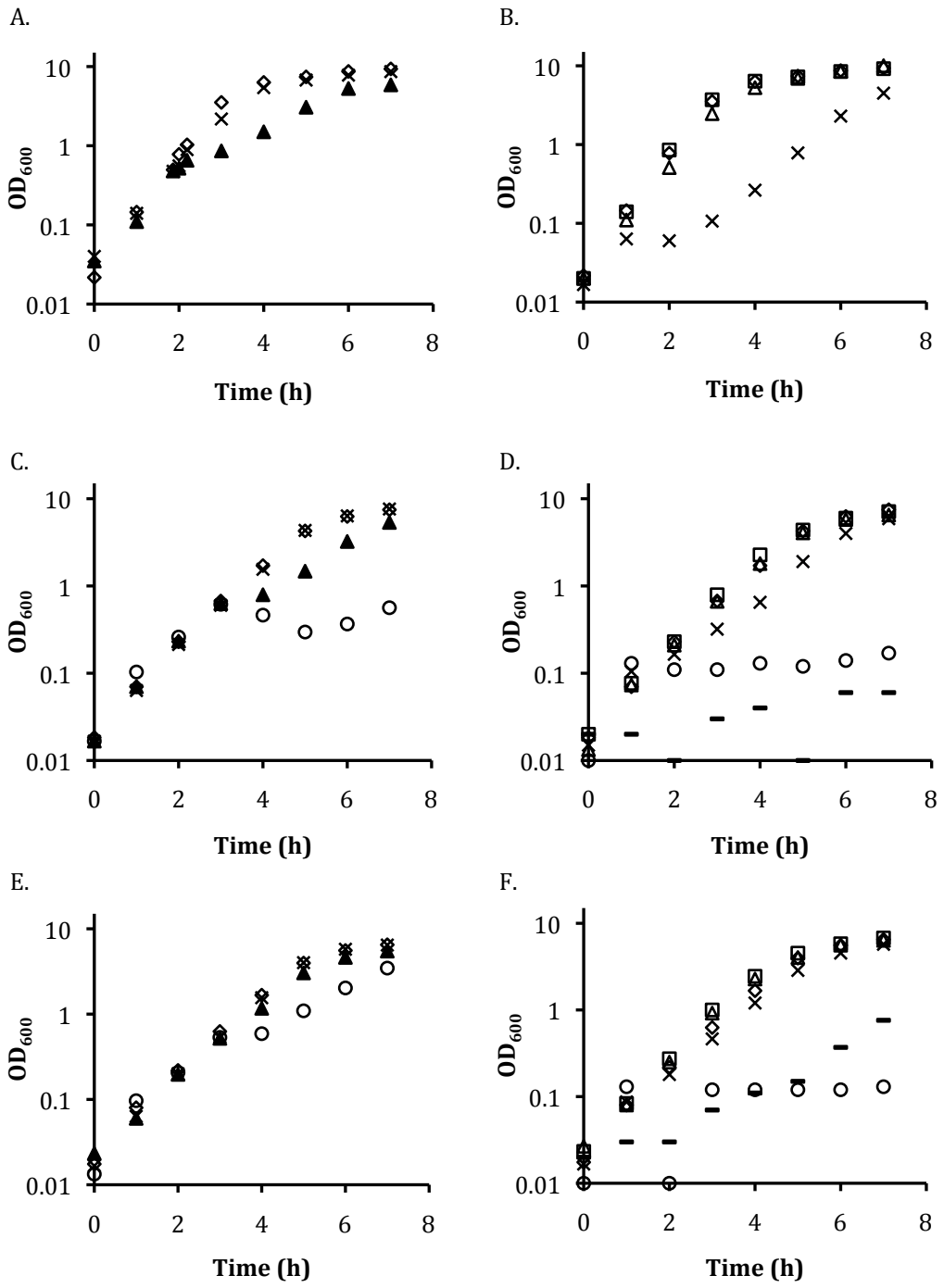


Figure 3.1 Growth of *S. aureus* and *S. epidermidis* strains in sapienic acid challenge or exposure conditions.

S. aureus Newman (A & B), *S. epidermidis* NCTC 1457 (C & D) and *S. epidermidis* Tü3298 (E & F) grown with sapienic acid (exposure conditions, right) or grown to OD_{600} 0.5 before addition of sapienic acid (challenge conditions, left). Concentrations of sapienic acid used were 0 μM (diamond), 1.5 μM (square), 3.75 μM (open triangle), 7.5 μM (cross), 11.25 μM (closed triangle), 15 μM (circle) and 37.5 μM (dash).

The ideal concentration of sapienic acid for RNA-Seq experiments would be one that is certainly affecting the cell growth to ensure the cells are responding to the sapienic acid. However, it is also desirable that the effect is subtle to reduce noise in transcriptional responses. The changes in growth rates following challenge with sapienic acid during mid-exponential growth indicate appropriate concentrations for RNA-Seq experiments would be 11.25 μM for Newman and NCTC 1457, and 15 μM for Tü3298. Under exposure conditions, 11.25 μM would be an appropriate concentration for NCTC 1457 and Tü3298, whilst 3.75 μM would be an appropriate concentration for Newman.

3.3.3 Comparison of survival on linoleic acid agar plates for *S. epidermidis* and *S. aureus*

Whilst MIC and growth assays provided good indications that *S. epidermidis* is more resistant to antimicrobial fatty acids than *S. aureus*, when looking at colonisation of skin, it is important to know if any enhanced resistance extends to sessile growth, as occurs on the skin. To assess if this trend also occurs during sessile growth, an agar plate based linoleic acid survival assay was used to test *S. aureus* and *S. epidermidis*. After overnight growth, cultures were serially diluted and inoculated onto agar plates containing milli-molar concentrations of linoleic acid. Linoleic acid was used since it is more readily available and affordable whilst sapienic acid is prohibitively expensive at the quantities required. There was good correlation between sapienic acid and linoleic acid MICs, so it was expected that results would be similar for both fatty acids.

S. epidermidis strains consistently showed at least one log fold greater survival than *S. aureus* at all concentrations of linoleic acid tested (Fig. 3.2, A, C, E, G and I). The exception to this was strain SH1000, which showed survival levels similar to the most resistant *S. epidermidis* strain O16, and *S. aureus* strain MRSA 252, which showed levels of survival similar to the least resistant *S. epidermidis* strains at ≤ 2.5 mM. The remainder of the *S. aureus* strains had more than 99.9 % reduction in survival at all linoleic acid concentrations.

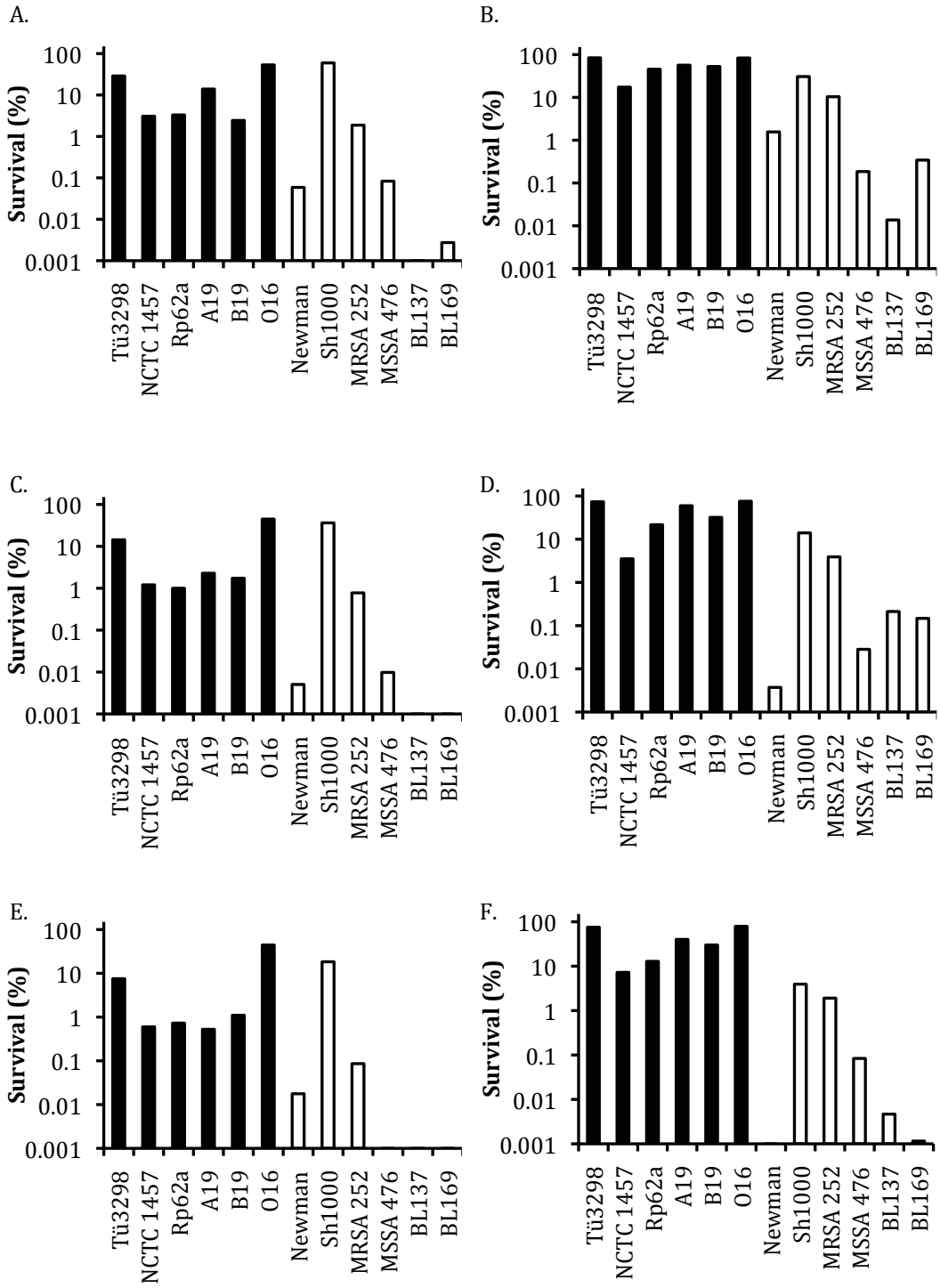


Figure 3.2 Survival of *S. epidermidis* and *S. aureus* on linoleic acid agar.

Strains of *S. epidermidis* (black bars) and *S. aureus* (white bars) were grown overnight with 0.01 mM linoleic acid (right) or without (left), then spotted onto agar containing 1 mM (A & B), 2.5 mM (C & D), 5 mM (E & F), 7.5 mM (G & H) or 10 mM (I & J) and incubated at 37 °C for 24 h. Survival was calculated as growth on linoleic acid containing plates as a percentage of growth on plates without linoleic acid.

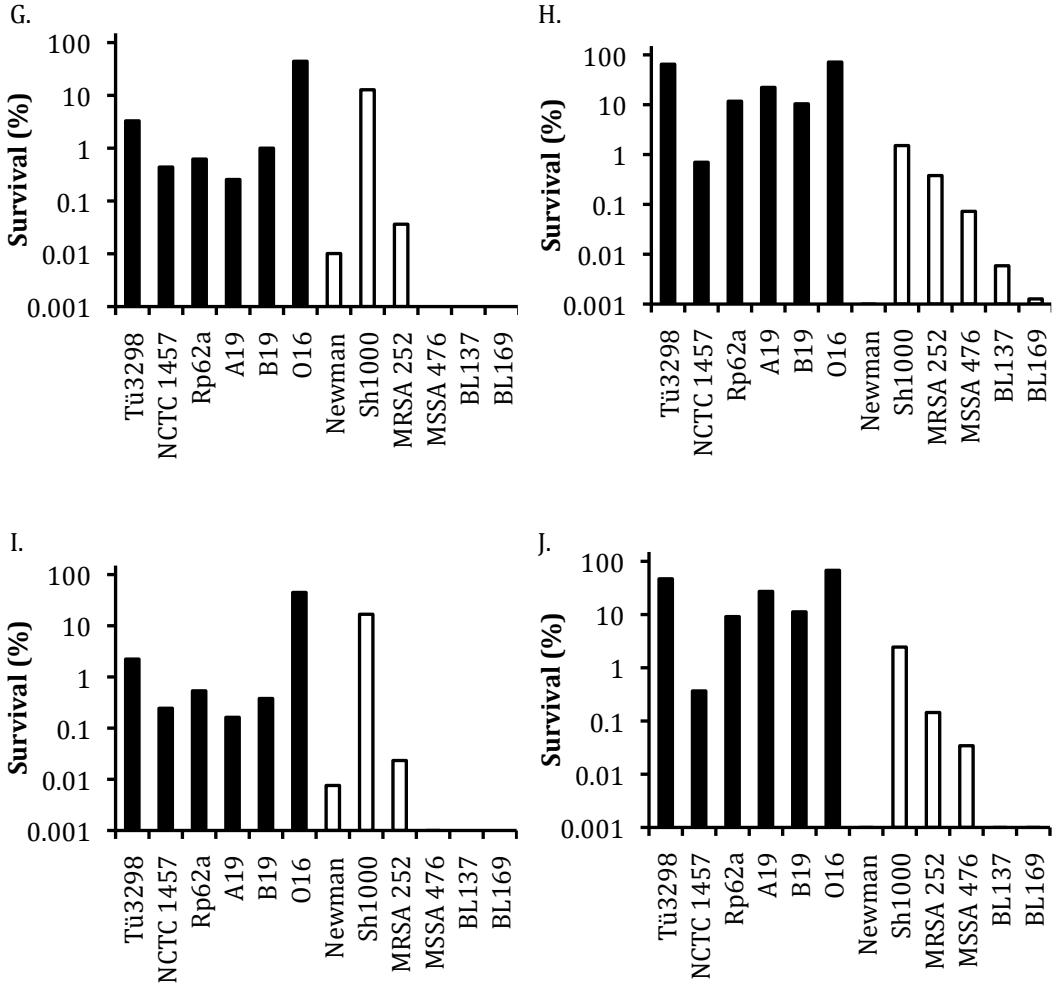


Figure 3.2 Survival of *S. epidermidis* and *S. aureus* on linoleic acid agar.

Strains of *S. epidermidis* (black bars) and *S. aureus* (white bars) were grown overnight with 0.01 mM linoleic acid (right) or without (left), then spotted onto agar containing 1 mM (A & B), 2.5 mM (C & D), 5 mM (E & F), 7.5 mM (G & H) or 10 mM (I & J) and incubated at 37 °C for 24 h. Survival was calculated as growth on linoleic acid containing plates as a percentage of growth on plates without linoleic acid.

From a transmission and colonisation point of view it is important to determine if *S. epidermidis* is better adapted to survive exposure and/or has a more effective response to antimicrobial fatty acids than *S. aureus*. To test this, overnight cultures were grown with 0.01 mM linoleic acid and used in the linoleic agar plate based assay. Cultures grown with the sub-inhibitory concentration of linoleic acid are referred to as pre-treated, whilst those grown without are referred to as untreated.

Survival of pretreated *S. epidermidis* strains and most *S. aureus* strains increases compared to untreated strains at the same concentration from an average of 10.7 % to 32.7 % survival for *S. epidermidis* strains and 2.4 % to 4.9 % survival for *S. aureus* strains. This increase in survival indicates a resistance mechanism is activated by incubating these staphylococci with the subinhibitory linoleic acid. SH1000 and Newman are the only strains inconsistent with this trend, showing a decrease in survival when pretreated at concentrations over 1 mM for Newman, and at all concentrations tested for SH1000.

3.4 Discussion

This study aimed to assess the antimicrobial effects of skin lipids upon staphylococcal species *S. epidermidis* and *S. aureus*. It was observed that D-sphingosine was the compound with the highest antimicrobial activity, and linoleic acid was the compound with the lowest antimicrobial activity for both *S. aureus* and *S. epidermidis*. The higher antimicrobial activity of D-sphingosine is consistent with previous findings that sphingosines are more active against *S. aureus* than fatty acids including sapienic acid (Fischer *et al*, 2012). It is not certain what causes this difference in potency, though it seems likely that it is due to the presence of the amine group and the extra hydroxyl group on sphingosines. These could potentially allow greater incorporation into the membrane, possibly increasing disorder and interference with membrane proteins more than the carboxyl group of fatty acids, as the the amine group and extra hydroxyl group should make sphingosines more hydrophilic than fatty acids.

The observed higher antimicrobial activity of sapienic acid compared to linoleic acid activity is also to be expected. The shorter of the medium or long chain fatty acids have greater antibacterial activity (Drake *et al*., 2008). Fatty acids with a kink, created by unsaturated bonds in the chain, are likely to have more antibacterial activity than straight fatty acids with the same chain length. This increased antimicrobial activity is likely due to their greater ability to interfere with phospholipid packing and hence membrane fluidity and order (Desbois and Smith, 2010). This correlates well with the observed difference between linoleic acid and sapienic acid; linoleic acid is 18 carbons and predicted to have only a slight kink in its chain, whereas sapienic acid is 16 carbons and is predicted to have a large kink (Fig. 1.2).

There was no difference between D-sphingosine MICs of *S. aureus* and *S. epidermidis*. This result would suggest D-sphingosine does not contribute to the dominance of *S. epidermidis* over *S. aureus* on the skin as has been previously inferred (Arikawa *et al*., 2002). However, it is possible that a major resistance mechanism to D-sphingosines is dependent on a co-factor present on the skin and absent in the MIC assay. Indeed, Bibel *et al*, (1992) showed that 200 μg per

cm^2 of sphinganine, another sphingosine found on skin, was required to reduce *S. aureus* on skin by > 3 log CFU, this is far above indications of MBC in broth. It would be useful to repeat MIC assays using a chemically defined medium with supplements that represent compounds typically found on the skin to give a better representation of how antimicrobial lipids affect bacteria *in vivo*. Another interesting test would be to determine if there if any synergistic or antagonistic activity between skin antimicrobials as has been observed previously for antimicrobial proteins found on skin (Chen *et al*., 2005).

There is no correlation between MIC values for D-sphingosine and either of the fatty acids, indicating resistance mechanisms to D-sphingosine are different to fatty acid resistance mechanisms. This supports growing evidence that, despite previous suggestions, the mechanism of action of D-sphingosines and fatty acids are quite distinct (see section 1.6 and 1.7 for more detail) (Cartron *et al*., 2014; Fischer *et al*., 2013; Parsons *et al*., 2012).

The antimicrobial fatty acid MICs of *S. epidermidis* were significantly higher than those of *S. aureus*. Some proposed fatty acid resistance factors present in *S. aureus*, such as carotenoids (Chamberlain *et al*., 1991), are absent in *S. epidermidis*; however these results indicate that *S. epidermidis* must possess mechanisms of resistance to fatty acids that *S. aureus* does not, or uses similar components to greater effect.

There was correlation between the linoleic acid and sapienic acid MIC values, which corroborates assumptions that the mechanism of action is universal between all fatty acids (Parsons *et al*., 2012; Desbois and Smith 2010). If this is the case, resistance mechanisms to fatty acids are also likely to be similar. It is also possible that this correlation simply reflects that both of the fatty acids are often seen together on the skin and that as such genes encoding resistance mechanisms are likely to be linked.

It is worth noting that whilst the fatty acid MICs for *S. epidermidis* are higher than those of *S. aureus* they are still low in comparison to another Gram-positive genus dominant on human skin- Corynebacteria. *Corynebacterium jeikeium* and *Corynebacterium striatum* sapienic acid MIC was >1.96 mM, which was much higher than that for *S. epidermidis* or *S. aureus* (Fischer *et al*., 2012). This

difference could reflect the different niches these genera are reported to inhabit on skin. Areas of high staphylococcal colonisation are inversely correlated to areas of high fatty acid levels on healthy skin (Lampe *et al*., 1983; Costello *et al*., 2009; Coates *et al*., 2014). This may mean that fatty acids control levels of all staphylococcal species to some degree either by direct growth inhibition, or by allowing the staphylococci to be outcompeted by lipophillic bacteria such as species of corynebacteria.

In the agar plate based assay, *S. epidermidis* strains had higher survival to linoleic acid than the majority of *S. aureus* strains. This trend was more pronounced when strains had been pre-exposed to linoleic acid, as all *S. epidermidis* strains showed enhanced survival when pre-exposed to linoleic acid, whilst the *S. aureus* strain with the highest resistance to linoleic acid, SH1000, showed decreased survival when pre-exposed. This decrease in survival was not typical, as all the remaining *S. aureus* strains except Newman did show enhanced survival when pre-exposed to linoleic acid. The mechanism for this reduction in growth after pre-exposure, or its significance, remains unclear.

The ability of *S. epidermidis* strains and the majority of *S. aureus* strains grown with a sub-inhibitory concentration of linoleic acid to increase their survival to linoleic acid indicates they can adapt to very low concentrations of linoleic acid. This could be through population variation; a subpopulation of the bacteria could be hypersensitive to linoleic acid and are inhibited from growing overnight, leaving the population dominated by bacteria with a higher level of resistance to linoleic acid. More likely, this occurs through alterations in gene expression. Either way, the low survival levels of *S. aureus* strains compared to *S. epidermidis* indicates *S. epidermidis* would have the competitive advantage on healthy skin. The decreases in survival levels in pre-exposed *S. aureus* SH1000 and Newman could indicate that they also adapt to linoleic acid, but that the mechanisms activated decrease their likelihood of survival.

Susceptibility to linoleic acid for *S. epidermidis* O16 was much lower in broth microdilution assays than in agar plate based assays. Other *S. epidermidis* strains showed survival levels in keeping with their MIC when they were not

pre-exposed to linoleic acid. O16 was a strain that had recently been isolated from human skin (Kelly, 2013), as a result, its survival mechanisms could rely quite strongly on a solid surface. Another explanation could be that this lower susceptibility in liquid culture could be due to a synergistic effect caused by the combination of ethanol and linoleic acid which has been observed previously (Drake *et al*., 2008).

Sapienic acid challenge caused growth inhibition or lysis at the higher concentrations tested, followed by reduced growth rate. This was similar to findings by Greenway and Dyke (1979) using linoleic acid. The growth inhibition at lower concentrations and lysis at higher concentrations is consistent with findings that, whilst at higher concentrations sapienic acid can cause lysis, at lower concentrations it affects cells through its activity as a protonophore (see section 1.6 for more detail) (Cartron *et al*., 2014).

Sapienic acid exposure caused inhibition of growth followed by recovery in *S. aureus* Newman, and increased growth rate followed by growth inhibition or reduced growth rate in *S. epidermidis* strains. It is interesting to note that the inhibitory effect of sapienic acid was rapid when cells were challenged during mid logarithmic growth, but only occurred after 1 h when exposed to sapienic acid from 0 h.

Growth experiments show striking reductions in growth rate at sub-MIC levels; this is likely a reflection of the differences in inoculum used for growth and MIC assays. The inoculum for growth assays was half the optical density of that for MIC assays. A 24 h time point would be required to determine if this reduction in inoculum would reduce MIC.

It was surprising that there was not a greater difference between the levels of sapienic acid required to inhibit growth of *S. epidermidis* and *S. aureus* in growth experiments, given the differences observed in fatty acid MICs and survival on linoleic acid plates. Despite this, Newman was still more susceptible to sapienic acid than the *S. epidermidis* strains.

As well as to define the effects sapienic acid had on growth, the growth experiments were designed to find growth conditions to use in future RNA-Seq experiments. Concentrations of sapienic acid selected for RNA-Seq work were

based on the lowest concentration that showed a visible deflection in growth. As previous studies using other antimicrobials had indicated that challenge conditions showed a more dramatic transcriptional response than exposure conditions (Kenny *et al*., 2009), challenge conditions were selected for RNA-Seq experiments. Under challenge conditions, the concentration of sapienic acid to be used for Newman and NCTC 1147 was 11.25 μM, and for Tü3298 was 15 μM. Due to its superior tolerance to sapienic acid under challenge conditions, Tü3298 was selected for the RNA-Seq work along with Newman. Though the concentrations of sapienic acid are different, it is expected that they will show equivalent transcriptional responses based on the growth effects observed. If the same concentration had been chosen but with different effects on growth, it is likely this would be reflected by the transcriptional responses.

The main aim of this study was to test the hypothesis that *S. aureus* is more susceptible to antimicrobial skin lipids than *S. epidermidis*. Results indicate that this hypothesis is true for the two fatty acids tested, by all three methods used, but not for the tested sphingosine.

Chapter 4 The transcriptional response to sapienic acid of *S. aureus* and *S. epidermidis*.

4.1 Introduction

4.1.1 Transcriptomics technologies

Transcriptomics is the study of all of the transcripts found within a collection of cells. In its simplest form, transcriptomics is used to determine what genes are being transcribed in different conditions. This can provide useful insights into how the cells react to a given stimulus.

Until recently, transcriptomic analysis was predominantly undertaken using microarray based methods. Microarray technologies use chips, which have an ordered grid structure with clusters of small fragments of DNA; each cluster of fragments on the grid represent transcripts from the organism of interest. Microarray chips are incubated with fluorescently labelled cDNA, synthesised from RNA, which hybridize to any corresponding DNA fragments on the microarray chip. Following hybridization, the fluorescence intensity for each arrayed fragment on the microarray chip gives a relative abundance of each transcript in the sample.

Microarray experiments are high throughput and relatively inexpensive (Dong and Chen, 2013), however, there are several concerns with microarray experiments. Firstly, for a transcript to be represented on the microarray, it must be already known, therefore this method is only suited to organisms with known and, ideally, well annotated genomes (Wang *et al*., 2009). Secondly, because transcripts may cross-hybridise to other DNA fragments on the microarray chip, results can be unreliable (Dong and Chen, 2013). Cross-hybridisation creates a lot of background noise, therefore low-level transcripts are often lost within this noise (Wang *et al*., 2009). Saturation of signals and hybridisation points means that the exact abundance of high-level transcripts cannot be determined. Together, background noise and saturation issues mean that microarrays have a very limited dynamic range (Wang *et al*., 2009).

Advances in sequencing that has led to high-throughput sequencing technologies, has led to advances in transcriptomics. In 2008, a number of studies used RNA-Seq to provide transcriptomics data in place of microarrays (Marioni *et al*., 2008; Sultan *et al*., 2008; Mortazavi *et al*., 2008; Oliver *et al*., 2009). In RNA-Seq, cDNA is sequenced using high-throughput sequencers; the quantity of each transcript should be proportional to the number of sequence reads for that transcript. There are several advantages of this method. Firstly, RNA-Seq does not require the genome to be sequenced or annotated (Wang *et al*., 2009), though this will enable easier data analysis. Secondly, the low background noise and inability to be saturated mean RNA-Seq has a very large dynamic range; comparative data indicates that RNA-Seq can detect 25 % more transcripts than microarrays (Wang *et al*., 2009; Dong and Chen, 2013; Sultan *et al*., 2008). RNA-Seq can also be used for several applications beyond quantification of transcripts, including determining transcriptional start sites, identifying splice variants in eukaryotes and investigation of non-coding RNA (Dong and Chen, 2013; Denoeud *et al*., 2008; Mortazavi *et al*., 2008). RNA-Seq has also been shown to be highly reproducible and accurate (Wang *et al*., 2009).

Despite the benefits that RNA-Seq provides over microarray, there are still some unresolved issues with RNA-Seq. Firstly, amplification steps used in cDNA production and library construction may create bias or PCR artefacts within results (Wang *et al*., 2009; Finotello *et al*., 2014). Secondly, fragmentation techniques used in library construction can create sequencing bias (Wang *et al*., 2009; Finotello *et al*., 2014). It is also believed there is a bias during sequencing towards genes with higher GC-contents (Finotello *et al*., 2014). Bioinformatics analysis tools often aim to correct these bias', however bioinformatics tools used to identify differentially expressed transcripts between samples can introduce their own bias. Different bioinformatics analysis pipelines run on the same datasets have been shown to dramatically alter results (Finotello *et al*., 2014; Robinson and Oshlack, 2010; Seyednasrollah *et al*., 2013).

4.1.2 Transcriptomics in staphylococci

Transcriptomics data from staphylococci has primarily focussed on differential gene expression between normal and treated cells (Campbell *et al*., 2012; Price-Whelan *et al*., 2013; Cuaron *et al*., 2013; Muthaiyan *et al*., 2012; Pietiainen *et al*., 2009). However, some reports have investigated differentially expressed (DE) genes between wild type and gene mutant cells (Truong-Bolduc *et al*., 2011), or between sensitive and resistant isolates (Song *et al*., 2013b) whilst others have focussed on non-coding small RNAs (Abu-Qatouseh *et al*., 2010).

The transcriptional response of bacteria to an antimicrobial can be used to indicate the antimicrobial's mode of action (Muthaiyan *et al*., 2008). An example of this approach comes from the many reports that have noted the activation of a cell-wall-stress response upon treatment with antimicrobials, such as oxacillin and vancomycin, that are known or predicted to inhibit cell wall biosynthesis (Campbell *et al*., 2012; Muthaiyan *et al*., 2008; Sass *et al*., 2008; Utaida, 2003). This response includes upregulation of the *vraRS*, that encodes a two-component regulatory system, and its downstream regulon, including cell wall metabolism, proteases, protein chaperones and members of the heat shock regulon (Campbell *et al*., 2012; Muthaiyan *et al*., 2008; Utaida, 2003; Sass *et al*., 2008). Upregulation of the heat shock regulon also leads to downregulation of cell division and autolysins in this response, which is believed to further preserve the cell wall (Campbell *et al*., 2012; Utaida, 2003). Activation of the cell-wall-stress response has been used to indicate that an antimicrobial affects cell wall production (Muthaiyan *et al*., 2008).

4.2 Aims

Sapienic acid is a key antimicrobial compound of human skin that plays a role in the observed inhibition of skin colonisation that affects *S. aureus* more than *S. epidermidis*. In Chapter three it was determined that *S. epidermidis* strains typically display higher levels of innate resistance to AFAs linoleic and sapienic acid than *S. aureus* strains. In this Chapter, the underlying genetic basis for this difference was investigated by determining the transcriptional response of *S. aureus* and *S. epidermidis* to a challenge with sub-MIC levels of sapienic acid.

The transcriptional responses were then compared with the aim of finding differences in responses or possible resistance determinants that could account for differences in survival. The transcriptional responses were then compared to transciptomics datasets from the literature to assess if there were similarities that could be suggestive of a mode of action for sapienic acid.

4.3 Results

To determine the transcriptional response of *S. aureus* and *S. epidermidis* to sapienic acid, RNA-Seq was performed on sapienic acid challenged and control samples of *S. aureus* Newman and *S. epidermidis* Tü3298. For this, sub-MIC levels of sapienic acid were added to strains during the mid-logarithmic phase of growth. The sub-MIC concentration for *S. aureus* and *S. epidermidis* was 11.25 μM and 15 μM respectively, as established in Chapter three. RNA was then purified from cells whose growth and transcriptional activity had been halted 20 minutes after addition of sapienic acid.

4.3.1 RNA quality control

Prior to sequencing, RNA samples were tested to ensure they were at levels of sufficient quality and concentration (Table 4.1). All RNA samples used for sequencing had low protein, salt and solvent contamination, as indicated by nanodrop 260/280 & 260/230 ratios over 1.8. All samples had sufficient concentration (≥ 30 ng ul^{-1}) and yield of RNA (≥ 3 μg) as determined by Qubit analysis. Samples were determined as sufficiently un-degraded by RNA integrity (RIN) scores > 7.0 and low evidence of degradation on bioanalyser traces (Fig. 4.1) as determined by the Agilent bioanalyser.

Table 4.1 QC values of RNA samples for sequencing.

To assess if samples were of sufficient quality for sequencing, quality control assessment was undertaken. Sample purity was assessed using the Agilent NanoDrop, concentration was measured using the Qubit and sample integrity was measured using the Agilent bioanalyser. Abbreviations: RIN = RNA integrity number, C = control condition, S = sapienic acid challenge condition.

Species	Sample	Nano-Drop 260/280	Nano-Drop 260/230	Concentration ng μl^{-1}	Sample volume (µl)	RIN
S. epidermidis	C1	2.1	2.5	1200	7	8.6
	C2	2.1	2.5	1036	11	8.1
	C3	2.1	2.4	1200	11	8.1
	S1	2.1	2.0	1200	7	9.4
	S2	2.1	2.1	554	11	7.5
	S3	2.1	2.3	945	11	9.4
S. aureus	C1	2.2	1.9	1100	11	9.2
	C2	2.1	2.4	1000	11	9.5
	C3	2.2	2.4	1200	11	9.5
	S1	2.2	2.4	580	13	7.8
	S2	2.2	2.0	450	13	9.0
	S3	2.0	1.8	455	11	8.6

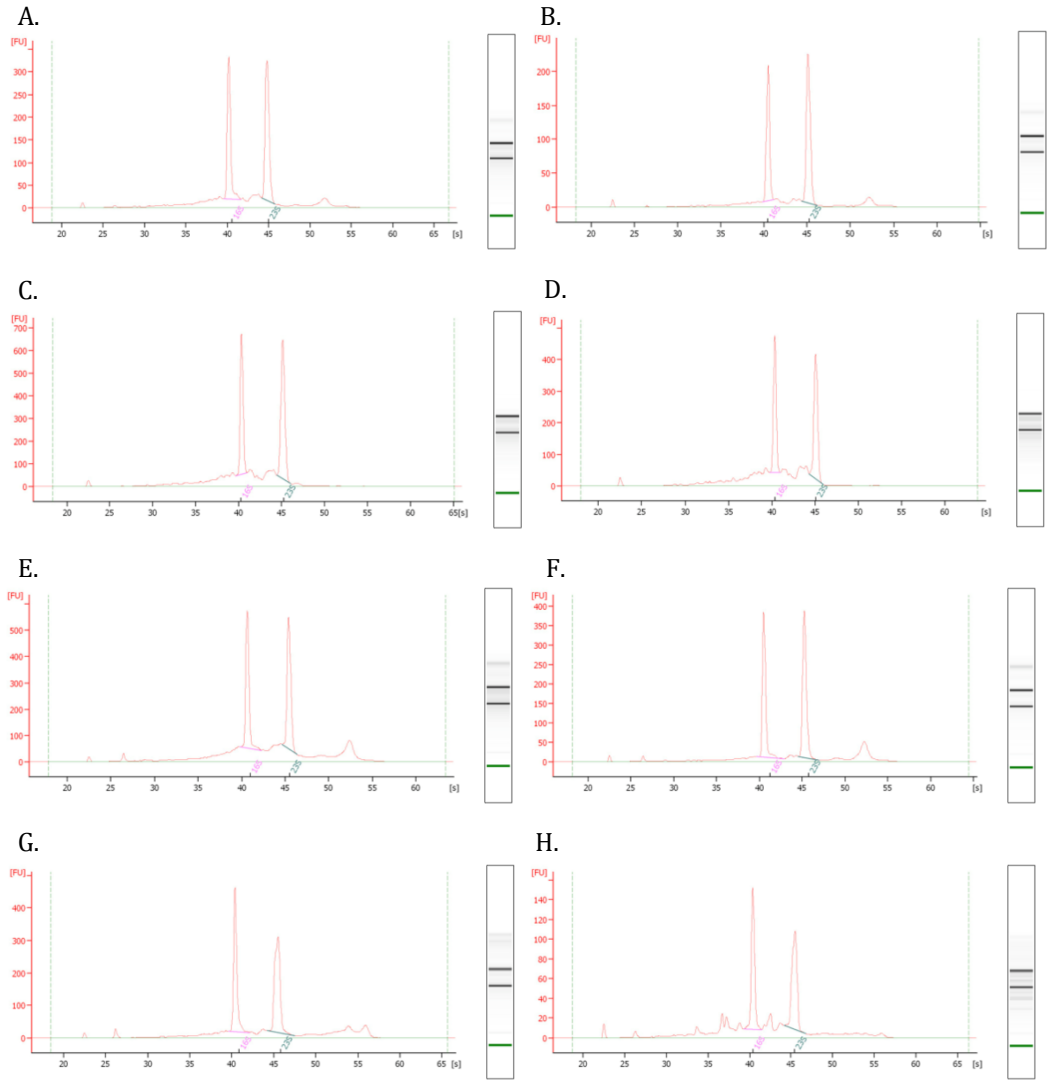


Figure 4.1 Bioanalyzer traces of RNA samples for sequencing.

The Agilent bioanalyser visual output for the determined RNA integrity showing the levels of particular sizes of RNA. Large peaks are expected (from right to left) for 23S and 16S, with a small peak for the ladder and small RNAs, other peaks and/or smaller 23S or 16S peaks are indicators of degradation. Samples are: *S. epidermidis* Tü3298 control 1, 2 and 3 (A, C & E respectively) and sapienic acid treated 1, 2 and 3 (B, D & F respectively) and *S. aureus* Newman control 1, 2 and 3 (G, I & K respectively) and sapeinic acid treated 1 2 and 3 (H, J & L respectively).

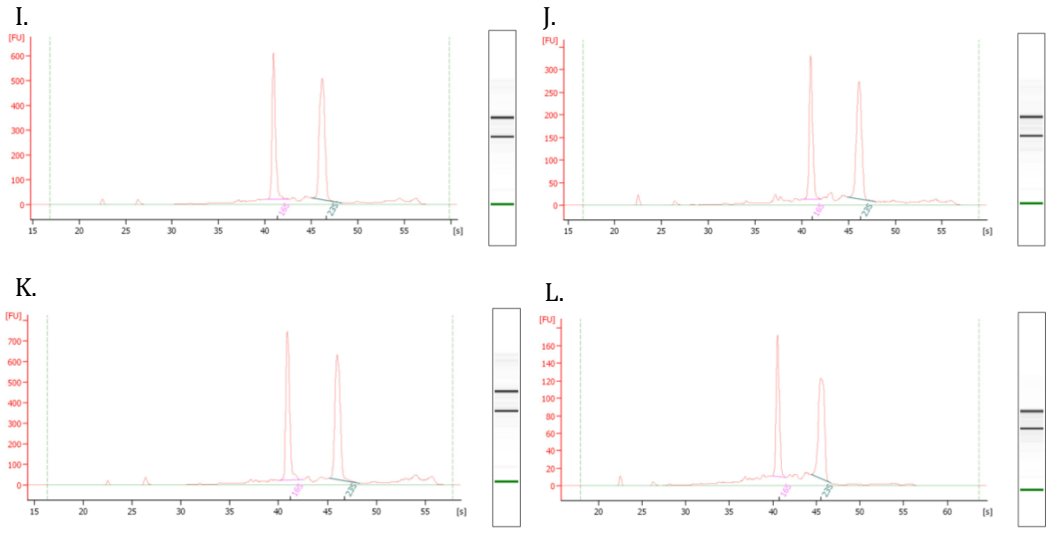


Figure 4.1 Bioanalyzer traces of RNA samples for sequencing.

The Agilent bioanalyser visual output for the determined RNA integrity showing the levels of particular sizes of RNA. Large peaks are expected (from right to left) for 23S and 16S, with a small peak for the ladder and small RNAs, other peaks and/or smaller 23S or 16S peaks are indicators of degradation. Samples are: *S. epidermidis* Tü3298 control 1, 2 and 3 (A, C & E respectively) and sapienic acid treated 1, 2 and 3 (B, D & F respectively) and *S. aureus* Newman control 1, 2 and 3 (G, I & K respectively) and sapeinic acid treated 1 2 and 3 (H, J & L respectively).

4.3.2 Comparison of *S. aureus* Newman and *S. epidermidis* Tü3298 transcriptional response to sapienic acid challenge

Following sequencing, sequence reads were analysed by the CGR to produce lists of genes for *S. epidermidis* Tü3298 and *S. aureus* Newman that were significantly differentially expressed (DE) between the control and sapienic acid treated samples as outlined in the methods (section 2.7.8). The genes for these strains were then compared to each other to find orthologous genes by the CGR. The transcriptional response of orthologous genes in each species could then be directly compared to assess differences between these species.

Results showed that *S. aureus* Newman had 1224 significantly DE genes in response to sapienic acid challenge; 630 of these were upregulated and 594 were downregulated. *S. epidermidis* Tü3298 had 1505 significantly DE genes in

response to sapienic acid challenge; 708 of these were upregulated whilst 797 were downregulated.

Of the DE genes, 1485 had a homologue in the alternate species, 690 genes were DE in both species, 545 were DE in *S. epidermidis* but not *S. aureus*, 249 were DE in *S. aureus* but not in *S. epidermidis* (Fig. 4.2). Of the 690 genes that were DE in both species, 166 were regulated in the opposite direction to their counterpart in the alternate species. This means that approximately 40 % of genes were similarly regulated in both species.

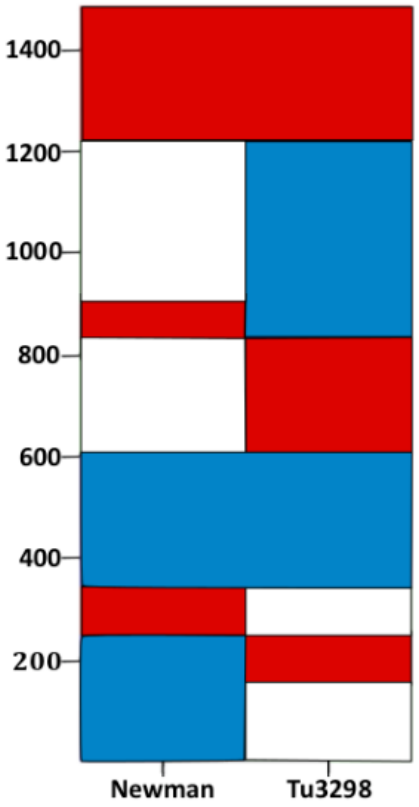


Figure 4.2 Comparison of differential expression in *S. aureus* Newman and *S. epidermidis* Tü3298 homologous genes when challenged with sapienic acid.

In this figure, red indicates upregulated genes, blue indicates downregulated genes and white indicates genes that are not significantly differentially expressed.

Some major differences between the datasets were immediately apparent from the DE gene lists (provided as a table on attached CD). There was strong upregulation of all 16 genes of the capsule biosynthesis *cap* operon in *S. aureus* (between 2.8 and 52 fold, with an average of 11.3 fold). Capsule may offer protection to *S. aureus* by preventing sapienic acid interacting with the cell.

Another upregulated operon in *S. aureus* Newman that has no homologue in *S. epidermidis* Tü3298 is the *mtl* operon involved in manitol utilisation. Genes *mtlA*, *mtlD* and *mtlF* were all upregulated between 1.7 and 6 fold.

S. epidermidis Tü3298, shows strong upregulation of a cluster of genes (*setu_00412 – 00425*) consisting of 7 hypothetical genes, 5 pathogenicity island proteins and a virulence-associated E family protein. Most of these genes have no homologue in *S. aureus*, whilst the others are not DE in *S. aureus*. These genes are upregulated between 2.5 and 33.8 fold; the degree of upregulation indicates these proteins are involved in sapienic acid resistance. The gene arrangement at this loci means it is unlikely that this gene cluster forms an operon or operons, though their proximity suggests that they could be within the same regulon.

4.3.3 Comparison of DE COGs

To assess the cellular pathways that were most affected in *S. epidermidis* and *S. aureus* following sapienic acid challenge, clusters of orthologous groups (COG) enrichment analysis was used. Using the WebMGA, bespoke perl scripts and the IMG database, it was possible to find the most frequent COG classes that DE genes were assigned to. It was also possible to determine if the number of DE genes in these orthologous classes were more or less than would be expected if DE genes were evenly distributed across the genome. If DE genes were evenly distributed across the genome, the number of DE genes in each COG class would be proportional to the number of genes on the genome in that class. This theory was used to find the COG classes with significantly more or less DE genes than expected.

COG analysis indicated that sapienic acid treatment of *S. aureus* led to COG classes O (post-translational modification, protein turnover and chaperones), E

(amino acid transport and metabolism) and Q (secondary metabolites biosynthesis, transport and catabolism) having significantly more upregulated genes than would be expected ($p < 0.05$ by Pearson's Chi^2 test) (Fig. 4.3). COG classes J (translation, ribosomal structure and biogenesis), L (replication, recombination and repair) and F (nucleotide transport and metabolism) were found to have significantly less upregulated genes than expected. COG classes J (translation, ribosomal structure and biogenesis) and F (nucleotide transport and metabolism) had significantly more downregulated genes than expected.

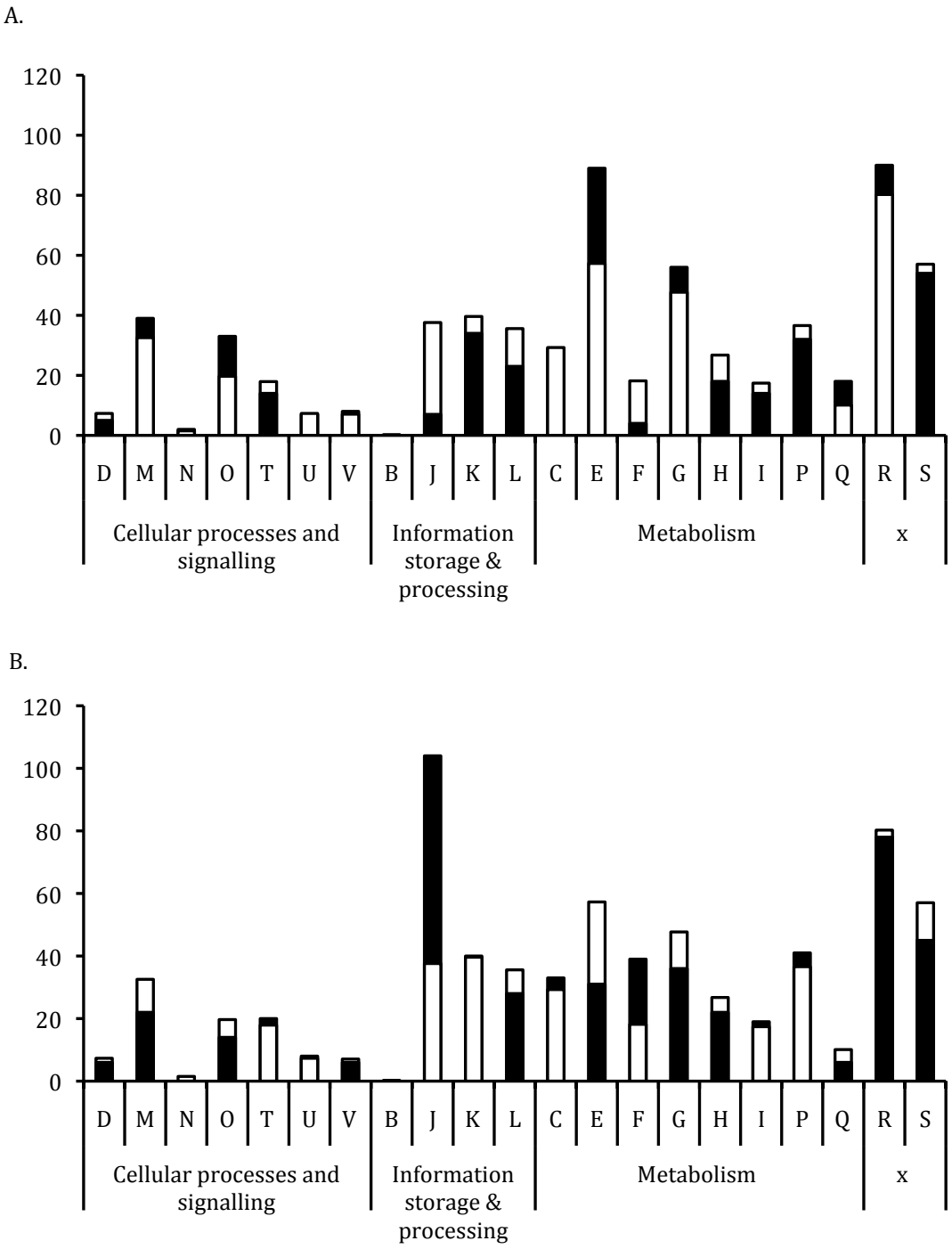
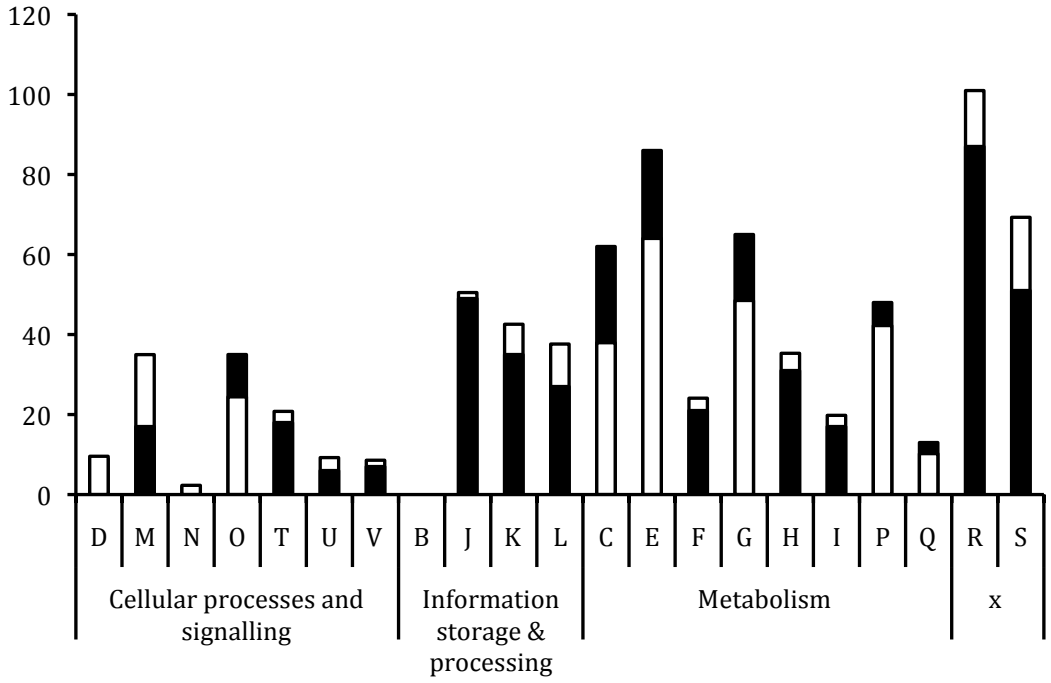


Figure 4.3 Number of *S. aureus* Newman DE genes per COG class.

Graphs show the expected number of genes if expression was uniform across the genome (white bars) and the observed number of genes per COG class (black bars), for upregulated DE genes (A) and downregulated DE genes (B). COG classes are further grouped into the higher functions: cellular processing and signalling, Information storage and processing, metabolism and poorly characterised (indicated by "X"). Genes were assigned to a COG class using WebMGA, the number of DE genes in each class were then calculated using a bespoke Perl script.

COG analysis of sapienic acid treated *S. epidermidis* DE genes showed that there were more upregulated genes than expected in COG classes O (post-translational modification, protein turnover and chaperones), C (energy production and conversion), E (amino acid transport and metabolism) and G (carbohydrate transport and metabolism)(Fig. 4.4). There were less upregulated genes than expected in COG classes M (cell wall, membrane or envelope biogenesis) and S (function unknown). There were more down regulated genes than expected in COG classes M (cell wall, membrane or envelope biogenesis) and F (nucleotide transport and metabolism). There were less down regulated genes than expected in COG classes C (energy production and conversion) and G (carbohydrate transport and metabolism).

A.



B.

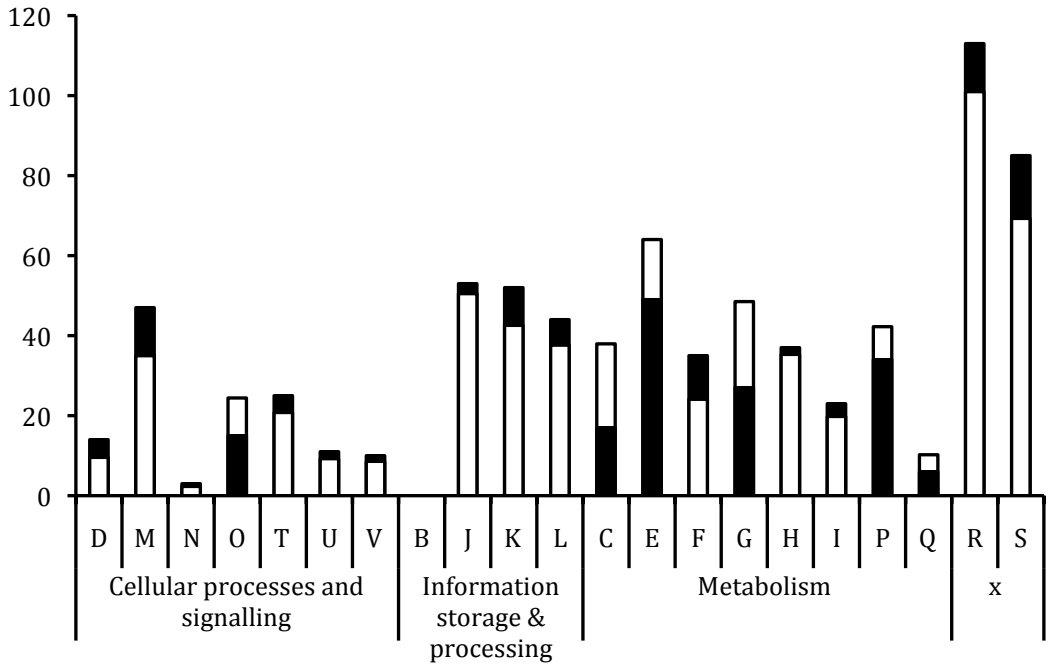


Figure 4.4 Number of *S. epidermidis* Tü3298 DE genes per COG class.

Graphs show the expected number of genes if expression was uniform across the genome (white bars) and the observed number of genes per COG class (black bars), for upregulated DE genes (A) and downregulated DE genes (B). COG classes are further grouped into the higher functions: cellular processing and signalling, Information storage and processing, metabolism and poorly characterised (indicated by "X"). Genes were assigned to a COG class using WebMGA, the number of DE genes in each class were then calculated using a bespoke Perl script.

S. aureus and *S. epidermidis* both up-regulate more genes than expected in post-translational modification, protein turnover and chaperones and amino acid transport and metabolism. Upregulation of amino acid production combined with the increase in expression of post-translational modification, protein turnover and chaperone genes suggests an increase in protein production and refolding. This could indicate protein damage is caused by sapienic acid challenge, or alternatively reflect the degree of protein remoldelling that occurs after sapienic acid challenge. Both species also downregulate more genes than expected in COG class F (nucleotide transport and metabolism). The overall downregulation in both species of nucleotide transport is suggestive of a reduction in DNA replication. This may be more pronounced in *S. aureus*, as nucleotide transport and metabolism and replication, recombination and repair also have significantly less genes upregulated than expected.

There are also a number of differences between the *S. aureus* and *S. epidermidis* enriched COGs. Whilst *S. epidermidis* significantly decreases cell wall, membrane and envelope biogenesis gene expression, this is not the case in *S. aureus*. This may suggest that *S. epidermidis* acts to repair membrane or cell wall damage whilst *S. aureus* does not, or that *S. aureus* does not sustain as much damage as *S. epidermidis* to the membrane and cell wall. A further difference between the two species is the significant overall upregulation of energy production and conversion and carbohydrate transport and metabolism in *S. epidermidis*, but not *S. aureus*. This could indicate that *S. epidermidis* has a greater need than *S. aureus* for energy after sapienic acid challenge, or that *S. epidermidis* has a more rapid or better adaptive response to sapienic acid. *S. aureus* shows overall downregulation of translation, ribosomal structure and biogenesis genes, which would suggest a decrease in protein biosynthesis.

4.3.4 Comparison of DE metabolic pathways

The lists of DE genes produced by the CGR were used in KEGG mapper version 2.1-search & color, an online programe that highlights the proteins within KEGG pathways based upon the users gene list (Kanehisa *et al*., 2012). The *S. epidermidis* Tü3298 genome is not an available reference genome for the KEGG

database, as such, *S. epidermidis* gene names were converted to homologous gene names from strain Rp62a prior to use in KEGG mapper. In this gene name conversion, 206 *S. epidermidis* DE genes had no homologue so were not used in this analysis. The coloured maps of pathways with up and downregulated genes are provided in the attached CD, labelled by pathway name and species. Analysis of these pathways enabled detection of metabolic processes with DE genes and speculative theories on the effect of sapienic acid on metabolism.

Both *S. epidermidis* and *S. aureus* upregulate genes associated with glycolysis and sugar uptake (Table 4.2). This upregulation of genes directly involved in glycolysis is accompanied by upregulation of genes involved in funnelling glyceraldehyde, glycerol and glycerone into glycolysis and away from glycerolipid and lipoteichoic acid production (Table 4.2). Genes that allow NADPH/NADP+ recycling are upregulated in both *S. aureus* and *S. epidermidis* (Table 4.2), though this is particularly apparent in *S. epidermidis*. Genes involved in pyruvate metabolism are also upregulated in both species (Table 4.2). Overall, there appears to be a trend in regulation to increase energy production.

Table 4.2 DE genes involved in energy production pathways production in sapienic acid challenged *S. aureus* and *S. epidermidis*.

DE genes highlighted by KEGG mapper analysis and the level of log_2 fold change in *S. aureus* Newman and *S. epidermidis* Tü3298 are shown. Genes that were not DE in one species are indicated by a dash in the relavant fold change column. Genes that were not present in one species are indicated by an 'X' in the relevant fold change column. Where gene names are different in each species, the *S. aureus* gene name is given first. Kegg mapper output pathways relevant to this table were glycolysis/gluconeogenesis, pentose phosphate pathway, fructose and mannose metabolism, galactose metabolism, amino sugar and nucleotide sugar metabolism, pyruvate metabolism, propanoate metabolism, butanoate metabolism, glutathione metabolism

Gene	Fold change *S. aureus*	Fold change *S. epidermidis*	Enzyme name
Sugar uptake and Glycolysis			
NWMN_0177 SETU_01006	+0.8	+1.3	2.7.1.69
NWMN_1333	+1.2	X	
pfkA	-	+0.6	2.7.1.11
fbaA	-	+0.5	4.1.2.13
fdaB	-	+1.8	
tpiA	+1.3	+2.4	5.3.1.1
gapA-1	+0.9	+2.3	1.2.1.12
gapA-2	-	+1.5	
pgk	+1.2	+2.4	2.7.2.3
pgm	+1.2	+2.3	5.4.2.12
gpmA	+2.0	+1.2	5.4.2.11
SETU_00031 NWMN_0367	-	-2.6	
eno	+0.8	+1.9	4.2.1.11
pyk		+1.2	2.7.1.40
SETU_01522 NWMN_1947	-	+0.7	2.7.1.4
nagA	+0.7	+0.5	3.5.1.25
nagB NWMN_0532	-	+1.1	3.5.99.6
pgi	+0.8	+0.9	5.3.1.9
NWMN_2224	+0.8	-	GlvBC
lacF	+2.3	+0.8	LacF
mtlA	+2.4	X	MtlA
mtlF	+0.8	-	
NWMN_0180	+2.6	X	GatA
NWMN_1619 SETU_01288	-1.6	+0.6	NagE

Table 4.2 continued DE genes involved in energy production pathways production in sapienic acid challenged *S. aureus* and *S. epidermidis*.

DE genes highlighted by KEGG mapper analysis and the level of log_2 fold change in *S. aureus* Newman and *S. epidermidis* Tü3298 are shown. Genes that were not DE in one species are indicated by a dash in the relavant fold change column. Genes that were not present in one species are indicated by an 'X' in the relevant fold change column. Where gene names are different in each species, the *S. aureus* gene name is given first. Kegg mapper output pathways relevant to this table were glycolysis/gluconeogenesis, pentose phosphate pathway, fructose and mannose metabolism, galactose metabolism, amino sugar and nucleotide sugar metabolism, pyruvate metabolism, propanoate metabolism, butanoate metabolism, glutathione metabolism

Gene	Fold change *S. aureus*	Fold change *S. epidermidis*	Enzyme name
Sugar uptake and glycolysis			
glcA	-2.5	-	Gamp
ptsG	-2.4	X	
fruA	-1.4	+1.7	FruA
Pyruvate metabolism			
Ldh		+1.7	1.1.1.27
Ldh1	-2.5	X	
NWMN_2459	+1.4	-	
poxB SETU_01970	+1.6	+2.0	1.2.3.3
ackA	-	-0.4	2.7.2.1
NWMN_1315 SETU_00980	+1.1	+1.1	3.6.1.7
AldA/aldA-1	+1.6	+0.4	1.2.1.3
NWMN_1858 AldA-2	+1.1	+1.8	
NWMN_2026 SETU_01602	+2.5	+2.0	
NWMN_2506	+2.4	-	
ald	-	+1.1	1.4.1.1
pycA/pyc	+1.1	+0.7	6.4.1.1
pdhA	-	+0.8	1.2.4.1
pdhB	-	+0.9	
pdhC	-	+0.9	2.3.1.12
SETU_02150	X	+0.5	
pdhD	-	+1.0	1.8.1.4
lpdA	-0.7	-	
pflB	-2.9	+1.4	2.3.1.54
citZ/gltA	2.5	+0.4	2.3.3.1

Table 4.2 continued DE genes involved in energy production pathways production in sapienic acid challenged *S. aureus* and *S. epidermidis*.

DE genes highlighted by KEGG mapper analysis and the level of log_2 fold change in *S. aureus* Newman and *S. epidermidis* Tü3298 are shown. Genes that were not DE in one species are indicated by a dash in the relavant fold change column. Genes that were not present in one species are indicated by an 'X' in the relevant fold change column. Where gene names are different in each species, the *S. aureus* gene name is given first. Kegg mapper output pathways relevant to this table were glycolysis/gluconeogenesis, pentose phosphate pathway, fructose and mannose metabolism, galactose metabolism, amino sugar and nucleotide sugar metabolism, pyruvate metabolism, propanoate metabolism, butanoate metabolism, glutathione metabolism

Gene	Fold change *S. aureus*	Fold change *S. epidermidis*	Enzyme name
Pyruvate metabolism			
sucA	-	+1.0	1.2.4.2
sucB	-	+1.1	2.3.1.61
ilvB	+3.3	+2.9	2.2.1.6
budB	-	+1.9	
SETU01538	X	+3.2	
BudA	-	+1.3	4.1.1.5
aldB	-0.8	-	
SETU_01888	X	+1.3	1.1.1.303/ 1.1.1.4
NWMN_0071 SETU_02090	+3.7	+0.7	1.1.1.304/ 1.1.1.76
SETU_02208	X	+4.3	
NADH recycling			
Ldh	-	+1.7	1.1.1.27
Ldh1	-2.5	-	
SETU_02044	X	+1.6	1.8.1.7
citC	+1.9	-	1.1.1.42
gnd	-	+0.7	1.1.1.44
Zwf	+1.1	+0.5	1.1.1.49
nadE	-	+0.6	6.3.1.5

Peptidoglycan (PG) biosynthesis was mainly downregulated in *S. epidermidis*, but was relatively unaffected in *S. aureus* (Table 4.3). The downregulation in both *S. aureus* and *S. epidermidis* of genes that produce PG precursors such as UDP-GlcNAc and UDP-Glc may also decrease PG biosynthesis (Table 4.3). Potentially, decreased PG biosynthesis may be a mechanism to conserve energy

and increase glycolysis, as UDP-GlcNAc or UDP-Glc production could reduce the pool of glucose available for glycolysis. A decrease in PG biosynthesis could also be suggestive of a decrease in cell growth and division.

Table 4.3 DE genes involved in peptidoglycan biosynthesis production in sapienic acid challenged *S. aureus* and *S. epidermidis*.

DE genes highlighted by KEGG color analysis and the level of $\log_2$ fold change in *S. aureus* Newman and *S. epidermidis* Tü3298 are shown. Genes that were not DE in one species are indicated by a dash in the relevant fold change column. Genes that were not present in one species are indicated by an 'X' in the relevant fold change column. Where gene names are different in each species, the *S. aureus* gene name is given first. Kegg mapper output pathway relevant to this table was peptidoglycan biosynthesis.

