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

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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.


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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 (Kluymans 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-hydroxy ceramides, 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-hydroxy ceramides 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 by 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.**

<table>
<thead>
<tr>
<th>Host receptor</th>
<th>Adhesins</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undetermined receptor on desquamated epithelial cell</td>
<td>Aap</td>
<td><em>S. epidermis</em></td>
<td>(Macintosh et al., 2009)</td>
</tr>
<tr>
<td>Undetermined receptor on desquamated nasal cells</td>
<td>SasG, ClfB, SdrC, SdrD, IsdA</td>
<td><em>S. aureus</em></td>
<td>(Corrigan et al., 2007; 2009)</td>
</tr>
<tr>
<td>Undetermined receptor on keratinocyte cell line</td>
<td>Spa, Coagulase, FnbpA/FnbA, FnbpB/FnbB, ClfA</td>
<td><em>S. aureus</em></td>
<td>(Mempel et al., 1998)</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>SdrF, GehD, Can, Eap/Map, Emp</td>
<td><em>S. epidermidis</em></td>
<td>(Arrecubieta et al., 2007; Bowden et al., 2002)</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>Sdrl</td>
<td><em>S. saprophyticus</em></td>
<td>(Sakinc et al., 2006)</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>Fbe/SdrG</td>
<td><em>S. epidermidis</em></td>
<td>(Snodgrass et al., 1987; Jonsson et al., 1991; Clarke et al., 2004; Hirschhausen et al., 2010; Hussain et al., 2001)</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>Embp, Fbe/SdrG, Aae</td>
<td><em>S. epidermidis</em></td>
<td>(Williams et al., 2002; Nilsson et al., 1998; Heilmann et al., 2003)</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>Aas, UafB, SdrI</td>
<td><em>S. saprophyticus</em></td>
<td>(Huff et al., 1998; King et al., 2011; Sakinc et al., 2009)</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>AtlC</td>
<td><em>S. caprae</em></td>
<td>(Allignet et al., 2002)</td>
</tr>
<tr>
<td>Involucrin</td>
<td>IsdA</td>
<td><em>S. aureus</em></td>
<td>(Clarke et al., 2009)</td>
</tr>
<tr>
<td>Loricrin</td>
<td>IsdA, CifB</td>
<td><em>S. aureus</em></td>
<td>(Clarke et al., 2009; Mulcahy et al., 2012)</td>
</tr>
<tr>
<td>Cytokeratin-10</td>
<td>IsdA, CifB</td>
<td><em>S. aureus</em></td>
<td>(Clarke et al., 2009; O'Brien et al., 2002)</td>
</tr>
<tr>
<td>Keratinocyte lipids/glycolipids</td>
<td>Pls</td>
<td><em>S. aureus</em></td>
<td>(Huesca et al., 2002)</td>
</tr>
</tbody>
</table>
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). Conversely, *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$_{1}$F$_{0}$-ATPase is a well-studied proton pump that mediates acid resistance (Cotter and Hill, 2003). F$_{1}$F$_{0}$-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$_{1}$F$_{0}$-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
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 $\alpha$-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 graRS, 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 (ChSO₄) 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).
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, whilst 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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain identifier</th>
<th>Isolation site</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. epidermidis</em></td>
<td>Rp62a</td>
<td>Intravascular catheter</td>
<td>(Christensen et al., 1982; 1985)</td>
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<tr>
<td><em>S. epidermidis</em></td>
<td>Tü3298</td>
<td>Unspecified</td>
<td>(Allgaier et al., 1986)</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>NCTC 1457</td>
<td>Intravenous catheter</td>
<td>(Mack et al., 1992)</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>A19</td>
<td>Skin (forearm)</td>
<td>(Kelly, 2013)</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>B19</td>
<td>Skin (forearm)</td>
<td>(Kelly, 2013)</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>O16</td>
<td>Skin (forearm)</td>
<td>(Kelly, 2013)</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>Newman</td>
<td>Osteomyelitis</td>
<td>(Duthie and Lorenz, 1952)</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>SH1000</td>
<td>Lab strain (8325 derivative)</td>
<td>(Horsburgh et al., 2002)</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>MSSA476</td>
<td>Osteomyelitis</td>
<td>(Holden et al., 2004)</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>MRSA252</td>
<td>Fatal bacteraemia</td>
<td>(Holden et al., 2004)</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>BL169</td>
<td>Nasal</td>
<td>(Libberton, 2011)</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>BL137</td>
<td>Nasal</td>
<td>(Libberton, 2011)</td>
</tr>
</tbody>
</table>
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 \(OD_{600} 0.2 \pm 0.005\) 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 \(1 \times 10^{-6}\) 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.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<th>9</th>
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<td>A</td>
<td>25</td>
<td>12.5</td>
<td>6.25</td>
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<td>1.56</td>
<td>0.78</td>
<td>0.39</td>
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<td>0.1</td>
<td>0.05</td>
<td>0.02</td>
<td>0.01</td>
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<td>2.29</td>
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<td>0.29</td>
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<td>0.04</td>
<td>0.02</td>
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<td>3.75</td>
<td>1.88</td>
<td>0.94</td>
<td>0.47</td>
<td>0.23</td>
<td>0.12</td>
<td>0.06</td>
<td>0.03</td>
<td>0.02</td>
<td>+</td>
</tr>
</tbody>
</table>

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 $1 \times 10^{-6}$; 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 \( \text{OD}_{600} \) 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 promoter 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) diethylypercarbonate 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 Chi² 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 sapenic 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 µg 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 µg 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 µg 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 1x10⁰ – 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<sub>50</sub>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.

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<th>MIC (µM)</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
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<td>-</td>
<td>CL</td>
<td>SH1000 &amp; Newman</td>
<td>(Clarke et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>-</td>
<td>TB</td>
<td>RN4220</td>
<td>(Parsons et al., 2012)</td>
</tr>
<tr>
<td>Sapienic acid (16:1∆6)</td>
<td>245.7*</td>
<td>-</td>
<td>MHB</td>
<td>ATCC 29213</td>
<td>(Fischer et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>196.5</td>
<td>-</td>
<td>CT-TSB</td>
<td>SH1000 &amp; Newman</td>
<td>(Clarke et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>39.3</td>
<td>-</td>
<td>CT-TSB</td>
<td>SH1000 &amp; Newman</td>
<td>(Clarke et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>-</td>
<td>TB</td>
<td>RN4220</td>
<td>(Parsons et al., 2012)</td>
</tr>
<tr>
<td>Capric acid (10:0)</td>
<td>58.1</td>
<td>-</td>
<td>CL</td>
<td>SH1000</td>
<td>(Clarke et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>232.2</td>
<td>-</td>
<td>CL</td>
<td>Newman</td>
<td>(Clarke et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>&gt;500</td>
<td>-</td>
<td>TB</td>
<td>RN4220</td>
<td>(Parsons et al., 2012)</td>
</tr>
<tr>
<td>Oleic acid (18:1∆9)</td>
<td>-</td>
<td>8.6</td>
<td>TSB</td>
<td>18Z</td>
<td>(Chamberlain et al., 1991)</td>
</tr>
<tr>
<td></td>
<td>&gt;500</td>
<td>-</td>
<td>TB</td>
<td>RN4220</td>
<td>(Parsons et al., 2012)</td>
</tr>
<tr>
<td>Linoleic acid (18:2∆9,12)</td>
<td>71.3</td>
<td>-</td>
<td>CL</td>
<td>Newman &amp; SH1000</td>
<td>(Clarke et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>-</td>
<td>TB</td>
<td>RN4220</td>
<td>(Parsons et al., 2012)</td>
</tr>
<tr>
<td>Palmitoleic acid (16:1∆9)</td>
<td>78.6</td>
<td>-</td>
<td>CL</td>
<td>Newman &amp; SH1000</td>
<td>(Clarke et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>-</td>
<td>TB</td>
<td>RN4220</td>
<td>(Parsons et al., 2012)</td>
</tr>
</tbody>
</table>
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 $\text{Ba}^{2+}$, $\text{Sr}^{2+}$, $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ 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, $\text{Fe}^{2+}$ and $\text{Fe}^{3+}$ have been shown to increase the potency of sapienic acid and linoleic respectively against $\text{S. aureus}$; 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 LD₅₀ 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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Linoleic acid (mM)</th>
<th>Sapienic acid (µM)</th>
<th>D-sphingosine (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>Tu3298</td>
<td>37</td>
<td>37</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>NCTC 1457</td>
<td>37</td>
<td>43</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>Rp62a</td>
<td>25</td>
<td>25</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>A19</td>
<td>25</td>
<td>50</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>B19</td>
<td>5</td>
<td>37</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>O16</td>
<td>5</td>
<td>15</td>
<td>98</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>Newman</td>
<td>5</td>
<td>11</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>SH1000</td>
<td>5</td>
<td>37</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>MRSA 252</td>
<td>0.6</td>
<td>5</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>MSSA 476</td>
<td>0.6</td>
<td>0.6</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>BL137</td>
<td>5</td>
<td>5</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>BL169</td>
<td>5</td>
<td>5</td>
<td>72</td>
</tr>
</tbody>
</table>

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<sub>600</sub> 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
cm² of sphinganine, another sphingosine found on skin, was required to reduce \textit{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 \textit{in vivo}. Another interesting test would be to determine if there is any synergistic or antagonistic activity between skin antimicrobials as has been observed previously for antimicrobial proteins found on skin (Chen \textit{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 \textit{et al.}, 2014; Fischer \textit{et al.}, 2013; Parsons \textit{et al.}, 2012).

The antimicrobial fatty acid MICs of \textit{S. epidermidis} were significantly higher than those of \textit{S. aureus}. Some proposed fatty acid resistance factors present in \textit{S. aureus}, such as carotenoids (Chamberlain \textit{et al.}, 1991), are absent in \textit{S. epidermidis}; however these results indicate that \textit{S. epidermidis} must possess mechanisms of resistance to fatty acids that \textit{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 \textit{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 \textit{S. epidermidis} are higher than those of \textit{S. aureus} they are still low in comparison to another Gram-positive genus dominant on human skin- \textit{Corynebacterium}. \textit{Corynebacterium jeikeium} and \textit{Corynebacterium striatum} sapienic acid MIC was >1.96 mM, which was much higher than that for \textit{S. epidermidis} or \textit{S. aureus} (Fischer \textit{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 biases, 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 transcriptomics 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.

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<th>Species</th>
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<td>1200</td>
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</tr>
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<td>1.8</td>
<td>455</td>
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<td>8.6</td>
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</table>
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 sapienic 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 sapienic 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 \textit{S. aureus} (between 2.8 and 52 fold, with an average of 11.3 fold). Capsule may offer protection to \textit{S. aureus} by preventing sapienic acid interacting with the cell.

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

\textit{S. epidermidis} Tü3298, shows strong upregulation of a cluster of genes (\textit{setu\textunderscore 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 \textit{S. aureus}, whilst the others are not DE in \textit{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.

\textbf{4.3.3 Comparison of DE COGs}

To assess the cellular pathways that were most affected in \textit{S. epidermidis} and \textit{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 \textit{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).
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 remodelling 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 programme 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 glycerol lipid 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 log₂ 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 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.

