UNIVERSITY OF LIVERPOOL

Staphylococcus aureus survival mechanisms from skin antimicrobials

Thesis submitted for the degree of Doctor of Philosophy by Munirah Aldayel

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Declaration

I declare that this thesis entitled ***Staphylococcus aureus*** **survival mechanisms from skin antimicrobial** is my own work, and that neither the whole nor part has been submitted to another university.

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April 2015

Abstract

S.aureus is highly adaptable to environmental conditions and has the ability to colonise and infect a range of tissues within the host. The ability to colonise skin requires survival mechanisms to counter an array of abiotic factors that includes epidermal and sebaceous skin lipids. This study sought to investigate the effects of cholesterol, an epidermal lipid produced in substantial quantities, on *S. aureus* growth and survival. Previous studies have reported that cholesterol addition reduces the growth inhibition of antimicrobial fatty acids (AFAs) and this phenomenon was investigated further to identify the underlying mechanism.

The addition of ethanol-solubilised cholesterol to broth cultures of *S. aureus* increased bacterial survival in the presence of growth inhibitory levels of linoleic acid and the lipid sphingosine. This effect was confirmed in strains SH1000 and Newman. The pigmentation of *S. aureus* when grown in the presence of ethanol-solubilised cholesterol was greatly reduced. Initially this study focused on these effects being mediated by cholesterol, however ethanol concentration was not controlled effectively when designing the experiments and ethanol could also be the major mediator of pigmentation changes. It was initially hypothesised that cholesterol would affect *S. aureus* cell membrane properties since it is known to be incorporated when added extracellularly. From this hypothesis, studies were designed to examine factors controlling survival and pigmentation changes in response to ethanol-solubilised cholesterol.

Separate screens of transposon libraries were performed to identify mutants that: i) produced pigment in the presence of ethanol-solubilised cholesterol; ii) did not show enhanced growth with ethanol-solubilised cholesterol supplementation in the presence of growth-inhibitory levels of linoleic acid. The majority of the transposon mutants identified and localised using arbitrary-primed PCR sequencing revealed insertions into genes previously associated with modulating activity of the accessory sigma factor σ^B. Subsequent experiments with ethanol controls indicated a clear solvent effect on pigment expression, confounding the previous hypothesis and contrary to published reports about the activation of σ^B.

To investigate the effects of ethanol and ethanol-solubilised cholesterol on pigment expression and σ^B activity a series of qRT-PCR assays were established. In the presence of ethanol-solubilised cholesterol the expression of *crtM* was decreased, whereas, extracellular protease, *aur* and *sspA* expression were increased in the presence of 0.3 mM ethanol-solubilised cholesterol. However the effect of ethanol, as a solvent control, was substantial resulting in decreased transcription of *sigB* and *crtM* while correspondingly *aur* and *sspA* transcription were increased.

These data from the study of ethanol and ethanol-solubilised cholesterol identify novel effects on the cell membrane of *S. aureus* that require further study to dissect the individual roles of each component and indicate that current literature reports of σ^B activity and regulation might be incomplete.

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There have been many late nights and early mornings, and after all the work I can finally conclude that the thesis is finished. Through all of this there have been several helpful people I would like to thank for their support and encourage.

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Abbreviations

BHI	Brain heart infusion
BLAST	Basic local alignment search tool
DIGE	Difference gel electrophoresis
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
et al.	*Et alia* (and others)
Fig.	Figure
LB	L broth
MW	Low molecular weight
LTA	Lipoteichoic acid
PAGE	Polyacrylamide gel electrophoresis
dH_20	Deuterated water
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Tet	Tetracycline
Lin	Lincomycin
Kan	Kanamycin
Ery	Erythromycin
OD	Optical density
LA	L agar
Tris	Tris (hydroxymethyl) aminomethane
WT	Wild type
σ	Sigma factor
Tn	Transposon Library
VLEC	Very low ethanol concentration
SAML	Skin antimicrobial lipids
AMP	Antimicrobial peptides
AFAs	Antimicrobial fatty acids
LTA	Lipoteichoic acid
LPS	Lipopolysaccharides

IsdA	Iron-responsive surface determinant A
FAME	Fatty acid modifying enzyme
NsaRS	Nisin susceptibility-associated response regulator and sensor
GraRS	Glycopeptides resistance associated-regulator and sensor
TCSTs	Two-component signal transduction system
HK	Histidine kinase
RR	Response regulator
TSST-1	Toxic-shock syndrome toxin 1
Sar	Staphylococcal accessory regulator
Sae	*S. aureus* exoprotein expression
MRSA	Methicillin resistant
PBPs	Penicillin-binding protein
PDT	Photodynamic therapy
DAP	Daptomycin
TMP-SMX	Trimethoprim/sulfamethoxazole
CHOL	Cholesterol
CER	Ceramide
i.e	*Id est* (that is)
rpm	Revolutions per minute

Units

%	Percentage
µg	Microgram
µl	Micro litre
µM	Micro molar
bp	Base pair
cfu	Colony forming units
Ml	Milliliter
G	Gram
H	Hour
V/V	Volume/volume
MIC	Minimum Inhibitory Concentration
l	Litre
M	Molar
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
Mw	Molecular weight
F	Fold
nm	Nanometre
W/V	weight for volume
rpm	Revolutions per minute
sec (s)	Second
ºC	Degree Celsius

CHAPTER 1:

1. General Introduction

1.1 The staphylococci: human and animal colonisers

All over the human body the indigenous microbiota (bacteria, fungi, and viruses) colonises surfaces, particularly the gastrointestinal tract and to a lesser degree the skin. This colonisation can take the form of three types of symbiotic interaction between the host and the microbes; mutualism which is useful for both; commensalism where one benefits and the other is not affected, and parasitism where the relationship is beneficial for one but harmful for the other, being pathogenic to the host (Wilson, 2008).

Staphylococci can be considered a good model to study their host and symbiotic relationship. The majority of the staphylococci successfully colonise their host commensally and are therefore considered as non-pathogenic; these species are coagulase-negative. Approximately 20 % of healthy humans are determined carriers of *S. aureus*, that is, they have the bacteria on their skin without any active infection or disease (known as colonisation only) and this originates from the carrier site in the nostrils.

A small proportion of staphylococci can be harmful to their human and/or animal host by changing the symbiotic relationship from commensal to parasitic (Morgan, 2008) and become pathogenic to their host. For example, *S. aureus* is known to colonise and infect both livestock and pets, including cats, rabbits, dogs, horses, cattle and pigs (Morgan, 2008). The pathogenic staphylococcal strains have the ability to clot the blood plasma, hence known

as coagulase-positive. Coagulase is an extra-cellular enzyme, which alters plasma fibrinogen to fibrin (Kloos and Musselwhite, 1975, Kloos and George, 1991, Witte, 2006). There are two coagulase-positive species distinguished as animal colonisers and pathogens which are *S. intermedius* and *S. hyicus*, and the *S. aureus* strain which is known in particular is a human pathogen but also infects dairy cattle (Kenneth, 2008).

Staphylococci are Gram-positive (retaining the crystal violet of the Gram stain), due to the presence of cytoplasmic membranes bordered by thick cell walls (Neuhaus and Baddiley, 2003). These bacteria form irregular clumps, and are usually micro-capsulate, and taxonomically belong to the family of *Staphylococcaceae* with approximate cellular diameters of 0.5 – 1.5 μm; they are facultative anaerobes, which can grow by fermentation as well as by aerobic respiration. The cell walls of staphylococcal bacteria are characterised by the presence of glycine cross-links in their peptidoglycan and therefore this makes them sensitive to the lytic enzyme lysostaphin (Fig1.1). Staphylococci are distributed widely in the environment and are easily found in the water, soil, sewage, and air. They adapt well to the surrounding environment, and tolerate high concentrations of salt (up to 1.7 M), and are typically heat-resistant up to 50 °C for nearly half an hour (Kloos and George, 1991, Wilkinson, 1997, Singh et al., 2012).

The mucous membrane, the skin, and skin glands of warm-blooded mammals are the natural habitat of the staphylococci, with limited presence in the upper respiratory tract and, mouth (Kloos and George, 1991). The nutrient needs of the staphylococci are generally not specific; however, some

vitamins of group B and some other essential amino acids are commonly important for their growth (Wilkinson, 1997, Harris et al., 2002b).

1.2 *Staphylococcus aureus*

S. aureus is the most characterised and well-studied species of the genus. The golden appearance of the colonies of the *S. aureus* species when grown on BHI agar led to the species name. The genome of *S. aureus* has an average size of around 2.8Mb (Kuroda et al., 2001). *S. aureus* is protected by a strong and thick (around 20 nm-40 nm) cell wall, where peptidoglycan is the main component, and located below the cell wall is the cytoplasmic membrane (Shockman and Barrett, 1983).

Teichoic acid, a phosphate-containing polymer of polysaccharides is an essential part of the cell wall and constitutes around 40 % of the mass of the *S. aureus* cell wall (Fig 1.1). There are two forms of teichoic acids: cell membrane-associated lipoteichoic acid, which is anchored in the cytoplasmic membrane, and cell wall teichoic acids, which are covalently bonded to the peptidoglycan N-acetyl muramic acid. Teichoic acids are partly responsible for the negative charge of the cell surface, due to the presence of the negatively charged acidic polysaccharides (Neuhaus and Baddiley, 2003, Matias and Beveridge, 2006). Teichoic acids act to efficiently facilitate the transfer of proteins, ions, nutrients and antibiotics via the cell wall, and in particular teichoic acids play an essential role in metal ion homeostasis. These unique structures provide elasticity, a physical barrier and modulate permeability (Neuhaus and Baddiley, 2003, Schaffer and Messner, 2005).

Teichoic acids are significant contributors to bacterial pathogenicity due to their ability to directly interact with host receptors for example, research studies using S. *aureus* deficient in teichoic acid have revealed significantly higher rates of sepsis and septic arthritis in mouse infection models, indicating a crucial role of teichoic acids in the virulence of *S. aureus* (Neuhaus and Baddiley, 2003).

Clinical strains of *S. aureus* show that around 90 % have the ability to produce polysaccharide capsules, which leads to decreased phagocytosis in *vitro*, and this contributes to the enhancement of S. *aureus* virulence (Wilkinson and Holmes, 1979, Thakker et al., 1998).

Until recently, *S. aureus* was observed as an completely extracellular pathogen, however, research evidence has shown it survives with macrophages and neutrophils, and has the ability to infect non-professional phagocytes such as epithelial cells and endothelial cells, promoted by fibronectin-binding proteins forming a fibronectin bridge to the α5β1 integrin on the host-cell surface. This increases resistance to killing by antimicrobial peptides (Schwarz-Linek et al., 2003, Schwarz-Linek et al., 2004, Peacock et al., 1999). *S. aureus* is capable to grow and persist by adherence to host tissues or prosthetic materials (Donlan and Costerton, 2002). *S. aureus* forms biofilms on these surfaces, allowing it to evade host responses and antimicrobial agents (Donlan and Costerton, 2002).

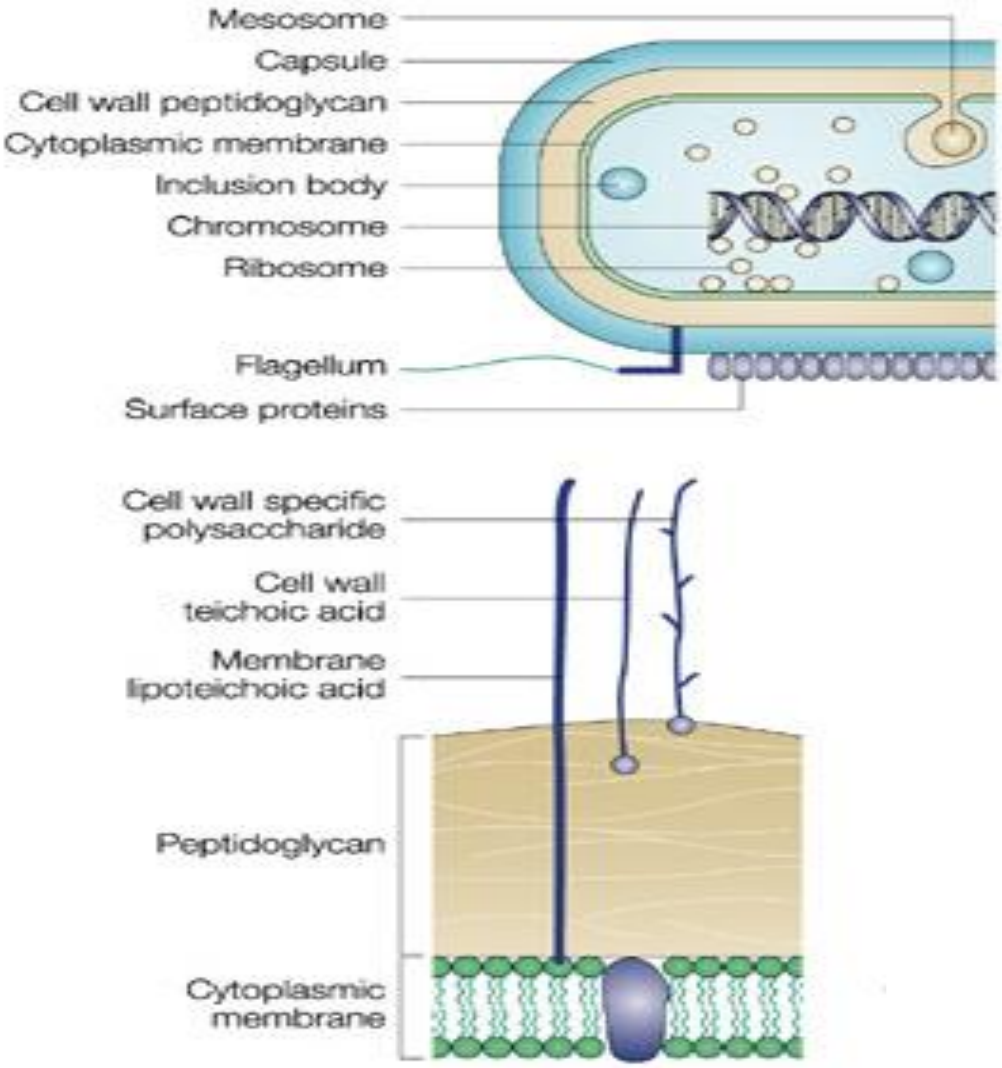


Figure 1.1. The structure of Gram-positive bacteria cell wall. This structure shows bacterial cell wall, which consists of peptidoglycan and Teichoic acid (Lolis and Bucala, 2003).

1.2.1. Virulence factors (determinants) of *S. aureus*

The term virulence refers to the degree of interaction that a pathogenic bacterium has with its host and the properties that can enable to establish colonisation and enhance the potential to cause and maintain disease via certain strategies; adhesion to the tissue surface, evading the host natural and immune responses, and invading and penetrating the target tissues and organs (Mekalanos, 1992). *S. aureus* expresses many cell surface-associated and secreted virulence factors, comprising proteins that perform specific transient functions including proteins that allow the bacteria to evade immune recognition and killing during different stages of infection. For example, the expression of the exo-toxin coagulase enzyme, which promotes blood clotting, provides protection from multiple components of the innate and acquired immune responses to enable it to disseminate the infection. *S. aureus* secretes extracellular enzymes such as hyaluronidase, lipases, proteases, and a nuclease that facilitate invasion and the destruction of tissue and spreading to other site of infection, as well as membrane-damaging toxins that cause cytolytic impacts on host cells and tissue harm (Dinges et al., 2000).

Genome sequencing and annotation of *S aureus* strains indicate around 120 virulence factors (Kuroda et al., 2001). Virulence factors are thought to function according to the requirements of the stage of the infection (Fig 1.1), where in the infection the factors that promote and facilitate attachment to the host cells and extracellular matrices are expressed and function early, while on the other hand factors that are associated with invasion of the tissues and evasion of the host responses are known to function during

advanced stages of the infection (Table 1.1 shows a list of virulence factors and their contribution stage for pathogenicity). Furthermore, it is most likely that some virulence factors are not limited for one particular role and therefore they may perform more than one function. The ability of *S. aureus* to adapt to ecological niche changes is important for growth and survival, and therefore the expression of the virulence factors can also be understood within this context, rather than for pathogenicity alone.

Table 1.1. Summary list of the *S. aureus* virulence factors expressed during different stages of host infection.

Virulence factors of *S. aureus*	Production during growth phase	Gene	Reference
1-Determinants of adhesion (Attachment) stage:			
Polysaccharide intracellular adhesin	Lag phase	*ica*	(Dobinsky et al., 2003)
Polysaccharide PS/adhesion A	Lag phase	*icaA*	(Gill et al., 2005)
Collagen-binding protein	Lag phase	*cna*	(Gill et al., 2005)
Collagen-binding protein	Lag phase	*sej*	(Jarraud et al., 2002)
Coagulase	Lag phase	*cga / coa*	(Moreillon et al., 1995)
Fibronectin-binding protein A	Lag phase	*fbpA*	(Chaffin et al., 2012)
Fibronectin-binding protein B	Lag phase	*fnbB*	(Gill et al., 2005)
Fibronectin-binding protein	Lag phase	*bpA*	(Finlay and Falkow, 1997)
Clumping factor	Lag phase	*clfA*	(Moreillon et al., 1995)
Putative adhesins	Lag phase	*sdrE*	(Sitkiewicz et al., 2011)
2- Determinants of evasion of host responses stage:			
Protein A spa	Lag phase	*spa*	(Gill et al., 2005)
Staphylococcal exotoxin-like proteins	x -Un-identified	*set* 1-5	
Enterotoxin A	Produced at constant rate in all stages (constitutively)	*sea*	(Gill et al., 2005)
Catalase	Post-exponential phase	*katA*	(Harris et al., 2002a)

Staphylokinase	Post-exponential phase	*sak*	(Gill et al., 2005)
Capsular polysaccharide type 8	Post-exponential phase	*cap8 locus*	(Gill et al., 2005)
Capsular polysaccharide type 5	Post-exponential phase	*Cap5 locus*	(Gill et al., 2005)
Capsular polysaccharide types 1	Post-exponential phase	*Cap1 locus*	(Gill et al., 2005)
Panton-Valentine leukocidin	Post-exponential phase	*lukPV, lukS-PV*	(Gill et al., 2005)
Fatty acid modifying enzyme (FAME)	Post-exponential phase	*fme*	(Chamberlain and Imanoel, 1996)
V8 protease (serine protease)	Post-exponential phase	*sspA*	(Gill et al., 2005)
Lipase	Post-exponential phase	*geh*	(Gill et al., 2005)
Exfoliative toxins A, B	Post-exponential phase	*eta, etb*	(Gill et al., 2005)
Toxic shock syndrome toxin-1 (TSST-1)	Post-exponential phase	*tst*	(Gloria Paniagua-Contreras, 2012)
Enterotoxins B, C 1-3, D, E, H	Post-exponential phase	*seb-h*	(Gloria Paniagua-Contreras, 2012)
3- Determinants of the host invasion and tissue destruction stage:	Post-exponential phase		
Hyaluronidase (hyaluronate lyase)	Post-exponential phase	*hysA*	(Gill et al., 2005)
Phospholipase C	Post-exponential phase	*plc*	(Goldstein et al., 2012)
Cysteine protease	Post-exponential phase	*sspB*	(Gustafsson and Oscarsson,

			2008)
Metalloprotease (aureolysin)	Post-exponential phase	*aurA*	(Gustafsson and Oscarsson, 2008)
y-haemolysin (y-toxin	Post-exponential phase	*h1gA, B, C*	(Gloria Paniagua-Contreras, 2012)
S-haemolysin (8-toxin)	Post-exponential phase	*hId*	(Gill et al., 2005)
ß-haemolysin (ß-toxin	Post-exponential phase	*hlb*	(Gill et al., 2005)
α -haemolysin (α –toxin	Post-exponential phase	*hla*	(Jarraud et al., 2002)

1.2.2. Regulation of the production of *S. aureus* virulence factors

The regulation of virulence is a complex process due to parameters such as host and environmental signals, growth phase dependency of virulence factors, and the redundancy in adhesions, exotoxins and proteases. The pattern of expression of *S. aureus* virulence factors is fully coordinated with the development of infection (Fig 1.2) and therefore it is precisely controlled and genetically mediated by global regulatory elements.

The expression of virulence factors that are more often associated in the attachment to host tissues (surface-associated proteins) is up-regulated through early exponential growth phase, whereas in the late exponential growth phase is down-regulated sharply. However, extracellular virulence factor expression, which is mainly involved in tissue destruction (required for nutrient up-take), and the evasion of host immune and natural responses is only up-regulated during both the post-exponential phase and the stationary phase (Cheung et al., 2004, Coleman et al., 1978). The ability of *S. aureus* to change the expression of virulence factors from one category during lag phase early growth (e.g. surface-associated proteins) to another during post-exponential phase (e.g. extracellular virulence factors) generally indicates the presence of a genetic switch. On the contrary, the expression of *S. aureus* coagulase, which is produced during logarithmic growth, and the enterotoxin A, which is produced constitutively, seems to be both exceptionally expressed highly (Tremaine et al., 1993). The majority of *S. aureus* extracellular proteins (which are important during pathogenesis) are

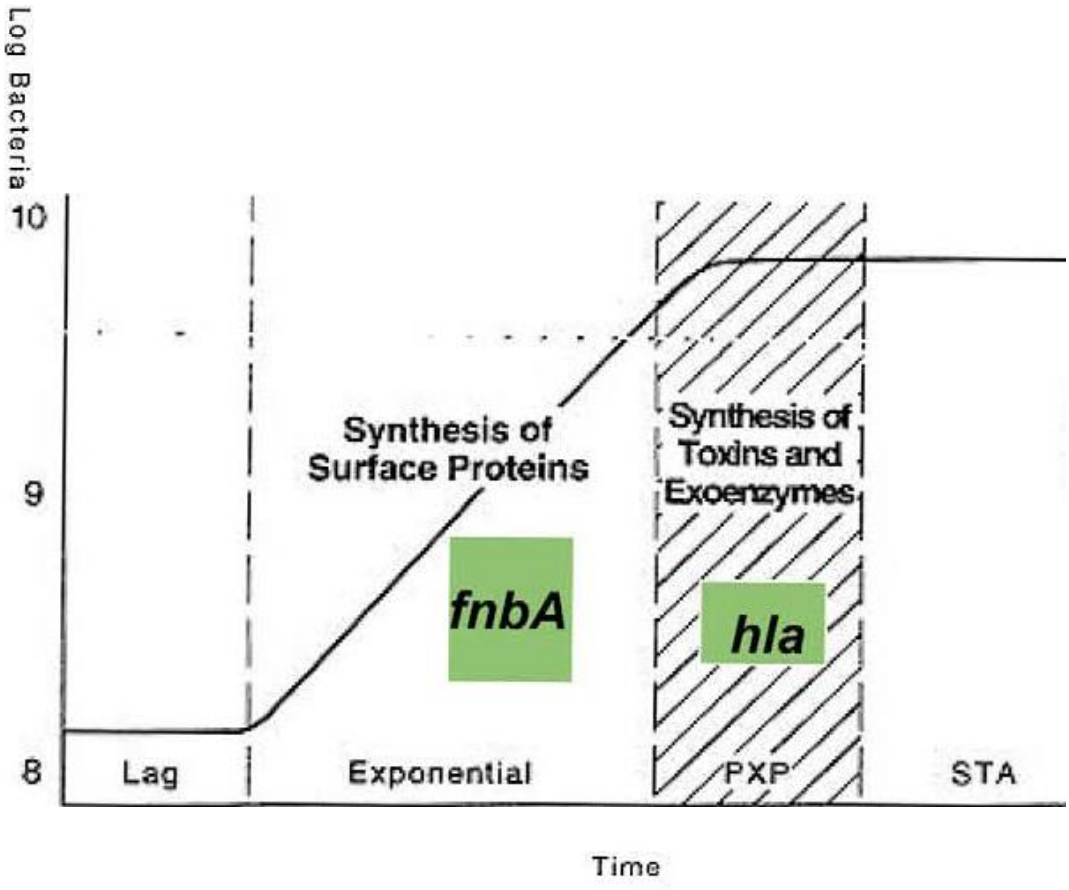


Figure 1.2. Hypothetical model of virulence factor expression in *S. aureus* infections. Initiation of infection during the lag phase, and the multiplication begin upon entering exponential phase where synthesis of virulence factors enables attachment to host cells (surface proteins). During the post-exponential phase (PXP) the activation of a density-sensing mechanism stimulates extracellular virulence factor production (resulting in the production of toxins and exoproteins) to allow the escape of *S. aureus* from the localised infection (abscess) during stationary (STA) phase and metastasis to new sites, enabling the cycle to be repeated. For example, cell surface adhesins such as fibronectin binding protein A-gene (*fnba*) are expressed during the exponential phase and the alpha-haemolysin toxin-gene (*hla*) are expressed during post-exponential phase (Cheung et al., 2004).

synthesised at the end of the exponential phase of growth in laboratory cultures (Abbas-ali and Coleman, 1977).

Bacterial growth and survival is competitive and mainly depends on the adaptability to environmental changes. In general, only small percentages of bacterial genes are considered housekeeping genes (often expressed constitutively), which are essential for growth and survival. However the majority of the genes are termed accessory, and are considered to be non-essential for growth and survival, and this includes *S. aureus* virulence factor expression, which is only advantageous during the infection periods. This accessory gene expression will normally divert the metabolic supplies away from the expression of the housekeeping genes. Therefore, a bacterial regulatory system has evolved to enable gene expression to occur only when required (Cooper and Feil, 2006).

1.2.2.1. The accessory gene regulator (*agr*) locus

The gene locus termed *agr* (accessory gene regulator), because the fact that *S. aureus* virulence factors are considered non-essential for growth and survival, is also a quorum-sensing gene cluster that up-regulates creation of secreted virulence factors and down-regulates production of cell-associated virulence factors in a growth phase– dependent manner (Lyon et al., 2000, Ji et al., 1997). Quorum-sensing is a form of cell-to-cell communication, which allows bacterial cells to migrate towards better nutrient supplies or adopt new modes of growth, such as biofilm formation for defence from the environment and antimicrobial agents (Yarwood and Schlievert, 2003). The *agr* system was identified to positively affect of other virulence factors expression,

including; the exoproteins associated with toxic-shock syndrome toxin 1 (TSST-1), enterotoxins (B, C, D), haemolysins (α,β,γ,δ), and lipase (Peng et al., 1988, Janzon and Arvidson, 1990, Recsei et al., 1986). The *agr* also blocks the production of many cell surface-associated adhesins, including surface protein A and fibronectin binding protein A (Tegmark et al., 2000, Novick, 2003, Novick et al., 1993).

The *agr* locus comprises of two divergently transcribed operons, RNAII and RNAIII (Fig 1.3). RNAII comprises the *agr* BDCA genes that encode the signal transducer (*Agr C*) and response regulators (*AgrA* and *AgrB* and *AgrD*) which modulate transcription and generate the quorum-sensing signal molecule (de Kievit and Iglewski, 2000). The RNAIII operon encodes delta-haemolysin and functions as a small regulatory RNA that plays a important role in the *agr* response (de Kievit and Iglewski, 2000). During *S. aureus* quorum sensing the *Agr C* signal transducer is auto-phosphorylated in response to the octapeptide signal molecule, known as auto inducing peptide (AIP), which leads to phosphorylation of the *agr* A response regulator (Ji et al., 1995). Thereafter, phosphorylated *agr* A stimulates transcription of RNAIII which, in turn up-regulates expression of the *agr* regulon (de Kievit and Iglewski, 2000).