Gene	Fold change *S. aureus*	Fold change *S. epidermidis*	Enzyme name
murF	-	-1.1	6.3.2.10
murE	-	-1.8	6.3.2.7
femA	-	-1.0	2.3.2.17
femX	-	+0.6	2.3.2.16
SETU_01292	-	-1.2	SgtA
NWMN_1766 SETU_01431	+2.2	-1.0	SgtB
pbp1	-	-0.8	PbpA
pbp3	-	-1.1	Pbp3
murZ	+0.9	-	-
murB	-0.8	-	1.3.1.98

Genes involved in nitrogen metabolism are upregulated in *S. epidermidis*, which may suggest another mechanism for energy production (Table 4.4). However, energy production may not be the sole goal. The genes upregulated within the nitrogen metabolism pathway would also lead to the generation of ammonia. In *S. epidermidis*, enzymes from various pathways that could lead to the production of ammonia are upregulated. These enzymes include nitrate reductase (*narG*, *narH*, *narJ*), nitrite reductase (*nirB*, *nirD*), carbamate kinase (*arcC*) and urease (*ureC*). Consistent with a shift towards ammonia production is the downregulation of genes that would reduce the pool of ammonia,

including nitric-oxide synthase (*SETU_01471*), argininosuccinate synthase (*argG*) and argininosuccinate lyase (*argH*).

A similar pattern is seen in *S. aureus*; urease is strongly upregulated (*ureA*, *ureB*, *ureC*), whilst nitric-oxide synthase is downregulated (*NWMN_1852*). However, in *S. aureus* there is downregulation of the ammonia producer carbamate kinase (*arcA1*), and no change in *narGHIJ*, *nirBD*, *argG* or *argH*. Increases in ammonia production may therefore be less pronounced in *S. aureus* Newman than *S. epidermidis* Tü3298.

Table 4.4 DE genes involved in ammonia production in sapienic acid challenged *S. aureus* and *S. epidermidis*.

DE genes highlighted by KEGG color analysis and the level of $\log_2$ fold change in *S. aureus* Newman and *S. epidermidis* Tü3298 are shown. Genes that were not DE in one species are indicated by a dash in the relevant fold change column. Genes that were not present in one species are indicated by an 'X' in the relevant fold change column. Where gene names are different in each species, the *S. aureus* gene name is given first. Kegg mapper output pathways relevant to this table were purine metabolism, arginine and proline metabolism, nitrogen metabolism and two-component system.

Gene	Fold change *S. aureus*	Fold change *S. epidermidis*	Enzyme name
gcvH	-	+0.4	GDCH
ureA	+3.7	-0.7	3.5.1.5
ureB	+3.7	-	
ureC	+3.6	+0.5	
arcA1/arcC	-1.0	+0.8	2.7.2.2
gluD	-	+0.4	1.4.1.2
narG	-	+1.4	NarG
narH	-	+1.8	NarH
narJ	-	+1.6	NarJ
NirB	-	+1.1	NirB
NirD	-	+1.0	NirD
Setu_01471 NWMN_1852	-1.1	-2.0	1.14.13.39
argG	-	-1.0	6.3.4.5
argH	-	-0.6	4.3.2.1

Pathways involved in production of amino acids are generally upregulated in *S. epidermidis* and *S. aureus* (Table 4.5). The upregulation of pyruvate metabolism mentioned above would also increase metabolites for amino acid production (Table 4.2). There is also downregulation of genes involved in conversion of the amino acid aspartate to the metabolite fumarate. Both species showed changes in RNA degradation (Table 4.6), indicating alteration in protein synthesis. Together this indicates an increase in amino acid production, possibly for increased protein synthesis or protein restructuring.

Table 4.5 DE genes involved in amino acid production in sapienic acid challenged *S. aureus* and *S. epidermidis*.

DE genes highlighted by KEGG color analysis and the level of $\log_2$ fold change in *S. aureus* Newman and *S. epidermidis* Tü3298 are shown. Genes that were not DE in one species are indicated by a dash in the relevant fold change column. Genes that were not present in one species are indicated by an 'X' in the relevant fold change column. Where gene names are different in each species, the *S. aureus* gene name is given first. Kegg mapper output pathways relevant to this table were histidine metabolism, glycine, serine and threonine metabolism, cysteine and methionine metabolism, lysine biosynthesis, valine, leucine and isoleucine biosynthesis and alanine, aspartate and glutamate metabolism.

Gene	Fold change *S. aureus*	Fold change *S. epidermidis*	Enzyme name
Amino acid biosynthesis			
hisG	-	+1.7	2.4.2.17
hisIE	+1.7	+0.9	3.6.1.31/ 3.5.4.19
hisA	+2.3	+1.0	5.3.1.16
hisF	+1.9	+0.9	HisF
hisH	+2.2	+1.5	HisH
hisB	+2.3	+1.7	4.2.1.19
hisC	+1.8	-	2.6.1.9
hisD	+2.1	+1.4	1.1.1.23
hutU	+1.5	X	4.2.1.49
hutG	+1.8	+1.1	3.5.3.8
thrA	+4.0	-	2.7.2.4
lysC	+3.2	-0.9	

Table 4.5 continued DE genes involved in amino acid production in sapienic acid challenged *S. aureus* and *S. epidermidis*.

DE genes highlighted by KEGG color analysis and the level of log_2 fold change in *S. aureus* Newman and *S. epidermidis* Tü3298 are shown. Genes that were not DE in one species are indicated by a dash in the relevant fold change column. Genes that were not present in one species are indicated by an 'X' in the relevant fold change column. Where gene names are different in each species, the *S. aureus* gene name is given first. Kegg mapper output pathways relevant to this table were histidine metabolism, glycine, serine and threonine metabolism, cysteine and methionine metabolism, lysine biosynthesis, valine, leucine and isoleucine biosynthesis and alanine, aspartate and glutamate metabolism.

Gene	Fold change *S. aureus*	Fold change *S. epidermidis*	Enzyme name
Amino acid biosynthesis			
asd	+3.5	+1.3	1.2.1.11
dapA	+3.6	+1.9	4.3.3.7
dapB	+3.6	+2.4	1.17.1.8
NWMN_0919 SETU_00643	+0.7	-0.4	2.6.1.-
dapD	+3.2	+2.4	2.3.1.89/ 23.1.1.17
NWMN_1929 SETU_02190	+3.2	+1.0	3.5.1.18
lysA	+1.4	+0.7	4.1.1.20
metL/ hom	+4.4	-	1.1.1.3
ilvA	+3.5	+2.4	4.3.1.19
leuB	+3.4	+3.3	1.1.1.85
leuC	+3.6	-	4.2.1.35/ 4.2.1.33
leuD	+3.6	+2.6	
ilvB	+3.3	+2.9	2.2.1.6
budB	-	+1.9	
SETU_01538	X	+3.2	
ilvC	+3.5	+3.2	1.1.1.86
ilvD	+2.7	+2.6	4.2.1.9
ilvE	+0.8	+0.5	2.6.1.42
leuA	+3.7	+3.3	2.3.3.13
NWMN_0351 SETU_00018	+2.1	+1.3	CGS
NWMN_0350 SETU_00017	+2.8	+1.5	CBL
metE	+2.0	+1.3	MET
NWMN_1643 dat	-	+0.4	2.6.1.21

Table 4.5 continued DE genes involved in amino acid production in sapienic acid challenged *S. aureus* and *S. epidermidis*.

DE genes highlighted by KEGG color analysis and the level of log_2 fold change in *S. aureus* Newman and *S. epidermidis* Tü3298 are shown. Genes that were not DE in one species are indicated by a dash in the relevant fold change column. Genes that were not present in one species are indicated by an 'X' in the relevant fold change column. Where gene names are different in each species, the *S. aureus* gene name is given first. Kegg mapper output pathways relevant to this table were histidine metabolism, glycine, serine and threonine metabolism, cysteine and methionine metabolism, lysine biosynthesis, valine, leucine and isoleucine biosynthesis and alanine, aspartate and glutamate metabolism.

Gene	Fold change *S. aureus*	Fold change *S. epidermidis*	Enzyme name
Amino acid biosynthesis			
alr-2 SETU_00973	2.3	+1.1	5.1.1.1
NWMN_2454	+1.0	-	1.2.1.88
gltB	+1.6	-	1.4.1.13/ 1.4.1.14
gltD	-	+1.2	
cysK	-	+1.4	2.5.1.47
Aspartate degradation			
purA	-1.6	-0.5	6.3.4.4
purB	-1.2	-0.5	4.3.2.2
argG	-	-1.0	6.3.4.5
argH	-	-0.6	4.3.2.1

Table 4.6 DE genes involved in RNA degradation in sapienic acid challenged *S. aureus* and *S. epidermidis*.

DE genes highlighted by KEGG color analysis and the level of log_2 fold change in *S. aureus* Newman and *S. epidermidis* Tü3298 are shown. Genes that were not DE in one species are indicated by a dash in the relevant fold change column. Genes that were not present in one species are indicated by an 'X' in the relevant fold change column. Where gene names are different in each species, the *S. aureus* gene name is given first. Kegg mapper output pathway relevant to this table was RNA degradation.

Gene	Fold change *S. aureus*	Fold change *S. epidermidis*	Enzyme name
eno	+0.8	+1.9	Enolase
pnp	+1.6	-0.6	PNPase
pnpA	-1.1	-	
NWMN_0749 SETU_00406	-0.7	+1.1	RNaseR
recQ	-	-0.6	Helicase
recQ-2	-	-0.7	
rho	-0.7	-0.6	Rho
NWMN_0956	-0.8	-	RNaseJ
dnaK	+2.8	+2.2	DnaK
groEL	+3.5	+1.3	GroEL
SETU_00874	-	+1.1	Hfq
ppk	X	-	PPK

Glycerophospholipid biosynthesis is downregulated in both *S. epidermidis* and *S. aureus* (Table 4.7). This, combined with upregulation of fatty acid degradation components, may support speculations by Parsons *et al.* (2012) that the bacteria are utilising the fatty acid as a source of membrane lipid rather than undergoing glycerophospholipid biosynthesis.

Genes involved in production of mevalonate and cardiolipin production in *S. epidermidis* are downregulated. Conversely, in *S. aureus*, cardiolipin production genes are upregulated, as are staphyloxanthin biosynthesis genes. Squalene, cardiolipin and staphyloxanthin are all proposed membrane stiffeners. Staphyloxanthin has previously been suggested as a resistance determinant for fatty acids in *S. aureus* (Chamberlain *et al*., 1991).

Table 4.7 DE genes involved in membrane lipid biosynthesis in sapienic acid challenged *S. aureus* and *S. epidermidis*.

DE genes highlighted by KEGG color analysis and the level of log_2 fold change in *S. aureus* Newman and *S. epidermidis* Tü3298 are shown. Genes that were not DE in one species are indicated by a dash in the relevant fold change column. Genes that were not present in one species are indicated by an 'X' in the relevant fold change column. Where gene names are different in each species, the *S. aureus* gene name is given first. Kegg mapper output pathways relevant to this table were glycerolipid metabolism, glycerophospholipid metabolism, fatty acid degradation, terpenoid backbone biosynthesis and carotenoid biosynthesis.

Gene	Fold change *S. aureus*	Fold change *S. epidermidis*	Enzyme name
Glycerol metabolism			
SETU_00365	-	+0.7	2.7.1.31
NWMN_2331 garK	+1.4	+0.7	
aldA	+1.6	+0.4	1.2.1.3
aldA-2	-	+1.8	
NWMN_1858 SETU_01602	+1.1	+2.0	
NWMN_2026	+2.5	X	
NWMN_0619	+1.6	X	2.7.1.29
NWMN_0620	+1.3	X	
gldA	X	+1.8	1.1.1.6
Glycerolipid biosynthesis			
plsX	-1.1	-1.7	2.3.1.15
NWMN_1266 Serp0924	-1.6	-1.9	
plsC	-2.0	X	2.3.1.51
murG ypfP	-	-1.5	2.4.1.157
Fatty acid degradation			
fadE		X	6.2.13
aldA	+1.6	+0.4	1.2.1.3
aldA-2	-	+1.8	
NWMN_1858 SETU_01602	+1.1	+2.0	
NWMN_2026	+2.5	X	
NWMN_0346 SETU_00013	-	+0.44	2.3.1.9
NWMN_2090 SETU_01661	+1.67	+2.67	1.1.1.1
NWMN_2091 SETU_01662	+2.30	+2.53	
NWMN_2272 SETU_01828	-	+0.82	

Table 4.7 continued DE genes involved in membrane lipid biosynthesis in sapienic acid challenged *S. aureus* and *S. epidermidis*.

DE genes highlighted by KEGG color analysis and the level of log_2 fold change in *S. aureus* Newman and *S. epidermidis* Tü3298 are shown. Genes that were not DE in one species are indicated by a dash in the relevant fold change column. Genes that were not present in one species are indicated by an 'X' in the relevant fold change column. Where gene names are different in each species, the *S. aureus* gene name is given first. Kegg mapper output pathways relevant to this table were glycerolipid metabolism, glycerophospholipid metabolism, fatty acid degradation, terpenoid backbone biosynthesis and carotenoid biosynthesis.

Gene	Fold change *S. aureus*	Fold change *S. epidermidis*	Enzyme name
Glycerophospholipid metabolism			
gpsA	-1.0	-0.6	1.1.1.94
plsX	-1.1	-1.7	2.3.1.15
NWMN_1266 SETU_00930	-1.6	-1.9	
plsC	-2.0	X	2.3.1.51
cdsA	-2.3	-1.2	2.7.7.41
pgsA	-1.1	-	2.7.8.5
NWMN_0164	-1.3	-	3.1.4.46
SETU_00717	-	-0.6	
SETU_01283	-	-0.5	
NWMN_1614	+1.4	-	
Carotenoid, cardiolipin, branched chain fatty acid and mevalonate biosynthesis			
CrtM	+2.0	X	CrtM
CrtN	+2.3	X	CrtN
CrtI	+1.7	X	CrtP
NWMN_2463	+1.9	X	CrtQ
mvk	-	-0.8	2.7.1.36
SETU_00217	-	-0.6	2.7.4.2
mvaD	-	-0.5	4.1.1.33
Idi/fni-2	-1.2	-0.6	5.3.3.2
NWMN_1230	+1.1	-	Cls
cls/cls-2	-1.1	-0.5	
ispA	-1.7	-	2.5.1.1/ 2.5.1.29
hepT SETU_01049	-1.0	-0.4	2.5.1.30
uppS	-1.8	-2.2	2.5.1.31
Idi/fni-2	-1.2	-0.6	5.3.3.2

Both *S. aureus* and *S. epidermidis* mainly downregulated genes involved in mismatch repair and homologous recombination (Table 4.8). Downregulation of these genes could be indicative of a reduced rate of DNA replication. Further, this indicates that sapienic acid does not cause DNA damage.

Table 4.8 DE genes involved in DNA repair pathways in sapienic acid challenged *S. aureus* and *S. epidermidis*.

DE genes highlighted by KEGG color analysis and the level of $\log_2$ fold change in *S. aureus* Newman and *S. epidermidis* Tü3298 are shown. Genes that were not DE in one species are indicated by a dash in the relevant fold change column. Genes that were not present in one species are indicated by an 'X' in the relevant fold change column. Where gene names are different in each species, the *S. aureus* gene name is given first. Kegg mapper output pathways relevant to this table were DNA replication, base excision repair and mismatch repair.

Gene	Fold change *S. epidermidis*	Fold change *S. aureus*	Enzyme name
mutS/hexA	-	-0.5	MutS
xseA		-1.3	ExoVII
recJ	-1.7	-1.1	RecJ
pcrA	-0.8	-	UVRD
NWMN_0358	-1.4	X	SSB
NWMN_1909	+2.5	X	
dnaN	-	+0.9	DpoIII
dnaX	-	-0.9	
holA	-1.0	-	
holB	-1.3	X	
NWMN_1832	+1.3		
Lig/ligA	-0.8	+0.6	Lig
NWMN_1520 SETU_01187	-0.9	-1.7	RecD
ruvA	-1.1	-	RuvA
ruvB	-1.3	-	RuvB
SETU_01223	-	-0.7	Tag
ung	-1.4	-1.1	Ung
NWMN_1760 mutY	-	-0.8	MutY
NWMN_1460 nfo	-1.6	+1.0	Nfo

Both *S. epidermidis* and *S. aureus* show altered expression of genes that are considered virulence factors (Table 4.9). This includes downregulation of *dlt*, *fmtC* (*mprF*) and *isdA*, which would most likely increase cell hydrophobicity. Several factors shown to directly affect immune responses are downregulated in *S. aureus*, such as *scn*, *spa* and *aur*. Factors involved in inhibition of complement evasion are upregulated in *S. aureus* including *set7nm* (SSL7) (Bestebroer *et al*., 2010). There is also differential expression in *S. aureus* in genes predicted to play a role in tissue adhesion specificity including *clfB*, *sdrC*, *sdrD* and *sdrE*. Together this could suggest that sapienic acid is an important regulator of niche adaptation in *S. aureus*. The only virulence-associated genes with altered expression in *S. epidermidis* were those in the *VraFGDE* operon.

Table 4.9 DE virulence factor genes in sapienic acid challenged *S. aureus* and *S. epidermidis*.

DE genes highlighted by KEGG color analysis and the level of $\log_2$ fold change in *S. aureus* Newman and *S. epidermidis* Tü3298 are shown. Genes that were not DE in one species are indicated by a dash in the relevant fold change column. Genes that were not present in one species are indicated by an 'X' in the relevant fold change column. Where gene names are different in each species, the *S. aureus* gene name is given first. Kegg mapper output pathway relevant to this table was staphylococcus aureus infection

Gene	Fold change *S. aureuss*	Fold change *S. epidermidis*	Enzyme name
dltA	-2.1	-1.2	Dlt
dltB	-1.8	-	
dltC	-2.0	-	
dltD	-1.9	-	
fmtC	-1.0	-0.7	MprF
aur/sepA	-	-1.0	Aur
spa	-1.0	X	SpA
scn	-1.3	X	
clfB	-1.6	X	ClfB
isdA	-2.3	X	IsdA
eta	-1.7	X	Eta

Table 4.9 continued DE virulence factor genes in sapienic acid challenged *S. aureus* and *S. epidermidis*.

DE genes highlighted by KEGG color analysis and the level of log_2 fold change in *S. aureus* Newman and *S. epidermidis* Tü3298 are shown. Genes that were not DE in one species are indicated by a dash in the relevant fold change column. Genes that were not present in one species are indicated by an 'X' in the relevant fold change column. Where gene names are different in each species, the *S. aureus* gene name is given first. Kegg mapper output pathway relevant to this table was staphylococcus aureus infection

Gene	Fold change *S. aureuss*	Fold change *S. epidermidis*	Enzyme name
vraF	-	+1.0	VraF
vraD	-	-0.6	
vraG	-	+1.3	VraG
hlgA	+2.0	X	Hlg/luc
clfA	+1.7	X	ClfA
set7nm	+1.3	X	SSL7
sdrC	+2.9	X	
sdrD	+1.0	X	SdrC/D
sdrE	+0.8	X	

From the above analysis, it is possible to see that though the homologous genes shared between *S. epidermidis* and *S. aureus* are not always being regulated in the same way, often the overall trend of how the pathway is regulated is the same. This suggests that the results using gene orthologs (section 4.3.2) are likely to exagerate differences in the transcriptional response of *S. epidermidis* compared to *S. aureus*.

4.3.5 Comparison of DE genes to other *S. aureus* transcriptome datasets

As discussed in Chapter one, the sapienic acid mechanism of action is still unclear. Comparison of DE genes in response to an antimicrobial to other transcriptome datasets has been used to indicate the mechanism of action of antimicrobials, for example daptomycin (Muthaiyan *et al*., 2008).

The online bioinformatics tool SAMMD (*S. aureus* microarray meta-database) is a searchable database of *S. aureus* microarray transcriptomics data for 250 experiments (Nagarajan and Elasri, 2007). This tool was utilised to investigate overlaps in gene regulation between the sapienic acid challenge datasets and published *S. aureus* transcriptomics datasets. The sapienic acid challenge datasets were compared to all datasets in SAMMD. Results for datasets for antimicrobials with a similar predicted mechanism of action to sapienic acid are shown. Where multiple datasets for the same condition were available, the data set using experimental conditions most similar to that used in this study were selected.

As SAMMD is based upon microarray data, the gene names used are from *S. aureus* COL, N315, MW2 or Mu50. Therefore, to enable the data to be used to search SAMMD it was necessary to convert the gene names into their homologues in strain *S. aureus* N315 using bespoke perl scripts. These scripts converted 1331/1506 *S. epidermidis* Tü3298 gene names and 1164/1225 *S. aureus* Newman gene names to their closest Blast match in *S. aureus* N315. Gene names that were not converted did not have a Blast hit to the *S. aureus* N315 genome with an E-value over 0.0001, so were not considered homologous.

None of the datasets in SAMMD had as many DE genes as the response of *S. aureus* Newman or *S. epidermidis* Tü3298 to sapienic acid. This is not surprising, as microarrays are known to be less sensitive than RNA-Seq. However, even considering the reduced sensitivity of microarrays compared to RNA-Seq, the only datasets with over 800 DE genes were: chlorination 10 min, epithelial cells -2h- post internalization, fosfomycin (4 μg/ml -40 min), lung adaptation, orange oil and stringent response.

To gain insight into the potential mechanism of action of sapienic acid, the sapienic acid challenge datasets were compared to those from antimicrobials with mechanisms of action similar to those previously proposed for sapienic acid. Previous reports and results from Chapter five suggest that sapienic acid may inhibit cell wall biosynthesis (CWB). Known CWB inhibitors vancomycin and oxicillin showed relatively low overlap to sapienic acid challenge gene expression of *S. aureus* (25 % and 19 % respectively) or *S. epidermidis* (28 %

and 26 % respectively) (Fig. 4.5). Similarly, daptomycin, predicted to inhibit CWB or disrupt the cell membrane, showed little overlap in gene expression to sapienic acid challenge of *S. aureus* (27 % and 26 % respectively).

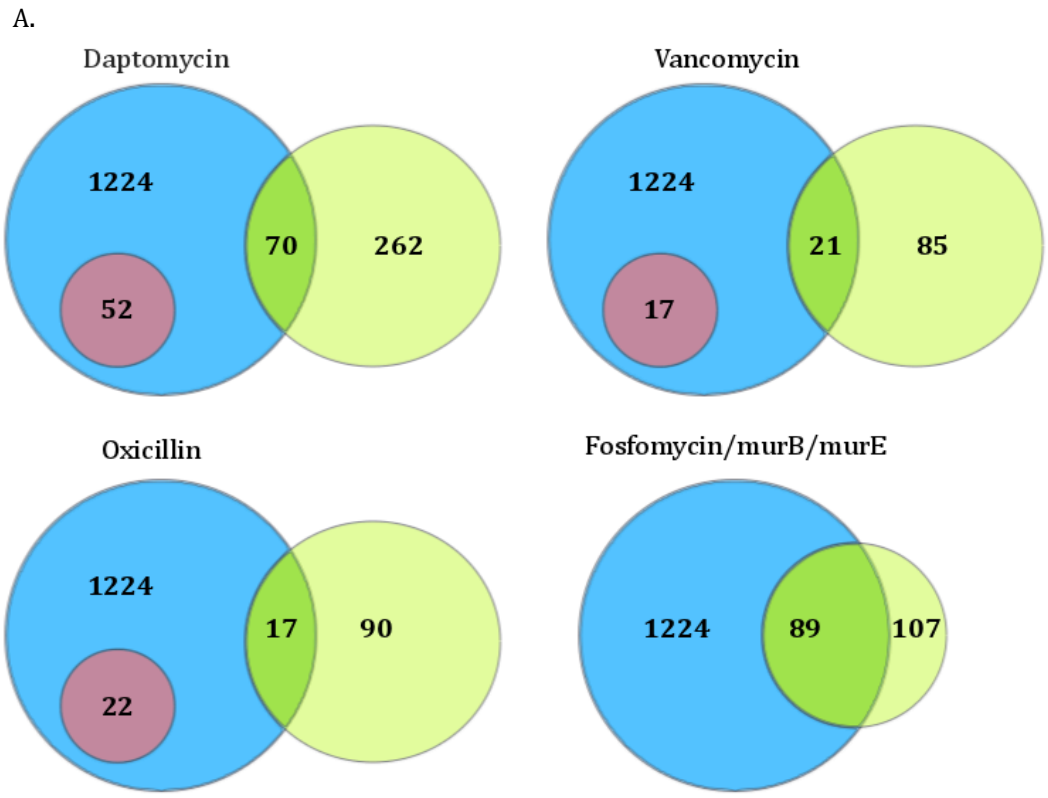


Figure 4.5 SAMMD comparison of sapienic acid transcriptomes to *S. aureus* cell wall biosynthesis inhibitor transcriptomes.

Comparison of *S. aureus* Newman (A) and *S. epidermidis* Tü3298 (B). Genes in the sapienic acid datasets are represented by the blue circles, whilst the comparison datasets are represented by the green circles, red circles indicate oppositely regulated genes between the datasets.

B.

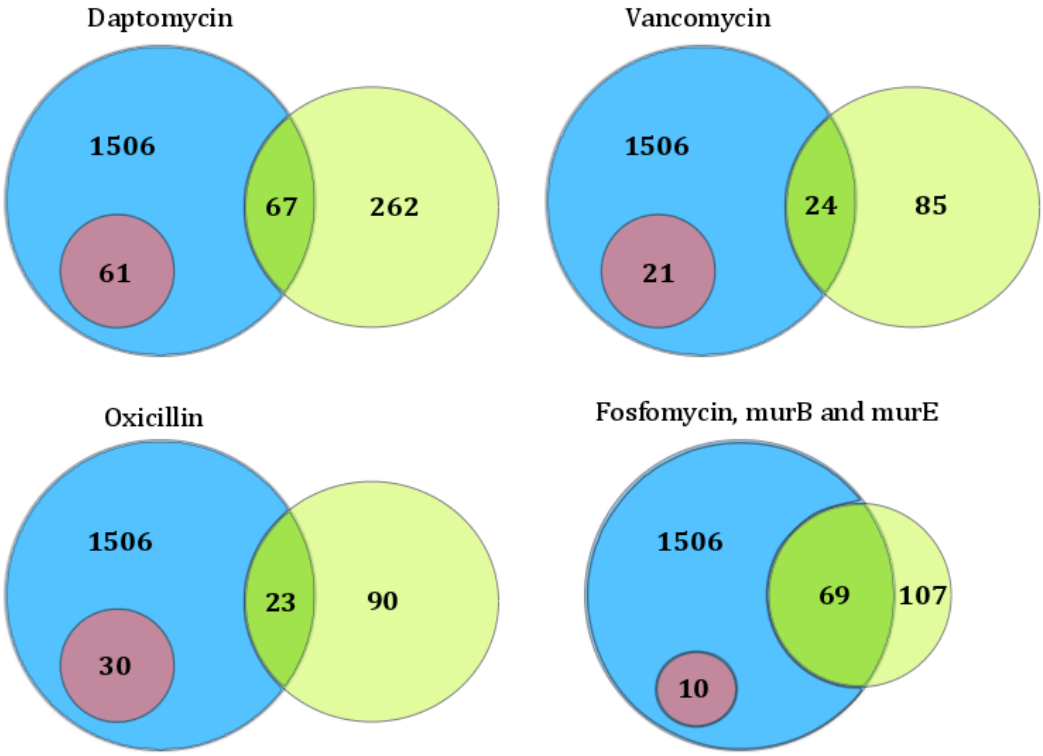


Figure 4.5 continued SAMMD comparison of sapienic acid transcriptomes to *S. aureus* cell wall biosynthesis inhibitor transcriptomes.

Comparison of *S. aureus* Newman (A) and *S. epidermidis* Tü3298 (B). Genes in the sapienic acid datasets are represented by the blue circles, whilst the comparison datasets are represented by the green circles, red circles indicate oppositely regulated genes between the datasets.

The fosfomycin, *murB* mutant and *murE* mutant combined data set, indicating a core expression profile in early cell wall biosynthesis (O'Neill *et al*., 2009), shared 83 % and 65 % of DE genes with *S. aureus* and *S. epidermidis* sapienic acid challenge datasets respectively. Both MurB and MurE are involved in early cell wall biosynthesis (CWB), whilst fosfomycin has been shown to inhibit early cell wall biosynthesis. The overlap between the CWB inhibited dataset and sapienic acid challenged *S. epidermidis* and *S. aureus* transcriptomes potentially indicates that either sapienic acid inhibits CWB directly, or that the downregulation of CWB as a response to sapienic acid results in the same pattern of gene regulation as seen in CWB inhibited *S. aureus*.

Membrane disruption is another mechanism through which sapienic acid is suggested to act. The cationic antimicrobial peptides (CAMPs) temporin and

ovispirin are known to cause membrane disruption. *S. aureus* and *S. epidermidis* sapienic acid challenge datasets showed a degree of similarity to *S. aureus* challenged with temporin (62 % and 44 % respectively) and ovisporin (60 % and 43 % respectively) (Fig. 4.6). However, this overlap did not extend to other membrane acting antimicrobials dermaseptin (23 % and 17 % respectively) or nisin (20 % and 23 % respectively).

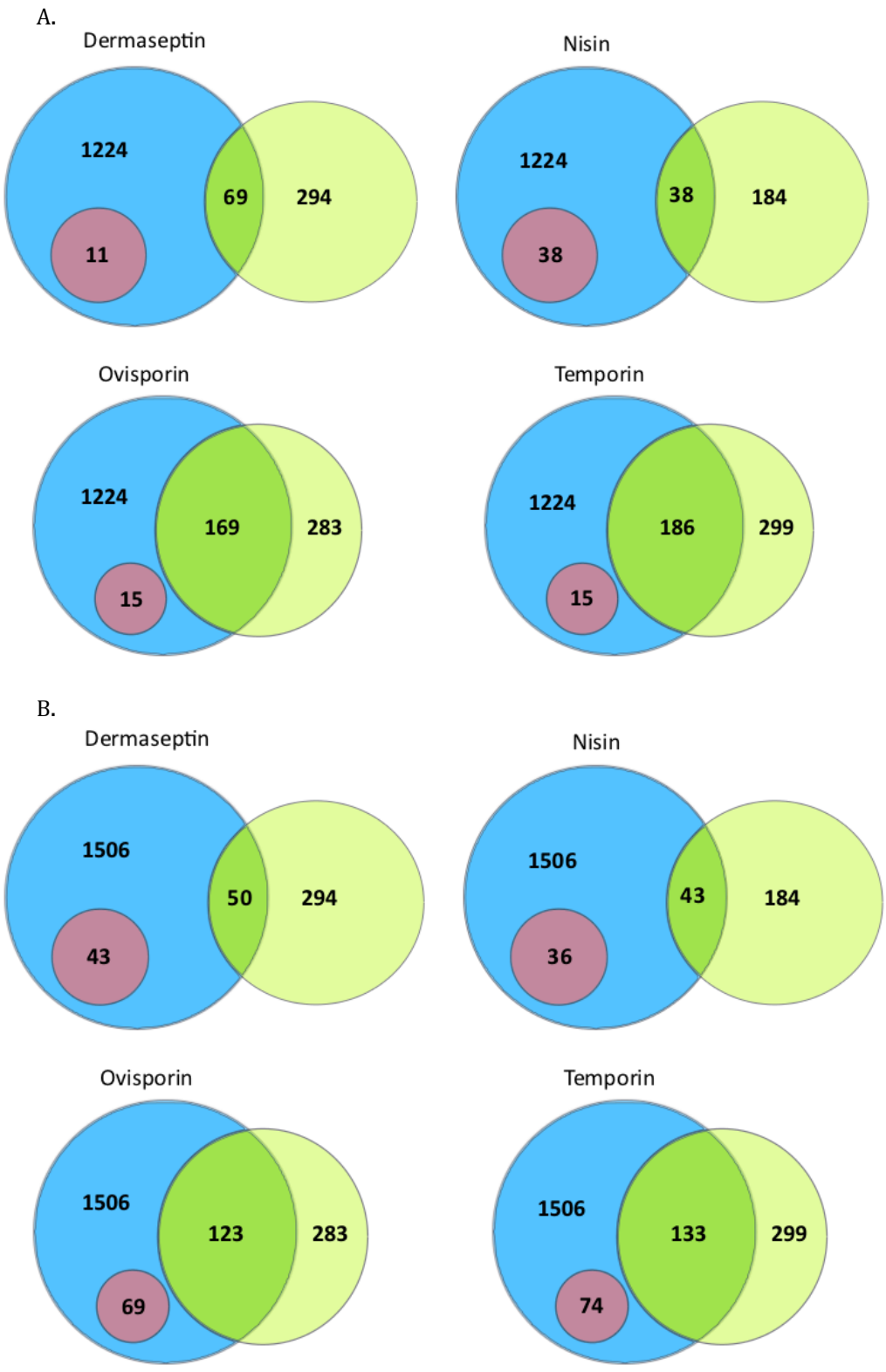


Figure 4.6 SAMMD comparison of sapienic acid transcriptomes to *S. aureus* membrane disrupting antimicrobials transcriptomes.

Comparison of *S. aureus* Newman (A) and *S. epidermidis* Tü3298 (B). Genes in the sapienic acid datasets are represented by the blue circles, whilst the comparison datasets are represented by the green circles, red circles indicate oppositely regulated genes between the datasets.

Protonophore activity is a suggested mechanism of action for sapienic acid. There was not a large overlap in gene regulation between *S. aureus* and *S. epidermidis* sapienic acid challenged cells and challenge of *S. aureus* ATCC 29213 with the protonophore CCCP (20 % and 23 % respectively) (Fig. 4.7).

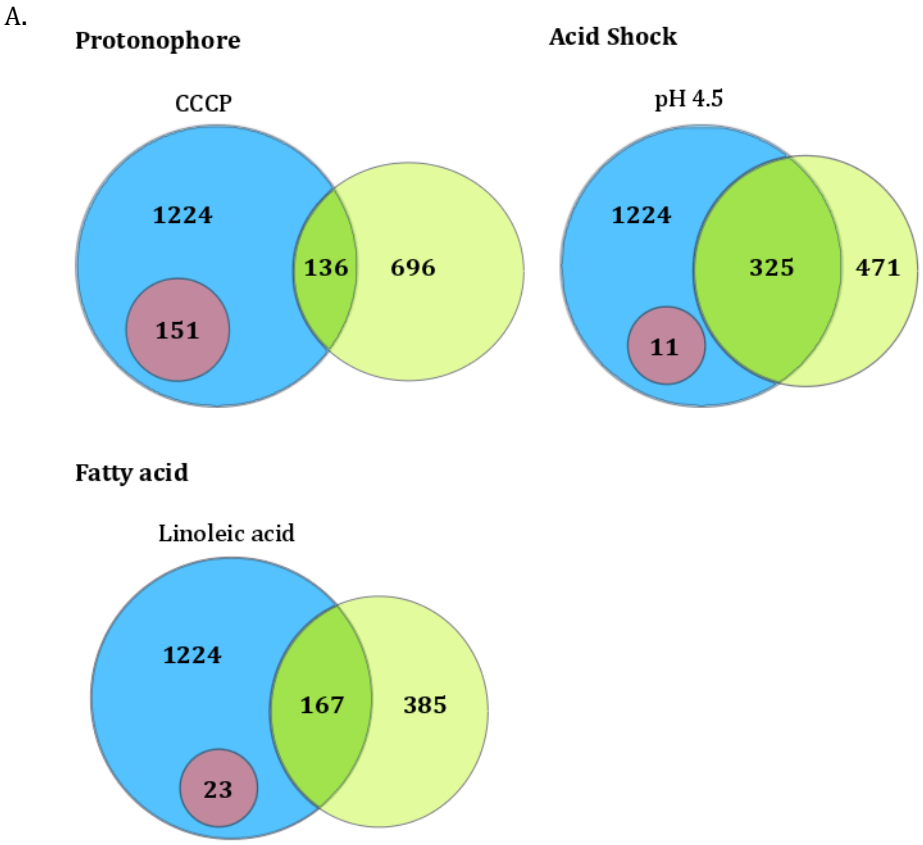


Figure 4.7 SAMMD comparison of sapienic acid transcriptomes to *S. aureus* protonophore, acid shock and linoleic acid transcriptomes.

Comparison of *S. aureus* Newman (A) and *S. epidermidis* Tü3298 (B). Genes in the sapienic acid datasets are represented by the blue circles, whilst the comparison datasets are represented by the green circles, red circles indicate oppositely regulated genes between the datasets.

B.

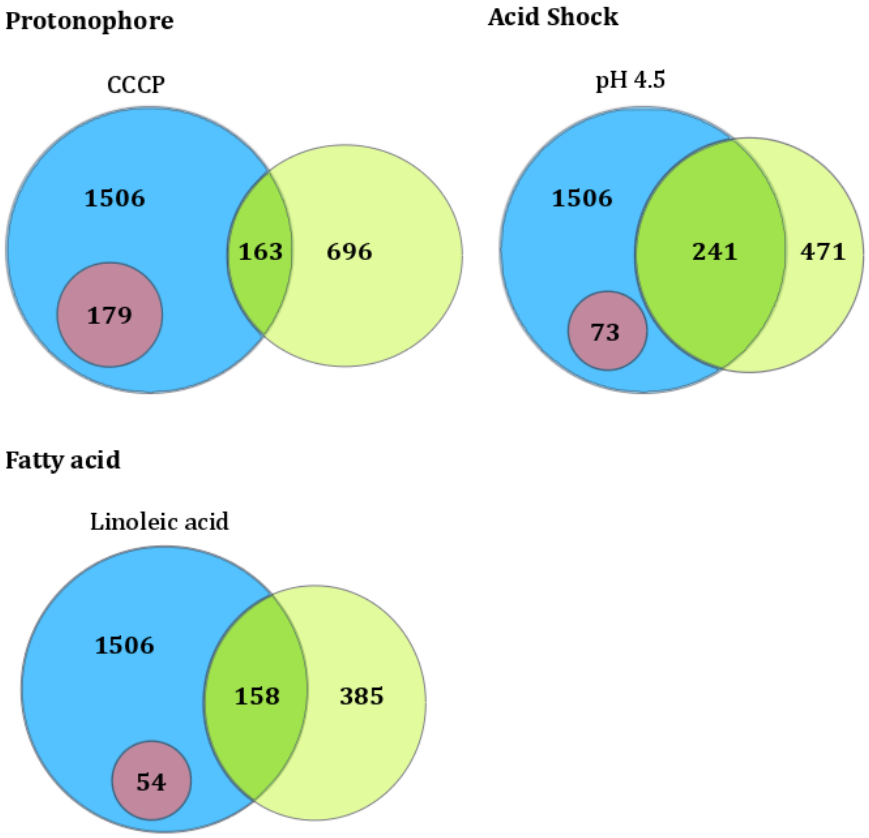


Figure 4.7 continued SAMMD comparison of sapienic acid transcriptomes to *S. aureus* protonophore, acid shock and linoleic acid transcriptomes.

Comparison of *S. aureus* Newman (A) and *S. epidermidis* Tü3298 (B). Genes in the sapienic acid datasets are represented by the blue circles, whilst the comparison datasets are represented by the green circles, red circles indicate oppositely regulated genes between the datasets.

Finally, sapienic acid has been suggested to act through its ability to lower pH. Acid-shocked *S. aureus* 50583 had a substantial overlap in gene expression to *S. aureus* and *S. epidermidis* sapienic acid challenge datasets (69 % and 51 % respectively) (Fig. 4.7). The similarity in gene expression indicates that sapienic acid challenged *S. aureus* Newman and *S. epidermidis S. epidermidis* regulate many genes in a similar way to that reported for acid shock conditions.

Though acid-shock, ovisporin, temporin and early CWB core data-sets had high overlap in gene expression to sapienic acid challenge datasets, there are still differences in regulation. These differences could indicate differences in regulatory pathways due to strain and species differences. This could explain

why *S. epidermidis* has fewer DE genes in common with the acid shock, ovisporin, temporin and early CWB core datasets, as it is more evolutionarily distant to the *S. aureus* strains used. However, the differences between the DE genes of sapienic acid challenge and other datasets will also be due to the different regulatory pathways activated by sapienic acid compared to other antimicrobials.

Investigation of publications describing each data set displaying considerable overlap in DE genes to that of sapienic acid challenge gave an insight into the DE genes that could account for the similarities observed.

In the corresponding journal article describing temporin and ovisporin challenge responses of *S. aureus* Newman, it was suggested that the main transcriptional changes are caused by induction of the VraSR regulon (Pietiainen *et al*., 2009). Upregulation of *VraR* was observed in both sapienic acid challenged *S. aureus* and *S. epidermidis* by 4.6 and 2.3 fold respectively. Other key transcriptional responses to temporin and ovisporin that were similar in sapienic acid challenge were upregulation of protein fate genes, components for oxidative stress response and amino acid biosynthesis.

The majority of transcriptional responses assessed to be key in the acid shock data set in the corresponding journal article are regulated similarly in the sapienic acid challenge data. Similar transcriptional responses include regulation of genes leading to increased urease production, proton efflux, cellular reducing power, conversion of acetate to alcohol, oxidative stress response, regulation of metal ion transport and protein fate proteins (Bore *et al*., 2007). Other transcriptional responses led to reduced protein synthesis, fatty acid metabolism, phospholipid metabolism, pyrimidine and purine biosynthesis (Bore *et al*., 2007). Bore *et al.* (2007), proposed that the upregulated processes were involved in acid resistance whilst the downregulated genes were linked to the observed decrease in cell growth.

Universally DE genes in the early CWB inhibited data set included the upregulation of glutamate biosynthesis, oligopeptide transport and amino acid biosynthesis, which O'Neill *et al*. (2009) mark as essential precursors for CWB.

The other main transcriptional response is upregulation of genes involved in the general stress response. CWB was downregulated in sapienic acid treated *S. aureus* and *S. epidermidis*, as were the processes mentioned in the early CWB inhibited data set. It is not possible to tell if this is because sapienic acid directly inhibits early CWB or if early CWB is simply downregulated in response to sapienic acid.

Perhaps surprisingly, comparison of sapienic acid challenged *S. aureus* and *S. epidermidis* showed relatively low similarity to the linoleic acid challenged *S. aureus* MRSA252 data set. Many of the processes highlighted as key responses by Kenny *et al*. (2009), including PG biosynthesis, pyruvate utilisation and sugar transport and phosphorylation are expressed differently under sapienic acid challenge. There is similarity in expression of amino acid biosynthesis genes, however in sapienic acid challenge data there is a general upregulation of all amino acid biosynthesis, whereas under linoleic acid challenge there is upregulation specifically those used in cell wall biosynthesis.

There are also some similarities not present in all three datasets. Sapienic acid challenged *S. aureus* Newman and linoleic acid challenged *S. aureus* MRSA252, both upregulate capsule and carotenoid genes, which are not present in *S. epidermidis*.

All three datasets had downregulation in the fatty acid biosynthesis pathway, though mainly through downregulation of different enzymes in each case (*fadD*, *fadX* and *plc* in *S. aureus* MRSA 252, *fabD*, *AccABCD* and *fabG* in *S. aureus* Newman, and *fabG* and *fabI* in *S. epidermidis* Tü3298).

Past studies looking at the similarities between strains of *S. aureus* to cell wall acting antibiotics also found large differences in gene expression between datasets (Campbell *et al*., 2012; Muthaiyan *et al*., 2008; Sass *et al*., 2008; Utaida, 2003). These data were used to determine a set of core genes that was typical of an *S. aureus* response to cell wall acting antimicrobials. To determine if there is a core set of genes DE in response to fatty acids all three datasets were compared using information from SAMMD and a bespoke perl script. Within this

core set of genes of fatty acid challenge stimulon, 59 genes were upregulated and 35 genes were downregulated (Table 4.10).

Table 4.10 The staphylococcal AFA challenge stimulon.

Compilation of homologous genes significantly differentially expressed during challenge with linoleic acid in *S. aureus* MRSA252 and sapienic acid in *S. aureus* Newman and *S. epidermidis* Tü3298. Where multiple annotations were available for homologues, the most likely was used.

Gene name			Description	Fold change		
Tü3298	Newman	MRSA 252		Tü3298	Newman	MRSA252
Upregulated						
fdaB	*NWMN_2503*	*fda*	Fructose-bisphosphate aldolase	3.53	2.35	2.02
gpmA	*gpmA*	*gpmA*	Phosphoglycerate mutase	2.3	4.06	2.06
SETU_00193	*NWMN_0533*	*SAR0574*	Hexulose-6P synthase	2.23	1.8	2.36
SETU_00242	*NWMN_0587*	*SAR0624*	Esterase/lipase	5.13	6.87	6.49
SETU_00243	*sarA*	*sarA*	Accessory regulator A	3.2	3.97	2.14
SETU_00102	*NWMN_0460*	*yabJ*	Translation initiation inhibitor, N: deiminase activity	4.79	2.23	3.65
SETU_00324	*NWMN_0673*	*SAR0757*	Glucosyl transferase	9.06	4.29	3.49
SETU_00394	*NWMN_0737*	*SAR0825*	Cell division inhibitor homologue	9.32	2.55	5.06
SETU_00401	*pgm*	*pgm*	2, 3-diphosphoglycerate independent phosphoglycerate mutase	4.96	2.35	2.64
SETU_00343	*NWMN_0690*	*SAR0775*	Osmoprotectant ABC transporter	2.13	3.43	2.13
SETU_00344	*NWMN_0691*	*SAR0776*	Osmoprotectant ABC transporter, permease	2.48	3.2	2.99
SETU_02108	*NWMN_2548*	*SAR2728*	SecA preprotein translocase homologue	1.39	3.23	3.85
SETU_02148	*NWMN_2089*	*opuD2*	Osmoprotectant transporter	1.38	8.17	4.42
SETU_02209	*NWMN_0048*	*SAR0109*	Putative transporter	1.88	7.78	2.37
SETU_00446	*NWMN_0771*	*SAR0859*	General stress protein	7.78	12.82	3.82
SETU_00468	*NWMN_0791*	*SAR0882*	Membrane protein/hemolysin	4.03	2.48	4.05

Table 4.10 continued The staphylococcal AFA challenge stimulon.

Compilation of homologous genes significantly differentially expressed during challenge with linoleic acid in *S. aureus* MRSA252 and sapienic acid in *S. aureus* Newman and *S. epidermidis* Tü3298. Where multiple annotations were available for homologues, the most likely was used.

Gene name			Description	Fold change		
Tü3298	Newman	MRSA 252		Tü3298	Newman	MRSA252
SETU_00641	*NWMN_0914*	*SAR1018*	Hydrolase/prolyl aminopeptidase	1.31	1.97	2.80
SETU_00673 *SETU_00674*	*NWMN_0050*	*SAR0111*	Streptococcal McrA homologue	5.39 5.74	7.36	5.96
SETU_00907	*thrB*	*thrB*	Homoserine kinase	7.21	16.8	2.20
SETU_00911	*katA*	*KatA*	Catalase	5.82	5.98	5.71
SETU_01152	*dnaJ*	*dnaJ*	Molecular chaperone	1.87	4.11	2.30
SETU_00522	*NWMN_0845*	*clpB*	Molecular chaperone	25.99	47.18	2.49
SETU_02130	*hisIE*	*HisIE*	Histidine biosynthesis protein	1.85	3.16	2.09
spoVG	*spoVG*	*spoVG*	Stage V sporulation protein G homologue	4.96	2.57	2.83
SETU_01602	*NWMN_2026*	*sar2210*	Aldehyde dehydrogenase	3.2	5.54	5.48
SETU_01786	*NWMN_2229*	*SAR2413*	Oxidoreductase, short chain dehydrogenase	18.38	15.03	4.63
SETU_01902	*NWMN_2369*	*SAR2559*	Glucose 1-dehydrogenase	18.38	9.38	6.85
SETU_00323	*NWMN_0672*	*SAR0756*	Aldo/keto reductase	8.28	4.17	2.96
SETU_01788	*hutG*	*hutG*	formiminoglutamase	2.17	3.39	2.83
SETU_01905	*NWMN_1929*	*dapE*	succinyl-diaminopimelate desuccinylase	2.58	9.19	4.89
tpiA	*tpi*	*tpiA*	Triosephosphate isomerase	5.35	2.41	2.22
trap	*NWMN_2507*	*SAR2688*	Signal transduction protein	1.64	4.03	7.55
prmA	*NWMN_1481*	*SAR1655*	Methyltransferase	1.96	3.94	2.25
SETU_00029	*NWMN_0366*	*SAR0392*	Hypothetical membrane transglycosylase	4.59	3.86	2.54
SETU_00194	*NWMN_0534*	*SAR0575*	6-phospho-3-hexuloisomerase	3.07	1.95	2.16
SETU_00452	*NWMN_0778*	*SAR0867*	TOPRIM domain-containing protein	2.77	3.20	2.54
SETU_01270	*NWMN_1601*	*SAR1785*	Metallo-beta lactamase superfamily protein	6.41	5.50	3.05

Table 4.10 continued The staphylococcal AFA challenge stimulon.

Compilation of homologous genes significantly differentially expressed during challenge with linoleic acid in *S. aureus* MRSA252 and sapienic acid in *S. aureus* Newman and *S. epidermidis* Tü3298. Where multiple annotations were available for homologues, the most likely was used.

Gene name			Description	Fold change		
Tü3298	Newman	MRSA 252		Tü3298	Newman	MRSA252
SETU_01432	*NWMN_1767*	*SAR1965*	ThiJ/Pfpl family protein	5.94	3.97	2.25
SETU_02097	*NWMN_2545*	*sasF*	Surface anchored protein	2.14	16.00	16.80
SETU_01785	*NWMN_2558*	*SAR2740*	Putative riboflavin reductase	6.19	3.18	2.05
SETU_01623	*NWMN_2048*	*SAR2232*	Oxidoreductase/epimerase	3.51	18.77	8.26
SETU_00389	*NWMN_0734*	*SAR0821*	Hypothetical membrane protein	1.32	4.56	3.19
SETU_00300	*NWMN_0649*	*SAR0733*	Decarboxylase	8.40	1.83	3.04
SETU_00301	*NWMN_0650*	*SAR0734*	Hypothetical protein	7.46	1.78	2.23
SETU_00435	*NWMN_0762*	*SAR0849*	Hypothetical protein	2.01	5.50	2.81
SETU_00436	*NWMN_0763*	*SAR0850*	Hypothetical protein	2.31	4.23	2.94
SETU_00443	*NWMN_0767*	*SAR0855*	Hypothetical protein	1.80	5.50	2.53
SETU_02103	*NWMN_2557*	*SAR2739*	Hypothetical protein	2.51	4.17	4.21
SETU_01659	*NWMN_2087*	*SAR2274*	Hypothetical membrane protein	4.08	8.00	4.59
SETU_01296	*NWMN_1631*	*SAR1816*	Hypothetical membrane protein	2.06	2.66	2.82
SETU_00041	*NWMN_0377*	*SAR0405*	Hypothetical protein	7.89	5.28	2.76
SETU_01439	*NWMN_1820*	*SAR1972*	Hypothetical exported protein	2.01	2.04	5.71
SETU_00677	*NWMN_0948*	*SAR1055*	Hypothetical protein	2.58	2.25	4.50
Downregulated						
galU	*gtaB*	*gtaB*	UTP-glucose-1-phosphate uridyltransferase	1.53	2.91	2.63
gmk	*gmk*	*gmk*	Guanylate kinase	3.14	2.16	2.78
ndk	*ndk*	*ndk*	Nucleoside diphosphate kinase	4.26	3.63	2.38
relA	*relA*	*relA*	GTP pyrophosphokinase	1.82	1.65	2.27
SETU_00180	*NWMN_0519*	*SAR0562*	Deoxypurine kinase	2.45	2.33	2.17

Table 4.10 continued The staphylococcal AFA challenge stimulon.

Compilation of homologous genes significantly differentially expressed during challenge with linoleic acid in *S. aureus* MRSA252 and sapienic acid in *S. aureus* Newman and *S. epidermidis* Tü3298. Where multiple annotations were available for homologues, the most likely was used.

Gene name			Description	Fold change		
Tü3298	Newman	MRSA 252		Tü3298	Newman	MRSA252
SETU_00261	*tagA*	*tagA*	Teichoic acid biosynthesis	1.75	2.06	2.78
SETU_00263	*tagG*	*tagG*	Teichoic acid tranlocation permease	3.51	1.75	2.38
SETU_00268	*NWMN_0614*	*SAR0655*	Pyrimidine transporter	2.33	3.43	2.17
SETU_02365	*NWMN_2412*	*SAR2594*	ABC transporter	4.17	8.46	2.38
SETU_01802	*NWMN_2248*	*SAR2432*	Divalent cation transporter	2.3	1.72	2.44
SETU_00139	*nupC*	*nupC*	Pyrimidine transporter	1.61	8.75	2.94
SETU_00333	*pabA*	*SAR0766*	Anthranilate synthase/glutamine amidotransferase	2.93	6.02	2.04
SETU_00713	*cyoD*	*ctaB*	Cytochrome caa3 oxidase	1.72	5.46	2.04
SETU_01049	*hepT*	*gerCC*	Heptaprenyl diphosphate synthase component II	1.30	1.99	2.78
SETU_01842	*NWMN_2286*	*sarZ*	Virulence regulator	1.92	2.16	2.22
SETU_01880	*lytS*	*lytS*	Two-component sensor histidine kinase	3.71	2.00	3.33
SETU_01881	*lytR*	*lytR*	Two-component response regulator	1.74	2.53	3.57
SETU_01554	*rsbU*	*rsbU*	SigmaB regulatory protein	2.97	1.62	2.56
SETU_01998	*pyrD*	*SAR2669*	Dihydroorotate dehydrogenase	2.20	2.06	2.86
ubiE	*gerCB*	*menH*	Menaquinone biosynthesis	1.36	1.68	2.27
ydjM	*NWMN_2073*	*SAR2263*	Hypothetical membrane protein	2.81	1.89	2.17
yutE	*NWMN_0799*	*SAR0890*	Hypothetical protein	1.40	1.73	2.56
SETU_01555	*NWMN_1974*	*SAR2156*	PemK family protein	2.04	1.59	3.03
SETU_01077	*NWMN_1411*	*SAR1581*	AtsA/ElaC family protein	2.31	3.43	2.86
SETU_01123	*NWMN_1453*	*SAR1627*	5-formyltetrahydrofolate cyclo-ligase family protein	1.78	2.87	2.78
SETU_01195	*NWMN_1530*	*SAR1708*	ThiF family protein	1.56	2.81	2.04
SETU_01305	*NWMN_1642*	*SAR1834*	Hypothetical leucyl-tRNA synthase	3.53	2.41	2.17

Table 4.10 continued The staphylococcal AFA challenge stimulon.

Compilation of homologous genes significantly differentially expressed during challenge with linoleic acid in *S. aureus* MRSA252 and sapienic acid in *S. aureus* Newman and *S. epidermidis* Tü3298. Where multiple annotations were available for homologues, the most likely was used.

Gene name			Description	Fold change		
Tü3298	Newman	MRSA 252		Tü3298	Newman	MRSA252
SETU_00372	*NWMN_0718*	SAR0803	DegV family protein	2.39	3.23	3.23
SETU_00570	*NWMN_0882*	SAR0979	Permease	2.08	3.36	2.50
SETU_00280	*NWMN_0632*	SAR0673	Phosphate transport regulator	3.92	3.71	2.70
dltX	*NWMN_0802*	SAR0893	D-Ala-teichoic acid biosynthesis regulator	6.63	4.03	2.13
SETU_01373	*NWMN_1739*	SAR1938	Transcriptional regulator	11.00	9.71	2.38
SETU_01609	*NWMN_2035*	SAR2219	Hypothetical protein	2.22	1.71	2.78

This gene list of the AFA challenge stimulon indicates common set of genes that are similarly regulated. Some of these genes encode proteins that have already been postulated as linoleic acid resistance factors, such as the LPXTG cell wall anchored protein SasF (Kenny *et al*., 2009; King *et al*., 2012). This work therefore suggests a role for these proteins in sapienic acid, and potentially all long chain unsaturated fatty acid resistance.

One factor reported to aid AFA resistance is WTA (Kohler *et al*., 2009), so it is worth noting that WTA biosynthesis genes are downregulated in the FA challenge stimulon. This suggests that WTA is not regulated to enhance AFA resistance.

Other processes shown to have similar regulation include pyruvate production, glycolysis, the CtsR stress regulon and cell wall regulators *lytSR* and *lrgA* (Brunskill and Bayles, 1996; Groicher *et al*., 2000).

Of note, two McrA homologue genes are shown as upregulated in *S. epidermidis*, this was initially thought to be a duplication of the McrA homologue. However, alignment of these two genes to the *S. epidermidis* Rp62a McrA homologue shows that *setu_00673* aligns with the beginning of the gene and *setu_00674*

aligns with the end, with a short gap between. This could indicate a mutation that introduces a premature stop signal, however, as both of these sequences are transcribed, it is probable that these genes are in fact one gene.

4.3.6 Quantitative PCR validation

To validate the RNA-Seq data, genes found to be DE in the sapienic acid challenge RNA-Seq data were quantified by qPCR. Using qPCR, the fold changes for each gene between control and sapienic acid challenge samples were determined. At least three biological and two technical qPCR replicates were used for each gene. RNA integrity was assessed by gel electrophoresis prior to conversion to cDNA for qPCR.

The *narH*, *sasF* and *clpB* transcript levels and *capB*, *pyrP* and the tetR regulator *nwmn_2452* transcript levels were assessed for *S. epidermidis* and *S. aureus* respectively. Reference genes used were *gyrB*, *hu* and *rpoB*; these genes gave single products for both *S. aureus* and *S. epidermidis*, and are recommended for *S. aureus* qPCR studies (Valihrach and Demnerova, 2012). All primers chosen had efficiency values between 90 % and 100 % and gave products of approximately 150 bp. The primers and their efficiency values are shown in Table 4.11.

Table 4.11 Primers for qPCR

Gene name	Primer sequences	Efficiency (%)	Reference
rpoB	F- GCGAACATGCAACGTCAAG	97.0	This study
	R- GACCTCTGTGCTTAGCTGTAATAGC		
hu	F- TTTACGTGCAGCACGTTCAC	90.3	(Duquenne *et al.*, 2010)
	R- AAAAAGAAGCTGGTTCAGCAGTAG		
gyrB (Tü3298)	F- AGAAAAGATGGGACGCCCTG	96.6	This study
	R- CACCATGAAGACCGCCAGAT		
gyrB (Newman)	F- ATCGACTTCAGAGAGAGGTTG	92.9	(Kenny *et al.*, 2009)
	R- CCGTTATCCGTTACTTTAATCCA		
capB	F- GCGATATGCGTAAGCCAACAC	91.5	This study
	R- GGTACAGGGCCAGCTGTTAG		
pyrP	F- CGATGTTTGGCGCAACAGTA	92.5	This study
	R- GCTGGTATTTGCGCCTTCG		
clpB	F- TGGTGCACCTCCAGGTTATG	99.0	This study
	R- AGAATCCGTAAGACGACCTTCA		
NWMN_2452	F- ACGCCAGCTGTGTGGATTAT	93.3	This study
	R- AACGACTGCGACCTTGATGT		
sasF	F- TCACTCTGCGATTGAAGGCA	95.0	This study
	R- TTTCCGGTGCCGAATGATCT		
narH	F- TGGCCTTTCCATTGCATCCT	93.6	This study
	R- TTCAGTGTCGCCAGCAGTTA		

The fold changes determined from the qPCR data were consistently lower than the fold changes from RNA-Seq (Fig. 4.8). However, all fold changes showed regulation in the same direction as the RNA-Seq data and were approximately proportional. This phenomenon may be due to underestimation of gene expression by qPCR, overestimation of gene expression by RNA-Seq or both. Overall, the qPCR results indicate that the directionality and general fold changes of RNA-Seq results are accurate.

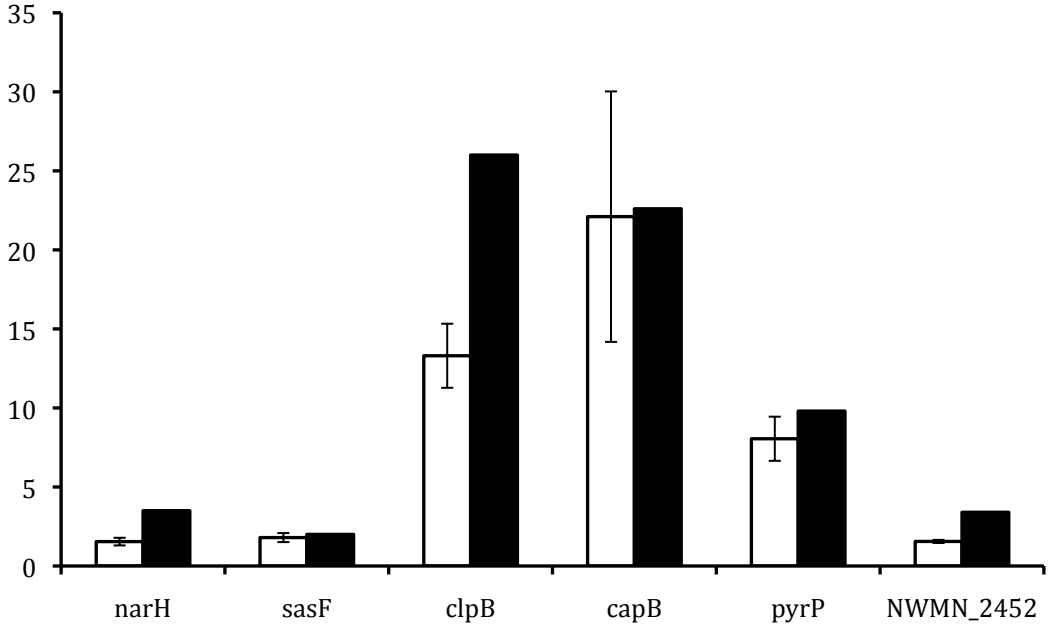


Figure 4.8 Differential expression of NarH, SasF and ClpB in *S. epidermidis* and CapB, PyrP and NWMN_2452 in *S. aureus* after challenge with sapienic acid, assessed by qPCR.

Black bars indicate the fold change in gene expression from RNA-Seq data while white bars indicate fold change in gene expression from qPCR data.

4.4 Discussion

Whilst initial comparisons of homologous DE genes in *S. aureus* and *S. epidermidis* indicated large differences in the transcriptional response to sapienic acid, subsequent COG and metabolic pathway analysis suggest a strong overlap in the transcriptional response. This included upregulation of COG groups 'post-translational modification, protein turnover and chaperones' and 'amino acid transport and metabolism' and down regulation of COG group 'nucleotide transport and metabolism'. Metabolic pathway analysis indicated that in both staphylococcal species there was upregulation of glycolysis, NADH recycling and amino acid biosynthesis, whilst glycerophospholipid biosynthesis and DNA recombination and repair were downregulated in both.

Differences in gene expression between *S. aureus* and *S. epidermidis* could explain the basis for increased AFA resistance of *S. epidermidis*. One potential resistance-associated locus strongly upregulated in *S. epidermidis* but not present in *S. aureus* is the gene cluster *setu_00412-00455*. This gene cluster consists of hypothetical genes and pathogenicity island proteins, and represents an interesting target for future investigations, but at present little can be said about its potential function in survival.

The remainder of the genes with clear differences in expression that may potentially explain the differences in resistance levels between *S. aureus* and *S. epidermidis* are pathways found in *S. aureus* but not *S. epidermidis*. These included carotenoid biosynthesis, capsule biosynthesis and and mannitol utilisation, which were all upregulated in response to sapienic acid challenge.

The biosynthesis pathway for the carotenoid staphyloxanthin was strongly upregulated in response to sapienic acid challenge in *S. aureus*. Previous work by Chamberlain *et al*., (1991) has indicated that carotenoid can enhance resistance to fatty acids, and is upregulated in response to linoleic acid challenge. Unsaturated fatty acids can increase the fluidity of membrane due to their ability to insert into the membrane, resulting in a less packed order. It is thought staphyloxanthin increases AFA resistance through its ability to create more rigid membranes, thereby negating the expected increase in fluidity

caused by insertion of fatty acids into the membrane (Mishra *et al*., 2011; Kenny *et al*., 2009; Chamberlain *et al*., 1991).