<table>
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<th>Gene</th>
<th>Fold change <em>S. aureus</em></th>
<th>Fold change <em>S. epidermidis</em></th>
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<td>+0.9</td>
<td>5.3.1.9</td>
</tr>
<tr>
<td><em>NWMN_2224</em></td>
<td>+0.8</td>
<td>-</td>
<td>GlvBC</td>
</tr>
<tr>
<td><em>lacF</em></td>
<td>+2.3</td>
<td>+0.8</td>
<td>LacF</td>
</tr>
<tr>
<td><em>mtlA</em></td>
<td>+2.4</td>
<td>X</td>
<td>MtlA</td>
</tr>
<tr>
<td><em>mtlF</em></td>
<td>+0.8</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>NWMN_0190</em></td>
<td>+2.6</td>
<td>X</td>
<td>GatA</td>
</tr>
<tr>
<td><em>NWMN_1619 SETU_01288</em></td>
<td>-1.6</td>
<td>+0.6</td>
<td>NagE</td>
</tr>
</tbody>
</table>
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\textsubscript{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 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change <strong>S. aureus</strong></th>
<th>Fold change <strong>S. epidermidis</strong></th>
<th>Enzyme name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sugar uptake and glycolysis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>glcA</em></td>
<td>-2.5</td>
<td>-</td>
<td>Gamp</td>
</tr>
<tr>
<td><em>ptsG</em></td>
<td>-2.4</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>fruA</em></td>
<td>-1.4</td>
<td>+1.7</td>
<td>FruA</td>
</tr>
<tr>
<td><strong>Pyruvate metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ldh</em></td>
<td></td>
<td>+1.7</td>
<td>1.1.1.27</td>
</tr>
<tr>
<td><em>Ldh1</em></td>
<td>-2.5</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>NWMN_2459</em></td>
<td>+1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>poxB SETU_01970</em></td>
<td>+1.6</td>
<td>+2.0</td>
<td>1.2.3.3</td>
</tr>
<tr>
<td><em>ackA</em></td>
<td>-</td>
<td>-0.4</td>
<td>2.7.2.1</td>
</tr>
<tr>
<td><em>NWMN_1315 SETU_00980</em></td>
<td>+1.1</td>
<td>+1.1</td>
<td>3.6.1.7</td>
</tr>
<tr>
<td><em>AldA/aldA-1</em></td>
<td>+1.6</td>
<td>+0.4</td>
<td>1.2.1.3</td>
</tr>
<tr>
<td><em>NWMN_185B AldA-2</em></td>
<td>+1.1</td>
<td>+1.8</td>
<td></td>
</tr>
<tr>
<td><em>NWMN_2026 SETU_01602</em></td>
<td>+2.5</td>
<td>+2.0</td>
<td></td>
</tr>
<tr>
<td><em>NWMN_2506</em></td>
<td>+2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>ald</em></td>
<td>-</td>
<td>+1.1</td>
<td>1.4.1.1</td>
</tr>
<tr>
<td><em>pycA/pyc</em></td>
<td>+1.1</td>
<td>+0.7</td>
<td>6.4.1.1</td>
</tr>
<tr>
<td><em>pdhA</em></td>
<td>-</td>
<td>+0.8</td>
<td>1.2.4.1</td>
</tr>
<tr>
<td><em>pdhB</em></td>
<td>-</td>
<td>+0.9</td>
<td></td>
</tr>
<tr>
<td><em>pdhC</em></td>
<td>-</td>
<td>+0.9</td>
<td>2.3.1.12</td>
</tr>
<tr>
<td><em>SETU_02150</em></td>
<td>X</td>
<td>+0.5</td>
<td></td>
</tr>
<tr>
<td><em>pdhD</em></td>
<td>-</td>
<td>+1.0</td>
<td>1.8.1.4</td>
</tr>
<tr>
<td><em>lpdA</em></td>
<td>-0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>pflB</em></td>
<td>-2.9</td>
<td>+1.4</td>
<td>2.3.1.54</td>
</tr>
<tr>
<td><em>citZ/gltA</em></td>
<td>2.5</td>
<td>+0.4</td>
<td>2.3.3.1</td>
</tr>
</tbody>
</table>
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 log2 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 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change <em>S. aureus</em></th>
<th>Fold change <em>S. epidermidis</em></th>
<th>Enzyme name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pyruvate metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>sucA</em></td>
<td>-</td>
<td>+1.0</td>
<td>1.2.4.2</td>
</tr>
<tr>
<td><em>sucB</em></td>
<td>-</td>
<td>+1.1</td>
<td>2.3.1.61</td>
</tr>
<tr>
<td><em>ilvB</em></td>
<td>+3.3</td>
<td>+2.9</td>
<td>2.2.1.6</td>
</tr>
<tr>
<td><em>budB</em></td>
<td>-</td>
<td>+1.9</td>
<td></td>
</tr>
<tr>
<td><em>SETU01538</em></td>
<td>X</td>
<td>+3.2</td>
<td></td>
</tr>
<tr>
<td><em>BudA</em></td>
<td>-</td>
<td>+1.3</td>
<td>4.1.1.5</td>
</tr>
<tr>
<td><em>aldB</em></td>
<td>-0.8</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>SETU,01888</em></td>
<td>X</td>
<td>+1.3</td>
<td>1.1.1.303/1.1.1.4</td>
</tr>
<tr>
<td><em>NWNN_0071 SETU,02090</em></td>
<td>+3.7</td>
<td>+0.7</td>
<td>1.1.1.304/1.1.1.76</td>
</tr>
<tr>
<td><em>SETU,02208</em></td>
<td>X</td>
<td>+4.3</td>
<td></td>
</tr>
<tr>
<td><strong>NADH recycling</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ldh</em></td>
<td>-</td>
<td>+1.7</td>
<td>1.1.1.27</td>
</tr>
<tr>
<td><em>Ldh1</em></td>
<td>-2.5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>SETU,02044</em></td>
<td>X</td>
<td>+1.6</td>
<td>1.8.1.7</td>
</tr>
<tr>
<td><em>citC</em></td>
<td>+1.9</td>
<td>-</td>
<td>1.1.1.42</td>
</tr>
<tr>
<td><em>gnd</em></td>
<td>-</td>
<td>+0.7</td>
<td>1.1.1.44</td>
</tr>
<tr>
<td><em>zwf</em></td>
<td>+1.1</td>
<td>+0.5</td>
<td>1.1.1.49</td>
</tr>
<tr>
<td><em>nadE</em></td>
<td>-</td>
<td>+0.6</td>
<td>6.3.1.5</td>
</tr>
</tbody>
</table>

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 log2 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change S. aureus</th>
<th>Fold change S. epidermidis</th>
<th>Enzyme name</th>
</tr>
</thead>
<tbody>
<tr>
<td>murF</td>
<td>-</td>
<td>-1.1</td>
<td>6.3.2.10</td>
</tr>
<tr>
<td>murE</td>
<td>-</td>
<td>-1.8</td>
<td>6.3.2.7</td>
</tr>
<tr>
<td>femA</td>
<td>-</td>
<td>-1.0</td>
<td>2.3.2.17</td>
</tr>
<tr>
<td>femX</td>
<td>-</td>
<td>+0.6</td>
<td>2.3.2.16</td>
</tr>
<tr>
<td>SETU_01292</td>
<td>-</td>
<td>-1.2</td>
<td>SgtA</td>
</tr>
<tr>
<td>NWML_1766 SETU_01431</td>
<td>+2.2</td>
<td>-1.0</td>
<td>SgtB</td>
</tr>
<tr>
<td>pbp1</td>
<td>-</td>
<td>-0.8</td>
<td>PbpA</td>
</tr>
<tr>
<td>pbp3</td>
<td>-</td>
<td>-1.1</td>
<td>Pbp3</td>
</tr>
<tr>
<td>murZ</td>
<td>+0.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>murB</td>
<td>-0.8</td>
<td>-</td>
<td>1.3.1.98</td>
</tr>
</tbody>
</table>

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, narf), 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 log₂ 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change S. aureus</th>
<th>Fold change S. epidermidis</th>
<th>Enzyme name</th>
</tr>
</thead>
<tbody>
<tr>
<td>gcvH</td>
<td>-</td>
<td>+0.4</td>
<td>GDCH</td>
</tr>
<tr>
<td>ureA</td>
<td>+3.7</td>
<td>-0.7</td>
<td>3.5.1.5</td>
</tr>
<tr>
<td>ureB</td>
<td>+3.7</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>ureC</td>
<td>+3.6</td>
<td>+0.5</td>
<td></td>
</tr>
<tr>
<td>arcA1/arcC</td>
<td>-1.0</td>
<td>+0.8</td>
<td>2.7.2.2</td>
</tr>
<tr>
<td>gluD</td>
<td>-</td>
<td>+0.4</td>
<td>1.4.1.2</td>
</tr>
<tr>
<td>narG</td>
<td>-</td>
<td>+1.4</td>
<td>NarG</td>
</tr>
<tr>
<td>narH</td>
<td>-</td>
<td>+1.8</td>
<td>NarH</td>
</tr>
<tr>
<td>narJ</td>
<td>-</td>
<td>+1.6</td>
<td>NarJ</td>
</tr>
<tr>
<td>NirB</td>
<td>-</td>
<td>+1.1</td>
<td>NirB</td>
</tr>
<tr>
<td>NirD</td>
<td>-</td>
<td>+1.0</td>
<td>NirD</td>
</tr>
<tr>
<td>Setu_01471 NWMN_1852</td>
<td>-1.1</td>
<td>-2.0</td>
<td>1.14.13.39</td>
</tr>
<tr>
<td>argG</td>
<td>-</td>
<td>-1.0</td>
<td>6.3.4.5</td>
</tr>
<tr>
<td>argH</td>
<td>-</td>
<td>-0.6</td>
<td>4.3.2.1</td>
</tr>
</tbody>
</table>
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 log₂ 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change <em>S. aureus</em></th>
<th>Fold change <em>S. epidermidis</em></th>
<th>Enzyme name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino acid biosynthesis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>hisG</em></td>
<td>-</td>
<td>+1.7</td>
<td>2.4.2.17</td>
</tr>
<tr>
<td><em>hisIE</em></td>
<td>+1.7</td>
<td>+0.9</td>
<td>3.6.1.31/3.5.4.19</td>
</tr>
<tr>
<td><em>hisA</em></td>
<td>+2.3</td>
<td>+1.0</td>
<td>5.3.1.16</td>
</tr>
<tr>
<td><em>hisF</em></td>
<td>+1.9</td>
<td>+0.9</td>
<td>HisF</td>
</tr>
<tr>
<td><em>hisH</em></td>
<td>+2.2</td>
<td>+1.5</td>
<td>HisH</td>
</tr>
<tr>
<td><em>hisB</em></td>
<td>+2.3</td>
<td>+1.7</td>
<td>4.2.1.19</td>
</tr>
<tr>
<td><em>hisC</em></td>
<td>+1.8</td>
<td>-</td>
<td>2.6.1.9</td>
</tr>
<tr>
<td><em>hisD</em></td>
<td>+2.1</td>
<td>+1.4</td>
<td>1.1.1.23</td>
</tr>
<tr>
<td><em>hutU</em></td>
<td>+1.5</td>
<td>X</td>
<td>4.2.1.49</td>
</tr>
<tr>
<td><em>hutG</em></td>
<td>+1.8</td>
<td>+1.1</td>
<td>3.5.3.8</td>
</tr>
<tr>
<td><em>thrA</em></td>
<td>+4.0</td>
<td>-</td>
<td>2.7.2.4</td>
</tr>
<tr>
<td><em>lysC</em></td>
<td>+3.2</td>
<td>-0.9</td>
<td></td>
</tr>
</tbody>
</table>
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 log2 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.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change <em>S. aureus</em></th>
<th>Fold change <em>S. epidermidis</em></th>
<th>Enzyme name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino acid biosynthesis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alr-2 SETU_00973</td>
<td>+2.3</td>
<td>+1.1</td>
<td>5.1.1.1</td>
</tr>
<tr>
<td>NWMN_2454</td>
<td>+1.0</td>
<td>-</td>
<td>1.2.1.88</td>
</tr>
<tr>
<td>gltB</td>
<td>+1.6</td>
<td>-</td>
<td>1.4.1.13/1.4.1.14</td>
</tr>
<tr>
<td>gltD</td>
<td>-</td>
<td>+1.2</td>
<td></td>
</tr>
<tr>
<td>cysK</td>
<td>-</td>
<td>+1.4</td>
<td>2.5.1.47</td>
</tr>
<tr>
<td><strong>Aspartate degradation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>purA</td>
<td>-1.6</td>
<td>-0.5</td>
<td>6.3.4.4</td>
</tr>
<tr>
<td>purB</td>
<td>-1.2</td>
<td>-0.5</td>
<td>4.3.2.2</td>
</tr>
<tr>
<td>argG</td>
<td>-</td>
<td>-1.0</td>
<td>6.3.4.5</td>
</tr>
<tr>
<td>argH</td>
<td>-</td>
<td>-0.6</td>
<td>4.3.2.1</td>
</tr>
</tbody>
</table>
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 log₂ 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.