The importance of *agr* in pathogenicity was confirmed by the fact that *agr* mutants have massively attenuated virulence in several animal models of infection, including osteomyelitis, mastitis and arthritis (Projan et al., 1989).

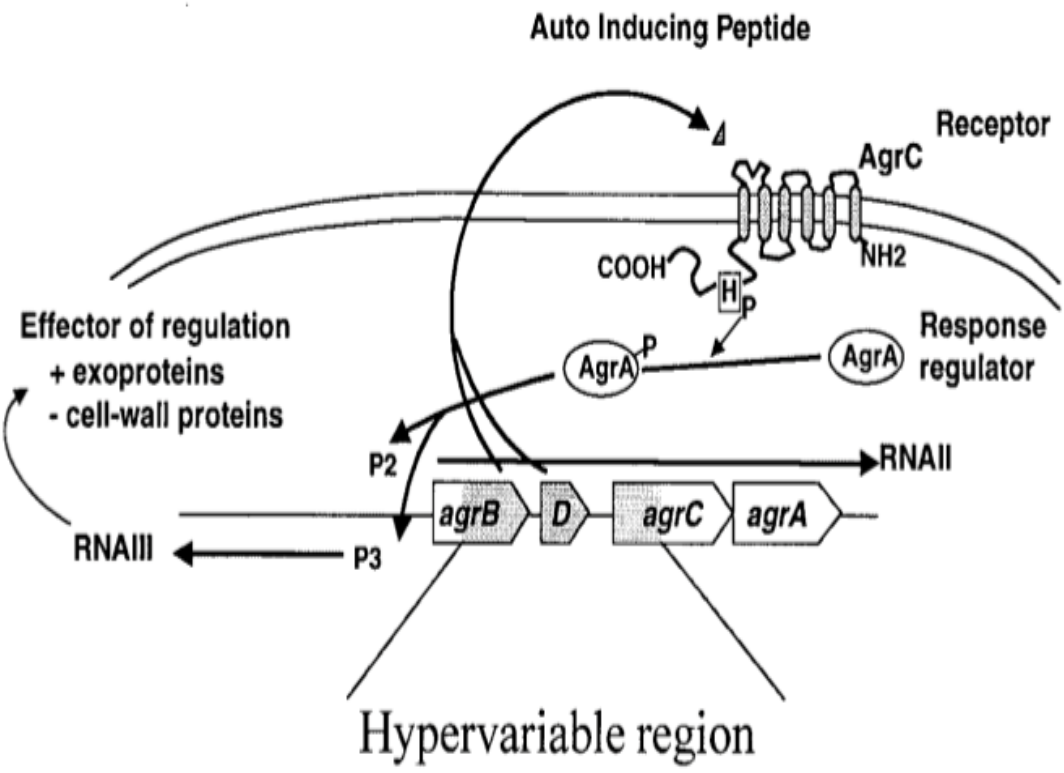


Figure 1.3. Schemetic of agr-depent regulation. The main role of the P2 operon is to control RNAIII transcription in a cell density dependent manner. AIP is auto-inducing peptide (Dufour et al., 2002)

Among staphylococci the *agr* system is conserved (Ji et al., 1997). However, a regional variation in the sequences of *agr B*, *agr C* and *agr D* was reported, together with highly variant AIP molecules. Based on the type of AIP that they produce*, S. aureus* strains can be divided into four groups. The AIP from one group can suppress the expression of *agr* in the other groups. This process is collectively known as bacterial interference, which is a suggested mechanism for isolating bacterial populations during *S. aureus* competition (Ji et al., 1997, Robinson et al., 2005).

1.2.2.2. The staphylococcal accessory regulator (*sar*) locus

The *sar* gene locus comprises three overlapping genes (A,B, and C), that originate from P1, P2, and P3 promoters respectively (Manna et al., 1998). During the *S. aureus* growth cycle the expression of these transcripts changes such that during exponential growth phase *sar A* and *sar B* are transcribed whereas during the post-exponential growth phase *sar C* is transcribed (Manna et al., 1998). The binding of the *sar* gene product SarA to the promoter of *agr* can partially mediate the activation of *agr*. A research study has also revealed the essential role of the *sar B* transcript for the full transcription of RNAII and RNA III of the *agr* locus (Manna et al., 1998). *SarA* as a transcription factor has been found to directly contribute to the formation of *S. aureus* biofilm mechanism through or linked to the production of polysaccharide intercellular adhesin (PIA) via enhancing *ica* operon transcription (Valle et al., 2003).

*Sar*A activates the expression of haemolysins (α-, β-, and δ–), TSST-1 and fibronectin binding protein A, while in contrast, the expression of serine

protease, metalloprotease, collagen adhesin and protein A is repressed (Wolz et al., 2000, Cheung et al., 1994, Chan and Foster, 1998, Cheung et al., 1997). Several studies in animal models of infection have confirmed the importance of *sar*A in virulence (Booth et al., 1997, Cheung et al., 1994, Gillaspy et al., 1995, Nilsson et al., 1997).

1.2.2.3. *S. aureus* exoprotein expression (*sae*) locus

The *sae* locus was discovered following the isolation of a *Tn551* mutant that displays reduced β-haemolysin activity (Giraudo et al., 1994). Subsequently, insertional inactivations of this locus revealed diminished production of DNase, protein A, α- and β-haemolysin, and coagulase (Giraudo et al., 1994, Goerke et al., 2001b). Pathogenicity experiments revealed slower mortality rate in mice for a *sae* mutant that loss of function compared to its isogenic parent (Giraudo et al., 1996).

1.2.3. Diseases and infections caused by *S. aureus*

S. aureus is frequently described as an opportunistic, versatile pathogenic bacterium because it is capable of causing a broad spectrum of human infections. *S. aureus* infections can be divided into three groups: superficial lesions, (e.g. small skin abscesses and wound infections, impetigo, cellulitis), systemic and potentially life-threatening conditions (e.g. pneumonia, endocarditis, osteomyelitis, meningitis, brain abscesses, and bacteraemia), and toxinoses (e.g. food poisoning, toxic shock syndrome, and scalded skin syndrome). The typical characteristic of *S. aureus* infection is the formation of abscess, containing pus which consists of living and dead bacteria, dead neutrophils, contents of lysed host and bacterial cells, and necrotic tissue

(Williamson et al., 2014). In most cases immunocompetent hosts will successfully clear the infection and drain the abscess, however, with immunocompromised individuals and some healthy hosts, the infection may progress into deeper tissues and become a potentially determinant invasive infection (Maddocks, 2014). Currently and for several decades, the most concerning issues of *S. aureus* infections is the methicillin resistant (MRSA) strains. *S. aureus* is the fourth most frequently isolated pathogen in hospital environments (Liu et al., 2011). *S. aureus* is also a major cause of community-acquired infections (e.g. skin and soft tissue infection). Following invasive hospital techniques, infection with *S. aureus* is a common complication, as skin damage enables *S. aureus* entry into the body. *S. aureus* was identified as the main cause of postoperative wound infections in 28 % of cases (Giacometti et al., 2000). Moreover, *Staphylococcus* frequently infects medical devices such as catheters and central venous lines (Otto, 2009) .

MRSA-related infections are mainly spread from person to person by direct physical contact with the skin, clothing, or an area (e.g. chair, sink, bench, and bed) that had current physical contact with a MRSA-infected person. A nosocomial infection is an infection whose improvement is favoured by a hospital environment, such as one obtained by a patient through a hospital visit from hospital staff. It can be caused by different microorganisms including, bacterial, viral, and fungal pathogens; the most common sorts of these infections are bloodstream infection, pneumonia, urinary tract infection, and surgical site infection. The fact that *S. aureus* can be carried by up to nearly 50 % of healthy individuals (Frank et al., 2010) has been an important

factor in the spread of *S. aureus*-associated diseases by hospital workers. The anterior nares are the main site of carriage, although *S. aureus* is also found on the skin and in faeces of carriers (Kloos and Bannerman, 1994). The capability of *S. aureus* to survive on abiotic surfaces, and to resist common disinfectants has also played an important role in allowing *S. aureus* to become a major cause of nosocomial infection.

In surgical site infections, *S. aureus* can evade host immune responses and the antibiotic treatment by forming a biofilm (conglomeration of microbial cells in a sessile multicellular community) on prosthetic surfaces; this type of infection is very difficult to eradicate (Bryers, 2008, Cucarella et al., 2001). Antimicrobial resistance rates in *S. aureus* are significantly higher among hospital-acquired infection isolates than for community-acquired infection isolates, indicating that there is a resident microflora in hospitals and that the hospital isolates are epidemiologically distinct from community isolates. In addition, these strains found an ecological niche in the hospital environment and are simply spread from doctor to patient and between patients (Livermore, 2000). The primary mechanism of host defence against *S. aureus* infection is the mucous membranes and intact skin. Therefore, patients who have acute or chronic disorders that disturb the integrity of the skin and mucous membrane are at a high risk for infection. Thus, inserted devices such as cardiac pacemakers, and accidentally or surgically generated wounds, as well as burns, also present an increased risk. Moreover, patients with disorders of neutrophil function or neutropenia are at an increased risk for *S. aureus* infection, due to the fact that neutrophils play a key role in the host immune response to *S. aureus* (Maddocks, 2014).

1.2.4. Antimicrobial chemotherapy of *S. aureus* infections

The antibiotic penicillin was introduced for the treatment of severe *S. aureus* infections in the 1940's. β-lactam antibiotics bind to penicillin-binding proteins (PBPs) in the bacterial cell wall, which disrupts the synthesis of the peptidoglycan layer and kills the bacterium. However, by the late 1940's the emergence of β–lactamase production allowed approximately 60 % of hospital-acquired strains to become penicillin-resistant (Daum and Seal, 2001). MRSA can produce β–lactamase, an enzyme which hydrolyses the β-lactam ring of penicillin-based antibiotics, hence its resistance to them. β-lactam antibiotics mimic the structure of D-alanyl-D-alanine and thus target the transepeptidation reaction of peptidoglycan synthesis (Daum and Seal, 2001). The β-lactam ring occupies the active site serine residue of PBPs, which catalyse the glycan polymerisation reactions, thus inhibiting their action (Navarre and Schneewind, 1999).

MRSA strains are typically resistant to all β-lactam antibiotics as with as others, including aminoglycosides. *S. aureus* strains resistant to generally used antibiotics such as tetracycline, streptomycin, and erythromycin were reported shortly after they began to be administered clinically. In the 1960's, the ß-lactamase- resistant antibiotic methicillin was introduced. However, strains of meticillin-resistant *S. aureus* (MRSA) possessing a novel form of a penicillin binding protein (PBP2A (MecA)) with reduced affinity for meticillin rapidly emerged (Hartman and Tomasz, 1984). MRSA infection problem has grown speedily since. Presently, MRSA results in more than one-half of the nosocomial infections with *S. aureus* strains in most countries (Jean and Hsueh, 2011). Over the last decade, the glycopeptide vancomycin has been

reserved to treat *S. aureus* infections that are resistant to the other major antibiotics. Although *S. aureus* strains displaying resistance to vancomycin were initially identified in Japan (Aish, 2003), and subsequently in the United States and France, the first case of vancomycin-resistant *S. aureus* (VRSA) was not reported until 2002 (Centres for Disease Control, 2002). Daptomycin (DAP) is a complex lipopeptide antibiotic synthesised by *Streptomyces roseosporus*. DAP was prescribed to treat skin and soft tissue infections of *S. aureus* and bacteremia, however, recently a number of clinical reports have identified resistance during treatment (Bayer et al., 2013). Daptomycin in combination with other antibiotics such as gentamicin, rifampin, beta-lactams, trimethoprim/sulfamethoxazole (TMP-SMX), or clarithromycin is regarded as an alternative new method for treatment (Nadrah and Strle, 2011). Linezolid is a new synthetic antibiotic that is utilised for infections by Gram-positive pathogens (Toh et al., 2007) and is used to treat MRSA nosocomial pneumonia (Walkey et al., 2011). Resistance to linezolid has been emerging gradually (Walkey et al., 2011).

1.3. The human skin

All living organisms have an outer protective surface separating endogenous and exogenous compartments. As such, human skin is considered part of the integumentary organ system (skin, nails, and hair), which is the outermost tissue of the body and the largest organ in terms of both weight and surface area, and is also a continuously self-renewing organ. The skin is a physical barrier to the greatest microorganisms, water, and most UV light. The acidic

surface (pH 4.0–6.8) inhibits the growth of most pathogens (Grice and Segre, 2011).

1.3.1. Development and structure

The skin of the human body is on average a surface covering nearly 1.6 m^2 with an average thickness of 1.6 -3.9 mm, and comprising around 10 % of the body weight of an adult person (Marieb. E, 2012). The skin is in direct contact with the external environment, and enables the skin to help to maintain four essential functions of the body: the retention of moisture, prevention of permeation or loss of other molecules, sensation ability and protection of the body from pathogenic microorganisms (Goldsmith, 1991). The skin can be divided into three main compartments, the epidermis, the outermost layer of skin, which has no veins and capillaries (avascular), stratified squamous epithelium mainly composed of terminally differentiating keratinocytes, and the dermis, which is made up of connective tissues with a large fraction of collagen and elastin fibres providing flexibility and strength (Fig 1.3) (Goldsmith, 1991). Below the dermis lies the hypodermis, a layer composed mostly of adipose tissue and collagen, ranging in thickness according to the person (Goldsmith, 1991).

The key function of the skin is to act as a first line of defense to protect the living interior compartments of the body from invasion by pathogenic microorganisms. This vital task is fulfilled via the collaboration of a biochemical barrier (e.g. hydrolytic enzymes, antibacterial fatty acids and antimicrobial peptides), a membrane-like physical barrier (stratum corneum), and an immunological barrier involving the effector cells of the immune systems (Proksch et al., 2008).

The skin involves different sorts of cells, where the key cells are keratinocyte, fibroblast, fat cell, melanocyte, erythrocyte (red blood cells), and Langerhans cells. These cells are distributed in diverse locations and have different structures and functions (Romani et al., 2003). Keratinocytes are the main essential cells in the epidermis. These cells create fibriform proteins called keratin, which contribute to the rigidity of the outermost layer of skin. Keratinocytes defend the body from the external environment, such as from stimulation, friction and viruses, while retaining moisture. Keratinocytes can be supplementary divided into four types of cells based on their structures and functions: basal cells, granular cells, prickle cells, and horny cells. Although these cells have the same origin, they have diverse functions, shapes, and subcellular level elements called organelles (Romani et al., 2003). Conversely, the horny cell, which mostly acts as a protector from the external environment, is a very flat and hard dead cell in which most organelles are degenerate (Romani et al., 2003).

The fibroblasts are long and narrow cells present in the dermis, the second skin layer underneath the epidermis. They produce collagen and elastin fibers, which are the primary components of the dermis. The fat cells are quantitatively the most abundant cells of the dermis (Romani et al., 2003). These cells accumulate fat and their sizes differ according to the volume of fat contained in them. On the other hand, melanocyte and erythrocyte cells, both of which contain chromospheres, mostly absorb light. Langerhans cells (also known as epidermal dendritic cells), which are the second most abundant sort of cell in the epidermis, are professional antigen presenting cells, which have a significant role in the skin immune defense (Romani et al.,

2003). The skin also contains many types of fibers including keratin, collagen and elastin. Keratin fibres are mostly found in the outer-level of epidermal cells, involving horny cells. These fibres protect the inner side of skin from the external environment. At the same time, they contribute to moisture-retention in skin by holding water. The length and diameter of these fibres depend on the volume of moisture they retain. The collagen fibres (0.5 to 3 μm in diameter) are the key constituents of the dermis. They represent about 70 % of the dermis in dry weight and these long fibres form vast and strong networks providing the dermis with elasticity, strength, and tension. The elastin fibres are also random coiled proteins that are also present in the dermis and thinner than collagen bundles (1 to 3 μm in diameter) and they occupy 2 to 4 % of the total weight of the dermis (Marieb. E, 2012).

The epidermis is a dynamic, constantly self-renewing tissue that is made up of four distinct structural and functional layers, and organised from superficial to deep into the following sub-layers, *stratum corneum, stratum granulosum, stratum spinosum*, and *stratum basale*. The *stratum corneum* is composed of terminally differentiated, enucleated keratinocytes, which are known as squames. Squames contain keratin fibrils and cross-linked, cornified envelopes embedded in lipid bilayers (Segre, 2006, Fuchs and Raghavan, 2002) (Fig 1.4).

Lamellar bodies which are found in the cells of the *stratum granulosum* are responsible for the release lipids which form the lipid layers that overlie the *stratum corneum* and play an important role in the prevention of water loss (Proksch et al., 2008)

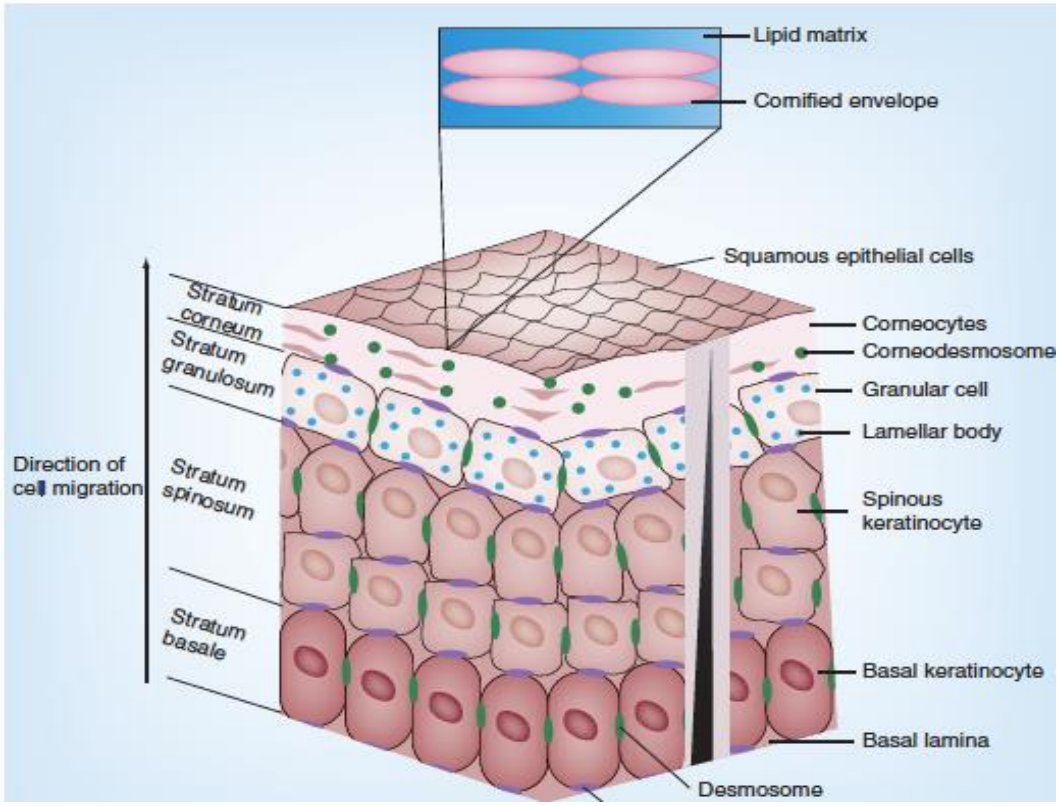


Figure 1.4. Structure of the skin, presenting the main region of the skin, the epidermis and the keratinization process of forming a strong and protective epidermis. Corneocytes yield lamellar bodies which produce antimicrobial peptides, organic acids, antimicrobial fatty acids, cholesterol, sphingosine and ceramides, that include the lipid matrix (Coates et al., 2014).

1.3.2. Chemical features making skin a niche for bacterial colonisation

The skin provides a diverse ecological niche for the growth of both commensal and pathogenic bacteria. The microenvironment at the skin surface has an effect on the type and number of commensal and pathogenic organisms that may colonise it. Studies have revealed that the commensal bacteria residing on the skin are most often Gram-positive (e.g. *Staphylococcus aureus*) species, but some Gram-negative bacteria (e.g. *Pseudomonas* spp) are known to colonise the skin as well (Grice et al., 2009). Factors like skin temperature and surface humidity vary from site to site and in response to both environmental and internal stimuli, potentially affecting bacterial colonisation (Mcewan Jenkinson, 2004). Although the pH of the skin may vary from person to person, in adults it is acidic (Teun Boekhout, 2010). The mantle is not only important in limiting the growth of certain bacteria on the skin such as *S. aureus*, but is required for the production of ceramides with enzymes required to produce ceramides optimal at an acidic pH (Lambers et al., 2006). The trapped pockets of carbon dioxide and oxygen in the outer layer of the epidermis provide a diverse environment for exploitation by microbes (Mcewan Jenkinson, 2004). It is known that keratin is not suitable for bacterial growth. The process of emulsion of both sweat (salt, water, enzymes)) and sebum (lipid-rich substance) in the stratum corneum may explain why bacteria are more commonly found within hair follicles and in the upper layers of the stratum corneum (Mcewan Jenkinson, 2004). The major lipid classes of human

stratum corneum are cholesterol, ceramides, and saturated long chain free fatty acids (Weerheim and Ponec, 2001).

1.3.3. The lipid matrix of human skin

1- Composition and structural aspects of the matrix

Corneocytes are surrounded by a crosslinked protein layer that is attached chemically to a monolayer of lipids. This lipid monolayer plays an important role in the stratum corneum where it serves as an interface between the lipophilic extracellular lipid matrix and the hydrophilic corneocytes. A characteristic of stratum corneum lipid is the broad distributions of saturated, long hydrocarbon chains and high cholesterol content lipid, with only a small amount of phospholipid is present, which is completely diverse from that of the cell membranes of living cells.

The three main classes of lipid on the stratum corneum are cholesterol (CHOL), ceramides (CER), and saturated long chain free fatty acids (FFA) are secreted from keratinocytes in lamellar bodies (the epidermal membrane bilayer-encircled secretory organelles). Ceramides are formed of sphingosine linked to a fatty acid molecule by an amide bond and account for 50 % of the lipids found in the stratum corneum (Paslin and Wertz, 2006). The length of the acyl chains in the CER differs between 16 and 33 carbons (Proksch et al., 2008, Weerheim and Ponec, 2001). There are nearly nine different known ceramides, of which ceramide A and ceramide B bind covalently to the protein cornified envelope through involucrin. Cholesterol is an essential structural component of the lipid membrane and accounts for 25 % of the lipid matrix, where it is required for membrane fluidity, permeability and

signalling cascades. In addition, it is also the third major lipid class in the stratum corneum that is necessary to keep the skin barrier hydrated (Proksch et al., 2008).

Cholesterol obtained from nutritional sources is transported around the body via blood circulation but only a small amount is absorbed in the stratum basale by keratinocytes (Fig 1.5). Most cholesterol in the epidermis is synthesised from acetate in the cells *in situ* (Proksch et al., 2008).

The lipid envelope in the stratum corneum also contains long chain (mainly 22 and 24 carbon chain lengths) free fatty acids, which are derived from phospholipids and triglycerides. These chain lengths are much longer than those of phospholipids that are present in plasma membranes of living cells, and account for 15% of the lipid matrix. Most saturated and monosaturated fatty acids can be synthesised in the human body, while some essential fatty acids such as linolenic acid and linoleic acids are not synthesised in the body, and therefore must be obtained from nutritional sources (Proksch et al., 2008). Covalently bound lipids form a lipid layer that has the dimensions of a typical bilayer matrix. In addition, the stratum corneum contains extremely flat, keratin-filled cells bounded by a cornified envelope and embedded in a lipid matrix. Therefore, this structure provides a permeability barrier that prevents desiccation, and as such, this represents the first line of defence against microbial colonisation and infection.

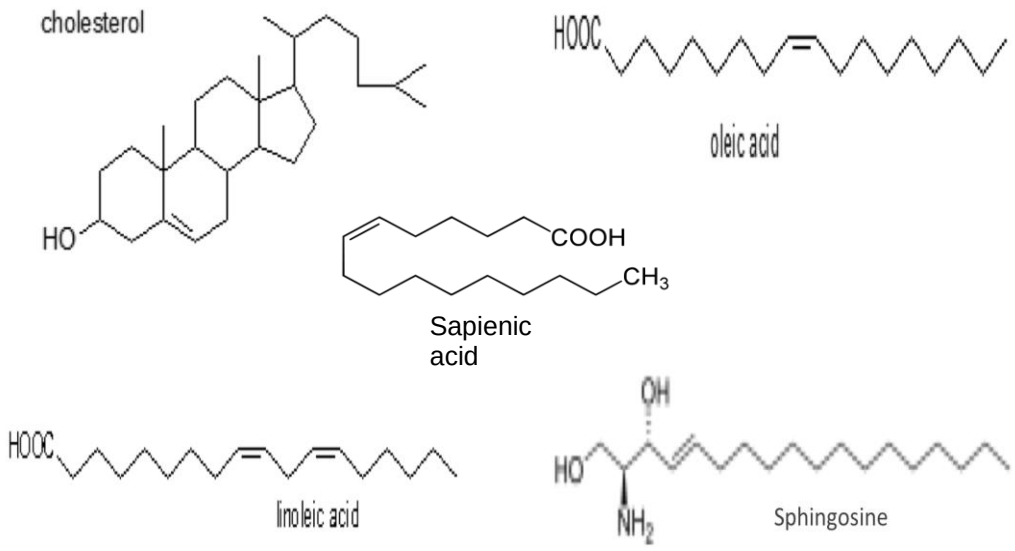


Figure 1.5. Chemical structures of the fatty acids, linoleic, oleic, sapienic and the lipids sphingosine, the steroid cholesterol. Fatty acids from (http://lipidlibrary.aocs.org/Lipids/whatlip/index.htm and sphingosine from http://en.wikipedia.org/wiki/Sphingosine) and cholesterol (http://www.cyberlipid.org/sterols/ster0003.htm)

Recently, Iwai *et al* reported the molecular organisation of the lipid matrix of the skin barrier (*stratum corneum*) in situ using electron microscopy and molecular modelling. The lipids are organised in stacked bilayers of fully extended ceramides with cholesterol molecules related with the Ceramide sphingoid moiety (Iwai et al., 2012).