S. epidermidis does not produce staphyloxanthin, so any required resistance against AFA induced membrane fluidity must be achieved through another method. A major mechanism of altering membrane fluidity in bacteria is through adjustment of branched chain fatty acid and saturated fatty acid content in the membrane. Branched chain fatty acids increase membrane fluidity, whilst saturated fatty acids decrease membrane fluidity (Singh *et al*., 2008; Schujman and de Mendoza, 2005). Branched chain fatty acids are synthesised by Lpd in staphylococci (Singh *et al*., 2008), *lpd*, is downregulated by a similar degree in *S. aureus* and *S. epidermidis* upon sapienic acid challenge. This could result in decreased membrane fluidity, and therefore potentially fatty acid resistance. However, this response occurs in *S. aureus* as well as *S. epidermidis*, meaning *S. epidermidis* would presumably still have less protection against fatty acid induced membrane fluidity than *S. aureus*. It is possible that this observed downregulation of branched chain fatty acids is a mechanism to preserve the pool of branched amino acids, which act as precursors for branched fatty acid biosynthesis.

Though there were no major transcriptional changes in genes regulating membrane fluidity in *S. epidermidis*, this may not mean there is no change in membrane fluidity. Several studies in Gram-positive bacteria have shown that alterations in membrane fluidity are not controlled at the transcriptional or translational level, but are predicted to be regulated at the protein level (Pesakhov *et al*., 2007; Fozo and Quivey, 2004; Schujman and de Mendoza, 2005). Membrane lipid analysis would therefore be required to confirm if there are any changes in membrane fluidity.

Staphyloxanthin may offer protection against fatty acids through an alternative mechanism than acting as a membrane stiffener. As carotenoid production requires fatty acid as a precursor molecule (Wieland *et al*., 1994), staphyloxanthin may act to utilise the extra fatty acids in a manor that detoxifies them. Otherwise, AFAs have been suggested to cause oxidative damage,

staphyloxanthin provides oxidative stress resistance through free radical scavenging (Desbois and Smith, 2010; Clauditz *et al*., 2006).

Whilst capsule is not normally strongly produced in logarithmic growth, the *cap* operon, encoding type 5 capsule biosynthesis proteins (Herbert *et al*., 2001; O'Riordan and Lee, 2004), was strongly upregulated in *S. aureus* upon sapienic acid challenge. Capsule biosynthesis is dependent on *yabJ-spoVG* (Meier *et al*., 2007), which are also upregulated in response to sapienic acid. Capsule may offer protection against sapienic acid by limiting sapienic acid penetrating the membrane. In support of this barrier theory, microencapsulated strains, such as type 5 capsule producers, are more resistant to opsonation and phagocytosis than un-encapsulated mutants (O'Riordan and Lee, 2004). Alternatively, capsule could prevent leakage of small solutes, potentially helping to restore membrane polarisation.

S. epidermidis does not possess the *cap* operon for polysaccharide capsule production, instead, capsule in *S. epidermidis* is the result of polysaccharide intercellular adhesin (PIA) production or poly-γ-glutamic acid (PGA) production (McKenney *et al*., 1998; Kocianova *et al*., 2005). PGA biosynthesis genes (*pgsACBD*) are not DE in *S. epidermidis*, indicating either they do not play a role in sapienic acid resistance, or that the basal level of PGA is sufficient to provide resistance. PGA mutants could be used to test the role of PGA in sapienic acid resistance. As strain Tü3298 does not contain the *ica* operon it does not produce PIA. PIA is known to enhance resistance to antimicrobial peptides and phagocytosis (Vuong *et al*., 2004). It would be interesting to test if PIA production was upregulated in a strain containing the *ica* operon, such as *S. epidermidis* NCTC 1457, in response to sapienic acid. Of note *yabJ-spoVG* were also upregulated in *S. epidermidis*, despite the lack of the *cap* operon. It is possible that *yabJ-spoVG* help coordinate the response to sapienic acid, this loci has previously been indicated to modulate the σ^B response to stress (Meier *et al*., 2007).

S. aureus may actually be at a disadvantage from producing staphyloxanthin and capsule as their production is energetically expensive. Sapienic acid challenged cells actively upregulate glycolysis and sugar transport, suggesting a need for

more energy production, possibly to replace lost energy in the form of ATP, that is released from the cell upon AFA treatment (Parsons *et al*., 2012). Energy costly processes may actually be detrimental to survival in AFA conditions. In *S. epidermidis,* more genes involved in energy production, carbohydrate transport and metabolism are upregulated than in *S. aureus*, as revealed by COG and metabolic pathway analysis. This suggests that *S. epidermidis* might be better able to replace lost energy rapidly, perhaps increasing its chance of survival.

Mannitol is used as an energy source by *S. aureus* but not by *S. epidermidis*. Mannitol (Mtl) and mannitol-1-phosphate (Mtl-p) accumulation in *S. aureus*, caused by *mtlD* mutation, resulted in increased sensitivity to AFAs (Kenny *et al*., 2013). Further, mannitol acted synergistically with linoleic acid against wild type *S. aureus* (Kenny *et al*., 2013). This suggests that mannitol accumulation may actually be deleterious to *S. aureus* when exposed to AFAs.

Mannitol utilisation genes *mtlA*, mtlD and mtlF were upregulated in *S. aureus* Newman in response to sapienic acid. Whilst the upregulation of *mtlD*, which converts Mtl-P to fructose-6P, should ensure that there is no accumulation of Mtl or Mtl-P, the upregulation of MtlA, involved in mannitol transport, would increase the amount of mannitol-phosphate entering the cell. This could potentially make *S. aureus* Newman more sensitive to AFAs if the sugar alcohol if it is not rapidly metabolised. As *S. epidermidis* does not have mannitol utilisation genes, mannitol is not expected to be transported into the cell, and would therefore be unlikely to act synergistically with AFAs. While this may confer a survival advantage to *S. epidermidis* against AFAs on skin, it does not account for the higher AFA MICs of *S. epidermidis* compared to *S. aureus* in broth without mannitol as observed in Chapter three.

As previously outlined, there are similarities between the *S. aureus* and *S. epidermidis* responses to sapienic acid challenge. There are indications that sapienic acid challenge induces a general stress response in both *S. aureus* and *S. epidermidis*, which may explain the widespread changes in transcription and the similarities of the response between these two species. This includes upregulation of CtsR regulon *dnaJ*, *dnaK* and *clpB* as well as strong upregulation of *ctsR* itself. The CtsR regulon is linked to regulation of protein fate and is

upregulated in protein damaging conditions such as heat shock (Derré *et al*., 1999; Elsholz *et al*., 2010).

Another DE pathway that points to a general stress response is the upregulation of amino acid biosynthesis. Sapienic acid is proposed to lead to loss of small molecules, including amino acids; therefore increased amino acid synthesis might be needed to replace losses. Alternatively, accumulation of amino acids following a shock is theorised to enable the cell to grow rapidly once it has overcome the stress (Durfee *et al*., 2008).

The amino acids could also be acting as osmoprotectants or osmoprotectant precursors. In support of this, several osmoprotectant transporters were upregulated in the AFA challenge response. Further, Ebh genes, encoding cell surface mega proteins known to provide osmotic stress tolerance (Kuroda *et al*., 2008), were also upregulated in *S. epidermidis* . It is plausible that the staphylococci would face osmotic stress after AFA challenge, due to the known leakage of solutes (Parsons *et al*., 2012). It is possible that this upregulation of amino acid biosynthesis is not related to a stress response; amino acid transport is inhibited by AFAs (Galbraith and Miller, 1973). Therefore, amino acid biosynthesis upregulation could be a response to the loss of external amino acids as an amino acid source.

The downregulation of phospholipid and membrane fatty acid biosynthesis pathways could form part of a general stress response. Phospholipid and fatty acid biosynthesis pathways are downregulated upon entering stationary phase when cellular division has ceased (Schujman and de Mendoza, 2005). It is possible that AFA challenge causes a brief halt in cellular division, as indicated by growth analysis in Chapter three. If cellular division were halted, downregulation of membrane lipid biosynthesis would be expected. Alternatively, downregulation of fatty acid biosynthesis may be directly caused by the presence of extracellular fatty acids. The presence of extracellular fatty acids can downregulate expression of fatty acid biosynthesis pathways as bacteria switch to utilise the extracellular lipids preferentially (Schujman and de Mendoza, 2005; Aguilar and de Mendoza, 2006). Incorporation of AFAs into cellular lipoproteins and phospholipids was suggested as a detoxification

mechanism (Desbois and Smith, 2010). However, inhibition of phospholipid biosynthesis in *S. aureus* did not affect FA toxicity (Parsons *et al*., 2012).

Finally, purine biosynthesis and import was downregulated in the AFA challenge response. The downregulation of pyrimidine metabolism combined with the upregulation of amino acid biosynthesis and stress response genes is somewhat similar to the stringent response (Durfee *et al*., 2008). The stringent response is a response to nutrient starvation; in this response cellular energy is directed away from growth and toward nutrient stocking (Durfee *et al*., 2008). However *rsh* (RelA/SpoT homologue), the main inducer of the stringent response in *S. aureus* (Geiger *et al*., 2010), is downregulated in the AFA challenge response. Further, there was only a small overlap of DE genes between sapienic acid challenge and the stringent response induced by mupirocin, as assessed using SAMMD. Together, this indicates sapienic acid challenge did not induce a typical stringent response in the staphylococci, though there is a clear tendency away from growth and towards nutrient stocking.

Despite there being little overlap with the vancomycin or oxicillin transcriptomes, the cell wall stress stimulon appears to be upregulated following sapienic acid challenge. The cell wall stress stimulon is normally induced by inhibition of cell wall biosynthesis (Campbell *et al*., 2012; Muthaiyan *et al*., 2008; Sass *et al*., 2008; Utaida, 2003). This stimulon is primarily induced by the two-component regulator, VraSR, and can enhance resistance to cell wall biosynthesis inhibitors (Gardete *et al*., 2006; Boyle-Vavra *et al*., 2006).

VraSR genes are upregulated in both *S. aureus* and *S. epidermidis* sapienic acid challenge conditions. Given that VraSR is positively auto-regulated this indicates there is upregulation of the VraSR regulon (Sengupta *et al*., 2012). Many of the genes predicted to be part of the VraSR regulon are concurrently upregulated within the sapienic acid challenge data, including cell wall biosynthesis genes *pbp2* and *sgtB*, and osmoprotectant transporters (Kuroda *et al*., 2004; Sengupta *et al*., 2012). Moreover, sapienic acid-challenged *S. aureus* shows upregulation of *murZ* and *fmtA* of the VraSR regulon. VraSR was found to be activated upon challenge with cell wall acting antibiotics, but not heat, high osmolarity or acidic

pH (Kuroda *et al*., 2004). This suggests VraSR responds to cell wall biosynthesis inhibition rather than damage, however membrane destabilising cationic antimicrobial peptides were also shown to induce VraSR (Pietiainen *et al*., 2009).

The induction of a cell well stress-like response by sapienic acid indicates it either causes cell wall biosynthesis inhibition or membrane destabilisation. This is more likely to be the latter because the sapienic acid challenge transcriptome had more DE genes overlapped with the *S. aureus* response to the membrane destabilisers temporin and ovisporin than cell wall active antimicrobials. However, the *S. aureus* response to other membrane destabilising agents dermaseptin and nisin showed limited similarity. This is perplexing, as dermaseptin and ovisporin both use the carpet mechanism of membrane disruption, whilst temporin and nisin are both pore forming. Nisin is likely to have two mechanisms of action, pore formation and cell wall biosynthesis inhibition (Wiedemann *et al*., 2001; Breukink *et al*., 1997); this could partially account for the differences in the responses.

Though expression of various components of the cell wall stress stimulon are upregulated, there are some transcriptional changes that indicate the response is not the same as a cell wall stress response. Firstly, *tagA* transcription is upregulated by VraSR, but is downregulated in the sapienic acid challenge data, indicating a secondary regulatory pathway for this protein. Secondly, the genes encoding the LytSR two-component regulator were downregulated in the AFA challenge stimulon, whilst upregulated in the cell wall stress stimulon. LytSR is known to supress autolysis in *S. aureus* and *S. epidermidis* by positively regulating murein hydrolase activity (Zhu *et al*., 2010; Groicher *et al*., 2000).

Interestingly, LytSR was found to be induced by dissipation of the cell membrane electrical potential (Patton *et al*., 2006). Exposure of *S. aureus* to CAMPs, known to disrupt the cell membrane electrical potential through membrane perturbation, resulted in LytS mediated upregulated expression of the LytSR regulon (Yang *et al*., 2013). The sapienic acid mechanism of action has similarly been indicated to be through membrane perturbation or protonophore activity, and overall dissipation of cell membrane electrical

potential (Cartron *et al*., 2014; Parsons *et al*., 2012). It is therefore surprising to note that the auto-induced *lytSR* is downregulated in response to AFA challenge. A possible explanation could be that the levels of AFA used in these challenge experiments were insufficient to result in disruption of the cell membrane electrical potential, which would suggest that inferences about the mechanism of action from these data may be unreliable.

A final regulatory response apparent in both *S. aureus* and *S. epidermidis* is transcriptional change of virulence-associated loci. For example, the staphylococcal accessory regulator gene, *sarA*, was upregulated in the fatty acid challenge stimulon. SarA induces expression of fibrinogen adhesins and FAME, whilst repressing expression of proteases (Chamberlain and Imanoel, 1996; Chamberlain and Brueggemann, 1997; Chan and Foster, 1998; Cheung *et al*., 2004). SarA was shown to positively regulate the expression of toxins, though the reverse has also been indicated, suggesting this regulation may be indirect or growth-phase dependent (Cheung *et al*., 2004; Oscarsson *et al*., 2006). In support of this theory, *sarA* mutants still produced α-hemolysin, though its induction was delayed compared to wild type (Chan and Foster, 1998). Another staphylococcal accessory regulator gene, *sarZ*, was downregulated in the fatty acid challenge stimulon. SarZ mutants had reduced α-hemolysin, β-hemolysin and RNAIII production, indicating that SarZ positively regulates these proteins (Kaito *et al*., 2006).

Expression of *traP* (target of RNAIII-activating peptide) was upregulated in the AFA challenge stimulon. TraP has been reported to induce *agr* expression, biofilm formation and toxin production, with inhibition of TraP phosphorylation resulting in reduced virulence (Korem *et al*., 2005; Balaban *et al*., 2007). However, Tsang *et al.* (2007) report that TraP regulation of *agr*, biofilm formation and toxin production is a strain specific phenomenon. As *agr* was not induced in this data whilst TraP was, it is likely that in the strains tested here TraP does not induce expression of *agr*. Indeed, it was seen that TraP mutants had higher rates of spontaneous Agr mutations, which probably explains the observed differences in *agr* expression and Agr regulated genes (Kiran and Balaban, 2009). Despite this, TraP expression is still associated with virulence,

and evidence suggests it protects against oxidative stress (Yang *et al*., 2006; Kiran and Balaban, 2009).

Virulence-associated proteins can promote infection by increasing the survival of the bacteria upon or within the host. The transcriptional changes within virulence-associated networks are therefore likely to indicate the bacterial adjustment to a new niche stimulated by AFA challenge. Indeed, many of the upregulated virulence-associated genes are involved in adhesion, which are vital for niche selection and persistence.

There are strong similarities in the pattern of regulation of factors proposed to act as AFA resistance determinants. SasF has previously been shown to enhance *S. aureus* resistance to linoleic acid, as was the homologue in *S. saprophyticus*, SssF (Kenny *et al*., 2009; King *et al*., 2012). Here, *sasF* and its homologue in *S. epidermidis* were found to be upregulated in the AFA challenge stimulon. SasF has homology to the myosin cross-reactive antigen (McrA) of *Steptococcus pyogenes*, this protein and homologues in other genera have been shown to act as fatty acid hydratases (Volkov *et al*., 2010; Joo *et al*., 2012; Bevers *et al*., 2009; Rosberg-Cody *et al*., 2011). This enzyme activity converts unsaturated fatty acids into their less toxic saturated counterparts, thereby providing resistance to AFAs (King *et al*., 2012).

SasF contains a G-related albumin-binding (GA) domain, which are known to bind albumin (Johannessen *et al*., 1997). Albumin is known to bind fatty acids, and addition of bovine serum albumin can reduce palmitic acid toxicity to *S. aureus* (Parsons *et al*., 2012; Kragh-Hansen, 1981). If the McrA homologues bind to albumin, they could potentially reduce AFA toxicity by reducing the amount of AFA that interacts with the membrane.

There is a second McrA homologue gene that is upregulated within the AFA challenge stimulon and this encodes an intracellular protein. It is plausible that this protein is required for resistance from antimicrobial fatty acids that enter the cytoplasm. Alternatively, this enzyme may function in desaturation of cell membrane fatty acids, which would likely lead to a closer packed, less fluid cell

membrane; thereby reducing the damage caused by AFAs. Such fatty acid desaturases have been described in several bacterial species e.g. *B. subtilis*.

In *S. epidermidis*, *ebh* was upregulated in response to sapienic acid challenge (up to 1.85 fold). Full-length Ebh protein contains many GA domains, so could act in a similar manor to that described for SasF above. If Ebh does act similarly, this could explain why *sasF* is not as highly upregulated in the *S. epidermidis* as *S. aureus* (2.14 and 16 fold respectively). There is no upregulation of *ebh* in *S. aureus* Newman or *S. aureus* MRSA 252 in response to AFA challenge. It is possible that this represents a key difference between *S. aureus* and *S. epidermidis* response, however, transcriptional data from more *S. epidermidis* strains would be needed to test this hypothesis.

In addition to the repeats of GA domains, Ebh contains many FIVAR domains, which enable sugar-binding. Ebh is known to bind host extracellular matrix components and is suggested to play a role in adhesion (Clarke *et al*., 2002). It is possible that Ebh could also allow formation of a capsule-like structure around the cell, using host derived or excreted sugars and proteins.

Another factor previously implicated in AFA resistance is the *S. aureus* RND transporter SAR2632 (Kenny *et al*., 2009). Similarly, the RND transporter homologue in RN4220 was upregulated in response to sapienic acid challenge (Parsons *et al*., 2012). The the predicted TetR-like regulator (NWMN_2452) of this RND transporter (NWMN_2451) (as described in Chapter five) is downregulated in *S. aureus* Newman in response to sapienic acid challenge, though no significant upregulation of the transporter was observed. As discussed in Chapter five, these transporters can act to remove harmful compounds from the membrane and can export lipids (Domenech *et al*., 2005; Putman *et al*., 2000). It is plausible that these transporters pump out AFAs to provide AFA resistance. There was no homologue in Tü3298, though there are homologues in other *S. epidermidis* strains including Rp62a (Serp_0245). It would be interesting to see if this transporter would be upregulated in Rp62a in response to sapienic acid challenge.

WTA mutants were shown to be more sensitive to antimicrobial fatty acids (Kohler *et al*., 2009). WTAs are hypothesised to reduce cell surface hydrophobicity, which in turn reduces interaction or incorporation of fatty acids into *S. aureus* and reduces leakage of low molecular weight compounds (Kohler *et al*., 2009; Parsons *et al*., 2012). WTA biosynthesis genes were shown to be downregulated in the AFA challenge stimulon, which should cause increased sensitivity to AFAs if WTA does represent a resistance determinant. WTA biosynthesis genes that were downregulated included *tagA*, which catalyses the second step in WTA biosynthesis, *tagG*, which transports WTA across the cell membrane and *dltX*, a positive regulator of WTA alanylation (Xia *et al*., 2010). Additionally, when challenged with sapienic acid, *tagB*, *tagF*/*tarF* and *dltA* are downregulated in *S. epidermidis*, and *dltA, dltB* and *dltC* are downregulated in *S. aureus*. *S. aureus* cell surface hydrophobicity was observed to be reduced in response to AFAs (Kenny *et al*., 2009), however this data indicates this is not achieved through WTA.

When considering the skin environment, there could be other deleterious results of down-regulating WTA biosynthesis. WTA plays a role in CAMP and lysozyme resistance, both antimicrobials of the skin (Xia *et al*., 2010; Bera *et al*., 2007; Perego *et al*., 1995; Peschel *et al*., 1999). Further, WTA is involved in adhesion to host endothelial cells (Burian *et al*., 2010; Weidenmaier *et al*., 2004).

Undecaprenylphosphate is the precursor for WTA, this compound is also the precursor for peptidoglycan and capsule biosynthesis (Xia *et al*., 2010). Though there is evidence PG biosynthesis is downregulated in *S. epidermidis*, parts of the pathway show upregulation in *S. aureus*. Capsule biosynthesis shows clear upregulation in *S. aureus*. It is possible that decreased WTA biosynthesis would enable greater production of capsule and PG in *S. aureus*, though this is not an explanation for *S. epidermidis*. Another explanation is that teichoic acids are known to stimulate the adaptive immune response, as WTA is considered a valid target for vaccine development (Schaffer and Lee, 2009; Takahashi *et al*., 2013). If sapienic acid acts as a signal indicating to the bacteria that it is on the

skin, the response of reducing WTA could aid in evasion of the immune response.

A curiosity in WTA biosynthesis is that *tagB, tagD, tagF, tarIJ* and *tagH* are essential unless *tagO* is knocked out (D'Elia *et al*., 2006). As such, studies on the role of WTA use *tagO* mutants. TagO is not downregulated under challenge with linoleic or sapienic acid; assuming *tagO* is not regulated at the translational or protein level, undecaprenylphosphate must still enter the WTA biosynthesis pathway. Downregulation of WTA biosynthesis in favour of capsule or PG biosynthesis is therefore unlikely. Instead, as *tagA* is downregulated, there would be an accumulation of N-acetylglucosamine-undecaprenylphosphate within the cell. Further, as the teichoic acid transporter *tagG* is also downregulated, any down-stream WTA intermediates that were produced would also accumulate intracellularly.

Mutants of genes for WTA biosynthesis beyond the initial stage are lethal; it is proposed that, once WTA biosynthesis has been initiated, it must be completed or lethal intermediates accumulate (D'Elia *et al*., 2006). Subsequent research has indicated that inhibition of late steps of WTA biosynthesis subvert undecaprenylphosphate from PG biosynthesis (D'Elia *et al*., 2009). Further, depletion of TagD in *B. subtillis* gave a similar transcriptional response to cell wall active antibiotics (D'Elia *et al*., 2009). This could indicate that the downregulation of *tagA* could be sequestering undecaprenylphosphate, and resulting in the observed increase in genes of the cell wall stress stimulon. This does not explain why WTA toxic intermediates are retained within the cell by downregulation of tagG. It is possible that these WTA intermediates actually facilitate an AFA resistance mechanism. This theory would be entirely consistent with the observed reduction in AFA resistance in TagO mutants. Treating staphylococci with a sub-inhibitory concentration of targocil, an inhibitor of TarG (Campbell *et al*., 2012), prior to AFA challenge could help to determine if WTA intermediates have any protective effect against AFAs.

Another gene that could affect WTA and lipoteichoic acid (LTA) synthesis is *gtaB*, which is downregulated in the AFA challenge stimulon. GtaB is required for the synthesis of UDP-glucose, used to glycosylate WTA and which is also a

precursor for glycolipid synthesis and diglucosyl-diacylglycerol, used as a cell membrane anchor for LTA (Grundling and Schneewind, 2007). Whist LTA is still found in the cell membrane of *gtaB* mutants, it is anchored to diacylglycerol rather than diglycosyl-diacylglycerol; this change can cause aberrant morphology and increased cell size (Grundling and Schneewind, 2007). In *B. subtillis*, intracellular accumulation of WTA intermediates through *tagD* depletion led to cells with bloated sphere morphology, cell division defects and thickening of the cell wall prior to death (Bhavsar *et al*., 2001).

One outcome of mutations in late WTA biosynthesis and *gtaB* is increased cell size. Increased volume to cell ratio has been described as a resistance mechanism against osmotic stress (de Goffau *et al*., 2009; de Goffau *et al*., 2011), and may potentially offer resistance against AFAs by effectively diluting the percentage of AFAs incorporated into the membrane.

A further resistance determinant considered important for acid resistance, though not AFA resistance, is ammonia production. Genes encoding urease were strongly upregulated in the *S. aureus* acid shock transcriptome (Bore *et al*., 2007) and the strain Newman sapienic acid challenge transcriptome. The authors suggest that ureases may offer acid resistance by neutralising the acid, as has been seen in *Helicobacter pylori* (Bore *et al*., 2007). In Tü3298, ureases are only slightly upregulated, whilst the nitrate reductase pathway and *arcC* of the ammonia deiminase pathway, which both produce ammonia, are upregulated. Ammonia production through arginine deiminase activity has previously been hypothesised to enhance survival on the skin by neutralising the acidic environment of the skin (Diep *et al*., 2006; Thurlow *et al*., 2013). As fatty acids are thought to acidify their environment, ammonia production could be acting to counteract this. Indeed, the overlap between transcription upon AFA challenge and acid challenge and upregulation of other acid resistance determinants, suggests AFAs are sensed similarly to acids in staphylococci.

Ammonia has also been shown to have a more direct effect on fatty acids. Ammonia reacts with fatty acids leading to the formation of less saturated, nitrogenous derivatives (Black *et al*., 1978). These nitrogenous derivatives have increased polarity making them more hydrophilic (Black *et al*., 1978). With the

consensus that a key mechanism of action of fatty acid is membrane insertion, these more hydrophilic derivatives are likely to be less potent. In addition, saturated fatty acids are less antimicrobial than their unsaturated counterparts (Galbraith and Miller, 1973; Desbois and Smith, 2010). This reaction was not reported to occur in anaerobic conditions, so would be less frequent at deeper layers of the skin.

Alternatively, the ammonia produced may not be a defence mechanism and could be recycled as a precursor for amino acid biosynthesis (Doroshchuk *et al*., 2006).

Ammonia production genes were also upregulated in *S. aureus* MRSA252 in linoleic acid exposure conditions (0.01 mM linoleic acid from inoculation to mid-log phase) through the arginine deiminase pathway of the arc operon (Kenny *et al*., 2009). MRSA252 showed an up to 20 fold increase in survival on plates containing linoleic acid following overnight growth with 0.01 mM linoleic acid (see section 3.3.3).

Another gene upregulated in the AFA challenge regulon that would be tempting to associate with AFA resistance or cell wall stress is the metallo-β lactamase superfamily protein (encoded by *setu_01270*, *nwmn_1601* and *sar1785*). Metallo-β lactamases provide resistance against β-lactam antibiotics, which inhibit cell wall biosynthesis, through enzymatic hydrolysis. Of note, the membrane bound β-lactamase penicillinase of *S. aureus* was found to enhance the antimicrobial effects of long chain unsaturated fatty acids through a direct interaction with the fatty acids (Greenway and Dyke, 1983; 1979). However, there are 16 documented sub-groups within the metallo-β lactamase superfamily, with diverse enzymatic functions ranging from DNA repair to oxidoreductase activity (Daiyasu *et al*., 2001). The metallo-β lactamase superfamily protein in this data is approximately 230 aa in length, with a β-lactamase domain spanning from approximately 10 to 200 aa. This makes this protein most similar to group 0 metallo-β lactamases, whose function is unknown (Daiyasu *et al*., 2001). It is tempting to speculate that AFAs may interact directly with these upregulated metallo-β lactamase superfamily

proteins, similar to their interaction with penicillinase. A direct interaction may result in resistance if the protein has some detoxifying activity, alternatively their upregulation could then be due to a feedback loop caused by inhibition of their activity by AFA interaction.

4.4.1 Summary

There were several aims for the analysis of the transcriptional response to sapienic acid challenge. The primary aim was to compare the DE genes of *S. aureus* and *S. epidermidis* challenged with sapienic acid to find potential resistance determinants. Overall, it was found that many of the resistance determinants utilised by *S. aureus* were also used by *S. epidermidis*. There were more described AFA resistance determinants in *S. aureus* than *S. epidermidis*, including those not common to both species, such as capsule and staphyloxanthin biosynthesis. There were no obvious functionally analogous alternatives to these resistance mechanisms in *S. epidermidis*, though as these mechanisms are energetically expensive, perhaps this is actually an advantage. It is probable that the factors leading to the enhanced AFA resistance of *S. epidermidis* are poorly characterised. Though the volume of poorly characterised DE genes particulary present in *S. epidermidis* hindered further investigations to find *S. epidermidis* AFA resistance determinant candidates, a strongly upregulated gene cluster of poorly characterised genes was found that seems a strong lead.

A second aim for this work was to give an indication of the sapienic acid mechanism of action. The data indicates that sapienic acid likely interacts with the membrane in a manner similar to CAMPs and acid shock, meaning it disrupts the electrochemical gradient and possibly intracellular pH. There is some evidence that AFAs also cause protein damage, though this could also be part of a general response to stress. If AFAs affected the intercellular pH as has been indicated previously (Cartron *et al*., 2014), there would likely be damage to proteins.

One particularly useful technique used to narrow down the genes of importance was generating an AFA challenge stimulon from comparison with previous work. However, the AFA challenge stimulon is likely to be missing some of the key responses, including downregulation of fatty acid biosynthesis, because genes in the same pathway are DE but not necessarily the same genes. The AFA challenge stimulon could be improved in future by producing RNA-seq data for more *S aureus* and *S. epidermidis* strains challenged with AFAs. Further, this would enable identification of the core response in *S. epidermidis* versus *S. aureus*, or high resistance versus low resistance isolates. This would likely enable the key genes in fatty acid resistance to be discovered.

Determining the genes not found in any other transcriptional response is another method to discover key genes or pathways involved in response to the condition being tested. This method could be employed in future studies to provide an indication of specific AFA or sapienic acid responses.

Chapter 5 Experimental evolution of *S. aureus* and *S. epidermidis* to skin lipids

5.1 Introduction

Whole genome comparison of antimicrobial-resistant strains and susceptible strains has been previously described as a method to identify resistance determinants. The technique is most powerful when a resistant isolate is compared to a susceptible isogenic or parent strain. This technique has been used to identify genetic determinants for vancomycin- and glycopeptide-intermediate *S. aureus* (VISA and GISA respectively) and daptomycin-resistant *S. aureus* (Mwangi *et al*., 2007; Howden *et al*., 2011; Renzoni *et al*., 2011; Peleg *et al*., 2012). Some of these reports have used isogenic clinical isolates (Mwangi *et al*., 2007; Howden *et al*., 2011), whilst others have used laboratory-evolved isolates (Renzoni *et al*., 2011; Song *et al*., 2013b) and still others have used a combination of both (Peleg *et al*., 2012).

Use of laboratory-evolved isolates has the advantage that the evolution of resistance observed is specific to the antimicrobial used. However, antimicrobial specific evolution could also be considered a disadvantage, as interaction of the antimicrobial with host factors may require different or multiple resistance determinants due to possible synergistic interactions. Clinically-evolved isolates can also gain resistance determinants through horizontal gene transfer from other members of the host microflora, whilst this cannot happen in typical experiments that generate laboratory-evolved isolates.

In the reports that generated laboratory-evolved resistance, isolates were developed by passaging *S. aureus* in the antimicrobial of interest either once or through serial passage with stepwise increases of the antimicrobial concentration (Renzoni *et al*., 2011; Song *et al*., 2013b). In the reports that studied clinical isolates, isolates were collected from patients during routine swabbing. Isolates that were sequenced were selected from patients with an antibiotic susceptible infection that developed into an antibiotic resistant infection. In both instances, increases in resistance were confirmed using MIC-

based methods. For clinical isolates, measures were taken to confirm the strains were isogenic.

Insights to be gained from experiments that compare genomes of antimicrobial resistant and susceptible isolates are multifold. Firstly, these experiments determine if it is possible for resistance to develop to the antimicrobial. Secondly, since mutations that lead to resistance frequently occur within the target of the antimicrobial, these experiments may help clarify the mechanism of action of the antimicrobial. Finally, these experiments enable discovery of genes and modification of genes that might act as resistance determinants.

Genes involved in resistance can provide protection through four main mechanisms. Firstly, resistance may be provided by alteration of the antimicrobial by a product of the bacteria, usually through enzymatic cleavage or modification of the antimicrobial. Enzyme-mediated resistance is typically specific to a given class of antimicrobial, such as the enzyme penicillinase which is active against β-lactam antibiotics (Chambers and Deleo, 2009).

Secondly, resistance may be provided by mechanisms that inhibit the antimicrobial accessing its target. For example, the proteins MprF and Dlt increase staphylococcal cell surface negative charge, thereby repelling cationic antimicrobials and preventing them from interacting with their target (Peschel *et al*., 2001; Peschel *et al*., 1999).

Thirdly, resistance may be gained by modification of the target so that the antimicrobial can no longer form a harmful interaction. If the antimicrobial target is a cell component this may mean alteration of the component through activity of an enzyme, as is seen in staphylococcal lysozyme resistance via OatA. OatA O-acetylates the C6-OH of peptidoglycan, so lysozyme can no longer interact and cleave peptidoglycan (Bera *et al*., 2005). If the antimicrobial target is a protein, the target may be altered through mutation of the gene or acquisition of a functionally redundant mutant version of the gene; this is seen in penicillin resistance with modification of the penicillin binding protein (PBP). Modified versions of PBP have lower affinity for penicillin than the native protein (Chambers *et al*., 1994).

Finally, resistance can be supplied through active expulsion of the antimicrobial. This mechanism of resistance is usually mediated by efflux pumps, such as the tetracycline efflux protein that provides resistance to tetracycline (Guay and Rothstein, 1993).

5.2 Aims

Skin colonisation by staphylococci must rely on some innate resistance to antimicrobial skin lipids. However, staphylococcal tolerance of skin lipids could be considered low, particularly in comparison to other dominant skin colonisers such as corynebacteria (Fischer *et al*., 2012). This comparably low skin lipid resistance raises the question of whether staphylococci are capable of evolving resistance to antimicrobial skin lipids. The data from Chapter three showed there was a significant range of resistance levels to sapienic acid, indicating that evolution of resistance to sapienic acid is possible. Data from Chapter three also showed there was not a significant range of resistance levels to D-sphingosine, suggesting that evolution of resistance to D-sphingosine may not occur naturally. In this Chapter, the aim was to test whether resistance could be evolved experimentally in strains of *S. epidermidis* to sapienic acid and *S. epidermidis* and *S. aureus* to D-sphingosine. This is an important consideration if these compounds are to be developed as therapeutic agents as has been suggested previously (Desbois and Smith, 2010; Vidal *et al*., 2014; Desbois and Lawlor, 2013).

The mechanism of action of antimicrobial lipids is still uncertain, particularly whether the mechanism of action is consistent across lipids or varies with their structural properties, as discussed in the introduction. A further aim of this Chapter was therefore to determine the foci of resistance selection to antimicrobial lipids for *S. aureus* and *S. epidermidis*. Determining the selective pressures of the antimicrobial lipids may help validate predicted mechanisms for these antimicrobials or give rise to new hypotheses.

The main aim of this Chapter was to identify genes or mutations in genes that act as resistance determinants to sapienic acid and D-sphingosine. As previously discussed, a degree of resistance to antimicrobial skin lipids, particularly to sapienic acid, is hypothesised to be a vital factor for survival on skin. Finding genes potentially responsible for variations in staphylococcal resistance to antimicrobial lipids will therefore aid our understanding of how staphylococci survive on human skin.

5.3 Results

This Chapter explores the evolution of genetic mutations that increase resistance of *S. aureus* and *S. epidermidis* to antimicrobial skin lipids. To achieve this, *S. epidermidis* Rp62a and *S. aureus* SH1000 were evolved through passage in broth containing stepwise increases in antimicrobial lipid concentration, described in more detail below. The genomes of selected isolates with increased resistance were sequenced along with their parental strain to identify Single Nucleotide Polymorphisms (SNPs) and INsertions and DELetions (INDELs) associated with the observed increase in resistance.

A second round of sequencing was undertaken for sapienic acid evolved *S. epidermidis* to compare the data available from sequencing individual evolved isolates versus sequencing a pool of isolates. Sequencing of pools enables more isolates to be sequenced without additional costs, potentially revealing more SNPs or INDELs. Sequencing a greater number of isolates should also help to highlight the SNPs that have been maintained in the population, and are therefore more likely to be involved in resistance.

S. epidermidis Rp62a was evolved to grow in high concentrations of D-sphingosine or sapienic acid. This was achieved by passage of the wild-type isolate in broth containing 5 % ethanol and antimicrobial lipid. The concentration of antimicrobial lipid in the broth was increased when the culture grew to an optical density comparable to growth of a control culture passaged in 5 % ethanol. Since Rp62a grew poorly in 5 % ethanol, the wild type was passaged once in 5 % ethanol prior to passages with antimicrobial lipids; the resultant strain was taken as the day 0 isolate. Samples of culture from different days of the subsequent evolution experiment were frozen at -80 °C in 15 % glycerol for future analysis.

A masters student within the lab group used a similar method to that described above to evolve *S. aureus* SH1000 to D-sphingosine (Campbell-Lee, 2012). There were some differences between the methods used for SH1000 evolution and Rp62a evolution that may impact on results. Firstly, SH1000 cultures were plated between some passages and an individual isolate selected for the subsequent passage. Secondly, the concentration of ethanol in SH1000 cultures

was not consistent across passages, so as the D-sphingosine concentration increased so did the ethanol concentration. The final major differences were that no ethanol control culture was passaged alongside the test cultures and the day 0 isolate was the same as the wild type SH1000.

Evolved isolates were named according to their strain, the antimicrobial used during the evolution, the number of days they had been passaged for and the specific isolate. Strains used were either *S. epidermidis* Rp62a or *S. aureus* SH1000, denoted by either Rp- or SH- respectively. The antimicrobials used were denoted by Ds for D-sphingosine, Sap for sapienic acid and Eth for the ethanol control; for the Rp62a day 0 isolate no classification was used. The number of days the isolates had been passaged for was represented numerically, whilst if multiple isolates had been collected a letter indicated the specific isolate. For example, the Rp62a day 0 isolate was called Rp-0, whilst the third SH1000 isolate from the third day of passage in D-sphingosine was called SH-Ds3C. Isolates that were pooled (selected from isolates Rp-Sap5F-Y) to create the pooled data set were referred to as Rp-SapPool after they had been pooled.

5.3.1 Susceptibility of experimentally evolved isolates to antimicrobial lipids

At the end of the *S. epidermidis* Rp62a and *S. aureus* SH1000 passages, these strains were growing in higher concentrations of D-sphingosine or sapienic acid than they were capable of growing in at the beginning of the experiment. It was necessary to assess if growth in increased concentrations of antimicrobial lipids was due to transcriptional changes or stable genome mutations. MIC values for individual isolates from each stored passage sample were determined using a microbroth dilution assay as used in Chapter three. Unchanged MIC levels would indicate transcriptional changes or unstable genome mutation, as these would be lost after a subsequent passage without antimicrobials. Increased MIC would indicate a stable genome mutation, as these would not be lost following a passage without antimicrobials.

D-sphingosine MIC levels increased up to 16 times the original value for SH1000 isolates experimentally evolved to D-sphingosine (Fig. 5.1A). This indicates that the increased resistance observed was due to genetic changes rather than adaptation. There were three observed stepwise increases in MIC, which might indicate that at least three key genetic changes occurred over the course of the experimental evolution.

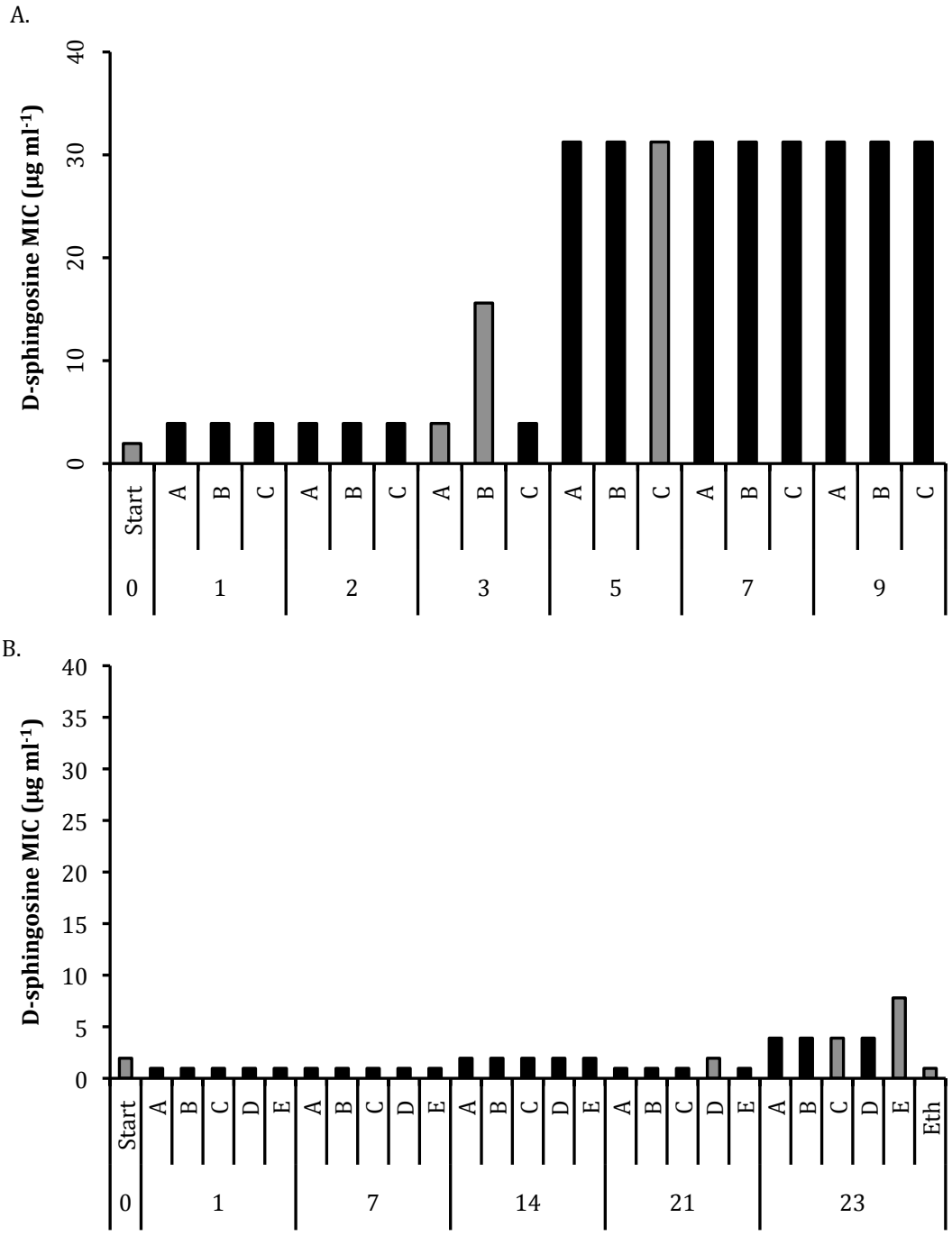


Figure 5.1 D-sphingosine MICs of D-sphingosine evolved *S. aureus* SH1000 and *S.epidermidis* Rp62a isolates.

A) *S. epidermidis* Rp62a, B) *S. aureus* SH1000. Passages are identified by a number and individual isolates are indicated by a letter. Isolates that were selected for sequencing are indicated by the grey bars. Eth indicates a 23-day isolate passaged in 5% ethanol.

D-sphingosine MIC levels for *S. epidermidis* experimentally evolved in D-sphingosine (Fig. 5.1B) initially decreased prior to an increase in resistance. This initial decrease was within the standard range of error for MIC tests. The observed increases of resistance levels in the day 23 isolates is suggestive of at least two genetic changes that increase Rp62a resistance to D-sphingosine up to 4 times the original MIC. The endpoint level of resistance observed in D-sphingosine evolved Rp62a isolates are still considerably lower than those observed in *S. aureus* D-sphingosine evolved isolates.

The sapienic acid MIC values observed for Rp62a isolates were higher than expected, with little variation using previously established concentrations (Fig. 5.2A). When higher concentrations were used for the assay, all Rp62a evolved strains tested showed higher resistance to sapienic acid than Rp-0 and Rp-Eth23 (Fig. 5.2B), however results were considered dubious because all MIC values were much higher than previously determined. This apparent increased resistance to sapienic acid may be a result of genetic changes caused by the initial passage in 5 % ethanol prior to experimental evolution with antimicrobial lipids, but this was not confirmed. These results indicate that the sapienic acid MIC method was not a reliable method for determining alterations in resistance. Subsequently, the previously described assay that assesses levels of survival on agar plates containing linoleic acid was used to determine changes in resistance to fatty acids.

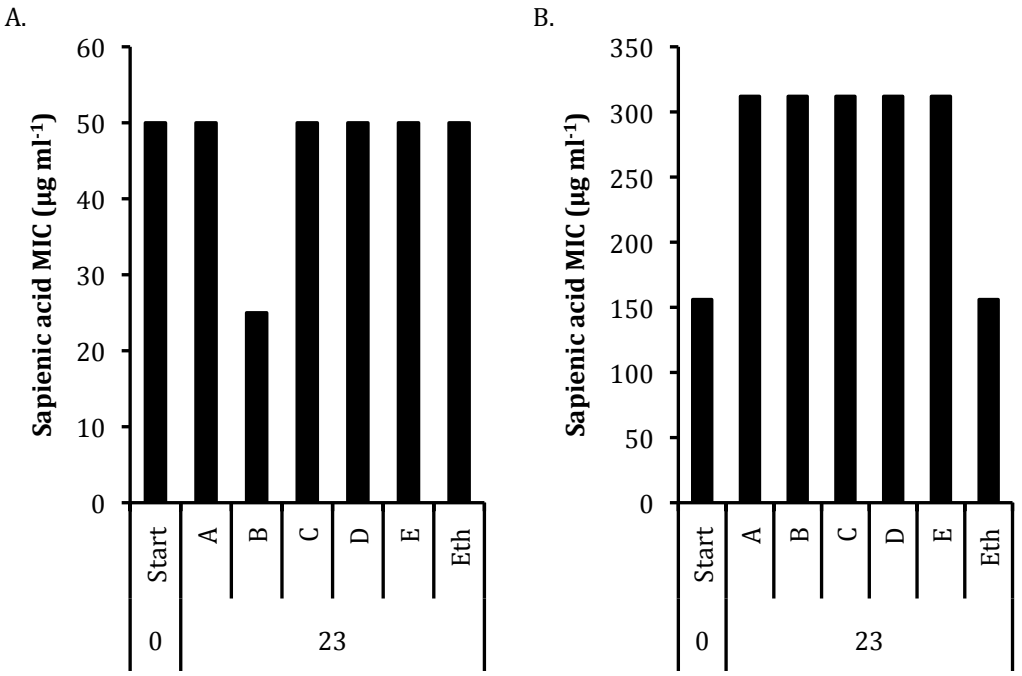


Figure 5.2 Observed MICs for *S. epidermidis* Rp62a sapienic acid evolved isolates

MICs were produced using a stock of (A) 400 µg/ml or (B) 2500 µg/ml. Eth indicates a 23-day isolate passaged in 5% ethanol.

Survival on linoleic acid agar plates was greatly increased for sapienic acid evolved Rp62a isolates from day 21 and 23, with some isolates from days 7 and 14 also showing appreciable, though lower, increases in survival (Fig. 5.3). This indicates that genetic changes leading to increased resistance to fatty acids occurred in sapienic acid evolved Rp62a with increasing passages. Evolved isolates Rp-sap21D and Rp-Ds-23C also displayed survival > 100 % (Fig. 5.3 and 5.4 respectively); this could be indicative of an evolved requirement for the presence of a lipid for cells to achieve optimal viability.

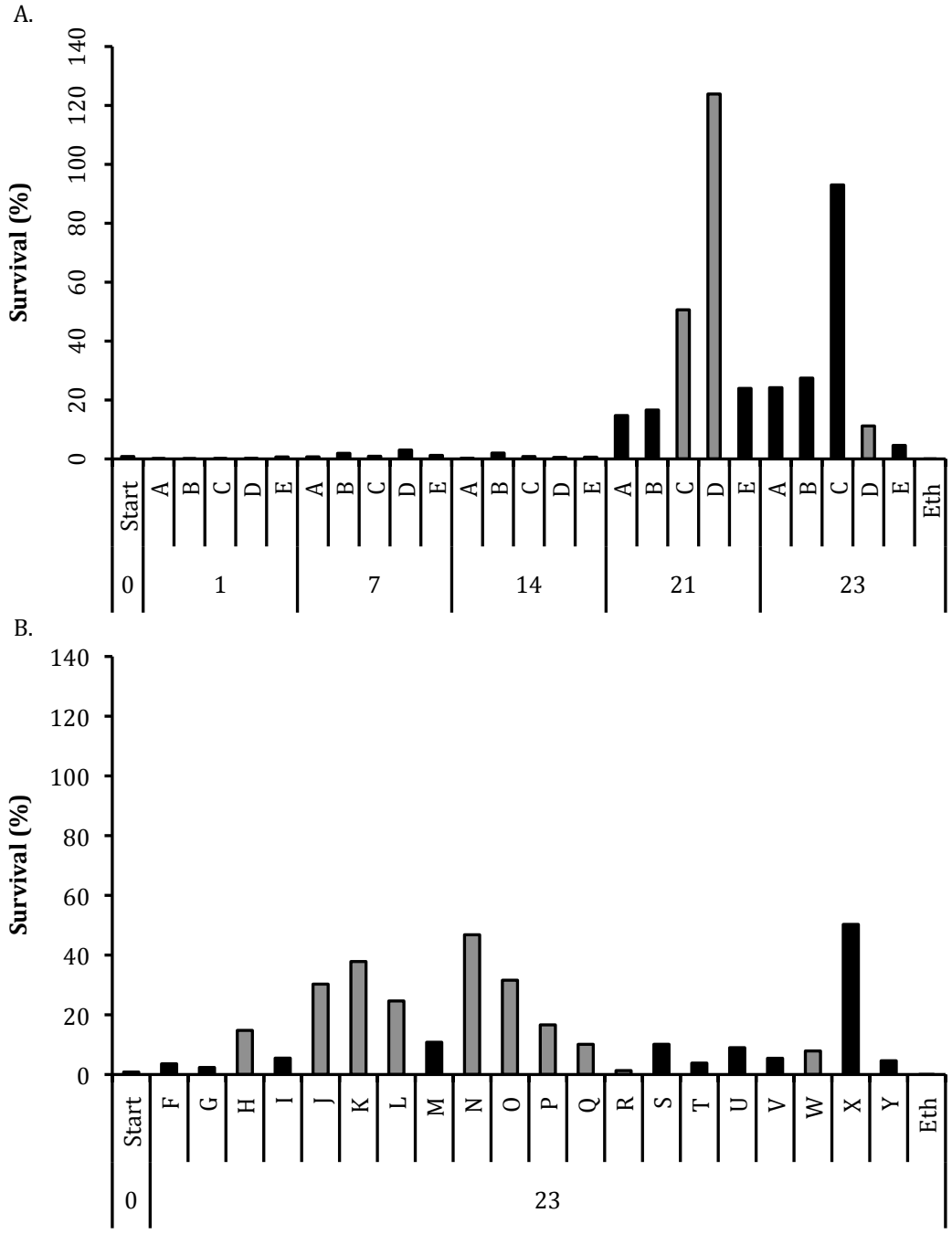


Figure 5.3 Survival of *S. epidermidis* Rp62a sapienic acid evolved isolates on 5 mM linoleic acid agar plates.

Serial dilutions of isolate overnight cultures were plated onto agar with or without 5 mM linoleic acid. The survival of these isolates on linoleic acid agar was compared as a percentage of growth on plates without linoleic acid. Isolates from the initial screen (A), and isolates screened for selection of mutants for the pooled isolates sample (B). Passages are identified by a number, and individual isolates are indicated by letter. Survival of isolates that were selected for sequencing are indicated by the grey bars, whilst resistance of un-sequenced isolates are indicated by black bars. Eth indicates a 23-day isolate passaged in 5 % ethanol.

Of note, D-sphingosine evolved SH1000 and Rp62a endpoint isolates (day 23) showed enhanced linoleic acid survival using the agar plate based assay (Fig. 5.4A and 5.5). However, sapienic acid evolved Rp62a endpoint isolates did not show any increased resistance to D-sphingosine, with the majority showing reduced resistance (Fig. 5.4B).

The Rp-Eth23 isolate, evolved in 5 % ethanol, had reduced D-sphingosine resistance in the microdilution assay, and over 2-log fold decreased survival in the linoleic acid agar plate-based assay compared to Rp-0. This control data supports the assertion that increased resistance is due to the presence of antimicrobial lipids in the experimental evolution experiment and not due to the presence of ethanol.

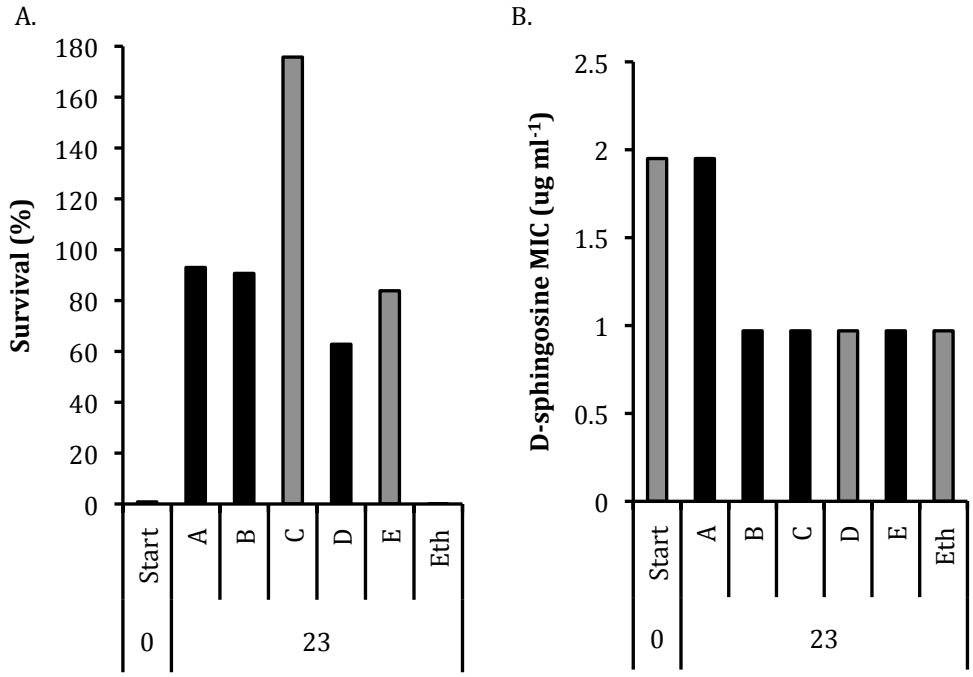


Figure 5.4 Tolerance of *S. epidermidis* Rp62a D-sphingosine or sapienic acid evolved isolates exposed to other antimicrobial lipids.

(A) Survival of Rp62a isolates evolved to D-sphingosine on 5 mM linoleic acid plates. (B) D-sphingosine MIC levels for Rp62a isolates evolved to sapienic acid. Passages are identified by a number, and individual isolates are indicated by letter. Isolates that were selected for sequencing are indicated by the grey bars.

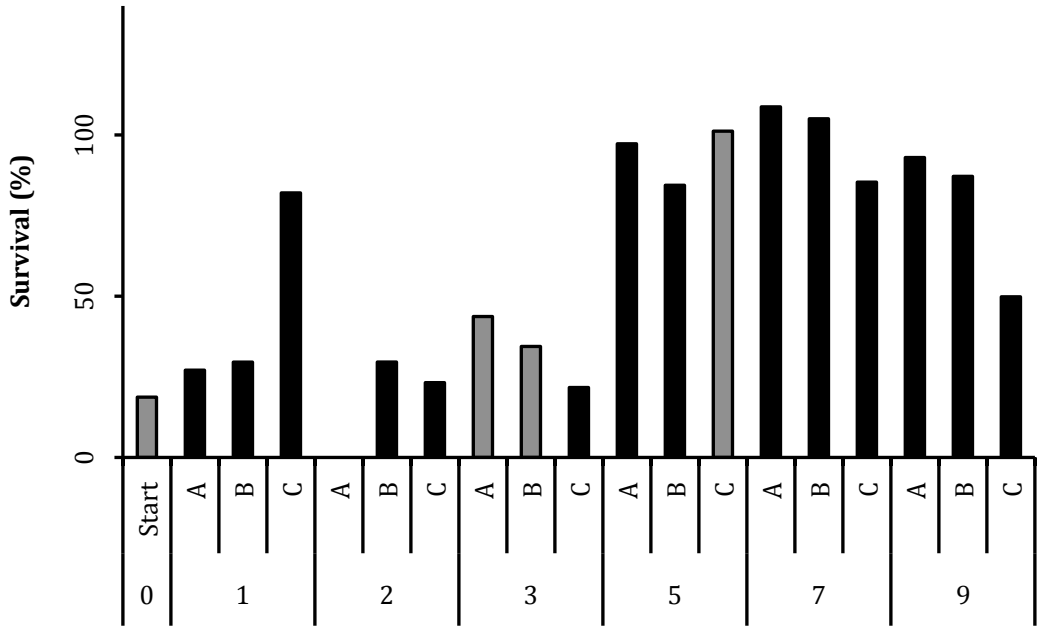


Figure 5.5 Survival of *S. aureus* SH1000 D-sphingosine evolved isolates on 5 mM linoleic acid agar plates.

Passages are identified by a number, and individual isolates are indicated by letter. Isolates that were selected for sequencing are indicated by the grey bars.

5.3.2 Sequencing QC and alignment statistics

Sequencing of isolates with increased resistance to antimicrobial lipids was performed to determine SNPs or INDELs that may account for changes in resistance and provide indications of potential resistance mechanisms. Further, genes containing SNPs or INDELs were expected to be involved in components or processes in the cell under pressure due to the presence of the antimicrobial lipids or within enzymes and components mediating resistance.

Prior to sequencing, DNA samples were checked to confirm they were of sufficient quality and quantity for sequencing. All genomic DNA samples used for sequencing had low protein, salt and solvent contamination, as indicated by 260/280 & 260/230 ratios greater than 1.8 (Table 5.1). DNA samples were also determined to contain a sufficient yield of intact gDNA, as indicated by Qubit reads and gel electrophoresis.

Table 5.1 Quality control analysis results for DNA samples of *S. aureus* and *S. epidermidis* submitted for sequencing.

Table shows the required quality checks for DNA prior to sequencing, these are concentration- as assessed by Qubit- and absorbances 260/280 and 260/230 as assessed by Nanodrop.

Strain	Isolate	DNA concentration (ng/µl)	Absorbance 260/280	Absorbance 260/230
S. aureus SH1000	SH1000	130	2.0	2.3
	SH-Ds3A	146	2.0	2.3
	SH-Ds3B	46.2	1.8	1.9
	SH-Ds5C	176	1.9	2.3
S. epidermidis Rp62a	Rp-Ds23E	37.7	1.9	2.1
	Rp-Ds23C	133	1.9	2.2
	Rp-Ds21D	37.8	1.9	2.1
	Rp-Sap23D	41.3	1.8	2.2
	Rp-Sap21D	59.6	1.9	1.9
	Rp-Sap21C	62	2.0	2.0
	Rp-0	56.6	2.0	2.0
	End	55.6	2.0	1.8
	RpSapPool	37.3	2.1	2.1

Sequence reads were aligned to reference genomes using the Burrows-Wheeler aligner (BWA). Analysis of the alignment data (Table 5.2) reveals more than 97 % of reads aligned to the reference genomes, with approximately 95 % of reads from each sample mapping to just one position in the reference genome (uniquely mapped reads). There was a considerable percentage of exactly duplicated reads in the Rp-SapPool sample, indicating PCR duplicates were sequenced multiple times which is to be expected as this sample was sequenced to higher depth than the other samples sequenced. The mean coverage of the sequencing was in excess of 100 X for all samples, which provides ample depth for SNP and INDEL identification.

Table 5.2 Mapping statistics for sequenced *S. aureus* and *S. epidermidis* evolved isolates.

Table shows the percentage of reads mapped before and after duplicate reads and multiply mapping reads were filtered, as well as some coverage per base and per genome statistics.

Strain	Isolate	Total no. reads	Total reads mapped (%)	Unique and Non-duplicate reads (%)	Mean coverage	Genome covered (%)
S. aureus SH1000	Eth con	2992974	98.25	69.85	193.45 ±26.59	94.81
	SH1000	1998524	99.08	79.96	145.7 ±18.9	94.61
	SH-Ds3A	2046926	99.14	79.57	148.43 ±21.02	94.62
	SH-Ds3B	1841456	99.05	81.30	135.67 ±17.68	94.59
	SH-Ds5C	2201392	98.98	78.61	158.27 ±19.11	94.6
S. epidermidis Rp62a	Eth con	1950152	98.63	78.74	146.23 ±17.24	98.14
	Rp-0	1827680	98.48	79.42	138.3 ±16.62	98.16
	Rp-Sap21C	1702790	98.38	79.99	129.15 ±17.4	98.1
	Rp-Sap21D	1370884	97.98	81.85	106.04 ±14.29	98.13
	Rp-Sap23D	1999958	98.89	77.26	157.5 ±26.05	91.15
	Rp-Ds21D	1853358	98.84	79.53	139.82 ±17.13	98.15
	Rp-Ds23C	2023980	99.28	78.39	150.24 ±17.99	98.15
	Rp-Ds23E	2110692	99.23	77.95	155.61 ±21.57	98.17
	Rp-SapPool	14981371	98.54	31.23	453.63 ±38.3	98.24

5.3.3 SNPs and INDELs

DNA from evolved Rp62a and SH1000 was sequenced and the sequence reads aligned to reference genomes. After alignment, SNPs and INDELs were called and filtered using a bespoke perl script, which utilised the SNPEff open source software. SNPs and INDELs that were synonymous or intergenic and more than 200 bp upstream of a transcriptional start site were filtered out. A second

bespoke perl script was used to filter SNPs or INDELs identical to any found in the Rp-0 or Rp-Eth23 for Rp62a evolved isolates, or SH1000 wild type for SH1000 evolved isolates. The remaining SNPs and INDELs were considered candidates for genes affecting resistance.

To assess the impact mutations may have upon proteins thought to be key in resistance levels, the original structure of these proteins was modelled and the location of the SNPs overlaid. Investigation of the roles of domains within the model where the SNPs occur was then used to further knowledge of how the SNP may affect the protein function.

5.3.4 *S. epidermidis* Rp62a sapienic acid induced SNPs and INDELs

As discussed above, Rp62a isolates experimentally evolved to sapienic acid were tested for increased linoleic acid resistance to fatty acids using the agar plate-based assay. This assay indicated that, of the sequenced strains, resistance to linoleic acid was in the order Rp-Sap21D>Rp-Sap21C>Rp-Sap23D>Rp-0>Rp-Eth23. Pooled samples survival levels were tested on a separate day, so cannot be directly compared due to the variability of the linoleic agar plate assay. However, though all pooled isolates showed higher resistance than Rp-0, resistance levels were not uniformly at the highest.

All of the sapienic acid evolved Rp62a isolates, including those from the Rp-SapPool sample, had a SNP in *yycG*, leading to YycG.R378H (Table 5.3). This is the only SNP in Rp-Sap21C within a coding region and not shared by Rp-0 or Rp-Eth23. It is therefore likely that YycG.R378H contributes to increased fatty acid resistance. YycG is part of an essential two-component regulator known to regulate cell wall biosynthesis proteins.

Table 5.3 Non-synonymous, homozygous SNPs and INDELs from Rp62a sapienic acid evolved isolates.

SNPs and INDELs from *S. epidermidis* Rp62a sequenced evolved isolates were determined using a bespoke perl script. All mutations were confirmed by at least 6x coverage and were classed as homozygous if > 80 % of reads had alternative bases to the reference genome. Intergenic SNPs were included if they were less than 200 bases upstream of the predicted translational start site.