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

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 log2 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change <em>S. aureus</em></th>
<th>Fold change <em>S. epidermidis</em></th>
<th>Enzyme name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glycerol metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>SETU_00365</em></td>
<td>-</td>
<td>+0.7</td>
<td>2.7.1.31</td>
</tr>
<tr>
<td><em>NWMMN_2331 garK</em></td>
<td>+1.4</td>
<td>+0.7</td>
<td></td>
</tr>
<tr>
<td><em>aldA</em></td>
<td>+1.6</td>
<td>+0.4</td>
<td>1.2.1.3</td>
</tr>
<tr>
<td><em>aldA-2</em></td>
<td>-</td>
<td>+1.8</td>
<td></td>
</tr>
<tr>
<td><em>NWMMN_1858 SETU_01602</em></td>
<td>+1.1</td>
<td>+2.0</td>
<td></td>
</tr>
<tr>
<td><em>NWMMN_2026</em></td>
<td>+2.5</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>NWMMN_0619</em></td>
<td>+1.6</td>
<td>X</td>
<td>2.7.1.29</td>
</tr>
<tr>
<td><em>NWMMN_0620</em></td>
<td>+1.3</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>gldA</em></td>
<td>X</td>
<td>+1.8</td>
<td>1.1.1.6</td>
</tr>
<tr>
<td><strong>Glycerolipid biosynthesis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>plsX</em></td>
<td>-1.1</td>
<td>-1.7</td>
<td>2.3.1.15</td>
</tr>
<tr>
<td><em>NWMMN_1266 Serp0924</em></td>
<td>-1.6</td>
<td>-1.9</td>
<td></td>
</tr>
<tr>
<td><em>plsC</em></td>
<td>-2.0</td>
<td>X</td>
<td>2.3.1.51</td>
</tr>
<tr>
<td><em>murG ypfP</em></td>
<td>-</td>
<td>-1.5</td>
<td>2.4.1.157</td>
</tr>
<tr>
<td><strong>Fatty acid degradation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>fadE</em></td>
<td></td>
<td>X</td>
<td>6.2.13</td>
</tr>
<tr>
<td><em>aldA</em></td>
<td>+1.6</td>
<td>+0.4</td>
<td>1.2.1.3</td>
</tr>
<tr>
<td><em>aldA-2</em></td>
<td>-</td>
<td>+1.8</td>
<td></td>
</tr>
<tr>
<td><em>NWMMN_1858 SETU_01602</em></td>
<td>+1.1</td>
<td>+2.0</td>
<td></td>
</tr>
<tr>
<td><em>NWMMN_2026</em></td>
<td>+2.5</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>NWMMN_0346 SETU_00013</em></td>
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</tr>
<tr>
<td><em>NWMMN_2090 SETU_01661</em></td>
<td>+1.67</td>
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<td>1.1.1.1</td>
</tr>
<tr>
<td><em>NWMMN_2091 SETU_01662</em></td>
<td>+2.30</td>
<td>+2.53</td>
<td></td>
</tr>
<tr>
<td><em>NWMMN_2272 SETU_01828</em></td>
<td>-</td>
<td>+0.82</td>
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</tr>
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</table>
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 log₂ 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change <em>S. aureus</em></th>
<th>Fold change <em>S. epidermidis</em></th>
<th>Enzyme name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glycerophospholipid metabolism</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gpsA</td>
<td>-1.0</td>
<td>-0.6</td>
<td>1.1.1.94</td>
</tr>
<tr>
<td>plsX</td>
<td>-1.1</td>
<td>-1.7</td>
<td>2.3.1.15</td>
</tr>
<tr>
<td>NWMN_1266 SETU_00930</td>
<td>-1.6</td>
<td>-1.9</td>
<td></td>
</tr>
<tr>
<td>plsC</td>
<td>-2.0</td>
<td>X</td>
<td>2.3.1.51</td>
</tr>
<tr>
<td>cdsA</td>
<td>-2.3</td>
<td>-1.2</td>
<td>27.7.41</td>
</tr>
<tr>
<td>pgsA</td>
<td>-1.1</td>
<td></td>
<td>27.8.5</td>
</tr>
<tr>
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<td>-1.3</td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td>-0.6</td>
<td>3.1.4.46</td>
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<tr>
<td>SETU_01283</td>
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<td></td>
</tr>
<tr>
<td>NWMN_1614</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Carotenoid, cardiolipin, branched chain fatty acid and mevalonate biosynthesis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CrtM</td>
<td>+2.0</td>
<td>X</td>
<td>CrtM</td>
</tr>
<tr>
<td>CrtN</td>
<td>+2.3</td>
<td>X</td>
<td>CrtN</td>
</tr>
<tr>
<td>CrtI</td>
<td>+1.7</td>
<td>X</td>
<td>CrtP</td>
</tr>
<tr>
<td>NWMN_2463</td>
<td>+1.9</td>
<td>X</td>
<td>CrtQ</td>
</tr>
<tr>
<td>mvk</td>
<td></td>
<td>-0.8</td>
<td>27.1.36</td>
</tr>
<tr>
<td>SETU_00217</td>
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<td>-0.6</td>
<td>27.4.2</td>
</tr>
<tr>
<td>mvaD</td>
<td></td>
<td>-0.5</td>
<td>41.1.33</td>
</tr>
<tr>
<td>idi/fni-2</td>
<td>-1.2</td>
<td>-0.6</td>
<td>5.3.3.2</td>
</tr>
<tr>
<td>NWMN_1230</td>
<td>+1.1</td>
<td></td>
<td>Cls</td>
</tr>
<tr>
<td>clp/clp-2</td>
<td>-1.1</td>
<td>-0.5</td>
<td></td>
</tr>
<tr>
<td>ispA</td>
<td>-1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hepT SETU_01049</td>
<td>-1.0</td>
<td>-0.4</td>
<td>25.1.30</td>
</tr>
<tr>
<td>uppS</td>
<td>-1.8</td>
<td>-2.2</td>
<td>25.1.31</td>
</tr>
<tr>
<td>idi/fni-2</td>
<td>-1.2</td>
<td>-0.6</td>
<td>5.3.3.2</td>
</tr>
</tbody>
</table>
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 log₂ 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change <em>S. epidermidis</em></th>
<th>Fold change <em>S. aureus</em></th>
<th>Enzyme name</th>
</tr>
</thead>
<tbody>
<tr>
<td>mutS/hexA</td>
<td>-</td>
<td>-0.5</td>
<td>MutS</td>
</tr>
<tr>
<td>xseA</td>
<td>-1.3</td>
<td>-</td>
<td>ExoVII</td>
</tr>
<tr>
<td>recJ</td>
<td>-1.7</td>
<td>-1.1</td>
<td>RecJ</td>
</tr>
<tr>
<td>pcrA</td>
<td>-0.8</td>
<td>-</td>
<td>UVRD</td>
</tr>
<tr>
<td>NWMN_0358</td>
<td>-1.4</td>
<td>X</td>
<td>SSB</td>
</tr>
<tr>
<td>NWMN_1909</td>
<td>+2.5</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>dnaN</td>
<td>-</td>
<td>+0.9</td>
<td>DpoIII</td>
</tr>
<tr>
<td>dnaX</td>
<td>-</td>
<td>-0.9</td>
<td></td>
</tr>
<tr>
<td>holA</td>
<td>-1.0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>holB</td>
<td>-1.3</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>NWMN_1832</td>
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<td></td>
<td></td>
</tr>
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<td>Lig/ligA</td>
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<td>+0.6</td>
<td>Lig</td>
</tr>
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<td>NWMN_1520 SETU_01187</td>
<td>-0.9</td>
<td>-1.7</td>
<td>RecD</td>
</tr>
<tr>
<td>ruvA</td>
<td>-1.1</td>
<td>-</td>
<td>RuvA</td>
</tr>
<tr>
<td>ruvB</td>
<td>-1.3</td>
<td>-</td>
<td>RuvB</td>
</tr>
<tr>
<td>SETU_01223</td>
<td>-</td>
<td>-0.7</td>
<td>Tag</td>
</tr>
<tr>
<td>ung</td>
<td>-1.4</td>
<td>-1.1</td>
<td>Ung</td>
</tr>
<tr>
<td>NWMN_1760 mutY</td>
<td>-</td>
<td>-0.8</td>
<td>MutY</td>
</tr>
<tr>
<td>NWMN_1460 nfo</td>
<td>-1.6</td>
<td>+1.0</td>
<td>Nfo</td>
</tr>
</tbody>
</table>
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 log₂ 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change <em>S. aureus</em></th>
<th>Fold change <em>S. epidermidis</em></th>
<th>Enzyme name</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>dltA</em></td>
<td>-2.1</td>
<td>-1.2</td>
<td></td>
</tr>
<tr>
<td><em>dltB</em></td>
<td>-1.8</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>dltC</em></td>
<td>-2.0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>dltD</em></td>
<td>-1.9</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><em>fmtC</em></td>
<td>-1.0</td>
<td>-0.7</td>
<td>MprF</td>
</tr>
<tr>
<td><em>aur/sepA</em></td>
<td>-</td>
<td>-1.0</td>
<td></td>
</tr>
<tr>
<td><em>spa</em></td>
<td>-1.0</td>
<td>X</td>
<td>SpA</td>
</tr>
<tr>
<td><em>scn</em></td>
<td>-1.3</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td><em>clfB</em></td>
<td>-1.6</td>
<td>X</td>
<td>ClfB</td>
</tr>
<tr>
<td><em>isdA</em></td>
<td>-2.3</td>
<td>X</td>
<td>IsdA</td>
</tr>
<tr>
<td><em>eta</em></td>
<td>-1.7</td>
<td>X</td>
<td>Eta</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change <em>S. aureus</em></th>
<th>Fold change <em>S. epidermidis</em></th>
<th>Enzyme name</th>
</tr>
</thead>
<tbody>
<tr>
<td>vraF</td>
<td>-</td>
<td>+1.0</td>
<td>VraF</td>
</tr>
<tr>
<td>vraD</td>
<td>-</td>
<td>-0.6</td>
<td>VraG</td>
</tr>
<tr>
<td>vraG</td>
<td>-</td>
<td>+1.3</td>
<td>VraG</td>
</tr>
<tr>
<td>hlgA</td>
<td>+2.0</td>
<td>X</td>
<td>Hlg/luc</td>
</tr>
<tr>
<td>clfA</td>
<td>+1.7</td>
<td>X</td>
<td>ClfA</td>
</tr>
<tr>
<td>set7nm</td>
<td>+1.3</td>
<td>X</td>
<td>SSL7</td>
</tr>
<tr>
<td>sdrC</td>
<td>+2.9</td>
<td>X</td>
<td>SdrC/D</td>
</tr>
<tr>
<td>sdrD</td>
<td>+1.0</td>
<td>X</td>
<td>SdrC/D</td>
</tr>
<tr>
<td>sdrE</td>
<td>+0.8</td>
<td>X</td>
<td>SdrC/D</td>
</tr>
</tbody>
</table>

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 dataset 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).

![Venn diagrams showing gene expression overlap](image)

**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.
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).

**A.**

**Protonophore**

**Acid Shock**

![Venn diagram showing gene regulation](image)

**Fatty acid**

**Linoleic acid**

![Venn diagram showing gene regulation](image)

**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.
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.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description</th>
<th>Tü3298</th>
<th>Newman</th>
<th>MRSA 252</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Upregulated</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><code>fdaB</code></td>
<td>Fructose-bisphosphate aldolase</td>
<td>3.53</td>
<td>2.35</td>
<td>2.02</td>
</tr>
<tr>
<td><code>gpmA</code></td>
<td>Phosphoglycerate mutase</td>
<td>2.3</td>
<td>4.06</td>
<td>2.06</td>
</tr>
<tr>
<td>SETU_00193</td>
<td>Hexulose-6P synthase</td>
<td>2.23</td>
<td>1.8</td>
<td>2.36</td>
</tr>
<tr>
<td>SETU_00242</td>
<td>Esterase/lipase</td>
<td>5.13</td>
<td>6.87</td>
<td>6.49</td>
</tr>
<tr>
<td>SETU_00243</td>
<td>Accessory regulator A</td>
<td>3.2</td>
<td>3.97</td>
<td>2.14</td>
</tr>
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<td>SETU_00102</td>
<td>Translation initiation inhibitor, N: deiminase activity</td>
<td>4.79</td>
<td>2.23</td>
<td>3.65</td>
</tr>
<tr>
<td>SETU_00324</td>
<td>Glucosyl transferase</td>
<td>9.06</td>
<td>4.29</td>
<td>3.49</td>
</tr>
<tr>
<td>SETU_00394</td>
<td>Cell division inhibitor homologue</td>
<td>9.32</td>
<td>2.55</td>
<td>5.06</td>
</tr>
<tr>
<td>SETU_00401</td>
<td>2,3-diphosphoglycerate independent phosphoglycerate mutase</td>
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<td>2.35</td>
<td>2.64</td>
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<td>Osmoprotectant ABC transporter</td>
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</tr>
<tr>
<td>SETU_00344</td>
<td>Osmoprotectant ABC transporter, permease</td>
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<td>2.99</td>
</tr>
<tr>
<td>SETU_02108</td>
<td>SecA preprotein translocase homologue</td>
<td>1.39</td>
<td>3.23</td>
<td>3.85</td>
</tr>
<tr>
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<td>Osmoprotectant transporter</td>
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<td>8.17</td>
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<td>SETU_02209</td>
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<td>General stress protein</td>
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<td>12.82</td>
<td>3.82</td>
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<tr>
<td>SETU_00468</td>
<td>Membrane protein/hemolysin</td>
<td>4.03</td>
<td>2.48</td>
<td>4.05</td>
</tr>
</tbody>
</table>
**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.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description</th>
<th>Tü3298</th>
<th>Newman</th>
<th>MRSA 252</th>
</tr>
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<td>Tü3298</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>NWMN_0914</td>
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<tr>
<td>SETU_00673</td>
<td>NWMN_0050</td>
<td>SAR0111</td>
<td>5.39</td>
<td>7.36</td>
</tr>
<tr>
<td>SETU_00674</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SETU_00907</td>
<td>thrB</td>
<td>thrB</td>
<td>7.21</td>
<td>16.8</td>
</tr>
<tr>
<td>SETU_00911</td>
<td>katA</td>
<td>KatA</td>
<td>5.82</td>
<td>5.98</td>
</tr>
<tr>
<td>SETU_01152</td>
<td>dnaJ</td>
<td>dnaJ</td>
<td>1.87</td>
<td>4.11</td>
</tr>
<tr>
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<td>NWMN_0845</td>
<td>clpB</td>
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<td>47.18</td>
</tr>
<tr>
<td>SETU_02130</td>
<td>hisiE</td>
<td>HisiE</td>
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</tr>
<tr>
<td></td>
<td>spoVG</td>
<td>spoVG</td>
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<td>2.57</td>
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<tr>
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<td>NWMN_2026</td>
<td>sar2210</td>
<td>3.2</td>
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<tr>
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<td>SAR1785</td>
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100
Table 4.10 continued The staphylococcal AFA challenge stimulan.