2- Skin antimicrobial lipids (SAML), and their mode of action

Human skin has an extraordinary power to clear its surface of harmful bacteria and to provide its endogenous bacterial flora. This capability to destroy exogenous microbes has been indicated to as the self-disinfecting power of the skin (Drake et al., 2008b). The antibacterial activities of the lipids at the skin surface (sapienic acid, lauric acid, long-chain bases sphingosine, dihydrosphingosine and 6-hydroxysphingosine) have been reported against a range of bacterial species including *S. aureus*. These potent antimicrobial molecules are generated through the action of ceramidases on ceramides from the stratum corneum. Free fatty acids, and probably some of the other polar lipids from epidermal stratum corneum, have antibacterial activity against a range of Gram-positive bacteria (Bibel et al., 1992). The organisms that are sensitive to fatty acids involve *S. aureus, S. pyogenes, S. epidermidis and Micrococcus sp*. Some studies have also established that certain fatty acids from the skin surface are more active than others. In particular, sapienic acid and lauric acid have the best activity against *S. aureus* bacteria (Drake et al., 2008b). These natural antimicrobials are thought to be part of the innate immune system of the skin, in addition to other peptides, acids, and lysozymes. They have the ability to interfere with

and disrupt the integrity of the bacterial cell membrane; hence they are potent bactericidal agents (Fischer et al., 2014). These lipids like other components of innate immunity provide first-line recognition of microbial pathogens (Medzhitov and Janeway, 2000b), and they contain infection prior to the induction of adaptive immune responses, which can take 3 to 5 days. Furthermore, innate responses control the activation of adaptive immunity and determine the sort of effector responses suitable for the bacterial pathogen (Medzhitov and Janeway, 2000a, Holmskov, 2000).

3- Skin antimicrobial peptides (AMPs)

In addition to the antimicrobial skin lipids, several small cationic peptides have been recognised as antimicrobial agents that act as part of the innate immune system. Skin keratinocytes are a main source of antimicrobial peptides (AMPs) (Fig 1.6). Both skin fatty acids and AMPs are induced in the skin upon microbial stimuli or injury through Toll-like receptor-dependent pathways (Schauber et al., 2007). These peptides are amphiphilic, relatively short (12 to 100 amino acids) and are positively charged. They show a broad-spectrum of activities, so can capably kill a wide range of bacteria, fungi, and viruses. One mode of action of AMPs includes disruption of bacterial membrane integrity, the first step in the disruption of membranes, and also for AMP internalisation, is the ability of AMPs to attach to the bacterial membranes. Because most AMPs have a positive (cationic) net charge in physiological pH and an amphipathic structure, this facilitates their electrostatic interaction with the negatively charged (anionic) bacterial membranes and allow the AMPs to come close and to aggregate on the

bacterial surface (Diamond et al., 2009). Other modes of action are targeting key cellular processes, including DNA and protein synthesis, cell wall synthesis, protein folding, and metabolic turnover (Brogden, 2005, Bevins and Salzman, 2011). It was determined that these peptides are synthesised by differentiating keratinocytes, and the most extensively studied among these are the cathelicidins and defensins (Ali et al., 2001). Their production is up-regulated in response to bacterial infection or wounding (Dorschner et al., 2001).

In the absence of a microbial challenge or epidermal damage, antimicrobial peptides production is limited. It has been noticed that after physical damage to the skin barrier, there is a rapid rise in antimicrobial peptide creation, however, under normal resting conditions, antimicrobial peptides in epidermis are produced near potential points of microbial entry, for example, around follicles, and during *S. aureus* infection defensins and cathelicidin are expressed at relatively high levels. In addition to direct antibacterial action, these peptides are also chemotactic and can attract leukocytes to sites of infection (Ong et al., 2002). Moreover, it has been proposed that the function of the innate immune system may not be to reduce infective bacteria, but to limit their growth while signalling and activating the adaptive immune system, and that AMP may act synergistically as antimicrobial agents (Ong et al., 2002).

The cationic and amphiphilic nature of AMP is related to their physiological activity. The general positive charge ensures accumulation at polyanionic microbial cell surfaces that comprise of acidic polymers, such as lipopolysaccharide of the Gram-negative bacteria, and wall-associated

teichoic acids of Gram-positive bacteria, and then transiting the outer membrane of these bacteira via self-promoted uptake. Consequently these peptides interact the anionic surface of the cytoplasmic membrane and insert in a manner such that they primarily straddle the interface of the hydrophilic head groups and the fatty acyl chains of membrane phospholipids. After insertion into the membrane, antimicrobial peptides act by either disrupting the physical integrity of the bilayer, through membrane thinning, transient poration and/or disruption of the barrier function, or translocate across the membrane and act on internal targets (Hancock and Lehrer, 1998).

Defensins are small and cationic polypeptides expressed in great amounts in humans. They are expressed in many different cells and tissues where bacterial infections are a threat, such as monocytes, macrophages, dendritic cells, keratinocytes and epithelial cells. In general, all defensins are expressed as pro-peptides and are cleaved to become active. Structurally, defensins are composed of three antiparallel β-sheets stabilized by three disulphide bonds. They are classified into α- defensins, β-defensins and θ-defensins depending on their number of amino acids and how the cysteine residues are linked (Selsted and Ouellette, 2005).

Cathelicidins in humans are expressed as pro-peptides, LL-37 is the only cathelicidin in humans and it is stored as a precursor called human cationic antimicrobial protein (hCAP-18). LL-37 displays a broad antimicrobial activity, as well as being chemotactic and promoting wound healing. Cathelicidin expression can be induced during infection as well as constitutive. LL-37 is expressed in neutrophils, lymphocytes, in keratinocytes and epithelial cells of

the skin, gastrointestinal tract and airways (Vandamme et al., 2012, Lehrer and Ganz, 2002)

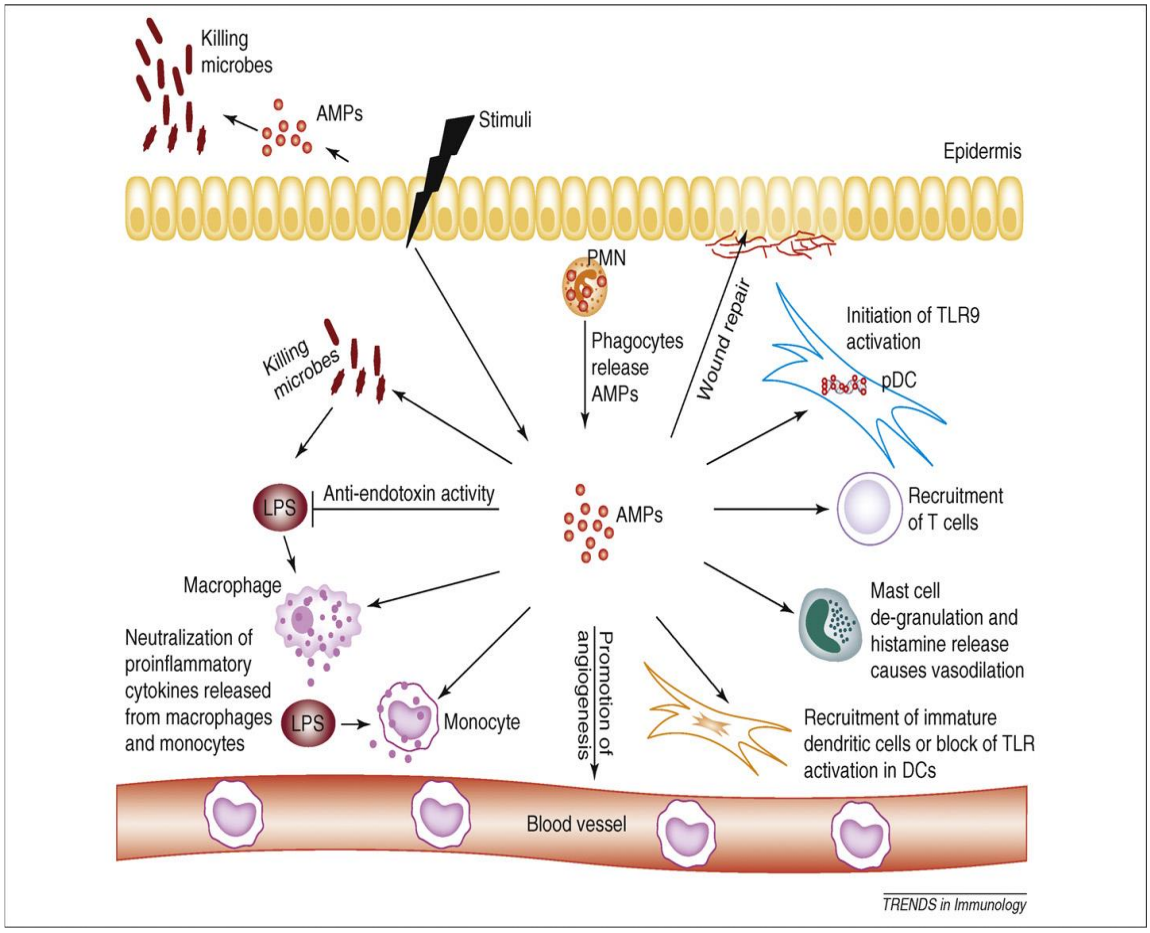


Figure 1.6. Functions performed by AMPs. AMPs are antimicrobial as well as having other additional features, such as wound repair, recruiting immune effector cells, neutralizing endotoxins and pro-inflammatory cytokines as well as promoting angiogenesis (Lai and Gallo, 2009).

4- Bacterial defence mechanisms against skin AMPs and fatty acids

Despite the availability of potentially formidable epidermal molecules secreted on the surface of the skin against microbial pathogens, which can eliminate them, certain opportunistic bacteria can still infect and cause disease. Bacteria employ different strategies to avoid being killed by the AMPs produced by the host. One of these strategies is the ability of a bacterial pathogen to alter cell surface charge. Since AMPs have to come in close contact with the bacteria to insert into the membrane, one defence strategy of the bacteria is to reduce the negative charge of their membrane, thereby weakening the electrostatical forces attracting the cationic AMPs. Some Gram-positive bacteria modify the negatively charged lipoteichoic acid (LTA) and Gram-negative bacteria can alter their lipopolysaccharides (LPS) resulting in a less negative membrane (Peschel et al., 2001).

Studies have also established highly regulated antimicrobial resistance mechanisms of cytoplasmic membrane modification in Gram-positive bacterial pathogens. It was revealed that both defensin-susceptible *S. aureus* mutants, *dltA* and *mprF*, accumulate relatively more cationic peptides on their surface than wild type cells (Peschel et al., 2001), The enzyme MprF creates lysinylated phospholipids, whose integration into the cytoplasmic membrane reductions the overall negative charge of this direct target structure for many AMPs. This indicates that decreasing the negative surface charge leads to repulsion and resistance to cationic AMPs. Another strategy utilises an iron uptake protein to evade cell death by AMPs. The iron-responsive surface determinant A (IsdA) protein which is induced in

response to iron restriction is anchored via its C terminus to the peptidoglycan of the *S. aureus* cell wall by the action of sortase enzymes (Mazmanian et al., 2002). Moreover, by reducing the overall hydrophobicity of the bacterium, IsdA blocks the action of numerous antibacterial molecules present in normal skin, including cathelicidin and beta defensin peptides, human sebum, and its constituent hydrophobic fatty acids, which then enable IsdA to promote *S. aureus* survival on human skin (Fig 1.7).

Capsule expression is a further strategy of survival adopted by certain bacteria, which provide electrostatic shielding against AMPs. Several virulence factors in bacteria such as *S. aureus* and *P. aeruginosa* rely upon elaboration of a capsule as a means of adherence to tissue and evasion of opsonisation and phagocytosis. Therefore, capsule production is a significant virulence factor particularly in pathogenic microbes that colonise or infect the skin, bloodstream, respiratory tract, and gastrointestinal mucosa. However, there is little information available from which to assess the role of pathogen capsule expression associated to resistance to AMPs. Compositions of capsule vary widely between different organisms. However, the capsule of a large number of microbial pathogens is often composed of an anionic complex of carbohydrate and phosphate. Thus, it is most likely that matrices such as these surround cationic AMPs, and consequently prevent them from reaching their intended targets (Yeaman and Yount, 2003).

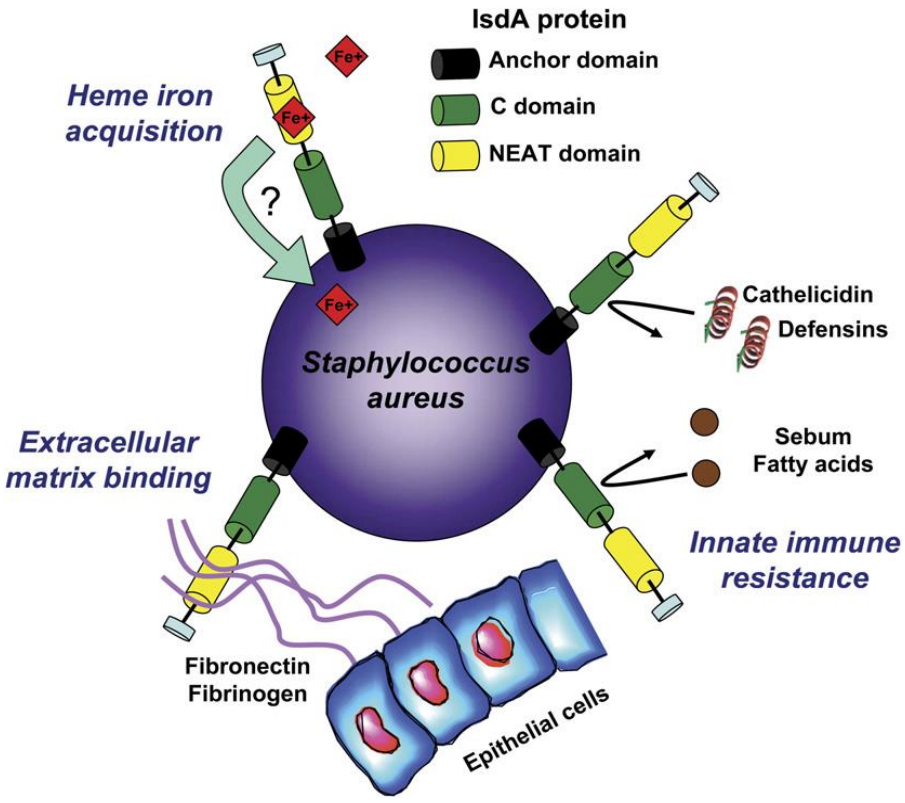


Figure 1.7. IsdA promotes *S. aureus* survival on human skin. IsdA resists AMPs as well as fatty acids, which then increase the pathogenicity (Zinkernagel and Nizet, 2007).

S. aureus secretes proteases that degrade AMPs. Lysozyme (found in several body fluids, such as mucosal secretions and tears) is an enzyme that cleaves the β-1,4 glycosidic bond in bacterial peptidoglycan between the *N*-acetylglucosamine and *N*-acetylmuramic acid residues (Chipman and Sharon, 1969). Lysozyme is created in skin cells, but only in the cytoplasm, and thus its contribution to cutaneous defense is unclear. Lysozyme has a wide variety of activity against Gram-negative as well as Gram-positive bacteria. However, a large number of staphylococcal species, involving *S. aureus* and *S. epidermidis*, have an enzyme that *O*-acetylates peptidoglycan, which provide resistance to lysozyme (Bera et al., 2006). This modification limits the cleavage of the β-1,4 glycosidic bond in peptidoglycan.

The secreted enzyme fatty acid modifying enzyme (FAME) catalyses the esterification of long chain free fatty acids to generate cholesterol, enhancing invasiveness and in abscesses it inactivates the bactericidal effects of host lipids in the skin (Chamberlain and Imanoel, 1996).

5- Regulation of *S. aureus* antimicrobial peptide defence

The success of *S. aureus* as a pathogen is in part because its precise regulation of genes needed to survive in different environments, including the host. Two-component systems (TCS) are one class of regulating element used by *S. aureus* to sense the environment and adapt to it accordingly. These systems consist of two components, a membrane spanning histidine kinase (HK) and an intracellular response regulator (RR). The histidine kinase acts as a sensor and the response regulator, which is normally a DNA-binding protein, is responsible for changing gene expression to aid in

survival of the bacterial cell (Parkinson, 1993). In *S. aureus* the regulation of the cationic AMP resistance systems is not fully known. There is evidence that *dltD*, one of the genes responsible for D-alanine transfer into teichoic acids, is down-regulated by the global regulator of virulence *agr* (Dunman et al., 2001), demonstrating that *S. aureus* cationic AMP resistance systems and other virulence factors are controlled in a coordinated manner.

The glycopeptide resistance associated- regulatory system (*GraRS*) of *S. aureus* is a vital system for its survival as pathogen. One of the main functions of GraRS is cationic AMP resistance; and as a result it has alternatively been called the antimicrobial peptide sensor (*ApsRS*). The cationic AMPs are positively charged and kill bacteria by binding to their negatively charged cell wall, where they form pores or enter the cell and reduce DNA, RNA and protein synthesis. The presence of cationic AMPs induces *GraSR*, which leads to changes in gene expression to facilitate resistance by the D-alanylation of teichoic acids and lysylination of phosphatidylglycerol. D-alanylation of teichoic acids increases the net positive charge of the cell and decreases the affinity of cationic AMP binding to pathogens target molecules. D-alanylation of teichoic acids is performed by the DltA enzyme, while MprF is responsible for the lysylination of phosphatidylglycerol. *GraRS* helps in disseminating the infection process by limiting the activity of host cationic AMPs and is important in several animal models of infection including Drosophila, and murine models (Kraus et al., 2008, Tabuchi et al., 2010).

A second TCS, *NsaRS*, senses cell-envelope stress, and exerts its effects by regulating over 200 different genes (Blake et al., 2011). The majority of these

genes are positively regulated, and cover a range of functions, including cell wall biosynthesis genes (*tagB*, *fmhA*, and *scdA*), osmoprotectants (*opuCA* and *cudT*), as well as other regulators (*sarS and rex*). Kolar and colleagues showed that *NsaRS* responds to disruption of the cell envelope and redirects gene expression to mediate resistance. The study demonstrated that *NsaRS* is essential for full biofilm formation across a large number of *S. aureus* strains, and contributes to survival through challenge by factors of the innate immune system (Kolar et al., 2011).

TCS in *S. aureus* can also respond to chemical changes in the environment, including potassium, iron, phosphate, oxygen and nitrogen and inactivation of this system has been revealed to decrease the expression of several virulence factors. This suggests that *S. aureus* determines its infectious state partially by sensing multiple external factors (Xue et al., 2011).

6- Bacterial competition

Symbiotic relationships between bacteria are common with their human or animal host. Bacterial competition, typically when one species out competes the other for resources by producing factors to kill or decrease expression of toxic products, is also known as bacterial interference. Factors produced and contributing to competition are not considered virulence determinants, but they might indirectly contribute to virulence where a more virulent strain can out-compete the less virulent strains (Otto, 2004).

S. aureus competes with other skin flora, involving members of Gram positive *Corynebacterium*, which manufacture lipases that break down the lipids in sweat to generate smaller molecules like butyric acid. These smaller

molecules are volatile, and give body odor its characteristic aroma (Rosline, 2012). Isovaleric acid (3-methyl butanoic acid) is also a source of body odor as a result of actions of *Staphylococcus epidermidis* (Ara et al., 2006). Propionic-bacteria reside in the sebaceous glands and derive energy from the fatty acids of sebum; this bacterium in adult sebaceous glands could turn its amino acids into propionic acid. The most well known disease related with *P. acnes* is the skin condition known as acne vulgaris. Bacteriocins have been suggested to enhance survival of the producer strains in a competitive fashion, and they are produced by many staphylococci (Jack et al., 1995). These bacteriocins often belong to the lantibiotic class that is characterised by lanthionine bridges, which make the peptide strongly resistant to proteolytic degradation (Bierbaum and Sahl, 2009). In general, the encoding genes for bacteriocins are coupled to genes that provide protection for the producer, therefore giving an extra advantage over other bacteria that are susceptible to that substance (Draper et al., 2008). The phenol soluble modulins (PSMs) are peptides produced by the majority of staphylococcal strains including *S. aureus.* PSMs are short amphipathic and α-helical peptides implicated in tissue invasion. PSMs share a common alpha helical region that is thought to disrupt cell membranes and induce host cell lysis (Wang et al., 2007). They lack the cationic character that is typical of AMPs, which would indicate that these molecules have no specific activity against bacteria and could be targeted mainly against host tissues (Dhople and Nagaraj, 1993).

7- Staphylococcal virulence/ staphyloxanthin and membrane fluidity

The majority of *Staphylococcus aureus* isolates (more than 90 %) from human infections are golden pigmented. This pigmentation is due to the biosynthesis and presence of staphyloxanthin (an orange-red triterpenoid membrane-bound carotenoid) on the *Staphylococcus* cellular membranes. Staphyloxanthin will be discussed in more detail in Chapter 4.

Staphyloxanthin affects the functional activity and chemical composition of the *S. aureus* cellular membrane by reducing membrane fluidity ensuring the membrane structure is stabilised. The change in membrane fluidity produces resistance to host cationic AMPs used by the host to eliminate bacterial pathogens (Mishra et al., 2011c). The enzymes of the staphyloxanthin biosynthetic pathway have been used as a target for drug development to help infection eradication. CrtM, the first enzyme in this pathway shares the same catalytic site structure as that of human squalene synthase, which is involved in the biosynthesis of cholesterol (Fig 1.8). A study by Liu et al showed that inhibitor molecules of the human squalene synthase might also prevent *S. aureus* production of pigment and therefore affects its survival in the host (Liu et al., 2008).

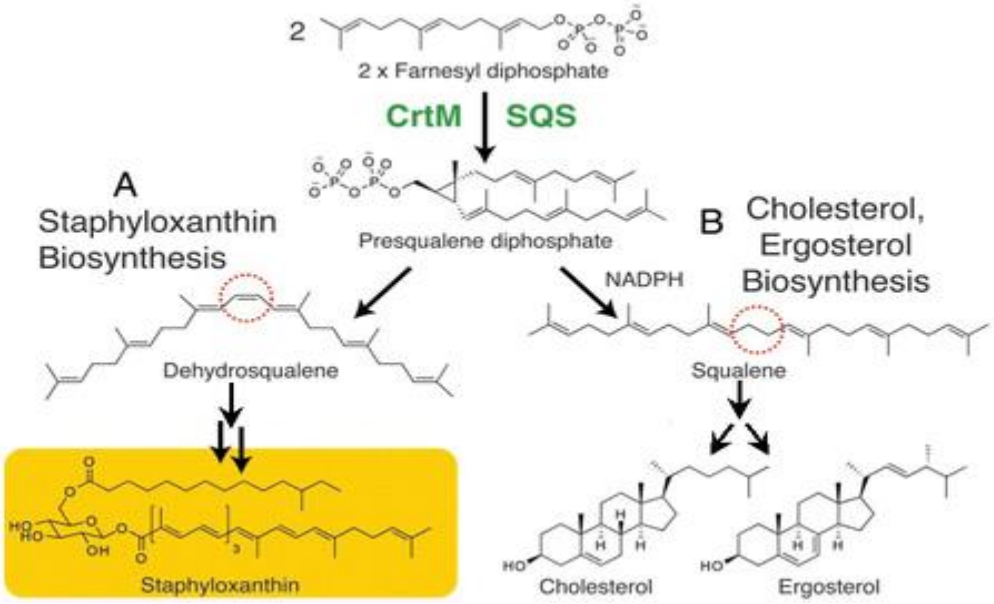


Figure 1.8 Staphyloxanthin and sterol biosynthetic pathways. (A) Staphyloxanthin biosynthesis in *S. aureus*. (B) Human cholesterol and ergosterol biosynthesis. Both biosynthetic pathways involve first formation of presqualene diphosphate, catalysed by CrtM (*S. aureus*) or by squalene synthase (SQS). In *S. aureus*, the NADPH decrease step is absent, resulting in production of dehydrosqualene (Liu et al., 2008).

8- Osmotic stress and stress resistance in *S. aureus*

The cell wall peptidoglycan of bacteria acts as a mechanical framework and is a major cell shape determinant. The peptidoglycan can expand or contract in response to changes in environmental osmolarity or pH. *S. aureus* is a highly adaptable bacterium, which survives in a variety of abiotic environments and causes disease in diverse areas of the human body. Therefore, as well as being regulated in a growth-phase dependent manner, the *S. aureus* virulence factors are regulated in response to many environmental parameters. Detection of environmental conditions enables *S. aureus* to determine whether it has entered the host and to identify its location inside the human body. Carotenoids within the host allow *S. aureus* to respond appropriately to pH, the concentration of metal ions (e.g. magnesium and iron), temperature, and the concentration of NaCl, glucose, and oxygen (Mekalanos, 1992). A key signal indicating entry into the host for many pathogenic bacteria is temperature. Whereas for many pathogens virulence genes are optimally expressed at 37 °C in *S. aureus* RNAIII and *hla* are expressed maximally at 42 °C (Ohlsen et al., 1997).

NaCl concentration is one of the most extensively studied environmental conditions affecting *S. aureus* growth. It is believed that *S. aureus* although a non-halophile, is among the most salt tolerant of bacteria, and is capable of growth in the presence of 3.5 M NaCl (Armstrong-Buisseret et al., 1995). This growth ability might relate to it encountering high concentrations of NaCl in abiotic environments and also when in contact with a host. The skin and anterior nares are the main sites of colonisation and these are high-NaCl concentration niches of the human body. In nasal secretions the

concentration of NaCl has been shown to be up to 225 mM and a maximum of 170 mM in sweat (Mangos, 1973). *S. aureus* is affected by NaCl at two levels; it affects general physiology of the cell, for example by stimulating an osmotic stress response, and the presence of NaCl alters the expression of virulence determinants.

The availability of water is a fundamental environmental parameter affecting the growth and survival of microorganisms. External osmolarity changes cause water to move along the osmotic gradient, and potentially result in either swelling or bursting in hypotonic environments, or dehydration and plasmolysis under hypertonic conditions. Therefore, it is vital that the cell possesses effective measures to counteract adverse changes in external osmolarity, and to allow a suitable level of cytoplasmic water and turgor pressure (the hydrostatic pressure difference between the inside and the outside of the cell) to be maintained (Meuillet et al., 2000). *S. aureus* contains a naturally high level of cytoplasmic K^+, which is relatively unaffected by osmotic stress (Graham & Wilkinson, 1992). This in fact enables *S. aureus* to possess a highly versatile osmotic stress response, which allows adaptation to environments containing both low and high osmolarity, and involves the accumulation of K^+ ions and a number of osmoprotective compounds. It has been reported that *S. aureus* is able to grow in the presence of NaCl concentrations of up to 3.5 M (Armstrong-Buisseret et al., 1995) by combining a more moderate level of cytoplasmic K with the accumulation of osmoprotective compounds, which can be released from the cell following a reduction in external osmolarity. The osmoprotectants are compounds that relieve the growth inhibition caused by

high osmolarity (Wilkinson, 1997). The accumulation of compatible solutes is an almost universal response to high osmolarity, and occurs in the cells of many bacteria, including *S. aureus* (Csonka, 1989). These compatible solutes are highly soluble polar molecules, and usually do not carry a net charge at physiological pH (Meuillet et al., 2000). Thus, when accumulated in up to molar concentrations, they lower the osmolarity of the cytoplasm and help to establish a suitable cytoplasmic water volume and turgor pressure. The compatible solutes comprise a wide range of compounds, although the main ones accumulated by *S. aureus* in response to osmotic stress include the trimethylammonium compound, glycine betaine and the amino acid L-proline (Smith et al., 1991, Graham and Wilkinson, 1992, Bae et al., 1993).