Official gene symbol	Gene function	Isolates	Position of change	Base change	Change	Position in protein
yycG	Histidine kinase, regulates cell wall turnover	Rp-SapPool, Rp-Sap21C, Rp-Sap21D, Rp-Sap23D	2589940	C/T	Substitution R/H	378 aa
gcvT	Glycine cleavage system aminomethyl-transferase T	Rp-Sap21D	1146571	G/A	Substitution S/F	74 aa
SERP1544	Phage lipoprotein	Rp-Sap21D	1596978	G/A	Substitution P/S	52 aa
SERP1061	Fur transcriptional regulator	Rp-Sap23D	1106479	C/A	Substitution D/Y	49 aa
SERP0499	Iron-sulphur cluster scaffold protein	Rp-Sap21D	489097	+ATG GCT	Insertion +MA	29 aa
SERP2127	FeoA family, likely Fe^{2+} transport	Rp-Sap21C	2152015	G/A	Upstream substitution	-42 bp
SERP0057	Hypothetical protein	Rp-SapPool Rp-Sap23D	46171	A/G	Upstream substitution	-102 bp
SERP0058	Hypothetical protein	Rp-SapPool Rp-Sap23D	46171	A/G	Upstream substitution	-41 bp

Since YycG.R378H potentially contributes to enhanced linoleic acid resistance, YycG was modelled and the location of the SNP investigated. As shown in Figure 5.6, YycG has a HAMP domain, a PAS domain and a histidine kinase domain, it also has two transmembrane domains and an extracellular domain not shown. The HAMP and PAS domains are involved in signal sensing, whilst the histidine kinase domain can activate YycF, a transcriptional regulator, if it is itself activated by the signal and dimerisation. The mutation YycG.R378H occurs in the hairpin of the histidine kinase domain, this hairpin is essential for

dimerisation. The R378H change from an arginine to a histidine residue alters the side chain and potentially the charge, dependent on the pH of the cytosol. These changes may destabilise the alpha helix or interactions between the alpha helices, potentially altering YycG dimerisation.

Figure 5.6 Protein model of YycG with highlighted SNP R378H.

The model was constructed using HHpred and coloured using Pymol. The image shows the protein domains: HAMP (yellow), PAS (green), histidine kinase ATPase domain (dark blue) and histidine kinase dimerisation domain (light blue). SNP R378H is highlighted in red.

Isolate Rp-Sap23D has one more coding SNP than Rp-Sap21C, SERP1061.D49Y. *serp1061* encodes the ferric uptake regulator (FUR) protein, which regulates iron transport by transcriptional repression when bound to iron. It is possible that this SNP accounts for the decrease in survival observed between Rp-Sap21C and Rp-23D. SNP SERP1061.D49Y is also present in approximately 30 % of the reads from the Rp-SapPool data, indicating approximately 3 of the 10 pooled isolates also had this SNP (Table 5.4). Isolate Rp-sap23D was also shown to have decreased resistance to D-sphingosine than the Rp-0 isolate. It was not tested if isolates Rp-Sap21C and Rp-Sap21D also have reduced resistance to D-sphingosine, so either SERP1061.D49Y or YycG.R378H are likely candidates for the observed reducion in D-sphingosine resistance levels.

Table 5.4 Non-synonymous, heterozygous SNPs and INDELs from Rp62a sapienic acid evolved isolates.

SNPs and INDELs identified from sequencing of *S. epidermidis* Rp62a evolved isolates were determined using a bespoke perl script that filtered out results from non-coding regions. All mutations were confirmed by at least 6x coverage and were classed as heterozygous if < 80 % of reads had alternative bases to the reference genome. Intergenic SNPs were included if they were less than 200 bases upstream of the predicted translational start site.

Official gene symbol	Gene function	Isolates	SNP position	Base change	Change	Codon no.	% of reads
mqo-3	Malate: quinone oxidoreductase	Rp-SapPool	2351205	A/G	Substitution D/G	402 aa	66.6
SERP1061	Fur transcriptional regulator	Rp-SapPool	1106479	C/A	Substitution D/Y	49 aa	31.0

If SERP1061.D49Y does not decrease the linoleic acid resistance of Rp-Sap23D compared to Rp-Sap21C, it is possible that intergenic SNPs in this isolate decrease resistance. Rp-Sap23D has an intergenic SNP in the potential promoter region for *serp0057* and *serp0058*, 108 and 42 bp upstream of the transcriptional start sites for these genes, respectively. Both of these proteins are hypothetical with unknown function, so it is difficult to suggest what involvement they may play. However, it is notable that SERP0057 is a 207 amino acid secreted protein, as determined by using the SignalP4.1 server (http://www.cbs.dtu.dk/services/SignalP/).

Alternatively, an intergenic SNP in Rp-Sap21C may increase the resistance of this isolate over that of Rp-Sap23D. Rp-Sap21C has a base change 42 bp upstream from the transcriptional start site of *serp2127*, a likely $Fe2^{+}$ transporter (FeoB)-associated protein. As SERP1061.D49Y and the upstream SNP from *serp0057* and *serp0058* all occur with some frequency in the pool, this could indicate that none of these SNPs reduce sapienic acid resistance. If so, the intergenic SNP upstream of *serp2127* is the most likely candidate for the increase in resistance between 23D and 21C.

Since SERP1061.D49Y may decrease resistance levels in the isolates, this protein was also modelled and the location of the SNP investigated. SERP1061 is

the ferric uptake regulator (Fur) protein and has two domains (Fig. 5.7), the N-terminal domain is a DNA binding domain, whilst the C-terminal domain is involved in dimerisation and binding of the ligand, the iron metal ion. The SNP SERP1061.D49Y occurs on the external edge of α3 of the DNA binding domain. The change results in a more hydrophobic residue, which may be energetically unfavourable for a residue on the outside of the protein, potentially resulting in some degree of destabilisation or altering the DNA affinity.

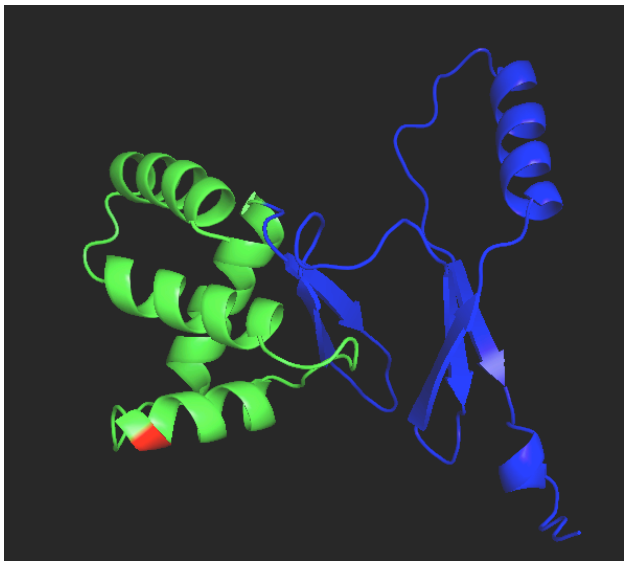


Figure 5.7 Protein model of SERP1061

Constructed using HHpred, coloured using Pymol. Images show the proteins DNA binding domain (green) and ligand binding domain (blue). The location of SNP D49Y is highlighted in red.

The Rp62a sapienic acid-evolved isolate with the highest observed linoleic acid resistance is Rp-Sap21D. This isolate contains two additional SNPs compared with the other evolved isolates, GcvT.S74F and SERP1544.P52S, plus an insertion, SERP0499.29_30insMA. GcvT is glycine cleavage system aminomethyltransferaseT, which leads to the production of ammonia and begins conversion of glycine to serine, SERP1544 is homologous to an SP β-phage lipoprotein and SERP0499 is homologous to iron-sulphur cluster scaffold-like proteins. It possible that one, or all three, of these mutations contribute to the enhanced resistance observed in Rp-Sap21D compared to Rp-Sap21C.

In addition to the SNPs already discussed, there was one more SNP present in 66.6 % of reads from the Rp-SapPool sample, Mqo-3.D402G (Table 5.4). This

SNP is likely to be present in 6-7 of the 10 isolates from the Rp-SapPool, indicating it is found in the majority of isolates, as such it is likely to contribute to fatty acid resistance. Mqo-3 is a malate:quinone oxidoreductase, involved in pyruvate metabolism.

5.3.5 *S. epidermidis* Rp62a D-sphingosine induced SNPs and INDELs

S. epidermidis Rp62a isolates experimentally evolved with D-sphingosine were tested for increased resistance to D-sphingosine using a microdilution broth assay. Isolate resistance levels according to MIC values were in the following order: Rp-Ds23E > Rp-Ds23C > Rp-Ds21D = Rp-0 > Rp-Eth23.

Sequencing identified that there were three coding SNPs, two SNPs within the potential promoter region of genes and one deletion in strain Rp-Ds21D (Table 5.5). Since isolate Rp-Ds21D exhibited the same level of resistance as the Rp-0 isolate, any SNPs found in this strain do not markedly enhance D-sphingosine resistance. However, it is possible that some of these mutations provide a subtle contribution to D-sphingosine resistance, particularly as resistance was observed to decrease initially. The SNP SERP2264.G482A is maintained through all of the sequenced D-sphingosine evolved isolates, this SNP may therefore play a role in D-sphingosine resistance. SERP2264 is the *S. epidermidis* homologue of the *S. aureus* protein, SasF, which has previously been indicated to play a role in linoleic acid resistance (see discussion).

Table 5.5 Non-synonymous, homozygous SNPs and INDELs from *S. epidermidis* D-sphingosine-evolved isolates.

SNPs and INDELs from *S. epidermidis* Rp62a sequenced evolved isolates were determined using a bespoke perl script to filter results. All mutations were confirmed by at least 6x coverage and were classed as homozygous if > 80 % of reads had alternative bases to the reference genome. Intergenic SNPs were included if they were less than 200 bases upstream of the predicted translational start site.

Official gene symbol	Gene function	Isolates	SNP position	Base change	Change	Position in protein
mnhA	Monovalent cation/H+ antiporter	Rp-Ds21D	521021	G/T	Substitution A/D	635 aa
SERP2264	SasF, cell wall protein homologue	Rp-Ds21D, Rp-Ds23C Rp-Ds23E	2298704	C/G	Substitution G/A	482 aa
yycG	Two-component histidine kinase	Rp-Ds21D	2590283	T/C	Substitution K/E	264 aa
SERP0245	RND transporter	Rp-Ds23C, Rp-Ds23E	254343	G/T	Substitution K/N	658 aa
SERP0247	Tet-R transcriptional regulator	Rp-Ds23C, Rp-Ds23E	256697	T/A	Substitution F/Y	110 aa
SERP0247	Tet-R transcriptional regulator	Rp-Ds23C, Rp-Ds23E	256748	G/T	Substitution R/L	127 aa
SERP1407	Hypothetical protein	Rp-Ds23C, Rp-Ds23E	1472010	G/C	Substitution A/G	116 aa
SERP2327	Acetoin dehydrogenase	Rp-Ds23C	2364609	G/C	Substitution A/G	137 aa
yycG	Two-component histidine kinase	Rp-Ds23C, Rp-Ds23E	2590084	T/A	Substitution D/V	330 aa
SERP0458	Ion transporter	Rp-Ds23E, Rp-Ds23C	462389	-A	Frameshift	260 aa
SERP2017	Amino acid permease	Rp-Ds21D	2035803	-A	Frameshift	296 aa
SERP0389	Acetaldehyde-CoA/alcohol dehydrogenase	Rp-Ds21D	392259	A/G	Upstream substitution	-41 bp
SERP1757	Hypothetical protein	Rp-Ds23C Rp-Ds23E	1794214	-G	Upstream deletion	-118 bp
SERP1758	Lytic regulatory protein	Rp-Ds23C Rp-Ds23E	1794214	-G	Upstream deletion	-15 bp

Isolate Rp-Ds23E was more resistant to D-sphingosine compared with isolate Rp-Ds23C, however the reverse is true for linoleic acid resistance. The majority of the intergenic and coding mutations between these two isolates are shared. The exceptions are SERP2327.A137G found in Rp-Ds23C and Aap.G198S found in 21 % of Rp-Ds23E reads (Table 5.6). Since Aap is a highly repetitive protein it is possible that SNPs could arise in this protein without any selective pressure, or that sequence reads were aligned to the wrong part of the gene sequence by BWA. The other option is that this alteration in 20 % of Aap sequences enhances D-sphingosine resistance.

If the SNP in Aap does not enhance D-sphingosine resistance, it is surprising that SERP2327.A137G, the only other SNP that differs between Rp-Ds23E and Rp-Ds23C, occurs in Rp-Ds23C, which was the least resistant to D-sphingosine of the two. This suggests that SERP2327.A137G reduces resistance to D-sphingosine. It would be unusual for a SNP to reduce resistance to the compound that was acting as the selective pressure. It is even more unusual as this SNP was observed to increase resistance to linoleic acid, a compound that was not present to provide any selective pressure. SERP2327 shares sequence similarity with dihydrolipoamide dehydrogenases, which reduce lipoamide or lipoic acid.

Table 5.6 Non-synonymous, heterozygous SNPs and INDELs from Rp62a D-sphingosine evolved isolates.

SNPs and INDELs from *S. epidermidis* Rp62a sequenced evolved isolates were determined using a bespoke perl script to filter results. All mutations were confirmed by at least 6x coverage and were classed as heterozygous if they < 80 % of reads had alternative bases to the reference genome.

Official gene symbol	Gene function	Isolates	SNP position	Base change	Change	Position in protein	% of reads
SERP2438	Cation transporter	Rp-Ds21D	2495588	T/A	Substitution L/F	299	24.5
aap	Accumulation associated protein	Rp-Ds23E	2461245	C/T	Substitution G/S	198	21.2

Eight SNPs and INDELs are shared between Rp-Ds23E and Rp-Ds23C, which are the Rp62a isolates with increased D-sphingosine resistance. It is not possible to assess how many of these mutations contribute to the increased D-sphingosine resistance without moving each individual mutation to a clean background.

One of these eight shared mutations occurred in YycG (YycG.D330V), a gene which also had SNPs in sapienic acid-evolved Rp62a isolates. A further SNP in YycG (YycG.K264E) occurs in Rp-Ds21D, which had no observed increase in D-sphingosine resistance. As the protein structure of Rp62a YycG was already modelled, the effects of these SNPs were investigated (Fig. 5.8). YycG.K264E and YycG.D330V both occur in the PAS domain, which are usually involved in sensing signals such as redox potential or light intensity. YycG.K264E occurs in the β-scaffold of the PAS domain, which is likely to be involved in ligand binding. YycG.D330V occurs in the linker region between the PAS domain and the HAMP domain; HAMP domains are usually involved in sensing membrane and cell wall stress signals.

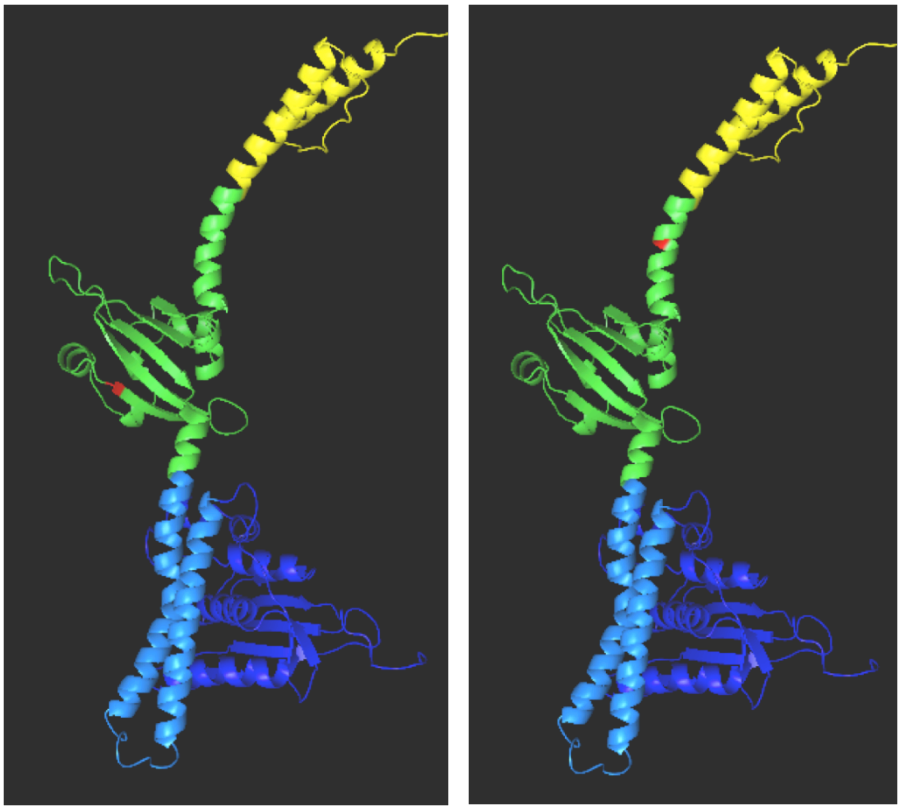


Figure 5.8 Protein models of YycG

Constructed using HHpred, coloured using Pymol. Images show the protein domains HAMP (yellow), PAS (green), histidine kinase ATPase domain (dark blue) and histidine kinase dimerisation domain (light blue). The location of SNPs K264E (left) and D330V (right) are highlighted in red.

5.3.6 *S. aureus* SH1000 D-sphingosine induced SNPs and INDELs

SH1000 isolates experimentally evolved with D-sphingosine were assayed to determine their relative resistance to D-sphingosine using the microdilution broth assay, and to linoleic acid using the agar plate-based method. The order of D-sphingosine resistance in isolates was SH-Ds5C>SH-Ds3B>SH-Ds3A>SH1000, while the order of linoleic acid resistance was the same except for SH-Ds3A having greater linoleic acid resistance than SH-Ds3B.

The only SNPs shared by all three SH1000 D-sphingosine evolved isolates are upstream of the transcriptional start sites for *rsbU* and *saouhsc_A02189* by 73 and 15 bp respectively (Table 5.7). As these SNPs are found in all three sequenced isolates, they are the most likely candidates for increasing SH1000 resistance to D-sphingosine. These SNPs could result in increased or decreased

transcription of these genes by affecting binding sites for promoters or transcription factors. RsbU makes a particularly compelling candidate as it positively regulates sigmaB activity (Palma and Cheung, 2001), and could therefore influence expression of the general stress response.

Table 5.7 Non-synonymous, homozygous SNPs from *S. aureus* D-sphingosine evolved isolates.

SNPs and INDELs from *S. aureus* SH1000 sequenced evolved isolates were determined using a bespoke perl script that filtered out results from non-coding regions. All described mutations had at least 6x coverage and were classed as homozygous if > 80 % of reads had alternative bases to the reference genome. Intergenic SNPs were included if they were less than 200 bases upstream of the predicted translational start site.

Official gene symbol	Gene function	Isolates	SNP position	Base change	Change	Position in protein
gltX	Glutamyl-tRNA synthatase	SH-Ds3A, SH-Ds5C	511636	C/T	Substitution R/C	151 aa
SAOUHSC_01130	Hypothetical membrane protein	SH-Ds3A	1084556	A/G	Substitution K/R	247 aa
SAOUHSC_01337	Transketalase	SH-Ds3A	1277240	A/G	Substitution T/A	193 aa
SAOUHSC_01895	Hypothetical protein	SH-Ds3A, SH-Ds5C	1807093	C/A	Substitution A/S	182 aa
SAOUHSC_02341	ATP synthase	SH-Ds3A, SH-Ds5C	2168000	G/C	Substitution P/A	426 aa
agrA	accessory gene regulator	SH-Ds3A	2096582	A/T	Premature Stop	193 aa
SAOUHSC_02587	Hypothetical CAAX protease	SH-Ds3A, SH-Ds5C	2379408	C/A	Substitution W/C	93 aa
SAOUHSC_02867	TetR transcriptional regulator	SH-Ds3A, SH-Ds5C	2641724	G/A	Substitution E/K	151 aa
SAOUHSC_01450	amino acid transporter	SH-Ds3B	1406715	T/C	Substitution I/V	418 aa
SAOUHSC_02867	TetR transcriptional regulator	SH-Ds3B	2641391	G/T	Substitution A/S	40 aa
SAOUHSC_02867	TetR transcriptional regulator	SH-Ds3B, SH-Ds5C	2641553	G/T	Substitution D/Y	94 aa
ebh	Extracellular matrix binding protein homologue	SH-Ds5C	1397392	A/G	Substitution L/S	2436 aa
fmhA	Methicillin resistance determinant	SH-Ds5C	2479727	G/A	Substitution M/I	174 aa
SAOUHSC_02866	RND transporter	SH-Ds5C	2640222	A/G	Substitution V/A	296 aa
SAOUHSC_01584	Hypothetical protein	SH-Ds3A	1511362	G/T	Premature stop	23 aa

Table 5.7 continued Non-synonymous, homozygous SNPs from *S. aureus* D-sphingosine evolved isolates.

SNPs and INDELs from *S. aureus* SH1000 sequenced evolved isolates were determined using a bespoke perl script that filtered out results from non-coding regions. All described mutations had at least 6x coverage and were classed as homozygous if > 80 % of reads had alternative bases to the reference genome. Intergenic SNPs were included if they were less than 200 bases upstream of the predicted translational start site.

Official gene symbol	Gene function	Isolates	SNP position	Base change	Change	Position in protein
SAOUHSC_02664	Transcriptional regulator	SH-Ds3B	2449509	+A	Frameshift	362 aa
SAOUHSC_02555	Pseudogene	SH-Ds3A	2349971	-A	Upstream deletion	80 aa
SAOUHSC_02555	Pseudogene	SH-Ds3A SH-Ds3B	2349963	-G	Upstream deletion	72 aa
SAOUHSC_02302 RsbU	sigmaB regulator	SH-Ds3A SH-Ds3B SH-Ds5C	2134726	C/A	upstream substitution	-73 bp
SAOUHSC_A02189	Hypothetical protein	SH-Ds3A SH-Ds3B SH-Ds5C	2134726	C/A	Upstream substitution	-15 bp
SAOUHSC_01840	Transglycosylase	SH-Ds3A SH-Ds5C	1746115	C/A	upstream substitution	-196 bp
SAOUHSC_01839	Tyrosyl-tRNA synthase	SH-Ds3A SH-Ds5C	1746115	C/A	upstream substitution	-186 bp
SAOUHSC_02809	gluconate operon repressor	SH-Ds3B	2588702	G/C	upstream substitution	-63 bp
SAOUHSC_00446	Hypothetical protein	SH-Ds3B	448086	T/G	upstream substitution	-86 bp
SAOUHSC_00325	Hypothetical protein	SH-Ds5C	337692	G/T	upstream substitution	-188 bp

Though no other SNPs are shared by all three isolates, they each have SNPs in *saouhsc_02867*, encoding a TetR-like transcriptional regulator (E151K in SH-Ds3A and SH-Ds5C, A40S in SH-Ds3B and G94T in SH-Ds3B and SH-Ds5C), with the neighbouring transporter in the most resistant isolate, SH-Ds5C, also having a SNP (SAOUHSC_02866.V296A).

The importance of this TetR-like regulator in D-sphingosine resistance is apparent from the volume of SNPs occurring in the gene. This is especially true for SH-Ds3B, the second most resistant D-sphingosine evolved isolate of SH1000, as this isolate has three SNPs in SAOUHSC_02867, and just one other SNP in a coding region. SAOUHSC_02867 and SAOUHSC_02866 of *S. aureus* SH1000, are homologous to the *S. epidermidis* Rp62a SERP0247 and SERP0245 genes, respectively. Both SERP0245 and SERP0247 were found to contain SNPs in Rp62a D-sphingosine-evolved isolates, supporting the likely importance of these components in D-sphingosine resistance.

Genome comparison of *S. aureus* SH1000, *S. epidermidis* Rp62a and other staphylococcal strains using the SEED, indicates that homologues of the SAOUHSC_02867 RND transporter are most often found near a TetR-like transcriptional regulator (Fig. 5.9). It seems probable that expression of these RND transporters are regulated by the adjacent TetR-like regulator. Intriguingly, this analysis also indicated that the gene arrangement around this transporter in *S. aureus* strains is quite different to that found in *S. epidermidis*, *S. hominis*, *S. haemolyticus*, *S. caprae*, *S. warneri* and *S. saprophyticus*. Of particular interest, in all species of staphylococci tested other than *S. aureus*, there is an adjacent, and possibly co-transcribed, osmoprotectant ABC transporter. It would be tempting to speculate that this protein may also play a role in D-sphingosine resistance.

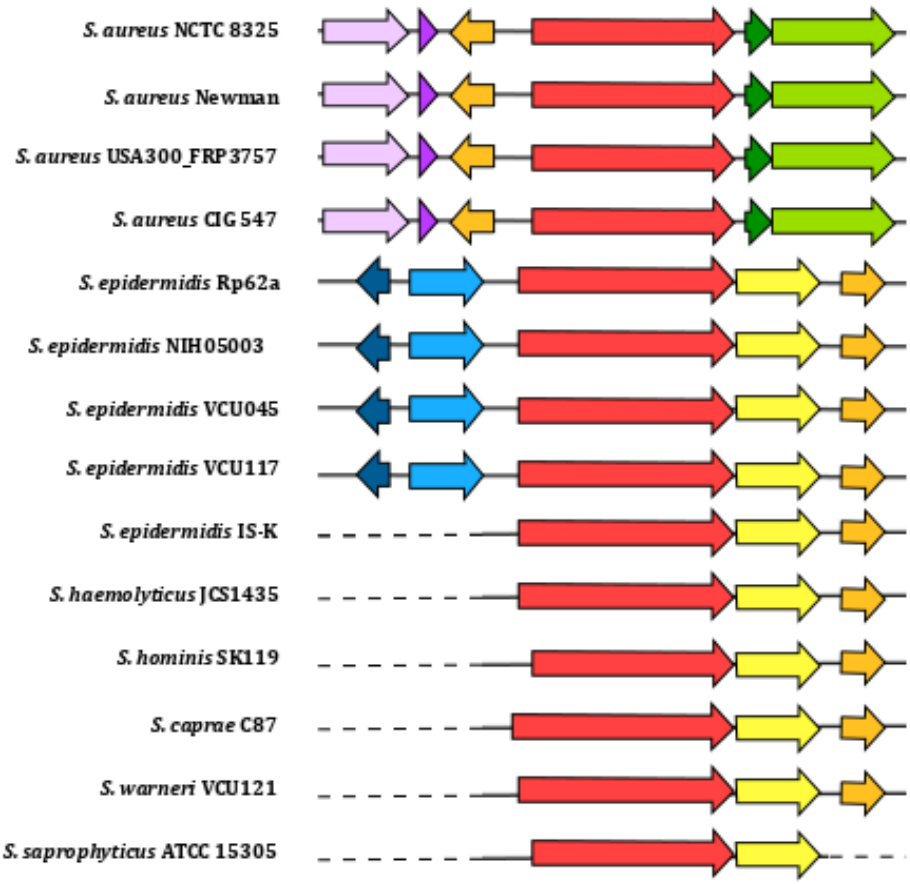


Figure 5.9 Synteny of SAOUHSC_02867 RND transporter homologue loci between staphylococcal species.

The coloured arrows are representative of homologous genes, red is the RND transporter, orange is the TetR family transcriptional regulator, pink is delta-1-pyrroline-5-carboxylate dehydrogenase, purple is a hypothetical protein, dark blue is a hypothetical protein, light blue is a oxidoreductase ion channel, dark green is a hypothetical protein, green is ferrous iron transport protein B and yellow is a osmoprotectant ABC transporter. Dotted lines indicates genes with no synteny to the other genomes. Diagram based upon SEED output (Overbeek *et al.*, 2005).

Due to the proposed importance of this TetR-like regulator and its proximally associated transporter, these proteins were modelled in both Rp62a and SH1000, and the SNP locations investigated (Fig. 5.10 and Fig. 5.11). In both SAOUHSC_02867 and SERP0247 the SNPs were positioned at the entrance to and within a pocket of the ligand-binding domain. These SNPs may affect ligand accessibility and binding.

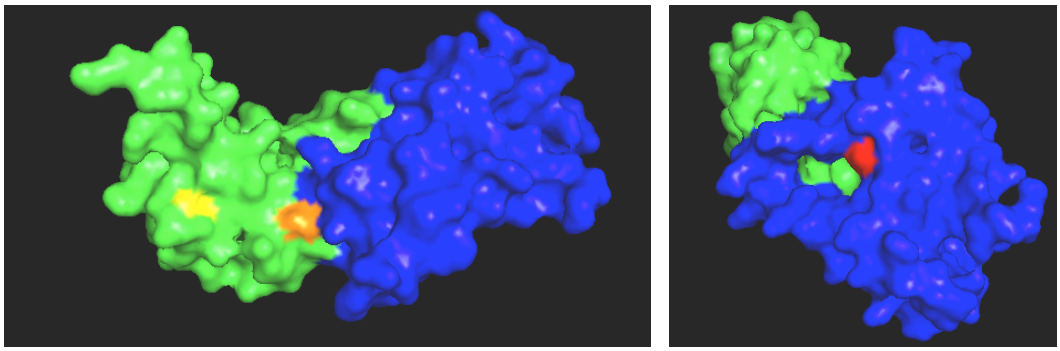


Figure 5.10 Two views of SNPs in the Protein model of SAOUHSC_02867.

The protein model was constructed using HHpred, then visualised and coloured using Pymol. Images show DNA binding domain (green) and ligand binding and dimerisation domain (blue). The left image shows location of SNPs A40S (yellow) and G94T (orange), whilst the right image shows SNP E151K (red) at the entrance to a pocket.

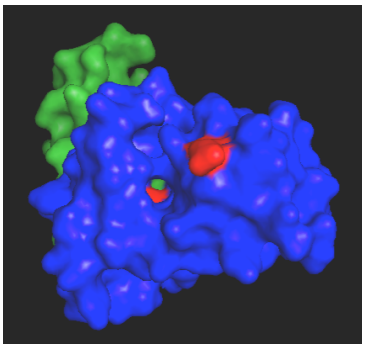


Figure 5.11 SNPs in the Protein model of SERP0247.

The protein model was constructed using HHpred, then visualised and coloured using Pymol. Image shows the DNA binding domain (green) behind the ligand binding and dimerisation domain (blue) with the location of the two SNPs (red) on the entrance to and inside surface of a pocket.

The SNPs in resistance-nodulation-division (RND) transporters SAOUHSC_02866 and SERP0245 occur in the transmembrane helices near to the extracellular domains (Fig. 5.12). Since RND transporter monomers have a degree of symmetry between transmembrane domains 1-6 and 7-12, these SNPs occur at approximately their symmetrically opposite location.

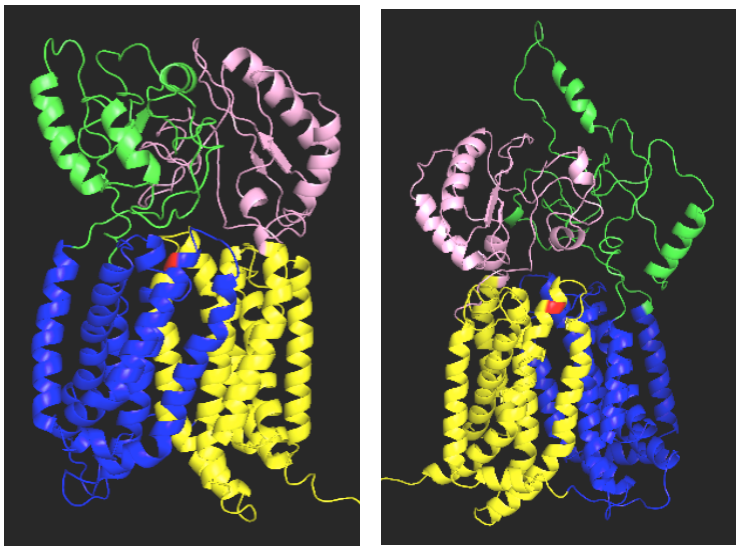


Figure 5.12 SNPs in Protein models of RND transporters SAOUHSC_02866 and SERP0245

SAOUHSC_02855 (left) and SERP0245 (right). Protein models were constructed in HHpred, then visualised and coloured using Pymol. Models show transmembrane helices 1-6 (blue) and 7-12 (yellow), extracellular domains 1 (green) and 2 (pink) and location of SNPs (red). Cytoplasmic tail domain not shown.

SH1000 D-sphingosine evolved isolate SH-Ds3A has four unique SNPs and six that are shared with SH-Ds5C. It is more likely that those found in both SH-Ds3A and SH-Ds5C would be involved in increasing D-sphingosine and linoleic acid resistance as both of these strains showed increased resistance to these two antimicrobials. However, the SNPs found only in SH-Ds3A cannot be discounted; only reproducing the SNPs in an otherwise clean genetic background could determine their involvement in the increased resistance observed.

Of the SNPs that only occur in SH1000 isolate SH-Ds3A, the most worthy of note are the two truncations, AgrA.K193X and SAOUHSC_01584.S23X. AgrA is part of a quorum sensing operon and the truncation removes part of the protein predicted to bind DNA, theoretically reducing the ability of AgrA to bind or release its consensus sequence. SAOUHSC_01584 is a conserved hypothetical protein; the truncation leaves a protein of only 23 aa.

Of the SNPs that are shared between SH1000 isolates SH-Ds3A and SH-Ds5C, besides the already described TetR-like transcriptional regulator, only SAOUHSC_02587 has a known role in antimicrobial resistance. SAOUHSC_02587

is a hypothetical protein with similarity to the CAAX proteases involved in bacteriocin resistance, though it is unclear how protease activity could affect antimicrobial lipid resistance.

Isolates SH-Ds3A and SG-Ds5C additionally share SNPs in GltX and upstream of SAOUHSC_01839, both involved in tRNA synthesis. This implies that an increase or decrease in tRNA synthesis may be a factor in D-sphingosine resistance.

The SH1000 D-sphingosine evolved isolate SH-Ds3B has six mutations besides those already mentioned. Though it is not possible to tell which, if any, of these mutations are important for D-sphingosine resistance, there are two with annotated functions that make them more pertinent. Firstly, the SNP containing SAOUHSC_01450, is a hypothetical protein annotated as a PotE-like amino acid transporter. As both the other sequenced D-sphingosine-evolved SH1000 isolates had SNPs in tRNA synthesis genes, this again implies a role for amino acid or protein synthesis in D-sphingosine resistance. Secondly, this isolate has an insertion resulting in a frameshift in SAOUHSC_02664. SAOUHSC_02664 is annotated as a transcriptional regulator and the frameshift would disrupt the ligand binding domain, and therefore likely disrupt any regulation this protein provides.

The D-sphingosine SH1000 isolate with the highest resistance to D-sphingosine and linoleic acid was SH-Ds5C. This isolate had four SNPs not found in any other isolate, including SAOUHSC_02866.V296A which has already been discussed. The other SNPs found in SH-Ds5C alone are Ebh.L2436S, FmhA.M174I and a SNP upstream of *saouhsc_00325*. Ebh is a membrane protein known to be involved in desiccation tolerance and binding of extracellular matrix components. FmhA is involved in peptidoglycan cross-linking and is a known determinant of methicillin resistance. SAOUHSC_00325 is a hypothetical protein. Any of these SNPs could account for the higher level of resistance in this strain, alternatively, the unique combination of SNPs from strains SH-Ds3A and SH-Ds3B could account for this increased resistance.

Heterogeneous SH1000 SNPs (Table 5.8) were not considered during analysis of SH1000 D-sphingosine evolved isolates due to the volume of homogenous SNPs already of interest.

Table 5.8 Non-synonymous, heterozygous SNPs from *S. aureus* D-sphingosine evolved isolates.

SNPs and INDELs from *S. aureus* SH1000 sequenced evolved isolates were determined using a bespoke perl script that filtered out results from non-coding regions. All described mutations had at least 6x coverage and were classed as heterozygous if < 80 % of reads had alternative bases to the reference genome.

Ordered locus name	Gene function	Isolates	SNP position	Base change	Change	Codon no.	% of reads
SAOUHSC_00113	Alcohol dehydrogenase	SH-Ds3A	116769	G/T	A/S	78	53.6
SAOUHSC_00196	Hypothetical protein	SH-Ds3A	216521	C/A	D/Y	401	20.1
SAOUHSC_00323	Hypothetical protein	SH-Ds3A	336879	T/A	I/N	78	22.2
SAOUHSC_00936	Hypothetical protein	SH-Ds3A	908994	C/G	A/G	186	36.1
SAOUHSC_01342	Hypothetical protein	SH-Ds3A	1282568	C/A	A/D	538	53.6
SAOUHSC_01716	Protease	SH-Ds3A	1621683	T/C	D/G	213	20
SAOUHSC_01407	5-bromo-4-chlorindolyl phosphate hydrolase	SH-Ds3B	1347090	T/G	V/G	15	61.3
SAOUHSC_01849	Deacetylase	SH-Ds3B	1756243	C/A	Q/K	389	32.5
SAOUHSC_01918	Hypothetical protein	SH-Ds3B	1826785	G/T	C/F	40	21.5
SAOUHSC_00217	Sorbitol dehydrogenase	SH-Ds5C	237713	C/A	D/E	195	33.9
SAOUHSC_01141	Hypothetical protein	SH-Ds5C	1091682	T/A	N/K	311	21.2
SAOUHSC_01918	Hypothetical protein	SH-Ds5C	1826794	C/T	S/F	43	20.2
SAOUHSC_01584	Hypothetical protein	SH-Ds5C	1511362	G/T	S/*	23	22.5

5.3.7 Phenotypic analysis of sequenced Rp62a sapienic acid-evolved isolates

SNPs in YycG have previously been reported to increase *S. aureus* resistance to vancomycin in VISA isolates (see discussion) (Howden *et al*., 2011). These YycG SNP mutants are often characterised by increased cell wall thickness, decreased biofilm formation and decreased autolysis. It is likely that if the SNPs in YycG in Rp62a have the same effect as the SNPs in VISA isolates, the evolved isolates would show similar phenotypic traits.

The Rp62a sapienic acid-evolved sequenced isolates were characterised to assess the impact of YycG mutations. Experiments to characterise the effects of mutations included biofilm assays and vancomycin resistance.

The results clearly indicate that biofilm formation capability is lost in all evolved isolates (Fig 5.13). Surprisingly, this includes the ethanol-evolved isolate. This isolate did not have any SNPs in YycG, indicating this loss of biofilm formation is not restricted to a mutation in YycG.

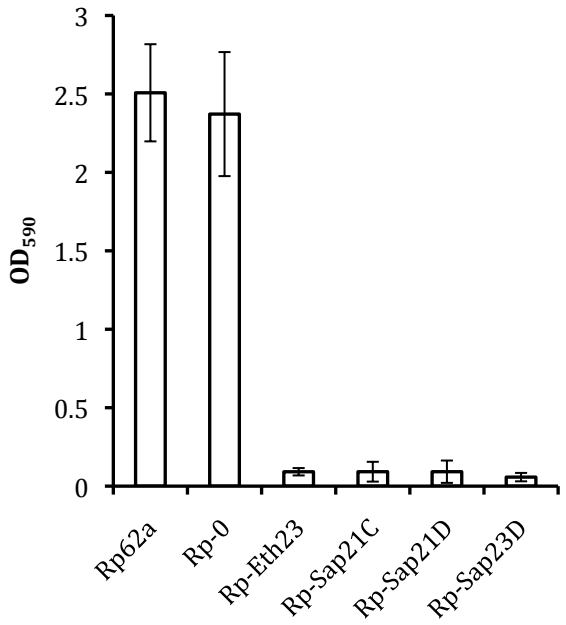


Figure 5.13 Biofilm forming capability of sequenced *S. epidermidis* Rp62a sapeinic acid-evolved isolates.

Isolates were grown for 24 h in 96 well plates, then washed to remove unattached cells. Attached cells were stained with crystal violet. The biofilm forming capability was determined by measuring the OD_{590} of the crystal violet re-dissolved in ethanol.

Preliminary data without replicates, indicated isolates Rp-Sap21C and Rp-Sap21D have vancomycin MICs of 12 μg ml^{-1}, whilst isolate Rp-Sap23D, the Rp-0 isolate and ethanol control isolate had MICs of 1.5 μg ml^{-1}. However, as these experiments were not performed using clinical laboratory standards they cannot inform as to the VISA status of these isolates. Since the SNP in YycG is the only synonymous, coding SNP in isolate Rp-Sap21C, this indicates that this SNP confers vancomycin resistance. The absence of resistance to vancomycin in isolate Rp-Sap23D may suggest that SERP1061.D49Y, which reduces linoleic acid resistance, also reduces vancomycin resistance.

MIC results for sapienic acid-evolved Rp62a did not indicate a difference between ethanol evolved and sapienic acid evolved isolates, as a result it was uncertain if these isolates are more resistant to sapienic acid. A concern was that differences in resistance might only be apparent under the same conditions as used when evolving the isolates. To compare growth of isolates in sapienic acid under the same conditions as used when evolving the isolates, growth under these conditions with or without 1.25 μM of sapienic acid was measured at 4 h and 24 h. It was found that after 4 h all isolates except Rp-Sap21D had viability levels below those prior to the addition of sapienic acid (Fig. 5.14, A). Isolate Rp-Sap21D showed growth in sapienic acid at 4 h, though not to the same degree as in control conditions. Isolate Rp-Sap23D, Rp-Sap21C and Rp-Eth23 showed viabilities higher than the Rp-0 isolate, indicating greater resistance compared to the parent strain. At 24 h all isolates had grown in the sapienic acid conditions, with growth following a similar, though less distinct, pattern to that observed at 4 h (Fig. 5.14, B). This indicates that all evolved isolates, including Rp-Eth23, had increased growth rates in media containing sapienic acid and ethanol, with Rp-Sap21D showing the greatest increase in growth rate under these conditions.

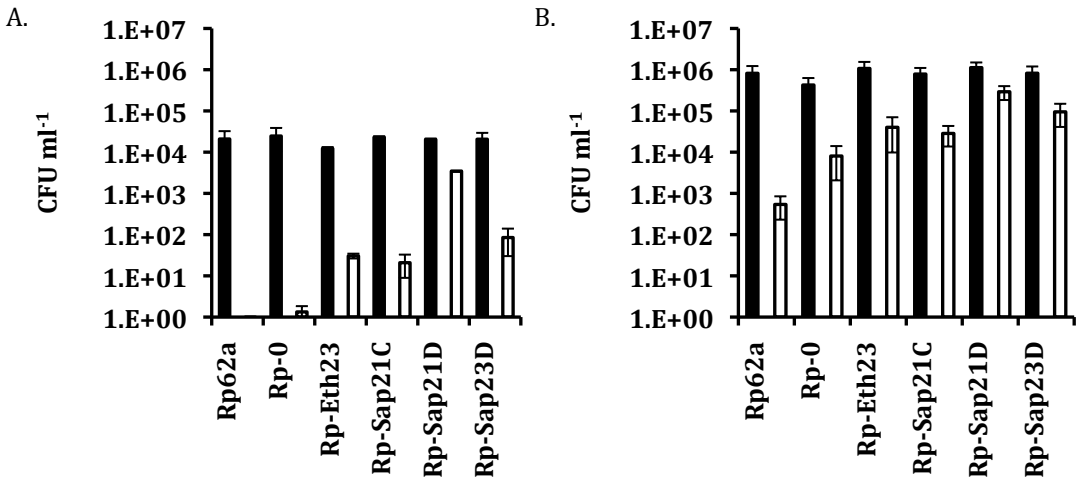


Figure 5.14 Growth of genome sequenced *S. epidermidis* Rp62a sapienic acid evolved isolates with and without sapienic acid.

Control THB (black bars), sapienic acid containing THB (white bars) at 4 h (A) or 24 h (B). Isolates were grown at 37 °C in universals with shaking. Control THB contained 5 % ethanol, whilst sapienic acid THB contained 1.25 μM sapienic acid and 5 % ethanol.

5.4 Discussion

In this Chapter, increased resistance was evolved to D-sphingosine and sapienic acid in staphylococcal species. This presents a potential setback for studies that suggest the use of antimicrobial lipids as novel topical antimicrobials (Desbois and Smith, 2010; Vidal *et al*., 2014; Desbois and Lawlor, 2013). Conversely, data from screening of resistance levels to antimicrobial lipid for *S. epidermidis* and *S. aureus* skin and nasal isolates (see Chapter three), suggests that evolution of antimicrobial lipid resistance does not occur to such an extent on skin. This leads to the question: what restricts evolution of resistance to antimicrobial lipids on skin? It is possible that components found on the skin prevent antimicrobial lipid resistance developing. Increasing antimicrobial lipid resistance may well decrease resistance to other antimicrobials found on the skin, as has been observed for some combinations of antibiotics (Sieradzki and Tomasz, 1997; Vignaroli *et al*., 2011). Alternatively, it may indicate that antimicrobial skin lipids come into contact with the bacteria at lower concentrations on skin than has been tested here, thereby rendering development of further resistance unnecessary.

The mutations that evolved as result of selective pressure from sapienic acid or D-sphingosine give indications of processes and components affected by sapienic acid and D-sphingosine treatment respectively. Considering both of these compounds are suggested to act through membrane perturbation, it is surprising that only one gene, YycG, developed SNPs when evolved with either sapienic acid or D-sphingosine in *S. epidermidis*.

YycG is the histidine kinase of the two-component regulator YycGF (also known as WalKR and VicKR), which has been shown to regulate cell wall turnover (Bisicchia *et al*., 2007; Dubrac *et al*., 2007). Single point mutations in YycG have previously been shown to increase staphylococcal resistance to vancomycin, a cell wall biosynthesis inhibitor, and daptomycin, which is predicted to disrupt the cell membrane (Howden *et al*., 2011; Jansen *et al*., 2007; Hafer *et al*., 2012; Friedman *et al*., 2006). This indicates that YycG mutations may provide resistance to a range of antimicrobials, particularly those that affect cell wall biosynthesis or disrupt cell membranes. The mutation of YycG in both sapienic

acid and D-sphingosine experiments may similarly suggest both antimicrobial lipids disrupt the cell membrane or cell wall biosynthesis, however it may simply be that this gene is hypermutable in Rp62a under stress.

YycG SNP-mediated resistance to vancomycin was reported to be the result of both more active and less active forms of YycG. In the work by Jansen *et al*. (2007), it was demonstrated that overexpression of YycGF or increased YycG kinase activity increased vancomycin resistance. However, Dubrac *et al*. (2007) showed that YycGF depletion led to increased vancomycin resistance**.** Reports indicate that some, but not all, YycGF SNPs increase daptomycin resistance (Hafer *et al*., 2012; Song *et al*., 2013b; Patel *et al*., 2011).

YycG primarily regulates cell wall turnover, so that more active YycG would increase cell wall turnover and less active YycG would decrease cell wall turnover (Turck and Bierbaum, 2012). Speculatively, it seems plausible that mutations leading to either more or less active YycG could result in vancomycin resistance. Vancomycin acts by binding to peptidoglycan thereby sterically inhibiting interactions with peptidoglycan cross-linking enzymes (Reynolds, 1989). Consequently, vancomycin blocks cross-linking in newly synthesised peptidoglycan, making the cell wall weak and likely to fail if growth continues (Reynolds, 1989). If the cell wall had a higher rate of turnover, vancomycin bound peptidoglycan would be shed and replaced more rapidly than in normal cells, potentially increasing vancomycin resistance. If the cell wall has a lower rate of turnover, the wall becomes thicker with more cross-linking, potentially increasing the number of targets thereby effectively diluting vancomycin or blocking vancomycin access (Cui *et al*., 2000; Srinivasan *et al*., 2002).

Daptomycin on the other hand, is predicted to act by interacting with the cell membrane rather than the cell wall. YycG mutations that increase cell wall turnover would be unlikely to offer protection against membrane targeting antimicrobials. YycG mutations that decrease cell wall turnover may be able to offer resistance to daptomycin by reducing its ability to interact with the membrane due to the increased cell wall thickness. This theory could account for why some YycG mutations give rise to both daptomycin and vancomycin resistance, whilst others only result in vancomycin resistance.

If YycG mutations that decrease its activity are responsible for daptomycin resistance, it is plausible that such mutations would also provide resistance to other membrane targeting antimicrobials. Since sapienic acid, linoleic acid and D-sphingosine are all predicted to be membrane targeting antimicrobials, this hypothesis would predict that the YycG mutations seen in sapienic acid and D-sphingosine-evolved isolates would reduce YycG activity.

YycG SNPs tested by Dubrac *et al*. (2007) led to vancomycin resistance as well as phenotypes of cell wall thickening, increased autolysis resistance and decreased biofilm formation. These phenotypes were the same as those observed for point mutations that increased vancomycin and daptomycin resisance (Howden *et al*., 2011). To test if YycG.R378H is likely to have decreased activity, strains containing this SNP were compared to their controls for biofilm formation. Isolates containing YycG.R378H showed decreased biofilm formation, though this result was inconclusive as the ethanol control isolate without YycG.R378H also had reduced biofilm formation compared to Rp-0. A more conclusive test may be to visualise the cell wall thickness or the daptomycin resistance of strains containing YycG.R378H compared to the wild type.

Isolates containing YycG.R378H were also tested for vancomycin resistance. Two of the three isolates showed increased vancomycin MIC values compared to the wild type strains. It is possible that YycG.R378H confers vancomycin resistance at a level sufficient to class these isolates as vancomycin intermediate (MIC of 8-16 μg/ml) (Srinivasan *et al*., 2002). Regardless of whether this increased resistance is due to YycG.R378H, these results suggest a concerning link between antimicrobial lipids and development of antibiotic resistance.

YycG.R378H, which is likely to increase linoleic acid resistance, occurs in the helical hairpin of the histidine kinase domain at the point that it joins to the PAS domain. This mutation could potentially affect the activation state or activity of the histidine kinase. The hairpin section of the histidine kinase domain is responsible for the dimerisation of histidine kinases, as such this mutation is more likely to affect YycG dimerisation. In the model CovS, two arginines are found in a comparable location in the hairpin structure (Arg208 and Arg206)

(Wang *et al*., 2013). This suggests a degree of conservation and may indicate a role in dimerisation. A change from arginine to histidine not only changes the side chain to a ring but may also change the charge from positive to neutral depending on the pH in the cytosol.

YycG.K264E and YycG.D330V that occur in D-sphingosine-evolved Rp62A isolates are in the alpha-chain connector between the PAS and HAMP domain and in the β-scaffold of the PAS domain respectively. PAS domains are well characterised, and examples have been found that act as intracellular sensors for light, oxygen or redox potential (Taylor and Zhulin, 1999). Signals interact with PAS domains within the β-scaffold, these interactions are limited by the N-terminal cap, which in YycG continues into the HAMP domain. HAMP domains are predicted to undergo conformational changes upon sensing of a signal within transmembrane, periplasmic or extracellular domains. YycG has two transmembrane domains linked by an extracellular loop that are theorised to sense turgor pressure within the membrane (Turck and Bierbaum, 2012). It is possible that YycG.K264E and YycG.D330V interfere with signal transduction and hence activation state or activity of the histidine kinase.

As well as cell wall biosynthesis, another factor that seems to come under selective pressure from sapienic acid in *S. epidermidis* are iron acquisition genes. Iron acuisition genes with mutations included *serp1061*, encoding the Fur iron acquisition transcriptional regulator, *serp0499*, encoding an iron sulphur scaffold protein and possible iron chelator, and *serp2127*, encoding a predicted Fe^{2+} transporter.

Iron has previously been seen to be play a role in antimicrobial fatty acid resistance (Clarke *et al*., 2007). Iron-starvation conditions promoted higher fatty acid resistance levels because it leads to upregulation of IsdA. IsdA knockouts exhibited low antimicrobial fatty acid MICs that did not increase under iron starvation (Clarke *et al*., 2007). IsdA production results in less hydrophobic cells; lower hydrophobicity caused reduced interaction with the antimicrobial fatty acids (Clarke *et al*., 2007). The SNPs observed in iron acquisition genes may make *S. epidermidis* less effective at acquiring iron, resulting in an upregulation of IsdA via reduced Fur repression. This seems the

most likely scenario as addition of iron did not enhance fatty acid MICs (Clarke *et al*., 2007).

The most frequent iron acquisition mutation identified in sapienic acid-evolved *S. epidermidis* was SERP1061.D49Y. SERP1061 is the Fur transcriptional regulator, known to regulate metabolism, siderophore synthesis and virulence determinants (Horsburgh *et al*., 2001; Fillat, 2014). The downregulation of Fur transcription in previous work has indicated this protein may help regulate responses in *S. aureus* to linoleic acid challenge (Kenny *et al*., 2009).

Fur transcriptional regulators form homo-dimers, with N-terminal DNA binding domains and C-terminal ion binding and dimerisation domains (Dian *et al*., 2011). The aa change resulting from the SNP occurs in the DNA binding domain of the protein, though is likely to occur outside of the regions that directly contact DNA. This aa change may affect the affinity of SERP1061 for DNA through protein conformational changes.

An *S. aureus* Fur inactivation mutant was observed to have reduced growth rate, reduced oxidative stress resistance and was unable to down-regulate siderophore biosynthesis in the presence of iron (Horsburgh *et al*., 2001). It may be possible to assess if the SNP in SERP1061 increases or decreases the affinity of SERP1061 for its consensus sequence by comparing oxidative stress resistance or siderophore biosynthesis in this isolate compared to the wild type.

There were four other genes associated with SNPs that may be involved in the observed increase in linoleic acid resistance, the better characterised of these genes were Mqo-3 and GcvT. Mqo genes are enzymes involved in pyruvate metabolism, whilst GcvT is an enzyme which cleaves glycine to produce ammonia and serine precursors (Okamura-Ikeda *et al*., 2010). Similarly, Mqo-3 may also be involved in amino acid biosynthesis, as disruption of Mqo in *Corynebacterium glutamicum* increased L-Lysine production (Mitsuhashi *et al*., 2014).

Increased D-sphingosine resistance in both *S. epidermidis* and *S. aureus* could be mediated through SNPs within a RND family protein and nearby TetR family protein. The RND family proteins encode secondary multidrug transporters that can transport a wide range of compounds including drugs, metals, solvents,

fatty acids, detergents and dyes (Domenech *et al*., 2005; Putman *et al*., 2000). TetR family proteins are transcriptional regulators that in the absense of their ligand, are negative regulators of gene expression for genes whose promoters they bind, typically efflux pumps (Ramos *et al*., 2005; Sevvana *et al*., 2012).

The RND family proteins are characterised by their structure of 12 transmembrane domains, with the 1st and 2nd, 7th and 8th being linked by extracytoplasmic/extracellular loops which are suggested to determine substrate specificity (Domenech *et al*., 2005; Varela *et al*., 2012). RND family proteins often form multimers that form a central pore that allows substrate transportation (Murakami *et al*., 2002). In the RND family protein AcrB of *E.coli*, transmembrane domains of the multimer are indicated to interact loosely, creating vestibules that would allow phospholipids and other membrane components access to the central pore (Murakami *et al*., 2002). The RND proteins discussed here are Mmpl-like proteins. Some Mmpl-like RND family proteins have been described to act as "membrane hoovers" that pump out harmful substrates from the membrane (Truong-Bolduc *et al*., 2014). The creation of vestibules through loose interactions of transmembrane domains would explain how harmful components inserted into the membrane could enter the pump.

Both SERP0245 and SAOUHSC_02866, have a single aa change facing outwards in an α-helix within the portion of the transmembrane domains nearest to the extracellular domains. Though the transmembrane domains of RND family proteins are predicted to contain proton interacting residues (Guan and Nakae, 2001), these SNPs do not occur in the previously predicted regions, and the SNP in SAOUHSC_02866 does not alter charge. The SNPs of SERP0245 and SAOUHSC_02866 occur in the 12th and 6th transmembrane domain respectively. These domains are thought to have a conserved sequence (Putman *et al*., 2000), and may therefore play roles in substrate interaction. Guay *et al.* (1994), found that amino acid changes in transmembrane helices of the RND transporter *tetA*(B) increased resistance to 9-(dimethylglyclamido)minocycline whilst decreasing tetracycline resistance. This change in resistance is likely due to differences in substrate specificity (Guay *et al*., 1994). Interestingly, these amino

acid mutations were predicted to occur at similar depths within the membrane as the aa changes observed in SAOUHSC_02866 and SERP0245, though in different helices.

All TetR family repressors with known structures form homodimers, with the N-terminal domains containing a DNA binding helix-turn-helix motif, and the c-teminal domain acting as the substrate binding domain (Ramos *et al*., 2005). TetR proteins have a relaxed state, capable of binding DNA, and a tensed state, which is not (Ramos *et al*., 2005). The tensed state occurs when TetR family proteins are bound by their substrates, and prevents DNA binding, thus enabling transcription from TetR-regulated promoters.

Both SAOUHSC_02867 and SERP0247 TetR proteins have aa changes around or in a pocket in the substrate-binding domain. It is likely that these aa changes affect how this repressor interacts with the substrate, or may alternatively affect the state of the protein regardless of whether it is bound by the substrate or not. The SNP SAOUHSC_02867.A40S occurs in the α-2 in the DNA binding domain, this is therefore likely to be involved in DNA binding and may directly affect how the protein interacts with the consensus sequence.

It is predicted here that the TetR family regulator SERP0247 and SAOUHSC_02866 regulate expression of the RND transporters SERP0245 and SAOUHSC_02867 respectively. The synteny observed between the genes for these proteins across staphylococcal species supports this theory. Taken together, the most likely explanation for any resistance gained through these SNPs, would be that aa changes in the regulator result in increased expression of the transporter. Meanwhile, SNPs in the transporter result in greater recognition or more efficient export of D-sphingosine. Alternatively, the transporter could import D-sphingosine, in which case the reverse would be true.

S. aureus and *S. epidermidis* also both have SNPs resulting in aa changes in GA domain containing proteins, namely Ebh and SasF respectively. GA domains are known to bind albumin, which in turn can bind lipids (Johannessen *et al*., 1997; Kragh-Hansen, 1981). This interaction could be harmful, enabling proximity of D-sphingosine to the cell membrane. Alternatively, these albumin bound

proteins may well form a protective barrier that binds the lipid D-sphingosine, preventing its interaction with the membrane. The SNPs may therefore either enhance or reduce albumin binding by these proteins, potentially making these proteins more or less specific to the type of albumin present in the media as observed in other GA domain proteins (Johansson *et al*., 2002). Expression of *sasF* was previously observed to enhance staphylococcal resistance to linoleic acid (Kenny *et al*., 2009; King *et al*., 2012). The involvement of SasF in linoleic acid resistance may support the role of the SNP increasing albumin binding. Another possibility is that the SNPs may alter the flexibility or orientation of the proteins, which could again affect the ability of D-sphingosine to interact with the cell.

One mutation that seems a likely candidate by contributing to increased D-sphingosine resistance arose in *S. aureus* but not in *S. epidermidis*. This was a SNP upstream of the gene encoding RsbU; RsbU has been shown to be is required for σ^B activation in *S. aureus*, and deletion of *S. aureus rsbU* reduces σ^B activity (Pane-Farre *et al*., 2006; Palma and Cheung, 2001). The alternative sigma factor σ^B regulates a number of stress resistance related pathways, including carotenoid biosynthesis, capsule biosynthesis, coagulase activity and clumping factor (Nicholas *et al*., 1999; Morikawa *et al*., 2001; Pane-Farre *et al*., 2006). Further, σ^B mediates resistance to a number of stresses including heat and acid (Chan and Foster, 1998). Overproduction of σ^B is known to lead to increased carotenoid production, increased PBP proteins production, cell wall thickening and increased resistance to lysostaphin, glycopeptides and β-lactams (Morikawa *et al*., 2001). With so many factors affected by σ^B activity, and therefore ultimately RsbU, a mutation affecting the transcription of *rsbU* could well enhance resistance.

Another process with mutations in *S. aureus* but not *S. epidermidis* in response to D-sphingosine passage was uptake and usage of amino acids. These mutations consist of a SNP upstream of *saouhsc_01839*, encoding the tyrosyl-tRNA synthase, a SNP within *gltX*, encoding the glutamyl tRNA synthase, and *saouhsc_01450*, encoding an amino acid transporter. Further testing would be

required to assess the effects of these SNPs and their impact upon D-sphingosine resistance.

Of note, *S. aureus* experimentally evolved to D-sphingosine had up to 8 times higher D-sphingosine MICs than the D-sphingosine most evolved *S. epidermidis* isolates. This higher D-sphingosine MIC corresponds well with the greater number of mutations observed in *S. aureus* than *S. epidermidis*. It remains unclear if this difference is due to strain or species variation or the differences in evolution methods used.

The data presented here shows interesting candidates for mutations that may lead to sapienic acid, linoleic acid and D-sphingosine resistance, and processes that may be under particular selective pressure from these antimicrobial lipids. Nevertheless, none of these mutations listed above can be said to definitely increase resistance to either antimicrobial lipid, since there was always multiple mutations per genome. Further, intergenic mutations outside of promoter regions were not analysed, but may potentially contribute to increases in resistance by affecting the chromosomal structure and therefore transcription. To confirm if mutations increase resistance to the antimicrobial it would be necessary to engineer the mutation in a clean background and test its antimicrobial resistance compared with wild type.

One issue of note with the sapienic acid-evolved *S. epidermidis* was that the sapienic acid MIC values were much higher than expected based on previous MICs in this strain. At first, it was suspected that there may be an issue with the batch of sapienic acid, however MICs using other strains gave results consistent with those previously determined. The increased MICs are therefore likely to be a result of the passage in 5 % ethanol, prior to evolution in antimicrobial lipids, which resulted in the "Rp-0" Rp62a isolate. Work by Drake *et al.* (2008), indicates there is a synergistic effect between ethanol and antimicrobial lipids. It is possible that genetic changes occurred between the stock strain of Rp62a and Rp-0 that enhanced this strain's tolerance of ethanol and thus, increased the MIC for all subsequent strains. An assay that compared sapienic acid growth or MIC of Rp62a to Rp-0 could confirm if Rp-0 has increased resistance compared to the wild type Rp62a. SNPs or INDELs that occurred between Rp-0 and the

Rp62a reference genome would be candidates for genes that may increase ethanol tolerance.

Despite concerns over ethanol interference, the ethanol evolved control Rp62a showed decreased survival to linoleic acid and D-sphingosine compared to the Rp-0 and previous Rp62a wild type levels. However, data from 4 h and 24 h growth in sapienic acid indicated that the ethanol evolved clone is more resistant to sapienic acid than the Rp-0 clone, having a similar level of resistance to two of the three sapienic acid-evolved clones. Together, this indicates passage in ethanol does not select for increased resistance to linoleic acid or D-sphingosine, but that it may with sapienic acid. This could be indicative of a greater synergistic interaction between ethanol and sapienic acid than previously thought. It has not been tested if this result is specific to Rp62a, and it is uncertain if passage in broth without ethanol would have similar outcomes.

Chapter 6 General discussion

There is speculation that the decrease in cutaneous antimicrobial lipids on the skin of AD patients is directly linked to the degree of *S. aureus* colonisation (Takigawa *et al*., 2005; Arikawa *et al*., 2002; Bibel *et al*., 1992; 1995). It was hypothesised that if antimicrobial skin lipids prevent *S. aureus* colonisation, *S. aureus* must be susceptible to antimicrobial skin lipids whilst dominant skin colonisers, such as *S. epidermidis*, should be less susceptible. It was found that *S. aureus* strains were significantly more susceptible to AFAs than *S. epidermidis*, but that there was no significant difference in susceptibility to D-sphingosine. This supports the hypothesis that AFAs can prevent *S. aureus* skin colonisation while allowing *S. epidermidis* to persist. It also indicates that AFAs may play an important role in preventing skin infections in AD patients.

Of note, *S. epidermidis* strains were still more susceptible to AFAs than has been reported for other dominant skin colonisers (Fischer *et al*., 2012). These findings agree with recent reports that not just *S. aureus*, but a range of staphylococcal species were increased on AD skin (Soares *et al*., 2013; Kong *et al*., 2012). This supports suggestions that AFAs control the levels of all staphylococcal colonisation (Coates *et al*., 2014).

The interaction between host and microbe leading to various skin complaints including acne vulgaris, rosacea, AD, seborrheic dermatitis, body malodour and psoriasis is a popular topic (Schommer and Gallo, 2013). The belief is that these conditions are caused or exacerbated by disruption of the normal skin flora and that, with sufficient understanding, skin care products that promote a beneficial microbiota could be produced (Schommer and Gallo, 2013; Nakatsuji and Gallo, 2014). Various reports have suggested the use of AFAs and sphingoid bases as topical treatments for skin disorders including AD (Takigawa *et al*., 2005; Arikawa *et al*., 2002; Bibel *et al*., 1992; 1995).