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.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description</th>
<th>Fold change Tü3298</th>
<th>Newman</th>
<th>MRSA252</th>
</tr>
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<tbody>
<tr>
<td>SETU_01432</td>
<td>Thil/Pfpl family protein</td>
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<td>Surface anchored protein</td>
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<td>16.00</td>
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<td>SETU_01785</td>
<td>Putative riboflavin reductase</td>
<td>6.19</td>
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<td>SETU_01623</td>
<td>Oxidoreductase/epimerase</td>
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<td>Hypothetical membrane protein</td>
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<td>Hypothetical protein</td>
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**Downregulated**

<table>
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<td>SAR0562</td>
<td>2.45</td>
<td>2.33</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description</th>
<th>Tü3298 Fold change</th>
<th>Newman Fold change</th>
<th>MRSA252 Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>SETU_00261</td>
<td><em>tagA</em></td>
<td>Teichoic acid biosynthesis</td>
<td>1.75</td>
<td>2.06</td>
</tr>
<tr>
<td>SETU_00263</td>
<td><em>tagG</em></td>
<td>Teichoic acid tranlocation permease</td>
<td>3.51</td>
<td>1.75</td>
</tr>
<tr>
<td>SETU_00268</td>
<td><em>NWMN_0614</em></td>
<td>Pyrimidine transporter</td>
<td>2.33</td>
<td>3.43</td>
</tr>
<tr>
<td>SETU_02365</td>
<td><em>NWMN_2412</em></td>
<td>ABC transporter</td>
<td>4.17</td>
<td>8.46</td>
</tr>
<tr>
<td>SETU_01802</td>
<td><em>NWMN_2248</em></td>
<td>Divalent cation transporter</td>
<td>2.3</td>
<td>1.72</td>
</tr>
<tr>
<td>SETU_00139</td>
<td><em>nupC</em></td>
<td>Pyrimidine transporter</td>
<td>1.61</td>
<td>8.75</td>
</tr>
<tr>
<td>SETU_00333</td>
<td><em>pabA</em></td>
<td>Anthranilate synthase/glutamine amidotransferase</td>
<td>2.93</td>
<td>6.02</td>
</tr>
<tr>
<td>SETU_00713</td>
<td><em>cyoD</em></td>
<td>Cytochrome c oxidase</td>
<td>1.72</td>
<td>5.46</td>
</tr>
<tr>
<td>SETU_01049</td>
<td><em>hepT</em></td>
<td>Heptaprenyl diphosphate synthase component II</td>
<td>1.30</td>
<td>1.99</td>
</tr>
<tr>
<td>SETU_01842</td>
<td><em>NWMN_2286</em></td>
<td>Virulence regulator</td>
<td>1.92</td>
<td>2.16</td>
</tr>
<tr>
<td>SETU_01880</td>
<td><em>lytS</em></td>
<td>Two-component sensor histidine kinase</td>
<td>3.71</td>
<td>2.00</td>
</tr>
<tr>
<td>SETU_01881</td>
<td><em>lytR</em></td>
<td>Two-component response regulator</td>
<td>1.74</td>
<td>2.53</td>
</tr>
<tr>
<td>SETU_01554</td>
<td><em>rsbU</em></td>
<td>SigmaB regulatory protein</td>
<td>2.97</td>
<td>1.62</td>
</tr>
<tr>
<td>SETU_01998</td>
<td><em>pyrD</em></td>
<td>Dihydroorotate dehydrogenase</td>
<td>2.20</td>
<td>2.06</td>
</tr>
<tr>
<td><em>ubiE</em></td>
<td><em>gerCB</em></td>
<td>MenAquinone biosynthesis</td>
<td>1.36</td>
<td>1.68</td>
</tr>
<tr>
<td><em>ydiM</em></td>
<td><em>NWMN_2073</em></td>
<td>Hypothetical membrane protein</td>
<td>2.81</td>
<td>1.89</td>
</tr>
<tr>
<td><em>yatE</em></td>
<td><em>NWMN_0799</em></td>
<td>Hypothetical protein</td>
<td>1.40</td>
<td>1.73</td>
</tr>
<tr>
<td>SETU_01555</td>
<td><em>NWMN_1974</em></td>
<td>PemK family protein</td>
<td>2.04</td>
<td>1.59</td>
</tr>
<tr>
<td>SETU_01077</td>
<td><em>NWMN_1411</em></td>
<td>AtsA/ElaC family protein</td>
<td>2.31</td>
<td>3.43</td>
</tr>
<tr>
<td>SETU_01123</td>
<td><em>NWMN_1453</em></td>
<td>5-formyltetrahydrofolate cyclo-ligase family protein</td>
<td>1.78</td>
<td>2.87</td>
</tr>
<tr>
<td>SETU_01195</td>
<td><em>NWMN_1530</em></td>
<td>ThIF family protein</td>
<td>1.56</td>
<td>2.81</td>
</tr>
<tr>
<td>SETU_01305</td>
<td><em>NWMN_1642</em></td>
<td>Hypothetical leucyl-tRNA synthase</td>
<td>3.53</td>
<td>2.41</td>
</tr>
</tbody>
</table>
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.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description</th>
<th>Fold change</th>
<th>Tü3298</th>
<th>Newman</th>
<th>MRSA252</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SETU_00372</strong></td>
<td>DegV family protein</td>
<td>2.39</td>
<td>3.23</td>
<td>3.23</td>
<td></td>
</tr>
<tr>
<td><strong>SETU_00570</strong></td>
<td>Permease</td>
<td>2.08</td>
<td>3.36</td>
<td>2.50</td>
<td></td>
</tr>
<tr>
<td><strong>SETU_00280</strong></td>
<td>Phosphate transport regulator</td>
<td>3.92</td>
<td>3.71</td>
<td>2.70</td>
<td></td>
</tr>
<tr>
<td><strong>dltx</strong></td>
<td>D-Ala-teichoic acid biosynthesis regulator</td>
<td>6.63</td>
<td>4.03</td>
<td>2.13</td>
<td></td>
</tr>
<tr>
<td><strong>SETU_01373</strong></td>
<td>Transcriptional regulator</td>
<td>11.00</td>
<td>9.71</td>
<td>2.38</td>
<td></td>
</tr>
<tr>
<td><strong>SETU_01609</strong></td>
<td>Hypothetical protein</td>
<td>2.22</td>
<td>1.71</td>
<td>2.78</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequences</th>
<th>Efficiency (%)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| rpoB      | F- GCGAACATGCAAGTCAAG  
           | R- GACCTCTGCTGTAGCTGTAATAGC | 97.0  | This study |
| hu        | F- TTTACGTGCAGCACGTTTCAC  
           | R- AAAAGAAGCTGTTGTTCACGAGTAG | 90.3  | (Duquenne et al, 2010) |
| gyrB (Tu3298) | F- AGAAAAGATGGGACGCCCTG  
                      | R- CACCATGAAAGACCAGCCAGAT | 96.6  | This study |
| gyrB (Newman) | F- ATCGACTTCAGAGAGGTGTTG  
                          | R- CGGTATTCGTACTTATAATCCA | 92.9  | (Kenny et al, 2009) |
| capB      | F- GCGATATGCGTAAGCCAACAC  
           | R- GGTACAGGGGCGAGCTGTAG | 91.5  | This study |
| pyrP      | F- CGATGTTTGGGGCAAGAAGTA  
           | R- GCTGTTATTTGGGCGCTTTCG | 92.5  | This study |
| clpB      | F- TGAGTCAGCTCCAGTATG  
           | R- AGAATCGTAAGACGACCTTCA | 99.0  | This study |
| NWMN_2452 | F- AGGCCAGCTTGCTGGATTA  
           | R- AACGACTCGAGCTTGTGATT | 93.3  | This study |
| sasF      | F- TCACCTGTGGATGAAAGGCA  
           | R- TTTCCGGTGCCGAAATGATCT | 95.0  | This study |
| narH      | F- TGGGCTTTCCATRCGATCT  
           | R- TTCAGTGTGCGGAGCAGTTA | 93.6  | This study |

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 σB 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 wall 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 suppress 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 Streptococcus 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. aureus* as *S. epidermidis* (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 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. subtilis 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 particularly 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.
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.

<table>
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<th>Isolate</th>
<th>DNA concentration (ng/µl)</th>
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</tr>
<tr>
<td></td>
<td>Rp-0</td>
<td>56.6</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>End</td>
<td>55.6</td>
<td>2.0</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>RpSapPool</td>
<td>37.3</td>
<td>2.1</td>
<td>2.1</td>
</tr>
</tbody>
</table>

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 100X 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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Isolate</th>
<th>Total no. reads</th>
<th>Total reads mapped (%)</th>
<th>Unique and Non-duplicate reads (%)</th>
<th>Mean coverage Genome covered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eth con</td>
<td>SH1000</td>
<td>2992974</td>
<td>98.25</td>
<td>69.85</td>
<td>193.45 ± 26.59</td>
</tr>
<tr>
<td>SH1000</td>
<td>1998524</td>
<td>99.08</td>
<td>79.96</td>
<td>145.7 ± 18.9</td>
<td>94.61</td>
</tr>
<tr>
<td>SH-Ds3A</td>
<td>2046926</td>
<td>99.14</td>
<td>79.57</td>
<td>148.43 ± 21.02</td>
<td>94.62</td>
</tr>
<tr>
<td>Eth con</td>
<td>SH-Ds3B</td>
<td>1841456</td>
<td>99.05</td>
<td>81.30</td>
<td>135.67 ± 17.68</td>
</tr>
<tr>
<td>S. aureus SH1000</td>
<td>SH-Ds5C</td>
<td>2201392</td>
<td>98.98</td>
<td>78.61</td>
<td>158.27 ± 19.11</td>
</tr>
<tr>
<td>Eth con</td>
<td>SH-Ds5C</td>
<td>1950152</td>
<td>98.63</td>
<td>78.74</td>
<td>146.23 ± 17.24</td>
</tr>
<tr>
<td>Rp-0</td>
<td>1827680</td>
<td>98.48</td>
<td>79.42</td>
<td>138.3 ± 16.62</td>
<td>98.16</td>
</tr>
<tr>
<td>Rp-Sap21C</td>
<td>1702790</td>
<td>98.38</td>
<td>79.99</td>
<td>129.15 ± 17.4</td>
<td>98.13</td>
</tr>
<tr>
<td>Rp-Sap21D</td>
<td>1370884</td>
<td>97.98</td>
<td>81.85</td>
<td>106.04 ± 14.29</td>
<td>98.13</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>Rp-Ds23D</td>
<td>1999958</td>
<td>98.89</td>
<td>77.26</td>
<td>157.5 ± 26.05</td>
</tr>
<tr>
<td>Rp-Ds21D</td>
<td>1853358</td>
<td>98.84</td>
<td>79.53</td>
<td>139.82 ± 17.13</td>
<td>98.15</td>
</tr>
<tr>
<td>Rp-Ds23C</td>
<td>1999958</td>
<td>99.28</td>
<td>78.39</td>
<td>150.24 ± 17.99</td>
<td>98.15</td>
</tr>
<tr>
<td>Rp-Ds23E</td>
<td>2110692</td>
<td>99.23</td>
<td>77.95</td>
<td>155.61 ± 21.57</td>
<td>98.17</td>
</tr>
<tr>
<td>Rp-SapPool</td>
<td>14981371</td>
<td>98.54</td>
<td>31.23</td>
<td>453.63 ± 38.3</td>
<td>98.24</td>
</tr>
</tbody>
</table>

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
A 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.

<table>
<thead>
<tr>
<th>Official gene symbol</th>
<th>Gene function</th>
<th>Isolates</th>
<th>Position of change</th>
<th>Base change</th>
<th>Change</th>
<th>Position in protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>yycG</em></td>
<td>Histidine kinase, regulates cell wall turnover</td>
<td>Rp-SapPool, Rp-Sap21C, Rp-Sap21D, Rp-Sap23D</td>
<td>2589940</td>
<td>C/T</td>
<td>Substitution</td>
<td>378 aa</td>
</tr>
<tr>
<td><em>gcvT</em></td>
<td>Glycine cleavage system aminomethyltransferase T</td>
<td>Rp-Sap21D</td>
<td>1146571</td>
<td>G/A</td>
<td>Substitution</td>
<td>74 aa</td>
</tr>
<tr>
<td>SERP1544</td>
<td>Phage lipoprotein</td>
<td>Rp-Sap21D</td>
<td>1596978</td>
<td>G/A</td>
<td>Substitution</td>
<td>52 aa</td>
</tr>
<tr>
<td>SERP1061</td>
<td>Fur transcriptional regulator</td>
<td>Rp-Sap23D</td>
<td>1106479</td>
<td>C/A</td>
<td>Substitution</td>
<td>49 aa</td>
</tr>
<tr>
<td>SERP0499</td>
<td>Iron-sulphur cluster scaffold protein</td>
<td>Rp-Sap21D</td>
<td>489097</td>
<td>+ATG</td>
<td>Insertion</td>
<td>29 aa</td>
</tr>
<tr>
<td>SERP2127</td>
<td>FeoA family, likely Fe^{2+} transport</td>
<td>Rp-Sap21C</td>
<td>2152015</td>
<td>G/A</td>
<td>Upstream</td>
<td>-42 bp</td>
</tr>
<tr>
<td>SERP0057</td>
<td>Hypothetical protein</td>
<td>Rp-SapPool, Rp-Sap23D</td>
<td>46171</td>
<td>A/G</td>
<td>Upstream</td>
<td>-102 bp</td>
</tr>
<tr>
<td>SERP0058</td>
<td>Hypothetical protein</td>
<td>Rp-SapPool, Rp-Sap23D</td>
<td>46171</td>
<td>A/G</td>
<td>Upstream</td>
<td>-41 bp</td>
</tr>
</tbody>
</table>

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 reduction in D-sphingosine resistance levels.
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.

<table>
<thead>
<tr>
<th>Official gene symbol</th>
<th>Gene function</th>
<th>Isolates</th>
<th>SNP position</th>
<th>Base change</th>
<th>Change</th>
<th>Codon no.</th>
<th>% of reads</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>mgo-3</em></td>
<td>Malate: quinone oxidoreductase</td>
<td>Rp-SapPool</td>
<td>2351205</td>
<td>A/G</td>
<td>Substitution</td>
<td>D/G</td>
<td>402 aa</td>
</tr>
<tr>
<td>SERP1061</td>
<td>Fur transcriptional regulator</td>
<td>Rp-SapPool</td>
<td>1106479</td>
<td>C/A</td>
<td>Substitution</td>
<td>D/Y</td>
<td>49 aa</td>
</tr>
</tbody>
</table>

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](image)

*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.