9- Regulation of stress resistance / SigB (accessory sigma factor)

The accessory sigma factors in bacteria are known to play an important role in regulating gene expression upon major environmental changes. Sigma factors are proteins that bind to core RNA polymerase to form holoenzymes which specify transcription directly from promoters with conserved sequence motifs (Sonenshein. A. L, 2002). A primary sigma factor is essential for the transcription of housekeeping genes whose products are essential for growth. A second group comprises the alternative sigma factors, which coordinate the transcription of specific sets of genes under particular cellular conditions (Dombroski et al., 1993). In S. *aureus* an alternative sigma factor, SigB, has been identified and its induction has been shown to occur through the stationary phase of growth and upon heat shock (Kullik et al., 1998). The

SigB of *S. aureus* modulates functions including peroxide resistance, production of lipase and thermo-nuclease, possibly alkali stress response, and cell aggregation (Kullik et al., 1998). The corresponding sigma factor of the model Gram-positive bacterium *Bacillus subtilis* has a complex regulatory network which controls gene expression in response to stress and certain stationary-phase-specific signals (Hecker et al., 1996). Transcription of the global regulator *Sar* of *S. aureus* is also partially controlled by *SigB* (Kullik et al., 1998). It is known that the Sar protein is essential for expression of the regulator *agr,* which is itself a global regulator involved in the expression of virulence genes. Therefore, it seems that *SigB* is a stress- and stationary phase specific global regulator which is directly and indirectly linked with the expression of virulence genes in particular (Kullik et al., 1998).

1.3.4 The rationale, objectives, and aims of this Thesis project

While some advances have been made in relation to the *S. aureus* response to certain lipids, including the identification of mutants with reduced survival and characterisation of the transcriptional response, the mechanisms that are used for protection from the diversity of skin lipids by *S. aureus* remain poorly understood.

Previous reports have proposed that cholesterol could ameliorate the growth reduction caused by the AFAs, linoleic and oleic acid. This report was confirmed and to characterise the potential survival mechanism from AFAs, further studies were performed to investigate potential mechanisms to explain the cholesterol enhancement of growth in the presence of AFAs.

The project hypothesis is that *S. aureus* has mechanisms that enable a response to skin lipids and furthermore it can utilise specific lipids such as cholesterol to ameliorate the action of fatty acids. These mechanisms and their regulation are proposed to be important for skin survival and they could potentially impact upon *S. aureus* transmission dynamics.

Therefore, the specific research aims are to:

1) Determine the effects that cholesterol has on the antimicrobial action of AFAs (linoleic acid) and D-sphingosine (from breakdown of ceramide) on *S. aureus*.
2) Characterise the effects of cholesterol on cell growth and survival to investigate its effect on *S. aureus* physiology.

CHAPTER 2:

2. Materials and Methods

2.1. Growth Media and Antibiotics

All solutions were prepared and made up in 1 liter of distilled (d), deionised water and were sterilised using autoclave at 121 °C for 40 min.

Brain heart infusion (BHI)

Brain heart infusion broth powder (BHI)	37 g /l

LK

Difco Bacto tryptone	10 g /l
Difco Bacto yeast extracts	5 g /l
KCl	7 g /l
Sodium Citrate	0.5 g /l

LB

LB	37 g /l

Antibiotics

Table 2.1 Concentrations of antibiotics used during this study

Antibiotic		**Working concentration ($\mu g\ ml^{-1}$)**
Erythromycin	(Ery)	5
Kanamycin	(Kan)	50
Lincomycin	(Lin)	25
Tetracycline	(Tet)	5
Chloramphenicl	(Cat)	5
Neomycin	(Neo)	50

The above antibiotics were used in broth and agar plates. Growth media was cooled to 50 °C before adding the antibiotics. The media stock solutions were then all filters sterilised and stored at -20 °C.

2.2. Buffers stock solutions

All buffers were prepared in dH_20 and were stored at room temperature.

Buffers

Catalase 10 mg/ml dissolved with phosphate buffer:

Phosphate buffer

pH 7	0.1 M
Di-potassium phosphate	174.2 g/l
Mono-potassium phosphate	136.08 g/l
Dissolved in water	

Tris-Edta buffer

Tris	1 M
EDTA	0.5 M

Ethanol buffer Tris pH 6.8

Ethanol	950 µl
Tris pH 6.8	50 µl

Phage buffer

$MgSO_4$	1 mM
$CaCl_2$	4 mM
Tris-HCl pH 7.8	50 mM

NaCl	5.9 g/L
Gelatin	1 g/L

DNA loading buffer (6x)

Glycerol	6 ml
Bromophenol blue	25 mg
Xylene cyanol FF	25 mg
Ficoll (MW 400,000)	1.5 g
dH_20	to 10 ml

Phosphate buffered saline (PBS)

SDS-PAGE solutions

1x SDS-PAGE sample buffer

dH_2O	0.8 ml
0.5 M Tris-HCl (pH 6.8)	0.2 ml
10 % (w/v) SDS	0.32 ml
Glycerol	160 µl
0.05 % (w/v) Bromophenol blue	40 µl

2.3. SDS-PAGE gel formulations and construction

The following components were added and mixed in a sterile 50 ml plastic universal:

Resolving gel components:

dH_2O	3.98 ml
30 % Acrylamide / Bis	3.33 ml
1.5 M Tris-HCl (pH 8.8)	2.5 ml

10 % (w/v) SDS	100 µl

The mixture was then swirled to remove bubbles from the mixed components. Then 100 µl 10 % (w/v) ammonium persulphate, and 15 µl Emed were then added. The contents were mixed before being pipetted into the gel casting apparatus. A layer of water was then over played onto the mixture. The gel was then allowed to solidify.

Stacking gel components:

dH_2O	2.27 ml
30 % Acrylamide	0.6 ml
0.5 M Tris-HCl (pH 6.8)	1 ml
10 % (w/v) SDS	40 µl

The stacking gel mixture was swirled and then 75 µl 10 % (w/v) ammonium persulphate and 15 µl of TEMED were added to the solution, and the components were mixed by swirling. The water on the overlay layer gel was removed before stacking gel added to casting. A plastic comb was placed in the gel to create wells and isolate the gel from the air. After that, the gel was left to solidify and was transferred to the gel tank and submerged in 1X SDS-PAGE buffer. The samples were loaded and subjected to electrophoresis of 170 mA for 40 minutes, through the stacking gel.

1x SDS-PAGE electrophoresis buffer:

The following components were dissolved in dH_2O:

Glycine	14.4 g/l
Tris base	3 g/l
SDS	10 g/l

Coomassie Blue staining solution

Methanol	50 % (v/v)
Acetic acid	10 % (v/v)
Coomassie Blue R-250	0.25 (w/v)

Destaining solution

Methanol	10 % (v/v)
Acetic acid	10 % (v/v)

Stock solutions

All solutions were dissolved in ethanol and were stored at -20 °C

Lipid stock solutions:

Linoleic acid	100 mM, 1 mM and 0.1 mM
D-sphingosine	10 mM
Cholesterol	100 mM
Lanosterol	0.1 mM
Desmosterol	0.1 mM
Ergosterol	0.1 mM

Lytic enzymes:

these enzymes were dissolved in PBS and were stored at -20 °C

Lysostaphin	6 mg/ml
Mutanolysin	50 uk/10 ml

2.4. Staphylococcus aureus strains

2.4.1 Bacterial Culture

The bacterial host strains SH1000 (Horsburgh et al., 2002) and Newman (Giachino et al., 2001) were used in this project. Brain heart infusion broth (BHI; Lab M Limited) (37 $g.l^{-1}$) was used for bacterial growth and agar (12 $g.l^{-1}$) was added for plates. Suitable antibiotics to preserve selection of bacterial resistance were also used. *S. aureus* strain SH1000 transposon mutant colonies were inoculated and grown overnight in 96-well plates to create mutant libraries. Glycerol (10 %) was added to each well by initial dilution in ethanol. These plates were then stored at -80 ℃.

Agar plates were stored at 4 °C. Standard *S. aureus* growth conditions were: For inocula cells were grown overnight in 30 ml sterile glass universals, containing 10 ml of media (BHI), and aerated on a rotary shaker at 250 rpm for experiments in liquid media. These cultures were utilised to inoculate 250 ml conical flasks containing 50 ml media (BHI) and were aerated on a rotary shaker at 250 rpm.

TABLE 2.2. List of *S. aureus* strains used in this study

Strain ID	Other Strain ID	Genus/ species/ strain name	Strain description	Drug resistance	Obtained from
Liv023	SH682	*S. aureus* SH1000	rsbU repaired strain of S. aureus 8325-4	None	Lab stock
Liv028	SH730	S.aureus Newman	Newman wild type of S. aureus	None	Lab stock
Liv038	SH1046	*S. aureus* SH1000 agr::tet	SH1001	tet 5	Lab stock
Liv083	P31323 228 AE	*S.aureus* WCUH29	2 component mutant in VraR/S SA1700	ery 5, lin 25	Lab stock
Liv084	P31316 270 AE	*S.aureus* WCUH29	2 component mutant arlS SA1246	tet 5	Lab stock
Liv085	P31317 247 AE	*S.aureus* WCUH29	2 component mutant phoR SA1515	tet 5	Lab stock
Liv086	P31318 248 AE	*S.aureus* WCUH29	2 component mutant SA2152	tet 5	Lab stock
Liv087	P31324 157 AE	*S.aureus* WCUH29	2 component mutant SA2180	ery 5, lin 25	Lab stock
Liv088	SB0364 250 AE	*S.aureus* WCUH29	2 component mutant SA0615	ery 5, lin 25	Lab stock
Liv089	SB0371 261 AE	*S.aureus* WCUH29	2 component mutant SA0660 SaeS	tet 5	Lab stock
Liv090	SB0536 249 AE	*S.aureus* WCUH29	2 component mutant SA2417	tet 5	Lab stock
Liv091	P31326 226 P1	*S.aureus* WCUH29	2 component mutant KdpD SA1882	ery 5, lin 25	Lab stock
Liv092	P31326 268 P1	*S.aureus* WCUH29	2 component mutant KdpD SA1882	ery 5, lin 25	Lab stock
Liv093	P31321 236 HR	*S.aureus* WCUH29	2 component mutant srhSR SA1322		Lab stock
Liv094	P31320 262 P1	*S.aureus* WCUH29	2 component mutant SA1158	ery 5, lin 25	Lab stock
Liv095	P31320 264 P1	*S.aureus* WCUH29	2 component mutant SA1158	ery 5, lin 25	Lab stock

Liv130		*S. aureus* sigB::tet	SH1000 sigB mutant	tet 5	Lab stock
Liv137	SH1002	*S.aureus* sarA::kan	SH1000 sar mutant	kan 50, neo 50	Lab stock
Liv140		*S.aureus* WCUH29	S. aureus WCUH29 wild-type	None	Lab stock
Liv226		*S.aureus* Newman	agr::tet	tet 5	Lab stock
LIV692		S. aureus SH1000	arcA::tet clone 1	tet 5	Lab stock
LIV694		*S. aureus* SH1000	sasF::tet clone 1	tet 5	Lab stock
LIV718		*S. aureus* SH1000	vra::tet clone 1	tet 5	Lab stock
LIV873		*S. aureus* RN4220	xxxxxxxxxx		Lab stock
Liv1022		*S. aureus* SH1000	sar2632::tet	tet 5	Lab stock
Liv1023		*S. aureus* SH1000	mtlD::tet	tet 5	Lab stock
Liv1025		*S. aureus* SH1000	vraE::tet	tet 5	Lab stock
Liv1082		*S.epidermidis* Tu3298		tet 5	Lab stock
Ben's 002	S.hominis				Lab stock
Ben's064	Kocuria SPP				Lab stock
Ben's080	S. pasterui				Lab stock
Ben's020	M.luteus				Lab stock
Ben's160	S.capitis				Lab stock
Ben's102	S.warneri				Lab stock

2.5 Methods

2.5.1 Determination of bacterial cell density

2.5.1.1 Spectrophotometry (OD_{600})

Optical density (600 nm) of cell cultures was measured during growth using a 1cm path length spectrophotometer or a plate reader (Wallac Victor) to define bacterial yield. The culture samples were diluted 1:10 ratio with sterile BHI, when appropriate.

2.5.1.2 Colony Forming Unit (CFU) or Viable Cell Counts

Colony forming units (CFU) were used as a determination of viability. Bacteria were serially diluted in BHI or PBS before spreading replicates of 20 μl and growing on BHI agar plates overnight at 37 °C after which that number of colony forming units was counted.

2.6 Minimum Inhibitory Concentration Test (MIC)

Strains were grown overnight in BHI broth. Linoleic acid (100 mM) and D-sphingosine (1 mg/ml) were prepared as stocks in ethanol. 96-well polystyrene microtitre plates were loaded stock with 100 μl of BHI and lipid was added as described in table 2.3.

Table 2.3 The volume of inhibitor inoculated in each well with BHI media

Well	linoleic acid (mM)	BHI media (µl)
A1, E1	100	0
B1, F1	86.7	13.3
C1, G1	73.3	26.7
D1, F1	60	40

The volumes of lipid listed in table 2 were added to the first column of wells then serially diluted across the microtitre plate, except wells D11, D12, H11 and H12 that were utilised as positive and negative controls. Overnight cultures were adjusted to OD600=0.2 then 100 µl was added to each one. Plates were then incubated overnight at 37 °C. When cholesterol was added stock solutions (100 mM) was prepared in ethanol with heating to 80°C, and then were added to BHI prior to its addition to microtitre plates (final concentrations of 3 mM, 0.3 mM and 0.03 mM). Growth was measured via optical density following 24 hours incubation. Viable counts were determined by serial dilution and plate counting on BHI agar.

2.7 Staphyloxanthin extraction and assay

An absorbance spectrum of methanol extracted carotenoid pigment, staphyloxanthin, was determined using a Fluostar Omega (BMG Labtech) plate reader and values were corrected for relative cell density (OD_{600}) differences between strains. Strains were grown for 48 hours in BHI broth with agitation in the presence or absence of cholesterol. Cholesterol was

prepared as stock in ethanol and was incorporated at different concentrations (0.03, 0.3, 3 mM, ethanol control). After growth cells were centrifuged for 10 min at 4000 rpm. The cell pellets were extracted with methanol (500 µl), vortexed and then incubated for 15 min at 37 ºC. The methanol extract was centrifuged again and 96-well polystyrene microtitre plates were loaded with 250 µl of extract in triplicate. Experiments were repeated on three separate occasions.

2.8 Cholesterol rescue screen using Tn*551*

Mutant clones were inoculated with (0.05 v/v) into 96-well microtitre plates containing a mixture of linoleic acid (0.1 mM)/ethanol solution 0.29 % and cholesterol (0.3 mM) in BHI broth. Plates were incubated overnight at 37 °C in aerobic conditions. Growth was then determined by absorbance (600 nm) using spectrophotometer or evaluated relative to the isogenic parent strain by growing selected mutants in BHI broth consisting of the same concentration of cholesterol and linoleic acid and incubated overnight at 37 °C in aerobic conditions. Next, each mutant culture was serially diluted from 10^{-1} up to 10^{-6} and viable cells counts determined whereby 20 µL of each well or batch culture was spotted on BHI agar and incubated 24 hours at 37 ℃ in aerobic conditions.

2.9 Cholesterol rescue screening

Transposon *Tn551* mutants were inoculated (20 µL) into 96 well plates containing a mixture of linoleic acid (0.1 mM)/ethanol solution (0.37 %) and cholesterol (0.3 mM)/BHI broth (0.37 %) in a 1:1 ratio. These plates were then incubated overnight (37 ℃) in aerobic conditions. To assess the

presence of viable cells in each well 0.05 % (v/v) of each mixture was spotted on BHI agar and incubated overnight (37 ℃). The survival phenotype was then assessed after 24 h through the presence or absence colonies.

2.10 Cholesterol and other sterols

The culture of Newman strain in BHI was done in universals (30 ml) supplemented with 1 mM of cholesterol or 1 mM of erygosterol (a sterol present in yeast membrane), lanosterol or desmosterol (precursors cholesterol biosynthesis) and grown for 48 hours with 125 rpm shaking at 37 °C.

2.11 Effect of cholesterol on carotenoid expression

Cultures of SH1000 were grown with cholesterol 0, 0 ethanol control, 0.03 mM, 0.3 mM and 3 mM for 48 hours at 37 °C with shaking 125 rmp. Samples were taken and methanol extraction was performed. Viable count of cells was conducted for all samples to enable sample values to be compared to relative cell numbers.

2.12 Effect of cholesterol on carotenoid expression with 1 mM of linoleic acid

Cultures of SH1000 were grown with cholesterol 0, 0 ethanol control, 0.03 mM, 0.3 mM and 3 mM in the presence of 1 mM of linoleic acid for 48 hours at 37 °C with shaking at 125 rpm. Samples were taken for methanol extraction and viable count as described above.

2.13 Carotenoid Expression in Linoleic acid Sensitive mutants

The SH1000 *SigB, Sar2632, sasF, crtM,* and *arcA* mutant strains were grown in BHI with cholesterol (0, 0.03 mM, 0.3 mM and 3 mM) and incubated for 48 hours at 37 °C with shaking (125 rpm). Then, the samples were extracted by methanol. Viable count of cells was conducted for all samples.

2.14 Methanol Extraction of staphyloxanthin

This method is based on that described by Liu *et al* (2005). Briefly, bacterial cultures (5 ml) were centrifuged and the pellet was washed with PBS. A 1 ml aliquot of methanol was added and vortexed. The mixture was incubated for 15 min at 37 °C then centrifuged and 250 μl of supernatant was added to a 96 well plate and the absorbance spectrum measured.

2.15 Cell membrane hydrophobicity

This method is based on that described in Kotzamanidis *et al* (2010). Cultures were grown for 18 hours at 37 °C. A 1 ml sample of each culture was centrifuged (4000 rpm, 4 °C for 5 minutes) and then the supernatant was discarded and the pellet was washed with sterile, distilled water three times. The bacteria were suspended in 10 ml of distilled water before the optical density was adjusted to OD440 =0.5. A 50 µl aliquot of the culture was used to perform a 1:10 serial dilution (50 µl : 450 µl of PBS buffer) between 1×10^{-1} and 1×10^{-6} to determine cell numbers. The culture (3 ml) was mixed with 500 µl of n-hexadencane for 1 minute before being incubated at room temperature for 3 min. The OD_{440} then recorded for 1 ml of the

aqueous (top) layer, and another 50 µl was taken to do a 1:10 serial dilution in a 450 of PBS buffer from 1×10^{-1} up to 1×10^{-6}. Cell surface hydrophobicity calculated as a percentage reduction of relative in OD or viability of CFU ml^{-1}.

2.16 Hydrogen Peroxide Resistance Assay

Cultures of *S.aureus* strains SH1000 and Newman were grown in BHI overnight under different conditions, ethanol control and with presence and absence of 0.3 mM of cholesterol. Catalase 10 mg/ml added to PBS and used for sample quenching and dilution. Hydrogen peroxide (7.5 mM) was added. The samples were then heated at a heat block at 37 °C. The samples were taken in 0 min replicate spotted in agar plate, and after 10 min the samples were taken replicate in agar plates and the same every 10 min until 1 hour.

2.17 Linoleic acid Resistance Assay

Cultures of *S. aureus* strain Newman were grown in BHI overnight with different conditions 0, ethanol control and 0.3 mM of cholesterol. A 1 mM of linoleic acid was added. The samples were taken in 0 min until 1 hour every 10 min as previous experiment.

2.18 Construction of Allelic Replacement Mutants

For the construction of allelic replacement mutants of *desR* (SA1158) bacterial cultured were grown in BHI overnight with different concentration of cholesterol (0, 0.03, 0.3, 3 mM), The *desR* gene was PCR amplified as two flanking segments for insertion of a tetracycline resistance gene cassette to create an allelic replacement vector. The *tet* gene was also amplified and all of the purified DNA fragments were co-ligated into the suicide plasmid

pMUTIN4. The ligated products were used to transform *E. coli* TOP10 cells and clones resistant to tetracycline. Genomic DNA (~100 ng) (3 µl) or plasmids (~10 ng) served as the template for the PCR amplification of *S. aureus* flanking or resistance cassettes.

The reaction was performed using 50 µl of Reddy Mix master solution (Thermo), 3 µl of the forward (F) primer (Sigma), 3 µl of the reverse (R) primer (Sigma), 40.5 µl of water and 1 µl Accuzyme (Bioline). The PCR program consisted of an initial denaturation step at 95°C for 3 minutes, followed by 10 cycles consisting of:

Denature	95 °C, 30 sec
Anneal	50 °C, 30 sec
Extension	72 °C, 1 min 50 sec

The above reaction was then followed by 20 cycles consisting of (Increasing by 20 s per cycle):

Denature	95 °C, 30 sec
Anneal	50 °C, 30 sec
Extension	72 °C, 1 min 50 sec

2.19 DNA purification techniques

2.19.1 Gel extraction purification of DNA

PCR products were separated by electrophoresis on 2 % agarose gels (Bioline) and the DNA fragments were purified from the gel using an agarose gel extraction kit (QIAGEN) as per manufacturer's instructions, as follows.

The DNA band was cut from the agarose gel 1.5 ml or 2 ml and placed in a clean microfuge tube. 650 µl of buffer gel solubiliser was added to DNA/gel. The mixture was incubated for 10 min at 50 °C, and vortexed. The binding optimizer was then added to the mixture, and vortexed and the mixture transferred to a clean spin column and bound by centrifugation (10,000 rpm, for 1 min). The flow through was discarded before 700 µl wash buffer A was added and centrifuged (10,000 rpm, 1 min). The flow through was again discarded and 700 µl wash buffer was added and the column centrifuged for a further 1 min (10,000 rpm). The DNA was eluted into a clean microfuge tube by adding 50 µl of buffer EB and centrifugation (6,000 rpm, 1 min). DNA was stored at -20 °C.

DNA fragments were digested with the relevant enzymes (*EcoR*I, *BamH*I, *Kpn*I or *Not*I), purified and ligated with *EcoR*I and *BamH*I digested pMUTIN4 overnight at 4 °C. Ligation mixes were used to transform *E. coli* TOP10 with selection on LB agar plates containing tetracycline (12.5 µg ml^{-1}) or kanamycin (30 µg ml^{-1}), and incubated at 37°C.

2.20 Plasmid Purification

Bacterial cultures were grown overnight in LB broth containing appropriate antibiotics and next day 0.5-5 ml was transferred into an appropriate tube. The tube containing the culture was centrifuged for 1 min at 14,000 rpm. The pellet was resuspended in 250 µl resuspension buffer then the lysis buffer B, 250 µl, was added, mixed by inverting and incubated for 5 min at room temperature. Neutralization buffer, 350 µl, was added and mixed by inverting 4-6 times. The mixture was centrifuged (14,000 rmp, 10 min). The sample

was transferred to a spin column and placed in a collection tube centrifuged for (10,000 rmp, 1 min). 500 μl of wash buffer AP was added and centrifuged at 10,000 rpm for 1 min before 700 μl of wash buffer BP was added and the mixture was centrifuged at (10,000 rmp, 1 min). The DNA was eluted into a clean microfuge tube after adding 50 μl of buffer EB follwed by centrifugation (10,000 rpm, 1 min), and DNA was stored at -20 °C.

2.21 Molecular Biology Techniques/Isolation of PCR Products

2.21.1 Agarose gel electrophoresis

A gel was prepared by adding 1 g agarose to 100 ml TAE buffer (Tris acetate 40 mM and EDTA 1mM, pH 8.3). Midori green DNA dye was added (2 µL) to molten agarose to enable visualisation of DNA fragments using UV. Gels were run submerged in TAE buffer at 80-100 V depending on the degree of band separation required and the samples loaded varied according to the sample and its use.

2.21.2 Genomic DNA preparation

Culture of *S. aureus* (1 mL) was centrifuged for 15 min at 4000 rpm and the supernatant was discarded. The cell pellet was resuspended in 180 µl lysis buffer (1 mM EDTA, 10 mM Tris pH8 and 10 % (v/v) Triton X-100), and 2 µl lysostaphin (5 mg ml^{-1}). The samples were placed in a heating block at 37 °C for 30 min until almost clear. This solution was used in conjunction with the DNeasy Gram-Positive Bacteria DNA extraction kit and protocol

(QIAGEN). A 200 μl aliquot of buffer AL was added along with 25 μl of Proteinase K (20 mg ml^{-1}, QIAGEN) and vortexed thoroughly before incubation at 56 °C for 30 min. 200 μl of 96-100 % (v/v) ethanol was added and mixed by vortexing. The cleared lysate was then applied to a DNeasy column (QIAGEN).

Genomic DNA was bound to the column through centrifugation for 1 min (11,000 rpm) and the flow through was discarded. The DNA was purified with two wash steps; 500 μl of buffer AW1 was applied to the column and centrifuged (8,000 rmp, 1 min) and the flow through was discarded, then a second wash was 500 μl of buffer AW2 was applied to the column and again centrifuged (14,000 rpm, 3 min) and the flow through was discarded. The genomic DNA was eluted into a clean microfuge tube using 2X100 μl of buffer AE followed by centrifugation for 1 min (8,000 rpm). The purity of each DNA sample was assessed using a 1000 Nanodrop Spectrophotometer. The genomic DNA was stored at 4 °C.

2.21.3 PCR procedure

2.21.3.1 Arbitrary primed PCR (Polymerase chain reaction) target amplification

For the purpose of determining the site of transposon Tn*917* insertion within the *S. aureus* genome, two consecutive PCR reactions were performed. DNA sample was added at 4 μL (30-50 ng μL^{-1}) into a PCR tube containing 25 μl BioMix red (Bioline), 19 μL sterile distilled water and 1 μL (50 ng μL^{-1})

of primers ARB1B, ARB3 or ARB5 with 1 µL of primer ODG29 (Garsin et al., 2004b).

PCR was conducted according to the following conditions for amplification; an initial treatment of 95 ℃ for 2 min was followed by 30 cycles of the PCR profile below as first stage. After cycling the sample was held at 72 ℃ for 5 min.

The PCR products from the first reaction were diluted 25-fold in distil water and 5 µl was added to a PCR tube with 15 µl BioMix Red (Bioline), 8.5 µl sterile distilled water, and 0.75 µl of the primers ARB2 and ODG30 (Garsin et al., 2004b). The second stage the sample was after incubation at 95 ℃ for 2 min, 40 cycles of the second stage PCR profile was applied below. Then, the sample was held at 72 ℃ for 5 min.

First stage

Denature	95 °C, 30 sec
Anneal	42 °C, 30 sec
Extension	72 °C, 2 min

Second stage

Denature	94 °C, 30 sec
Anneal	45 °C, 50 sec
Extension	72 °C, 2 min

After gel electrophoresis to confirm amplicons had been generated, selected PCR products were cleaned up to remove primers and nucleotides by adding

ExoSAP-IT (2 µL) to 10 µL of each selected PCR product with incubation at 37 °C for 60 min and then 80 °C, both for 20 min. The samples were stored at 4 °C between steps. For sequencing primer ODG31 was sent with the reaction products to GATC-biotech.com and the products were sequenced. Analysis of sequenced samples was performed using nucleotide basic alignment tool (BLAST) on the Aureolist genome browser – (http://genolist.pasteur.fr/AureoList/index.html) identifying the nucleotide insertion point.

Table 2.4. The primers and genes were used in Arbitrary PCR and Qrt-PCR.