We may already be affecting our microbiota through skincare, possibly even promoting a detrimental microbiota. Ceramides are frequently added to moisurisers and body wash products as they improve skin hydration

(Meckfessel and Brandt, 2014). The breakdown of these ceramides on skin will increase the skin content of free fatty acids and sphingosines. On skin, whilst long chain saturated fatty acids are predominant, it is thought that the level of short-medium chain unsaturated fatty acids increases closer to the surface, likely due to sebaceous contributions (Ananthapadmanabhan *et al*., 2013). Short-medium chain unsaturated fatty acids are more antimicrobial than their long chain or saturated counterparts (Desbois and Smith, 2010), as such, this gradient is likely to increase the antimicrobial potential at the skin surface, potentially acting as a barrier against microbial challenge. Addition of ceramides to the skin will effectively dilute surface antimicrobial lipids, which could disrupt the normal skin flora. Skin surface lipids, particularly free fatty acids, are also removed during cleansing due to the surfactant activity of most wash products (Ananthapadmanabhan *et al*., 2013).

Addition of non-native antimicrobial lipids, such as tea tree or orange essential oils, to skincare is also likely to impact the skin microflora. Indeed orange oil and tea tree oil are known to be antimicrobial to *S. aureus*, and have been suggested as novel antimicrobials to control MRSA or as a topical acne treatment respectively (Muthaiyan *et al*., 2012; Cuaron *et al*., 2013). The antimicrobial activity of these oils is due mainly to terpenes (Lee *et al*., 2013). Antimicrobial activity is broad, with Gram-positive bacteria, Gram-negative bacteria, yeasts and fungi all being susceptible (Carson *et al*., 1998). The impact of these and other antimicrobial oils on the skin microbiome could therefore be profound.

Of concern, it was found here that evolution with sapienic acid led to vancomycin intermediate resistance in *S. epidermidis*, with vancomycin-intermediate associated SNPs also developing in *yycG* in *S. epidermidis* evolved with D-sphingosine. Passage of staphylococci, as well as other pathogenic bacteria, in sub-lethal concentrations of tea tree oil led to small increases in MICs to a range of antimicrobials including vancomycin, though results varied in significance and were sometime temporary (Thomsen *et al*., 2013; McMahon *et al*., 2007; 2008). The use of AFAs, sphingosines and other antimicrobial lipids to

control microbiota should therefore be initialised with care, as it could cause a rise in antibiotic resistance.

Emergence of vancomycin intermediate resistance staphylococcal isolates is not unique to treatment with antimicrobial lipids. A recent study found VISA isolates developed when *S. aureus* was grown in an Mg^{2+} induced biofilm for 5 days (Koch *et al*., 2014). In this study, the Mg^{2+} resulted in σ^B deficient strains with increased Bsa bacteriocin production; this drove evolution of Bsa and vancomycin intermediate resistant isolates from the wild type (Koch *et al*., 2014). These VISA isolates had mutations in genes encoding YycGF, GraRS and VraSR, all loci typically associated with VISA isolates (Koch *et al*., 2014; Mwangi *et al*., 2007; Howden *et al*., 2011; Renzoni *et al*., 2011; Peleg *et al*., 2012). Bsa and vancomycin both interfere with bacterial cell wall biosynthesis, so it is not entirely surprising that mutations that lead to resistance to one give rise to resistance to the other.

SNPs in *yycG* have been linked to reduced daptomycin susceptibility and VISA. Vancomycin and daptomycin are considered last resort antibiotics for staphylococcal infections (Boneca and Chiosis, 2003; Boger, 2001). VISA and GISA isolates are known to arise spontaneously in the host from susceptible isolates (Mwangi *et al*., 2007; Howden *et al*., 2011; Renzoni *et al*., 2011; Peleg *et al*., 2012). The work here indicates that VISA strains may be selected for by antimicrobial lipids on skin, perhaps forming a reservoir of resistance.

Semi-synthetic glycopeptides, such as the Food and Drug Administration (FDA) approved telavancin (commercially known as Vibativ), with increased activity against VISA and VRSA isolates provide the next generation of last resort antibiotics against MRSA (Boneca and Chiosis, 2003; Boger, 2001; Yim *et al*., 2014). Though there are many potential modifications that could be used to increase susceptibility of glycopeptides (Yim *et al*., 2014), the targets of these antibiotics are still the cell wall, strains with intermediate resistance to these next generation glycopeptides could develop through similar means as to vancomycin. This effect could be compounded if antimicrobial lipids were to be used as a treatment for AD patients, particularly as their skin is known to harbour higher levels of *S. aureus* than is typical of healthy skin.

During this study, transcriptional profiling and experimental evolution were both used to identify genes that may be involved in antimicrobial lipid resistance. A number of the genes containing SNPs or INDELs had homologues that were differentially expressed in *S. aureus* or *S. epidermidis* upon sapienic acid challenge (Table 6.1). This overlap may give an indication as to the nature of the SNP or INDEL. For example, in an *S. epidermidis* isolate evolved to sapieinic acid *serp0057*, predicted to encode an extracellular protein, had a SNP upstream of the translational start site that potentially affects transcription. This gene was two-fold downregulated in *S. epidermidis* upon sapienic acid change, suggesting this SNP could further downregulate transcription of this gene. Alternatively, the SNP could reduce the repression of this gene in sapienic acid conditions. Only expression analysis would really answer the effect of this SNP, if any.

Table 6.1 *S. aureus* and *S. epidermidis* genes that contain SNPs or INDELs after evolution with sapienic acid or D-sphingosine with homologues that are DE after sapienic acid challenge.

Genes not differentially expressed are noted by a dash, and genes without a homologue in the corresponding data set are marked by an "X".

SNP gene	Gene function	*S. aureus* $\log_2$ fold change	*S. epidermidis* $\log_2$ Fold Change
Sapienic acid evolved *S. epidermidis*			
Serp0499	NifU homologue	-	+0.55
Serp2127	Hypothetical Fe^{2+} transporter	X	+1.64
Serp0057	Hypothetical protein	-	-1.44
Mqo-3	Malate:quinone oxidoreductase	-0.95	-
D-sphingosine evolved *S. epidermidis*			
mnhA	Monovalent cation transporter	+2.22	+2.19
Serp2264	sasF homologue	+4.00	+1.10
Serp0247	TetR-like transcriptional regulator	-1.75	-
Serp1407	Hypothetical protein	+1.71	+1.10
Serp2327	Acetoin dehydrogenase	-0.72	-
Serp0458	Ion transporter	+1.25	-1.17
Serp1758	Lytic regulatory protein	-	-0.91
Serp2438	Cation transporter	+3.36	+2.76
D-sphingosine evolved *S. aureus*			
gltX	Glutamyl tRNA synthase	-1.2	-
SAOUHSC_01130	Hypothetical membrane protein	+1.15	-
SAOUHSC_01337	Transketalase	+0.97	-
SAOUHSC_01895	Hypothetical protein	-0.98	-1.62
SAOUHSC_02341	ATP synthase	-1.20	-
SAOUHSC_02587	Hypothetical CAAX protease	-1.42	-2.37
SAOUHSC_01450	Amino acid transporter	-	-1.27
Ebh	Extracellular matrix binding protein homologue	-	+0.41 - +1.36
FmhA	Methicillin resistance determinant	+1.60	-
SAOUHSC_02664	Transcriptional regulator	-0.74	-1.24
RsbU	Sigma B regulator	-0.70	-1.57
SAOUHSC_01840	Transglycosylase	-	-1.23
SAOUHSC_01839	Tyrosyl tRNA synthase	-1.61	-
SAOUHSC_00325	Hypothetical protein	+1.60	-

Table 6.1 continued *S. aureus* and *S. epidermidis* genes that contain SNPs or INDELs after evolution with sapienic acid or D-sphingosine with homologues that are DE after sapienic acid challenge.

Genes not differentially expressed are noted by a dash, and genes without a homologue in the corresponding data set are marked by an "X".

SNP gene	Gene function	*S. aureus* $\log_2$ fold change	*S. epidermidis* $\log_2$ Fold Change
D-sphingosine evolved *S. aureus*			
SAOUHSC_00196	Hypothetical protein	-	+0.71
SAOUHSC_00323	Hypothetical protein	+1.98	-
SAOUHSC_00936	Hypothetical protein	-	+0.97
SAOUHSC_01716	Protease	-1.17	-
SAOUHSC_01407	5-bromo-4-chlorindolyl phosphate hydrolase	+1.21	+1.38
SAOUHSC_00217	Sorbitol dehydrogenase	+1.72	+1.25
SAOUHSC_01141	Hypothetical protein	+1.15	-
SAOUHSC_01584	Hypothetical protein	+3.71	-

The genes that contain D-sphingosine evolved SNPs and are differentially expressed after sapienic acid challenge could indicate genes that are important in resistance to both sphingosines and AFAs. These genes included factors already shown to be important in linoleic acid resistance such as SasF. Most noteworthy of these genes are *ebh* and *saouhsc_00325*, as homozygous SNPs in these genes occur only in isolates with increased resistance to D-sphingosine and linoleic acid and these genes are upregulated after sapienic acid challenge. These proteins would be interesting targets for future study.

One of the aims of this study was to find indicators of the mechanisms of action (MOA) for sapienic acid and D-sphingosine. In Chapter three it was observed that there was no correlation between AFA and D-sphingosine MICs, suggesting that the mechanism of action and/or resistance determinants for these compounds are different.

An emerging theme between the SNP and RNA-Seq data that could be indicative of a MOA for sapienic acid was observed in factors associated with cell wall stress and cell wall stress resistance. There was upregulation of members of the cell wall stress stimulon such as VraSR, Fmt and MurZ, and the aforementioned

development of SNPs in YycG. This is suggestive that sapienic acid could interfere with cell wall biosynthesis, however no studies on the mechanism of action of antimicrobial lipids have found any evidence of cell wall interference. Indeed, studies generally agree that antimicrobial lipids target the membrane where they cause depolarisation and loss of cell solutes (Greenway and Dyke, 1979; Cartron *et al*., 2014; Parsons *et al*., 2012). Whilst members of the cell wall stress stimulon were upregulated, key members of this stimulon, such as *tagA* and *lytSR*, were not upregulated. Further doubt is cast upon this MOA by the development of *yycG* SNPs in D-sphingosine evolved *S. epidermidis* as well as sapienic acid evolved *S. epidermidis*. This could indicate this gene is a means to rapidly evolve resistance to many antimicrobials or stresses, therefore increasing the likelihood of a mutation in this gene. This apparent correlation may therefore be more indicative of a resistance mechanism to antimicrobial lipids rather than MOA.

There are indications in the RNA-Seq data (Chapter four) that sapienic acid induces an acid stress response with upregulation of NADH dehydrogenases, Na^+/H^+ antiporters and ammonia biosynthesis genes. Though sapienic acid has been linked to decreasing pH, the amount used in the RNA-seq experiment does not alter the pH of the medium. Acidic conditions decrease the reducing power available to the cell (Bore *et al*., 2007), if sapienic acid causes membrane depolarisation this could similarly decrease the reducing power. Alternatively, membrane permeability could directly alter the intracellular pH to that of the media as has been indicated previously (Cartron *et al*., 2014). This supports the theory that the MOA of sapienic acid could be through membrane depolarisation.

In D-sphingosine evolved *S. epidermidis* there were several SNPs in genes encoding cation transporters, suggesting cation transport may be important in D-sphingosine resistance. This may indicate D-sphingosine also interferes with the reducing power of the cell. Alternatively, this may help to neutralise pH changes caused by D-sphingosine, which is basic.

Membrane permeabilisation has been stated as a mechanism of action for D-sphingosine and sapienic acid. The upregulation of osmoprotectant transporters

and amino acid biosynthesis after sapienic acid challenge could support this theory, as osmotic stress and loss of amino acids could be challenges faced by the cell following membrane permealisation. Similarly, SNPs developed in *ebh*, encoding an osmotic stress resistance determinant, and *saouhsc_01450*, encoding an amino acid uptake protein during D-sphingosine evolution in *S. aureus* (Chapter five).

Taken together, cellular response data and experimental evolution data appears to suggest that sapienic acid and D-sphingosine have very similar modes of action. This would suggest that the lack of correlation between MIC is the result of differences in resistance mechanisms to sapienic acid and D-sphingosine. This indicates that sapienic acid and D-sphingosine resistance is mediated by different cellular components. D-sphingosine MICs did not show as much variation as sapienic acid MICs, and D-sphingosine was much more potent. Resistance mechanisms to D-sphingosine may therefore be less specific than those to sapienic acid, suggesting that sapienic acid plays a greater role in control of staphylococci on skin than D-sphingosine.

The final aim of this study was to identify genes and pathways that could act as resistance determinants in *S. epidermidis* and *S. aureus* to sapienic acid and D-sphingosine. An overview of the speculative resistance determinants and hypothesised mechanisms are shown in Figure 6.1. Further investigations are required to confirm the involvement of the factors that are novelly associated with antimicrobial lipid resistance in this study.

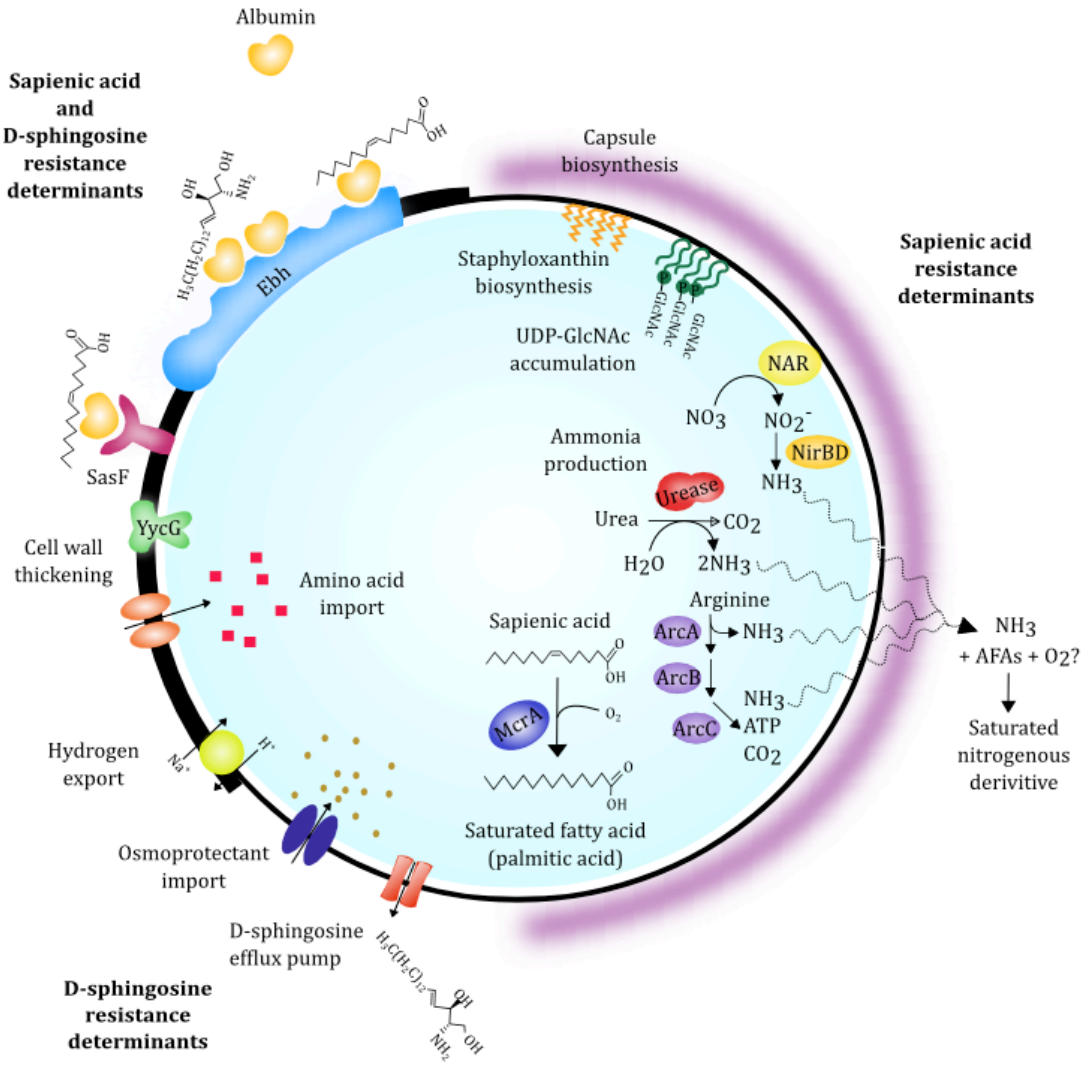


Figure 6.1 Proposed cutaneous antimicrobial lipid resistance determinants of *S. aureus* and *S. epidermidis.*

AFA: antimicrobial fatty acid, UDP-GlcNAc: undecaprenylphosphate N-acetyl glucosamine.

Over the course of this investigation, two factors that could limit the validity of results became evident. The first was the use of ethanol to dilute the antimicrobial lipids as ethanol may have had unexpected effects on staphylococcal responses, despite the low concentrations used. One report found that very low ethanol concentrations (VLEC), as low as 0.075 % affected post-stationary phase growth, metabolism and morphology (Chatterjee *et al.*, 2006). *S. aureus* appeared to have weakened cell wall structures that led to lysis after 48 h when grown in 0.1 % ethanol in microaerobic conditions, though not in aerobic conditions (Chatterjee *et al.*, 2006). Cultures grown with ≥ 0.075 %

ethanol showed delayed catabolism of acetate and delayed accumulation of ammonia; delayed ammonia production was linked to inhibition of amino acid uptake (Chatterjee *et al*., 2006). As ammonia production has in this study been speculated to act as a resistance determinant against fatty acids, the impact of ethanol could be considerable. Interestingly, supplementation with arginine reversed the effects of ethanol on the cells and the arginine deiminase pathway was upregulated under VLEC with arginine conditions (Chatterjee *et al*., 2006).

A number of the transcriptional changes observed in *S. aureus* exposed to 2.4 % ethanol for 24 h were also observed upon sapienic acid challenge. These similarities included upregulation of capsule biosynthesis, urease, vraS, purine and pyrimidine biosynthesis, H^+ antiporters and oxidative stress response genes (Korem *et al*., 2010). In the sapienic acid challenge RNA-seq experiments, the concentrations of ethanol were < 0.002 %, and control cultures were treated with the same concentration of ethanol as the treated cultures. Since the concentrations used were well below 0.075 % it is unlikely that this would impact transcription, also because the control was treated with the same concentration, any changes should have been present in both cultures so would not have registered as a change in transcription. The genes with similar transcriptional regulation in sapienic acid challenge as ethanol treatment are linked to the ability of ethanol to reduce the intracellular pH (Korem *et al*., 2010). This may explain why there are some similarities in gene expression as sapienic acid may also decrease intracellular pH, or decrease the reducing potential as discussed above.

Cells were grown with 5 % ethanol during the experimental evolution experiment, which is above the level of ethanol that is known to affect *S. aureus*. Cells were grown for 24 h, which would take cells into post-stationary phase growth. These experiments are therefore likely to be impacted by ethanol effects. Interestingly, the study by Chatterjee *et al*. (2006) did not show any increase in ethanol MIC following passage in VLEC. In our study, SNPs were detected in strains passaged in ethanol, and there was some evidence that strain Rp-0, which was grown for 24 h in 5 % ethanol, did develop increased resistance to ethanol, though this was never directly tested.

Beyond direct responses of staphylococci, ethanol may also have a synergistic effect on the antimicrobial activity of sapienic acid (Drake *et al*., 2008). One mechanism for synergy would be for ethanol to increase the solubility of sapienic acid in broth, thereby increasing its capability to interact with the cells. However, Drake *et al*. (2008) state that pretreatment of cells with ethanol prior to fatty acid treatment led to the same increased inhibition, though no supporting data was presented. Ethanol has been found to act synergistically with a number of antimicrobials including daptomycin and minocycline (Estes *et al*., 2013). The former requires solvents to dissolve but the latter dissolves in water, supporting the idea that the synergistic effect can be achieved without increased solubility. This suggests that ethanol affects a target on the cell, such as the membrane, that is then further attacked upon sapienic acid treatment. Ethanol synergism would mean the true MICs for sapienic acid, linoleic acid and possibly D-sphingosine are higher than observed in Chapter three. Irrespective, the trends observed are likely to hold true. This synergism may have implications for SNPs and INDELs described in Chapter five, further tests without the use of ethanol as described by Fischer *et al.* (2012) may aid clarification of this topic.

The second concern that may affect the impact of results is one that plagues many studies; how results gained *in vitro* may differ *in vivo*. It is reported that human serum and proteins such as albumin increase the MIC of AFAs (Lacey and Lord, 1981; Parsons *et al*., 2012). A compound present on the skin that has been observed to decrease the activity of sphingosine is cholesterol sulphate (Payne *et al*., 1995). The water concentration of skin is also considerably lower than those used in the assays described here, given that antimicrobial lipids are amphipathic molecules this could have large ramifications for results.

Use of defined medium may address and assess some of the concerns described above. Such mediums create large differences in gene expression compared to rich media (Krismer *et al*., 2014). They could be used here to study the impact of genes indicated to have roles in antimicrobial lipid resistance in a more *in vivo* like environment. Alternatively, the use of a 3-D organotropic human skin

model as described previously (Popov *et al*., 2014) may provide more insight into the role of these genes on skin.

Chapter 7 References

ABU-QATOUSEH, L., CHINNI, S., SEGGEWISS, J., PROCTOR, R., BROSIUS, J., ROZHDESTVENSKY, T., PETERS, G., VON EIFF, C. & BECKER, K. 2010. Identification of differentially expressed small non-protein-coding RNAs in *Staphylococcus aureus* displaying both the normal and the small-colony variant phenotype. *Journal of Molecular Medicine,* 88**,** 565-75.

AGUILAR, P. & DE MENDOZA, D. 2006. Control of fatty acid desaturation: a mechanism conserved from bacteria to humans. *Molecular Microbiology,* 62**,** 1507-14.

ALLGAIER, H., JUNG, G., WERNER, R., SCHNEIDER, U. & ZÄHNER, H. 1986. Epidermin: sequencing of a heterodet tetracyclic 21-peptide amide antibiotic *European Journal of Biochemistry,* 160**,** 9-22.

ALLIGNET, J., ENGLAND, P., OLD, I. & EL SOLH, N. 2002. Several regions of the repeat domain of the *Staphylococcus caprae* autolysin, At1C, are involved in fibronectin binding. *FEMS Microbiology Letters,* 213**,** 193-97.

ALTSCHUL, S., GISH, W., MILLER, W., MYERS, E. & LIPMAN, D. 1990. Basic Local Alignment Search Tool. *Journal of Molecular Biology,* 215**,** 403-10.

AMIN, U., LASH, T. & WILKINSON, B. 1995. Proline betaine is a highly effective osmoprotectant for *Staphylococcus aureus*. *Archives of Microbiology,* 163**,** 138-42.

ANANTHAPADMANABHAN, K., MUKHERJEE, S. & CHANDAR, P. 2013. Stratum corneum fatty acids: their critical role in preserving barrier integrity during cleansing. *International Journal of Cosmetic Science,* 35**,** 337-45.

ANDERSON, K., WONG, J., POLYAK, K., ARONZON, D. & ENERBACK, C. 2009. Detection of psoriasin/S100A7 in the sera of patients with psoriasis. *British Journal of Dermatology,* 160**,** 325-32.

ARIKAWA, J., ISHIBASHI, M., KAWASHIMA, M., TAKAGI, Y., ICHIKAWA, Y. & IMOKAWA, G. 2002. Decreased levels of sphingosine, a natural antimicrobial agent, may be associated with vulnerability of the stratum corneum from patients with atopic dermatitis to colonization by *Staphylococcus aureus*. *Journal of Investigative Dermatology,* 119**,** 433-9.

ARRECUBIETA, C., LEE, M., MACEY, A., FOSTER, T. & LOWY, F. 2007. SdrF, a *Staphylococcus epidermidis* surface protein, binds type I collagen. *Journal of Biological Chemistry,* 282**,** 18767-76.

ASWANI, V., TREMBLAY, D., MOINEAU, S. & SHUKLA, S. 2011. *Staphylococcus epidermidis* bacteriophages from the anterior nares of humans. *Applied and Environment Microbiology,* 77**,** 7853-5.

BALABAN, N., CIRIONI, O., GIACOMETTI, A., GHISELLI, R., BRAUNSTEIN, J., SILVESTRI, C., MOCCHEGIANI, F., SABA, V. & SCALISE, G. 2007. Treatment of *Staphylococcus aureus* biofilm infection by the quorum-sensing inhibitor RIP. *Antimicrobial Agents & Chemotherapy,* 51**,** 2226-9.

BARNS, K. & WEISSHAAR, J. 2013. Real-time attack of LL-37 on single *Bacillus subtilis* cells. *Biochimica et Biophysica Acta,* 1828**,** 1511-20.

BERA, A., BISWAS, R., HERBERT, S., KULAUZOVIC, E., WEIDENMAIER, C., PESCHEL, A. & GOTZ, F. 2007. Influence of wall teichoic acid on lysozyme resistance in *Staphylococcus aureus*. *Journal of Bacteriology,* 189**,** 280-3.

BERA, A., HERBERT, S., JAKOB, A., VOLLMER, W. & GOTZ, F. 2005. Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*. *Molecular Microbiology,* 55**,** 778-787.

BERGSSON, G., ARNFINNSSON, J., STEINGRIMSSON, O. & THORMAR, H. 2001. Killing of Gram-positive cocci by fatty acids and monoglycerides. *Acta Pathologica Microbiologica et Immunologica,* 109**,** 670-8.

BESTEBROER, J., AERTS, P., ROOIJAKKERS, S., PANDEY, M., KÖHL, J., VAN STRIJP, J. & DE HAAS, C. 2010. Functional basis for complement evasion by staphylococcal superantigen-like 7. *Cellular Microbiology,* 12**,** 1506-16.

BEVERS, L., PINKSE, M., VERHAERT, P. & HAGEN, W. 2009. Oleate hydratase catalyzes the hydration of a nonactivated carbon-carbon bond. *Journal of Bacteriology,* 191**,** 5010-2.

BHAVSAR, A., BEVERIDGE, T. & BROWN, E. 2001. Precise deletion of tagD and controlled depletion of its product, glycerol 3-phosphate cytidylyltransferase, leads to irregular morphology and lysis of *Bacillus subtilis* grown at physiological temperature. *Journal of Bacteriology,* 183**,** 6688-93.

BIBEL, D., ALY, R. & SHINEFIELD, H. 1992. Antimicrobial activity of sphingosines. *Journal of Investigative Dermatology,* 98**,** 269-73.

BIBEL, D., ALY, R. & SHINEFIELD, H. 1995. Topical sphingolipids in antisepsis and antifungal therapy. *Clinical and Experimental Dermatology,* 20**,** 395-400.

BIEBER, T. 2008. Mechanisms of disease: Atopic dermatitis. *New England Journal of Medicine,* 358**,** 1483-94.

BISICCHIA, P., NOONE, D., LIOLIOU, E., HOWELL, A., QUIGLEY, S., JENSEN, T., JARMER, H. & DEVINE, K. 2007. The essential YycFG two-component system controls cell wall metabolism in *Bacillus subtilis*. *Molecular Microbiology,* 65**,** 180-200.

BLACK, L., SPENCER, G. & BREKKE, O. 1978. Reactions of lipids in corn with ammonia. *Journal of American Oil Chemistry,* 55**,** 526-9.

BOGER, D. 2001. Vancomycin, teicoplanin, and ramoplanin: synthetic and mechanical studies. *Medical Research Reviews,* 21**,** 356-81.

BONECA, I. & CHIOSIS, G. 2003. Vancomycin resistance: occurance, mechanisms and strategies to combat it. *Expert Opinion on Therapeutic Targets,* 7**,** 311-328.

BORE, E., LANGSRUD, S., LANGSRUD, O., RODE, T. & HOLCK, A. 2007. Acid-shock responses in *Staphylococcus aureus* investigated by global gene expression analysis. *Microbiology,* 153**,** 2289-303.

BOWDEN, M., VISAI, L., LONGSHAW, C., HOLLAND, K., SPEZIALE, P. & HOOK, M. 2002. Is the GehD lipase from *Staphylococcus epidermidis* a collagen binding adhesin? *Journal of Biological Chemistry,* 277**,** 43017-23.

BOYLE-VAVRA, S., YIN, S. & DAUM, R. 2006. The VraS/VraR two-component regulatory system required for oxacillin resistance in community-acquired methicillin-resistant *Staphylococcus aureus*. *FEMS Microbiology Letters,* 262**,** 163-71.

BREUKINK, E., VAN KRAAIJ, C., DEMEL, R., SIEZEN, R., KUIPERS, O. & DE KRUIJFF, B. 1997. The C-terminal region of nisin is responsible for the

initial interaction of nisin with the target membrane. *Biochemistry,* 36**,** 6968-76.
BROGDEN, N., MEHALICK, L., FISCHER, C., WERTZ, P. & BROGDEN, K. 2012. The emerging role of peptides and lipids as antimicrobial epidermal barriers and modulators of local inflammation. *Skin Pharmacology and Physiology,* 25**,** 167-81.
BROOKER, B. & FULLER, R. 1984. The adhesion of coagulase negative staphylococci to human skin and its relevance to the bacterial flora of milk. *Journal of Applied Bacteriology,* 57**,** 325-32.
BRUNSKILL, E. & BAYLES, K. 1996. Identification of LytSR-regulated genes from *Staphylococcus aureus*. *Journal of Bacteriology,* 178**,** 5810-2.
BURIAN, M., RAUTENBERG, M., KOHLER, T., FRITZ, M., KRISMER, B., UNGER, C., HOFFMANN, W., PESCHEL, A., WOLZ, C. & GOERKE, C. 2010. Temporal expression of adhesion factors and activity of global regulators during establishment of *Staphylococcus aureus* nasal colonization. *Journal of Infectious Diseases,* 201**,** 1414-21.
BURKE, F., DI POTO, A., SPEZIALE, P. & FOSTER, T. 2011. The A domain of fibronectin-binding protein B of *Staphylococcus aureus* contains a novel fibronectin binding site. *FEBS Journal,* 278**,** 2359-71.
CALLEWAERT, C., KERCKHOF, F., GRANITSIOTIS, M., VAN GELE, M., VAN DE WIELE, T. & BOON, N. 2013. Characterization of *Staphylococcus* and *Corynebacterium* clusters in the human axillary region. *PLoS One,* 8**,** e70538.
CAMPBELL, I., CROZIER, D., PAWAGI, A. & BUIVIDS, I. 1983. *In vitro* response of *Staphylococcus aureus* from Cystic Fibrosis patients to combinations of linoleic and oleic acids added to the nutrient medium. *Journal of Clinical Microbiology,* 18**,** 408-15.
CAMPBELL, J., SINGH, A., SWOBODA, J., GILMORE, M., WILKINSON, B. & WALKER, S. 2012. An antibiotic that inhibits a late step in wall teichoic acid biosynthesis induces the cell wall stress stimulon in *Staphylococcus aureus*. *Antimicrobial Agents & Chemotherapy,* 56**,** 1810-20.
CAMPBELL-LEE, S. 2012. The *Staphylococcus aureus* response to D-sphingosine and implications in atopic dermatitis. *MSc dissertation, University of Liverpool. UK*.
CANDI, E., SCHMIDT, R. & MELINO, G. 2005. The cornified envelope: a model of cell death in the skin. *Nature Reviews Molecular Cell Biology,* 6**,** 328-40.
CARSON, C., RILEY, T. & COOKSON, B. 1998. Efficacy and safety of tea tree oil as a topical antimicrobial agent. *Journal of Hospital Infection,* 40**,** 175-8.
CARTRON, M., ENGLAND, S., CHIRIAC, A., JOSTEN, M., TURNER, R., RAUTER, Y., HURD, A., SAHL, H., JONES, S. & FOSTER, S. 2014. Analysis of the bactericidal activity of the human skin fatty acid, cis-6-hexadecanoic cid on *Staphylococcus aureus*. *Antimicrobial Agents & Chemotherapy,* 58**,** 3599-609.
CHAIBENJAWONG, P. & FOSTER, S. 2011. Desiccation tolerance in *Staphylococcus aureus*. *Archives of Microbiology,* 193**,** 125-35.
CHAMBERLAIN, N. & BRUEGGEMANN, S. 1997. Characterisation and expression of fatty acid modifying enzyme produced by *Staphylococcus epidermidis*. *Journal of Medical Microbiology,* 46**,** 693-7.

CHAMBERLAIN, N. & IMANOEL, B. 1996. Genetic regulation of fatty acid modifying enzyme from *Staphylococcus aureus*. *Journal of Medical Microbiology,* 44**,** 125-9.
CHAMBERLAIN, N., MEHRTENS, B., XIONG, Z., KAPRAL, F., BOARDMAN, J. & REARICK, J. 1991. Correlation of carotenoid production, decreased membrane fluidity and resistance to oleic acid killing in *Staphylococcus aureus* 18Z. *Infection and Immunity,* 59**,** 4332-7.
CHAMBERS, H. & DELEO, F. 2009. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nature Reviews: Microbiology,* 7**,** 629-41.
CHAMBERS, H., SACHDEVA, M. & HACKBARTH, C. 1994. Kinetics of penicillin binding to penicillin-binding proteins of *Staphylococcus aureus*. *Journal of Biochemistry,* 301**,** 139-44.
CHAN, P. & FOSTER, S. 1998. Role of SarA in virulence determinant production and environmental signal transduction in *Staphylococcus aureus*. *Journal of Bacteriology,* 180**,** 6232-41.
CHATTERJEE, I., SOMERVILLE, G., HEILMANN, C., SAHL, H., MAURER, H. & HERRMANN, M. 2006. Very low ethanol concentrations affect the viability and growth recovery in post-stationary-phase *Staphylococcus aureus* populations. *Applied and Environment Microbiology,* 72**,** 2627-36.
CHEN, X., NIYONSABA, F., USHIO, H., OKUDA, D., NAGAOKA, I., IKEDA, S., OKUMURA, K. & OGAWA, H. 2005. Synergistic effect of antibacterial agents human beta-defensins, cathelicidin LL-37 and lysozyme against *Staphylococcus aureus* and *Escherichia coli*. *Journal of Dermatological Science,* 40**,** 123-32.
CHEUNG, A., BAYER, A., ZHANG, G., GRESHAM, H. & XIONG, Y. 2004. Regulation of virulence determinants in vitro and in vivo in *Staphylococcus aureus*. *FEMS Immunology & Medical Microbiology,* 40**,** 1-9.
CHO, J., XUAN, C. & MILLER, L. 2010. Lucky number seven: Rnase 7 can prevent *Staphylococcus aureus* skin colonization. *Journal of Investigative Dermatology,* 130**,** 2703-6.
CHO, S., STRICKLAND, I., BOGUNIEWICZ, M. & LEUNG, D. 2001. Fibronectin and fibrinogen contribute to the enhanced binding of *Staphylococcus aureus* to atopic skin. *Journal of Allergy and Clinical Immunology,* 108**,** 269-74.
CHRISTENSEN, G. & BRUGGEMANN, H. 2014. Bacterial skin commensals and their role as host guardians. *Beneficial Microbes,* 5**,** 201-15.
CHRISTENSEN, G., SIMPSON, W., BISNO, A. & BEACHEY, E. 1982. Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. *Infection and Immunity,* 37**,** 318-26.
CHRISTENSEN, G., SIMPSON, W., YOUNGER, J., BADDOUR, L., BARRETT, F., MELTON, D. & BEACHEY, E. 1985. Adherence of coagulase negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *Journal of Clinical Microbiology,* 22**,** 966-1006.
CINGOLANI, P., PLATTS, A., WANG LE, L., COON, M., NGUYEN, T., WANG, L., LAND, S., LU, X. & RUDEN, D. 2012. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. *Fly (Austin),* 6**,** 80-92.

CLARKE, S., ANDRE, G., WALSH, E., DUFRENE, Y., FOSTER, T. & FOSTER, S. 2009. Iron-regulated surface determinant protein A mediates adhesion of *Staphylococcus aureus* to human corneocyte envelope proteins. *Infection and Immunity,* 77**,** 2408-16.

CLARKE, S., HARRIS, L., RICHARDS, R. & FOSTER, S. 2002. Analysis of Ebh, a 1.1-megadalton cell wall-associated fibronectin-binding protein of *Staphylococcus aureus*. *Infection and Immunity,* 70**,** 6680-7.

CLARKE, S., MOHAMED, R., BIAN, L., ROUTH, A., KOKAI-KUN, J., MOND, J., TARKOWSKI, A. & FOSTER, S. 2007. The *Staphylococcus aureus* surface protein IsdA mediates resistance to innate defenses of human skin. *Cell Host and Microbe,* 1**,** 199-212.

CLARKE, S., WILTSHIRE, M. & FOSTER, S. 2004. IsdA of *Staphylococcus aureus* is a broad spectrum, iron-regulated adhesin. *Molecular Microbiology,* 51**,** 1509-19.

CLAUDITZ, A., RESCH, A., WIELAND, K., PESCHEL, A. & GOETZ, F. 2006. Staphyloxanthin plays a role in the fitness of *Staphylococcus aureus* and its ability to cope with oxidative stress. *Infection and Immunity,* 74**,** 4950-3.

COATES, R., MORAN, J. & HORSBURGH, M. 2014. Staphylococci: colonizers and pathogens of human skin. *Future Microbiology,* 9**,** 75-91.

COGEN, A., NIZET, V. & GALLO, R. 2008. Skin microbiota: a source of disease or defence? *British Journal of Dermatology,* 158**,** 442-55.

COGEN, A., YAMASAKI, K., MUTO, J., SANCHEZ, K., CROTTY ALEXANDER, L., TANIOS, J., LAI, Y., KIM, J., NIZET, V. & GALLO, R. 2010a. *Staphylococcus epidermidis* antimicrobial delta-toxin (phenol-soluble modulin-gamma) cooperates with host antimicrobial peptides to kill group A Streptococcus. *PloS One,* 5**,** e8557.

COGEN, A., YAMASAKI, K., SANCHEZ, K., DORSCHNER, R., LAI, Y., MACLEOD, D., TORPEY, J., OTTO, M., NIZET, V., KIM, J. & GALLO, R. 2010b. Selective antimicrobial action is provided by phenol-soluble modulins derived from *Staphylococcus epidermidis*, a normal resident of the skin. *Journal of Investigative Dermatology,* 130**,** 192-200.

CORRIGAN, R., MIAJLOVIC, H. & FOSTER, T. 2009. Surface proteins that promote adherence of *Staphylococcus aureus* to human desquamated nasal epithelial cells. *BMC Microbiology,* 9.

CORRIGAN, R., RIGBY, D., HANDLEY, P. & FOSTER, T. 2007. The role of *Staphylococcus aureus* surface protein SasG in adherence and biofilm formation. *Microbiology-SGM,* 153**,** 2435-46.

COSTELLO, E., LAUBER, C., HAMADY, M., FIERER, N., GORDON, J. & KNIGHT, R. 2009. Bacterial community variation in human body habitats across space and time. *Science,* 326**,** 1694-97.

COTTER, P. & HILL, C. 2003. Surviving the acid test: responses of Gram-positive bacteria to low pH. *Microbiology and Molecular Biology Reviews,* 67**,** 429-53.

CUARON, J., DULAL, S., SONG, Y., SINGH, A., MONTELONGO, C., YU, W., NAGARAJAN, V., JAYASWAL, R., WILKINSON, B. & GUSTAFSON, J. 2013. Tea tree oil-induced transcriptional alterations in *Staphylococcus aureus*. *Phytotherapy Research,* 27**,** 390-6.

CUI, L., MURAKAMI, H., KUWAHARA-ARAI, K., HANAKI, H. & HIRAMATSU, K. 2000. Contribution of a thickened cell wall and Its glutamine nonamidated component to the vancomycin resistance expressed by *Staphylococcus aureus* Mu50. *Antimicrobial Agents and Chemotherapy,* 44**,** 2276-85.

D'ELIA, M., MILLAR, K., BHAVSAR, A., TOMLJENOVIC, A., HUTTER, B., SCHAAB, C., MORENO-HAGELSIEB, G. & BROWN, E. 2009. Probing teichoic acid genetics with bioactive molecules reveals new interactions among diverse processes in bacterial cell wall biogenesis. *Chemistry and Biology,* 16**,** 548-56.

D'ELIA, M., PEREIRA, M., CHUNG, Y., ZHAO, W., CHAU, A., KENNEY, T., SULAVIK, M., BLACK, T. & BROWN, E. 2006. Lesions in teichoic acid biosynthesis in *Staphylococcus aureus* lead to a lethal gain of function in the otherwise dispensable pathway. *Journal of Bacteriology,* 188**,** 4183-9.

DAIYASU, H., OSAKA, K., ISHINO, Y. & TOH, H. 2001. Expansion of the zinc metallo-hydrolase family of the beta-lactamase fold. *FEBS Letters,* 503**,** 1-6.

DE GOFFAU, M., VAN DIJL, J. & HARMSEN, H. 2011. Microbial growth on the edge of desiccation. *Environmental Microbiology,* 13**,** 2328-35.

DE GOFFAU, M., YANG, X., VAN DIJL, J. & HARMSEN, H. 2009. Bacterial pleomorphism and competition in a relative humidity gradient. *Environmental Microbiology,* 11**,** 809-22.

DENOEUD, F., AURY, J., DA SILVA, C., NOEL, B., ROGIER, O., DELLEDONNE, M., MORGANTE, M., VALLE, G., WINCKER, P., SCARPELLI, C., JAILLON, O. & ARTIGUENAVE, F. 2008. Annotating genomes with massive-scale RNA sequencing. *Genome Biology,* 9**,** R175.

DERRÉ, I., RAPOPORT, G. & MSADEK, T. 1999. CtsR, a novel regulator of stress and heat shock response, controls *clp* and molecular chaperone gene expression in Gram-Positive bacteria. *Molecular Microbiology,* 31, 117-31.

DESBOIS, A. & LAWLOR, K. 2013. Antibacterial activity of long-chain polyunsaturated fatty acids against *Propionibacterium acnes* and *Staphylococcus aureus*. *Marine Drugs,* 11**,** 4544-57.

DESBOIS, A. & SMITH, V. 2010. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. *Applied Microbiology and Biotechnology,* 85**,** 1629-42.

DIAN, C., VITALE, S., LEONARD, G., BAHLAWANE, C., FAUQUANT, C., LEDUC, D., MULLER, C., DE REUSE, H., MICHAUD-SORET, I. & TERRADOT, L. 2011. The structure of the *Helicobacter pylori* ferric uptake regulator Fur reveals three functional metal binding sites. *Molecular Microbiology,* 79**,** 1260-75.

DIEP, B., GILL, S., CHANG, R., PHAN, T., CHEN, J., DAVIDSON, M., LIN, F., LIN, J., CARLETON, H., MONGODIN, E., SENSABAUGH, G. & PERDREAU-REMINGTON, F. 2006. Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant *Staphylococcus aureus*. *Lancet,* 367**,** 731-39.

DOMENECH, P., REED, M. & BARRY, C., 3RD 2005. Contribution of the *Mycobacterium tuberculosis* MmpL protein family to virulence and drug resistance. *Infection and Immunity,* 73**,** 3492-501.

DONG, Z. & CHEN, Y. 2013. Transcriptomics: advances and approaches. *Science China Life Sciences,* 56**,** 960-7.
DOROSHCHUK, N., GELFAND, M. & RODIONOV, D. 2006. Regulation of nitrogen metabolism in Gram-positive bacteria. *Molecular Biology,* 40**,** 829-36.
DRAKE, D., BROGDEN, K., DAWSON, D. & WERTZ, P. 2008. Antimicrobial lipids at the skin surface. *Journal of Lipid Research,* 49**,** 4-11.
DUBRAC, S., GOMPERTS BONECA, I., POUPEL, O. & MSADEK, T. 2007. New insights into the WalK/WalR (YycG/YycF) essential signal transduction pathway reveal a major role in controlling cell wall metabolism and biofilm formation in *Staphylococcus aureus*. *Journal of Bacteriology,* 189**,** 8257-69.
DUQUENNE, M., FLEUROT, I., AIGLE, M., DARRIGO, C., BOREZÉE-DURANT, E., DERZELLE, S., BOUIX, M., DEPERROIS-LAFARGE, V. & DELACROIX-BUCHET, A. 2010. Tool for quantification of staphylococcal enterotoxin gene expression in cheese. *Applied and Environmental Microbiology,* 76**,** 1367-74.
DURFEE, T., HANSEN, A., ZHI, H., BLATTNER, F. & JIN, D. 2008. Transcription profiling of the stringent response in *Escherichia coli*. *Journal of Bacteriology,* 190**,** 1084-96.
DUTHIE, E. & LORENZ, L. 1952. Staphylococcal coagulase: mode of action and antigenicity. *Journal of General Microbiology,* 6**,** 95-107.
ELSHOLZ, A. K., MICHALIK, S., ZUHLKE, D., HECKER, M. & GERTH, U. 2010. CtsR, the Gram-positive master regulator of protein quality control, feels the heat. *EMBO Journal,* 29**,** 3621-9.
ERNST, C. & PESCHEL, A. 2011. Broad-spectrum antimicrobial peptide resistance by MprF-mediated aminoacylation and flipping of phospholipids. *Molecular Microbiology,* 80**,** 290-9.
ESTES, R., THEUSCH, J., BECK, A., PITRAK, D. & MULLANE, K. 2013. Activity of daptomycin with or without 25 percent ethanol compared to combinations of minocycline, EDTA, and 25 percent ethanol against methicillin-resistant *Staphylococcus aureus* isolates embedded in biofilm. *Antimicrobial Agents & Chemotherapy,* 57**,** 1998-2000.
EUCAST & ESCMID 2000. Determination of antimicrobial susceptibility test breakpoints. *Clinical Microbiology and Infection,* 6**,** 570-2.
FILLAT, M. 2014. The FUR (ferric uptake regulator) superfamily: diversity and versatility of key transcriptional regulators. *Archives of Biochemistry and Biophysics,* 546**,** 41-52.
FINOTELLO, F., LAVEZZO, E., BIANCO, L., BARZON, L., MAZZON, P., FONTANA, P., TOPPO, S. & DI CAMILLO, B. 2014. Reducing bias in RNA sequencing data: a novel approach to compute counts. *BMC Bioinformatics,* 15 Suppl 1**,** S7.
FISCHER, C., DRAKE, D., DAWSON, D., BLANCHETTE, D., BROGDEN, K. & WERTZ, P. 2012. Antibacterial activity of sphingoid bases and fatty acids against Gram positive and Gram negative bacteria. *Antimicrobial Agents and Chemotherapy,* 56**,** 1157-61.
FISCHER, C., WALTERS, K., DRAKE, D., BLANCHETTE, D., DAWSON, D., BROGDEN, K. & WERTZ, P. 2013. Sphingoid bases are taken up by *Escherichia coli* and *Staphylococcus aureus* and induce ultrastructural damage. *Skin Pharmacology and Physiology,* 26**,** 36-44.

FLOCK, J., FROMAN, G., JONSSON, K., GUSS, B., SIGNAS, C., NILSSON, B., RAUCCI, G., HOOK, M., WADSTROM, T. & LINDBERG, M. 1987. Cloning and expression of the gene for a fibronectin-binding protein from *Staphylococcus aureus*. *EMBO Journal,* 6**,** 2351-7.

FLUHR, J., KAO, J., JAIN, M., AHN, S., FEINGOLD, K. & ELIAS, P. 2001. Generation of free fatty acids from phospholipids regulates stratum corneum acidification and integrity. *Journal of Investigative Dermatology,* 117**,** 44-51.

FOSTER, T. 2009. Colonization and infection of the human host by staphylococci: adhesion, survival and immune evasion. *Veterinary Dermatology,* 20**,** 456-70.

FOZO, E. & QUIVEY, R. J. 2004. The fabM gene product of *Streptococcus mutans* is responsible for the synthesis of monounsaturated fatty acids and is necessary for survival at low pH. *Journal of Bacteriology,* 186**,** 4152-8.

FRANK, D., FEAZEL, L., BESSESEN, M., PRICE, C., JANOFF, E. & PACE, N. 2010. The human nasal microbiota and *Staphylococcus aureus* carriage. *PloS One,* 5**,** e10598.

FRIEDMAN, L., ALDER, J. & SILVERMAN, J. 2006. Genetic changes that correlate with reduced susceptibility to daptomycin in *Staphylococcus aureus*. *Antimicrobial Agents & Chemotherapy,* 50**,** 2137-45.

GALBRAITH, H. & MILLER, T. 1973. Effect of long-chain fatty acids on bacterial respiration and amino acid uptake. *Journal of Applied Bacteriology,* 36**,** 659-75.

GAMON, M., MOREIRA, E., SOARES DE OLIVEIRA, S., TEIXEIRA, M. & DO CARMO DE FREIRE BASTOS, M. 1999. Characterisation of a novel bacteriocin-encoding plasmid found in clinical isolates of *Staphylococcus aureus*. *Antonie Van Leeuwenhoek,* 75**,** 233-43.

GAO, Z., TSENG, C., PEI, Z. & BLASER, M. 2007. Molecular analysis of human forearm superficial skin bacterial biota. *PNAS,* 104**,** 2927-32.

GARDETE, S., WU, S., GILL, S. & TOMASZ, A. 2006. Role of VraSR in antibiotic resistance and antibiotic-induced stress response in *Staphylococcus aureus*. *Antimicrobial Agents & Chemotherapy,* 50**,** 3424-34.

GEIGER, T., GOERKE, C., FRITZ, M., SCHAFER, T., OHLSEN, K., LIEBEKE, M., LALK, M. & WOLZ, C. 2010. Role of the (p)ppGpp synthase RSH, a RelA/SpoT homolog, in stringent response and virulence of *Staphylococcus aureus*. *Infection and Immunity,* 78**,** 1873-83.

GIARDINE, B., RIEMER, C., HARDISON, R. C., BURHANS, R., ELNITSKI, L., SHAH, P., ZHANG, Y., BLANKENBERG, D., ALBERT, I., TAYLOR, J., MILLER, W., KENT, W. J. & NEKRUTENKO, A. 2005. Galaxy: a platform for interactive large-scale genome analysis. *Genome Research,* 15**,** 1451-5.

GLASER, R., HARDER, J., LANGE, H., BARTELS, J., CHRISTOPHERS, E. & SCHRODER, J. 2005. Antimicrobial psoriasin (S100A7) protects human skin from *Escherichia coli* infection. *Nature Immunology,* 6**,** 57-64.

GOECKS, J., NEKRUTENKO, A., TAYLOR, J. & GALAXY, T. 2010. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biology,* 11**,** R86.

GREENWAY, D. & DYKE, K. 1979. Mechanism of the inhibitory action of linoleic acid on the growth of *Staphylococcus aureus*. *Journal of General Microbiology,* 115**,** 233-45.
GREENWAY, D. & DYKE, K. 1983. The effect of membrane-bound beta-lactamase on linoleic acid sensitivity in *Staphylococcus aureus*. *Journal of General Microbiology,* 129**,** 2457-65.
GRICE, E., KONG, H., CONLAN, S., DEMING, C., DAVIS, J., YOUNG, A., BOUFFARD, G., BLAKESLEY, R., MURRAY, P., GREEN, E., TURNER, M. & SEGRE, J. 2009. Topographical and temporal diversity of the human skin microbiome. *Science,* 324**,** 1190-2.
GROICHER, K., FIREK, B., FUJIMOTO, D. & BAYLES, K. 2000. The *Staphylococcus aureus lrgAB* operon modulates murein hydrolase activity and penicillin tolerance. *Journal of Bacteriology,* 182**,** 1794-801.
GRUNDLING, A. & SCHNEEWIND, O. 2007. Genes required for glycolipid synthesis and lipoteichoic acid anchoring in *Staphylococcus aureus*. *Journal of Bacteriology,* 189**,** 2521-30.
GUAN, L. & NAKAE, T. 2001. Identification of essential charged residues in transmembrane segments of the multidrug transporter MexB of *Pseudomonas aeruginosa*. *Journal of Bacteriology,* 183**,** 1734-9.
GUAY, G. & ROTHSTEIN, D. 1993. Expression of the *Tetk* gene from *Staphylococcus aureus* in *Escherichia coli*: comparison of substrate specificities of TetA(B), TetA©, and TetK efflux proteins. *Antimicrobial Agents and Chemotherapy,* 37**,** 191-8.
GUAY, G., TUCKMAN, M. & ROTHSTEIN, D. 1994. Mutations in the tetA(B) gene that cause a change in substrate specificity of the tetracycline efflux pump. *Antimicrobial Agents and Chemotherapy,* 38**,** 857-60.
HAFER, C., LIN, Y., KORNBLUM, J., LOWY, F. & UHLEMANN, A. 2012. Contribution of selected gene mutations to resistance in clinical isolates of vancomycin-intermediate *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy,* 56**,** 5845-51.
HANSEN, U., HUSSAIN, M., VILLONE, D., HERRMANN, M., ROBENEK, H., PETERS, G., SINHA, B. & BRUCKNER, P. 2006. The anchorless adhesin Eap (extracellular adherence protein) from *Staphylococcus aureus* selectively recognizes extracellular matrix aggregates but binds promiscuously to monomeric matrix macromolecules. *Matrix Biology,* 25**,** 252-60.
HARTFORD, O., O'BRIEN, L., SCHOFIELD, K., WELLS, J. & FOSTER, T. 2001. The Fbe (SdrG) protein of *Staphylococcus epidermidis* HB promotes bacterial adherence to fibrinogen. *Microbiology-SGM,* 147**,** 2545-52.
HEILMANN, C. 2011. Adhesion mechanisms of staphylococci. *Bacterial Adhesion: Chemistry, Biology and Physics,* 715**,** 105-23.
HEILMANN, C., HARTLEIB, J., HUSSAIN, M. & PETERS, G. 2005. The multifunctional *Staphylococcus aureus* autolysin Aaa mediates adherence to immobilized fibrinogen and fibronectin. *Infection and Immunity,* 73**,** 4793-802.
HEILMANN, C., THUMM, G., CHHATWAL, G., HARTLEIB, J., UEKOTTER, A. & PETERS, G. 2003. Identification and characterization of a novel autolysin (Aae) with adhesive properties from *Staphylococcus epidermidis*. *Microbiology-SGM,* 149**,** 2769-78.

HELL, W., MEYER, H. & GATERMANN, S. 1998. Cloning of aas, a gene encoding a *Staphylococcus saprophyticus* surface protein with adhesive and autolytic properties. *Molecular Microbiology,* 29**,** 871-81.

HERBERT, S., BERA, A., NERZ, C., KRAUS, D., PESCHEL, A., GOERKE, C., MEEHL, M., CHEUNG, A. & GOTZ, F. 2007. Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci. *PloS Pathogens,* 3**,** 981-94.

HERBERT, S., NEWELL, S., LEE, C., WIELAND, K., DASSY, B., FOURNIER, J., WOLZ, C. & DORING, G. 2001. Regulation of *Staphylococcus aureus* type 5 and type 8 capsular polysaccharides by CO(2). *Journal of Bacteriology,* 183**,** 4609-13.

HIGAKI, S., MOROHASHI, M., YAMAGISHI, T. & HASEGAWA, Y. 1999. Comparative study of staphylococci from the skin of atopic dermatitis patients and from healthy subjects. *International Journal of Dermatology,* 38**,** 265-9.

HIRSCHHAUSEN, N., SCHLESIER, T., SCHMIDT, M., GOETZ, F., PETERS, G. & HEILMANN, C. 2010. A novel staphylococcal internalization mechanism involves the major autolysin Atl and heat shock cognate protein Hsc70 as host cell receptor. *Cellular Microbiology,* 12**,** 1746-64.

HOLDEN, M., FEIL, E., LINDSAY, J., PEACOCK, S., DAY, N., ENRIGHT, M., FOSTER, T., MOORE, C., HURST, L., ATKIN, R., BARRON, A., BASON, N., BENTLEY, S., CHILLINGWORTH, C., CHILLINGWORTH, T., CHURCHER, C., CLARK, L., CORTON, C., CRONIN, A., DOGGETT, J., DOWD, L., FELTWELL, T., HANCE, Z., HARRIS, B., HAUSER, H., HOLROYD, S., JAGELS, K., JAMES, K., LENNARD, N., LINE, A., MAYES, R., MOULE, S., MUNGALL, K., ORMOND, D., QUAIL, M., RABBINOWITSCH, E., RUTHERFORD, K., SANDERS, M., SHARP, S., SIMMONDS, M., STEVENS, K., WHITEHEAD, S., BARRELL, B., SPRATT, B. & PARKHILL, J. 2004. Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *PNAS,* 101**,** 9786-91.

HORSBURGH, M., AISH, J., WHITE, I., SHAW, L., LITHGOW, J. & FOSTER, S. 2002. Sigma B Modulates Virulence Determinant Expression and Stress Resistance: Characterization of a Functional rsbU Strain Derived from *Staphylococcus aureus* 8325-4. *Journal of Bacteriology,* 184**,** 5457-67.

HORSBURGH, M., INGHAM, E. & FOSTER, S. 2001. In *Staphylococcus aureus*, fur is an interactive regulator with PerR, contributes to virulence, and Is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis. *Journal of Bacteriology,* 183**,** 468-75.

HOWDEN, B., MCEVOY, C., ALLEN, D., CHUA, K., GAO, W., HARRISON, P., BELL, J., COOMBS, G., BENNETT-WOOD, V., PORTER, J., ROBINS-BROWNE, R., DAVIES, J., SEEMANN, T. & STINEAR, T. 2011. Evolution of multidrug resistance during *Staphylococcus aureus* infection involves mutation of the essential two component regulator WalKR. *Plos Pathogens,* 7**,** e1002359.

HUANG, W. C., TSAI, T. H., CHUANG, L. T., LI, Y. Y., ZOUBOULIS, C. C. & TSAI, P. J. 2014. Anti-bacterial and anti-inflammatory properties of capric acid against Propionibacterium acnes: a comparative study with lauric acid. *Journal of Dermatological Science,* 73**,** 232-40.

HUANG, Y., LIN, Y., CHANG, T., WU, S., LEE, Y., CHANG, M., CHEN, C., WU, S. & LIAO, Y. 2007. The flexible and clustered lysine residues of human ribonuclease 7 are critical for membrane permeability and antimicrobial activity. *Journal of Biological Chemistry,* 282**,** 4626-33.

HUESCA, M., PERALTA, R., SAUDER, D., SIMOR, A. & MCGAVIN, M. 2002. Adhesion and virulence properties of epidemic Canadian methicillin-resistant *Staphylococcus aureus* strain 1: Identification of novel adhesion functions associated with plasmin-sensitive surface protein. *Journal of Infectious Diseases,* 185**,** 1285-96.

HUSSAIN, M., BECKER, K., VON EIFF, C., SCHRENZEL, J., PETERS, G. & HERRMANN, M. 2001. Identification and characterization of a novel 38.5 kilodalton cell surface protein of *Staphylococcus aureus* with extended spectrum binding activity for extracellular matrix and plasma proteins. *Journal of Bacteriology,* 183**,** 6778-86.

IRVINE, A. & MCLEAN, W. 2006. Breaking the (un)sound barrier: filaggrin is a major gene for atopic dermatitis. *Journal of Investigative Dermatology,* 126**,** 1200-2.

ISHIKAWA, J., NARITA, H., KONDO, N., HOTTA, M., TAKAGI, Y., MASUKAWA, Y., KITAHARA, T., TAKEMA, Y., KOYANO, S., YAMAZAKI, S. & HATAMOCHI, A. 2010. Changes in the ceramide profile of atopic dermatitis patients. *Journal of Investigative Dermatology,* 130**,** 2511-4.

IWASE, T., UEHARA, Y., SHINJI, H., TAJIMA, A., SEO, H., TAKADA, K., AGATA, T. & MIZUNOE, Y. 2010. *Staphylococcus epidermidis* Esp inhibits *Staphylococcus aureus* biofilm formation and nasal colonization. *Nature,* 465**,** 346-9.

JANSEN, A., TURCK, M., SZEKAT, C., NAGEL, M., CLEVER, I. & BIERBAUM, G. 2007. Role of insertion elements and *yycFG* in the development of decreased susceptibility to vancomycin in *Staphylococcus aureus*. *International Journal of Medical Microbiology,* 297**,** 205-15.

JEZEK, P., ENGSTOVÁ, H., ZÁCKOVA, M., VERCESI, A., COSTA, A., ARRUDA, P. & GARLID, K. 1998. Fatty acid cycling mechanism and mitochodrial uncoupling proteins. *Biochimica et Biophysica Acta,* 1365**,** 319-27.

JINNESTAL, C., BELFRAGE, E., BÄCK, O., SCHMIDTCHEN, A. & SONESSON, A. 2014. Skin barrier impairment correlates with cutaneous *Staphylococcus aureus* colonization and sensitization to skin-associated microbial antigens in adult patients with atopic dermatitis. *International Journal of Dermatology,* 53**,** 27-33.

JOHANNESSEN, M., CHÂTEAU, M., WIKSTRÖM, M., FORSÉN, S., DRAKENBERG, T. & BJÖRCK, L. 1997. Solution structure of the albumin-binding GA module: a versatile bacterial protein domain. *Journal of Molecular Biology,* 266**,** 859-65.

JOHANNESSEN, M., SOLLID, J. & HANSSEN, A. 2012. Host- and microbe determinants that may influence the success of *S. aureus* colonization. *Frontiers in Cellular and Infection Microbiology,* 2**,** 56.

JOHANSSON, M., NILSSON, H., EVENÄS, J., FORSÉN, S., DRAKENBERG, T., BJÖRCK, L. & WIKSTRÖM, M. 2002. Differences in backbone dynamics of two homologous bacterial albumin-binding modules:implications for binding specificity and bacterial adaptation. *Journal of Molecular Biology,* 316**,** 1083-99.

JONSSON, K., SIGNAS, C., MULLER, H. & LINDBERG, M. 1991. Two different genes encode fibronectin binding proteins in *Staphylococcus aureus* – the complete nucleotide sequence and characterization of the 2nd gene. *European Journal of Biochemistry,* 202**,** 1041-8.

JOO, Y., JEONG, K., YEOM, S., KIM, Y., KIM, Y. & OH, D. 2012. Biochemical characterization and FAD-binding analysis of oleate hydratase from *Macrococcus caseolyticus*. *Biochimie,* 94**,** 907-15.

JORGENSEN, J. & FERRARO, M. 2009. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clinical Infectious Diseases,* 49**,** 1749-55.

KAITO, C., MORISHITA, D., MATSUMOTO, Y., KUROKAWA, K. & SEKIMIZU, K. 2006. Novel DNA binding protein SarZ contributes to virulence in *Staphylococcus aureus*. *Molecular Microbiology,* 62**,** 1601-17.

KANEHISA, M. & GOTO, S. 2000. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Research,* 28**,** 27-30.

KANEHISA, M., GOTO, S., SATO, Y., FURUMICHI, M. & TANABE, M. 2012. KEGG for integration and interpretation of large-scale molecular datasets. *Nucleic Acids Research,* 40**,** D109-14.

KAPRAL, F., SMITH, S. & LAL, D. 1992. The esterification of fatty acids by *Staphylococcus aureus* fatty acid modifying enzyme (FAME) and its inhibition by glycerides. *Journal of Medical Microbiology,* 37**,** 235-7.

KELLY, J. 2013. Genomic and metagenomic analysis of the skin microbiota. *PhD thesis, University of Liverpool: UK*.

KENNY, J., MORAN, J., KOLAR, S., ULANOV, A., LI, Z., SHAW, L., JOSEFSSON, E. & HORSBURGH, M. 2013. Mannitol utilisation is required for protection of *Staphylococcus aureus* from human skin antimicrobial fatty acids. *Plos One,* 8**,** e67698.

KENNY, J., WARD, D., JOSEFSSON, E., JONSSON, I., HINDS, J., REES, H., LINDSAY, J., TARKOWSKI, A. & HORSBURGH, M. 2009. The *Staphylococcus aureus* response to unsaturated long-chain free fatty acids: survival mechanisms and virulence implications. *PloS One,* 4**,** e4344.