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

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

<table>
<thead>
<tr>
<th>Official gene symbol</th>
<th>Gene function</th>
<th>Isolates</th>
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<th>Base change</th>
<th>Change</th>
<th>Position in protein</th>
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<tr>
<td>gltX</td>
<td>Glutamyl-tRNA synthetase</td>
<td>SH-Ds3A, SH-Ds5C</td>
<td>51 1636</td>
<td>C/T</td>
<td>Substitution R/C</td>
<td>151 aa</td>
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<td><em>SAOUHSC</em> 01130</td>
<td>Hypothetical membrane protein</td>
<td>SH-Ds3A</td>
<td>1084556</td>
<td>A/G</td>
<td>Substitution K/R</td>
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<tr>
<td><em>SAOUHSC</em> 01337</td>
<td>Transketalase</td>
<td>SH-Ds3A</td>
<td>1277240</td>
<td>A/G</td>
<td>Substitution T/A</td>
<td>193 aa</td>
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<td>1807093</td>
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<td>Substitution A/S</td>
<td>182 aa</td>
</tr>
<tr>
<td><em>SAOUHSC</em> 02341</td>
<td>ATP synthase</td>
<td>SH-Ds3A, SH-Ds5C</td>
<td>2168000</td>
<td>G/C</td>
<td>Substitution P/A</td>
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</tr>
<tr>
<td>agrA</td>
<td>accessory gene regulator</td>
<td>SH-Ds3A</td>
<td>2096582</td>
<td>A/T</td>
<td>Premature Stop</td>
<td>193 aa</td>
</tr>
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<td><em>SAOUHSC</em> 02587</td>
<td>Hypothetical CAAX protease</td>
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<td>C/A</td>
<td>Substitution W/C</td>
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<td>TetR transcriptional regulator</td>
<td>SH-Ds3A, SH-Ds5C</td>
<td>2641724</td>
<td>G/A</td>
<td>Substitution E/K</td>
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</tr>
<tr>
<td><em>SAOUHSC</em> 01450</td>
<td>amino acid transporter</td>
<td>SH-Ds3B</td>
<td>1406715</td>
<td>T/C</td>
<td>Substitution I/V</td>
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<td>TetR transcriptional regulator</td>
<td>SH-Ds3B, SH-Ds5C</td>
<td>2641553</td>
<td>G/T</td>
<td>Substitution D/Y</td>
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<td><em>ebh</em></td>
<td>Extracellular matrix binding protein homologue</td>
<td>SH-Ds5C</td>
<td>1397392</td>
<td>A/G</td>
<td>Substitution L/S</td>
<td>2436 aa</td>
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<td><em>fmhA</em></td>
<td>Methicillin resistance determinant</td>
<td>SH-Ds5C</td>
<td>2479727</td>
<td>G/A</td>
<td>Substitution M/I</td>
<td>174 aa</td>
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<tr>
<td><em>SAOUHSC</em> 02866</td>
<td>RND transporter</td>
<td>SH-Ds5C</td>
<td>2640222</td>
<td>A/G</td>
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<td><em>SAOUHSC</em> 01584</td>
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<td>G/T</td>
<td>Premature stop</td>
<td>23 aa</td>
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</table>
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.

<table>
<thead>
<tr>
<th>Official gene symbol</th>
<th>Gene function</th>
<th>Isolates</th>
<th>SNP position</th>
<th>Base change</th>
<th>Change</th>
<th>Position in protein</th>
</tr>
</thead>
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<td>SH-Ds3B</td>
<td>2449509</td>
<td>+A</td>
<td>Frameshift</td>
<td>362 aa</td>
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<tr>
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<td>Pseudogene</td>
<td>SH-Ds3A</td>
<td>2349971</td>
<td>-A</td>
<td>Upstream deletion</td>
<td>80 aa</td>
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<td>Pseudogene</td>
<td>SH-Ds3A, SH-Ds3B</td>
<td>2349963</td>
<td>-G</td>
<td>Upstream deletion</td>
<td>72 aa</td>
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<tr>
<td>SAOUHSC_02302 Rsbb</td>
<td>sigmaB regulator</td>
<td>SH-Ds3A, SH-Ds3B, SH-Ds5C</td>
<td>2134726</td>
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<td>SAOUHSC_A02189</td>
<td>Hypothetical protein</td>
<td>SH-Ds3A, SH-Ds3B, SH-Ds5C</td>
<td>2134726</td>
<td>C/A</td>
<td>upstream substitution</td>
<td>-15 bp</td>
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<tr>
<td>SAOUHSC_01840</td>
<td>Glycosylase</td>
<td>SH-Ds3A, SH-Ds5C</td>
<td>1746115</td>
<td>C/A</td>
<td>upstream substitution</td>
<td>-196 bp</td>
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<tr>
<td>SAOUHSC_01839</td>
<td>Tyrosyl-tRNA synthase</td>
<td>SH-Ds3A, SH-Ds5C</td>
<td>1746115</td>
<td>C/A</td>
<td>upstream substitution</td>
<td>-186 bp</td>
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<tr>
<td>SAOUHSC_02809</td>
<td>gluconate operon repressor</td>
<td>SH-Ds3B</td>
<td>2588702</td>
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<td>SAOUHSC_00446</td>
<td>Hypothetical protein</td>
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<td>448086</td>
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<tr>
<td>SAOUHSC_00325</td>
<td>Hypothetical protein</td>
<td>SH-Ds5C</td>
<td>337692</td>
<td>G/T</td>
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<td>-188 bp</td>
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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.
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.

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.
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 \textit{S. aureus} D-sphingosine evolved isolates.

SNPs and INDELs from \textit{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.

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<td>A/S</td>
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<td>23</td>
<td>22.5</td>
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</table>
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 sapienic acid-evolved isolates.](image)

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\textsubscript{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 YycG 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 resistance (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 acquisition 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 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 absence 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-terminal 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 $\alpha$-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 θ activation in S. aureus, and deletion of S. aureus rsbU reduces θ activity (Pane-Farre et al., 2006; Palma and Cheung, 2001). The alternative sigma factor θ 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, θ mediates resistance to a number of stresses including heat and acid (Chan and Foster, 1998). Overproduction of θ 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 θ 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 moisturisers 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 \(\sigma^B\) 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 sapienic 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".

<table>
<thead>
<tr>
<th>SNP gene</th>
<th>Gene function</th>
<th>S. aureus log₂ fold change</th>
<th>S. epidermidis log₂ Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serp0499</td>
<td>NifU homologue</td>
<td>-</td>
<td>+0.55</td>
</tr>
<tr>
<td>Serp2127</td>
<td>Hypothetical Fe⁺ transporter</td>
<td>X</td>
<td>+1.64</td>
</tr>
<tr>
<td>Serp0057</td>
<td>Hypothetical protein</td>
<td>-</td>
<td>-1.44</td>
</tr>
<tr>
<td>Mgo-3</td>
<td>Malate:quinone oxidoreductase</td>
<td>-0.95</td>
<td>-</td>
</tr>
<tr>
<td><strong>Sapienic acid evolved S. epidermidis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mnhA</td>
<td>Monovalent cation transporter</td>
<td>+2.22</td>
<td>+2.19</td>
</tr>
<tr>
<td>Serp2264</td>
<td>sasF homologue</td>
<td>+4.00</td>
<td>+1.10</td>
</tr>
<tr>
<td>Serp0247</td>
<td>TetR-like transcriptional regulator</td>
<td>-1.75</td>
<td>-</td>
</tr>
<tr>
<td>Serp1407</td>
<td>Hypothetical protein</td>
<td>+1.71</td>
<td>+1.10</td>
</tr>
<tr>
<td>Serp2327</td>
<td>Acetoin dehydrogenase</td>
<td>-0.72</td>
<td>-</td>
</tr>
<tr>
<td>Serp0458</td>
<td>Ion transporter</td>
<td>+1.25</td>
<td>-1.17</td>
</tr>
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<td>Serp1758</td>
<td>Lytic regulatory protein</td>
<td>-</td>
<td>-0.91</td>
</tr>
<tr>
<td>Serp2438</td>
<td>Cation transporter</td>
<td>+3.36</td>
<td>+2.76</td>
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<td><strong>D-sphingosine evolved S. epidermidis</strong></td>
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<tr>
<td>gltX</td>
<td>GlutamyltRNA synthase</td>
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<td>-</td>
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<tr>
<td>SAOUHSC_01130</td>
<td>Hypothetical membrane protein</td>
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<td>-</td>
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<td>Ebb</td>
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<td>-1.57</td>
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<tr>
<td>SAOUHSC_01840</td>
<td>Transglycosylase</td>
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<tr>
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<td>TyrosyltRNA synthase</td>
<td>-1.61</td>
<td>-</td>
</tr>
<tr>
<td>SAOUHSC_00325</td>
<td>Hypothetical protein</td>
<td>+1.60</td>
<td>-</td>
</tr>
</tbody>
</table>
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</tr>
</thead>
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<tr>
<td>SAOUHSC_00196</td>
<td>Hypothetical protein</td>
<td>-</td>
<td>+0.71</td>
</tr>
<tr>
<td>SAOUHSC_00323</td>
<td>Hypothetical protein</td>
<td>+1.98</td>
<td>-</td>
</tr>
<tr>
<td>SAOUHSC_00936</td>
<td>Hypothetical protein</td>
<td>-</td>
<td>+0.97</td>
</tr>
<tr>
<td>SAOUHSC_01716</td>
<td>Protease</td>
<td>-1.17</td>
<td>-</td>
</tr>
<tr>
<td>SAOUHSC_01407</td>
<td>5-bromo-4-chlorindolyl phosphate hydrolase</td>
<td>+1.21</td>
<td>+1.38</td>
</tr>
<tr>
<td>SAOUHSC_00217</td>
<td>Sorbitol dehydrogenase</td>
<td>+1.72</td>
<td>+1.25</td>
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<tr>
<td>SAOUHSC_01141</td>
<td>Hypothetical protein</td>
<td>+1.15</td>
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</tr>
<tr>
<td>SAOUHSC_01584</td>
<td>Hypothetical protein</td>
<td>+3.71</td>
<td>-</td>
</tr>
</tbody>
</table>

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 novelty 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


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initial interaction of nisin with the target membrane. Biochemistry, 36, 6968-76.


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ability of *Staphylococcus aureus* to colonize skin. *Journal of Infectious Diseases*, 209, 1485-93.


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.

<table>
<thead>
<tr>
<th>Script name</th>
<th>Description</th>
<th>Command</th>
<th>Output files</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mpileup_SNPs_v2.pl</td>
<td>Calls SNPs for data with $6x$ coverage and presents only coding SNPs in the main output (SNP_output.bcf)</td>
<td><code>Perl mpileup_SNPs_v2.pl &lt;input.bcf&gt; SNPEff_database min.coverage &lt;reference.gff&gt; chromosome_name</code></td>
<td>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 -&gt; new base 5- Codon number 6- Effect (e.g. 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)</td>
<td>Josephine Moran</td>
</tr>
</tbody>
</table>
**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.

<table>
<thead>
<tr>
<th>Script name</th>
<th>Description</th>
<th>Command</th>
<th>Output files</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>snpEff_useful.txt</strong></td>
<td>tab-delimited SnpEff output file with 24 columns, those relevant to this study are described below</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>files</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Author</strong></td>
<td></td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Script name</th>
<th>Description</th>
<th>Command</th>
<th>Output files</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unique_SNPs_bwa.pl</td>
<td>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)</td>
<td>Perl unique_SNPs_bwa.pl &lt;input_1.txt&gt; &lt;input_2.txt&gt; &lt;output_name&gt; (where input files are the SNP_output.txt from Mpileup_SNPs_v2.pl)</td>
<td>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)</td>
<td>Josephine Moran</td>
</tr>
</tbody>
</table>
| 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.

<table>
<thead>
<tr>
<th>Script name</th>
<th>Description</th>
<th>Command</th>
<th>Output files</th>
<th>Author</th>
</tr>
</thead>
</table>
| 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 mismatches  
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.

<table>
<thead>
<tr>
<th>Script name</th>
<th>Description</th>
<th>Command</th>
<th>Output files</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sammd_compare.pl</td>
<td>Finds genes common in two gene lists</td>
<td><code>Perl sammd_compare.pl &lt;gene_list1&gt; &lt;gene_list2&gt; output_name</code></td>
<td><code>Output.txt</code> contains the lines from the second file where the gene name in both input files matched.</td>
<td>Josephine Moran</td>
</tr>
<tr>
<td>coverageStatsSplitByChr_v2.pl</td>
<td>Calculates the coverage statistics for mapped reads</td>
<td><code>Perl coverageStatsSplitByChr_v2.pl -I &lt;input.bam&gt; &gt; output_name</code></td>
<td>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</td>
<td>Kevin Ashelford</td>
</tr>
</tbody>
</table>
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.
Mannitol Utilisation is Required for Protection of *Staphylococcus aureus* from Human Skin Antimicrobial Fatty Acids

John G. Kenny, Josephine Moran, Stacey L. Kolar, Alexander Ulanov, Zhong Li, Lindsay N. Shaw, Elisabeth Josefsson, Malcolm J. Horsburgh

1Institute for Integrative Biology, University of Liverpool; 2Department of Cell Biology, Microbiology & Molecular Biology, University of South Florida, Tampa, Florida, United States of America; 3Ray C. Crowder Biotechnology Center, University of Illinois, Urbana-Champaign, Illinois, United States of America; 4Department of Rheumatology and Inflammation Research, University of Gothenburg, Gothenburg, Sweden

Abstract

Mannitol (M6) 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 mifT, encoding M6-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 potent catalytic action for this membrane-acting antimicrobial. Analysis of cellular metabolites revealed that, during exponential growth, the mifT mutant accumulated high levels of M6 and M6-P. The latter metabolite was not detected in its isogenic parent strain or a deletion mutant of the entire mifT and M6 operon. In addition, the mifT mutant strain exhibited a decreased MIC for H2O2, however virulence was unaffected in a model of septic arthritis.