Oligonucleotide	Sequence (5' to 3')	Reference
crtM_For	TGATGACAGTATAGATGTTTATGG	(Kenny et al., 2009b)
crtM_Rev	ACATGCTGAAGGGCCATCATG	(Kenny et al., 2009b)
16S rRNA For	CGGTCCAGACTCCTACGGGAGGCAGCA	(Takle et al., 2007)
16S rRNA Rev	GCGTGGACTACCAGGGTATCTAATCC	(Takle et al., 2007)
ARB1B	GGCCACGCGTCGACTAGTACNNNNNNNNNNGT AAT	(Cao et al., 2007)
ODG29	GCAATAACCGTTACCTGTTTGTGC	(Garsin et al., 2004a)
ARB2	GGCCACGCGTCGACTAGTAC	(Cao et al., 2007)
ARB3	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT	(Tetsuka et al., 1997)
ARB5	GGCCACGCGTCGACTAGTACNNNNNNNNNGTTAC	(Lestrate et al., 2003)
ODG31	GATGTCACCGTCAAGTTAAATGTACAAA ATAACAGCG	(Garsin et al., 2004a)
ODG30	GAAAACTGTACCACTAATAACTCACAATAGAGAGATGTC	(Garsin et al., 2004a)
SigB For	TCAGCGGTTAGTTCATCGCTCACT	(Kenny et al., 2009b)
SigB Rev	GTCCTTTGAACGGAAGTTTGAAGCC	(Kenny et al., 2009b)
ftsZ For	ATCCAAATCGGTGAAAAATTAACAC	(Duquenne et al., 2010)
ftsZ Rev	CCATGTCTGCACCTTGGATTG	(Duquenne et al., 2010)
sspA For	TGA TAC ACA GCA TAT CCT CAT GCA	(Rice et al., 2001)
sspA Rev	TGG TCG CGA AGT GCC AAT A	(Rice et al., 2001)
aur For	ACC GTG TGT TAA TTC GTG TGC TA	(Valle et al., 2003)
aur Rev	ATG GTC GCA CAT TCA CAA GTT T	(Valle et al., 2003)

2.22 Protein analysis techniques

2.22.1 Preparation of protein samples from *S. aureus* cultures

S. aureus cell were cultured overnight in 50 ml BHI at 37 °C with shaking (250 rpm) until stationary phase (approx. 15 h) in the absence or presence of cholesterol (0.3 mM) in a 250 ml flask. Samples were taken to determine 5 OD_{600} units of culture were removed, and centrifuged (14,000 g, 10 min) and the supernatant was kept.

2.22.2 Preparation of protein samples from *S. aureus* cultures for challenge Experiment

Culture was grown overnight in BHI at 37 °C (SH1000 and Newman). The culture was inoculated OD_{600} 0.05 in 250ml flask with water bath shaking (125 rpm) at 37 °C until the OD_{600} reached 0.6. The cholesterol was added to two samples 0.3 mM and 3 mM and the third sample was cholesterol free, also another sample with ethanol only was acted as a control after 1 h. The samples were ready to take for the next step of exoproteins preparation.

2.23 Sample Preparation

2.23.1 Exoproteins

Trichloroacetic acid 100 % (w/v) was added (110 µL) to 1 ml of culture supernatant and mixed by vortexing. The mixture was incubated 30 min in ice, and was centrifuged (10 min, 14,000 rpm) to recover the precipitated proteins. The supernatant was discarded and the pellet was washed with ethanol buffer Tris pH 6.8, followed by centrifugation (14,000 rpm, 10 min)

and the supernatant was removed and the pellet air-dried. Sample buffer was added according to 5 OD/30 μl sample buffer and vortexed. The sample was then incubated at 80 °C for 10 min, then centrifuged (14,000 rpm, 2 min) before 2.5 μl was loaded onto an SDS-PAGE gel. Samples were placed at -20 °C for long-term storage.

2.24 RNA purification techniques

2.24.1 RNA extraction

The *S. aureus* strain SH1000 and Newman were inoculated into BHI media and grown (50 ml in 250 ml flask) in a water bath at 37 °C with shaking 125 rpm until OD_{600} 0.6. Cholesterol was added (0.3 mM) or solvent as control. After 30 min samples were taken for RNA extraction. Cells (0.5 ml) were harvested by centrifugation (4,000 rpm; 5 min; 4 °C) and re-suspended in 2 volumes RNAlater and incubated overnight at 4 °C. The samples were centrifuged and the pellet re-suspended with 42 μl lysostaphin (6 mg/ml), 33 μl mutanolysin (50 ku/10 ml) and 10 μl 100x TE. The mixtures were incubated (10-15 min) at 37 °C with mixing by flicking every 5 min, after which 25 μl of proteinase K was added and incubated for 30 min at 37 °C. Total RNA was then extracted from the cells using the RNAeasy kit (QIAGEN) according to the manufacturer's instructions, as follows. The mixtures were added to a spin column and centrifuged (10,000 rpm; 15 sec), and washed twice with 700 μl RW1 were added and centrifuged (10,000 rpm; 15 sec). The column was washed with 500 μl RPE three times and the mixture was vortexed before the layers were then separated again by

centrifugation (10,000 rpm; 15 sec). The upper layer was again removed to a clean column1 min before leaving to air-dry 2-5 min. The RNA was eluted into a clean microfuge tube after adding 2x30 μl of prewarmed DEPC water, by centrifugation (10,000 rpm, 2 min), 0.5 μl RNase was added. A 2 μl of the samples was used for Qubit analysis. DNase buffer was added 6.2 μl and 1 μl turbo DNase. The mixtures were incubated at 37 °C for 30 min. 350 μl of RLT 10 % was added, mixed well followed by 250 μl 0f 100 % (v/v) ethanol, and mixed well. The mixtures in the column were centrifuged into a clean up tube (10,000 rpm, 15 sec). The top layers were washed by adding 500 μl of RPE (10,000 rpm, 15 sec) then adding 500 μl of 80 % (v/v) ethanol (10,000 rpm, 15 sec) and centrifuging for 5 min. The RNA was eluted into a clean microfuge tube by adding 20 μl of prewarmed DEPC water with centrifugation (10,000 rpm, 1 min). A 1 μl of the samples were taken for Qubit analysis and then stored at –80 °C.

2.25 Phage techniques

2.25.1 Preparation of phage lysates

S. aureus strain SH1000 was grown into BHI (5 ml in a 30 ml universal), containing antibiotics where appropriate, and cells were harvested by centrifuging 3 ml of cells and re-suspending in 5 ml of broth to OD_{600} =0.1. 5 ml of phage buffer was added with 30 μl ($\geq 10^9$ pfu/ml) of stock lysate phage Φ11 was added. The mixtures were incubated at RT overnight. The lysate was filter sterilised and stored at 4 °C.

2.25.2 Phage transduction

S. aureus strains were grown in 20 ml LK in universal overnight. Culture was centrifuged at 5,000 rpm for 10 min and the cells were re-suspended in 1 ml LK Broth**.** Samples and control were prepared as follows:

Sample	**Control**
500 µl cells	500 µl cells
1 ml LK + 10 mM $CaCl_2$	1.5 ml LK + 10 mM $CaCl_2$
500 µl phage lysate	no phage

Mixtures were incubated at 37 °C for 25 min then 1 ml of ice cold 0.02 M Na citrate was added on ice for 5 min. Mixture were centrifuged at 5 K for 10 min and all supernatants were removed. The mixture was suspended in 1 ml of 0.02 M Na citrate and incubated on ice for 30 min to 2 h. 100 µl and 200 ul aliquots were spread on to LK cit plates containing selective drug 5 $mgml^{-1}$ ery, 5 kanamycin or 5 tetracycline plus 0.05 % (w/v) Na citrate. Plates were incubated at 37 °C for 1 to 2 hours. Plates were overlaid with 5 ml of LK Top agar containing selective antibiotics. Incubate for 24-48 hours at required 37 °C until colonies were visible.

2.26 Relative Quantitation Real Time PCR techniques (Qrt-PCR)

2.26.1 cDNA Synthesis

The cDNA was harvest from Tetro cDNA Synthesis Kit (BIOLINE) as followed, total RNA (up to 5 µg). The 1 µl of Primer Oligo was added with 1 µl 10 mM dNTP mix, 4 µl 5x RT buffer, 1 µl Ribosafe RNase Inhibitor, 1 µl Tetro Reverse Transcriptase (200 u/ µl) and DEPC-trated water to 20 µl

The mixture was spin by pipetting then PCR was conducted according to the following conditions:

45 °C for 30 min

85 °C for 5 min

The samples were chilled on ice and the reaction was stored in -20 °C or proceeds to Qrt-PCR immediately.

2.26.2 Primer Check

To check the primers the 10 µl sensifast was added with 5 µl distill water, 2 µl forward primer, 2 µl reverse primer and 0.01 % (v/v) diluted cDNA 10 fold -5. Then Qrt-PCR was set up to Quantitative standard curve.

To compare the different conditions 10 µl sensifast was added with 5 µl distill water, 2 µl forward primer, 2 µl reverse primer and 1 µl cDNA.

The mixtures were added in PCR 96 wells plate. The plate was centrifuged 10,000 rpm for 1 min; the Qrt-PCR machine was set up to comparative (ΔΔct). Qrt-PCR was conducted according to the following conditions for amplification; an initial treatment of 95 °C for 2 min was followed 40 cycles of the following

Denature	95 °C, 0.05 sec
Anneal	50 °C, 0.10 sec
Extension	72 °C, 0.05 min

Then followed by 1 cycle of melt curve as

55 °C, 0.02 sec

95 °C, 0.10 sec

CHAPTER 3- Characterisation of Cholesterol Rescue of Antimicrobial Fatty Acid

3.1 Introduction

S. aureus is a successful coloniser in a wide range of environments due to its ability to cope with stress and adapt to different conditions (Clements and Foster, 1999). *S. aureus* golden pigmentation is due to the C30 triterpenoid carotenoid staphyloxanthin. The biosynthetic genes form an operon, *crtOPQMN,* controlled by the accessory sigma factor σ^B (Bischoff et al., 2004, Pelz et al., 2005). There is no essential growth role for staphyloxanthin in *S. aureus,* however pigment increases survival of the microorganism in its host and resists certain antimicrobial functions of the immune system (Pelz et al., 2005). Previous studies have determined that the membrane characteristics of prokaryotes are modified by polar carotenoids in a manner comparable to cholesterol produced in eukaryotes (Rohmer et al., 1979).

The host employs a wide range of defence mechanisms to prevent infections by *S. aureus* and the skin is the first line of defence providing protection for the host from bacterial infections and disrupting and/or killing many microbial organisms (Krishna and Miller, 2012). Specific lipids are biosynthesised in the epidermis and are carried to the surface as cells differentiate, releasing a set of epidermal lipids. Yet other lipids are secreted onto the surface from the sebaceous glands (Drake et al., 2008a). Within the epidermal lipid pool, free sphingoid bases possess antimicrobial activity, and are created by enzymatic hydrolysis of epidermal ceramides (Drake et al., 2008a). Skin antimicrobial

free fatty acids (AFAs) are associated with the sebaceous lipid pool and have a critical role in preventing the growth of transient, and opportunistic microorganisms including *S. aureus* (Desbois et al., 2008). The presence of the skin bacterial microbiota also helps generate free fatty acids (FFAs) on the skin (Kenny et al., 2009b, Georgel et al., 2005, Takigawa et al., 2005). Skin lacking in FFAs is more susceptible to colonisation by *S. aureus,* an association that exacerbates atopic dermatitis (Takigawa et al., 2005, Georgel et al., 2005). In addition to inhibiting and killing bacteria, FFAs create conditions that restrict the growth of bacteria on the skin surface, by maintaining its acidic pH (Desbois and Smith, 2010). FFAs are reported to affect virulence factor expression that is vital for bacterial infection possibly via disordering bacterial signaling. (Kankaanpaa et al., 2004, Won et al., 2007, Osawa et al., 2001, Stenz et al., 2008, Davies and Marques, 2009).

It has been demonstrated that fatty acids are antimicrobial agents that interfere with the function of the membrane of *S. aureus*, leading to increased fluidity and they introduce disorder and increase permeability. Production of the carotenoid pigment staphyloxanthin increases membrane rigidity; thus potentially counteracting one of the modes of action of antimicrobial fatty acids and a *crtM* mutant has reduced survival when challenged with AFAs (Drake et al., 2008c, Kenny et al., 2009b). Other feasible antimicrobial mechanisms are that fatty acids disrupt the bacterial cell wall and cell metabolism, leading to reduced survival rates (Fischer et al., 2012). The fatty acid modifying enzyme (FAME) described in section 1.3.2.4 counteracts the presence of long chain unsaturated fatty acids by esterifaction with cholesterol; thereby reducing amphipathic toxicity torwards

the bacterial membrane (Mortensen et al., 1992b). The relative contribution of FAME to antimicrobial fatty acid defence is unknown as the gene remains to be identified.

Little is known about the role of epidermal lipids and environmental cholesterol with respect to *S. aureus*, other than that FAME can utilise it during esterification. It has been shown in an earlier study that cholesterol found in eye secretions affects the growth of *S. aureus* isolated from chronic eyelid infections (Shine et al., 1993b).

3.2 Specific aims

Published studies have determined that skin sebaceous fatty acids have antimicrobial effects on *S. aureus.* The specific aims for this study were to investigate the reported effect of cholesterol on the protection of *S.aureus* from antimicrobial fatty acid toxicity and to investigate whether cholesterol can ameliorate the effect of other antimicrobial lipids. Subsequently, a further aim was to investigate the effects of cholesterol on the growth and physiology of *S. aureus*.

3.3 Results

3.3.1 Effect of ethanol-solubilised cholesterol on survival from linoleic acid

Cholesterol was previously proposed to rescue the growth reduction caused by AFAs such as linoleic acid (Shine et al., 1993b). Thus, experiments were designed to investigate the impact of cholesterol on bacterial survival from linoleic acid.

Comparative growth was examined by culturing *S. aureus* in the presence of linoleic acid and cholesterol using microtitre 96-well plates. Both SH1000 and Newman strains were cultured in BHI broth containing 1 mM of linoleic acid in the absence and presence of a range of concentrations of ethanol-solubilised cholesterol (0, 0.03 mM, 0.3 mM, 3 mM) overnight at 37 °C.

Measuring growth yield by absorbance, the presence of ethanol-solubilised cholesterol at higher concentrations (3 mM) decreased the growth inhibition caused by linoleic acid. The yield of strain SH1000 increased around 300-fold (Fig 3.1 A) and strain Newman by around 40,000 fold (Fig 3.2 A). Viable counting confirmed these increased growth yields observed by absorbance measurement (Fig 3.1 B & Fig 3.2 B), and revealed increased survival was cholesterol concentration-dependent. The protective effect of cholesterol was more evident for strain Newman than SH1000. This appears to be due to strain Newman having a greater growth reduction in the presence of linoleic acid alone.

The growth yields of both strain SH1000 and Newman, in the presence of linoleic acid increased with an increasing concentration of supplemented ethanol-solubilised cholesterol (Figure 3.1 & 3.2). In the absence of cholesterol (orange symbols) there was a pronounced growth reduction caused by linoleic acid. With increasing ethanol-solubilised cholesterol across 0.03, 0.3 and 3 mM concentrations (dark yellow, yellow, beige respectively), there was a marked increase in yield such that growth was greatest at the highest concentrations of cholesterol despite the presence of growth-inhibitory concentrations of linoleic acid. The highest concentrations of cholesterol increased culture turbidity leading to erratic absorbance measurements (Fig 3.1), but the general observation of cholesterol increasing cellular protector was clearly demonstrated by viable counting (Fig 3.2).

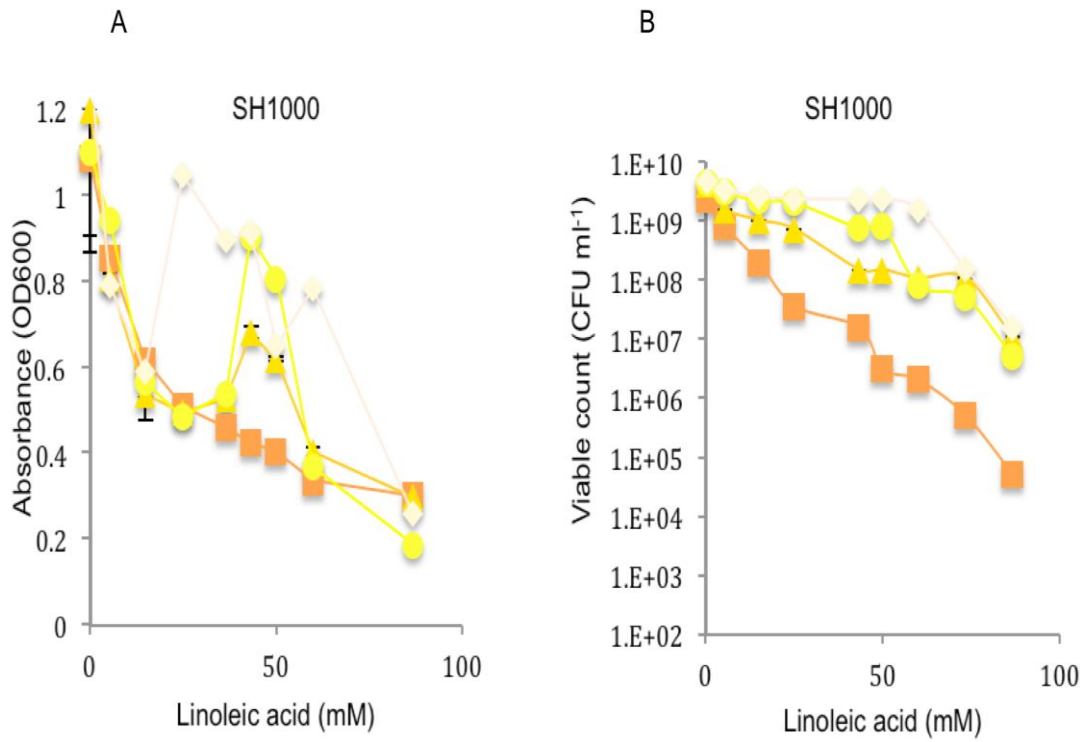


Figure 3.1. AFA survival of *S. aureus* in the presence of ethanol-solubilised cholesterol and linoleic acid. Absorbance of *S. aureus* SH1000 (A) and viable counts (B) following growth in BHI containing a range of concentrations of linoleic acid, in the presence (0.03 mM, dark yellow; triangle (0.029 % ethanol), 0.3 mM, yellow; circle (0.29 % ethanol), 3 mM, beige; diamond (2.89 % ethanol)) and absence of ethanol-solubilised cholesterol (orange; square), ethanol control, light orange; line (2.89 % ethanol). These data are representative of three experiments. Error bars represent standard error of the mean.

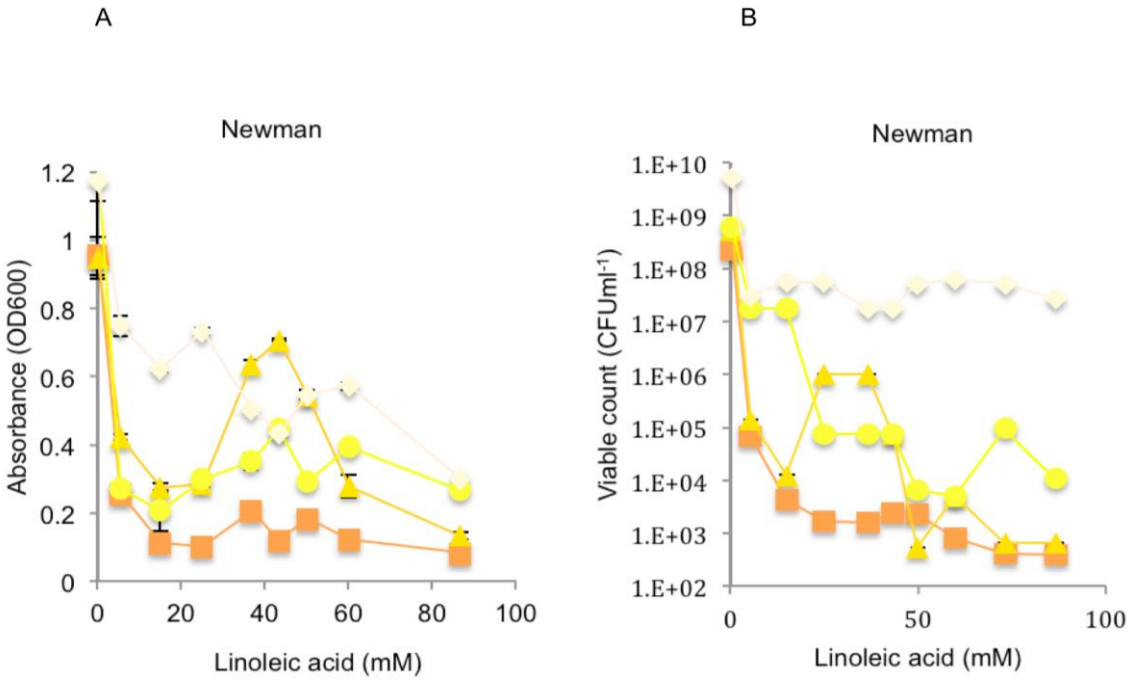


Figure 3.2. AFA survival of *S. aureus* in the presence of ethanol-solubilised cholesterol and linoleic acid. Absorbance of *S. aureus* Newman (A) and viable counts (B) following growth in BHI containing a range of concentrations of linoleic acid in the presence (0.03 mM, dark yellow; triangle (0.029 % ethanol), 0.3 mM, yellow; circle (0.29 % ethanol), 3 mM, beige; diamond (2.89 % ethanol)) and absence of ethanol-solubilised cholesterol (orange; square), ethanol control, light orange; line (2.89 % ethanol). These data are representative of three experiments. Error bars represent standard error of the mean.

3.3.2 Effects of ethanol-solubilised cholesterol on survival from D-sphingosine

A further investigation was undertaken to determine whether the growth-promoting effect of ethanol-solubilised cholesterol extended to a different chemical group of lipids. The amino alcohol D-sphingosine (2-amino-4-octadecene-1,3-diol) (chemical structure see (Fig 1.4)), has an aliphatic carbon chain and, like AFAs this lipid can insert into the bacterial membrane bilayer. Free sphingoid bases are widely recognised to have antimicrobial activity, and are created by enzymatic hydrolysis of epidermal ceramides. Antimicrobial lipids from the human skin surface include sapienic acid, lauric acid, sphingosine, dihydrosphingosine and 6-hydroxysphingosine (Drake et al., 2008a). Previous reports proposed that of the stratum corneum sphingolipids and phospholipids, only sphingosine has a clear and profound antimicrobial impact against *S. aureus* (Bibel et al., 1992). Sphingosine is recognised to exert effective antimicrobial activity on *S. aureus* at physiological levels, and sphingosine may play a considerable role in the defence mechanisms of healthy skin (Arikawa et al., 2002). Experiments were designed to establish whether the antimicrobial effect of D-sphingosine was modified by inclusion of cholesterol in the growth media, similar to the effect shown with AFA and cholesterol.

The *S. aureus* strain Newman was cultured in BHI broth with a range of concentrations of D-sphingosine (0-20 μg ml^{-1}) and ethanol-solubilised cholesterol (0, 0.03 mM, 0.3 mM, 3 mM). Growth was assayed using microtitre 96-well plates by measuring optical density after overnight culture at 37 °C.

D-sphingosine reduced *S. aureus* Newman survival, while the inclusion of ethanol-solubilised cholesterol in the media produced a concentration-dependent increase in survival as measured by absorbance, which was confirmed by viable count (Fig 3.3 B). The increased survival with ~6 μg ml^{-1} D-sphingosine was approximately 88 fold greater in the presence 3 mM of ethanol-solubilised cholesterol compared to growth in its absence (Figure 3.3 A). The growth promoting effect of the ethanol-solubilised cholesterol was more clearly observed with D-sphingosine compared with linoleic acid when using absorbance measurements. This was due to the solubility of D-sphingosine not contributing to turbidity at the concentrations used.

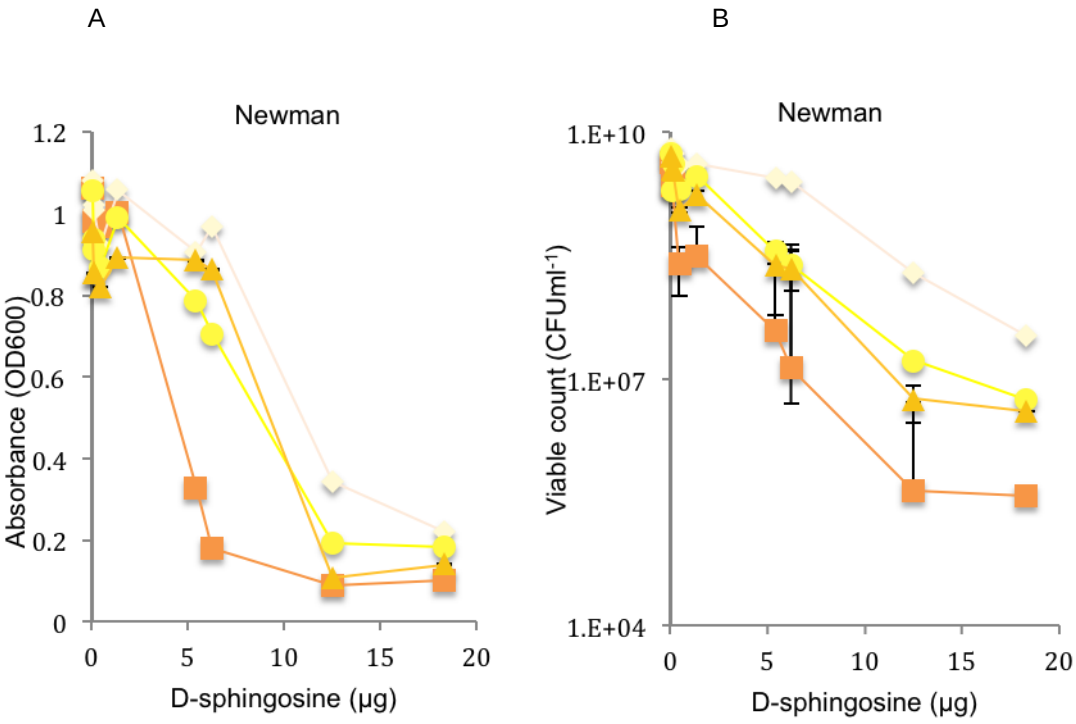


Figure 3.3. Growth-promoting effect of ethanol-solubilised cholesterol upon *S. aureus* Newman grown in the presence of D-sphingosine. Absorbance and viable count after growth in the presence of range concentrations of D-sphingosine in the presence (0.03 mM, light orange (0.02 % ethanol), triangle; 0.3 mM, yellow, circle (0.2 % ethanol); 3 mM, beige, diamond (2 % ethanol)) and absence of ethanol-solubilised cholesterol (orange, square). Growth was monitored by OD600 measurement (A) and confirmed using viable counts (B). These data are representative of three experiments. Error bars represent standard error of the mean.