KING, N., BEATSON, S., TOTSIKA, M., ULETT, G., ALM, R., MANNING, P. & SCHEMBRI, M. 2011. UafB is a serine-rich repeat adhesin of *Staphylococcus saprophyticus* that mediates binding to fibronectin, fibrinogen and human uroepithelial cells. *Microbiology-SGM,* 157**,** 1161-75.

KING, N., SAKINC, T., BEN ZAKOUR, N., TOTSIKA, M., HERAS, B., SIMERSKA, P., SHEPHERD, M., GATERMANN, S., BEATSON, S. & SCHEMBRI, M. 2012. Characterisation of a cell wall-anchored protein of *Staphylococcus saprophyticus* associated with linoleic acid resistance. *BMC Microbiology,* 12.

KIRAN, M. & BALABAN, N. 2009. TRAP playes a role in stress response in *Staphylococcus aureus*. *International Journal of Artificial Organs,* 32**,** 592-9.

KLOOS, W. & MUSSELWHITE, M. 1975. Distribution and persistance of *Staphylococcus* and *Micrococcus* species and other aerobic bacteria on human skin. *Applied Microbiology,* 30**,** 381-95.

KLOOS, W. & SCHLEIFER, K. 1975. Isolation and characterization of staphylococci from human skin. *International Journal of Systematic Bacteriology,* 25**,** 62-79.
KLUYTMANS, J. & WERTHEIM, H. 2005. Nasal carriage of *Staphylococcus aureus* and prevention of nosocomial infections. *Infection,* 33**,** 3-8.
KOCH, G., YEPES, A., FORSTNER, K., WERMSER, C., STENGEL, S., MODAMIO, J., OHLSEN, K., FOSTER, K. & LOPEZ, D. 2014. Evolution of Resistance to a Last-Resort Antibiotic in *Staphylococcus aureus* via Bacterial Competition. *Cell,* 158**,** 1060-71.
KOCIANOVA, S., VUONG, C., YAO, Y., VOYICH, J., FISCHER, E., DELEO, F. & OTTO, M. 2005. Key role of poly-gamma-DL-glutamic acid in immune evasion and virulence of *Staphylococcus epidermidis*. *Journal Clinical Investigation,* 115**,** 688-94.
KOHLER, T., WEIDENMAIER, C. & PESCHEL, A. 2009. Wall teichoic acid protects *Staphylococcus aureus* against antimicrobial fatty acids from human skin. *Journal of Bacteriology,* 191**,** 4482-4.
KONG, H. 2011. Skin microbiome: genomics-based insights into the diversity and role of skin microbes. *Trends in Molecular Medicine,* 17**,** 320-8.
KONG, H., OH, J., DEMING, C., CONLAN, S., GRICE, E., BEATSON, M., NOMICOS, E., POLLEY, E., KOMAROW, H., PROGRAM, N. C. S., MURRAY, P., TURNER, M. & SEGRE, J. 2012. Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. *Genome Research,* 22**,** 850-9.
KOREM, M., GOV, Y., KIRAN, M. & BALABAN, N. 2005. Transcriptional profiling of target of RNAIII-activating protein, a master regulator of staphylococcal virulence. *Infection and Immunity,* 73**,** 6220-8.
KOREM, M., GOV, Y. & ROSENBERG, M. 2010. Global gene expression in *Staphylococcus aureus* following exposure to alcohol. *Microbial Pathogenesis,* 48**,** 74-84.
KRAGH-HANSEN, U. 1981. Molecular aspects of ligand binding to serum albumin. *Pharmacological Reviews,* 33**,** 17-53.
KRAUS, D., HERBERT, S., KRISTIAN, S., KHOSRAVI, A., NIZET, V., GOTZ, F. & PESCHEL, A. 2008. The GraRS regulatory system controls *Staphylococcus aureus* susceptibility to antimicrobial host defenses. *BMC Microbiology,* 8.
KRISMER, B., LIEBEKE, M., JANEK, D., NEGA, M., RAUTENBERG, M., HORNIG, G., UNGER, C., WEIDENMAIER, C., LALK, M. & PESCHEL, A. 2014. Nutrient limitation governs *Staphylococcus aureus* metabolism and niche adaptation in the human nose. *PloS Pathogens,* 10**,** e1003862.
KURODA, M., KURODA, H., OSHIMA, T., TAKEUCHI, F., MORI, H. & HIRAMATSU, K. 2004. Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. *Molecular Microbiology,* 49**,** 807-21.
KURODA, M., TANAKA, Y., AOKI, R., SHU, D., TSUMOTO, K. & OHTA, T. 2008. *Staphylococcus aureus* giant protein Ebh is involved in tolerance to transient hyperosmotic pressure. *Biochemical and Biophysical Research Communications,* 374**,** 237-41.
LACEY, R. & LORD, V. 1981. Sensitivity of staphylococci to fatty acids: novel inactivation of linolenic acid by serum. *Journal of Medical Microbiology,* 14**,** 41-9.

LAI, Y., COGEN, A., RADEK, K., PARK, H., MACLEOD, D., LEICHTLE, A., RYAN, A. F., DI NARDO, A. & GALLO, R. 2010. Activation of TLR2 by a small molecule produced by *Staphylococcus epidermidis* increases antimicrobial defense against bacterial skin infections. *Journal of Investigative Dermatology,* 130**,** 2211-21.

LAI, Y., DI NARDO, A., NAKATSUJI, T., LEICHTLE, A., YANG, Y., COGEN, A., WU, Z., HOOPER, L., SCHMIDT, R., VON AULOCK, S., RADEK, K., HUANG, C., RYAN, A. & GALLO, R. 2009. Commensal bacteria regulate Toll-like receptor 3-dependent inflammation after skin injury. *Nature Medicine,* 15**,** 1377-82.

LAI, Y., VILLARUZ, A., LI, M., CHA, D., STURDEVANT, D. & OTTO, M. 2007. The human anionic antimicrobial peptide dermcidin induces proteolytic defence mechanisms in staphylococci. *Molecular Microbiology,* 63**,** 497-506.

LAMPE, M., BURLINGAME, A., WHITNEY, J., WILLIAMS, M., BROWN, B., ROITMAN, E. & ELIAS, P. 1983. Human stratum corneum lipids-characterization and regional variations. *Journal of Lipid Research,* 24**,** 120-30.

LANGMEAD, B., TRAPNELL, C., POP, M. & SALZBERG, S. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology,* 10**,** R25.

LEE, C., CHEN, L., CHEN, L., CHANG, T., HUANG, C., HUANG, M. & WANG, C. 2013. Correlations of the components of tea tree oil with its antibacterial effects and skin irritation. *Journal of Food and Drug Analysis,* 21**,** 169-76.

LI, H. & DURBIN, R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics,* 25**,** 1754-60.

LI, H. & DURBIN, R. 2010. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics,* 26**,** 589-95.

LI, H., HANDSAKER, B., WYSOKER, A., FENNELL, T., RUAN, J., HOMER, N., MARTH, G., ABECASIS, G., DURBIN, R. & GENOME PROJECT DATA PROCESSING, S. 2009. The Sequence Alignment/Map format and SAMtools. *Bioinformatics,* 25**,** 2078-9.

LI, K., BIHAN, M. & METHE, B. 2013. Analyses of the stability and core taxonomic memberships of the human microbiome. *PloS One,* 8**,** e63139.

LI, K., XING, R., LIU, S., QIN, Y., YU, H. & LI, P. 2014. Size and pH effects of chitooligomers on antibacterial activity against *Staphylococcus aureus*. *International Journal of Biological Macromolecules,* 64**,** 302-5.

LIBBERTON, B. 2011. The ecology of *Staphylococcus aureus*. *PhD thesis, University of Liverpool: UK*.

LIBBERTON, B., COATES, R., BROCKHURST, M. & HORSBURGH, M. 2014. Evidence that intraspecific trait variation among nasal bacteria shapes the distribution of *Staphylococcus aureus*. *Infection and Immunity,* 82**,** 3811-5.

LONG, J., HART, J., ALBERS, W. & KAPRAL, F. 1992. The production of fatty acid modifying enzyme (FAME) and lipase by various staphylococcal species. *Journal of Medical Microbiology,* 37**,** 232-4.

LU, T., PARK, J., PARNELL, K., FOX, L. & MCGUIRE, M. 2012. Characterization of fatty acid modifying enzyme activity in staphylococcal mastitis isolates and other bacteria. *BMC Research Notes,* 5**,** 323.

LU, Y. & MCEWAN, N. 2007. Staphylococcal and micrococcal adherence to canine and feline corneocytes: quantification using a simple adhesion assay. *Veterinary Dermatology,* 18**,** 29-35.
MACINTOSH, R., BRITTAN, J., BHATTACHARYA, R., JENKINSON, H., DERRICK, J., UPTON, M. & HANDLEY, P. 2009. The terminal A domain of the fibrillar accumulation-associated protein (Aap) of *Staphylococcus epidermidis* mediates adhesion to human corneocytes. *Journal of Bacteriology,* 191**,** 7007-16.
MACK, D., SIEMSSEN, N. & LAUFS, R. 1992. Parallel induction by glucose of adherence and a polysaccharide antigen specific for plastic-adherent *Staphylococcus epidermidis*: evidence for functional relation to intercellular adhesin. *Infection and Immunity,* 60**,** 2048-57.
MADISON, K. 2003. Barrier function of the skin: "La Raison d'Etre" of the epidermis. *Journal of Investigative Dermatology,* 121**,** 231-41.
MARIONI, J., MASON, C., MANE, S., STEPHENS, M. & GILAD, Y. 2008. RNA-seq: an assessment of technical reproducibility and comparison with gene expression arrays. *Genome Research,* 18**,** 1509-17.
MARRAFFINI, L. & SONTHEIMER, E. 2008. CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science,* 322**,** 1843-5.
MARTIN, M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal,* 17**,** 10-2.
MASSEY, R., HORSBURGH, M., LINA, G., HOOK, M. & RECKER, M. 2006. The evolution and maintenance of virulence in *Staphylococcus aureus*: a role for host-to-host transmission? *Nature Reviews Microbiology,* 4**,** 953-8.
MATOUSEK, J. & CAMPBELL, K. 2002. A comparative review of cutaneous pH. *Veterinary Dermatology,* 13**,** 293-300.
MCDEVITT, D., FRANCOIS, P., VAUDAUX, P. & FOSTER, T. 1994. Molecular characterization of the clumping factor (fibrinogen receptor) of *Staphylococcus aureus*. *Molecular Microbiology,* 11**,** 237-48.
MCEWAN, N., MELLOR, D. & KALNA, G. 2006. Adherence by *Staphylococcus intermedius* to canine corneocytes: a preliminary study comparing noninflamed and inflamed atopic canine skin. *Veterinary Dermatology,* 17**,** 151-4.
MCKENNEY, D., HÜBNER, J., MULLER, E., WANG, Y., GOLDMANN, D. & PIER, G. 1998. The ica locus of *Staphylococcus epidermidis* encodes production of the capsular polysaccharide/adhesin. *Infection and Immunity,* 66**,** 4711-20.
MCMAHON, M., BLAIR, I., MOORE, J. & MCDOWELL, D. 2007. Habituation to sub-lethal concentrations of tea tree oil (Melaleuca alternifolia) is associated with reduced susceptibility to antibiotics in human pathogens. *Journal of Antimicrobial Chemotherapy,* 59**,** 125-7.
MCMAHON, M., TUNNEY, M., MOORE, J., BLAIR, I., GILPIN, D. & MCDOWELL, D. 2008. Changes in antibiotic susceptibility in staphylococci habituated to sub-lethal concentrations of tea tree oil (Melaleuca alternifolia). *Letters in Applied Microbiology,* 47**,** 263-8.
MECKFESSEL, M. & BRANDT, S. 2014. The structure, function, and importance of ceramides in skin and their use as therapeutic agents in skin-care products. *Journal of the American Academy of Dermatology,* 71**,** 177-84.

MEIER, S., GOERKE, C., WOLZ, C., SEIDL, K., HOMEROVA, D., SCHULTHESS, B., KORMANEC, J., BERGER-BACHI, B. & BISCHOFF, M. 2007. Sigma B and the sigma B-dependent *arlRS* and *yabJ-spoVG* loci affect capsule formation in *Staphylococcus aureus*. *Infection and Immunity,* 75**,** 4562-71.

MELNIK, B. 2006. Disturbances of antimicrobial lipids in atopic dermatitis. *Journal der Deutschen Dermatologischen Gesellschaft,* 4**,** 114-23.

MEMPEL, M., SCHMIDT, T., WEIDINGER, S., SCHNOPP, C., FOSTER, T., RING, J. & ABECK, D. 1998. Role of *Staphylococcus aureus* surface-associated proteins in the attachment to cultured HaCaT keratinocytes in a new adhesion assay. *Journal of Investigative Dermatology,* 111**,** 452-6.

MISHRA, N., LIU, G., YEAMAN, M., NAST, C., PROCTOR, R., MCKINNELL, J. & BAYER, A. 2011. Carotenoid-related alteration of cell membrane fluidity impacts *Staphylococcus aureus* susceptibility to host defense peptides. *Antimicrobial Agents and Chemotherapy,* 55**,** 526-31.

MITSUHASHI, S., HAYASHI, M., OHNISHI, J. & IKEDA, M. 2014. Disruption of malate:quinone oxidoreductase increases L-lysine production by *Corynebacterium glutamicum*. *Bioscience, Biotechnology and Biochemistry,* 70**,** 2803-6.

MORIKAWA, K., MARUYAMA, A., INOSE, Y., HIGASHIDE, M., HAYASHI, H. & OHTA, T. 2001. Overexpression of sigma factor, sigma(B), urges Staphylococcus aureus to thicken the cell wall and to resist beta-lactams. *Biochemical and Biophysical Research Communications,* 288**,** 385-9.

MORTAZAVI, A., WILLIAMS, B., MCCUE, K., SCHAEFFER, L. & WOLD, B. 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nature Methods,* 5**,** 621-8.

MOSS, B. & SQUIRE, J. 1948. Nose and skin carriage of *Staphylococcus aureus* in patients receiving penicillin. *Lancet,* 1**,** 320-5.

MULCAHY, M., GEOGHEGAN, J., MONK, I., O'KEEFFE, K., WALSH, E., FOSTER, T. & MCLOUGHLIN, R. 2012. Nasal colonisation by *Staphylococcus aureus* depends upon clumping factor B binding to the squamous epithelial cell envelope protein loricrin. *PloS Pathogens,* 8**,** e1003092.

MURAKAMI, M., OHTAKE, T., DORSCHNER, R., SCHITTEK, B., GARBE, C. & GALLO, R. 2002. Cathelicidin anti-microbial peptide expression in sweat, an innate defense system for the skin. *Journal of Investigative Dermatology,* 119**,** 1090-5.

MUTHAIYAN, A., MARTIN, E., NATESAN, S., CRANDALL, P., WILKINSON, B. & RICKE, S. 2012. Antimicrobial effect and mode of action of terpeneless cold-pressed Valencia orange essential oil on methicillin-resistant *Staphylococcus aureus*. *Journal of Applied Microbiology,* 112**,** 1020-33.

MUTHAIYAN, A., SILVERMAN, J., JAYASWAL, R. & WILKINSON, B. 2008. Transcriptional profiling reveals that daptomycin induces the *Staphylococcus aureus* cell wall stress stimulon and genes responsive to membrane depolarization. *Antimicrobial Agents & Chemotherapy,* 52**,** 980-90.

MWANGI, M., WU, S., ZHOU, Y., SIERADZKI, K., DE LENCASTRE, H., RICHARDSON, P., BRUCE, D., RUBIN, E., MYERS, E., SIGGIA, E. & TOMASZ, A. 2007. Tracking the in vivo evolution of multidrug resistance in *Staphylococcus aureus* by whole-genome sequencing. *PNAS,* 104**,** 9451-6.

NAGAMACHI, E., HIRAI, Y., TOMOCHIKA, K. & KANEMASA, Y. 1992. Studies on osmotic stability of liposomes prepared with bacterial membrane lipids by carboxyfluorescein release. *Microbiology and Immunology,* 36**,** 231-41.
NAGARAJAN, V. & ELASRI, M. 2007. SAMMD: *Staphylococcus aureus* microarray meta-database. *BMC Genomics,* 8**,** 204-10.
NAGASE, N., SASAKI, A., YAMASHITA, K., SHIMIZU, A., WAKITA, Y., KITAI, S. & KAWANO, J. 2002. Isolation and species distribution of staphylococci from animal and human skin. *Journal of Veterinary Medical Science,* 64**,** 245-50.
NAKATSUJI, T. & GALLO, R. 2014. Dermatological therapy by topical application of non-pathogenic bacteria. *Journal of Investigative Dermatology,* 134**,** 11-14.
NI EIDHIN, D., PERKINS, S., FRANCOIS, P., VAUDAUX, P., HOOK, M. & FOSTER, T. 1998. Clumping factor B (ClfB), a new surface-located fibrinogen-binding adhesin of *Staphylococcus aureus*. *Molecular Microbiology,* 30**,** 245-57.
NICHOLAS, R., LI, T., MCDEVITT, D., MARRA, A., SUCOLOSKI, S., DEMARISH, P. & GENTRY, D. 1999. Isolation and characterization of a sigB deletion mutant of *Staphylococcus aureus*. *Infection and Immunity,* 67**,** 3667-9.
NILSSON, M., BJERKETORP, J., GUSS, B. & FRYKBERG, L. 2004. A fibrinogen-binding protein of *Staphylococcus lugdunensis*. *FEMS Microbiology Letters,* 241**,** 87-93.
NILSSON, M., FRYKBERG, L., FLOCK, J., PEI, L., LINDBERG, M. & GUSS, B. 1998. A fibrinogen-binding protein of *Staphylococcus epidermidis*. *Infection and Immunity,* 66**,** 2666-73.
NIYONSABA, F. & OGAWA, H. 2005. Protective roles of the skin against infection: implication of naturally occurring human antimicrobial agents beta-defensins, cathelicidin LL-37 and lysozyme. *Journal of Dermatological Science,* 40**,** 157-68.
NOLAN, T., HANDS, R. & BUSTIN, S. 2006. Quantification of mRNA using real-time RT-PCR. *Nature Protocols,* 1**,** 1559-82.
O'BRIEN, L., WALSH, E., MASSEY, R., PEACOCK, S. & FOSTER, T. 2002. *Staphylococcus aureus* clumping factor B (ClfB) promotes adherence to human type I cytokeratin 10: implications for nasal colonization. *Cellular Microbiology,* 4**,** 759-70.
O'NEILL, A., LINDSAY, J., GOULD, K., HINDS, J. & CHOPRA, I. 2009. Transcriptional signature following inhibition of early-stage cell wall biosynthesis in *Staphylococcus aureus*. *Antimicrobial Agents & Chemotherapy,* 53**,** 1701-4.
O'RIORDAN, K. & LEE, J. 2004. *Staphylococcus aureus* capsular polysaccharides. *Clinical Microbiology Reviews,* 17**,** 218-34.
OHNIWA, R., KITABAYASHI, K. & MORIKAWA, K. 2013. Alternative cardiolipin synthase Cls1 compensates for stalled Cls2 function in *Staphylococcus aureus* under conditions of acute acid stress. *FEMS Microbiology Letters,* 338**,** 141-6.
OKAMURA-IKEDA, K., HOSAKA, H., MAITA, N., FUJIWARA, K., YOSHIZAWA, A., NAKAGAWA, A. & TANIGUCHI, H. 2010. Crystal structure of aminomethyltransferase in complex with dihydrolipoyl-H-protein of the glycine cleavage system: implications for recognition of lipoyl protein

substrate, disease-related mutations, and reaction mechanism. *Journal of Biological Chemistry,* 285**,** 18684-92.
OLIVER, H., ORSI, R., PONNALA, L., KEICH, U., WANG, W., SUN, Q., CARTINHOUR, S., FILIATRAULT, M., WIEDMANN, M. & BOOR, K. 2009. Deep RNA sequencing of L. monocytogenes reveals overlapping and extensive stationary phase and sigma B-dependent transcriptomes, including multiple highly transcribed noncoding RNAs. *BMC Genomics,* 10**,** 641.
ONG, P., OHTAKE, T., BRANDT, C., STRICKLAND, I., BOGUNIEWICZ, M., GANZ, T., GALLO, R. & LEUNG, D. 2002. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *New England Journal of Medicine,* 347**,** 1151-60.
OSCARSSON, J., KANTH, A., TEGMARK-WISELL, K. & ARVIDSON, S. 2006. SarA is a repressor of hla (alpha-hemolysin) transcription in *Staphylococcus aureus*: its apparent role as an activator of hla in the prototype strain NCTC 8325 depends on reduced expression of sarS. *Journal of Bacteriology,* 188**,** 8526-33.
OVERBEEK, R., BEGLEY, T., BUTLER, R., CHOUDHURI, J., CHUANG, H., COHOON, M., DE CRECY-LAGARD, V., DIAZ, N., DISZ, T., EDWARDS, R., FONSTEIN, M., FRANK, E., GERDES, S., GLASS, E., GOESMANN, A., HANSON, A., IWATA-REUYL, D., JENSEN, R., JAMSHIDI, N., KRAUSE, L., KUBAL, M., LARSEN, N., LINKE, B., MCHARDY, A., MEYER, F., NEUWEGER, H., OLSEN, G., OLSON, R., OSTERMAN, A., PORTNOY, V., PUSCH, G., RODIONOV, D., RUCKERT, C., STEINER, J., STEVENS, R., THIELE, I., VASSIEVA, O., YE, Y., ZAGNITKO, O. & VONSTEIN, V. 2005. The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes. *Nucleic Acids Research,* 33**,** 5691-702.
PALMA, M. & CHEUNG, A. 2001. Sigma(B) activity in *Staphylococcus aureus* is controlled by RsbU and an additional factor(s) during bacterial growth. *Infection and Immunity,* 69**,** 7858-65.
PANE-FARRE, J., JONAS, B., FORSTNER, K., ENGELMANN, S. & HECKER, M. 2006. The sigma B regulon in *Staphylococcus aureus* and its regulation. *International Journal of Medical Microbiology,* 296**,** 237-58.
PARK, B., IWASE, T. & LIU, G. 2011. Intranasal application of *Staphylococcus epidermidis* prevents colonization by methicillin-resistant *Staphylococcus aureus* in mice. *PloS One,* 6**,** e25880.
PARSONS, J., YAO, J., FRANK, M., JACKSON, P. & ROCK, C. 2012. Membrane disruption by antimicrobial fatty acids releases low-molecular-weight proteins from *Staphylococcus aureus*. *Journal of Bacteriology,* 194**,** 5294-304.
PATEL, D., HUSAIN, M., VIDAILLAC, C., STEED, M., RYBAK, M., SEO, S. & KAATZ, G. 2011. Mechanisms of *in-vitro*-selected daptomycin-non-susceptibility in *Staphylococcus aureus*. *International Journal of Antimicrobial Agents,* 38**,** 442-6.
PATEL, R. & JAIN, M. 2012. NGS QC toolkit: a toolkit for quality control of next generation sequencing data. *PloS One,* 7**,** e30619.
PATTON, T., YANG, S. & BAYLES, K. 2006. The role of proton motive force in the expression of the *Staphylococcus aureus cid* and *lrg* operons. *Molecular Microbiology,* 59**,** 1395-404.

PAYNE, C., RAY, T. & DOWNING, D. 1995. Cholesterol sulfate protects *Candida albicans* from inhibition by sphingosine *in vitro*. *Journal of Investigative Dermatology,* 105**,** 549-52.

PELEG, A., MIYAKIS, S., WARD, D., EARL, A., RUBIO, A., CAMERON, D., PILLAI, S., MOELLERING, R., JR. & ELIOPOULOS, G. 2012. Whole genome characterization of the mechanisms of daptomycin resistance in clinical and laboratory derived isolates of *Staphylococcus aureus*. *PloS One,* 7**,** e28316.

PEREGO, M., GLASER, P., MINUTELLO, A., STRAUCH, M., LEOPOLD, K. & FISCHER, W. 1995. Incorporation of D-Alanine into lipoteichoic acid and wall teichoic acid in *Bacillus subtilis* – identification of genes and regulation. *Journal of Biological Chemistry,* 270**,** 15598-606.

PERIASAMY, S., CHATTERJEE, S., CHEUNG, G. & OTTO, M. 2012. Phenol-soluble modulins in staphylococci: What are they originally for? *Communicative and Integrative Biology,* 5**,** 275-7.

PESAKHOV, S., BENISTY, R., SIKRON, N., COHEN, Z., GOMELSKY, P., KHOZIN-GOLDBERG, I., DAGAN, R. & PORAT, N. 2007. Effect of hydrogen peroxide production and the Fenton reaction on membrane composition of *Streptococcus pneumoniae*. *Biochimica et Biophysica Acta,* 1768**,** 590-7.

PESCHEL, A., JACK, R., OTTO, M., COLLINS, L., STAUBITZ, P., NICHOLSON, G., KALBACHER, H., NIEUWENHUIZEN, W., JUNG, G., TARKOWSKI, A., VAN KESSEL, K. & VAN STRIJP, J. 2001. *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with L-lysine. *Journal of Experimental Medicine,* 193**,** 1067-76.

PESCHEL, A., OTTO, M., JACK, R., KALBACHER, H., JUNG, G. & GOTZ, F. 1999. Inactivation of the dlt operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. *Journal of Biological Chemistry,* 274**,** 8405-10.

PIETIAINEN, M., FRANCOIS, P., HYYRYLAINEN, H., TANGOMO, M., SASS, V., SAHL, H., SCHRENZEL, J. & KONTINEN, V. 2009. Transcriptome analysis of the responses of *Staphylococcus aureus* to antimicrobial peptides and characterization of the roles of vraDE and vraSR in antimicrobial resistance. *BMC Genomics,* 10**,** 429.

POPOV, L., KOVALSKI, J., GRANDI, G., BAGNOLI, F. & AMIEVA, M. 2014. Three-dimensional human skin models to understand *Staphylococcus aureus* skin colonization and infection. *Frontiers in Immunology,* 5**,** 41.

POTTER, A., CEOTTO, H., COELHO, M., GUIMARAES, A. & BASTOS MDO, C. 2014. The gene cluster of aureocyclicin 4185: the first cyclic bacteriocin of *Staphylococcus aureus*. *Microbiology,* 160**,** 917-28.

POTTS, M. 1994. Desiccation tolerance in prokaryotes. *Microbiological Reviews,* 58**,** 755-805.

PRICE-WHELAN, A., POON, C., BENSON, M., EIDEM, T., ROUX, C., BOYD, J., DUNMAN, P., TORRES, V. & KRULWICH, T. 2013. Transcriptional profiling of *Staphylococcus aureus* during growth in 2 M NaCl leads to clarification of physiological roles for Kdp and Ktr K+ uptake systems. *Mbio,* 4.

PROKSCH, E., BRANDNER, J. & JENSEN, J. 2008. The skin: an indispensable barrier. *Experimental Dermatology,* 17**,** 1063-72.

PULLMANOVÁ, P., STANKOVÁ, K., POSPÍSILOVÁ, M., SKOLOVÁ, B., ZBYTOVSKÁ, J. & VÁVROVÁ, K. 2014. Effects of sphingomyelin/ceramide ratio on the permeability and microstructure of model stratum corneum lipid membranes. *Biochimica et Biophysica Acta*, 2115-26.

PUTMAN, M., VAN VEEN, H. & KONINGS, W. 2000. Molecular properties of bacterial multidrug transporters. *Microbiology and Molecular Biology Reviews,* 64, 672-93.

RAMIA, N., TANG, L., COCOZAKI, A. & LI, H. 2014. *Staphylococcus epidermidis* Csm1 is a 3'-5' exonuclease. *Nucleic Acids Research,* 42, 1129-38.

RAMOS, J., MARTINEZ-BUENO, M., MOLINA-HENARES, A., TERAN, W., WATANABE, K., ZHANG, X., GALLEGOS, M., BRENNAN, R. & TOBES, R. 2005. The TetR family of transcriptional repressors. *Microbiology and Molecular Biology Reviews,* 69, 326-56.

REIMER, L., STRATTON, C. & RELLER, L. 1981. Minimum inhibitory and bactericidal concentrations of 44 anti-microbial agents against 3 standard control strains in broth with and without human-serum. *Antimicrobial Agents and Chemotherapy,* 19, 1050-5.

REMMERT, M., BIEGERT, A., HAUSER, A. & SÖDING, J. 2011. Hhblits: lightning-fast iterative protein sequence searching by HMM-HMM alignment. *Nature Methods,* 9, 173-5.

RENZONI, A., ANDREY, D., JOUSSELIN, A., BARRAS, C., MONOD, A., VAUDAUX, P., LEW, D. & KELLEY, W. 2011. Whole genome sequencing and complete genetic analysis reveals novel pathways to glycopeptide resistance in *Staphylococcus aureus*. *PloS One,* 6, e21577.

REYNOLDS, P. 1989. Structure, biochemistry and mechanism of action of glycopeptide antibiotics. *European Journal of Clinical Microbiology and Infectious Diseases,* 8, 943-50.

RIEG, S., STEFFEN, H., SEEBER, S., HUMENY, A., KALBACHER, H., DIETZ, K., GARBE, C. & SCHITTEK, B. 2005. Deficiency of dermcidin-derived antimicrobial peptides in sweat of patients with atopic dermatitis correlates with an impaired innate defense of human skin *in vivo*. *Journal of Immunology,* 174, 8003-10.

RIPPKE, F., SCHREINER, V., DOERING, T. & MAIBACH, H. 2004. Statum corneum pH in atopic dermatitis: impact on skin barrier function and colonization with *Staphylococcus aureus*. *American Journal of Clinical Dermatology,* 5, 217-23.

RIPPKE, F., SCHREINER, V. & SCHWANITZ, H. 2002. The acidic milieu of the horny layer: new findings on the physiology and pathophysiology of skin pH. *American Journal of Clinical Dermatology,* 3, 261-72.

ROBERSON, E. & FIRESTONE, M. 1992. Relationship between desiccation and exopolysaccharide production in a soil pseudomonas species. *Applied and Environmental Microbiology,* 58, 1284-91.

ROBINSON, M., MCCARTHY, D. & SMYTH, G. 2010. EdgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics,* 26, 139-40.

ROBINSON, M. & OSHLACK, A. 2010. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biology,* 11, R25.

ROCHE, F., MEEHAN, M. & FOSTER, T. 2003. The *Staphylococcus aureus* surface protein SasG and its homologues promote bacterial adherence to human desquamated nasal epithelial cells. *Microbiology-SGM,* 149**,** 2759-67.
ROGERS, K., RUPP, M. & FEY, P. 2008. The presence of icaADBC is detrimental to the colonization of human skin by *Staphylococcus epidermidis*. *Applied and Environment Microbiology,* 74**,** 6155-7.
ROMANTSOV, T., GUAN, Z. & WOOD, J. 2009. Cardiolipin and the osmotic stress responses of bacteria. *Biochimica Et Biophysica Acta-Biomembranes,* 1788**,** 2092-100.
ROSBERG-CODY, E., LIAVONCHANKA, A., GOBEL, C., ROSS, R., O'SULLIVAN, O., FITZGERALD, G., FEUSSNER, I. & STANTON, C. 2011. Myosin-cross-reactive antigen (MCRA) protein from Bifidobacterium breve is a FAD-dependent fatty acid hydratase which has a function in stress protection. *BMC Biochemistry,* 12**,** 9.
ROTH, R. & JAMES, W. 1988. Microbial ecology of the skin. *Annual Review of Microbiology,* 42**,** 441-64.
RYU, S., SONG, P., SEO, C., CHEONG, H. & PARK, Y. 2014. Colonization and infection of the skin by *S. aureus*: immune system evasion and the response to cationic antimicrobial peptides. *International Journal of Molecular Sciences,* 15**,** 8753-72.
SAKINC, T., KLEINE, B. & GATERMANN, S. 2006. SdrI, a serine-aspartate repeat protein identified in *Staphylococcus saprophyticus* strain 7108, is a collagen-binding protein. *Infection and Immunity,* 74**,** 4615-23.
SAKINC, T., KLEINE, B., MICHALSKI, N., KAASE, M. & GATERMANN, S. 2009. SdrI of *Staphylococcus saprophyticus* is a multifunctional protein: localization of the fibronectin-binding site. *FEMS Microbiology Letters,* 301**,** 28-34.
SASS, P., JANSEN, A., SZEKAT, C., SASS, V., SAHL, H. G. & BIERBAUM, G. 2008. The lantibiotic mersacidin is a strong inducer of the cell wall stress response of *Staphylococcus aureus*. *BMC Microbiology,* 8**,** 186.
SCHAFER, L. & KRAGBALLE, K. 1991. Abnormalities in epidermal lipid-metabolism in patients with Atopic dermatitis. *Journal of Investigative Dermatology,* 96**,** 10-15.
SCHAFFER, A. & LEE, J. 2009. Staphylococcal vaccines and immunotherapies. *Infectious Disease Clinics of North America,* 23**,** 153-71.
SCHIELKE, S., SCHMITT, C., SPATZ, C., FROSCH, M., SCHUBERT-UNKMEIR, A. & KURZAI, O. 2010. The transcriptional repressor FarR is not involved in meningococcal fatty acid resistance mediated by the FarAB efflux pump and dependent on lipopolysaccharide structure. *Applied and Environment Microbiology,* 76**,** 3160-9.
SCHMID-WENDTNER, M. & KORTING, H. 2006. The pH of the skin surface and its impact on the barrier function. *Skin Pharmacology and Physiology,* 19**,** 296-302.
SCHOMMER, N. & GALLO, R. 2013. Structure and function of the human skin microbiome. *Trends in Microbiology,* 21**,** 660-8.
SCHRODER, J. & HARDER, J. 2006. Antimicrobial skin peptides and proteins. *Cellular and Molecular Life Sciences,* 63**,** 469-86.
SCHUJMAN, G. E. & DE MENDOZA, D. 2005. Transcriptional control of membrane lipid synthesis in bacteria. *Current Opinion in Microbiology,* 8**,** 149-53.

SENGUPTA, M., JAIN, V., WILKINSON, B. & JAYASWAL, R. 2012. Chromatin immunoprecipitation identifies genes under direct VraSR regulation in *Staphylococcus aureus*. *Canadian Journal of Microbiology,* 58**,** 703-8.
SENYUREK, I., PAULMANN, M., SINNBERG, T., KALBACHER, H., DEEG, M., GUTSMANN, T., HERMES, M., KOHLER, T., GOTZ, F., WOLZ, C., PESCHEL, A. & SCHITTEK, B. 2009. Dermcidin-derived peptides show a different mode of action than the cathelicidin LL-37 against *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy,* 53**,** 2499-509.
SEVVANA, M., GOETZ, C., GOEKE, D., WIMMER, C., BERENS, C., HILLEN, W. & MULLER, Y. 2012. An exclusive alpha/beta code directs allostery in TetR-peptide complexes. *Journal of Molecular Biology,* 416**,** 46-56.
SEYEDNASROLLAH, F., LAIHO, A. & ELO, L. 2013. Comparison of software packages for detecting differential expression in RNA-seq studies. *Briefings in Bioinformatics*.
SHUBBAR, E., VEGFORS, J., CARLSTROM, M., PETERSSON, S. & ENERBACK, C. 2012. Psoriasin (S100A7) increases the expression of ROS and VEGF and acts through RAGE to promote endothelial cell proliferation. *Breast Cancer Research and Treatment,* 134**,** 71-80.
SIERADZKI, K. & TOMASZ, A. 1997. Inhibition of cell wall turnover and autolysis by vancomycin in a highly vancomycin-resistant mutant of *Staphylococcus aureus*. *Journal of Bacteriology,* 179**,** 2557-66.
SIMANSKI, M., KOTEN, B., SCHRODER, J., GLASER, R. & HARDER, J. 2012. Antimicrobial Rnases in cutaneous defense. *Journal of Innate Immunity,* 4**,** 241-7.
SINGH, V., HATTANGADY, D., GIOTIS, E., SINGH, A., CHAMBERLAIN, N., STUART, M. & WILKINSON, B. 2008. Insertional inactivation of branched-chain alpha-keto acid dehydrogenase in *Staphylococcus aureus* leads to decreased branched-chain membrane fatty acid content and increased susceptibility to certain stresses. *Applied and Environment Microbiology,* 74**,** 5882-90.
SLANETZ, L. & JAWETZ, E. 1941. Isolation and characteristics of bacteriophages for staphylococci of bovine mastitis. *Journal of Bacteriology,* 41**,** 447-55.
SNODGRASS, J., MOHAMED, N., ROSS, J., SAU, S., LEE, C. & SMELTZER, M. 1999. Functional analysis of the *Staphylococcus aureus* collagen adhesin B domain. *Infection and Immunity,* 67**,** 3952-9.
SOARES, J., LOPES, C., TAVARIA, F., DELGADO, L. & PINTADO, M. 2013. A diversity profile from the staphylococcal community on atopic dermatitis skin: a molecular approach. *Journal of Applied Microbiology,* 115**,** 1411-9.
SODING, J. 2005. Protein homology detection by HMM-HMM comparison. *Bioinformatics,* 21**,** 951-60.
SODING, J., BIEGERT, A. & LUPAS, A. 2005. The Hhpred interactive server for protein homology detection and structure prediction. *Nucleic Acids Research,* 33**,** W244-8.
SOLLID, J., FURBERG, A., HANSSEN, A. & JOHANNESSEN, M. 2014. *Staphylococcus aureus*: determinants of human carriage. *Infection, Genetics and Evolution,* 21**,** 531-41.
SONG, C., WEICHBRODT, C., SALNIKOV, E., DYNOWSKI, M., FORSBERG, B., BECHINGER, B., STEINEM, C., GROOT, B., ZACHARIAE, U. & ZETH, K.

2013a. Crystal structure and functional mechanism of a human antimicrobial membrane channel. *PNAS,* 110**,** 4586-91.
SONG, Y., RUBIO, A., JAYASWAL, R., SILVERMAN, J. & WILKINSON, B. 2013b. Additional routes to *Staphylococcus aureus* daptomycin resistance as revealed by comparative genome sequencing, transcriptional profiling, and phenotypic studies. *PloS One,* 8**,** e58469.
SPIEGEL, S. & MILSTIEN, S. 2000. Sphingosine-1-phosphate: signaling inside and out. *FEBS Letters,* 476**,** 55-7.
SRINIVASAN, A., DICK, J. & PERL, T. 2002. Vancomycin Resistance in Staphylococci. *Clinical Microbiology Reviews,* 15**,** 430-8.
SULTAN, M., SCHULZ, M., RICHARD, H., MAGEN, A., KLINGENHOFF, A., SCHERF, M., SEIFERT, M., BORODINA, T., SOLDATOV, A., PARKHOMCHUK, D., SCHMIDT, D., O'KEEFFE, S., HAAS, S., VINGRON, M., LEHRACH, H. & YASPO, M. 2008. A global view of gene activity and alternative splicing by deep sequencing of the human transcriptome. *Science,* 321**,** 956-60.
TAKAHASHI, K., KUROKAWA, K., MOYO, P., JUNG, D., AN, J., CHIGWESHE, L., PAUL, E. & LEE, B. 2013. Intradermal immunization with wall teichoic acid (WTA) elicits and augments an anti-WTA IgG response that protects mice from methicillin-resistant *Staphylococcus aureus* infection independent of mannose-binding lectin status. *PloS One,* 8**,** e69739.
TAKIGAWA, H., NAKAGAWA, H., KUZUKAWA, M., MORI, H. & IMOKAWA, G. 2005. Deficient production of hexadecenoic acid in the skin is associated in part with the vulnerability of atopic dermatitis patients to colonization by *Staphylococcus aureus*. *Dermatology,* 211**,** 240-8.
TATHAM, L. 2011. Analysis of the inhibitory activity and mode of action of novel antimicrobial organic nanoparticles. *PhD thesis, University of Liverpool: UK*.
TAYLOR, B. & ZHULIN, I. 1999. PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiology and Molecular Biology Reviews,* 63**,** 479-506.
TAYLOR, D., DAULBY, A., GRIMSHAW, S., JAMES, G., MERCER, J. & VAZIRI, S. 2003. Characterization of the microflora of the human axilla. *International Journal of Cosmetic Science,* 25**,** 137-45.
THOMSEN, N., HAMMER, K., RILEY, T., VAN BELKUM, A. & CARSON, C. 2013. Effect of habituation to tea tree (Melaleuca alternifolia) oil on the subsequent susceptibility of *Staphylococcus* spp. to antimicrobials, triclosan, tea tree oil, terpinen-4-ol and carvacrol. *International Journal of Antimicrobial Agents,* 41**,** 343-51.
THURLOW, L., JOSHI, G., CLARK, J., SPONTAK, J., NEELY, C., MAILE, R. & RICHARDSON, A. 2013. Functional modularity of the arginine catabolic mobile element contributes to the success of USA300 methicillin-resistant *Staphylococcus aureus*. *Cell Host & Microbe,* 13**,** 100-7.
TRUONG-BOLDUC, Q., DUNMAN, P., EIDEM, T. & HOOPER, D. 2011. Transcriptional profiling analysis of the global regulator NorG, a GntR-like protein of *Staphylococcus aureus*. *Journal of Bacteriology,* 193**,** 6207-14.
TRUONG-BOLDUC, Q., VILLET, R., ESTABROOKS, Z. & HOOPER, D. 2014. Native efflux pumps contribute resistance to antimicrobials of skin and the

ability of *Staphylococcus aureus* to colonize skin. *Journal of Infectious Diseases,* 209**,** 1485-93.
TSAI, M., OHNIWA, R., KATO, Y., TAKESHITA, S., OHTA, T., SAITO, S., HAYASHI, H. & MORIKAWA, K. 2011. *Staphylococcus aureus* requires cardiolipin for survival under conditions of high salinity. *BMC Microbiology,* 11.
TSANG, L. H., DAILY, S. T., WEISS, E. C. & SMELTZER, M. S. 2007. Mutation of *traP* in *Staphylococcus aureus* has no impact on expression of agr or biofilm formation. *Infection and Immunity,* 75**,** 4528-33.
TURCK, M. & BIERBAUM, G. 2012. Purification and activity testing of the full-length YycFGHI proteins of *Staphylococcus aureus*. *PloS One,* 7**,** e30403.
UNIPROT, C. 2014. Activities at the Universal Protein Resource (UniProt). *Nucleic Acids Research,* 42**,** D191-8.
UTAIDA, S. 2003. Genome-wide transcriptional profiling of the response of *Staphylococcus aureus* to cell-wall-active antibiotics reveals a cell-wall-stress stimulon. *Microbiology,* 149**,** 2719-32.
VALE, P. & LITTLE, T. 2010. CRISPR-mediated phage resistance and the ghost of coevolution past. *Proceedings of the Royal Society: Biological Sciences,* 277**,** 2097-103.
VALIHRACH, L. & DEMNEROVA, K. 2012. Impact of normalization method on experimental outcome using RT-qPCR in *Staphylococcus aureus*. *Journal of Microbiological Methods,* 90**,** 214-6.
VAN DER OOST, J., JORE, M., WESTRA, E., LUNDGREN, M. & BROUNS, S. 2009. CRISPR-based adaptive and heritable immunity in prokaryotes. *Trends in Biochemical Sciences,* 34**,** 401-7.
VAN SMEDEN, J., JANSSENS, M., GOORIS, G. & BOUWSTRA, J. 2014. The important role of stratum corneum lipids for the cutaneous barrier function. *Biochimica et Biophysica Acta,* 1841**,** 295-313.
VARELA, C., RITTMANN, D., SINGH, A., KRUMBACH, K., BHATT, K., EGGELING, L., BESRA, G. & BHATT, A. 2012. MmpL genes are associated with mycolic acid metabolism in mycobacteria and corynebacteria. *Chemistry and Biology,* 19**,** 498-506.
VIDAL, L., THUAULT, V., MANGAS, A., COVENAS, R., THIENPONT, A. & GEFFARD, M. 2014. Lauryl-poly-L-lysine: a new antimicrobial agent? *Journal of Amino Acids,* 2014**,** 672367.
VIGNAROLI, C., RINALDI, C. & VARALDO, P. 2011. Striking "seesaw effect" between daptomycin nonsusceptibility and beta-lactam susceptibility in *Staphylococcus haemolyticus*. *Antimicrobial Agents & Chemotherapy,* 55**,** 2495-6.
VOLKOV, A., LIAVONCHANKA, A., KAMNEVA, O., FIEDLER, T., GOEBEL, C., KREIKEMEYER, B. & FEUSSNER, I. 2010. Myosin cross-reactive antigen of *Streptococcus pyogenes* M49 encodes a fatty acid double bond hydratase that plays a role in oleic acid detoxification and bacterial virulence. *Journal of Biological Chemistry,* 285**,** 10353-61.
VUONG, C. & OTTO, M. 2002. *Staphylococcus epidermidis* infections. *Microbes and Infection,* 4**,** 481-9.
VUONG, C., VOYICH, J., FISCHER, E., BRAUGHTON, K., WHITNEY, A., DELEO, F. & OTTO, M. 2004. Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the innate immune system. *Cellular Microbiology,* 6**,** 269-75.

WALKENHORST, W., KLEIN, J., VO, P. & WIMLEY, W. 2013. pH Dependence of microbe sterilization by cationic antimicrobial peptides. *Antimicrobial Agents & Chemotherapy,* 57**,** 3312-20.
WANG, C., SANG, J., WANG, J., SU, M., DOWNEY, J., WU, Q., WANG, S., CAI, Y., XU, X., WU, J., SENADHEERA, D., CVITKOVITCH, D., CHEN, L., GOODMAN, S. & HAN, A. 2013. Mechanistic insights revealed by the crystal structure of a histidine kinase with signal transducer and sensor domains. *PloS Biol,* 11**,** e1001493.
WANG, Y., KUO, S., SHU, M., YU, J., HUANG, S., DAI, A., TWO, A., GALLO, R. & HUANG, C. 2014. *Staphylococcus epidermidis* in the human skin microbiome mediates fermentation to inhibit the growth of *Propionibacterium acnes*: implications of probiotics in acne vulgaris. *Applied Microbiology and Biotechnology,* 98**,** 411-24.
WANG, Z., GERSTEIN, M. & SNYDER, M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews: Genetics,* 10**,** 57-63.
WANN, E., GURUSIDDAPPA, S. & HOOK, M. 2000. The fibronectin-binding MSCRAMM FnbpA of *Staphylococcus aureus* is a bifunctional protein that also binds to fibrinogen. *Journal of Biological Chemistry,* 275**,** 13863-71.
WEIDENMAIER, C., KOKAI-KUN, J., KRISTIAN, S., CHANTURIYA, T., KALBACHER, H., GROSS, M., NICHOLSON, G., NEUMEISTER, B., MOND, J. & PESCHEL, A. 2004. Role of teichoic acids in *Staphylococcus aureus* nasal colonization, a major risk factor in nosocomial infections. *Nature Medicine,* 10**,** 243-5.
WEIDENMAIER, C., KOKAI-KUN, J., KULAUZOVIC, E., KOHLER, T., THUMM, G., STOLL, H., GOTZ, F. & PESCHEL, A. 2008. Differential roles of sortase-anchored surface proteins and wall teichoic acid in *Staphylococcus aureus* nasal colonization. *International Journal of Medical Microbiology,* 298**,** 505-13.
WERTHEIM, H., WALSH, E., CHOUDHURRY, R., MELLES, D., BOELENS, H., MIAJLOVIC, H., VERBRUGH, H., FOSTER, T. & VAN BELKUM, A. 2008. Key role for clumping factor B in *Staphylococcus aureus* nasal colonization of humans. *PloS Medicine,* 5**,** 104-12.
WIEDEMANN, I., BREUKINK, E., VAN KRAAIJ, C., KUIPERS, O., BIERBAUM, G., DE KRUIJFF, B. & SAHL, H. 2001. Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. *Journal of Biological Chemistry,* 276**,** 1772-9.
WIELAND, B., FEIL, C., GLORIA-MAERCKER, E., THUMM, G., LECHNER, M., BRAVO, J., PORALLA, K. & GÖTZ, F. 1994. Genetic and biochemical analysis of the biosynthesis of the yellow carotenoid 4,4'-diaponeurosporene of *Staphylococcus aureus*. *Journal of Bacteriology,* 176**,** 7719-26.
WILLIAMS, R., HENDERSON, B., SHARP, L. & NAIR, S. 2002. Identification of a fibronectin-binding protein from *Staphylococcus epidermidis*. *Infection and Immunity,* 70**,** 6805-10.
WOOLLEY, K., KELLY, R., FAZAKERLEY, J., WILLIAMS, N., NUTTALL, T. & MCEWAN, N. 2008. Reduced *in vitro* adherence of *Staphylococcus* species to feline corneocytes compared to canine and human corneocytes. *Veterinary Dermatology,* 19**,** 1-6.

WOS-OXLEY, M., PLUMEIER, I., VON EIFF, C., TAUDIEN, S., PLATZER, M., VILCHEZ-VARGAS, R., BECKER, K. & PIEPER, D. 2010. A poke into the diversity and associations within human anterior nare microbial communities. *ISME Journal,* 4**,** 839-51.

WU, S., ZHU, Z., FU, L., NIU, B. & LI, W. 2011. WebMGA: a customizable web server for fast metagenomic sequence analysis. *BMC Genomics,* 12**,** 444.

XIA, G., KOHLER, T. & PESCHEL, A. 2010. The wall teichoic acid and lipoteichoic acid polymers of *Staphylococcus aureus*. *International journal of Medical Microbiology,* 300**,** 148-54.

YANG, S., BAYER, A., MISHRA, N., MEEHL, M., LEDALA, N., YEAMAN, M., XIONG, Y. & CHEUNG, A. 2012. The *Staphylococcus aureus* two-component regulatory system, GraRS, senses and confers resistance to selected cationic antimicrobial peptides. *Infection and Immunity,* 80**,** 74-81.

YANG, S., XIONG, Y., YEAMAN, M., BAYLES, K., ABDELHADY, W. & BAYER, A. 2013. Role of the LytSR two-component regulatory system in adaptation to cationic antimicrobial peptides in *Staphylococcus aureus*. *Antimicrobial Agents & Chemotherapy,* 57**,** 3875-82.

YANG, X., LI, N., CHEN, J., OU, Y., JIN, H., LU, H., ZHU, Y., QIN, Z., QU, D. & YANG, P. 2006. Comparative proteomic analysis between the invasive and commensal strains of *Staphylococcus epidermidis*. *FEMS Microbiology Letters,* 261**,** 32-40.

YE, J., COULOURIS, G., ZARETSKAYA, I., CUTCUTACHE, I., ROZEN, S. & MADDEN, T. 2012. Primer-BLAST: a tool to design target-specific primers for a polymerase chain reaction. *BMC Bioinformatics,* 13.

YIM, G., THAKER, M., KOTEVA, K. & WRIGHT, G. 2014. Glycopeptide antibiotic biosynthesis. *Journal of Antibiotics (Tokyo),* 67**,** 31-41.

ZEEUWEN, P., BOEKHORST, J., VAN DEN BOGAARD, E., DE KONING, H., VAN DE KERKHOF, P., SAULNIER, D., VAN SWAM, I., VAN HIJUM, S., KLEEREBEZEM, M., SCHALKWIJK, J. & TIMMERMAN, H. 2012. Microbiome dynamics of human epidermis following skin barrier disruption. *Genome Biology,* 13.

ZERBINO, D. & BIRNEY, E. 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Research,* 18**,** 821-9.

ZHANG, H., ZHANG, L., PENG, L., DONG, X., WU, D., WU, V. & FENG, F. 2012. Quantitative structure-activity relationships of antimicrobial fatty acids and derivatives against *Staphylococcus aureus*. *Journal of Zhejiang University Science B,* 13**,** 83-93.

ZHENG, C., YOO, J., LEE, T., CHO, H., KIM, Y. & KIM, W. 2005. Fatty acid synthesis is a target for antibacterial activity of unsaturated fatty acids. *FEBS Letters,* 579**,** 5157-62.

ZHU, T., LOU, Q., WU, Y., HU, J., YU, F. & QU, D. 2010. Impact of the *Staphylococcus epidermidis* LytSR two-component regulatory system on murein hydrolase activity, pyruvate utilization and global transcriptional profile. *BMC Microbiology,* 10**,** 287.

Appendix

Appendix Table 1 Description and usage instructions for all bespoke Perl scripts used in this thesis. All scripts are provided in the attached CD.

Script name	Description	Command	Output files	Author
Mpileup_SNPs_v2.pl	Calls SNPs for data with > 6x coverage and presents only coding SNPs in the main output (SNP_output.bcf)	*Perl mpileup_SNPs_v2.pl <input.bcf> SNPEff.database min.coverage <reference.gff> chromosome_name*	**SNP_output.txt** tab-delimited file output containing only coding SNPs/INDELs with 13 columns: 7- Gene ID 2- Gene name 3- Homozygous or heterozygous change 4- Old base -> new base 5- Codon number 6- Effect (eg synonymous/non-synonymous) 7- Old amino acid/new amino acid 8- Base data 9- Coverage 10- Alternative base coverage (%) 11- Reference base coverage (%) 12- Position of SNP in chromosome 13- Warnings	Josephine Moran

Appendix Table 1 continued Description and usage instructions for all bespoke Perl scripts used in this thesis. All scripts are provided in the attached CD.

Script name	Description	Command	Output files	Author
			snpEff_useful.txt tab-delimited SnpEff output file with 24 columns, those relevant to this study are described below 2- Position of SNP/INDEL on chromosome (bp) 3- Nucleotide on reference genome 4- Nucleotide on input genome 5- Change type (SNP or INDEL) 8- Coverage for base 9- Warnings 10- Affected gene ID 11- Affected gene name 13- Affected transcript ID 16- Effect of SNP/INDEL 17- old AA/new AA 18- Old codon/New codon 19- Codon number 21- Coding sequence size	

Appendix Table 1 continued Description and usage instructions for all bespoke Perl scripts used in this thesis. All scripts are provided in the attached CD.

Script name	Description	Command	Output files	Author
Unique_SNPs_bwa.pl	Sorts SNPs/INDELs present in the input 2 file but not the input 1 file into output 1 (output.txt), sorts SNPs/INDELs present in both into output 2 (matches.txt)	*Perl unique_SNPs_bwa.pl <input_1.txt> <input_2.txt> output_name* (where input files are the SNP_output.txt from Mpileup_SNPs_v2.pl)	**Output.txt** and **matches.txt** tab-delimited files with 14 columns, the 13 described above and a final column containing a descriptor either: NO match (SNP/INDEL not preset in input 1), matches (SNP/INDEL present in both) or different aa substitution (SNP/INDEL at same chromosome location in both files but with different amino acid change)	Josephine Moran
DNA_fasta_to_protein_fasta.pl	Converts a multi-fasta file of gene sequences into multi-fasta file of protein sequences	*Perl DNA_fasta_to_protein_fasta.pl <input.fa> output_name*	**Output.txt** multi-fasta of protein sequences	Josephine Moran
Replace_names.pl	Replaces gene names in whole genome COG list from genome feature file (gff)	*Perl replace_names.pl <reference_genome.gff> <input_COG_list.txt>* (where input is the output from WebMGA function annotation (COG) tool)	**Input.ammended.txt** tab-delimited file with 6 columns 7- Official gene name 2- Gene start_end 3- COG 4- COG description 5- COG class 6- COG class description	Josephine Moran
Label_cogs.pl	Labels a gene list with COGs from whole genome COG list	*Perl label_cogs.pl <gene_list.txt> <input.ammended.txt> output_name*	**Output.txt** contains data as input_ammended .txt (see above) but with genes not present in the gene list removed	Josephine Moran

Appendix Table 1 continued Description and usage instructions for all bespoke Perl scripts used in this thesis. All scripts are provided in the attached CD.

Script name	Description	Command	Output files	Author
Counting_cogs.pl	Counts the genes assigned to a class from a gene list and genome, output as figures and percentages	*Perl counting_cogs.pl <input_ammended.txt> <input.txt> output_name* (where input is the output from label_cogs.pl)	**Output.txt** tab-delimited file with 8 columns 7- COG class 2- Percent of genome comprised of COG class 3- Percent of gene list comprised of COG class 4- Percent of upregulated genes in list 5- Percent of downregulated genes in list 6- Total number of genes in list in COG class 7- Total number of genes upregulated in list in COG class 8- Total number of genes downregulated in list in COG class	Josephine Moran
Convert_to_SA_homologs.pl	Converts gene list into list of possible homologs from a BLAST database using BLAST using the E-value as a cutoff	*Perl convert_to_SA_homologs.pl <SA_blast_database> <multi_fasta> output_name <reference_gff> e_value_cutoff*	**Output.csv** comma separated file with 7 columns: 7- Query gene name 2- BLAST hit gene name 3- Percent identity 4- Number of mistmatches 5- Gap openings 6- e-value 7- Bit score	Josephine Moran

Appendix Table 1 continued Description and usage instructions for all bespoke Perl scripts used in this thesis. All scripts are provided in the attached CD.

Script name	Description	Command	Output files	Author
Sammd_compare.pl	Finds genes common in two gene lists	*Perl sammd_compare.pl <gene_list1> <gene_list2> output_name*	**Output.txt** contains the lines from the second file where the gene name in both input files matched.	Josephine Moran
coverageStatsSplitBy Chr_v2.pl	Calculates the coverage statistics for mapped reads	*Perl coverageStatsSplitByChr_v2.pl –I <input.bam> > output_name*	Tab-delimited file with 13 columns: 1- Reference ID 2- Length of reference (bp) 3- Length of mapped reference (bp) 4- Percent of reference mapped to 5- Mean coverage 6- Median coverage 7- Standard deviation of coverage 8- Quartile 1 coverage 9- Quartile 2 coverage 10- 2.5 % coverage 11- 97.5 % coverage 12- Minimum coverage Maximum coverage	Kevin Ashelford

Supporting papers

Statement of contribution:

My contribution to the paper Kenny *et al*. (2013) included performance of antimicrobial susceptibility and mannitol protection assays and editing of the manuscript.

My contribution to the paper Coates *et al*. (2014) included writing of the sections and figures relating to adhesion, antimicrobial lipids and desiccation, as well as contributing to other sections. I was heavily involved in the editing of the manuscript.

OPEN ACCESS Freely available online

PLOS ONE

Mannitol Utilisation is Required for Protection of *Staphylococcus aureus* from Human Skin Antimicrobial Fatty Acids

John G. Kenny[1☯], Josephine Moran[1☯], Stacey L. Kolar[2], Alexander Ulanov[3], Zhong Li[3], Lindsey N. Shaw[2], Elisabet Josefsson[4], Malcolm J. Horsburgh[1*]

1 Institute of Integrative Biology, University of Liverpool, Liverpool, Merseyside, United Kingdom, **2** Department of Cell Biology, Microbiology & Molecular Biology, University of South Florida, Tampa, Florida, United States of America, **3** Roy J. Carver Biotechnology Center, University of Illinois, Urbana-Champaign, Illinois, United States of America, **4** Department of Rheumatology and Inflammation Research, University of Gothenburg, Göteborg, Sweden

Abstract

Mannitol (Mtl) fermentation, with the subsequent production of acid, is a species signature of *Staphylococcus aureus*, and discriminates it from most other members of the genus. Inactivation of the gene *mtlD*, encoding Mtl-1-P dehydrogenase was found to markedly reduce survival in the presence of the antimicrobial fatty acid, linoleic acid. We demonstrate that the sugar alcohol has a potentiating action for this membrane-acting antimicrobial. Analysis of cellular metabolites revealed that, during exponential growth, the *mtlD* mutant accumulated high levels of Mtl and Mtl-P. The latter metabolite was not detected in its isogenic parent strain or a deletion mutant of the entire *mtlABFD* operon. In addition, the *mtlD* mutant strain exhibited a decreased MIC for H_2O_2, however virulence was unaffected in a model of septic arthritis.

Citation: Kenny JG, Moran J, Kolar SL, Ulanov A, Li Z, et al. (2013) Mannitol Utilisation is Required for Protection of *Staphylococcus aureus* from Human Skin Antimicrobial Fatty Acids. PLoS ONE 8(7): e67698. doi:10.1371/journal.pone.0067698

Editor: Michael Otto, National Institutes of Health, United States of America

Received October 23, 2012; **Accepted** May 21, 2013; **Published** July 4, 2013

Funding: This study was supported by BBSRC grant BB/D003563/1 (MJH) and in part AI090350 (LNS) from the National Institute of Allergies and Infectious Diseases. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: MJH has funding from Unilever Plc to investigate skin survival mechanisms of skin staphylococci. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: M.J.Horsburgh@liverpool.ac.uk

☯ These authors contributed equally to this work.

Introduction

S. aureus is a common skin and soft tissue pathogen capable of causing more severe infections including sepsis, osteomyelitis, and endocarditis [1]. The range of infections is due to a multitude of encoded virulence factors and nasopharyngeal carriage is frequent and a risk factor [2,3]. The spread of antibiotic-resistant strains and the emergence of community-acquired MRSA have increased the impact of *S. aureus* on public health and it has necessitated the development of new therapeutics plus a better understanding of transmission and skin survival [4].

Several different barrier functions are proposed to retard the survival of *S. aureus* on human skin, these include the antimicrobial peptides cathelicidin LL-37 and human β-defensin 2, as well as dermicidin, psoriasin, RNase3 and RNase7. One focus for study of survival is the antimicrobial activity of long chain (typically C≥16) unsaturated free fatty acids that generate the acid mantle on skin [5,6,7,8,9]. These antimicrobial fatty acids (AFAs) are components of the innate immune system that function on skin and in abscesses [9,10,11,12,13,14,15,16,17,18]. The amphipathic properties of AFAs are proposed to disrupt membrane function by altering permeability and fluidity and this is supported by transcriptional analyses of linoleic acid-treated *S. aureus* [6]. Cells exposed to sub-inhibitory concentrations of linoleic acid respond by upregulating transcription of genes encoding capsule, peptidoglycan and carotenoid biosynthetic enzymes and pathways for stress resistance [6]; glycolysis and fermentation pathway genes are concomitantly upregulated. In *S. aureus* protection against AFAs is afforded by reducing cell surface hydrophobicity [6,7,19] and the described transcriptional upregulation of cell surface components is proposed to mediate this effect [6]. The transcript encoding the cell surface protein SasF is upregulated >30 fold after addition of linoleic acid and inactivation of the gene decreases survival, but not via detectable changes to surface hydrophobicity [6]. In contrast, cell wall teichoic acid (WTA) and the iron-regulated surface protein IsdA increase survival from AFAs by decreasing surface hydrophobicity [7,19]. Inhibitory concentrations of AFAs cause leakage of proteins and inhibit respiration [20,21,22,23].

In this study, extended screening of *S. aureus* mutants with reduced survival from AFAs identified identical clones with defective mannitol (Mtl) metabolism. Since the capacity of staphylococci to ferment Mtl is most frequently associated with the pathogens *S. aureus*, *S. saprophyticus* and *S. haemolyticus*, we sought to determine the nature of the survival defect in the context of cellular resistance and virulence.

Materials and Methods

Bacterial Strains, Plasmid and Growth Conditions

Strains and plasmids used in this study are listed in Table 1. Bacteria were grown in brain heart infusion broth (BHI) (Lab M) at 37°C with shaking at 125 rpm, unless indicated otherwise.

Mannitol broth contained: peptone 10 g l^{-1}, Mtl 10 g l^{-1}, beef extract 1 g l^{-1}, NaCl 10 g l^{-1}; fructose broth contained fructose in place of Mtl. Cultures were incubated at 37°C with shaking at 250 rpm and growth was monitored by measuring OD_{600}. When included, antibiotics were added at the following concentrations: erythromycin, 5 µg ml^{-1}; lincomycin, 25 µg ml^{-1}; tetracycline, 5 µg ml^{-1}, chloramphenicol 5 µg ml^{-1}. Antibiotics were not included in comparative growth experiments.