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 dermcidin, porcinin, RNaseA and RNase7. One focus for study of survival is the antimicrobial activity of long chain (typically C8-16) unsaturated free fatty acids that generate the acid mantle on skin [5,6,2,25]. 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 amphiphilic 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 coordinately upregulated. In *S. aureus* protection against AFAs is afforded by reducing cell surface hydrophobicity [6,7,10] and the described transcriptional upregulation of cell surface components is proposed to mediate this effect [6]. The transcript encoding the cell surface protein Saa1 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 (CTA) and the iron-regulated surface protein IsaA increase survival from AFAs by decreasing surface hydrophobicity [7,19]. Inhibitory concentrations of AFAs cause leakage of protease and inhibit replication [20,21,22,23].

In this study, extended screening of *S. aureus* mutants with reduced survival from AFAs identified identical clones with defective mannitol (M6) metabolism. Since the capacity of *staphylococci* to ferment M6 is most frequently associated with the pathogen *S. aureus*, *S. pseudintermedius* and *S. hominis*, 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, M1 10 g l⁻¹, beef extract 1 g l⁻¹, NaCl 10 g l⁻¹; fracture broth contained fracture in place of M1. Cultures were incubated at 37°C with shaking at 250 rpm and growth was monitored by measuring OD₅₀₀. When included, antibiotics were added at the following concentrations: erythromycin, 5 μg ml⁻¹; fosfomycin, 25 μg ml⁻¹; tetracycline, 5 μg ml⁻¹; chloramphenicol 5 μg ml⁻¹. Antibiotics were not included in comparative growth experiments.

Construction of mtI Mutants and Complementation Plasmids

Construction of mtID and mtIDARF alleles replacement mutants was performed using methods described previously [24]. Amplification of mtID for allelic replacement used upstream and downstream primer pairs, mtIDflaR/flaR CGACGGATCC-GATGGTTGATGGCAACACATC with mtID_Nhr, AATACTGGGCGCGCACTGAAATGTACGG and mtID reshape CGCGTGACATACGGTGAATTAATGTACGG with mtIDARF flasR ACATGGTCTGATATCACTGACAGGGTACGTG and for mtIDARF operon allelic replacement the primer pair mldRki ARF CAGCCGATCTTACCAATCTCGTGTGTCG and mtID_Nhr, AATACTGGGCGCGTCGTCACATTGGTCGACGGTACGG. The downstream operon fragment was amplified using mldRki ARF and mtIDARF. The tetracycline resistance gene (tet) was amplified from pBG1513 [25] followed by simultaneous cloning of tet disrupted alleles into pUTIN4 [24, 26] and the resultant plasmids pJK1 and pJK2 containing the mtID-ARF and mtIDARF-ARF insert, respectively, were used to generate allelic replacement mutants in strain SH1000. Plasmids to complement the mtI mutations were made by ligating the mtIDARF operon, amplified using mtID_SalI ACCGGGTCCAGC-GAAGTTCCGCAATGTCG with mtIDARF_SalI and ligated prior to directly transforming Liv1019 (RN4220 mtID::aph, selecting for acid production on M1 salt agar containing 5 μg ml⁻¹ chloramphenicol. The selected plasmid, pM(HF1), was purified and used to transform Liv1021 (SH1000 mtID::aph and Liv1019 (SH1000 mtIDARF::aph).

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 BH agar containing millimolar concentrations of AFA, prior to viable counting. Minimum inhibitory concentrations (MIC) of AFA were performed in 96 well plates using ethanol as a solvent.

Table 1. Strains and plasmids used in the study.

<table>
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<th>Features</th>
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<td>Invitrogen</td>
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<tr>
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<td>Top10-pMut4-mtID</td>
<td>This Study</td>
</tr>
<tr>
<td>Liv1011</td>
<td>Top10-pMut4-mtIDARF</td>
<td>This Study</td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
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</tr>
<tr>
<td>SH1000</td>
<td>Functional mld derivative of B325-4 mldA+</td>
<td>Lab strain</td>
</tr>
<tr>
<td>RN4220</td>
<td>Restriction deficient strain</td>
<td>Lab strain</td>
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<tr>
<td>SH1000 mtID::aph</td>
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<td>Liv1027</td>
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<td>Liv1091</td>
<td>RN4220 mlsC::aph pM(KP1)</td>
<td>This Study</td>
</tr>
<tr>
<td>Liv1093</td>
<td>SH1000 mlsC::aph pM(KP1)</td>
<td>This Study</td>
</tr>
<tr>
<td>Liv1098</td>
<td>SH1000 mlsC::aph pM(KP1)</td>
<td>This Study</td>
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<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pLTV1</td>
<td>Temperature sensitive plasmid harbouring Tra77</td>
<td>[24]</td>
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<tr>
<td>pUTIN4</td>
<td>Insertional inactivation vector</td>
<td>[26]</td>
</tr>
<tr>
<td>pO6.131</td>
<td>pML12 derivative, tetracycline resistant</td>
<td>[25]</td>
</tr>
<tr>
<td>pKX3632</td>
<td>Low copy number shuttle plasmid</td>
<td>[27]</td>
</tr>
<tr>
<td>pMKH10</td>
<td>pKX3632 containing mld</td>
<td>This Study</td>
</tr>
<tr>
<td>pMKH17</td>
<td>pKX3632 containing mtIDARF</td>
<td>This Study</td>
</tr>
</tbody>
</table>

DOI:10.1371/journal.pone.0007698.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 ZetaPak Nano (Malvern Instruments) with the detector positioned at a 17° scattering angle. The data were analyzed and interpreted using the associated software. All charges were recorded as the mean of 3 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 suvB23 were respensed 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/pdfs/pm_1.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 suvB23), and LIV1024 (SH1000 suvB23-2::Km) were harvested by centrifugation and washed 3 times in PBS, before being resuspended in 3 ml of PBS. Cells were lyzed 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 30 min at 30°C with 80 µl of methoxime hydrochloride in pyridine (20 mg ml⁻¹) following a 60 min treatment at 50°C with 80 µl MISTFA. Five µl of an internal standard (CS1 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 330°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 70.0 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), WILEY10 (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 MetaPA enrichment pathway analysis web application [31].

Experimental Septic Arthritis

A previously described mouse model of septic arthritis was used to test the in vivo role of mtd 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 Gothenburg, Sweden. All mice were maintained according to the local ethics 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 mtd AFA Survival Mutant

A screen of S. aureus Tn917 library transposants identified multiple clones with greatly reduced survival on BHI agar containing 1 mM linoleic acid (C18:2Δ9,12). In addition to those mutants described previously [6]. DNA sequence determination by arbitrary-primed PCR [6] revealed these clones were identical, with Tn917 inserted in mtd at a nucleotide position 517/1107. The mtdA/B/FD operon encodes the Mtd-specific phosphotransferase system (PTS) transporter (MtdAB) and the operon transcriptional repressor (MtdF). MtdA/B/F encode a 3-acylhydrolase, encoded by mtdB, catalyzes the conversion of Mtd-1-P to fructose-6-P (Figure 1). The mtdD mutant strain SH1000 mtdD/Tn917 selected for further study showed clearly reduced survival on linoleic acid agar compared to its isogenic parent strain (Figure 2). Transduction of suvB23 into S. aureus Newman (LV772; Table 1) identified a proportionately similar reduction in linoleic acid survival in this distinct strain background (data not shown).

Figure 1. Methyl uptake pathway in S. aureus. The mtdA/B/FD operon encodes the Mtd-specific PTS (MtdAB) and the operon transcriptional repressor (MtdF). MtdA/B/F encode a 3-acylhydrolase, encoded by mtdB, catalyzes the conversion of Mtd-1-P to fructose-6-P which enters into the Embden-Meyerhoff and hexosemonophosphate glycolytic pathways.

0611b.3719/journal.pone.0067668.g001

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S. aureus Maltose Utilisation and Survival

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. SuwB24 (SH1000 midD::Tn917) and Liv1023 (SH1000 midD::tet) displayed >500-fold reduced survival on linoleic acid relative to wild type (SH1000). Liv1024 (SH1000 midABFD::tet) and the complemented mutant strain Liv1098 (SH1000 midABFD::tet pMH71).

doi:10.1371/journal.pone.0067998.p002

Culture Phenotypes of mid Mutants

To investigate the role of the midD gene product in host cell physiology and to help explain the mechanism for reduced linoleic acid agar survival, growth of the suwB24 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, phosphorous and sulphur compounds and a variety of amino acids, peptide nitrogen sources, osmolytes and pH ranges [28] identified that reduced Ml metabolism was the only significantly altered phenotype (data not shown).

To confirm the role of the Ml PTS operon in S. aureus cell survival, allelic replacement mutants were generated for midD, Liv1023 (SH1000 midD::tet) and for the entire midABFD operon, Liv1024 (SH1000 midABFD::tet) (Figure 3), using methods described previously [34,39,36]. Two complementation vectors were also generated by cloning the midD gene and the midABFD operon into the low copy shuttle vector pSK363Z, producing plasmids pMH70 and pMH71, respectively. Cloning of the midABFD operon was achieved by transforming ligated products into strain Liv1021 (RN1922 midD::tet) selecting for fermentation on mannitol salt agar (MSA), since cloning of the operon in S. cerevisiae TOP10 was not successful, potentially due to toxicity. Complementation with midD alone did not restore Ml fermentation on MSA due to the absence of a promoter for this halted gene; consequently complementation experiments were performed using pMH71. Culture of Liv1023 (SH1000 midD::tet) and Liv1024 (SH1000 midABFD::tet) on MSA at 37°C demonstrated the inability of these mutants to ferment Ml 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 midABFD::tet pMH71) and Liv1098 (SH1000 midD::tet pMH71) (Figure 5). Transduction of the mid and midABFD inactivations into S. aureus Newman (Liv1027 and Liv1028, respectively) confirmed the absence of Ml fermentation in both mutants (data not shown).

Comparative growth assays of the allelic replacement mutants on linoleic acid agar confirmed that Liv1023 (SH1000 midD::tet) had an AFA growth defect similar to suwB24 (SH1000 midD::Tn917) with greater than 5-log reduction in survival (Figure 5). Similarly reduced levels of survival were observed following growth on agar supplemented with millimolar concentrations of oleic acid (C18:1ω9c) or sapienic acid (C18:1ω9c) (data not shown) demonstrating that inactivation of midD caused reduced survival to multiple AFA. Allelic replacement of the midABFD operon did not impair survival from AFA, in contrast to inactivation of midD alone. Proportionally reduced AFA survival was observed with an midD but not an midABFD inactivation in S. aureus Newman (Liv1027 and Liv1028, respectively; Table 1) [data not shown]. Reduced survival of the midD mutant was fully complemented with the entire midABFD operon present on pMH71 using strain Liv1098 (SH1000 midD::tet pMH71) (Figure 2). The reduced survival of Liv1023 (SH1000 midD::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 midD::tet exhibited a profound growth defect when cultured in broth containing Ml as the carbohydrate source (peptone 10 g L⁻¹, Ml 10 g L⁻¹, beef extract 1 g L⁻¹, NaCl 10 g L⁻¹) (Figure 6A). Substituting the sugar alcohol Ml for the
sugars fructose or glucose restored normal growth, demonstrating the Mi-specific defect (data not shown). *S. aureus* accumulates intracellular Mti following inoculation in the presence of glucose.

To test if this accumulation affected survival from AFAs, the relative survival of exponential cells (OD₆₀₀ = 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 mtIDΔtet) when cultured in BHI broth at 25°C (Figure 5B). This defect was specific to inactivation of mtID but not for deletion of the complete operon. Starvation survival with limiting glucose was not impaired in mti 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 5).

**Analysis of Cellular Metabolites**

A comparative metabolomics analysis was undertaken to identify the intracellular metabolites of exponentially growing cells of strains SH1000, Liv1023 (SH1000 mtIDΔtet) and Liv1024 (SH1000 mtABFDΔtet). This revealed that inactivation of mtID resulted in an accumulation of MtID and MtID-P, the latter being undetectable in both SH1000 and Liv1024 (Table 2 and supplementary table 1). The total relative levels of MtID species were over 20-fold greater in Liv1023 (SH1000 mtIDΔtet) than SH1000. The near absence of MtID in strain Liv1024 supports data that the MtABFDT transporter is the main portal for MtID uptake [31]. Inactivation of mtDFD resulted in the absence of cellular Sorbitol-6-P (Table 2). Further clear differences in metabolite levels were evident in strain Liv1024 (mtIDΔtet) relative to SH1000 and Liv1024 (Table S1).

![Figure 3. Schematic representation of the mtIDABFD locus](image_url)

*Figure 3. Schematic representation of the mtIDABFD locus. Position of the transposon insertion and allelic replacements created during this study.*

doi:10.1371/journal.pone.006798.g003

![Figure 4. Mti fermentation capability of *S. aureus* strains. Mti fermentation is revealed by acid formation and colour change of the pH indicator to yellow. Liv1023 (SH1000 mtIDΔtet) and Liv1024 (SH1000 mtABFDΔtet) do not ferment Mti and this capability was restored by complementation with the entire locus in strains Liv1098 (SH1000 mtIDΔtet pMti1171) and Liv1097 (SH1000 mtABFDΔtet pMti1171). Weak growth of Liv1023 was observed.](image_url)

doi:10.1371/journal.pone.006798.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 mtIDΔtet) (filled squares) and Liv1024 (SH1000 mtABFDΔtet) (open triangles) and Liv1098 (SH1000 mtDFDΔtet pMti1171) were diluted in PBS and equivalent volumes were plated onto the agar. Each experiment was performed three times using triplicate experiments.](image_url)

doi:10.1371/journal.pone.006798.g005
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-ΔΔ) extended beyond AFAcs. MICs for Liv1023 (SH1000 mtlD-ΔΔ) and SH1000 in the presence of a range of concentrations of NaCl, laurel sarcosine, SDS, dichlorophenol and the human cathelicidin LL-37 (Sigma). Liv1023 (SH1000 mtlD-ΔΔ) was observed to exhibit a lower MIC for HCl (1 mM) compared to SH1000 (4 mM) and Liv1024 (SH1000 mtlABFD-ΔΔ) (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).