3.3.3 Linoleic acid survival mutants and cholesterol rescue

A staphyloxanthin biosynthesis mutant (*crtM*) of *S. aureus* strain Newman has altered properties with respect to membrane order that affect its resistance to antimicrobials including cationic antimicrobial peptides (Mishra et al., 2011c). Its reduced survival from linoleic acid (Kenny et al., 2009b) was used for investigation into the growth-promoting effect of ethanol-solubilised cholesterol on the *crtM* mutant strain in the presence of AFA. The aim of this experiment was to determine the proportionate effect of cholesterol on the growth of the *crtM* mutant in the presence of linoleic acid.

The *crtM* mutant was cultured overnight at 37°C in BHI with 1 mM of linoleic acid, supplemented with different concentrations of ethanol-solubilised cholesterol (0, 0.03 mM, 0.3 mM, 3 mM).

The growth-promoting effect of cholesterol was clearly observed as linoleic acid concentrations increased and the cell yield was markedly raised, to levels similar to those of the wild-type Newman strain (Figure 3.4 A). There was up to a 1,000-fold improvement in the yield of the *crtM* mutant in the presence of ethanol-solubilised cholesterol (0.03 mM). Growth yield recovery was confirmed by viable count (Fig 3.4 B).

In addition, the Newman *crtM* strain was also cultured in BHI broth with a range of D-sphingosine and with different concentrations of ethanol-solubilised cholesterol (0, 0.03 mM, 0.3 mM, 3 mM). Growth yield was assayed using microtitre 96-well plates by comparing optical density after overnight culture at 37 °C. The reduced survival of the Newman *crtM* strain in the presence of D-sphingosine was reversed by the addition of ethanol-

solubilised cholesterol across a range of concentrations; with the survival increasing about 130 fold in 3 mM of cholesterol (Figure 3.5). The large increase in growth yield of *crtM* mutant indicates that addition of ethanol-solubilised cholesterol has a protective effect. This protection might occur at the cell surface, or potentially linoleic acid could be interacting with the cholesterol independent of the cell surface.

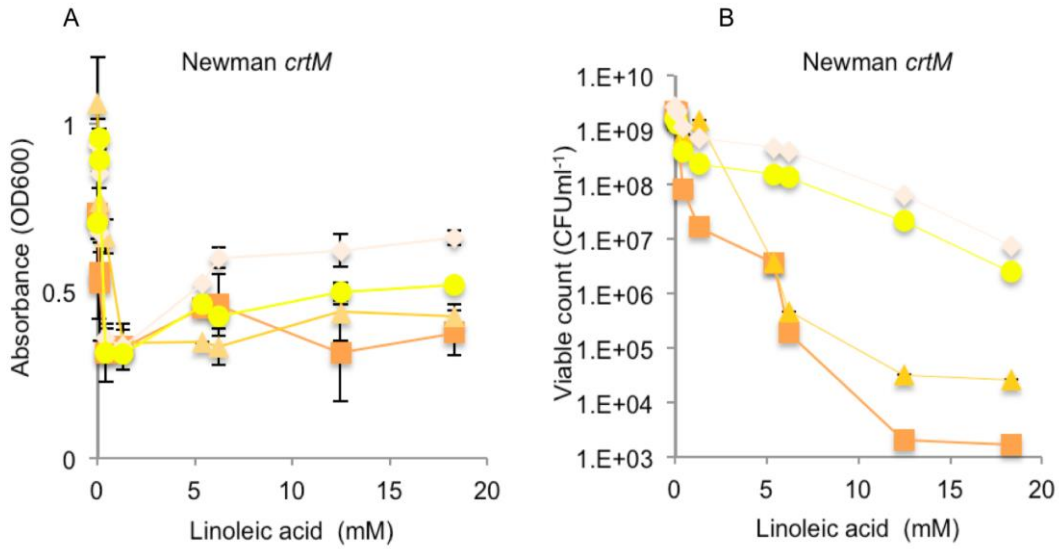


Figure 3.4. Growth yield of *S. aureus* Newman *crtM* in the presence of ethanol-solubilised cholesterol and linoleic acid. Newman *crtM* growth in BHI containing a range of concentration of linoleic acid in the presence (0.03 mM, dark yellow; triangle (0.029 % ethanol), 0.3 mM, yellow; circle (0.29 % ethanol), 3 mM, beige; diamond (2.89 % ethanol)) and absence of ethanol-solubilised cholesterol (orange; square). (A). Viable count of Newman *crtM* (B). This experiment is representative of three experiments. Error bars represent standard error of the mean.

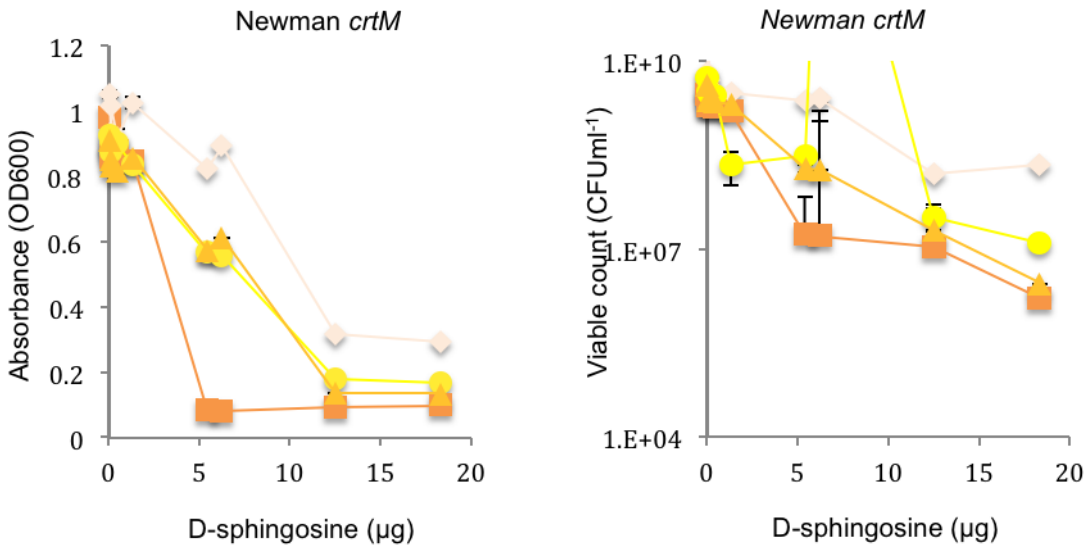


Figure 3. 5. Growth yield of *S. aureus* Newman *crtM* in the presence of amino alcohol D-sphingosine and ethanol-solubilised cholesterol. Absorbance and viable count following growth with a range of concentrations of D-sphingosine in the presence (0.03 mM, triangle, light orange (0.02 % ethanol); 0.3 mM, circle, yellow (0.2 % ethanol); 3 mM, diamond; beige (2 % ethanol)) and absence of ethanol-solubilised cholesterol (orange; square). Growth was monitored by OD600 measurement and confirmed using viable counts. This experiment is representative of three experiments. Error bars represent standard error of the mean.

3.3.4 The effects of ethanol-solubilised cholesterol on other staphylococci

The previous experiments determined that *S. aureus* strains could grow in the presence of antimicrobial concentrations of linoleic acid when supplemented with ethanol-solubilised cholesterol, and that growth was also rescued in a *S. aureus crtM* mutant. To address the potential role of staphyloxanthin in the survival of the organism and to establish future lines of investigation of the cell membrane roles of staphyloxanthin, the growth of the non-pigmented species *S. epidermidis* was investigated.

S. epidermidis TÜ3298 was cultured overnight at 37 °C in BHI with 1 mM of linoleic acid and was supplemented with a range of concentrations of ethanol-solubilised cholesterol (0, 0.03 mM, 0.3 mM, 3 mM) using microtitre 96-well plates.

The growth yield of *S. epidermidis TÜ3298* was reduced in the presence of linoleic acid but it progressively recovered with increasing concentrations of ethanol-solubilised cholesterol in the broth. In the absence of cholesterol (orange, square) and ethanol control (light orange, line) there are a clear growth reduction caused by linoleic acid. With supplemented cholesterol at 0.03, 0.3 and 3 mM (dark yellow; triangle, yellow; circle, beige; diamond) respectively, there was a marked increase in viability, such that there was growth at the highest concentrations of cholesterol despite the presence of a growth-inhibitory concentration of linoleic acid. *S. epidermidis TÜ3298* survival from linoleic acid increased 100-fold when supplemented with 3 mM

cholesterol in the presence of 60 mM linoleic acid, compared with the absence of cholesterol (Fig 3.6).

A B

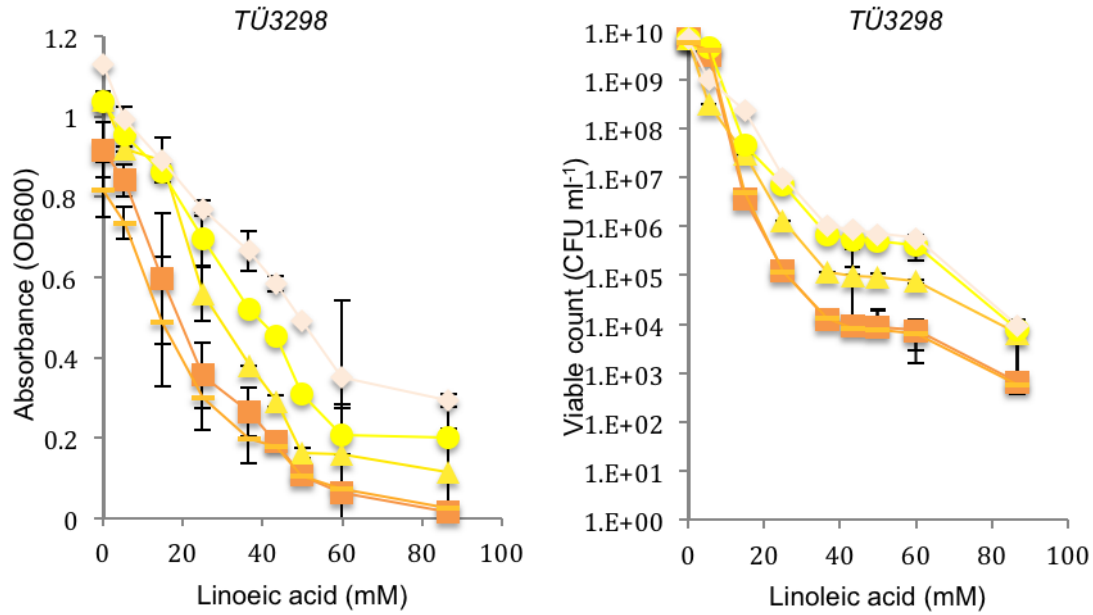


Figure 3.6. Growth yield of *S.epidermidis TÜ3298* in the presence of etanol-solubilised cholesterol and linoleic acid. Growth of *S.epidermidis* in the presence of different concentration of linoleic acid and cholesterol (0.03 mM, dark yellow; triangle (0.029 % ethanol), 0.3 mM, yellow; circle (0.29 % ethanol), 3 mM, beige; diamond (2.89 % ethanol)) and absence of ethanol-solubilised cholesterol (orange; square). (A). Viable count of *TÜ3298* (B). This experiment is representative of three experiments. Error bars represent standard error of the mean.

3.3.5 Roles of cholesterol in survival from linoleic acid

The exoprotein fatty acid-modifying enzyme (FAME) is produced by both *S. aureus* and *S. epidermidis* and esterifies lipids with cholesterol to reduce their cellular toxicity (Chamberlain and Brueggemann, 1997, Kapral et al., 1992).

FAME production is regulated by *agr* and *sarA* in *S. aureus* (Chamberlain and Imanoel, 1996, Cheung et al., 2004) and there is a correlation between the lipase activity of the organism and the production of FAME (Chamberlain and Brueggemann, 1997). Expression of FAME activity was reported to be minimal in both *agr* and *sarA* mutants. The aim of this experiment was to determine whether ethanol-solubilised cholesterol could rescue the mutant strains *agr* and *sar* in the presence of linoleic acid. This would indicate the extent to which FAME participated in the cholesterol-dependent survival described previously.

For the experiment, SH1000 *agr*, Newman *agr* and Newman *sar* were cultured overnight at 37 °C in BHI with 1 mM of linoleic acid and supplemented with different concentrations of ethanol-solubilised cholesterol (0, 0.03 mM, 0.3 mM, 3 mM). The data revealed that *S. aureus Newman agr* displayed increased survival from linoleic acid when supplemented with increasing concentrations of ethanol-solubilised cholesterol, and the survival was about 64-fold greater in 3 mM of cholesterol compared with survival in the absence of cholesterol (Fig 3.7). *Newman sarA* was also revealed to have increased survival from linoleic acid by adding increasing cholesterol concentrations but only with about 2-fold increase with 3 mM of added

cholesterol compared with the absence of cholesterol (Fig 3.8). *SH1000 agr* showed increased survival compared with its isogenic wild-type parent, but less than strain Newman *agr* with a 9-fold increase in survival in 3 mM of cholesterol compared with the absence of cholesterol (Fig 3.9); *SH1000 sarA* was not tested. The gene encoding FAME is not known and it is hard to directly examine the contribution of FAME, but the data suggests that in the presence of linoleic acid the *agr* and *sar* mutants still display recovery with supplemented cholesterol somewhat similar to wild type, although FAME activity is reported to be minimal in both mutants. This might indicate a minor role of FAME in the observed recovery with ethanol-solubilised cholesterol recovery.

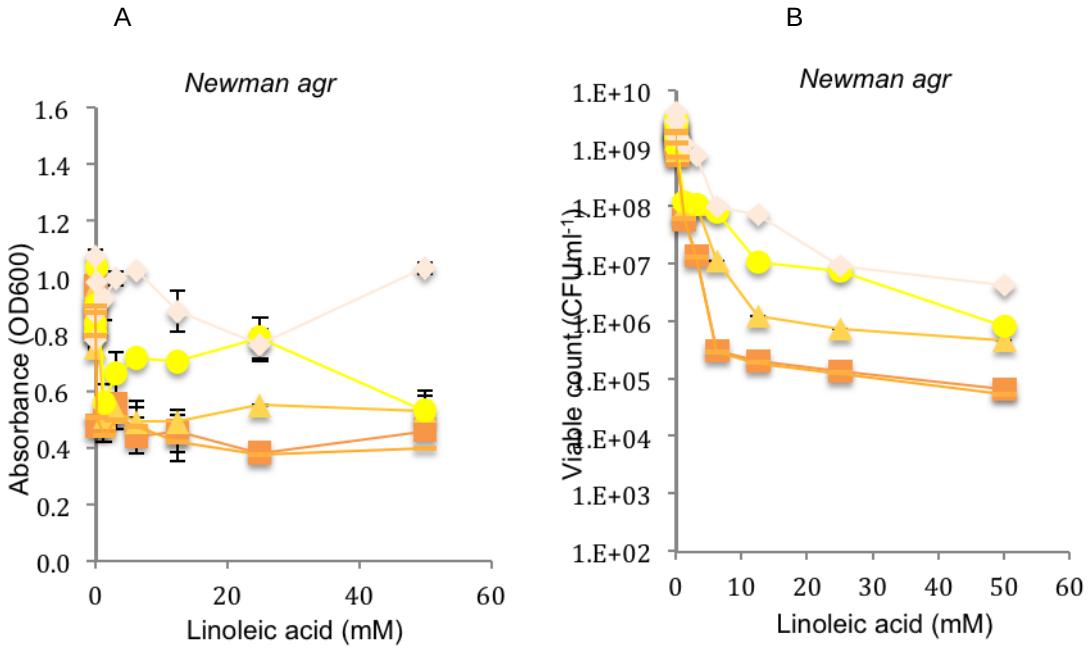


Figure 3.7. Growth yield of *S. aureus Newman agr* in the presence of linoleic acid. Linoleic acid growth and cholesterol rescue in *Newman agr* in the presence of a range of concentration of linoleic acid and cholesterol (0.03 mM, triangle, dark yellow (0.29 % ethanol); 0.3 mM, circle, yellow (0.29 % ethanol); 3 mM, diamond, beige (2.89 % ethanol)) and absence of cholesterol (square, orangem). (A). Viable count of *Newman agr* (B). This experiment is representative of three experiments. Error bars represent standard error of the mean.

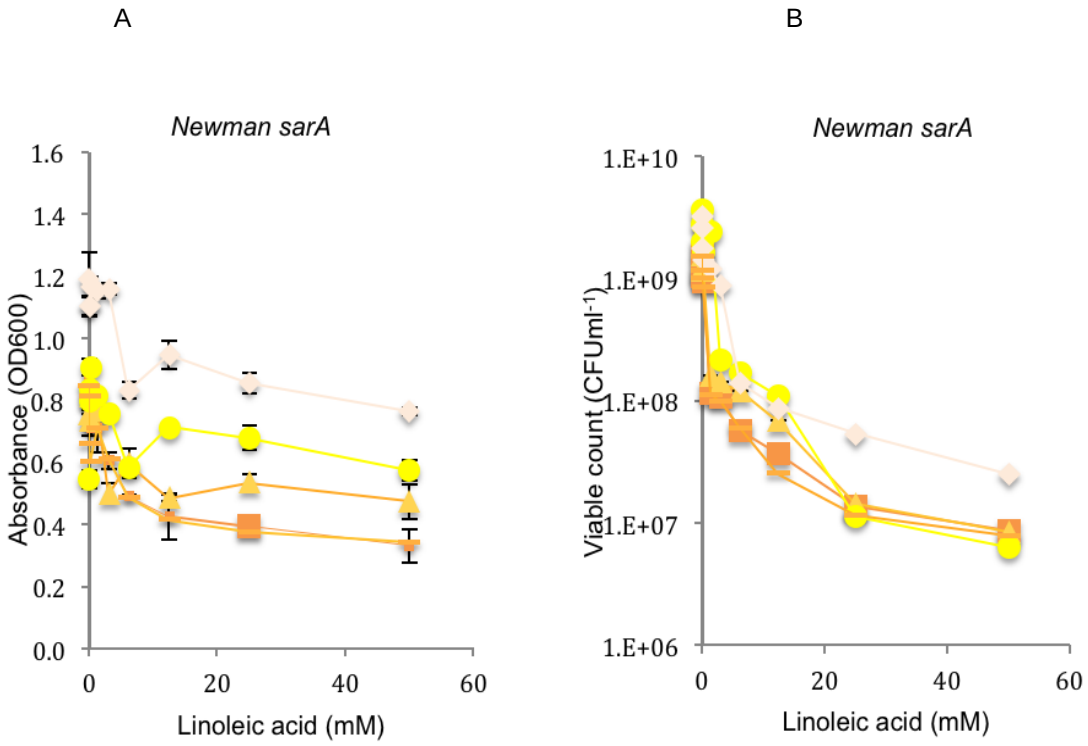


Figure 3.8. Growth yield of *S. aureus Newman sarA* in the presence of linoleic acid. Linoleic acid survival and cholesterol rescue in *Newman sarA* in the presence of (0.03 mM, triangle, dark yellow (0.029 % ethanol); 0.3 mM, circle, yellow (0.29 % ethanol); 3 mM, diamond, beige (2.89 % ethanol)) and absence of cholesterol (square, orange). (A). Viable count of *Newman sarA* (B). This experiment is representative of three experiments. Error bars represent standard error of the mean.

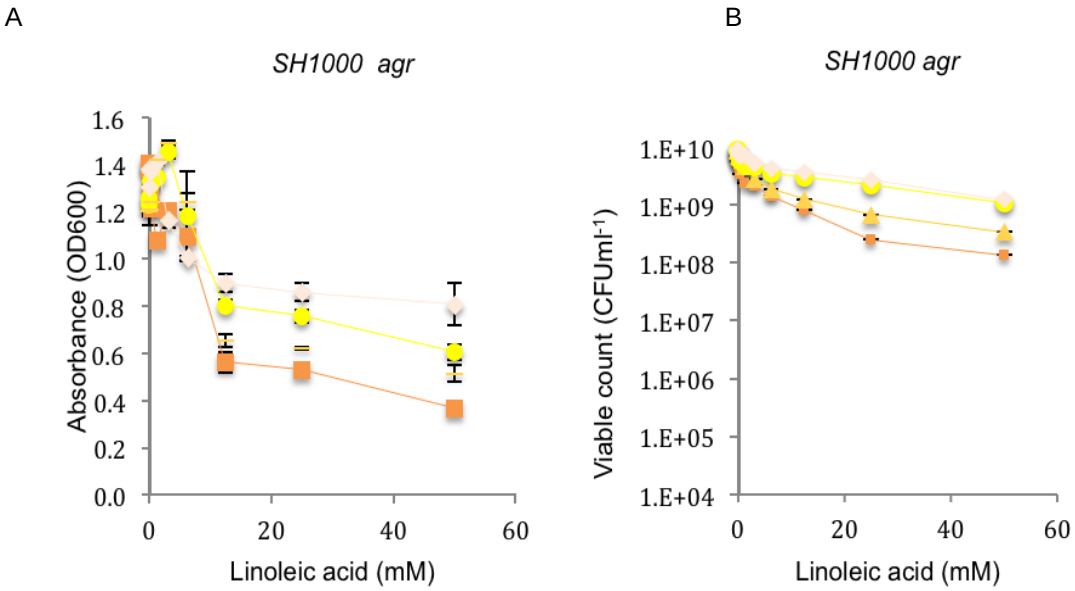


Figure 3.9. Growth yield of *S. aureus SH1000 agr* in the presence of linoleic acid. Survival of *S. aureus SH1000 agr* in the presence of cholesterol (ethanol control, line (2.89 % ethanol), light orange; 0.03 mM, triangle (0.029 % ethanol), dark yellow; 0.3 mM, circle (0.29 % ethanol), yellow; 3 mM, diamond, beige (2.89 % ethanol)) and absence of cholesterol (square, orange) (A). Viable count of *SH1000 agr* (B). This experiment is representative of three experiments. Error bars represent standard error of the mean.

3.3.6 Effect of ethanol-solubilised cholesterol on staphyloxanthin expression

Staphyloxanthin is a characteristic pigment of *S. aureus* and is associated with virulence by acting to protect cells from reactive oxygen species and changes in membrane fluidity (Liu et al., 2008, Liu et al., 2005a, Clauditz et al., 2006).

Targeted mutagenesis of the genes from the *crtOPQMN* operon revealed that staphyloxanthin expression is vital for resistance of *S. aureus* to oxidative stress, with transcription controlled by the alternative sigma factor *SigB* (Clauditz et al., 2006). Staphyloxanthin also impacts on the *S. aureus* cellular membrane by decreasing membrane fluidity, which is required to ensure resistance to cationic AMPs utilised by the host to reduce bacterial pathogen colonisation (Mishra et al., 2011c).

From the previous data in this study there are at least two possible scenarios whereby *S. aureus* might interact extracellularly with linoleic acid and cholesterol. Cholesterol may interact with linoleic acid in broth and increase *S. aureus* survival since less of the AFA will interact with the cell. Alternatively, cholesterol may be incorporated into the membrane and stabilise order to counteract the impact of linoleic acid.

SH1000 was cultured in 10 ml of BHI in the absence or presence of different concentrations of ethanol-solubilised cholesterol (0.03 mM, 0.3 mM and 3 mM). A staphyloxanthin expression assay was performed for this experiment whereby 10 ml of each culture was centrifuged for 15 min and the supernatant was discarded. Methanol was added to the samples and incubated for 15 min

at 37 °C. The methanol extract was centrifuged again and supernatant samples were loaded into 96 well plates with 250 µl of extract measured in triplicate.

From this experiment it was observed that the carotenoid pigmentation of the cells was reduced as the ethanol-solubilised cholesterol concentration was increased. Cells grown in the solubilised cholesterol had reduced staphyloxanthin expression causing loss of colour from their usual golden yellow (Fig 3.10 SH1000 and Newman). A concentration-dependent reduction in the staphyloxanthin content of the *S. aureus* cell membranes was clearly observed in the presence of added solubilised cholesterol (Fig 3.11 for SH1000 & Fig 3.13 for Newman). The viable count established reduced growth for both strains when supplemented with higher concentrations of solubilised cholesterol and was used to adjust spectra relative to growth yield (Fig 3.12 & Fig 3.14)

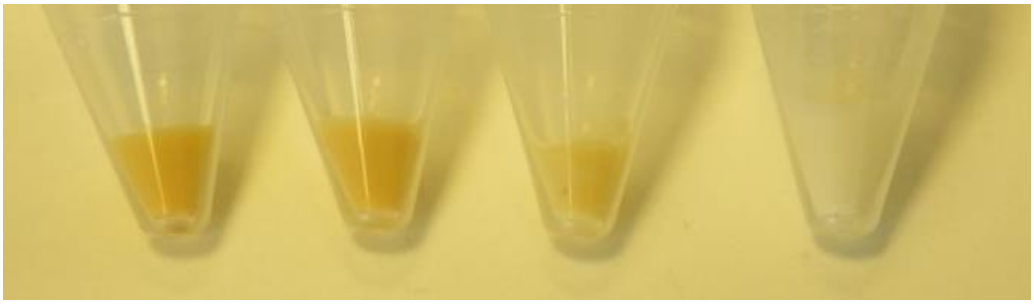
The observation from the previous experiment that cholesterol promotes growth of *S. aureus* in the presence of AFAs was reconsidered to determine the effect that linoleic acid had on the cholesterol-dependent reduction of staphyloxanthin in cell membranes. SH1000 was cultured in 10 ml of BHI in the absence or presence of different concentrations of cholesterol: 0.03 mM, 0.3 mM and 3 mM plus linoleic acid (1 mM). Pigmentation screening was performed as previously described for methanol extraction.

In the presence of linoleic acid, the addition of ethanol-solubilised cholesterol reduced expression of staphyloxanthin, but not to the same extent as with cholesterol alone at 3 mM. Supplementation with 0.3 mM

cholesterol reduced staphyloxanthin but little effect was noticeable with 0.03 mM (Figure 3.15). Viable counts were used to confirmed relative growth and adjust spectra for cell number (Fig 3.16).

A

SH1000

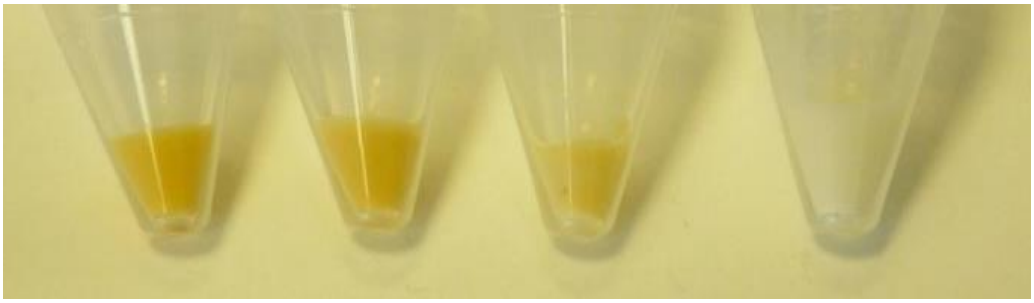


0 0.03 0.3 3

Cholesterol concentration (mM)

B

Newman



0 0.03 0.3 3

Cholesterol concentration (mM)

Figure 3.10. Staphyloxanthin expression with ethanol-solubilised cholesterol. (A) SH1000 and (B) Newman cell pellets after growth in solubilised cholesterol (0.03 mM, yellow; 0.3 mM, light yellow; 3 mM, white; compared to cells grown in the absence of cholesterol (orange). This experiment is representative of three experiments.