Construction of *mtl* Mutants and Complementation Plasmids

Construction of *mtlD* and *mtlABFD* allelic replacement mutants was performed using methods described previously [24]. Amplification of *mtlD* for allelic replacement used upstream and downstream primer pairs, *mtlD_BamHI* CGACGGATCCGATGTTGATGGCAACACATC with *mtlD_NotI* ATAACTGCGGCCGCCAGCACCAAAGTGAACTGC and *mtlD_KpnI* CCGGTACCTAGCCGATGAAATAATTG with *mtlD_EcoRI* ACATGAATTCAACTAATGACAAGGTTGC and for *mtlABFD* operon allelic replacement the primer pair *mtlA_BamHI* CGACGGATCCTAACTTCTGTATCTGTTTCTG and *mtlA_NotI* ATAACTGCGGCCGCTCTCTTCAGTTTGTGACATG. The downstream operon fragment was amplified using *mtlD_KpnI* and *mtlD_EcoRI*. The tetracycline resistance gene (*tet*) was amplified from pDG1513 [25] followed by simultaneous cloning of *tet* disrupted alleles into pMUTIN4 [24,26] and the resultant plasmids pJK1 and pJK2 containing the *mtlD-tet* and *mtlABFD-tet* inserts, respectively, were used to generate allelic replacement mutants in strain SH1000. Plasmids to complement the *mtl* mutations were made by ligating the *mtlABFD* operon, amplified using *mtlA_SalI* ACGCGTCGACCGAACTTTCCCCCTTTCC and *mtlD_BamHI* ACGCGGATCCGAACTACTACATTATTACTGATTG or *mtlD_SalI* with *mtlD_BamHI*. The amplicons and pSK5632 [27] were digested and ligated prior to directly transforming Liv1019 (RN4220 *mtlD::tet*), selecting for acid production on Mtl salt agar containing 5 µg ml^{-1} chloramphenicol. The selected plasmid, pMJH71, was purified and used to transform Liv1023 (SH1000 *mtlD::tet*) and Liv1024 (SH1000 *mtlABFD::tet*).

Antimicrobial Fatty Acid Survival and MICs

An agar plate assay for AFA survival described previously [6] was used to measure comparative growth. Serial dilutions of the mutant strains were plated onto BHI agar containing millimolar concentrations of AFA, prior to viable counting. Minimum inhibitory concentrations (MIC) of AFAs were performed in 96 well plates using ethanol as a solvent.

Table 1. Strains and plasmids used in the study.

Strain or Plasmid	Features	Reference or Source
Strains:		
E. coli:		
Top10	F- *mcrAΔ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ (ara leu) 7697 galU galK rpsL (strR) endA1 nupG*	Invitrogen
Liv1008	Top10-pMutin4-*mtlD*	This Study
Liv1011	Top10-pMutin4-*mtlABFD*	This Study
S. aureus:		
SH1000	Functional *rsbU* derivative of 8325-4 *rsbU+*	Lab strain
RN4220	Restriction-deficient strain	Lab strain
suvB24	SH1000 *mtlD::Tn917*	This Study
Liv772	Newman *mtlD::Tn917*	This Study
Liv1019	RN4220-pMutin4-*mtlD*	This Study
Liv1020	RN4220-pMutin4-*mtlABFD*	This Study
Liv1023	SH1000 *mtlD::tet*	This Study
Liv1024	SH1000 *mtlABFD::tet*	This Study
Liv1027	Newman *mtlD::tet*	This Study
Liv1028	Newman *mtlABFD::tet*	This Study
Liv1090	RN4220 *mtlD::tet* pMJH70	This Study
Liv1091	RN4220 *mtlD::tet* pMJH71	This Study
Liv1097	SH1000 *mtlABFD::tet* pMJH71	This Study
Liv1098	SH1000 *mtlD::tet* pMJH71	This Study
Plasmids		
pLTV1	Temperature sensitive plasmid harbouring Tn*917*	[24]
pMUTIN4	Insertional inactivation vector	[26]
pDG1513	pMTL22 derivative, tetracycline resistant	[25]
pSK5632	Low copy number shuttle plasmid	[27]
pMJH70	pSK5632 containing *mtlD*	This study
pMJH71	pSK5632 containing *mtlABFD*	This study

doi:10.1371/journal.pone.0067698.t001

Zeta potential and Hexadecane Partitioning

Zeta potential was determined using electrophoretic light scattering (ELS) in which the velocity of charged particles under the influence of an applied electric field is measured by monitoring the frequency shift of the scattered light from the particles. Culture (~800 µl) was injected into a capillary cell and measured using a Zetasizer Nano (Malvern Instruments) with the detector positioned at a 17° scattering angle. The data were analysed and interpreted using the associated software. All charges were recorded as the mean of 5 consecutive measurements. Hexadecane partitioning was performed as previously described [6].

BioLog Phenotypic Arrays

BioLog phenotypic arrays were used to monitor growth of bacterial strains in 96 well plates under a wide range of conditions using redox levels within the growth media as a measure of bacterial growth [28]. Strains SH1000 or suvB24 were resuspended from BHI plates to a transmittance of 81% using a BioLog turbidometer then added to the appropriate inoculation fluid for each assay plate. Comprehensive details of the growth factors tested using these assay plates PM1-PM10 can be found at http://www.biolog.com/pdf/pm_lit/PM1-PM10.pdf. Following inoculation the array plates were incubated at 37°C and monitored for turbidity using the OmniLog plate reader at 30 min intervals over a 47 hour period. The assay was performed in triplicate and the mean values were used to compare growth.

Metabolite Analysis

Four hour cultures of strains SH1000, Liv1023 (SH1000 *mtlD*::*tet*) and Liv1024 (SH1000 *mtlABFD*::*tet*) were harvested by centrifugation and washed 3 times in PBS, before being resuspended in 3 ml of PBS. Cells were lysed using a bead-beater for three 1 min intervals at 4°C, with chilling between breakages. Cytoplasmic fractions were centrifuged, and metabolic reactions quenched via the addition of methanol. Samples were then dried down and derivatized as described previously [29,30] with the following modifications. Samples were incubated for 90 min at 50°C with 80 µl of methoxyamine hydrochloride in pyridine (20 mg ml^{-1}) following a 60 min treatment at 50°C with 80 µl MSTFA. Five µl of an internal standard (C31 fatty acid) was added prior to trimethylsilylation, and sample volumes of 1 µL were injected with a split ratio of 7:1. The GC-MS system consisted of an Agilent 7890A (Agilent Inc, Palo Alto, CA, USA) gas chromatograph, an Agilent 5975C mass selective detector and Agilent 7683B autosampler. Gas chromatography was performed on a 60 m HP-5MS column with 0.25 mm inner diameter and 0.25 µm film thickness (Agilent Inc, Palo Alto, CA, USA), and an injection temperature of 250°C. The interface was set to 250°C, and the ion source adjusted to 230°C. Helium carrier gas was set at a constant flow rate of 1.5 ml min^{-1}. The temperature program was 5 min isothermal heating at 70°C, followed by an oven temperature increase of 5°C min^{-1} to 310°C, and a final 20 min at 310°C. The mass spectrometer was operated in positive electron impact mode (EI) at 69.9 eV ionization energy in a m/z 30–800 scan range. The spectra of all chromatogram peaks were compared with electron impact mass spectrum libraries NIST08 (NIST, MD, USA), WILEY08 (Palisade Corporation, NY, USA), and a custom library. To allow comparison between sample sets, all data were normalized to internal standards in each chromatogram, and the weights of each sample. The chromatograms and mass spectra were evaluated using the MSD ChemStation (Agilent, Palo Alto, CA, USA) and AMDIS (NIST, Gaithersburg, MD, USA) programs. The retention time and mass spectra were implemented within the AMDIS method formats. The resulting data from triplicate samples (with less than 10% variability) was analyzed using a t-test. Samples with a $p<0.05$ and greater than 2-fold variation were then analyzed using the MetPA enrichment pathway analysis web application (http://metpa.metabolomics.ca/) [31].

Experimental Septic Arthritis

A previously described mouse model of septic arthritis was used to test the *in vivo* role of *mtlD* in virulence [32,33]. Seven week female NMRI mice were obtained from Charles River Laboratories (Sulzfeld, Germany) and maintained in the animal facility of the Department of Rheumatology and Inflammation Research, University of Göteborg, Sweden. All mice were maintained according to the local ethic board animal husbandry standards. The mice were housed 10 to a cage under standard conditions of temperature and light and were fed standard laboratory chow and water *ad libitum*. Mice were inoculated in the tail vein with 0.2 ml of bacterial suspension cultured and bacteria in kidney abscesses were enumerated after 14 days as described previously [6]. Presented data represent CFU per kidney pair.

Results

Identification of a *mtlD* AFA Survival Mutant

A screen of *S. aureus* Tn*917* library transposants identified multiple clones with greatly reduced survival on BHI agar containing 1 mM linoleic acid ($C_{18:2\Delta9\Delta12}$), in addition to those mutants described previously [6]. DNA sequence determination by arbitrary-primed PCR [6] revealed these clones were identical, with Tn*917* inserted in *mtlD* at nucleotide position 317/1107. The *mtlABFD* operon encodes the Mtl-specific phosphotransferase system (PTS) trasnsporter (MtlAB) and the operon transcriptional repressor (MtlF); Mtl-1-P 5-dehydrogenase, encoded by *mtlD*, catalyses the conversion of Mtl-1-P to fructose-6-P (Figure 1). The *mtlD* mutant suvB24 (SH1000 *mtlD*::Tn*917*) selected for further study showed clearly reduced survival on linoleic acid agar compared to its isogenic parent strain (Figure 2). Transduction of suvB24 into *S. aureus* Newman (Liv772; Table 1) identified a proportionately similar reduction in linoleic acid survival in this distinct strain background (data not shown).

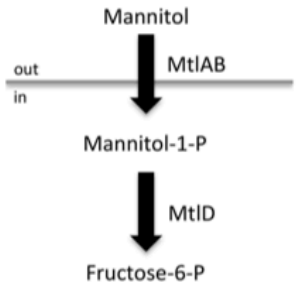


Figure 1. Mannitol uptake pathway in *S. aureus*. The *mtlABFD* operon encodes the Mtl-specific PTS (MtlAB) and the operon transcriptional repressor (MtlF); Mtl-1-P 5-dehydrogenase, encoded by *mtlD*, catalyses the conversion of Mtl-1-P to fructose-6-P which enters into the Embden-Meyerhoff and hexosemonophosphate glycolytic pathways.
doi:10.1371/journal.pone.0067698.g001

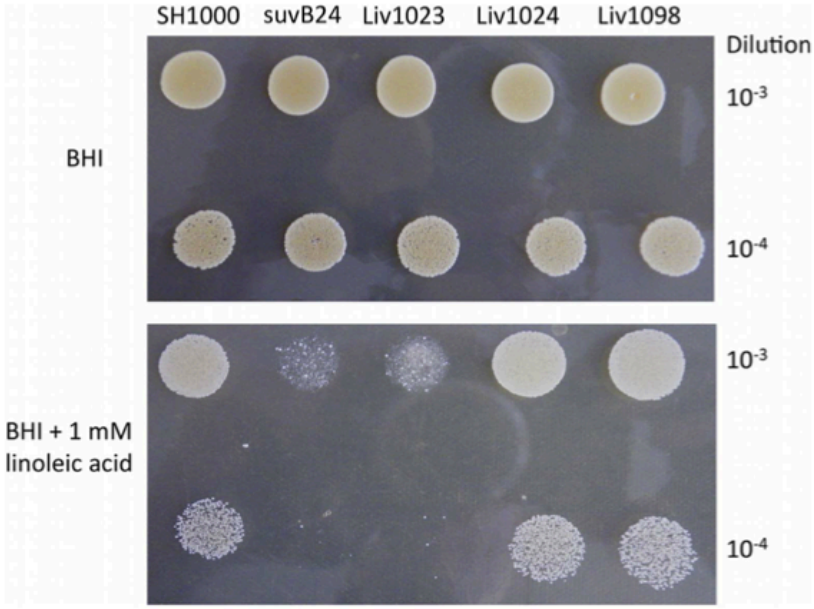


Figure 2. Comparative survival of *S. aureus* strains. Growth of dilutions from overnight cultures on BHI agar in the presence and absence of 1 mM linoleic acid. SuvB24 (SH1000 *mtlD*::Tn*917*) and Liv1023 (SH1000 *mtlD*::*tet*) displayed >500-fold reduced survival on linoleic acid relative to wild type (SH1000), Liv1024 (SH1000 *mtlABFD*::*tet*) and the complemented mutant strain Liv1098 (SH1000 *mtlD*::*tet* pMJH71).
doi:10.1371/journal.pone.0067698.g002

Culture Phenotypes of *mtl* Mutants

To investigate the role of the *mtlD* gene product in host cell physiology and to help explain the mechanism for reduced linoleic acid agar survival, growth of the suvB24 mutant was compared with its isogenic parental strain using a Biolog phenotype array (Biolog Inc. California, USA). Comparative growth arrays in the presence of various carbon, nitrogen, phophorous and sulphur compounds and a variety of amino acids, peptide nitrogen sources, osmolytes and pH ranges [28] identified that reduced Mtl metabolism was the only significantly altered phenotype (data not shown).

To confirm the role of the Mtl PTS operon in *S. aureus* cell survival, allelic replacement mutants were generated for *mtlD*, Liv1023 (SH1000 *mtlD*::*tet*) and for the entire *mtlABFD* operon, Liv1024 (SH1000 *mtlABFD*::*tet*) (Figure 3), using methods described previously [34,35,36]. Two complementation vectors were also generated by cloning the *mtlD* gene and the *mtlABFD* operon into the low copy shuttle vector pSK5632, producing plasmids pMJH70 and pMJH71, respectively. Cloning of the *mtlABFD* operon was achieved by transforming ligation products into strain Liv1021 (RN4220 *mtlD*::*tet*) selecting for fermentation on mannitol salt agar (MSA), since cloning of the operon in *E. coli* TOP10 was not successful, potentially due to toxicity. Complementation with *mtlD* alone did not restore Mtl fermentation on MSA due to the absence of a promoter for this distal gene; consequently complementation experiments were performed using pMJH71. Culture of Liv1023 (SH1000 *mtlD*::*tet*) and Liv1024 (SH1000 *mtlABFD*::*tet*) on MSA at 37°C demonstrated the inability of these mutants to ferment Mtl to produce acid (Figure 4). Weak growth was observed for Liv1023 on MSA agar in contrast to Liv1024, which grew similarly to the wild-type SH1000 strain. Metabolism was restored in the complemented strains Liv1097 (SH1000 *mtlABFD*::*tet* pMJH71) and LIV1098 (SH1000 *mtlD*::*tet* pMJH71) (Figure 4). Transduction of the *mtlD* and *mtlABFD* inactivations into *S. aureus* Newman (Liv1027 and Liv1028, respectively) confirmed the absence of Mtl fermentation in both mutants (data not shown).

Comparative growth assays of the allelic replacement mutants on linoleic acid agar confirmed that Liv1023 (SH1000 *mtlD*::*tet*) had an AFA growth defect similar to suvB24 (SH1000 *mtlD*::Tn*917*) with greater than 3-log reduction in survival (Figure 5). Similarly reduced levels of survival were observed following growth on agar supplemented with millimolar concentrations of oleic acid ($C_{18:1\Delta9}$) or sapienic acid ($C_{16:1\Delta6}$) (data not shown) demonstrating that inactivation of *mtlD* caused reduced survival to multiple AFAs. Allelic replacement of the *mtlABFD* operon did not impair survival from AFAs, in contrast to inactivation of *mtlD* alone. Proportionately reduced AFA survival was observed with an *mtlD* but not an *mtlABFD* inactivation in *S. aureus* Newman (Liv1027 and Liv1028, respectively; Table 1) (data not shown). Reduced survival of the *mtlD* mutant was fully complemented with the entire *mtlABFD* operon present on pMJH71 using strain Liv1098 (SH1000 *mtlD*::*tet* pMJH71) (Figure 2). The reduced survival of Liv1023 (SH1000 *mtlD*::*tet*) on linoleic acid agar was supported with a significantly reduced linoleic acid MIC (0.45±0.02 mM) ($p<0.004$) in BHI medium, compared to SH1000 (0.9±0.04 mM), Liv1024 (0.69±0.02 mM) and Liv1098 (0.85±0.03 mM).

Strain Liv1023 (SH1000 *mtlD*::*tet*) exhibited a profound growth defect when cultured in broth containing Mtl as the carbohydrate source (peptone 10 g l^{-1}, Mtl 10 g l^{-1}, beef extract 1 g l^{-1}, NaCl 10 g l^{-1}) (Figure 6A). Substituting the sugar alcohol Mtl for the

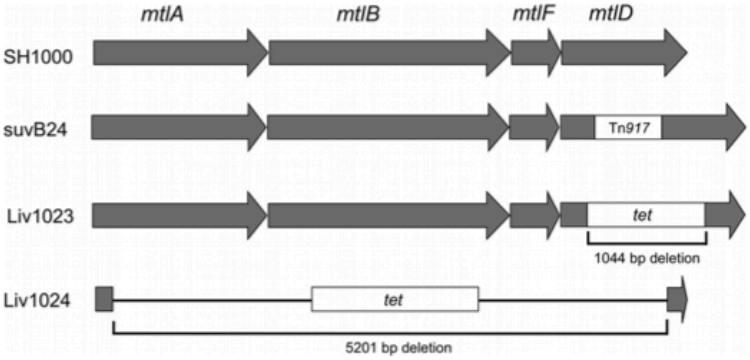


Figure 3. Schematic representation of the *mtlABFD* locus. Position of the transposon insertion and allelic replacements created during this study.
doi:10.1371/journal.pone.0067698.g003

sugars fructose or glucose restored normal growth, demonstrating the Mtl-specific defect (data not shown). *S. aureus* accumulates intracellular Mtl following incubation in the presence of glucose. To test if this accumulation affected survival from AFAs, the relative survival of exponential cells ($OD_{600} = 1$) of SH1000 incubated in PBS containing 1% (w/v) glucose was determined after growth on 1 mM linoleic acid agar. No clear difference in survival of the strains was observed.

All strains grew equally well at 37°C in BHI broth (data not shown), however a pronounced reduction in growth rate was observed for strain Liv1023 (SH1000 *mtlD*::*tet*) when cultured in BHI broth at 25°C (Figure 6B). This defect was specific to inactivation of *mtlD* but not for deletion of the complete operon. Starvation survival with limiting glucose was not impaired in *mtl* mutant strains [35,37]. Growth of *S. aureus* SH1000 was tested in the absence or presence of mannitol (0.1 M, 0.5 M), with or without 1 mM linoleic acid to test for synergy. Mannitol was shown to have similar properties as ethanol [13], by acting synergistically with linoleic acid as evident by the reduced viable count with increasing mannitol concentration (Figure 7).

Analysis of Cellular Metabolites

A comparative metabolomics analysis was undertaken to identify the intracellular metabolites of exponentially growing cells of strains SH1000, Liv1023 (SH1000 *mtlD*::*tet*) and Liv1024 (SH1000 *mtlABFD*::*tet*). This revealed that inactivation of *mtlD* resulted in an accumulation of Mtl and Mtl-P, the latter being undetectable in both SH1000 and Liv1024 (Table 2 and supplementary table 1). The total relative levels of Mtl species were over 20-fold greater in Liv1023 (SH1000 *mtlD*::*tet*) than SH1000. The near absence of Mtl in strain Liv1024 supports data that the MtlAB PTS transporter is the main portal for Mtl uptake [38]. Inactivation of *mtlD* and *mtlABFD* resulted in the absence of cellular Sorbitol-6-P (Table 2). Further clear differences in metabolite levels were evident in strain Liv1023 (*mtlD*::*tet*) relative to SH1000 and Liv1024 (Table S1).

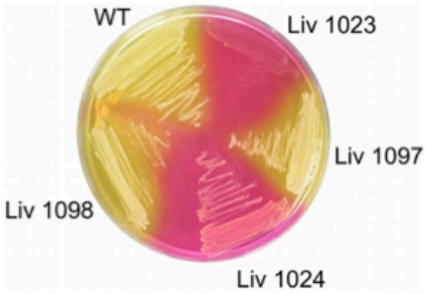


Figure 4. Mtl fermentation capability of *S. aureus* strains. Mtl fermentation is revealed by acid formation and colour change of the pH indicator to yellow. Liv1023 (SH1000 *mtlD*::*tet*) and Liv1024 (SH1000 *mtlABFD*::*tet*) do not ferment Mtl and this capability was restored by complementation with the entire locus in strains Liv1098 (SH1000 *mtlD*::*tet* pMJH71) and Liv1097 (SH1000 *mtlABFD*::*tet* pMJH71). Weak growth of Liv1023 was observed.
doi:10.1371/journal.pone.0067698.g004

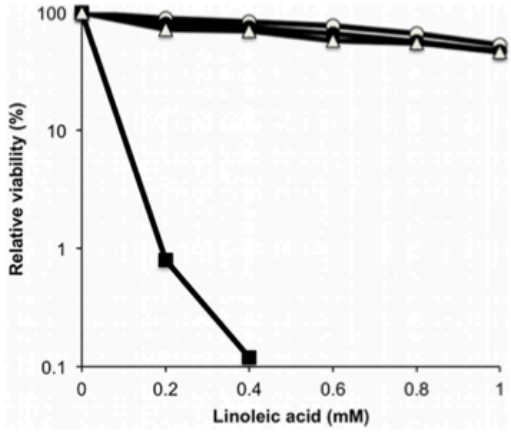


Figure 5. Survival on linoleic acid agar. Comparative survival of strains on BHI agar supplemented with 1 mM linoleic acid. Strains SH1000 (open circles), Liv1023 (SH1000 *mtlD*::*tet*) (filled squares), Liv1024 (SH1000 *mtlABFD*::*tet*) (open triangles) and Liv1098 (SH1000 *mtlD*::*tet* pMJH71) were diluted in PBS and equivalent volumes were plated onto the agar. SE from triplicate experiments is shown with error bars inside symbols.
doi:10.1371/journal.pone.0067698.g005

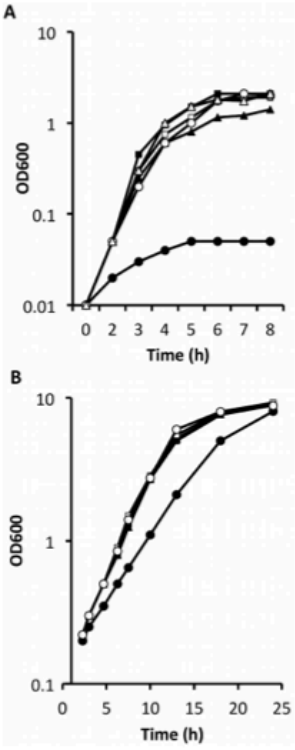


Figure 6. Growth phenotype of *mtlD* inactivated *S. aureus*. (A) Culture of strains in broth containing Mtl at 37°C. Liv1023 (SH1000 *mtlD::tet*) (●) had a significantly reduced growth rate ($P<0.01$, Student's t-test) compared to wild-type (SH1000) (■), Liv1024 (SH1000 *mtlABFD::tet*) (▲), strains Liv1098 (SH1000 *mtlD::tet* pMJH71) (□) and Liv1097 (SH1000 *mtlABFD::tet* pMJH71) (○). Calculated doubling times between 2 h and 3 h of growth: SH1000 = 0.33, Liv1023 (SH1000 *mtlD::tet*) = 1.7. Error bars indicate 1 SEM (n = 3). (B) Culture of strains in BHI broth at 25°C. Liv1023 (SH1000 *mtlD::tet*) (●) has a significantly reduced growth rate ($P<0.001$, Student's t-test) compared to wild-type (SH1000) (■), Liv1024 (SH1000 *mtlABFD::tet*) (▲), strains Liv1098 (SH1000 *mtlD::tet* pMJH71) (□) and Liv1097 (SH1000 *mtlABFD::tet* pMJH71) (○). Calculated doubling times between 5 h and 13 h of growth: SH1000 = 2.46, Liv1023 = 3.13. Representative dataset from triplicate assay.
doi:10.1371/journal.pone.0067698.g006

Resistance and Cell Surface Properties of *mtl* Mutants

A range of antimicrobial agents were tested to determine if the observed reduced resistance of Liv1023 (SH1000 *mtlD::tet*) extended beyond AFAs. Growth and MICs were comparable between Liv1023 (SH1000 *mtlD::tet*) and SH1000 in the presence of a range of concentrations of NaCl, lauroyl sarcosine, SDS, dichlorophenyl and the human cathelicidin LL37 (Sigma). Liv1023 (SH1000 *mtlD::tet*) was observed to exhibit a lower MIC for H_2O_2 (1 mM) compared to SH1000 (4 mM) and Liv1024 (SH1000 *mtlABFD::tet*) (4 mM).

The hydrophobicity and zeta potential of all of the strains was similar when tested using either hexadecane partitioning or measured using a zetasizer (Malvern, UK), respectively (data not shown). The levels of carotenoid in cell membranes were similar between SH1000 and the *mtl* mutants, as judged by spectrophotometric analysis of methanol-extracted cells from overnight and 2 day-old cultures (data not shown).

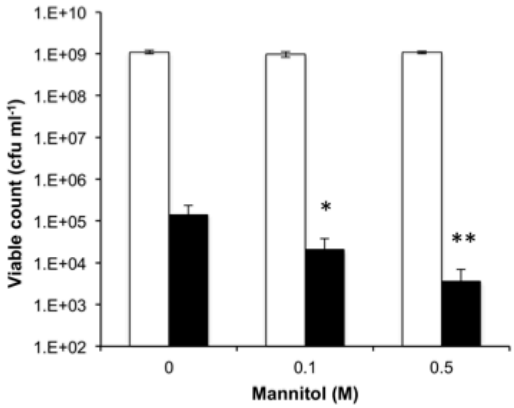


Figure 7. Growth of *S. aureus* in the presence of mannitol and linoleic acid. Bacteria were cultured on BHI agar containing either no or added mannitol (0.1 M, 0.5 M) in the presence (black bars) or absence (white bars) of 1 mM linoleic acid. Differences in viable cells recovered in the presence of mannitol were significantly reduced compared to the absence of mannitol ($P = 0.03$ and $P = 0.001$ for 0.1 M and 0.5 M, respectively).
doi:10.1371/journal.pone.0067698.g007

Virulence of *mtlD* Mutant

The decreased *in vitro* AFA survival and reduced H_2O_2 MIC of the *mtlD* mutant prompted testing of its virulence compared to the isogenic parent strain using a previously described model of experimental septic arthritis (Figure 8). This model was tested to determine whether inactivation of the *mtlABFD* locus affected virulence, since its contribution to metabolism *in vivo* is unknown and the model generates abscesses where AFAs accumulate [14]. This revealed that SH1000 *mtlD* did not have reduced virulence, at least under the conditions studied [6,32,33].

Table 2. Sugar alcohols present in *S. aureus* strains.

	Relative mean concentration		
Metabolite	**SH1000**	**Liv1023**	**Liv1024**
Arabitol	116.1 (3.9)	107.5 (19.7)	51.4 (7.9)
Mannitol	417.6 (29.5)	1351.4 (82.5)	5.9 (0.9)
Mannitol-P	ND	8161.3 (119.7	ND
Ribitol	240 (22.3)	214.8 (17.6)	272.2 (27.1)
Sorbitol-6-P	149.4 (18.2)	ND	ND

GC-MS was used to analyse cytoplasmic fractions from exponential growth phase cells. 131 unique metabolites were compared and chromatograms and mass spectra were evaluated as described previously [8,32] using the MSD ChemStation (Agilent, Palo Alto, CA, USA) and AMDIS (NIST, Gaithersburg, MD, USA) programs. The resulting data from triplicate samples (with less than 10% variability) were analyzed using a t-test. Samples with greater than 2-fold variation ($p<0.05$) were analyzed using the MetPA enrichment pathway analysis web application (http://metpa.metabolomics.ca/) [45]. ND, not detectable.
doi:10.1371/journal.pone.0067698.t002

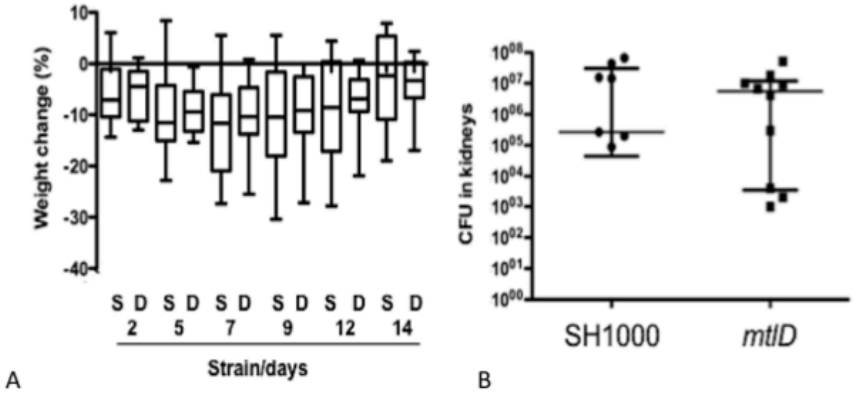


Figure 8. Virulence of *mtlD* in a murine infection. (A) Effect of WT SH1000 or Liv1023 (SH1000 *mtlD::tet*) on percentage change in weight of infected mice. There were no significant differences using Dunn's test. (B) Effect of mutations of *mtlD* on cfu of *S. aureus* SH1000 in kidneys of infected mice. There were no significant differences using the Mann Whitney Test.
doi:10.1371/journal.pone.0067698.g008

Discussion

The intrinsic importance of *S. aureus* carriage and transmission in relation to disease and its hypothesized link with virulence [39] requires that determinants are identified and characterised that promote survival in its primary niche and during its transient residence on human skin. From the study of gene mutants *S. aureus* defence from AFAs is achieved via a variety of surface components (IsdA, WTA, SasF) and regulation of peptidoglycan biosynthesis (VraRS, VraE), where a reduction in hydrophobicity to minimize access of the AFA to the membrane explains the contribution of several of these components to survival [6,7,19]. In addition, the arginine deiminase pathway increases survival [6], where its various contributions to metabolic versatility and its potential to modify local pH could explain its role.

Determining that an Mtl-1-P-dehydrogenase mutant, but not an *mtlABFD* transport operon mutant, has greatly reduced survival from AFAs implicates the accumulation of Mtl-1-P as being the causative factor. As the most abundant natural hexitol, Mtl is a carbon source for staphylococci and the inducible oxidation of Mtl-1-P generates fructose-6-P for entry into the Embden-Meyerhoff and hexosemonophosphate glycolytic pathways [38,40]. All strains of *S. aureus* accumulate Mtl, despite not all being capable of using it for metabolism during aerobic growth. In *S. aureus* the cellular accumulation of Mtl was identified in resting cells when incubated in glucose or cultured in media without added carbohydrate [38]. Mtl accumulation was proposed to enhance metabolic versatility in *S. aureus*, however its mechanistic role is incompletely understood [41]. Following stress, such as after exposure to AFAs, utilisation of the pathway for Mtl conversion to fructose-6-P would regenerate NADH, thereby alleviating the pressure upon regenerating reactions downstream of pyruvate. In our previous studies [6], exposure of *S. aureus* to linoleic acid caused downregulated transcription of the *mtlABFD* locus, which suggests, either that reduced levels of intracellular Mtl is a preferred metabolic state following exposure to AFAs, or that lower amounts of Mtl-1-P arising from metabolism (concomitantly regenerating NADH) limited induction of the operon. A potential explanation for the reduced AFA survival of the *mtlD* mutant is its reduced adaptive capacity due to an inability to metabolise Mtl. The near wild-type AFA survival of the *mtlABFD* operon mutant argues against this Mtl metabolism hypothesis, however, unless there is an alternative metabolic reserve. 3-phosphoglycerate could serve as just such an alternative metabolic source and substrate for regenerating NADH, and of note there is 3-fold reduced 3-PGA in the *mtlD* mutant.

Metabolite analysis of the *S. aureus mtlD* mutant, when compared to the *mtlABFD* transport mutant and the parental strain, revealed that 14% of the total Mtl that accumulated intracellularly was not phosphorylated. Since the EIIMtl mannitol transporter (encoded by *mtlA*) phosphorylates the imported Mtl and since the Mtl-1-P-dehydrogenase activity is ablated in the *mtlD* mutant, the conversion of Mtl-1-P to Mtl in the *mtlD* mutant is likely to arise from phosphotransferase reactions as described by Saier and Newman [42]. Alternatively, an undescribed phosphatase activity might account for the presence of Mtl. In *Lactobacillus plantarum* a hypothetical phosphatase activity of EIIMtl was proposed to explain the apearance of Mtl in engineered strains [43]. In the study of Mtl overproducing strains of *L. lactis* a Mtl-1-phosphatase activity was proposed to explain the presence of unphosphorylated Mtl, where *mtlA* was absent, and thus an EIIMtl activity, could not be present. Analysis of the metabolites of growing cells of the *S. aureus mtlD* mutant cultured in BHI broth, when compared to the *mtlABFD* transport mutant and the parental strain, revealed further differences aside from sugar alcohol content (Table S1). These metabolite changes e.g. aminoadipic acid, 3-phosphoglycerate, hydroxypentanoic acid, heptanoic acid and tetradecanoylglycerol, do not indicate a clearly defined mechanistic explanation for decreased AFA MIC.

Growth of the *mtlD* mutant was strongly retarded in media containing mannitol, highlighting the deleterious effects resulting from the likely unrestricted accumulation of Mtl/Mtl-1-P. A direct link between the intracellular accumulation of Mtl/Mtl-1-P and reduced resistance to AFAs in *S. aureus* currently lacks an evidence-based mechanism. However, several features of the *mtlD* phenotype could result from a membrane-associated effect. Alcohol has a well-described potentiating mechanism with respect to AFAs and their membrane activity [13], since it is capable of solubilising membrane lipids due to its polarity and lipophilicity. Intracellularly accumulated sugar alcohol, Mtl, might act similarly to potentiate AFA action, since it was demonstrated in this study that Mtl acted synergistically with linoleic acid when added externally

in BHI agar. Two further phenotypes point towards a membrane-specific alteration in the mtlD mutant; the reduced growth rate that was oberved for the *mtlD* mutant at 25°C and the reduced MIC for H_2O_2 which did not result from differences in catalase specific activity (data not shown). A perturbation in peroxide permeability at the membrane is consistent with the reduced MICs observed and might arise via Mtl potentiating the linoleic acid by virtue of the polarity and lipophilicity of alcohols affecting diffusion across the membrane, but this was not investigated further. No differences were observed between the staphyloxanthin levels in methanol extracts of any of the strains, which might be expected if the intracellular accumulation of Mtl altered membrane fluidity (data not shown) [44]. Mtl is frequently included in membrane preparations as a stabilising entity, either through direct effects or via osmotic stabilisation. The expression of a bacterial *mtlD* in *Saccharomyces cerevisiae* was sufficient to generate mannitol which was proposed to act as an osmolyte and was sufficient to rescue the phenotypes of a glycerol deficient mutant, producing an increased resistance to high salt and H_2O_2 [45]. Mtl is also proposed to function as an osmoprotectant in cells of petunia as well as improving cold tolerance [46].

The observed phenotype of reduced survival in the presence of AFAs did not translate to a reduction in virulence in a murine arthritis model or reduced MIC levels to a range of other membrane-acting agents. Thus, the changes to cellular physiology in the *mtlD* mutant are discrete, at least in this model of infection tested and other disease models, such as skin survival, remain to be tested.

Supporting Information

Table S1 Metabolites present in *S. aureus* strains. Metabolites were identified using GC-MS analysis of cytoplasmic fractions from exponential growth phase cells. 131 unique metabolites were compared and chromatograms and mass spectra were evaluated as described previously [8,32] using the MSD ChemStation (Agilent, Palo Alto, CA, USA) and AMDIS (NIST, Gaithersburg, MD, USA) programs. The resulting data from triplicate samples (with less than 10% variability) were analyzed using a t-test. Samples with greater than 2-fold variation ($p<0.05$) were analyzed using the MetPA enrichment pathway analysis web application (http://metpa.metabolomics.ca/) [45]. ND, not detectable.
(XLSX)

Acknowledgments

We thank Ing-Marie Jonsson (Gothenburg) for technical help with virulence studies.

Author Contributions

Conceived and designed the experiments: JGK JM ZL EJ LNS MJH. Performed the experiments: JGK JM SLK AU EJ MJH. Analyzed the data: JGK JM SLK AU EJ MJH. Contributed reagents/materials/analysis tools: ZL LNS EJ MJH. Wrote the paper: JM MJH.

References

1. Lowy FD (1998) *Staphylococcus aureus* infection. N Engl J Med 339: 520–532.
2. Foster TJ (2005) Immune evasion by staphylococci. Nat Rev Microbiol 3: 948–58.
3. Gorwitz RJ, Kruszon-Moran D, McAllister SK, McQuillan G, McDougal LK, et al (2008) Changes in the prevalence of nasal colonization with *Staphylococcus aureus* in the United States, 2001–2004. J Infect Dis 197: 1226–34.
4. Deleo FR, Otto M, Kreiswirth BN, Chambers HF (2010) Community-associated methicillin-resistant *Staphylococcus aureus*. Lancet 375: 1557–1568.
5. Kelsey JA, Bayles KW, Shafii B, McGuire MA (2006) Fatty acids and monoacylglycerols inhibit growth of *Staphylococcus aureus* Lipids 41: 951–961.
6. Kenny JG, Ward D, Josefsson E, Jonsson IM, Hinds J, et al. (2009) The *Staphylococcus aureus* response to unsaturated long chain free fatty acids: survival mechanisms and virulence implications. PLoS ONE 4: e4344.
7. Kohler T, Weidenmaier C, Peschel A (2009) Wall teichoic acid protects *Staphylococcus aureus* against antimicrobial fatty acids from human skin. J Bacteriol 191: 4482–4484.
8. Campbell IM, Crozier DN, Pawagi AB, Buivids IA (1983) In vitro response of *Staphylococcus aureus* from cystic fibrosis patients to combinations of linoleic and oleic acids added to nutrient medium. J Clin Microbiol 18: 408–415.
9. Zheng CJ, Yoo JS, Lee TG, Cho HY, Kim YH, et al. (2005) Fatty acid synthesis is a target for antibacterial activity of unsaturated fatty acids. FEBS Lett 579: 5157–5162.
10. Ansari MN, Nicolaides N, Fu HC (1970) Fatty acid composition of the living layer and stratum corneum lipids of human sole skin epidermis. Lipids 5: 838–845.
11. Bergsson G, JArnfinnsson J, Steingrimsson O, Thormar H (2001) Killing of Gram-positive cocci by fatty acids and monoglycerides. APMIS. 109: 670–678.
12. Do TQ, Moshkani S, Castillo P, Anunta S, Pogosyan A, et al (2008) Lipids including cholesteryl linoleate and cholesteryl arachidonate contribute to the inherent antibacterial activity of human nasal fluid. J Immunol 181: 4177–4187.
13. Drake DR, Brogden KA, Dawson DV, Wertz PW (2008) Thematic review series: skin lipids. Antimicrobial lipids at the skin surface. J Lipid Res. 49: 4–11.
14. Dye ES, Kapral FA (1981) Characterization of a bactericidal lipid developing within staphylococcal abscesses. Infect Immun 32: 98–104.
15. Engler HD, Kapral FA (1992) The production of a bactericidal monoglyceride in staphylococcal abscesses. J Med Microbiol. 37: 238–244.
16. Nieman C (1954) Influence of trace amounts of fatty acids on the growth of microorganisms. Bacteriol Rev 18: 147–161.
17. Pappas A, Anthonavage M, Gordon JS (2002) Metabolic fate and selective utilization of major fatty acids in human sebaceous gland. J Invest Dermatol 118: 164–171.
18. Won SR, Hong MJ, Kim YM, Li CY, Kim JW, et al. (2007). Oleic acid: an efficient inhibitor of glucosyltransferase. FEBS Lett 581: 4999–5002.
19. Clarke SR, Mohamed R, Bian L, Routh AF, Kokai-Kun JF, et al. (2007) The *Staphylococcus aureus* surface protein IsdA mediates resistance to innate defenses of human skin. Cell Host Microbe 1: 199–212.
20. Galbraith H, Miller TB (1973) Effect of long chain fatty acids on bacterial respiration and amino acid uptake. J Appl Bacteriol 36: 659–675.
21. Galbraith H, Miller TB (1973) Effect of metal cations and pH on the antibacterial activity and uptake of long-chain fatty acids. J Appl Bacteriol 36: 635–646.
22. Galbraith H, Miller TB (1973) Physicochemical effects of long-chain fatty acids on bacterial cells and their protoplasts. J Appl Bacteriol 36: 647–658.
23. Greenway DL, Dyke KG (1979) Mechanism of the inhibitory action of linoleic acid on the growth of *Staphylococcus aureus*. J Gen Microbiol 115: 233–245.
24. Horsburgh MJ, Wiltshire MD, Crossley H, Ingham E, Foster SJ (2004) PheP, a putative amino acid permease of *Staphylococcus aureus*, contributes to survival in vivo and during starvation. Infect Immun 72: 3073–3076.
25. Guerot-Fleury AM, Shazand K, Frandsen N, Stragier P (1995) Antibiotic resistance cassettes for *Bacillus subtilis*. Gene 167: 335–336.
26. Vagner V, Dervyn E, Ehrlich ED (1998) A vector for systematic inactivation in *Bacillus subtilis*. Microbiology 144: 3097–3104.
27. Grkovic S, Brown MH, Hardie KM, Firth N, Skurry RA (2003) Stable low-copy-number *Staphylococcus aureus* shuttle vectors. Microbiology 149: 785–794.
28. Bochner BR, Gadzinski P, Panomitros E (2001) Phenotype microarrays for high-throughput phenotypic testing and assay of gene function. Gen Res 11: 1246–1255.
29. Dawe AL, Van Voorhies WA, Lau TA, Ulanov AV, Li Z (2009) Major impacts on the primary metabolism of the plant pathogen *Cryphonectria parasitica* by the virulence-attenuating virus CHV1-EP713. Microbiology 155: 3913–3921.
30. Roessner U, Wagner C, Kopka J, Trethewey RN, Willmitzer L (2000) Technical advance: simultaneous analysis of metabolites in potato tuber by gas chromatography-mass spectrometry. Plant J 23: 131–42.
31. Xia J, Wishart DS (2010) MetPA: a web-based metabolomics tool for pathway analysis and visualization. Bioinformatics 26: 2342–2344.
32. Bremell T, Lange S, Yacoub A, Ryden C, Tarkowski A (1991) Experimental *Staphylococcus aureus* arthritis in mice. Infect Immun 59: 2615–2623.
33. Josefsson E, Kubica M, Mydel P, Potempa J, Tarkowski A (2008) In vivo sortase A and clumping factor A mRNA expression during *Staphylococcus aureus* infection. Microb Pathog 44: 103–110.
34. Horsburgh MJ, Aish JL, White IJ, Shaw L, Lithgow JK, et al. (2002) SigmaB modulates virulence determinant expression and stress resistance: characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325–4. J Bacteriol 184: 5457–5467.
35. Horsburgh MJ, Clements MO, Crossley H, Ingham E, Foster SJ (2001) PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus*. Infect Immun 69: 3744–3754.

36. Horsburgh MJ, Thackray PD, Moir A (2001) Transcriptional responses during outgrowth of *Bacillus subtilis* endospores. Microbiology 147: 2933–2941.
37. Watson SP, Clements MO, Foster SJ (1998) Characterization of the starvation-survival response of *Staphylococcus aureus*. J Bacteriol 180: 1750–1758.
38. Edwards KG, Blumenthal HJ, Khan M, Slodki ME (1981) Intracellular mannitol, a product of glucose metabolism in staphylococci. J Bacteriol 146: 1020–1029.
39. Massey RC, Horsburgh MJ, Lina G, Höök M, Recker M (2006) The evolution and maintenance of virulence in *Staphylococcus aureus*: a role for host-to-host transmission? Nat Rev Microbiol 4: 953–8.
40. Watanabe S, Hamano M, Kakeshita H, Bunai K, Tojo S, et al. (2003) Mannitol-1-phosphate dehydrogenase (MtlD) is required for mannitol and glucitol assimilation in *Bacillus subtilis*: possible cooperation of *mtl* and *gut* operons. J Bacteriol 185: 4816–4824.
41. Solomon PS, Waters ODC, Oliver RP (2007) Decoding the mannitol enigma in filamentous fungi. Trends Microbiol 15: 257–252.
42. Saier MH Jr, Newman MJ (1976) Direct transfer of the phosphoryl moiety of mannitol 1-phosphate to [14C]mannitol catalyzed by the enzyme II complexes of the phosphoenolpyruvate: mannitol phosphotransferase systems in *Spirochaeta aurantia* and *Salmonella typhimurium*. J Biol Chem 251: 3834–7.
43. Wisselink HW, Moers AP, Mars AE, Hoefnagel MH, de Vos WM, et al. (2005) Overproduction of heterologous mannitol 1-phosphatase: a key factor for engineering mannitol production by *Lactococcus lactis*. Appl Environ Microbiol 71: 1507–14.
44. Mishra NN, Liu GY, Yeaman MR, Nast CC, Proctor RA (2011) Carotenoid-related alteration of cell membrane fluidity impacts *Staphylococcus aureus* susceptibility to host defense peptides. Antimicrob Agents Chemother 55: 526–31.
45. Chaturvedi V, Bartiss A, Wong B (1997) Expression of bacterial *mtlD* in *Saccharomyces cerevisiae* results in mannitol synthesis and protects a glycerol-defective mutant from high-salt and oxidative stress. J Bacteriol 179: 157–62.
46. Chiang Y, Stushnoff C, McSay AE (2005) Overexpression of mannitol-1-phosphate dehydrogenase increases mannitol accumulation and adds protection against chilling injury in petunia. J Amer Soc Hort Sci 130: 605–610.

REVIEW

For reprint orders, please contact: reprints@futuremedicine.com

Staphylococci: colonizers and pathogens of human skin

Rosanna Coates[‡1], Josephine Moran[‡1] & Malcolm J Horsburgh[*1]

ABSTRACT: Staphylococci are abundant bacteria of the human skin microbiome. Several species, particularly *Staphylococcus aureus* and *Staphylococcus epidermidis*, are opportunistic pathogens and cause significant disease. The human skin serves many functions and here we review its role as an antimicrobial barrier and the staphylococcal mechanisms to colonize and counteract the various stresses present in this niche. Successful colonization is achieved using a diversity of adhesins, surface proteins and secreted enzymes to counteract the antimicrobial peptides, enzymes and lipid matrix components present in the acid mantle. Further mechanisms enable these bacteria to overcome osmotic and acid stresses and desiccation in order to survive the exacting demands of an ever-changing landscape.

KEYWORDS
• adhesin • antimicrobial peptide • commensal • desiccation • fatty acid • pathogen • skin colonization • sphingosine • *Staphylococcus*

The skin is a vital barrier against infection with many defenses to prevent invasion, yet many organisms thrive within this hostile environment. Resident bacteria of the skin microbiome, including certain species of staphylococci, supplement the barrier function of the epidermis by inhibiting the colonization of skin pathogens such as *Staphylococcus aureus* through nutrient competition and the production of antimicrobial peptides (AMPs) [1]. The importance of the human microbiome in health and disease is increasingly being recognized and is driving efforts to model the community structure of the resident microbiota [2–5]. Such studies highlight the diversity of the resident skin community across the human body over time, between individuals and ages [3,6–9].

Staphylococci are the most studied of the resident skin flora owing to their ubiquitous colonization of human skin and the wide spectrum of diseases they cause. The dominant *Staphylococcus* species on skin is *S. epidermidis*, which is considered to be a universal colonizer and part of a pan-microbiota [10]. Across different body sites a substantial proportion of the skin community comprises coagulase-negative staphylococci (CoNS), including *S. hominis, S. haemolyticus, S. saprophyticus, S. capitis, S. warneri, S. simulans* and *S. cohnii*. Coagulase-positive *S. aureus* is not considered to be part of the natural skin microbiota and nasal decolonization treatments reduce its frequency on skin [11,12]. While most CoNS interactions with skin are likely to be commensal, they can cause opportunistic infections [13]. Staphylococcal diseases range from common skin ailments, such as boils and impetigo, to urinary tract infections and more serious diseases, including postsurgical infections, device-associated disease, toxic shock syndrome and systemic infection [14]. *S. aureus* colonization has been linked as a risk factor of certain infections, such as bacteremia [15].

Structure of the human epidermis

To understand the challenges faced by bacteria on human skin, it is important to first consider the features of this niche and its barrier function.

[1]Microbiology Research Group, Institute of Integrative Biology, University of Liverpool, Liverpool, Merseyside, L69 7ZB, UK
*Author for correspondence: Tel.: +44 151 795 4569; Fax: +44 151 795 4410; m.j.horsburgh@liverpool.ac.uk
[‡]Authors contributed equally

Future Medicine part of fsg

The human epidermis comprises many pilosebaceous units, containing a hair follicle, sebaceous gland and interfollicular epidermis. These units enable the skin to perform its roles in thermoregulation and protection. Each unit contains a pool of stem cells that maintain cell number throughout skin homeostasis and in the event of injury. The interfollicular epidermis has a multilayered structure consisting of keratinocytes, which move closer to terminal differentiation as they migrate through the layers (**Figure 1**) [16]. Suprabasal cell division occurs during formation of the epidermis; these progeny cells differentiate into suprabasal spinous keratinocytes, then granular cells and finally corneocytes to complete development of the stratified structure [17]. Corneocytes are attached to one another through corneodesmosomes that, when proteolyzed, are shed from the upper layer of the epidermis, a process known as desquamation [18]. This outer skin layer is replaced by differentiation of the underlying granular cells into corneocytes. Migration of keratinocytes is accompanied by a series of morphological changes. Their round appearance changes to flattened and polygonal, and the plasma membrane becomes permeable. Following enucleation, development ends with the maturation of a cornified envelope [19]. Lipids such as ceramide are synthesized and secreted from lamellar bodies [20] and form covalent attachments to the proteins of the cornified envelope [21].

The skin extracellular matrix (ECM) has an important role in tissue organization since

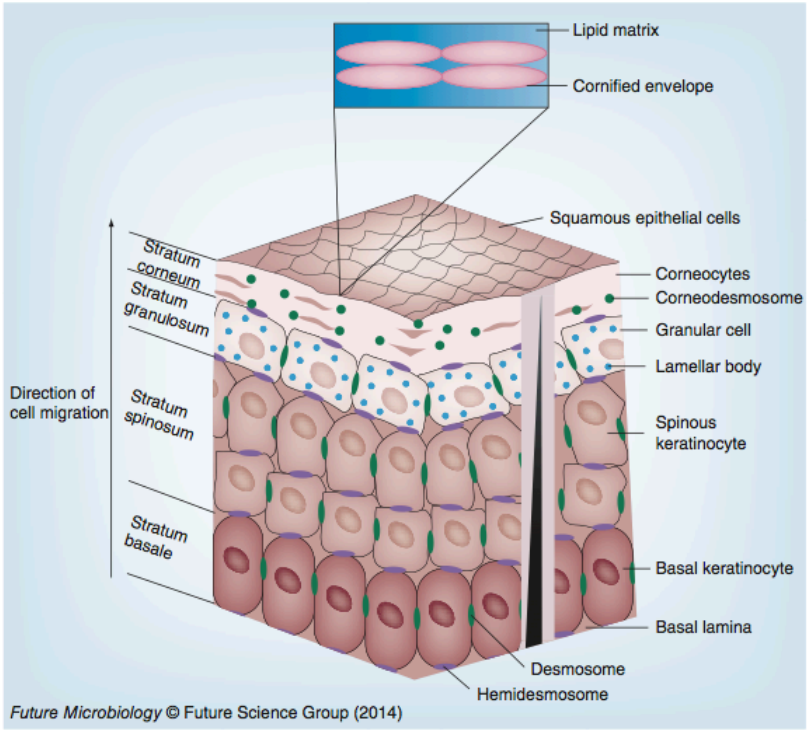


Figure 1. Human epidermis. The structural organization of the epidermis is derived from basal keratinocytes that migrate through the layers of the epidermis and undergo differentiation. This migration is accompanied by morphological changes resulting in the flattened, enucleated corneocytes of the stratum corneum. Corneocytes produce lamellar bodies, which are storage vesicles that secrete antimicrobial peptides, organic acids, antimicrobial fatty acids, cholesterol, sphingosine and ceramides, which in turn comprise the lipid matrix. Proteases are also secreted from the lamellar bodies resulting in desquamation of the upper layer of the epidermis.

different profiles of ECM molecules and adhesins create different environments that may influence the recruitment of different cell types. The ECM also plays an important role in both wound healing and aging [22]. The stratum corneum ECM is comprised mostly of skin lipids, with ceramides, free fatty acids and cholesterol, which in a ratio of 1:1:1, are organized in a stacked bilayer [23].

Colonization & persistence: the role of adhesins

A primary requirement for colonizing the skin surface is adherence to the flattened, cornified keratinocytes. Microbial adhesins specific for receptors on the skin minimize loss by mediating strong attachments to prevent detachment by sheer forces and ensuring reattachment before desquamation. It is unknown whether adhesion to the uppermost surface of the skin is sufficient for long-term skin survival or whether penetration and adhesion to deeper layers of the skin is necessary **(Figure 1)**. Purported methods for sampling different layers of the skin include skin swab (topmost layer), skin scrape (upper layers) or skin punch biopsy (all skin layers). Zeeuwen *et al.* recently proposed that bacteria found at a depth of several cell layers represent the indigenous microbiome rather than those of superficial skin layers [2]. Their study used a tape-stripping method to remove superficial skin layers prior to taking swabs of the area, then 16S rDNA sequencing was performed to compare the microbiota with an area without tape-stripping. However, this method does not account for skin surface irregularities, which may result in parts of the superficial layers not being removed, while in an adjacent area, potentially removing many more layers at the same time, resulting in swabs not necessarily sampling equivalent layers. By contrast, Grice *et al.* reported that skin swab, scrape and punch samples shared 97.2% of operational taxonomic units, revealing few differences in the microbiome between skin depths [24].

There is good evidence that adhesins contribute differentially during distinct stages of *S. aureus* nasal epithelium colonization. The nonproteinaceous adhesin wall teichoic acid is important during initial colonization [25–28], (revealed by the inability of wall teichoic acid biosynthesis mutants to colonize [28]), whereas the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) IsdA and ClfB were more important for colonization and persistence [25,26]. It is not known if equivalent adhesins are important for skin colonization across the staphylococci.

Adhesins facilitate niche selection

The breadth of adhesins and their differential specificity accounts for host and tissue specificity **(Table 1)**. The *S. epidermidis* RP62A genome encodes 12 MSCRAMMS, whereas *S. aureus* can express up to 20 [29]. This varied repertoire could explain colonization differences between the otherwise similar *S. aureus* and *S. epidermidis* [30]. For example, the homologous MSCRAMMs Aap of *S. epidermidis* and SasG of *S. aureus* mediate adhesion to different niches. While Aap can bind buccal epithelial cells and desquamated corneocytes, SasG binds desquamated nasal cells, but not buccal epithelial or keratinocyte cells [31,32]. It is likely that the receptor is the same, since recombinant Aap could block SasG adhesion [31]; however, Aap must be more promiscuous in its binding. This difference could indicate that Aap is adapted for colonization of skin and nares; however, it could also be an artifact caused by different methodologies and cell origin.

A study of adhesion to layers of the stratum corneum by *S. epidermidis* identified three patterns of binding. First binding to superficial, fully differentiated corneocytes and hairs; second, binding to deeper, less differentiated epithelia at cell–cell junctions; and finally universal binding to all layers across the entire surface of the cell [33]. Binding to different layers of the stratum corneum owing to receptor variation between skin layers could affect long-term colonization or survival.

Variation between cell layer receptors is an important consideration since most studies examine adhesion to desquamated corneocytes or less differentiated keratinocyte cell lines, but not both together. Human skin components known to act as staphylococcal adhesin receptors are listed in **Table 1**. Of these receptors, loricrin is a major component of the cornified envelope, and as such, is present on the periphery of corneocytes in the stratum corneum **(Figure 1)**. Another of these receptors, involucrin, is expressed at the cell periphery in the stratum spinosum, stratum granulosum and stratum corneum [34]. Differences in receptor expression between layers of the skin highlights the importance of choice of cell type in bacterial adhesion assays.

Table 1. Staphylococcal adhesins with known receptors for skin.

Host receptor	Adhesin	Species	Ref.
Collagen type I	Cna	*Staphylococcus aureus*	[130]
	Eap/Map	*S. aureus*	[131]
	Emp	*S. aureus*	[132]
	GehD	*Staphylococcus epidermidis*	[133]
	SdrF	*S. epidermidis*	[134]
	SdrI	*Staphylococcus saprophyticus*	[135]
Cytokeratin-10	ClfB	*S. aureus*	[136]
	IsdA	*S. aureus*	[137]
Fibrinogen	Aaa	*S. aureus*	[138]
	Atl	*S. aureus*	[139]
	ClfA	*S. aureus*	[140]
	ClfB	*S. aureus*	[141]
	Eap/Map	*S. aureus*	[132]
	Emp	*S. aureus*	[132]
	Fbe/SdrG	*S. epidermidis*	[142]
	Fbl	*Staphylococcus lugdunensis*	[143]
	FnbpA/FnbA	*S. aureus*	[144]
	FnbpB/FnbB	*S. aureus*	[145]
	IsdA	*S. aureus*	[146]
	UafB	*S. saprophyticus*	[147]
Fibronectin	Aaa	*S. aureus*	[138]
	Aae	*S. epidermidis*	[148]
	Aas	*S. saprophyticus*	[149]
	Atl	*S. aureus*	[139]
	AtlC	*Staphylococcus caprae*	[150]
	Eap/Map	*S. aureus*	[132]
	Embp	*S. epidermidis*	[151]
	Emp	*S. aureus*	[132]
	Fbe/SdrG	*S. epidermidis*	[152]
	FnbpA/FnbA	*S. aureus*	[153,154]
	FnbpB/FnbB	*S. aureus*	[154]
	IsdA	*S. aureus*	[146]
	SdrI	*S. saprophyticus*	[155]
	UafB	*S. saprophyticus*	[147]
Involucrin	IsdA	*S. aureus*	[137]
Keratinocyte lipids/glycolipids	Pls	*S. aureus*	[156]
Loricrin	ClfB	*S. aureus*	[157]
	IsdA	*S. aureus*	[137]
Undetermined receptor on desquamated epithelial cell	Aap	*S. epidermidis*	[32]
Undetermined receptor on desquamated nasal cells	ClfB	*S. aureus*	[158]
	IsdA	*S. aureus*	[158]
	SdrC	*S. aureus*	[158]
	SdrD	*S. aureus*	[158]
	SasG	*S. aureus*	[31,159]

Initial studies of staphylococcal adhesion focused on binding to the extracellular matrix and plasma proteins. More recently, studies have investigated adhesins for human keratinocytes and their ligands. Many receptor interactions are scarcely characterized, including loricrin, desmoglein-1, desmocollin-1 and keratinocyte lipids. Loricrin comprises up to 80% of protein on terminally differentiated keratinocyte cell surfaces, desmoglein-1 and desmocollin-1 are involved in intercellular keratinocyte adhesion, and keratinocyte lipids form an envelope around the terminally differentiated keratinocyte cell surfaces. Alternative acronyms are shown in the adhesin column.

Table 1. Staphylococcal adhesins with known receptors for skin (cont.).

Host receptor	Adhesin	Species	Ref.
Undetermined receptor on keratinocyte cell line	Coagulase	*S. aureus*	[160]
	ClfA	*S. aureus*	[160]
	FnbpA/FnbA	*S. aureus*	[160]
	FnbpB/FnbB	*S. aureus*	[160]
	Spa	*S. aureus*	[160]

Initial studies of staphylococcal adhesion focused on binding to the extracellular matrix and plasma proteins. More recently, studies have investigated adhesins for human keratinocytes and their ligands. Many receptor interactions are scarcely characterized, including loricrin, desmoglein-1, desmocollin-1 and keratinocyte lipids. Loricrin comprises up to 80% of protein on terminally differentiated keratinocyte cell surfaces, desmoglein-1 and desmocollin-1 are involved in intercellular keratinocyte adhesion, and keratinocyte lipids form an envelope around the terminally differentiated keratinocyte cell surfaces. Alternative acronyms are shown in the adhesin column.

Intraspecies variation between adhesins explains staphylococcal host and target cell range. In a study of 28 *S. epidermidis* isolates of human origin, there was no significant adhesion to bovine udder stratum corneum, whereas the majority (26/28) adhered to human stratum corneum [33]. Staphylococci of canine, feline or bovine origin were shown to adhere to a similar extent to human and canine skin, but differed significantly in adhesion to feline skin [35]. Skin diseases affect colonization. Most notably, *S. aureus* and *S. intermedius* exhibit increased binding to atopic human and canine skin, respectively [36,37]. In humans, enhanced binding was primarily due to the enhanced levels of fibronectin present in atopic skin, compared with healthy or psoriatic skin, and was mediated by the *S. aureus* MSCRAMMs FnbpA, FnbpB, ClfA and ClfB [37].

AMPs protect the mammalian epidermis

In concert with gaining a foothold on the epidermis, staphylococci are challenged to maintain colonization in the face of antimicrobial defense mechanisms. The cationic and anionic AMPs, together with lysozyme and RNases (see section below) form part of the host's first line of defense, the innate immune system.

Epithelial tissues produce the two major classes of cationic AMPs (cAMPs), the defensins and cathelicidins. The largest reservoir of which is in the terminal layers of skin, potentially owing to the calcium gradient in human epidermis allowing the function of kallikrein, a cathelicidin processing enzyme in differentiated keratinocytes [38]. Human β-defensins (HBDs) 1 and 2 were found localized to sweat glands through immunohistochemistry [39,40]; however, there is uncertainty over their activity and relevance in this environment [41]. Cathelicidins are secreted from sweat glands, where they are active at very low concentrations despite low pH and high salt conditions [40]. Defensins and cathelicidins are amphipathic peptides that bind to the negatively charged cell membrane of staphylococci owing to electrostatic interactions. Subsequent aggregation of the peptides results in the formation of channels affecting membrane permeability [42]. Activity of the anionic dermcidin is independent of both staphylococcal membrane charge and peptide charge; dermcidin inhibits synthesis of RNA and protein [41,43]. The mechanism was proposed to depend on ion channel formation in the bacterial membrane stabilized by zinc ions [44,45].

Lysozyme is present in many human fluids and secretions, and is produced by epithelial cells [46]. Many staphylococcal species are resistant to its *N*-acetylmuramoylhydrolase activity, including *S. aureus*, *S. epidermidis*, *S. xylosus* and *S. lugdunensis*. This resistance may be important for colonization of the skin [47]. Mutagenesis studies determined that the enzyme OatA, which *O*-acetylates peptidoglycan, is the major factor contributing to lysozyme resistance [47]. Moreover, *oatA* and *dltA* double mutants are highly sensitive to lysozyme and other cAMPs owing to the additional lack of D-alanine bound to teichoic acids [48].

The RNase A superfamily

Humans synthesize 13 RNaseA superfamily proteins and many exhibit antimicrobial activity, among other functions (reviewed by Gupta *et al.* [49]); however, only RNase 5 and 7 are expressed in keratinocytes [50,51].

RNase 5

RNase 5 has a well-described role in angiogenesis; however, Hooper *et al.* attributed its primary role as being antimicrobial defense of the epidermis since its gene expression does not correlate temporally with angiogenesis [52]. High mutation rates of RNase 5 are consistent with those of

other antimicrobial factors under selective pressure [53]. Hooper *et al.* observed that RNase 5 significantly reduced viability of *Candida albicans* and *Streptococcus pneumoniae* [52]. Although this potency was not easily reproduced by others [54], narrow spectrum bactericidal activity was confirmed [55], as was the capacity of the human epidermis to produce RNase 5 [56]. RNase 5 functions under similar physiological conditions, such as pH and salt concentration, as other AMPs [52]. Overall, the extent to which RNase 5 contributes to the epidermal barrier remains unclear.

RNase 7

Compared with RNase 5, the antimicrobial activity of RNase 7 is considerably broader. RNase 7 is expressed and active in epithelial tissues, including the human epidermis where it is active at low concentrations across a wide temperature range. High levels of RNase 7 in healthy skin were associated with protection against infection by *S. aureus* [57]. Further evidence for the physiological relevance of RNase7 comes from Simanski *et al.* who demonstrated that antibody blocking of RNase 7 activity in skin explants impairs killing activity against *S. aureus* [50]. RNase 7 is cationic with an abundance of lysine residues, and exhibits ribonuclease activity [58].