Virulence of mtlD Mutant

The decreased in vivo AFA survival and reduced H2O2 MIC of the mtlD mutant prompted testing of in virulence compared to theogenic 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 vitro is unknown and the model generates abscesses where AFAcs accumulate [14]. This revealed that SH1000 mtlD did not have reduced virulence, at least under the conditions studied [6,32,33].

Table 1. Sugar alcohols present in S. aureus strains.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>SH1000</th>
<th>Liv1023</th>
<th>Liv1024</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arbutin</td>
<td>116.1 (3.9)</td>
<td>197.5 (19.7)</td>
<td>51.4 (7.9)</td>
</tr>
<tr>
<td>Maltol</td>
<td>472.6 (29.1)</td>
<td>1251.4 (82.5)</td>
<td>3.0 (3.9)</td>
</tr>
<tr>
<td>Mannitol P</td>
<td>ND</td>
<td>8161.3 (119.7)</td>
<td>ND</td>
</tr>
<tr>
<td>Ribitol</td>
<td>240.2 (2.3)</td>
<td>214.8 (7.7)</td>
<td>272.2 (27.3)</td>
</tr>
<tr>
<td>Sorbitol-6-P</td>
<td>149.4 (18.2)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

GC-MS was used to analyze cytopathic fractions from exponential growth phase cells. 11 unique metabolites were compared and chromatograms and mass spectra were evaluated as described previously [8,32] using the MS2 Chemstation (Agilent, Palo Alto, CA, USA) and AMOS (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 egascent pathway analysis web application (http://metpa.meb.unimi.it) (AS). ND, not detectable.
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 characterized 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 AFA is achieved via a variety of surface components [HslA, WTA, SasF] and regulation of peptidoglycan biosynthesis [VpxRS, VrpE], 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 ultimate downpathway increases survival [6], where its various contributions to metabolic versatility and its potential to modify local pH could explain its role.

Determining that an *Md-l-P*-dehydrogenase mutant, but not an *mdABD* transport operon mutant, has greatly reduced survival from AFA implicates the accumulation of *Md-l-P* as being the causative factor. As the most abundant natural hexose, *Md* is a carbon source for metabolically oxidizing and the inducible oxidation of *Md-l-P* generates fructose-6-P for entry into the Embden- Meyerhoff and hexosemonophosphate glycolytic pathways [38,40]. All strains of *S. aureus* accumulate *Md*, despite not all being capable of using it for metabolism during aerobic growth. In *S. aureus* the cellular accumulation of *Md* was identified in resting cells when incubated in glucose or cultured in media without added carbohydrate [38]. *Md* accumulation was proposed to enhance metabolic versatility in *S. aureus*, however its mechanistic role is incompletely understood [41]. Following these studies, such as after exposure to AFA, utilization of the pathway for *Md* conversion to fructose-6-P would regenerate NADH, thereby alleviating the pressure upon regenerating reactions downstream of pyruvate. In our previous studies [8], exposure of *S. aureus* to lactic acid caused downregulated transcription of the *mdABD* locus, which suggests, either that reduced levels of intracellular *Md* is a preferred metabolic state following exposure to AFA, or that lower amounts of *Md-l-P* arising from metabolism (concomitantly regenerating NADH) limited induction of the operon. A potential explanation for the reduced AFA survival of the *mdABD* mutant is its reduced adaptive capacity due to an inability to metabolize *Md*. The near wild-type AFA survival of the *mdABD* operon mutant argues against this *Md* 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 *mdABD* mutant.

Metabolic analysis of the *S. aureus* *mdABD* mutant, when compared to the *mdABD* transport mutant and the parental strain, revealed that 14% of the total *Md* that accumulated intracellularly was not phosphorylated. Since the EIImp mannitol transporter (encoded by *mdABD*) phosphaferizes the imported *Md* and since the *Md-l-P*-dehydrogenase activity is inhibited in the *mdABD* mutant, the conversion of *Md-l-P* to *Md* in the *mdABD* 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 *Md* in *Lactobacillus fermentum* a hypothetically phosphaferizing activity of EIImp was proposed to explain the appearance of *Md* in engineered strains [13]. In the study of *Md* overproducing strains of *L. fermentum* a *Md-l-P*-phosphatase activity was proposed to explain the presence of unphosphorylated *Md*, where *mdABD* was absent, and thus an EIImp activity, could not be present. Analysis of the metabolites of growing cells of the *S. aureus* *mdABD* mutant cultured in BHI broth, when compared to the *mdABD* transport mutant and the parental strain, revealed further differences aside from sugar alcohol content (Table S1). These metabolic changes e.g. aminoacidic acid, 3-phosphoglycerate, hydroxypropanoic acid, heparonic acid and trehalose-6-glycerol, do not indicate a clearly defined mechanistic explanation for decreased AFA MIC.

Growth of the *mdABD* mutant was strongly retarded in media containing mannitol, highlighting the deleterious effects resulting from the likely unrestricted accumulation of *Md*/*Md-l-P*. A direct link between the intracellular accumulation of *Md*/*Md-l-P* and reduced resistance to AFA in *S. aureus* currently lacks an evidence-based mechanism. However, several features of the *mdABD* phenotype could result from a membrane-associated effect. Alcohol has a well-described potentiating mechanism with respect to AFA and their membrane activity [13], since it is capable of solubilizing membrane lipids due to its polarity and lipophility, intracellularly accumulated sugar alcohol, *Md*, might act similarly to potentiate AFA action, since it was demonstrated in this study that *Md* acted synergistically with lactic acid when added extracellularly.
in LBH agar. Two further phenotypes point towards a membrane-specific alteration in the mldD mutant; the reduced growth rate that was observed for the mldD mutant at 25°C and the reduced MIC for H₂O₂ which did not result from differences in catalase specific activity (data not shown). A perturbation in porin permeability at the membrane in consistent with the reduced MICs observed and might arise via Mld potentiating the lipophilic acid by virtue of the polarity and lipophilicity of the acid. Studies with the membrane, but this was not investigated further. No differences were observed between the naphthalene levels in methanol extracts of any of the strains, which might be expected if the intracellular accumulation of Mld altered membrane fluidity (data not shown) [44]. Mld is frequently included in membrane preparations as a stabilising entity, either through direct effects or via osmotic stabilisation. The expression of a bacterial mld in S. marcescens was sufficient to generate a phenotype which was similar to that observed for an mld mutant and was sufficient to rescue the phenotypes of a glycol deficient mutant, producing an increased resistance to high salt and H₂O₂ [45]. Mld is also proposed to function as an osmoprotectant in cells of peptin as well as improving cold tolerance [46].

The observed phenotype of reduced survival in the presence of APAs did not translate to a reduction in virulence in a murine arthritis model or reduced MIC levels to a range of other membrane-active agents. Thus, the changes to cell wall physiology in the mldD mutant are discrete, at least in this model of infection and other disease models, such as skin survival, remain to be tested.

References

Supporting information
Table S1 Metabolites present in S. aureus strains
Metabolites were identified using GC-MS analysis of co-plate fractions from exponentially growth phase cells. 131 unique metabolites were compared and chromatograms and mass spectra were evaluated as described previously [8,92] using the MSD Chemstation (Agilent, Falo Alto, CA, USA) and AMDIS (NIH, Carbondale, 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 MetaPCA enrichment pathway analysis web application. [http://www.metasystems.net/][5]. Not detectable.

Acknowledgments
We thank Ingmar Jost (Göteborg) for technical help with virulence studies.

Author Contributions
Conceived and designed the experiments: JGR JM ZL EJ LNS MJHL.Performed the experiments: JGR JM SLK AU MJHL. Analyzed the data: JGR JM SLK AU MJHL. Contributed reagents/materials/analysis tools: ZL LNS EJ MJHL. Wrote the paper: JM MJHL.

PLOS ONE | www.plosone.org 8 July 2013 | Volume 8 | Issue 7 | e67698
Staphylococci: colonizers and pathogens of human skin

Rosanna Coates*, Josephine Moran** & Malcolm J Horsburgh***

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.

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 [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 [6]. 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.

KEYWORDS
- adhesins
- antimicrobial peptide
- commensal
- desiccation
- fatty acid
- pathogen
- skin colonization
- sphingosine
- Staphylococcus

*Microbiology Research Group, Institute of Integrative Biology, University of Liverpool, Liverpool, Merseyside L69 7QH, UK
**Author for correspondence. Tel: +44 151 795 4189; fax: +44 151 795 4412; m.j.horsburgh@liverpool.ac.uk
***Authors contributed equally.
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 multi-layered 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 cornecytes to complete development of the stratified structure [17]. Cornecytes are attached to one another through corneodesmosomes that, when proteolized, 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 cornecytes. 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 cornecytes of the stratum corneum. Cornecytes 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 [32]. 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 [33].

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 shear 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). Purposive methods for sampling different layers of the skin include skin swab (topmost layer), skin scrape (upper layer) or skin punch biopsy (all skin layers). Zoegweng 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 [34]. 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, Grce 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 [35].

There is good evidence that adhesins contribute differentially during distinct stages of *S. aureus* nasal epithelium colonization. The nonproteinaceous adhesive wall teichoic acid is important during initial colonization [35-37], revealed by the inability of wall teichoic acid biosynthesis mutants to colonize [38], whereas the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) IsdA and CifB were more important for colonization and persistence [39,40]. 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 [41]. This varied repertoire could explain colonization differences between the otherwise similar *S. aureus* and *S. epidermidis*. 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 [42,43]. It is likely that the receptor is the same, since recombinant Aap could block SasG adhesion [44]; however, Aap must be more promiscuous in its binding. This difference could indicate that Aap is adapted for colonization of skin and nare; 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 halos; 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 adhesion 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.

<table>
<thead>
<tr>
<th>Host receptor</th>
<th>Adhesin</th>
<th>Species</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen type I</td>
<td>Cna</td>
<td><em>S. aureus</em></td>
<td>[319]</td>
</tr>
<tr>
<td></td>
<td>Eap/Map</td>
<td><em>S. aureus</em></td>
<td>[331]</td>
</tr>
<tr>
<td></td>
<td>Emp</td>
<td><em>S. aureus</em></td>
<td>[322]</td>
</tr>
<tr>
<td></td>
<td>GenD</td>
<td><em>Staphylococcus epidermidis</em></td>
<td>[333]</td>
</tr>
<tr>
<td></td>
<td>SdrF</td>
<td><em>S. epidermidis</em></td>
<td>[314]</td>
</tr>
<tr>
<td></td>
<td>SdrG</td>
<td><em>Staphylococcus saprophyticus</em></td>
<td>[332]</td>
</tr>
<tr>
<td>Cytokeratin-10</td>
<td>CfrB</td>
<td><em>S. aureus</em></td>
<td>[314]</td>
</tr>
<tr>
<td></td>
<td>IsdA</td>
<td><em>S. aureus</em></td>
<td>[327]</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Aaa</td>
<td><em>S. aureus</em></td>
<td>[326]</td>
</tr>
<tr>
<td></td>
<td>Aid</td>
<td><em>S. aureus</em></td>
<td>[319]</td>
</tr>
<tr>
<td></td>
<td>CfrA</td>
<td><em>S. aureus</em></td>
<td>[348]</td>
</tr>
<tr>
<td></td>
<td>CfrB</td>
<td><em>S. aureus</em></td>
<td>[341]</td>
</tr>
<tr>
<td></td>
<td>Eap/Map</td>
<td><em>S. aureus</em></td>
<td>[332]</td>
</tr>
<tr>
<td></td>
<td>Emp</td>
<td><em>S. aureus</em></td>
<td>[332]</td>
</tr>
<tr>
<td></td>
<td>Foe/SdrG</td>
<td><em>S. epidermidis</em></td>
<td>[342]</td>
</tr>
<tr>
<td></td>
<td>Fod</td>
<td><em>Staphylococcus lugdunensis</em></td>
<td>[243]</td>
</tr>
<tr>
<td></td>
<td>FnbA/FnbA</td>
<td><em>S. aureus</em></td>
<td>[244]</td>
</tr>
<tr>
<td></td>
<td>FnbB/FnbB</td>
<td><em>S. aureus</em></td>
<td>[145]</td>
</tr>
<tr>
<td></td>
<td>IsdA</td>
<td><em>S. aureus</em></td>
<td>[344]</td>
</tr>
<tr>
<td></td>
<td>UalB</td>
<td><em>S. saprophyticus</em></td>
<td>[147]</td>
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<tr>
<td>Fibronectin</td>
<td>Aaa</td>
<td><em>S. aureus</em></td>
<td>[338]</td>
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<td><em>S. epidermidis</em></td>
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<td>Aid</td>
<td><em>S. aureus</em></td>
<td>[329]</td>
</tr>
<tr>
<td></td>
<td>AIFC</td>
<td><em>Staphylococcus caprae</em></td>
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<td>Emp</td>
<td><em>S. epidermidis</em></td>
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<td></td>
<td>Emp</td>
<td><em>S. aureus</em></td>
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<tr>
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<td>Foe/SdrG</td>
<td><em>S. epidermidis</em></td>
<td>[352]</td>
</tr>
<tr>
<td></td>
<td>FnbA/FnbA</td>
<td><em>S. aureus</em></td>
<td>[354]</td>
</tr>
<tr>
<td></td>
<td>FnbB/FnbB</td>
<td><em>S. aureus</em></td>
<td>[344]</td>
</tr>
<tr>
<td></td>
<td>IsdA</td>
<td><em>S. aureus</em></td>
<td>[344]</td>
</tr>
<tr>
<td></td>
<td>SdrF</td>
<td><em>S. saprophyticus</em></td>
<td>[156]</td>
</tr>
<tr>
<td></td>
<td>UalB</td>
<td><em>S. saprophyticus</em></td>
<td>[157]</td>
</tr>
<tr>
<td>Involucrin</td>
<td>IsdA</td>
<td><em>S. aureus</em></td>
<td>[327]</td>
</tr>
<tr>
<td>Keratinocyte lipids/glycolipids</td>
<td>Pls</td>
<td><em>S. aureus</em></td>
<td>[154]</td>
</tr>
<tr>
<td>Loricrin</td>
<td>CfrB</td>
<td><em>S. aureus</em></td>
<td>[357]</td>
</tr>
<tr>
<td></td>
<td>IsdA</td>
<td><em>S. aureus</em></td>
<td>[327]</td>
</tr>
<tr>
<td>Undetermined receptor on desquamated epithelial cell</td>
<td>Aap</td>
<td><em>S. epidermidis</em></td>
<td>[32]</td>
</tr>
<tr>
<td>Undetermined receptor on desquamated nasal cells</td>
<td>CfrB</td>
<td><em>S. aureus</em></td>
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<tr>
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<td><em>S. aureus</em></td>
<td>[154]</td>
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<td><em>S. aureus</em></td>
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<td><em>S. aureus</em></td>
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</tr>
<tr>
<td></td>
<td>SdrG</td>
<td><em>S. aureus</em></td>
<td>[359]</td>
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</tbody>
</table>