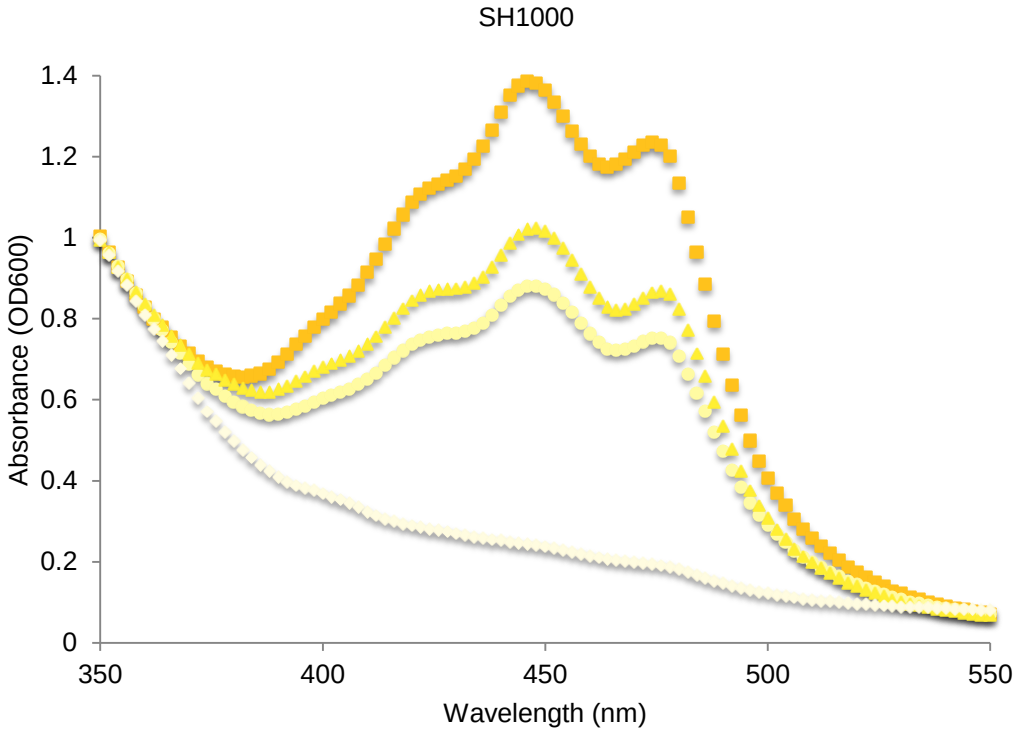


Figure 3.11. Staphyloxanthin expression in cholesterol-treated *S. aureus* SH1000. Absorbance spectrum of cells treated with ethanol-solubilised cholesterol (0.03 mM, yellow; triangle (0.029 % ethanol); 0.3 mM, light yellow; circle (0.29 % ethanol) and 3 mM, beige; diamond (2.89 % ethanol); compared to cells grown in the absence of cholesterol orange (square). This experiment is representative of three experiments.

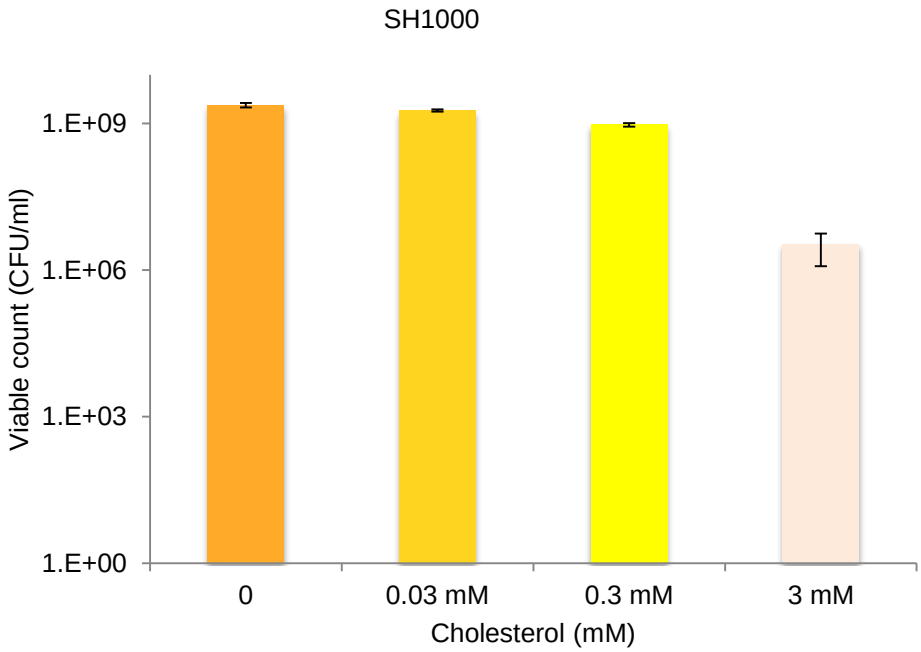


Figure 3.12. Viable count of SH1000 cells cultured with ethanol-solubilised cholesterol. The numbers of cells from different concentrations of solubilised cholesterol 0.03 mM (0.029 % ethanol); dark yellow, 0.3 mM (0.29 % ethanol); yellow and 3 mM (2.89 % ethanol) and in the absent of cholesterol; orange. This experiment is representative of three experiments. Error bars represent standard error of the mean.

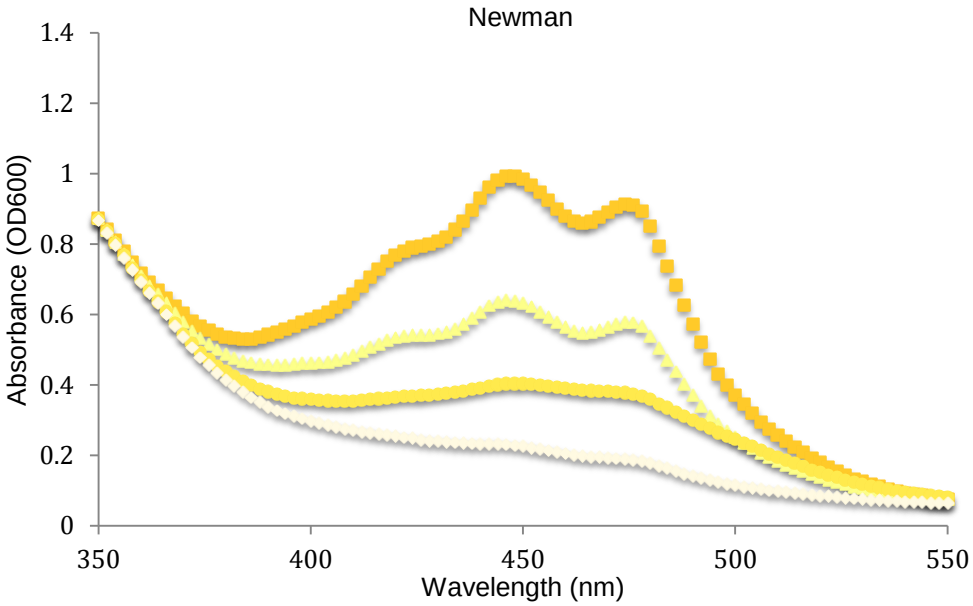


Figure 3.13. Staphyloxanthin expression of cholesterol-treated *S. aureus* Newman. Absorbance spectrum of cells treated with solubilised cholesterol (0.03 mM, yellow; triangle (0.029 % ethanol); 0.3 mM, light yellow; circle (029 % ethanol); 3 mM, beige; diamond (2.89 % ethanol) compared to cells grown in the absence of cholesterol (orange) (square). This experiment is representative of three experiments.

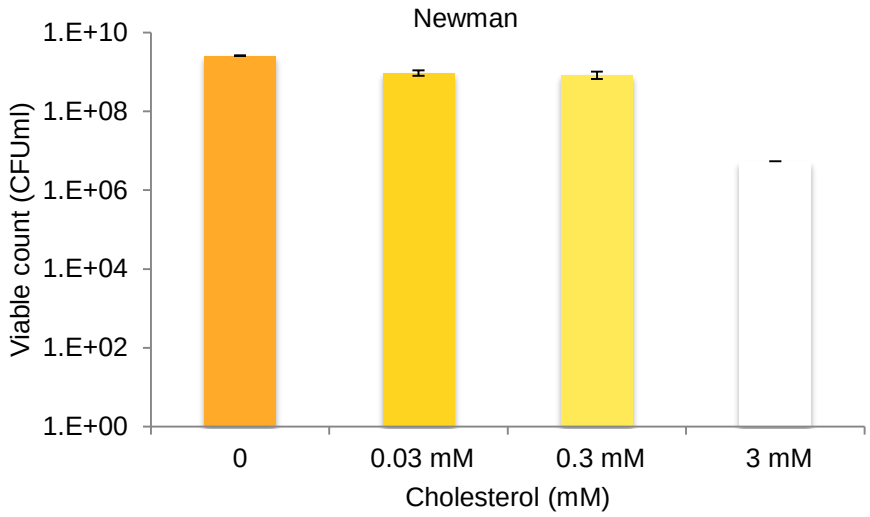


Figure 3.14. Viable count of Newman cells cultured with solubilised cholesterol. The number of cells in different concentration of cholesterol 0.03 mM (0.029 % ethanol); dark yellow, 0.3 mM; yellow (0.29 % ethanol) and beige 3 mM (2.89 % ethanol) and in the absent of cholesterol; orange. This experiment is representative of three experiments. Error bars represent standard error of the mean.

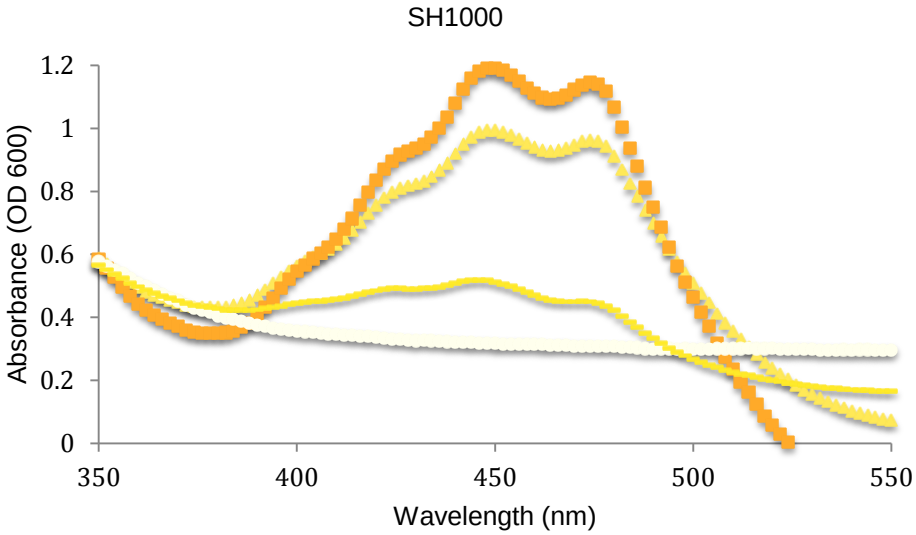


Figure 3.15. Staphyloxanthin expression of solubilised cholesterol-treated *S. aureus* SH1000. Absorbance spectrum of cells cultured in the presence of linoleic acid (1 mM) (0.9 % ethanol) in the absence or presence of solubilised cholesterol (0.03 mM, yellow (0.037 %); triangle, 0.3 mM, light yellow, circle (0.37 % ethanol); 3 mM, beige; diamond (3.79 % ethanol); ethanol control; line (3.79 % ethanol) compared to cells grown in the absence of cholesterol (orange) (square). This experiment is representative of three experiments.

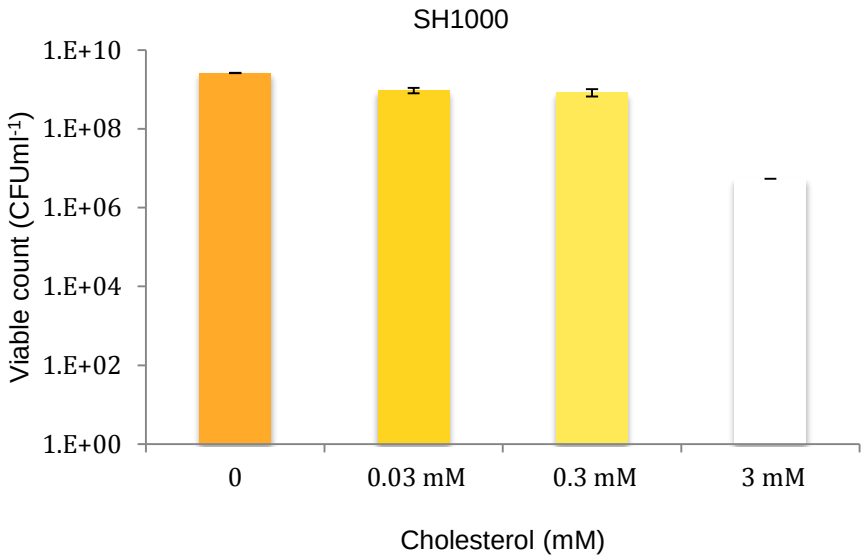


Figure 3.16. Viable counts of *S. aureus* cultured with ethanol-solubilised cholesterol plus linoleic acid. Viable count of cells shows the number of the cells in different concentration of cholesterol in the original cultures. The cultures were 0.03 mM; dark yellow (0.037 % ethanol), 0.3 mM; yellow (0.37 % ethanol) and 3 mM; beige (3.79 % ethanol) and in the absent of cholesterol and in the presence of 1 mM of linoleic acid; orange (0.9 % ethanol). This experiment is representative of three experiments. Error bars represent standard error of the mean.

3.3.7 Carotenoid expression in the presence of solubilised cholesterol in other pigmented bacteria

With the exception of *S. epidermidis* most staphylococci that colonise human skin possess genes for the production of staphyloxanthin. Several common Gram-positive pigmented species, whose colonies display a yellow colour, have been isolated from the skin (Kaur et al., 2011). Among these are *S. aureus* (Lindberg et al., 2004) isolated from the nares (Aswani et al., 2011), *S. capitis* and *S. hominis* commonly isolated from the head and arms and *S. cohnii* and *S. warneri*, which are infrequently isolated species from skin (Kloos and Musselwhite, 1975). *Micrococcus luteus* is the predominant of the micrococci isolated from the skin (Kloos and Musselwhite, 1975), while *Kocuria rosea* is classified as part of the family Micrococcaceae and is less frequently isolated from mammalian skin (Kaur et al., 2011, Sims et al., 1986, Reddy et al., 2003).

The specific purpose of this exploration was to investigate species of pigmented skin bacteria with the aim of determining whether cholesterol impacted upon these pigmented species in the same way as that observed with *S. aureus*. The pigmented strains tested included *S. capitis, S. warneri* and *S. hominis and* these staphylococci were compared with the pigmented micrococci, *K.rosea,* and *M. luteus*.

S. capitis, S. warneri, S. hominis, Kocuria rosea, and *Micrococcus luteus* were cultured in 10 ml of BHI in the absence and presence of different concentrations of solubilised cholesterol (0.0 mM, 0.03 mM, 0.3 mM and 3.0 mM) as described previously in the pigmentation screening methods.

S. hominis and *S. capitis* exhibited reduced staphyloxanthin expression at higher concentrations of cholesterol, similar to SH1000 (Fig. 3.17 & 3.18). The effect was less clear for *S. warneri,* since the strain tested expressed little extractable staphyloxanthin (Fig. 3.19). Members of the other genera, such as *Micrococcus luteus* and *Kocuria rosea,* appear to express a methanol extractable pigment with similar spectral characteristic to staphyloxanthin. The expression of this pigment was regulated in a similar manner to the staphylococci, in response to solubilised cholesterol added to the media (Fig. 3.20 & 3.21).

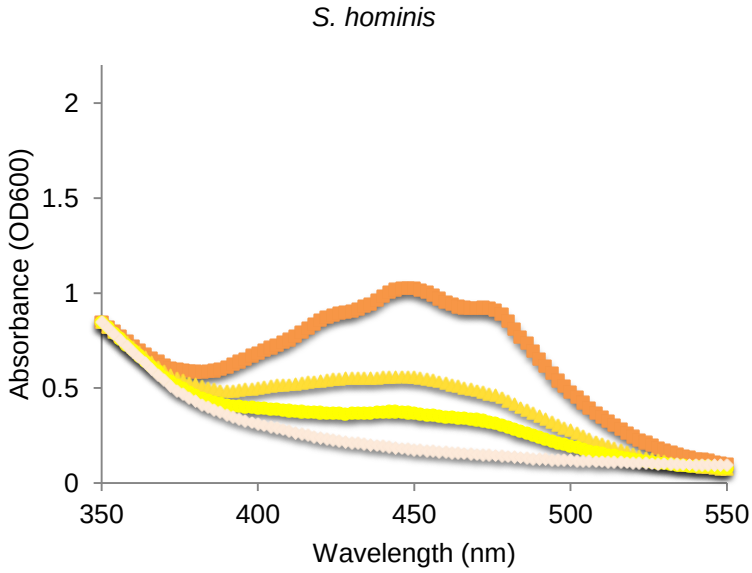


Figure 3.17. Staphyloxanthin expression of *S. hominis* in the presence of solubilised cholesterol. Absorbance spectrum of cells treated with cholesterol 0.03 mM triangle, yellow (0.029 % ethanol); 0.3 mM, circle light yellow (0.29 % ethanol); 3 mM, diamond, white (2.89 % ethanol)) compared to cells grown in the absence of cholesterol (orange square). This figure is representative of three experiments.

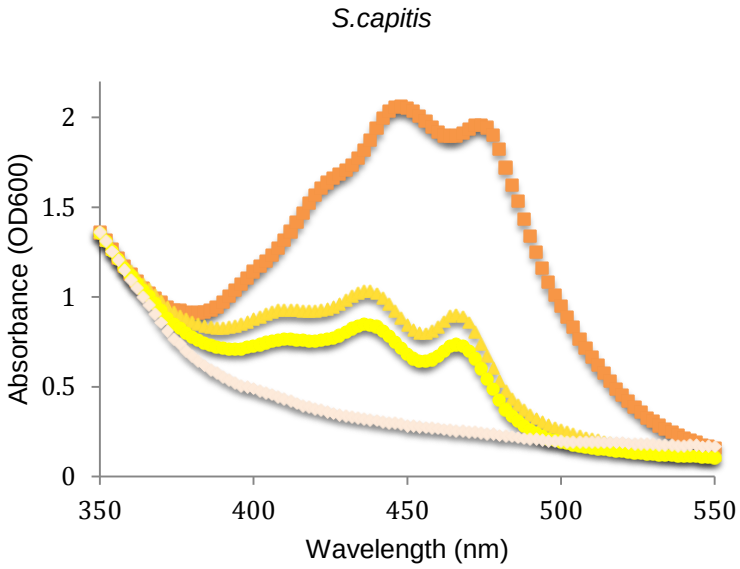


Figure 3.18. Staphyloxanthin expression of *S. capitis* in the presence of solubilised cholesterol. Absorbance spectrum of cells treated with cholesterol (0.03 mM triangle, yellow (0.029 % ethanol); 0.3 mM, circle light yellow (0.29 % ethanol); 3 mM, diamond, white (2.89 % ethanol)) compared to cells grown in the absence of cholesterol (orange square). This figure is representative of three experiments.

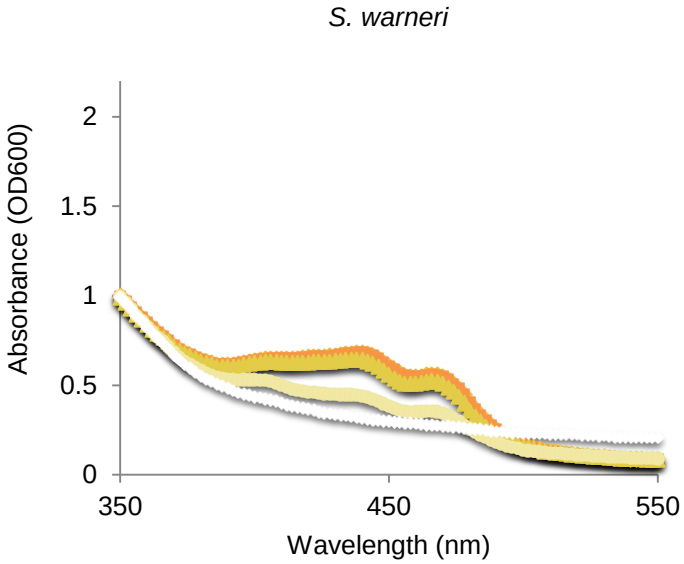


Figure 3.19. Staphyloxanthin expression of *S. warneri* in the presence of solubilised cholesterol. Absorbance spectrum of cells treated with cholesterol 0.03 mM triangle, yellow (0.029 % ethanol); 0.3 mM, circle light yellow (0.29 % ethanol); 3 mM, diamond, white (2.89 % ethanol)) compared to cells grown in the absence of cholesterol (orange square). This figure is representative of three experiments.

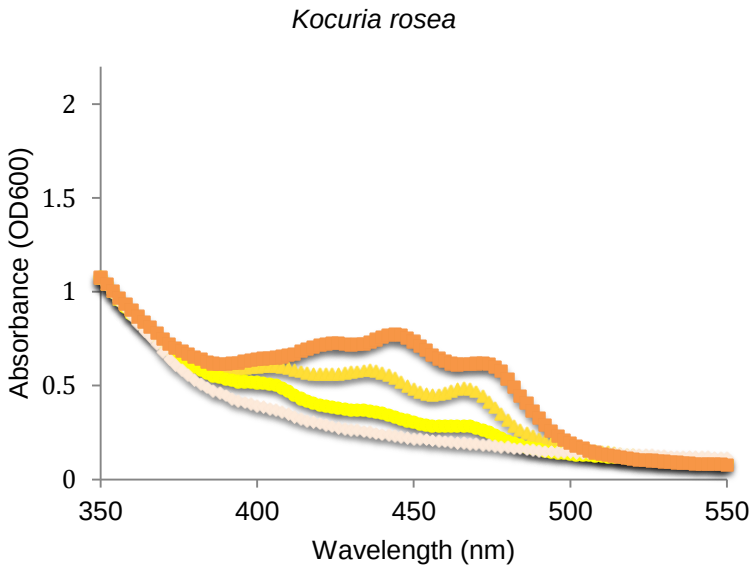


Figure 3.20. Staphyloxanthin expression of *Kocuria rosea* in the presence of solubilised cholesterol. Absorbance spectrum of cells treated with cholesterol light orange; 0.03 mM triangle (0.029 % ethanol), yellow; 0.3 mM, circle (0.29 % ethanol); light yellow; 3 mM, diamond, white (2.89 % ethanol)) compared to cells grown in the absence of cholesterol (orange square). This figure is representative of three experiments.

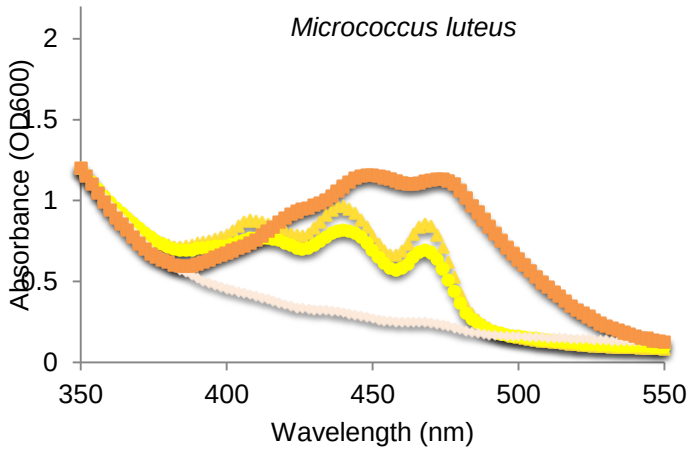


Figure 3.21. Staphyloxanthin expression of *Micrococcus luteus* in the presence of solubilised cholesterol. Absorbance spectrum of cells treated with cholesterol 0.03 mM triangle, yellow (0.029 % ethanol); 0.3 mM, circle light yellow (0.29 % ethanol); 3 mM, diamond, white (2.89 % ethanol)) compared to cells grown in the absence of cholesterol (orange square). This figure is representative of three experiments.

3.3.8 Exploring sterol regulation of staphyloxanthin expression

While *S. aureus'* characteristic golden colour comes from staphyloxanthin a glycosylated carotenoid biosynthesised from a pathway very similar to the early steps of sterol biosynthesis (Walsh and Fischbach, 2008) in yeast membranes the membrane-active sterols include ergosterol ($C_{28}H_{44}O$), campesterol, β-sitosterol, stigmasterol (Rebolj et al., 2006) and desmosterol (Huster et al., 2005). In mammalian cell membranes lanosterol ($C_{30}H_{50}O$) and desmosterol ($C_{27}H_{44}O$) are the immediate precursors of cholesterol biosynthesis (Valenza et al., 2007).

In human tissues, squalen is changed to lanosterol and lastly to cholesterol (Smith and Thiboutot, 2008). A large number of sterols are intermediate in the pathway of cholesterol biosynthetic such as desmosterol, lathosterol, and lanosterol (Wang et al., 2008b).

Desmosterol is direct precursor of cholesterol and related fewer avidly than cholesterol with detergent-resistant in mammalian cell membranes. Atomic scale molecular dynamics imitations presented that the double bond provides increase stress at the end and generating structure rigidity between C24 and C27 and supporting tilting of desmosterol from cholesterol (Vainio et al., 2006).

Ergosterol share such properties with cholesterol that stimulate the same properties and complete basically equivalent functions in membranes (Urbina et al., 1995). Several membrane transport proteins functional reconstitution

seems reliant on not only the sort of phospholipid present in the reconstitution mixture, but also on sterol composition (Urbina et al., 1995). The effects of sterols more than cholesterol and in specific of ergosterol on the phospholipid bilayer membranes physical properties (Urbina et al., 1995).

Having established that addition of ethanol-solubilised cholesterol reduces the levels of staphyloxanthin that can be extracted from cell membranes of *S. aureus* strain SH1000, further experiments were conducted to investigate whether this effect could be attributed to other sterols.

S. aureus SH1000 was cultured in BHI supplemented with ethanol-solubilised cholesterol, lanosterol, desmosterol or ergosterol at a concentration of 0.3 mM. Relative pigment expression determination required 10 ml of each culture, centrifuged as described previously in the pigmentation screening methods.

A similar reduction in extractable staphyloxanthin for cultures grown in cholesterol, was also observed in washed cells, cultured in either lanosterol, desmosterol, or ergosterol (Fig. 3.22). Viable counts showed a reduced number of cells when compared to growth in the absence of sterols (Fig. 3.23).