Concentration-dependent growth inhibition of *S. aureus* was observed in cells transformed with an RNase 7-expressing plasmid, with near-complete killing at micromolar enzyme concentrations [58]. While its antimicrobial mechanism of action is uncertain, Huang *et al.* credit the cationic properties of RNase 7 with its ability to pervade the bacterial membrane causing permeability [59]. Mutation and NMR analysis of the enzyme identified a flexible cluster of lysine residues integral to ribonuclease, but not antimicrobial activity [59].

Sensing & repelling cationic AMPs

Although the human epidermis secretes an arsenal of AMPs to prevent infection, the colonizing and pathogenic bacteria have evolved countermeasures (**Figure 2**). The ApsSR sensing system of *S. epidermidis* is specific to cAMPs. The presence of a negatively charged extracellular loop domain in the ApsS sensor is required for selectivity and gene deletion mutants have reduced survival to hBD3 [60]. The cAMP response induces resistance in *S. epidermidis* through regulation of the *dlt* and *mprF* operons [60,61]. The homologous APS system of *S. aureus*, GraRS, has a substitution of a serine for a proline residue in the extracellular loop, leading to altered expression of the *dlt* and *mprF* operons [62]. This potentially accounts for the different responses to AMPs by *S. aureus* and *S. epidermidis* [62]. Sensing and resistance of cAMPs through the GraRS system in *S. aureus* requires the contribution of GraX and the VraFG ABC transporter [48,63–65].

MprF modulates cell surface charge by the addition of lysine to the membrane lipid via lysyl-phosphatidylglycerol synthetase activity [66,67]. An *mprF* mutant of *S. aureus* has an increased susceptibility to killing by cAMPs [68], owing to perturbation of the cell surface charge, which reduces its ability to repel the cAMPs [69]. Similarly, the *dlt* operon protects *S. aureus* from AMPs by modifying cell surface charge via covalent attachment of D-alanine to wall teichoic acids [70]. Mutations in *dltA* increase susceptibility to defensins and cathelicidins owing to the greater negative charge of the cell surface [71].

Secreted proteases

The induced expression of secreted proteases forms a key defense from AMPs in staphylococci. SepA metalloprotease increases *S. epidermidis* persistence by degradation of dermcidin [72]. *S. epidermidis* also upregulates *agr*, *sarA* and *saeRS* in response to the peptide dermcidin [43], which corroborates findings that *sepA* is under the control of these global regulatory systems [72]. *S. aureus* metalloprotease aureolysin disables the human cathelicidin LL-37, which aids skin persistence and contributes to disease by limiting phagocytosis due to complement inhibition and increasing intracellular persistence [73–75].

S. aureus is the etiological agent of protease-linked disease of the epidermis, such as staphylococcal scalded-skin syndrome. Epidermolytic toxins (ET) are a group of serine proteases that degrade the epidermal cadherin desmoglein-1 in the stratum granulosum [76]; desmoglein-3 compensates for breakdown of desmoglein-1 in other skin layers. Another serine protease, glutamylendopeptidase SspA (V8), shares both cleavage site and sequence similarity with ET and hydrolyzes desmoglein-1 [77]. The application of SspA leads to a reduction in corneocyte abundance on the skin in a mouse model [78] resulting in speculation that ET and V8 proteases undermine the barrier function of the skin, allowing invasion [79]. In this way, they might act in concert with β-hemolysin promoting

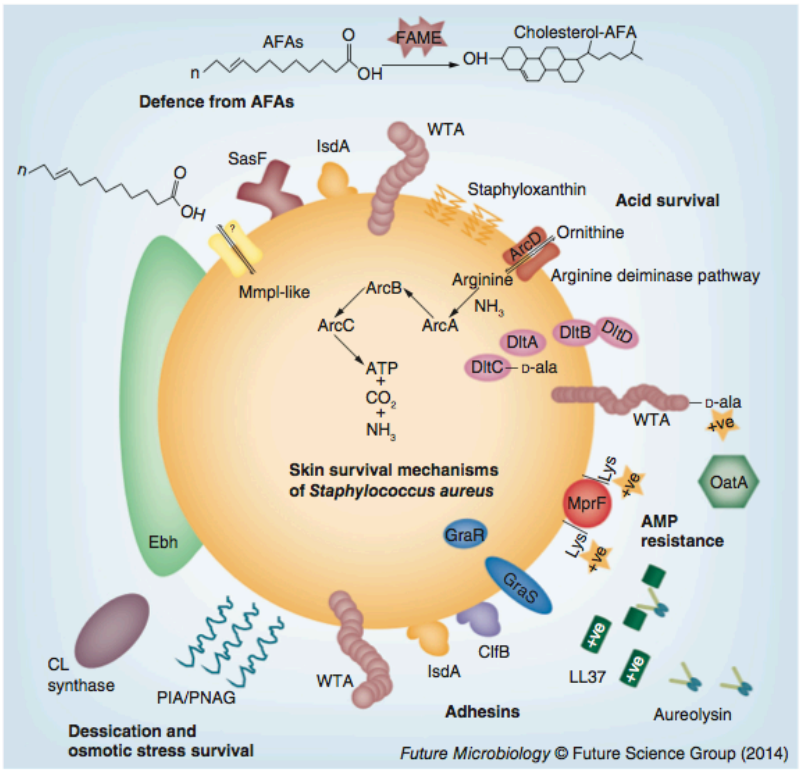


Figure 2. Proposed skin survival mechanisms of *Staphylococcus aureus*. Selected factors of *Staphylococcus aureus* that are known or proposed to contribute to human skin survival.
+ve: Positive; AFA: Antimicrobial fatty acid; AMP: Antimicrobial peptide; CL: Cardiolipin; FAME: Fatty acid-modifying enzyme; PIA: Polysaccharide intercellular adhesion; PNAG: Poly *N*-acetyl glucosamine; WTA: Wall teichoic acid.

colonization, and this exoprotein is cytotoxic to primary keratinocytes [80].

Cysteine proteases also impact upon staphylococcal skin persistence. Staphopain A and B in *S. aureus* and Ecp in *S. epidermidis* catalyze the breakdown of both elastin and collagen, which is an abundant polymer on the skin [81,82].

Competitive exclusion

Several studies have identified correlated species distributions, including the absence of staphylococci from skin or nasal environments colonized by *Propionibacterium acnes* and *Corynebacterium* spp. [5,10,83]. Intrageneric colonization distribution correlations were also shown in several studies for *S. epidermidis* and *S. aureus*, which were infrequently co-isolated from the nasal epithelium [10], although this was not consistent for all studies [84] and might indicate that trait variation is important. These reports support likely exclusion mechanisms between colonizing microbiota and, for *Corynebacterium* spp., a proposed mechanism for *S. aureus* exclusion was competition for nutrients or adhesin receptors [83]. No mechanism has yet been proposed for *P. acnes* and *S. aureus* competitive exclusion; although the ability of *P. acnes* to exacerbate *S. aureus* infection [85] may indicate an immunological contribution.

The exclusion mechanisms between *S. epidermidis* and *S. aureus* have been investigated and revealed that secreted proteins of *S. epidermidis*, such as the serine protease Esp, stimulates keratinocyte production of hBD2 and hBD3

antimicrobials active against *S. aureus*, but not *S. epidermidis* [84,86]. Esp-secreting *S. epidermidis* could clear *S. aureus* colonization when applied intranasally. Esp disrupts *S. aureus* biofilms and acts synergistically with hBD2 to decrease viability of *S. aureus* [84,87].

Additional exoproteins of staphylococci with antimicrobial activity targeting *S. aureus* and other resident and transient skin microbiota include the *S. epidermidis* phenol-soluble modulins (PSMs)-δ and -γ (δ-hemolysin), which are active against Group A *Streptococcus*, *S. aureus* and *Escherichia coli* [88,89]. Antimicrobial activity of PSM-δ and PSM-γ was synergistic with each other, with LL-37 and, in the case of PSM-γ, with CRAMP, hBD2 and hBD3 [88,89]. PSM-γ is abundant on healthy human skin and reduced bacterial load in a mouse Group A *Streptococcus* skin wound infection model, indicating physiological relevance [88]. *S. aureus* PSMs possess limited antimicrobial activity; instead, their role is linked to biofilm structuring, hemolysis, spreading over wet surfaces and possibly promoting skin colonization [90,91]. It remains to be determined whether other staphylococcal PSMs resemble *S. epidermidis* or *S. aureus* in their potential to promote colonization through antagonism.

Antimicrobial fatty acids protect human epidermis

In addition to AMPs, antimicrobial fatty acids (AFAs) protect skin against invasion by pathogenic bacteria. AFAs, together with lactic acid and amino acids in sweat, contribute to establishing and maintaining the acid mantle, by producing the acid pH that is a signature of the surface of the human epidermis. The acid mantle is important for the maintenance of the cohesion and integrity of the stratum corneum and contributes to the permeability homeostasis of the outer layers of the skin [92]. Enzymes required for the production of ceramides, the lipids integral to barrier homeostasis, function optimally at acidic pH [93]; whereas serine proteases, capable of degrading corneodesmosomes and compromising the integrity of the epidermis, are inhibited [94].

Structural organization studies recently demonstrated a highly regular 1:1:1 arrangement of fatty acid, sphingosine and cholesterol into multilamellar structures [23]. AFAs, including sapienic acid (16:1Δ9) and linoleic acid (18:2Δ9, Δ12), are abundant on healthy mammalian skin. An apparent inverse correlation between levels of staphylococcal colonization and AFAs [6,95] supports an AFA contribution to colonization inhibition. Atopic dermatitis sufferers have lower AFA levels than normal on their skin, and while *S. aureus* is isolated from <5% of individuals with healthy skin, it is routinely isolated from atopic dermatitis patients [96–98]. This indicates that AFAs are potentially important contributing factors in the differential survival of *S. epidermidis* and *S. aureus* on human skin.

The range and proposed mechanisms of AFA activity were recently reviewed elsewhere [99]. AFAs have a broad range of activity owing to their lipophilic properties, interacting with most organisms bearing lipid bilayer structures. Most theorized antimicrobial mechanisms of AFAs derive from their ability to insert into membranes, whereby unsaturated long chain fatty acids cause increased membrane disorder (fluidity) due to their shape reducing packing density. In *S. aureus*, inhibitory concentrations of linoleic acid (18:2Δ9, Δ12) cause protein leakage and interfere with metabolic pathways, such as the electron transport chain and nutrient uptake [100,101]. Recent studies have confirmed this observation and demonstrated that, while oleic acid (18:1Δ9) was a substrate for phospholipid biosynthesis, the AFA sapienic acid (16:1Δ9) required elongation for its incorporation into cellular pathways [102]. Arsic *et al.* determined that in some *S. aureus* strains AFAs, in addition to other long chain unsaturated fatty acids tested, variably induced protease expression [103].

AFA defense

Wall teichoic acids and the MSCRAMM IsdA contribute to AFA resistance of *S. aureus* by reducing hydrophobicity to limit AFA interaction **(Figure 2)** [102,104,105]. The reduction of cell surface hydrophobicity is a response to AFAs [106]. Challenge with AFAs increased transcription of capsule and peptidoglycan genes [106], which might contribute to the reduced hydrophobicity in response to AFAs. In addition to IsdA, the homologous MSCRAMMs SasF and SssF contribute to protection from AFAs [106,107]. In *S. aureus*, *SasF* transcription was upregulated 30-fold following exposure to linoleic acid (18:2Δ9, Δ12) and a replacement mutant had reduced AFA survival [106]. SssF complemented a *S. aureus sssF* mutant and clinical isolates of *S. saprophyticus* bearing SssF were more resistant

to linoleic acid (18:2Δ9, Δ12) compared with those without SssF [107]. No discrete enzyme activity has yet been reported for these proteins.

S. aureus biosynthesis of the carotenoid staphyloxanthin aids survival from AFA-generated membrane disorder [108]. Staphyloxanthin also contributes to antioxidant defense and counteracts AMP-mediated membrane fluidity [109,110]. The inactivation of *crtM* decreases *S. aureus* survival from AFAs and transcriptomic studies revealed upregulated *crtM* transcription after linoleic acid (18:2Δ9, Δ12) exposure [106].

The exoprotein fatty acid-modifying enzyme (FAME) is produced by *S. aureus* and *S. epidermidis* and esterifies lipids with cholesterol or primary alcohols to reduce their cellular toxicity [111,112]. Of 51 *S. epidermidis* isolates tested, the majority (88.2%) expressed FAME activity, demonstrating that FAME is widespread but potentially inessential for skin survival [111]. FAME activity is inhibited by triglycerides and diglycerides, which may explain why most staphylococci isolated from skin have lipase activity [112]. Lipases hydrolyze triglycerides and diglycerides releasing free fatty acids as substrates for FAME. Lipase and FAME are expressed by other staphylococcal species, being found particularly frequently in strains of *S. aureus*, *S. saprophyticus*, *S. schleiferi* and *S. cohnii*, but less often in strains of *S. warneri*, *S. epidermidis*, *S. caprae*, *S. hominis*, *S. simulans* and *S. capitis*. Neither activity was present in assays of *S. lugdunensis* or *S. haemolyticus* [113]. Notably, FAME was found frequently in *S. simulans* strains in the absence of lipase activity [113], which might indicate community interactions.

FAME could represent just one of several staphylococcal enzymes to detoxify AFAs. Campbell *et al.* observed *S. aureus* activity for the saturation of linoleic acid (18:2Δ9, Δ12) into the less-toxic oleic and stearic acids; other strains converted linoleic acid (18:2Δ9, Δ12) to mysteric and steric acids [114]. Recent studies identified that the myosin cross-reactive antigen protein of *S. pyogenes* is a fatty acid hydratase and a myosin cross-reactive antigen homolog is encoded by *S. aureus* [115]. Additional factors reported to contribute to AFA survival include the arginine deiminase pathway, VraE permease, and an Mmpl-like transporter (SAR2632) **(Figure 2)** [106]; however, the contributing mechanisms increasing survival have not been investigated.

Sphingosines

A major end product of epidermal cell differentiation is ceramide, an amide-linked fatty acid and sphingoid base that can be sphingosine, phytosphingosine, dihydrosphingosine and hydroxysphingosine to produce 16 isomers [116,117]. Ceramide constitutes up to 50% of stratum corneum lipids, differing in chain length and sphingoid base structure. It has important roles in skin barrier and mammalian cell physiology. On healthy skin, sphingosine is present at approximately 270 µM, whereas it is present at 140–160 µM on atopic skin [118]. *S. aureus* is rapidly killed by sphingosine and a similar mode of action to that of AFAs has been reported [102]. The effects of sphingosines on other staphylococci or staphylococcal resistance mechanisms to sphingosines are currently uncharacterized.

Arginine catabolic mobile element & skin survival

The arginine catabolic mobile element (ACME) encoding arginine deiminase activity is found in various species of staphylococci. Its evolutionary origins are speculated to have been in the CoNS, possibly *S. epidermidis* [119]. The gene clusters best characterized on the ACME are the oligopeptide permease operon (*opp*) and the *arc* operon, recognized as bacterial virulence genes [119]. The enzymes encoded by the *arc* operon form a metabolic pathway, whereby L-arginine is transported into the cell (ArcD) and is converted by ArcA to NH_3 and citrulline, which is then converted by ArcB to ornithine (exported by ArcD) and carbamoyl phosphate, and is finally converted by ArcC to CO_2 and NH_3, with the production of ATP. The presence of ACME in *S. epidermidis* did not alter its pathogenicity or host inflammatory responses [120]. The appearance of ACME within the hypervirulent USA300 lineage of community-acquired methicillin-resistant *S. aureus* [119] duplicates activity. However, the arginine deiminase operon of ACME is constitutively transcribed with markedly increased transcript abundance, compared with the anaerobiosis-induced, core genome operon [121]. Diep *et al.* first proposed that ACME carriage and its expressed arginine metabolism might allow commensal bacteria, such as *S. epidermidis* and *S. haemolyticus*, to survive more easily in an acidic environment [119]. The ACME element promotes acid

tolerance in USA300 and when plasmid-borne in strain Newman [121]. Arginine deiminase activity is proposed to counteract acidity from lactic acid in sweat [121] and may similarly contribute to AFA defense, as attributed to the core genome-encoded *arc* operon [106]. ACME-driven arginine metabolism results in elevated host polyamines, which are integral to host healing and clearance of *S. aureus*. As a result, the presence of *speG* is required to offset the increased polyamine levels caused by the constitutive arginine metabolism of ACME [121]. Increased survival of USA300 from skin antimicrobial barriers identifies both a skin survival mechanism and the potential for increased transmission. Biofilm formation of ACME-positive *S. epidermidis* isolates is reduced compared with ACME-negative isolates [120]. Similarly, *S. epidermidis* commensal isolates have a lower carriage rate of the *ica* locus (involved in biofilm formation) than disease isolates [122]. Increased transmission might explain why the ability to form a biofilm is sacrificed in favor of skin survival.

Box 1. Models of staphylococcal skin colonization.

- Many subcutaneous disease models have been described for staphylococci. In addition, there are various, recently described colonization models for normal and diseased skin, including:

Cutaneous colonization by tape-stripping of shaved murine skin

- Model uses a stretch plaster for mild or strong tape-stripping of shaved skin to generate defects in the epithelial barrier. The intact epidermis was inoculated with 10^7 *Staphylococcus aureus* [161]. The model was used to assess bioburden and measure the inflammatory response

Pruritic disease model of murine skin

- Model generates pruritic dermatitis following the injection of capsaicin that has similarities to atopic dermatitis. Model used for study of inflammatory mediators and filaggrin immunohistochemistry [162]

Allergic immune response model of murine skin

- Established the use of human-SCID mice for the study of inflammation responses as a model of atopic dermatitis [163]

Human volar forearm colonization–survival model

- Model used for determining comparative survival of an isogenic, unmarked *isdA* mutant of *S. aureus* by viable counting of bacteria after inoculation [105]

Murine ear colonization model

- Through the application of *S. aureus* to the ear of BALB/c-nu/nu mice, the role of β-hemolysin in skin colonization was determined [80]

Murine nasal colonization model

- This model established temporal studies of *S. aureus* colonization and was used to differentiate survival of isogenic mutants [164]. Use of loricrin-deficient mice extended the model to demonstrate the role of ClfB binding to squamous epithelial cells [157]

SCID: Severe combined immunodeficiency.

Life on the human epidermis leaves bacteria at risk of desiccation

Bacterial survival and growth on skin is limited by low relative humidity (RH) and high salt conditions produce osmotic stress. Both *S. aureus* and *S. epidermidis* can grow at low RH levels. Whilst *Bacillus subtilis*, *E. coli* and *Pseudomonas fluorescens* require 92–94.5% RH to grow, *S. aureus* can grow at 87% RH and *S. epidermidis* requires only 81–84% RH [123]. The extent to which this differential growth capability provides *S. epidermidis* with a competitive advantage is not known.

Desiccation, osmotic & matric stress resistance mechanisms

Desiccation tolerance is important for staphylococcal transmission and may affect survival on the skin if the RH falls below the threshold for growth. When *S. aureus* or *S. epidermidis* are grown at RH levels approaching their growth limit they become larger, form thicker cell walls and form cuboidal packs of eight cells rather than their typical grape-like clusters [123,124]. Similar observations were made for staphylococcal autolysin mutants, revealing that this morphological switch is likely to be autolysin controlled [124]. These morphology changes could limit water loss and osmotic stress by reducing surface area:volume ratio to maintain turgor pressure. Staphylococci grown at low RH are more hydrophilic, which aids water acquisition [124]. Desiccation tolerance, or its recovery from being subjected to this stress, requires antioxidant activity (catalase, alkylhydroperoxide reductase), regulatory activity via accessory σ factor B and the ClpX protease [125].

The ability to produce the polysaccharide intercellular adhesin (PIA) in staphylococci might contribute to desiccation tolerance. de Goffau *et al.* identified high tolerance to low RH in the PIA-producer RP62a [123]. PIA might act similarly to *Pseudomonas aeruginosa* exopolysaccharide (EPS) to limit desiccation [126]. EPS slows the rate of water loss to increase the time for bacteria to adapt; EPS also slows rehydration to aid adaptation [126].

Ebh is a giant (~1.1 MDa) cell wall-associated protein found in *S. aureus* and *S. epidermidis*. Ebh mutants exposed to high salt conditions reveal invaginated vacuoles along their septum within the first 30 min, suggesting that this contributes to initial osmotic stress

resistance via a role in cell wall and membrane architecture [127].

Membrane zwitterionic phospholipids decrease in conditions of increasing salinity in both Gram-positive and -negative bacteria. Cardiolipin (CL) contributes to osmotic stress resistance and CL synthase of *E. coli* is induced in response to osmotic stress and catalyzes the conversion of peptidoglycan into CL and glycerol. *S. aureus* encodes two CL synthase genes, and mutation of both genes reduced long-term survival in high salinity media [128]; however, levels of CL did not significantly increase in response to 2.7 M NaCl. In its annular phase, CL is theorized to regulate the mechanosensitive channel MscL and the osmosensory transporter ProP [129]. CL also decreases membrane fluidity in the bulk lipid phase [128].

Conclusion & future perspective

The increasing number of CoNS infections and the emergence of more virulent *S. aureus* strains highlight a need to understand how staphylococci exploit the human epidermis as a niche. Understandably, previous studies have used *in vitro* models and focused on mechanistic aspects. In the future, a more holistic approach is required in order to interrogate interactions between the staphylococci and the skin. The combined use of systems biology with emerging models of colonization (**Box 1**) is required to understand the complex interactions between

EXECUTIVE SUMMARY

Staphylococci are ubiquitous colonizers of human skin

- The staphylococci are an important cohort of the skin microbiome and many species are commensals that add to barrier defense. Several species are clinically important and cause a variety of diseases.

Role of adhesins in persistence and colonization

- Extensive variation in breadth and specificity of surface adhesins encoded by different staphylococcal species may explain host and tissue tropism.
- *Staphylococcus epidermidis* exhibits different patterns of colonization to skin epithelial layers, which may contribute to long-term colonization.

Sensing & repelling antimicrobial peptides

- ApsRS/GraRS antimicrobial peptide-sensing systems in *S. epidermidis* and *Staphylococcus aureus*, respectively, modulate cell surface charge through increased transcription of the *dlt* and *mprF* operons.

Protection against antimicrobial fatty acids

- The microbial surface components recognizing adhesive matrix molecules IsdA and SasF increase survival of *S. aureus* from antimicrobial fatty acids (AFAs) through different mechanisms.
- Multiple additional components contribute to defense from AFAs, including staphyloxanthin, wall teichoic acid, arginine deiminase activity and fatty acid-modifying enzyme.
- IsdA and wall teichoic acid alter surface hydrophobicity for AFAs defense.
- Esterification of AFAs to reduce toxicity is mediated by fatty acid-modifying enzyme and this activity is widespread, but not universal, in *S. epidermidis*, and is present in other staphylococci tested.

Arginine catabolic mobile element (ACME) may contribute to prolonged skin survival

- Arginine catabolic mobile element is thought to have its evolutionary origins in coagulase-negative staphylococci.
- Constitutive expression of the arginine deiminase pathway present on arginine catabolic mobile element in *S. aureus* promotes survival from acidic pH.

Resistance to desiccation & osmotic stress

- Morphological changes, which limit osmotic stress, are proposed to increase desiccation tolerance. The exopolysaccharide, polysaccharide intercellular adhesin, is postulated to aid survival through surface area reduction and regulating water levels.
- The gigantic Ebh protein contributes to early osmotic stress resistance in *S. aureus*.

multiple bacterial components and skin. An explosion of genome and metagenome data from studies of skin microbiota, from different body sites and individuals, will soon provide new insights into the evolution of skin survival. From a genomics perspective, the pan-genome analysis of the CoNS will reveal the generic and species-specific genes and the potential supply routes for enhanced versatility via interspecies horizontal gene transfer. The menacing emergence of the ACME mobile element in community-acquired methicillin-resistant *S. aureus* revealed transferable resources for enhanced versatility within the genus that impact upon *S. aureus* virulence. The surge in staphylococcal genome sequencing will enable single nucleotide resolution of the genetic basis for fitness and adaptation. The additional context of metagenomic data combined with studies of phenotypic variation will further add to our understanding of the staphylococci colonizing skin. Just as the secreted immune evasion proteins of *S. aureus* have provided useful tools to dissect innate immunity, the staphylococci are likely to provide resources to better understand the biology of skin and its antimicrobial barrier functions.

Acknowledgements

The late David Taylor is remembered for his enthusiastic discussions on the subject of this review.

Financial & competing interests disclosure

R Coates and J Moran are supported by BBSRC-Unilever CASE studentships BB/I532161/1 and BB/J500768/1 to MJ Horsburgh. The funders had no role in data collection and analysis, decision to publish or preparation of the manuscript. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

Papers of special note have been highlighted as:

- ∘ of interest
- ∘∘ of considerable interest

1 Cogen A, Nizet V, Gallo R. Skin microbiota: a source of disease or defence? *Br. J. Dermatol.* 158(3), 442–455 (2008).

2 Zeeuwen P, Boekhorst J, van den Bogaard E *et al.* Microbiome dynamics of human epidermis following skin barrier disruption. *Genome Biol.* 13(11), R101 (2012).

∘ **Uses a metagenomics approach to explore recolonization following wounding of the stratum corneum and the role of the microbiota at deeper skin layers.**

3 Grice E, Kong H, Conlan S *et al.* Topographical and temporal diversity of the human skin microbiome. *Science* 324(5931), 1190–1192 (2009).

∘∘ **Investigates microbial diversity at different body sites using 16S ribosomal RNA gene sequences.**

4 Gao Z, Tseng C, Pei Z, Blaser M. Molecular analysis of human forearm superficial skin bacterial biota. *Proc. Natl Acad. Sci. USA* 104(8), 2927–2932 (2007).

5 Taylor D, Daulby A, Grimshaw S, James G, Mercer J, Vaziri S. Characterization of the microflora of the human axilla. *Int. J. Cosmetic Sci.* 25(3), 137–145 (2003).

6 Costello E, Lauber C, Hamady M, Fierer N, Gordon J, Knight R. Bacterial community variation in human body habitats across space and time. *Science* 326(5960), 1694–1697 (2009).

∘∘ **Human microbiome population data identifies temporal and spatial variation using next-generation technology.**

7 Capone K, Dowd S, Stamatas G, Nikolovski J. Diversity of the human skin microbiome early in life. *J. Invest. Dermatol.* 131(10), 2026–2032 (2011).

8 Somerville D. Normal flora of skin in different age groups. *Br. J. Dermatol.* 81(4), 248–258 (1969).

9 Oh J, Conlan S, Polley E, Segre J, Kong H. Shifts in human skin and nares microbiota of healthy children and adults. *Genome Med.* 4(10), 77 (2012).

10 Frank D, Feazel L, Bessesen M, Price C, Janoff E, Pace N. The human nasal microbiota and *Staphylococcus aureus* carriage. *PLoS ONE* 5(5), e10598 (2010).

11 Kluytmans J, Wertheim H. Nasal carriage of *Staphylococcus aureus* and prevention of nosocomial infections. *Infection* 33(1), 3–8 (2005).

12 Moss B, Squire J. Nose and skin carriage of *Staphylococcus aureus* in patients receiving penicillin. *Lancet* 1(6496), 320–325 (1948).

13 Cherifi S, Byl B, Deplano A, Nonhoff C, Denis O, Hallin M. Comparative epidemiology of *Staphylococcus epidermidis* isolates from patients with catheter-related bacteremia and from healthy volunteers. *J. Clin. Microbiol.* 51(5), 1541–1547 (2013).

14 Cho J, Xuan C, Miller L. Lucky number seven: RNase 7 can prevent *Staphylococcus aureus* skin colonization. *J. Invest. Dermatol.* 130(12), 2703–2706 (2010).

15 von Eiff C, Becker K, Machka K, Stammer H, Peters G, Study G. Nasal carriage as a source of *Staphylococcus aureus* bacteremia. *N. Engl. J. Med.* 344(1), 11–16 (2001).

16 Segre J. Epidermal differentiation complex yields a secret: mutations in the cornification protein filaggrin underlie ichthyosis vulgaris. *J. Invest. Dermatol.* 126(6), 1202–1204 (2006).

17 Blanpain C, Fuchs E. Epidermal homeostasis: a balancing act of stem cells in the skin. *Nat. Rev. Mol. Cell Biol.* 10(3), 207–217 (2009).

18 Jonca N, Leclerc E, Caubet C, Simon M, Guerrin M, Serre G. Corneodesmosomes and corneodesmosin: from the stratum corneum cohesion to the pathophysiology of genodermatoses. *Eur. J. Dermatol.* 21, 35–42 (2011).

19 Rice R, Green H. Cornified envelope of terminally differentiated human epidermal keratinocytes consists of cross-linked protein. *Cell* 11(2), 417–422 (1977).

20 Schmitz G, Müller G. Structure and function of lamellar bodies, lipid-protein complexes involved in storage and secretion of cellular lipids. *J. Lipid Res.* 32(10), 1539–1570 (1991).

21 Candi E, Schmidt R, Melino G. The cornified envelope: a model of cell death in the skin. *Nat. Rev. Mol. Cell Biol.* 6(4), 328–340 (2005).

22 Watt F, Fujiwara H. Cell-extracellular matrix interactions in normal and diseased skin. *Cold Spring Harb. Perspect. Biol.* 3(4), doi:10.1101/cshperspect.a005124 (2011).

23 Iwai I, Han H, den Hollander L *et al.* The human skin barrier is organized as stacked bilayers of fully extended ceramides with cholesterol molecules associated with the ceramide sphingoid moiety. *J. Invest. Dermatol.* 132(9), 2215–2225 (2012).

- **Uses very high magnification cryoelectron microscopy to elucidate the arrangement of skin lipids into a stacked bilayer conformation.**

24 Grice E, Kong H, Renaud G *et al.* A diversity profile of the human skin microbiota. *Genome Res.* 18(7), 1043–1050 (2008).

25 Burian M, Rautenberg M, Kohler T *et al.* Temporal expression of adhesion factors and activity of global regulators during establishment of *Staphylococcus aureus* nasal colonization. *J. Infect. Dis.* 201(9), 1414–1421 (2010).

26 Burian M, Wolz C, Goerke C. Regulatory adaptation of *Staphylococcus aureus* during nasal colonization of humans. *PLoS ONE* 5(3), e10040 (2010).

27 Weidenmaier C, Kokai-Kun J, Kristian S *et al.* Role of teichoic acids in *Staphylococcus aureus* nasal colonization, a major risk factor in nosocomial infections. *Nat. Med.* 10(3), 243–245 (2004).

28 Weidenmaier C, Kokai-Kun J, Kulauzovic E *et al.* Differential roles of sortase-anchored surface proteins and wall teichoic acid in *Staphylococcus aureus* nasal colonization. *Int. J. Med. Microbiol.* 298(5–6), 505–513 (2008).

29 Heilmann C. Adhesion mechanisms of staphylococci. *Bacterial Adhesion: Chem. Biol. Phys.* 715, 105–123 (2011).

30 Ben Zakour N, Guinane C, Fitzgerald J. Pathogenomics of the staphylococci: insights into niche adaptation and the emergence of new virulent strains. *FEMS Microbiol. Lett.* 289(1), 1–12 (2008).

31 Roche F, Meehan M, Foster T. The *Staphylococcus aureus* surface protein SasG and its homologues promote bacterial adherence to human desquamated nasal epithelial cells. *Microbiology SGM* 149, 2759–2767 (2003).

32 Macintosh R, Brittan J, Bhattacharya R *et al.* The terminal A domain of the fibrillar accumulation-associated protein (Aap) of *Staphylococcus epidermidis* mediates adhesion to human corneocytes. *J. Bacteriol.* 191(22), 7007–7016 (2009).

33 Brooker B, Fuller R. The adhesion of coagulase negative staphylococci to human skin and its relevance to the bacterial flora of milk. *J. Appl. Bacteriol.* 57(2), 325–332 (1984).

34 Kassar S, Charfeddine C, Zribi H *et al.* Immunohistological study of involucrin expression in Darier's disease skin. *J. Cutan. Pathol.* 35(7), 635–640 (2008).

35 Woolley K, Kelly R, Fazakerley J, Williams N, Nuttall T, McEwan N. Reduced *in vitro* adherence of *Staphylococcus* species to feline corneocytes compared with canine and human corneocytes. *Vet. Dermatol.* 19(1), 1–6 (2008).

36 McEwan N, Mellor D, Kalna G. Adherence by *Staphylococcus intermedius* to canine corneocytes: a preliminary study comparing noninflamed and inflamed atopic canine skin. *Vet. Dermatol.* 17(2), 151–154 (2006).

37 Cho S, Strickland I, Boguniewicz M, Leung DY. Fibronectin and fibrinogen contribute to the enhanced binding of *Staphylococcus aureus* to atopic skin. *J. Allergy Clin. Immunol.* 108(2), 269–274 (2001).

38 Morizane S, Yamasaki K, Kabigting F, Gallo R. Kallikrein expression and cathelicidin processing are independently controlled in keratinocytes by calcium, vitamin D-3, and retinoic acid. *J. Invest. Dermatol.* 130(5), 1297–1306 (2010).

39 Ali R, Falconer A, Ikram M, Bissett C, Cerio R, Quinn A. Expression of the peptide antibiotics human beta defensin-1 and human beta defensin-2 in normal human skin. *J. Invest. Dermatol.* 117(1), 106–111 (2001).

40 Murakami M, Ohtake T, Dorschner R, Schittek B, Garbe C, Gallo R. Cathelicidin anti-microbial peptide expression in sweat, an innate defense system for the skin. *J. Invest. Dermatol.* 119(5), 1090–1095 (2002).

41 Rieg S, Steffen H, Seeber S *et al.* Deficiency of dermcidin-derived antimicrobial peptides in sweat of patients with atopic dermatitis correlates with an impaired innate defense of human skin *in vivo*. *J. Immunol.* 174, 8003–8010 (2005).

42 Ganz T. Defensins: antimicrobial peptides of innate immunity. *Nat. Rev. Immunol.* 3(9), 710–720 (2003).

43 Senyurek I, Paulmann M, Sinnberg T *et al.* Dermcidin-derived peptides show a different mode of action than the cathelicidin LL37 against *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 53(6), 2499–2509 (2009).

44 Paulmann M, Arnold T, Linke D *et al.* Structure–activity analysis of the dermcidin-derived peptide DCD1L, an anionic antimicrobial peptide present in human sweat. *J. Biol. Chem.* 287(11), 8434–8443 (2012).

45 Song C, Weichbrodt C, Salnikov E *et al.* Crystal structure and functional mechanism of a human antimicrobial membrane channel. *PNAS* 110(12), 4586–4591 (2013).

46 Chen V, France D, Martinelli G. *De nevo* synthesis of lysozyme by human epidermal cells. *J. Invest. Dermatol.* 87(5), 585–587 (1986).

47 Bera A, Herbert S, Jakob A, Vollmer W, Gotz F. Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan *O*-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*. *Mol. Microbiol.* 55(3), 778–787 (2005).

48 Herbert S, Bera A, Nerz C *et al.* Molecular basis of resistance to muramidase and cationic antimicrobial peptide activity of lysozyme in staphylococci. *PLoS Pathog.* 3(7), 981–994 (2007).

49 Gupta S, Haigh B, Griffin F, Wheeler T. The mammalian secreted RNases: mechanisms of action in host defence. *Innate Immunol.* 19(1), 86–97 (2013).

50 Simanski M, Dressel S, Glaeser R, Harder J. RNase 7 protects healthy skin from *Staphylococcus aureus* colonization. *J. Invest. Dermatol.* 130(12), 2836–2838 (2010).

51 Harder J, Schroder J. RNase 7, a novel innate immune defense antimicrobial protein of healthy human skin. *J. Biol. Chem.* 277(48), 46779–46784 (2002).

52 Hooper L, Stappenbeck T, Hong C, Gordon J. Angiogenins: a new class of microbicidal proteins involved in innate immunity. *Nat. Immunol.* 4(3), 269–273 (2003).

53 Cho S, Beintema J, Zhang J. The ribonuclease A superfamily of mammals and birds: identifying new members and tracing evolutionary histories. *Genomics* 85(2), 208–220 (2005).

54 Avdeeva S, Chernukha M, Shaginyan I, Tarantul V, Naroditsky B. Human angiogenin lacks specific antimicrobial activity. *Curr. Microbiol.* 53(6), 477–478 (2006).

55 Abtin A, Eckhart L, Mildner M *et al.* Degradation by stratum corneum proteases prevents endogenous RNase inhibitor from blocking antimicrobial activities of RNase 5 and RNase 7. *J. Invest. Dermatol.* 129(9), 2193–2201 (2009).

56 Rendl M, Mayer C, Weninger W, Tschachler E. Topically applied lactic acid increases spontaneous secretion of vascular endothelial growth factor by human reconstructed epidermis. *Br. J. Dermatol.* 145(1), 3–9 (2001).

57 Zanger P, Holzer J, Schleucher R, Steffen H, Schittek B, Gabrysch S. Constitutive expression of the antimicrobial peptide RNase 7 is associated with *Staphylococcus aureus* infection of the skin. *J. Infect. Dis.* 200(12), 1907–1915 (2009).

• **Draws a link between high baseline expression of RNase 7 and protection from *Staphylococcus aureus* infection.**

58 Zhang J, Dyer K, Rosenberg H. Human RNase 7: a new cationic ribonuclease of the RNase A superfamily. *Nucleic Acids Res.* 31(2), 602–607 (2003).

59 Huang Y, Lin Y, Chang T *et al.* The flexible and clustered lysine residues of human ribonuclease 7 are critical for membrane permeability and antimicrobial activity. *J. Biol. Chem.* 282(7), 4626–4633 (2007).

60 Li M, Lai Y, Villaruz A, Cha D, Sturdevant D, Otto M. Gram-positive three-component antimicrobial peptide-sensing system. *Proc. Natl Acad. Sci. USA* 104(22), 9469–9474 (2007).

61 Matsuo M, Oogai Y, Kato F, Sugai M, Komatsuzawa H. Growth-phase dependence of susceptibility to antimicrobial peptides in *Staphylococcus aureus*. *Microbiology-SGM* 157, 1786–1797 (2011).

62 Li M, Cha D, Lai Y, Villaruz A, Sturdevant D, Otto M. The antimicrobial peptide-sensing system aps of *Staphylococcus aureus*. *Mol. Microbiol.* 66(5), 1136–1147 (2007).

63 Meehl M, Herbert S, Gotz F, Cheung A. Interaction of the GraRS two-component system with the VraFG ABC transporter to support vancomycin-intermediate resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 51(8), 2679–2689 (2007).

64 Falord M, Karimova G, Hiron A, Msadek T. GraXSR proteins interact with the VraFG ABC transporter to form a five-component system required for cationic antimicrobial peptide sensing and resistance in *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 56(2), 1047–1058 (2012).

65 Yang S, Bayer A, Mishra N *et al.* The *Staphylococcus aureus* two-component regulatory system, GraRS, senses and confers resistance to selected cationic antimicrobial peptides. *Infect. Immun.* 80(1), 74–81 (2012).

• **Investigates the regulation of GraRS and its role in sensing cationic antimicrobial peptides through activation of the *dlt* operon and *mprF*.**

66 Oku Y, Kurokawa K, Ichihashi N, Sekimizu K. Characterization of the *Staphylococcus aureus* mprF gene, involved in lysinylation of phosphatidylglycerol. *Microbiology-SGM* 150, 45–51 (2004).

67 Staubitz P, Neumann H, Schneider T, Wiedemann I, Peschel A. MprF-mediated biosynthesis of lysylphosphatidylglycerol, an important determinant in staphylococcal defensin resistance. *FEMS Microbiol. Lett.* 231(1), 67–71 (2004).

68 Peschel A, Jack R, Otto M *et al.* *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with L-lysine. *J. Exp. Med.* 193(9), 1067–1076 (2001).

69 Andrae J, Goldmann T, Ernst C, Peschel A, Gutsmann T. Multiple peptide resistance factor (MprF)-mediated resistance of *Staphylococcus aureus* against antimicrobial peptides coincides with a modulated peptide interaction with artificial membranes comprising lysyl-phosphatidylglycerol. *J. Biol. Chem.* 286(21), 18692–18700 (2011).

70 Perego M, Glaser P, Minutello A, Strauch M, Leopold K, Fischer W. Incorporation of D-alanine into lipoteichoic acid and wall teichoic acid in *Bacillus subtilis* – identification of genes and regulation. *J. Biol. Chem.* 270(26), 15598–15606 (1995).

71 Peschel A, Otto M, Jack R, Kalbacher H, Jung G, Gotz F. Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. *J. Biol. Chem.* 274(13), 8405–8410 (1999).

72 Lai Y, Villaruz A, Li M, Cha D, Sturdevant D, Otto M. The human anionic antimicrobial peptide dermcidin induces proteolytic defence mechanisms in staphylococci. *Mol. Microbiol.* 63(2), 497–506 (2007).

73 Kubica M, Guzik K, Koziel J *et al.* A potential new pathway for *Staphylococcus aureus* dissemination: the silent survival of *S. aureus* phagocytosed by human monocyte-derived macrophages. *PLoS ONE* 3(1), e1409 (2008).

74 Garzoni C, Kelley W. *Staphylococcus aureus*: new evidence for intracellular persistence. *Trends Microbiol.* 17(2), 59–65 (2009).

75 Laarman A, Ruyken M, Malone C, van Strijp J, Horswill A, Rooijakkers S. *Staphylococcus aureus* metalloprotease aureolysin cleaves complement C3 to mediate immune evasion. *J. Immunol.* 186(11), 6445–6453 (2011).

76 Kato F, Kadomoto N, Iwamoto Y, Bunai K, Komatsuzawa H, Sugai M. Regulatory mechanism for exfoliative toxin production in *Staphylococcus aureus*. *Infect. Immun.* 79(4), 1660–1670 (2011).

77 Nemoto T, Ohara-Nemoto Y, Ono T *et al.* Characterization of the glutamyl endopeptidase from *Staphylococcus aureus* expressed in *Escherichia coli*. *FEBS J.* 275(3), 573–587 (2008).

78 Hirasawa Y, Takai T, Nakamura T *et al.* *Staphylococcus aureus* extracellular protease causes epidermal barrier dysfunction. *J. Invest. Dermatol.* 130(2), 614–617 (2010).

•• **Identifies the role of *S. aureus* proteases in disrupting the protective surface of skin.**

79 Koziel J, Potempa J. Protease-armed bacteria in the skin. *Cell Tissue Res.* 351(2), 325–337 (2013).

• **Discusses the role of proteases in bacterial defense against antimicrobial peptides on the skin.**

80 Katayama Y, Baba T, Sekine M, Fukuda M, Hiramatsu K. Beta-hemolysin promotes skin colonization by *Staphylococcus aureus*. *J. Bacteriol.* 195(6), 1194–1203 (2013).

81 Ohbayashi T, Irie A, Murakami Y *et al.* Degradation of fibrinogen and collagen by staphopains, cysteine proteases released from *Staphylococcus aureus*. *Microbiology* 157, 786–792 (2011).

82 Oleksy A, Golonka E, Banbula A *et al.* Growth phase-dependent production of a cell wall-associated elastinolytic cysteine proteinase by *Staphylococcus epidermidis*. *Biol. Chem.* 385(6), 525–535 (2004).

83 Uehara Y, Nakama H, Agematsu K *et al.* Bacterial interference among nasal inhabitants: eradication of *Staphylococcus aureus* from nasal cavities by artificial implantation of *Corynebacterium* sp. *J. Hosp. Infect.* 44(2), 127–133 (2000).

84 Iwase T, Uehara Y, Shinji H *et al.* *Staphylococcus epidermidis* Esp inhibits *Staphylococcus aureus* biofilm formation and nasal colonization. *Nature* 465(7296), 346–349 (2010).

85 Lo C, Lai Y, Liu Y, Gallo R, Huang C. *Staphylococcus aureus* hijacks a skin commensal to intensify its virulence: immunization targeting beta-hemolysin and CAMP factor. *J. Invest. Dermatol.* 131(2), 401–409 (2011).

86 Lai Y, Cogen A, Radek K *et al.* Activation of TLR2 by a small molecule produced by *Staphylococcus epidermidis* increases antimicrobial defense against bacterial skin infections. *J. Invest. Dermatol.* 130(9), 2211–2221 (2010).

87 Park B, Iwase T, Liu G. Intranasal application of *S. epidermidis* prevents colonization by methicillin-resistant *Staphylococcus aureus* in mice. *PLoS ONE* 6(10), e25880 (2011).

88 Cogen A, Yamasaki K, Muto J *et al.* *Staphylococcus epidermidis* antimicrobial delta-toxin (phenol-soluble modulin-gamma) cooperates with host antimicrobial peptides to kill group A *Streptococcus*. *PLoS ONE* 5(1), e8557 (2010).

89 Cogen A, Yamasaki K, Sanchez K *et al.* Selective antimicrobial action is provided by phenol-soluble modulins derived from *Staphylococcus epidermidis*, a normal resident of the skin. *J. Invest. Dermatol.* 130(1), 192–200 (2010).

90 Periasamy S, Chatterjee S, Cheung G, Otto M. Phenol-soluble modulins in staphylococci: what are they originally for? *Commun. Integr. Biol.* 5(3), 275–277 (2012).

91 Tsompanidou E, Denham E, Becher D *et al.* Distinct roles of phenol-soluble modulins in spreading of *Staphylococcus aureus* on wet surfaces. *Appl. Environ. Microbiol.* 79, 886–895 (2013).

92 Hachem J, Crumrine D, Fluhr J, Brown B, Feingold K, Elias P. pH directly regulates epidermal permeability barrier homeostasis, and stratum corneum integrity/cohesion. *J. Invest. Dermatol.* 121(2), 345–353 (2003).

93 Feingold K. The role of epidermal lipids in cutaneous permeability barrier homeostasis. *J. Lipid Res.* 48(12), 2531–2546 (2007).

94 Hachem J, Man M, Crumrine D *et al.* Sustained serine proteases activity by prolonged increase in pH leads to degradation of lipid processing enzymes and profound alterations of barrier function and stratum corneum integrity. *J. Invest. Dermatol.* 125(3), 510–520 (2005).

95 Lampe M, Burlingame A, Whitney J *et al.* Human stratum corneum lipids – characterization and regional variations. *J. Lipid Res.* 24(2), 120–130 (1983).

96 Noble W, Valkenburg HA, Wolters C. Carriage of *Staphylococcus aureus* in random samples of a normal population. *J. Hyg. Cambridge* 65(4), 567–573 (1967).

97 Leyden J, Marples R, Kligman A. *Staphylococcus aureus* in lesions of atopic dermatitis. *Br. J. Dermatol.* 90(5), 525–530 (1974).

98 Breuer K, Haussler S, Kapp A, Werfel T. *Staphylococcus aureus*: colonizing features and influence of an antibacterial treatment in adults with atopic dermatitis. *Br. J. Dermatol.* 147(1), 55–61 (2002).

99 Desbois A, Smith V. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. *Appl. Microbiol. Biotechnol.* 85(6), 1629–1642 (2010).

• **Discusses the various modes of action of antimicrobial free fatty acids and focuses on their commercial and biomedical uses as an alternative to antibiotics.**

100 Galbrait H, Miller T. Effect of long-chain fatty acids on bacterial respiration and amino acid uptake. *J. Appl. Bacteriol.* 36(4), 659–675 (1973).

101 Greenway D, Dyke K. Mechanism of the inhibitory action of linoleic acid on the growth of *Staphylococcus aureus*. *J. Gen. Microbiol.* 115(1), 233–245 (1979).

102 Parsons J, Yao J, Frank M, Jackson P, Rock C. Membrane disruption by antimicrobial fatty acids releases low-molecular-weight proteins from *Staphylococcus aureus*. *J. Bacteriol.* 194(19), 5294–5304 (2012).

•• **Confirms that unsaturated fatty acids cause protein leakage from *S. aureus* and identifies the mode of action of antimicrobial fatty acids.**

103 Arsic B, Zhu Y, Heinrichs D, McGavin M. Induction of the staphylococcal proteolytic cascade by antimicrobial fatty acids in community acquired methicillin resistant *Staphylococcus aureus*. *PLoS ONE* 7(9), e45952 (2012).

104 Kohler T, Weidenmaier C, Peschel A. Wall teichoic acid protects *Staphylococcus aureus* against antimicrobial fatty acids from human skin. *J. Bacteriol.* 191(13), 4482–4484 (2009).

105 Clarke S, Mohamed R, Bian L *et al.* The *Staphylococcus aureus* surface protein IsdA mediates resistance to innate defenses of human skin. *Cell Host Microbe* 1(3), 199–212 (2007).

•• **Discovering that IsdA modulates surface hydrophobicity to increase skin survival from lipid matrix components.**

106 Kenny J, Ward D, Josefsson E *et al.* The *Staphylococcus aureus* response to unsaturated long-chain free fatty acids: survival mechanisms and virulence implications. *PLoS ONE* 4(2), e4344 (2009).

•• **Transcriptomic and proteomic approaches suggest that antimicrobial activity of unsaturated long chain fatty acids involves cell membrane disruption.**

107 King N, Sakinc T, Ben Zakour N *et al.* Characterisation of a cell wall-anchored protein of *Staphylococcus saprophyticus* associated with linoleic acid resistance. *BMC Microbiol.* 12, 8 (2012).

•• **Details the characterization of the microbial surface components recognizing adhesive matrix molecules SssF, a cell wall anchored protein of *Staphylococcus saprophyticus* that promotes linoleic acid survival.**

108 Chamberlain N, Mehrtens B, Xiong Z, Kapral F, Boardman J, Rearick J. Correlation of carotenoid production, decreased membrane fluidity and resistance to oleic acid killing in *Staphylococcus aureus* 18Z. *Infect. Immun.* 59(12), 4332–4337 (1991).

109 Clauditz A, Resch A, Wieland K, Peschel A, Goetz F. Staphyloxanthin plays a role in the fitness of *Staphylococcus aureus* and its ability to cope with oxidative stress. *Infect. Immun.* 74(8), 4950–4953 (2006).

110 Mishra N, Liu G, Yeaman M *et al.* Carotenoid-related alteration of cell membrane fluidity impacts *Staphylococcus aureus* susceptibility to host defense peptides. *Antimicrob. Agents Chemother.* 55(2), 526–531 (2011).

111 Chamberlain N, Brueggemann S. Characterisation and expression of fatty acid modifying enzyme produced by *Staphylococcus epidermidis*. *J. Med. Microbiol.* 46(8), 693–697 (1997).

112 Kapral F, Smith S, Lal D. The esterification of fatty acids by *Staphylococcus aureus* fatty acid modifying enzyme (FAME) and its inhibition by glycerides. *J. Med. Microbiol.* 37(4), 235–237 (1992).

113 Long J, Hart J, Albers W, Kapral F. The production of fatty acid modifying enzyme (FAME) and lipase by various staphylococcal species. *J. Med. Microbiol.* 37(4), 232–234 (1992).

114 Campbell I, Crozier D, Pawagi A, Buivids I. *In vitro* response of *Staphylococcus aureus* from cystic fibrosis patients to combinations of linoleic and oleic acids added to the nutrient medium. *J. Clin. Microbiol.* 18(2), 408–415 (1983).

115 Volkov A, Liavonchanka A, Kamneva O *et al.* Myosin cross-reactive antigen of *Streptococcus pyogenes* M49 encodes a fatty acid double bond hydratase that plays a role in oleic acid detoxification and bacterial virulence. *J. Biol. Chem.* 285(14), 10353–10361 (2010).

• **Reports enzyme activity for hydratase that contributes to virulence and fatty acid modification.**

116 Pruett S, Bushnev A, Hagedorn K *et al.* Thematic review series: sphingolipids - biodiversity of sphingoid bases ("sphingosines") and related amino alcohols. *J. Lipid Res.* 49(8), 1621–1639 (2008).

117 Shirakura Y, Kikuchi K, Matsumura K, Mukai K, Mitsutake S, Igarashi Y. 4,8-sphingadienine and 4-hydroxy-8-sphingenine activate ceramide production in the skin. *Lipids Health Dis.* 11, 108 (2012).

118 Arikawa J, Ishibashi M, Kawashima M, Takagi Y, Ichikawa Y, Imokawa G. Decreased levels of sphingosine, a natural antimicrobial agent, may be associated with vulnerability of the stratum corneum from patients with atopic dermatitis to colonization by *Staphylococcus aureus*. *J. Invest. Dermatol.* 119(2), 433–439 (2002).

119 Diep B, Gill S, Chang R *et al.* Complete genome sequence of USA300, an epidemic clone of community-acquired methicillin-resistant *Staphylococcus aureus*. *Lancet* 367(9512), 731–739 (2006).

120 Granslo H, Klingenberg C, Fredheim E, Ronnestad A, Mollnes T, Flaegstad T. Arginine catabolic mobile element is associated with low antibiotic resistance and low pathogenicity in *Staphylococcus epidermidis* from neonates. *Pediatr. Res.* 68(3), 237–241 (2010).

•• **Explores the implications of arginine catabolic mobile element (ACME) on the physiology of *Staphylococcus epidermidis*, concluding that ACME does not result in this organism being more harmful.**

121 Thurlow L, Joshi G, Clark J *et al.* Functional modularity of the arginine catabolic mobile element contributes to the success of USA300 methicillin-resistant *Staphylococcus aureus*. *Cell Host Microbe* 13(1), 100–107 (2013).

•• **Proposes that ACME-encoded Arc is involved in acid tolerance of USA300 on the skin and that SpeG is essential to offset the resulting increase in host polyamines.**

122 Koskela A, Nilsdotter-Augustinsson A, Persson L, Soderquist B. Prevalence of the *ica* operon and insertion sequence IS256 among *Staphylococcus epidermidis* prosthetic joint infection isolates. *Eur. J. Clin. Microbiol. Infect. Dis.* 28(6), 655–660 (2009).

123 de Goffau M, Yang X, van Dijl J, Harmsen H. Bacterial pleomorphism and competition in a relative humidity gradient. *Environ. Microbiol.* 11(4), 809–822 (2009).

•• **Reports the morphological changes of bacteria at humidity growth limits.**

124 de Goffau M, van Dijl J, Harmsen H. Microbial growth on the edge of desiccation. *Environ. Microbiol.* 13(8), 2328–2335 (2011).

• **Examines the water activity of various bacteria including the skin resident *S. epidermidis* and discusses morphological changes resulting from nearing the water activity growth limit.**

125 Chaibenjawong P, Foster S. Desiccation tolerance in *Staphylococcus aureus*. *Arch. Microbiol.* 193(2), 125–135 (2011).

126 Roberson E, Firestone M. Relationship between desiccation and exopolysaccharide production in a soil *pseudomonas* species. *Appl. Environ. Microbiol.* 58(4), 1284–1291 (1992).

127 Kuroda M, Tanaka Y, Aoki R, Shu D, Tsumoto K, Ohta T. *Staphylococcus aureus* giant protein Ebh is involved in tolerance to transient hyperosmotic pressure. *Biochem. Biophys. Res. Commun.* 374(2), 237–241 (2008).

128 Tsai M, Ohniwa R, Kato Y *et al.* *Staphylococcus aureus* requires cardiolipin for survival under conditions of high salinity. *BMC Microbiol.* 11(13), (2011).

• **Outlines the involvement of cardiolipin in the survival of *S. aureus* under long-term high-salt conditions.**

129 Romantsov T, Guan Z, Wood J. Cardiolipin and the osmotic stress responses of bacteria. *Biochim. Biophys. Acta Biomembranes* 1788(10), 2092–2100 (2009).

130 Snodgrass J, Mohamed N, Ross J, Sau S, Lee C, Smeltzer M. Functional analysis of the *Staphylococcus aureus* collagen adhesin B domain. *Infect. Immun.* 67(8), 3952–3959 (1999).

131 Hansen U, Hussain M, Villone D *et al.* The anchorless adhesin Eap (extracellular adherence protein) from *Staphylococcus aureus* selectively recognizes extracellular matrix aggregates but binds promiscuously to monomeric matrix macromolecules. *Matrix Biol.* 25(4), 252–260 (2006).

132 Hussain M, Becker K, von Eiff C, Schrenzel J, Peters G, Herrmann M. Identification and characterization of a novel 38.5 kilodalton cell surface protein of *Staphylococcus aureus* with extended spectrum binding activity for extracellular matrix and plasma proteins. *J. Bacteriol.* 183(23), 6778–6786 (2001).

133 Bowden M, Visai L, Longshaw C, Holland KT, Speziale P, Hook M. Is the GehD lipase from *Staphylococcus epidermidis* a collagen binding adhesin? *J. Biol. Chem.* 277(45), 43017–43023 (2002).

134 Arrecubieta C, Lee M, Macey A, Foster T, Lowy F. SdrF, a *Staphylococcus epidermidis* surface protein, binds type I collagen. *J. Biol. Chem.* 282(26), 18767–18776 (2007).

135 Sakinc T, Kleine B, Gatermann S. SdrI, a serine–aspartate repeat protein identified in *Staphylococcus saprophyticus* strain 7108, is a collagen-binding protein. *Infect. Immun.* 74(8), 4615–4623 (2006).

136 O'Brien L, Walsh E, Massey R, Peacock S, Foster T. *Staphylococcus aureus* clumping factor B (ClfB) promotes adherence to human type I cytokeratin 10: implications for nasal colonization. *Cell. Microbiol.* 4(11), 759–770 (2002).

137 Clarke S, Andre G, Walsh E, Dufrene Y, Foster T, Foster S. Iron-regulated surface determinant protein A mediates adhesion of *Staphylococcus aureus* to human corneocyte envelope proteins. *Infect. Immun.* 77(6), 2408–2416 (2009).

138 Heilmann C, Hartleib J, Hussain M, Peters G. The multifunctional *Staphylococcus aureus* autolysin Aaa mediates adherence to immobilized fibrinogen and fibronectin. *Infect. Immun.* 73(8), 4793–4802 (2005).

139 Hirschhausen N, Schlesier T, Schmidt M, Goetz F, Peters G, Heilmann C. A novel staphylococcal internalization mechanism involves the major autolysin Atl and heat shock cognate protein Hsc70 as host cell receptor. *Cell. Microbiol.* 12(12), 1746–1764 (2010).

140 McDevitt D, Francois P, Vaudaux P, Foster T. Molecular characterization of the clumping factor (fibrinogen receptor) of *Staphylococcus aureus*. *Mol. Microbiol.* 11(2), 237–248 (1994).

141 Ni Eidhin D, Perkins S, Francois P, Vaudaux P, Hook M, Foster T. Clumping factor B (ClfB), a new surface-located fibrinogen-binding adhesin of *Staphylococcus aureus*. *Mol. Microbiol.* 30(2), 245–257 (1998).

142 Hartford O, O'Brien L, Schofield K, Wells J, Foster T. The Fbe (SdrG) protein of *Staphylococcus epidermidis* HB promotes bacterial adherence to fibrinogen. *Microbiology* 147, 2545–2552 (2001).

143 Nilsson M, Bjerketorp J, Guss B, Frykberg L. A fibrinogen-binding protein of *Staphylococcus lugdunensis*. *FEMS Microbiol. Lett.* 241(1), 87–93 (2004).

144 Wann E, Gurusiddappa S, Hook M. The fibronectin-binding MSCRAMM FnbpA of *Staphylococcus aureus* is a bifunctional protein that also binds to fibrinogen. *J. Biol. Chem.* 275(18), 13863–13871 (2000).

145 Burke F, Di Poto A, Speziale P, Foster T. The A domain of fibronectin-binding protein B of *Staphylococcus aureus* contains a novel fibronectin binding site. *FEBS J.* 278(13), 2359–2371 (2011).

146 Clarke S, Wiltshire M, Foster S. IsdA of *Staphylococcus aureus* is a broad spectrum, iron-regulated adhesin. *Mol. Microbiol.* 51(5), 1509–1519 (2004).

147 King N, Beatson S, Totsika M *et al.* UafB is a serine-rich repeat adhesin of *Staphylococcus saprophyticus* that mediates binding to fibronectin, fibrinogen and human uroepithelial cells. *Microbiology* 157, 1161–1175 (2011).

148 Heilmann C, Thumm G, Chhatwal G, Hartleib J, Uekotter A, Peters G. Identification and characterization of a novel autolysin (Aae) with adhesive properties from *Staphylococcus epidermidis*. *Microbiology* 149, 2769–2778 (2003).

149 Hell W, Meyer H, Gatermann S. Cloning of aas, a gene encoding a *Staphylococcus saprophyticus* surface protein with adhesive and autolytic properties. *Mol. Microbiol.* 29(3), 871–881 (1998).

150 Allignet J, England P, Old I, El Solh N. Several regions of the repeat domain of the *Staphylococcus caprae* autolysin, At1C, are involved in fibronectin binding. *FEMS Microbiol. Lett.* 213(2), 193–197 (2002).

151 Williams R, Henderson B, Sharp L, Nair S. Identification of a fibronectin-binding protein from *Staphylococcus epidermidis*. *Infect. Immun.* 70(12), 6805–6810 (2002).

152 Nilsson M, Frykberg L, Flock J, Pei L, Lindberg M, Guss B. A fibrinogen-binding protein of *Staphylococcus epidermidis*. *Infect. Immun.* 66(6), 2666–2673 (1998).

153 Flock J, Froman G, Jonsson K *et al.* Cloning and expression of the gene for a fibronectin-binding protein from *Staphylococcus aureus*. *EMBO J.* 6(8), 2351–2357 (1987).

154 Jonsson K, Signas C, Muller H, Lindberg M. Two different genes encode fibronectin binding proteins in *Staphylococcus aureus* – the complete nucleotide sequence and characterization of the 2nd gene. *Eur. J. Biochem.* 202(3), 1041–1048 (1991).

155 Sakinc T, Kleine B, Michalski N, Kaase M, Gatermann S. Sdrl of *Staphylococcus saprophyticus* is a multifunctional protein: localization of the fibronectin-binding site. *FEMS Microbiol. Lett.* 301(1), 28–34 (2009).

156 Huesca M, Peralta R, Sauder D, Simor A, McGavin M. Adhesion and virulence properties of epidemic Canadian methicillin-resistant *Staphylococcus aureus* strain 1: Identification of novel adhesion functions associated with plasmin-sensitive surface protein. *J. Infect. Dis.* 185(9), 1285–1296 (2002).

157 Mulcahy M, Geoghegan J, Monk I *et al.* Nasal colonisation by *Staphylococcus aureus* depends upon clumping factor B binding to the squamous epithelial cell envelope protein loricrin. *PLoS Pathog.* 8(12), e1003092 (2012).

158 Corrigan R, Miajlovic H, Foster T. Surface proteins that promote adherence of *Staphylococcus aureus* to human desquamated nasal epithelial cells. *BMC Microbiol.* 9 (2009).

159 Corrigan R, Rigby D, Handley P, Foster T. The role of *Staphylococcus aureus* surface protein SasG in adherence and biofilm formation. *Microbiology* 153, 2435–2446 (2007).

160 Mempel M, Schmidt T, Weidinger S *et al.* Role of *Staphylococcus aureus* surface-associated proteins in the attachment to cultured HaCaT keratinocytes in a new adhesion assay. *J. Invest. Dermatol.* 111(3), 452–456 (1998).

161 Wanke I, Skabytska Y, Kraft B, Peschel A, Biedermann T, Schittek B. *Staphylococcus aureus* skin colonization is promoted by barrier disruption and leads to local inflammation. *Exp. Dermatol.* 22(2), 153–155 (2013).

- **Damage to the skin surface by tape-stripping reduces barrier defense, increasing *S. aureus* persistence and expression of proinflammatory cytokines.**

162 Back S, Jeong K, Li C, Lee J, Lee S, Na H. Chronically relapsing pruritic dermatitis in the rats treated as neonate with capsaicin; a potential rat model of human atopic dermatitis. *J. Dermatol. Sci.* 67(2), 111–119 (2012).

163 Herz U, Schnoy N, Borelli S *et al.* A human-SCID mouse model for allergic immune responses: bacterial superantigen enhances skin inflammation and suppresses IgE production. *J. Invest. Dermatol.* 110(3), 224–231 (1998).

164 Kiser K, Cantey-Kiser J, Lee J. Development and characterization of a *Staphylococcus aureus* nasal colonization model in mice. *Infect. Immun.* 67(10), 5001–5006 (1999).