In situ studies of staphylococcal adhesions 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 fibrils. Loricrin complexes up to 80% of protein on terminally differentiated keratinocyte cell surfaces; desmoglein-1 and desmocollin-1 are involved in intercellular keratinocyte adhesion, and keratinocyte fibrils form an envelope around the terminally differentiated keratinocyte cell surface. Alternative apossions 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 [35]. Staphylococcal adhesion of canine, feline or bovine origin was 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 [38,39]. 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, Gfa and Gfb [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 lysosome 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 (KAMPs), 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 [42]. 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 defensin dermcidin is independent of both staphylococcal membrane charge and peptide charge; dermcidin inhibits synthesis of RNA and protein [43,44]. The mechanism was proposed to depend on ion channel formation in the bacterial membrane stabilised by zinc ions [44,45].

Lysosome is present in many human fluids and secretions, and is produced by epithelial cells [46]. Many staphylococcal species are resistant to its N-acetylmuramoyl-L-alanine activity, including S. aureus, S. epidermidis, S. hominis and S. lugdunensis. This resistance may be important for colonization of the skin [47]. Muraggression studies determined that the enzyme OatA, which O-acetylates peptidoglycan, is the major factor contributing to lysosome resistance [47]. Moreover, oatA and allA double mutants are highly sensitive to lysosome and other AMPs owing to the additional lack of D-alanine bound to threonine acids [48].

**The RNase A superfamily**

Humans synthesize 13 RNaseA superfamily proteins. 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 (35). Hooper et al. observed that RNase 5 significantly reduced viability of Candida albicans and Streptococcus pneumoniae (82). Although this potency was not easily reproduced by others (84), narrow spectrum bactericidal activity was confirmed (85), as was the capacity of the human epidermis to produce RNase 5 (86). RNase 5 functions under similar physiological conditions, such as pH and salt concentration, as other AMPs (87). 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 (86). Further evidence for the physiological relevance of RNase 7 comes from Simanowski et al. who demonstrated that antibody blocking of RNase 7 activity in skin explants impairs killing activity against S. aureus (86). RNase 7 is cationic with an abundance of lysine residues, and exhibits nucleolytic activity (86).

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 (86). 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 (89). Mutation and NMR analysis of the enzyme identified a flexible cluster of lysine residues integral to ribonuclease, but not antimicrobial activity (86).

**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 ApsS 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 mpfP operons (60,65). The homologous APS system of S. aureus, GraR, has a substitution of a serine for a proline residue in the extracellular loop, leading to altered expression of the dlt and mpfP operons (62). This potentially accounts for the different responses to AMPs by S. aureus and S. epidermidis (61). Sensing and resistance of cAMPs through the GraRS system in S. aureus requires the contribution of GraX and the VraFG ABC transporter (64,65).

MpfP modulates cell surface charge by the addition of lysine to the membrane lipid via lysyl-phosphatidylglycerol synthetase activity (66,67). An mpfP mutant of S. aureus has an increased susceptibility to killing by AMPs (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 (70).

**Secreted proteases**

The induced expression of secreted proteases forms a key defense from AMPs in staphylococci. SepA metalloprotease increases S. epidermidis persistence in biofilms (72). S. epidermidis also upregulates ageR, seeC and sellK in response to the peptide dermaidin (41), which corroborates findings that sepA is under the control of these global regulatory systems (73). 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 (74,75).

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, glutamylinephtidase SpaA (V8), shares both cleavage site and sequence similarity with ET and hydrodyes desmoglein-1 (77). The application of SpaA 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
colonization, and this exoprotein is cytotoxic to primary keratinocytes [88].

Cysteine proteases also impact upon staphylococcal skin persistence. Staphylospain 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 [32,82].

### Competitive exclusion

Several studies have identified species distributions, including the absence of staphylococci from skin or nasal environments colonized by *Propionibacterium acnes* and *Cornebacterium* spp. [31,83]. Intragenic colonization distribution correlations were also shown in several studies for *S. epidermidis* and *S. aureus*, which were infrequently co-isolated from the nasal epithelium [31], although this was not consistent for all studies [84] and might indicate that strain variation is important. These reports support likely exclusion mechanisms between colonizing microorganisms, for *Cornebacterium* spp., a proposed mechanism for *S. aureus* exclusion was competition for nutrients or adhesion receptors [85]. 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 proteases 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 exoprotoxins of staphylococci with antimicrobial activity targeting *S. aureus* and other resident and transient skin microorganisms include the *S. epidermidis*-derived, heat-stable metalloprotease (PSM)-6 and -7 (6-hemolysin), which are active against Group A *Streptococcus*, *S. aureus* and *Escherichia coli* [38,88]. Antimicrobial activity of PSM-6 and PSM-7 was synergistic with each other, with LL-37 and, in the case of PSM-1, with CRAMP, hBD2 and hBD3 [88,89]. PSM-7 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 structures, hemolysis, spreading over wet surfaces and possibly promoting skin colonization [80,90]. 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 (AFA) 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 [91]. Enzymes required for the production of ceramides, the lipid integral to barrier homeostasis, function optimally at acidic pH [92] whereas sAA p
tases, capable of degrading corneodesmosomes and compromising the integrity of the epithelium, are inhibited [94].

Structural organization studies recently demonstrated a highly regular 1:1:1 arrangement of fatty acid, sphingosine and cholesterol into multimellar structures [81]. 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 [81] supports an AFA contribution to colonization inhibition. AFAs differ in their ability to adhere to and form AFA levels on normal skin, and while *S. aureus* isolated from <5% of individuals with healthy skin, it is routinely isolated from atopic dermatitis patients [98-99]. This indicates that AFAs are potentially important contributing factors in the 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 AFA, in addition to other long chain unsaturated fatty acids tested, variably induced protease expression [103].

### AFA defense

Walnolic acids and the MSCRAMP InSA 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 [105], which might contribute to the reduced hydrophobicity in response to AFAs. In addition to InSA, the homologous MSCRAMPs SasF and SscF 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]. SasF complemented a *S. aureus* mssA mutant and clinical isolates of *S. saprophyticus* bearing SasF were more resistant
to linoleic acid (18:2Δ9, Δ12) compared with those without SsaF [107]. No discrete enzyme activity has yet been reported for these proteins.

*S. aureus* biosynthesis of the C16:0 sphingolipid staphylophanthin aids survival from APA-generated membrane disorder [108]. Staphylophanthin also contributes to antioxidant defense and counteracts AMP-mediated membrane fluidity [109,110]. The inactivation of *erid* decreases *S. aureus* survival from APAs and transcriptomic studies revealed upregulated *erid* transcription after linoleic acid (18:2Δ9, Δ12) exposure [111].

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 essential 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. capitis*, *S. hominis*, *S. simulans* and *S. caprae*. 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 APAs. 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 myristic and stearic 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 APA survival include the arginine delinase pathway, VaP perma- nase, and an Mmp-like transporter (SAR2632) (figure 2) [116], 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 sphingo- sine, phosphorylcholine, dihydroxyphosphatidylcholine and hydroxyphosphatidylcholine 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 APAs has been reported [105]. 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 delinase 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 arginine is transported into the cell (ArcD) and is converted by ArcA to NH₃ and citrulline, which is then converted by ArcB to ornithine (exported by ArcD) and carbamoyl phosphate, and is finally converted by ArcC to CO₂ and NH₃, 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* [120] duplicates activity. However, the arginine delinase operon of ACME is constitutively transcribed with markedly increased transcript abundance, compared with the anaerobiosis-induced, core genome operon [122]. Deep *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 [123]. The ACME element promotes acid...
tolerance in USA300 and when planktonic-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 [104]. 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 arc gene 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 [126]. 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 10⁵ Staphylococcus aureus [138]. 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 flaggin immunohistochemistry [112]
  - 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 [105]
  - Human volar forearm colonization/survival model
    - Model used for determining comparative survival of an isogenic, unmarked (ΔS) mutant of S. aureus viable counting of bacteria after inoculation [190]
  - 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 [110]
  - Murine nasal colonization model
    - This model established temporal studies of S. aureus colonization and was used to differentiate survival of isogenic mutants [184]. Use of forficile-deficient mice extended the model to demonstrate the role of CF8 binding to squamous epithelial cells [137]

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**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 producing osmotic stress. Both S. aureus and S. epidermidis can grow at low RH levels. While Bacillus subtilis, E. coli, and Pseudomonas aeruginosa require 92–94.5% RH to grow, S. aureus can grow at 87% RH and 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 change is likely by autolysin controlled [125]. 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 hydrophobic, which aids water acquisition [126]. Desiccation tolerance, or its recovery from being subjected to this stress, requires antioxidant activity (catalase, alkylhydroperoxide reductase), regulatory activity via accessory factor B and the ClpX protease [124].

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].

Ebb is a giant (~1.1 MDa) cell wall-associated protein found in S. aureus and S. epidermidis. Ebb exposed to high salt conditions reveals intravacuoles along their septum within the first 30 min, suggesting that this contributes to initial osmotic stress.
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/GroRS antimicrobial peptide-sensing systems in *S. epidermidis* and *Staphylococcus aureus*, respectively, modulate cell surface charge through increased transcription of the *dit* 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*.

**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...
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 generic 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 Daniel 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-UNIVERSE CASE studentships BB/157421/1 and BB/150078/1 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**

Depts of special note have been highlighted as:

*•* of interest

**•** of considerable interest


• Uses a metagenomics approach to explore recognition following wounding of the stratum corneum and the role of the microbiota at deeper skin layers.


• Investigates microbial diversity at different body sites using 16S ribosomal RNA gene sequences.


• Human microbiome population data identify temporal and spatial variation using next-generation technology.


Staphylococcal colonizers & pathogens of human skin


24. Use very high magnification cryosection microscopy to elucidate the arrangement of skin lipids into a stacked bilayer conformation.


- Draws a link between high baseline expression of RNase 7 and protection from Staphylococcus aureus infection.


- Investigates the regulation of GraRS and its role in sensing cationic antimicrobial peptides through activation of the all-sperme and mprF.


- Identifies the role of S. aureus proteases in disrupting the protective surface of the skin.


- Discusses the role of proteases in bacterial defense against antimicrobial peptides on the skin.


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- Details the characterization of the microbial surface components recognizing adhesive matrix molecules (SCRAM) ligand, a cell wall anchored protein of *Staphylococcus aureus* that promotes leukocidic acid survival.


98 Discusses the various modes of action of anticancer-free fatty acids and focuses on their commercial and biotechnological uses as an alternative to antibiotics.


102 Confirms that unsaturated fatty acids cause protein leakage from S. aureus and identifies the mode of action of antimicrobial fatty acids.


106 Discovering that InA mediates surface hydrophobicity to increase skin survival from lipid matrix components.


108 Transcriptomic and proteomic approaches suggest that antimicrobial activity of unsaturated long-chain fatty acids involves cell membrane disruption.


118 Reports enzyme activity for hydratase that contributes to virulence and fatty acid modification.


121 ** Explores the implications of angiostatic cellular mobile elements (ACME) in the physiology of Staphylococcus epidermidis, concluding that ACME do not result in this organism being more harmful.


123 ** 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 polymers.


126 ** Reports the morphological changes of bacteria under humidity growth limits.


128 Examine the water activity of various bacteria including the skin resident St. epidermidis and discusses morphological changes resulting from nearing the water activity growth limits.


133 ** Outlines the involvement of cellulose in the survival of S. aureus under long-term high-salt conditions.


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147 King N, Beaman S, Tesnica M et al. UAB is a serine-rich repeat adhesin of Staphylococcus saprophyticus that mediates binding to fibronectin, fibronectin and human urethral cells. Microbiology 157, 1165–1179 (2011).


162 Damage to the skin surface by tape-stripping reduces barrier defense, increasing S. aureus persistence and expression of proinflammatory cytokines.