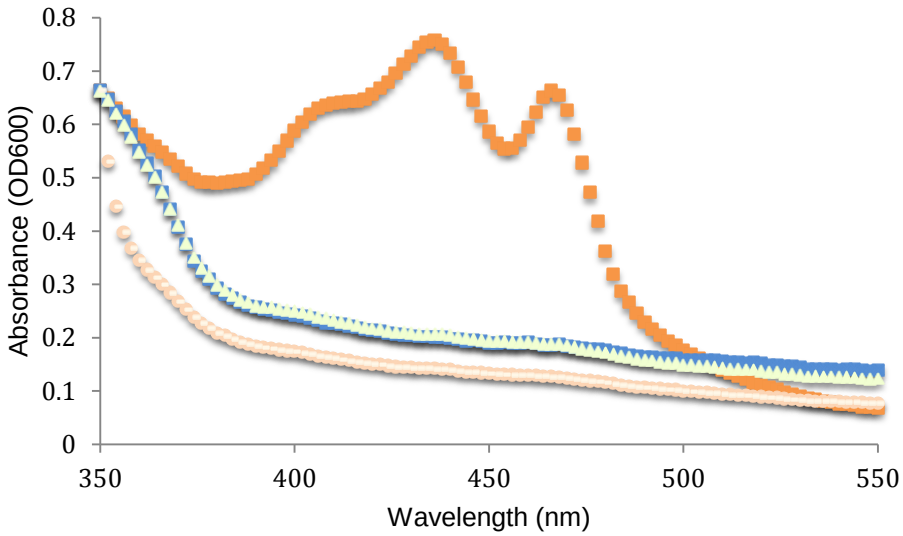


Figure 3.22. Carotenoid expression of SH1000 in the presence of cholesterol and other sterols (desmosterol, lanosterol and ergosterol). Absorbance spectrum showing reduced carotenoid in cells treated with 0.3 mM of cholesterol (beige); circle (0.29 % ethanol), ergosterol (white); line (0.29 % ethanol), Desmosterol (light green); triangle (0.29 % ethanol) and lanosterol (blue) compared to cells grown in the absence of sterols (orange, square). This experiment is representative of three experiments.

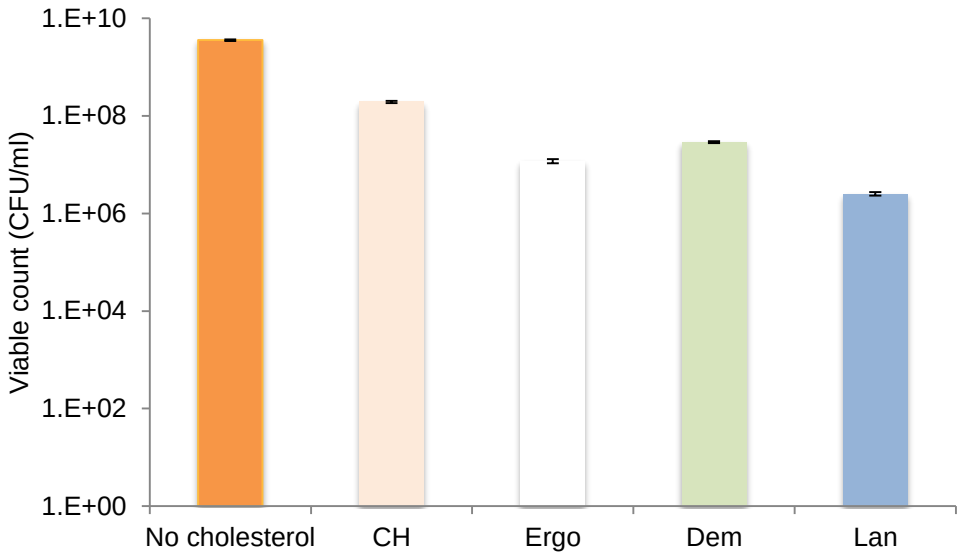


Figure 3.23. Viable count of *S. aureus* SH1000 cultured in media supplemented with sterols. Viable count of cells shows the number of the cells in 0.3 mM of ethanol-solubilised cholesterol, ergosterol, lanosterol and desmosterol (0.29 % ethanol). This experiment is representative of three experiments. Error bars represent standard error of the mean.

3.4 Discussion

3.4.1 Roles of cholesterol in the plasma membrane

Cholesterol is one of the principal essential components of the plasma membrane of eukaryotic animal cells (Harris, 2010). Cholesterol plays a key role in human health. High levels of cholesterol are considered a risk factor for cardiovascular diseases such as atherosclerosis, human coronary heart disease, stroke and plaque build up on the artery walls (Anandharaj M, 2014).

Sterols such as cholesterol, and in fungi ergosterol, are important structural components of all eukaryotic membranes. Their functions are principally involved in governing membrane fluidity and permeability (Volkman, 2003). Cholesterol is incorporated into phospholipid bilayers and monolayers and the presence of cholesterol in membranes may result in specific interactions with the phospholipids rather than with membrane proteins (Haberland and Reynolds, 1973). Cholesterol also controls the physical state of the phospholipid bilayer and impacts upon the activity of many membrane proteins (Socaciu et al., 2000).

Although evidence suggests that extracellular cholesterol can be incorporated into bacterial membranes, there are key differences between the composition of bacterial and mammalian membranes. These differences account for the selective action of antimicrobial peptides (Porcelli et al., 2004). For example, human beta-defensin 3 is a highly charged cationic peptide, which binds negatively charged lipids such as cardiolipin on the face of the *S. aureus* membrane, leading to up-regulation by the bacterium of

genes dealing with cell wall stress (Sass et al., 2008). This is in contrast to the human cell membrane wherein negatively charged phospholipid headgroups are oriented to the inner leaflet of the plasma membranes, resulting in weaker binding of the antimicrobial peptide.

Cholesterol was reported to incorporate into the cellular membranes of prokaryotes; specifically with the study of both *Micrococcus lysodeikticus* and *Lactobacillus acidophilus* when grown in the presence of cholesterol (NOH et al., 1997). Cholesterol also incorporates into the cellular membranes of *Bifidobacterium longum,* a species extracted from faeces and originating from normal human intestinal sources (Dambekodi and Gilliland, 1998). Few prokaryotic species require or synthesise cholesterol. The cell wall lacking *Mycoplasma* species fall into two categories in relation to sterols; those that require it for growth and survival, and those that do not. Investigations by Razin (1975) revealed that when sterol non-requiring species were grown in cholesterol containing media, they incorporated it into the plasma membrane. However the proportion of sterols in the plasma membrane was far less than that in the plasma membranes of the sterol-requiring cells. This indicates that even species, which do not require cholesterol, may incorporate the molecule, possibly for a biological function.

In relation to the results of the study here, it was determined that ethanol-solubilised cholesterol had a biological effect. It rescued *S. aureus* strains Newman and SH1000, plus the *crtM* mutant from reduced growth in the presence of AFAs (Fig 3.1, 3.3, 3.4, and 3.5); increased growth was also observed with *S. epidermidis* (Fig 3.6). When *agr* and *sarA* mutants were

grown in the presence of cholesterol and linoleic acid, this growth rescue phenomenon was also observed (Fig 3.7, 3.8 and 3.9). Previous studies determined that these mutants have minimal FAME and lipase activity. FAME is a secreted enzyme (Lu et al., 2012) that catalyses esterification of lipids with cholesterol in the extracellular environment and reveals an evolved interaction between staphylococci and cholesterol. The increased survival of the *agr* and *sarA* mutants in the assay suggests that the protective effect of solubilised cholesterol might be independent of FAME. However this assertion requires further experiments and ideally the identification of the FAME gene. Other sterols were tested for their ameliorative effect, including lanosterol, desmosterol, and ergosterol and these confirmed a general molecular structural effect of the lipid. These lipids also reduced the viable cell count of *S. aureus* (SH1000 strain, Fig 3.23) at the concentrations used. The action of cholesterol and related sterols affects cell growth and the physiological and biophysical effects of these lipids requires further study.

Antimicrobial peptides (AMPs) destroy the structure of the lipid bilayer. AMPs, which are mostly cationic, have been shown to interact with the lipid bilayer, such that upon binding they undergo a conformational change, causing organisational changes in the membrane. Thereafter they insert into the membrane, polymerize into structures such as pores and cause irrevocable alterations in packing of the lipid bilayer, resulting in the disintegration of the membrane (Hall et al., 2014). The presence of cholesterol in bacterial membranes decreases the binding of AMPs (Feigin et al., 1995, Matsuzaki et al., 1995, Tytler et al., 1995, Raghuraman and Chattopadhyay, 2004, Glukhov et al., 2005, Verly et al., 2008, Wu et al.,

2010). Therefore, one role of cholesterol when present in the bacterial membrane would be to prevent the binding of AMP. It is proposed that dehydration of the lipid bilayer phosphate head group region (M'Baye et al., 2008) partly accounts for this effect of cholesterol (Brender et al., 2012). Studies on membrane models have shown that peptides interact with the membrane at the water/lipid interface, and non-polar amino acids insert into the membrane (Bi et al., 2014). AMPs orientate into the membrane so that they interact with either the hydrophobic layer or the water/lipid interface (Grage et al., 2010). Therefore it may be that cholesterol dehydration of the lipid bilayer disrupts the interactions of AMPs with the bilayer, helping to protect the bacterium against AMPs. Staphyloxanthin is structurally similar to cholesterol and therefore may act in the same way. Thus when cholesterol is present, *S. aureus* expression of staphyloxanthin might be reduced whereby cholesterol would mediate this possible mechanism (Fig 3.13). There is also reduced expression of pigment in other pigmented bacteria as the extracellular concentration of cholesterol is increased (Fig 3.17, 3.18. 3.19, 3.20 and 3.21).

3.4.2 Role of carotenoids in the plasma membrane

Despite the presence of carotenoids as a minor component of higher eukaryotic membranes, these may act as modulators of the phase transition in the fluidity, polarisation and permeability of the membrane. Hence these can affect the physiology and pathology of the membrane (Socaciu et al., 2000). Staphyloxanthin is a carotenoid pigment, which was shown to have

antioxidant properties. It can also change the rigidity of the membrane, which helps defend bacteria from host AMPs (Mishra et al., 2011a).

Entrance into the cell membrane by polar carotenoids (Blasko et al., 2008) and cholesterol causes dose-dependent effects of rigidification in the bacterial membrane, similar to the eukaryotic membrane (Socaciu et al., 2000), whereby the expansion of order is detected by a change in anisotropy. The hydrophobicity of the membrane is defined by the polarity of cholesterol and carotenoids (Socaciu et al., 2000). Due to this effect, bacteria that express polar carotenoids may reduce their level of expression when another reagent, such as cholesterol, which increases membrane rigidification is present. This was found to be the case in a series of investigations (Fig 3.13, 3.17, 3.18. 3.19, 3.20 and 3.21).

3.4.3 Role of lipids on the skin

Previous reports determined that linoleic acid reduced *S. aureus* survival and this was correlated with an increase in the bacterial membrane permeability (Coates et al., 2014). In this study, the antimicrobial effect was confirmed by the inhibition of growth of two lab strains (SH1000 and Newman) in the presence of linoleic acid. However, taking this forward for this study, when *S. aureus* was supplemented with a range of concentrations of ethanol-solubilised cholesterol (0-3 mM) an increased level of growth was noted. It was determined that the highest concentration of ethanol-solubilised cholesterol used experimentally (3 mM) showed the greatest increase in growth yield, while concentrations less than 3 mM showed more modest

increases. A second antimicrobial lipid, D-sphingosine, also reduces bacterial growth yield, but supplementing cultures with increasing concentrations of ethanol-solubilised cholesterol improved the growth yield proportionately. These findings may indicate that the membrane was influenced by cholesterol since each experiment demonstrated similarly increased survival rates in the presence of cholesterol; i.e. cholesterol recovery. These growth enhancements are potentially due to the nature of cholesterol, in particular its relation to, and its interaction with cell membranes.

The sphingolipid metabolite, sphingosine, is a potent antimicrobial agent against *S. aureus* at physiologic levels; it is probable that this plays an important role in the bacterial defence mechanisms of healthy skin. This may be related to the observed susceptibility to colonisation by *S. aureus* noted in patients with atopic dermatitis (Arikawa et al., 2002). The investigation here revealed that cholesterol rescued the growth of *S. aureus* in the presence of D-sphingosine in a concentration-dependent manner. This is similar to its effect in the presence of linoleic acid. Therefore the mechanism of action of these two lipids at the bacterial cell surface may be similar, or the interaction of cholesterol distinct from the mode of action might similarly lower the antimicrobial action of each lipid.

3.4.4 Cholesterol and bacterial growth rescue, and the mechanism of action of antimicrobial lipids

Bacterial membranes are composed of proteins, which are integral within a lipid matrix that closely resembles a phospholipid bilayer. The survival of bacteria relies on the balance of membrane lipids and on their capacity to control the composition of lipids in order to adapt to, or enhance growth in, various environments (Zhang and Rock, 2008). Fatty acids are the most energetically expensive membrane lipid factors to synthesise. Phospholipid acyl chains affect several crucial membrane-associated functions, such as the passive permeability of hydrophobic molecules, active solute transport and protein–protein interactions. Therefore bacteria require sophisticated mechanisms to regulate gene expression as a response to the formation of fatty acids, to change existing fatty acyl chains, and to alter the biophysical properties of fatty acids (Zhang and Rock, 2008).

There are several possible explanations for the assistance of ethanol-solubilised cholesterol to the growth of *S. aureus* in the presence of linoleic acid. Kenny *et al* (2009) have speculated that linoleic acid could raise the fluidity of the *S. aureus* cell membrane, whilst pigment production makes the membrane more rigid (Kenny et al., 2009b). Potentially, cholesterol may act in the same manner as the pigment, in effect reducing membrane fluidity by enhancing the order in the bilayer membrane (Shine et al., 1993b). Staphyloxanthin is a hydrobrophobic pigment integral to the membrane that aids survival of the bacteria under stress conditions. It may be that when a similar molecule such as cholesterol is present extracellularly; the bacterium reduces its metabolic load by incorporating cholesterol in the plasma

membrane, and synthesises less staphyloxanthin. This could explain the reduction in pigmentation noted in *S. aureus* grown in the presence of cholesterol (Fig 3.10 and 3.11). This reduction is less when linoleic acid is present (Fig 3.10 and 3.13), therefore staphyloxanthin may also have a role in protecting the bacterium from AFAs.

Cholesterol may also interact with extracellular linoleic acid to reduce its availability to cell membranes. Other studies have shown steroids can be incorporated into the bacterial cell membrane helping to reinforce, thus making the cells them more resistant to the action of free lipids (Shimomura et al., 2009). Golden pigmentation is a characteristic of *S. aureus* (Liu et al., 2008, Liu et al., 2005a) and it acts to protect against changes to membrane fluidity (Bischoff et al., 2004, Pelz et al., 2005). The reduction of pigmentation noted in the *S. aureus* SH1000 and Newman strains (Fig 3.11, 3.12 and 3.15) indicate that this reduction may not be required in the presence of cholesterol. This could be due to cholesterol itself incorporating in the membrane to reduce the membrane fluidity.

3.4.5 Mode of cholesterol action at the bacterial membrane in response to findings of the study

It is interesting to note that in all cases of this study, ethanol-solubilised cholesterol reduced the expression of staphyloxanthin especially at high concentration of cholesterol (3 mM) as the carotenoid pigmentation that can be extracted from cell membranes of *S. aureus* was decreased. Other sterols

may also affect pigmentation of *S. aureus*. in the same manner as cholesterol, as evidenced by the decreased staphyloxanthin expression of the SH1000 strain when grown in other sterol containing media (Figure 3.22). This indicates that the effect of other sterols may be either similar in the extracellular environment, at the membrane or cause similar changes in bacterial expression and intracellular signalling.

Additionally, not only did cholesterol have an impact on *S. aureus* to reduce pigmentation, but also affected other pigmented skin microbes such as *Kocuria rosea, S. capitis, Micrococcus luteus, S. warneri, and S. hominis* (Fig 3.12). As previously discussed this may be due to cholesterol acting in the same way as carotenoid pigments in these bacteria. This role would need further investigation.

There is evidence to support that cholesterol enters the cell membrane of several bacteria including *S. aureus*. This raises an important question; does this explain what was observed in all experiments? The interaction mechanism of cholesterol with the *S.aureus* membrane has not yet been characterised, however this may be similar to its interaction with the eukaryotic membrane. In the presence of cholesterol in the extracellular environment, antimicrobial lipids may not interact with the pathogen cell membrane. This suggests that it might be of significant biological importance to investigate further the mechanism of cholesterol in *S. aureus*. It is known that cholesterol and other lipids when in water can form micelles above threshold concentrations. Therefore, when AFAs are present with cholesterol, micelle formation may help prevent interaction of the AFAs with the cell

membrane or affect entry into the bacterial cell. The specific action of AFAs at the cell surface requires further investigation.

Ethanol is simple to use in biological studies as a solvent, as cell media are mainly aqueous, while cholesterol has poor solubility in water. To overcome this solubility problem ethanol is frequently used as a solubilising agent. However, ethanol itself can have a biological effect on cell membranes, such that it increases the membrane's fluidity. Studies have also shown a range of other biological effects on bacteria (Bischoff et al., 2004, Pelz et al., 2005).

After conducting the investigation it was noted that the experimental controls for the growth and expression of *S. aureus* in the presence of cholesterol were not effectively designed. This meant that while increasing the concentration of cholesterol there was an accompanied increase in the concentration of ethanol. The failure to control for this severely limits the experiments reported above and requires further study to resolve. The reported difference in growth and staphyloxanthin expression of the strains tested could result from cholesterol, ethanol or a combined effect of the increasing concentration of both reagents. This unfortunate experimental design error was noted late during the study and could not be completely corrected in the remaining time of study, especially since subsequent work was based upon the perceived effects that cholesterol might be having as a mediator of change to *S. aureus* cell properties.

CHAPTER 4- Physiological effects of ethanol-solubilised cholesterol on *S. aureus*

4.1 Introduction

S. aureus utilises various mechanisms that enable it to survive in changing environments and support its resistance to external pressures, for example, to changes in pH, temperature and oxidative stress (Clements and Foster, 1999). This flexibility enables *S. aureus* to colonise and then adapt to the innate immune defences that are found deeper within the skin of the host (Wilson et al., 2002).

Previous reports identified that the carotenoid pigment is a survival component, which is used to resist environmental stress, and revealed that the pigment of *S. aureus* may be considered a virulence factor (Mishra et al., 2011c). Two principal functions of staphyloxanthin in *S. aureus* are that it maintains cell membrane fluidity, by increasing order similar to sterols like cholesterol; additionally it protects cells against oxidative stress (Mishra et al., 2011b, Lang et al., 2000, Liu et al., 2005b). The ability to compensate for changes in membrane fluidity allows for greater resistance to host cationic AMPs, used by the host to eliminate bacterial pathogens.

The synthesis of staphyloxanthin consists of a five-step enzymatic process involving the five enzymes encoded by the polycistronic *crtOPQMN* operon, which is transcribed from a single σ^B promoter (Pelz et al., 2005). From this series of enzyme reactions, staphyloxanthin is the product of the final step,

where the condensation of farnesyl diphosphate is the initial step of the process (Wieland et al., 1994) see section (1.3.3.7). It was proposed that there is the possibility to identify therapeutic agents that could interfere with the production of this pigmentation by blocking the unique final steps in the synthesis of staphyloxanthin (Fitzgerald-Hughes et al., 2012). CrtM, the first enzyme in this pathway, was shown to share the same catalytic site structure to that of human squalene synthase, which catalyses the biosynthesis of cholesterol. A reported study by Liu *et al* (2008) demonstrated that human squalene synthase inhibitor molecules, such as statins, prevent *S. aureus* producing pigment and might therefore affect its survival in the host.

The study of Clauditz et al. (2006) used targeted mutagenesis of the genes from the *crtOPQMN* operon to demonstrate the importance of staphyloxanthin for resistance of *S. aureus* to oxidative stress. Their study revealed that a wild type pigment-producing strain resisted exposure to higher concentrations of H_2O_2 than an isogenic, *crtM* mutant (Clauditz et al., 2006). Moreover, a similar study reported that the golden pigment helps *S. aureus* evade the innate immune response of neutrophil killing, via its anti-oxidative properties, which in turn promotes virulence and contributes significantly to the development of subcutaneous abscesses in an animal model of infection (Liu et al., 2005a).

Lan *et al* (2010a) reported that the production of staphyloxanthin and the expression of virulence genes are both affected by the metabolism of *S. aureus*. A range of metabolism gene mutants including purine biosynthesis and oxidative phosphorylation mutants had reduced pigmentation and a

significant degree of attenuated virulence in a murine abscess model of infection (Lan et al., 2010).

Aside from *Mycobacterium tuberculosis,* of the pathogenic bacteria there is scant information about the biology of cholesterol interactions and its effects on cell physiology and metabolism, particularly with respect to extracellular cholesterol (Miner et al., 2009).

4.2 Specific aims

Having identified clear effects of ethanol-solubilised cholesterol on growth and pigmentation of *S. aureus* there was a need to identify the implications for the cell. The specific aims for this chapter were to identify effects of ethanol-solubilised cholesterol on the physiology of *S. aureus*. This would be achieved by investigating environmental stresses such as temperatures, hydrogen peroxide and antimicrobial lipids in the absence and presence of cholesterol.

A further aim was to identify key regulators of the *S. aureus* response to ethanol-solubilised cholesterol by screening transposon mutants for reduced survival in its presence. By identifying the components that are contributing to the response or phenotypic change of pigment expression, it should be possible to explain the response to the ethanol-solubilised cholesterol. These aims were determined prior to questions about the individual roles of cholesterol and the carrier solvent ethanol being raised. Although these concerns are intimated in the previous chapter, this chapter describes concurrent experiments and does not directly deal with effects due to ethanol.

4.3 Results

4.3.1 The effects of ethanol-solubilised cholesterol on growth at different temperatures

Previous studies have demonstrated that *S. aureus* utilises multiple mechanisms that enable it to adapt and survive in an ever-changing environment (Clements and Foster, 1999). Since cholesterol is reported to assimilate into bacterial membranes, including those of staphylococci (Razin, 1975), it is likely to change physiological features of the response to temperature derived from membrane order (fluidity) changes. With this in mind, *S. aureus* was cultured to examine the impact of ethanol-solubilised cholesterol on growth at different temperatures.

Comparative growth was examined by culturing cells with or without cholesterol using microtitre 96-well plates. Both SH1000 and Newman strains were cultured in BHI broth in the absence and presence of cholesterol (0, 0.03 mM, 0.3 mM, 3 mM) and optical density was measured throughout growth at different temperatures (25 °C, 37 °C, 42 °C). Ethanol-solubilised cholesterol was shown to have a clear effect upon growth yield, with a reduction at its highest concentrations (0.3 mM and 3 mM) at 25 °C and 42 °C. In contrast, at 37 °C there was only a slightly reduced growth yield when cholesterol was added to the growth media (Fig 4.1A (A, B, C).

Increasing the concentration of ethanol-solubilised cholesterol reduced the growth rate at 25 °C compared with its the absence. A similar effect was obreserved at 42 °C, where there was also a reduced growth rate when the

higher concentrations of ethanol-solubilised cholesterol were added. Similar results were obtained with the Newman strain (Fig 4.1 B (D, E & F)), revealing that the effects were likely to be species-specific.

A

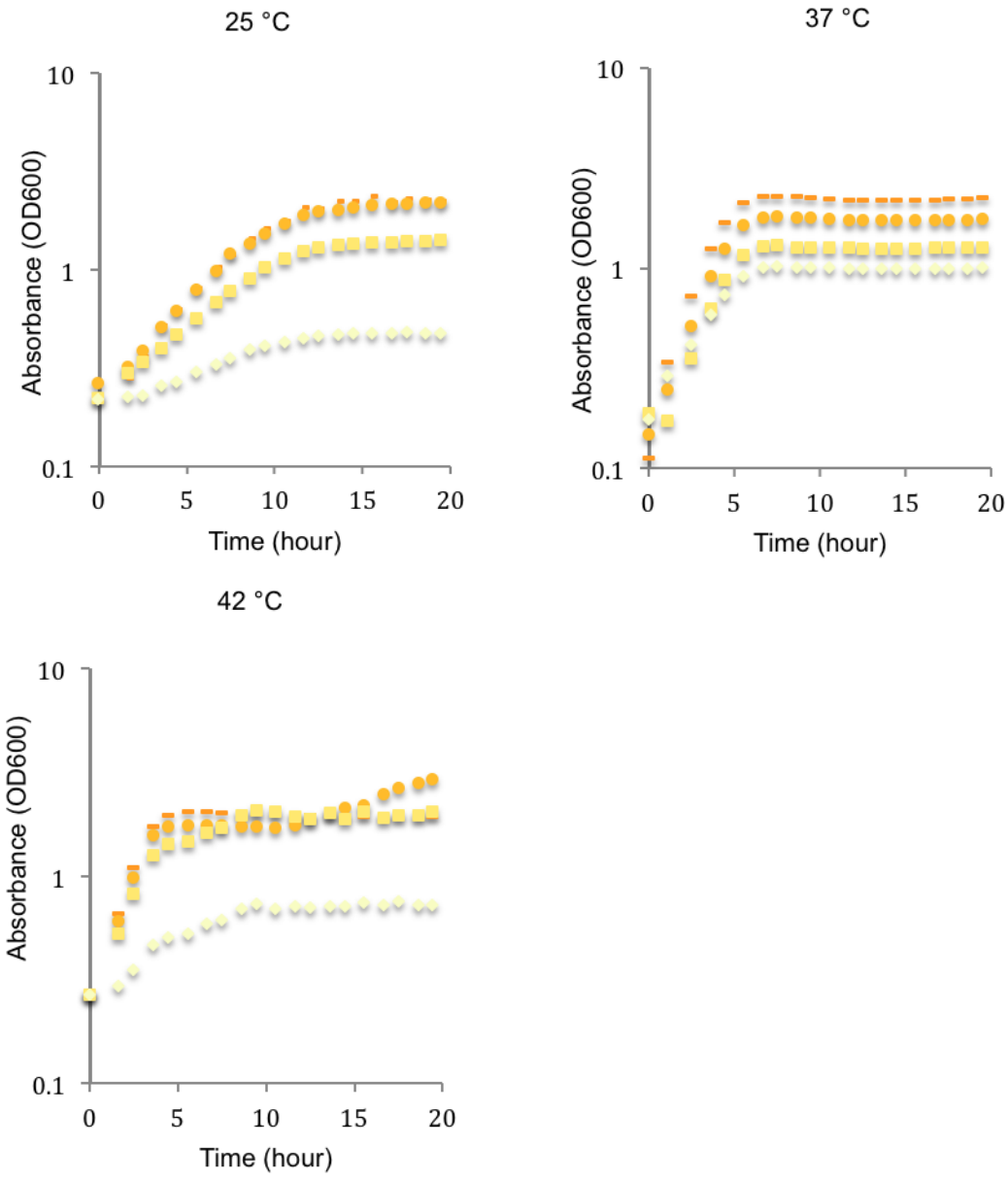


Figure 4.1 A. Growth of *S. aureus* SH1000 at different temperatures in the presence or absence of ethanol-solubilised cholesterol. Growth was determined at temperatures 25 °C, 37 °C and 42 °C. Wild type bacteria, no ethanol (orange; line), bacteria with ethanol control (light orange; circle), bacteria with cholesterol 0.3 mM (light yellow; square), and bacteria with cholesterol 3 mM (cream yellow; diamond). This experiment is representative of three experiments.

B

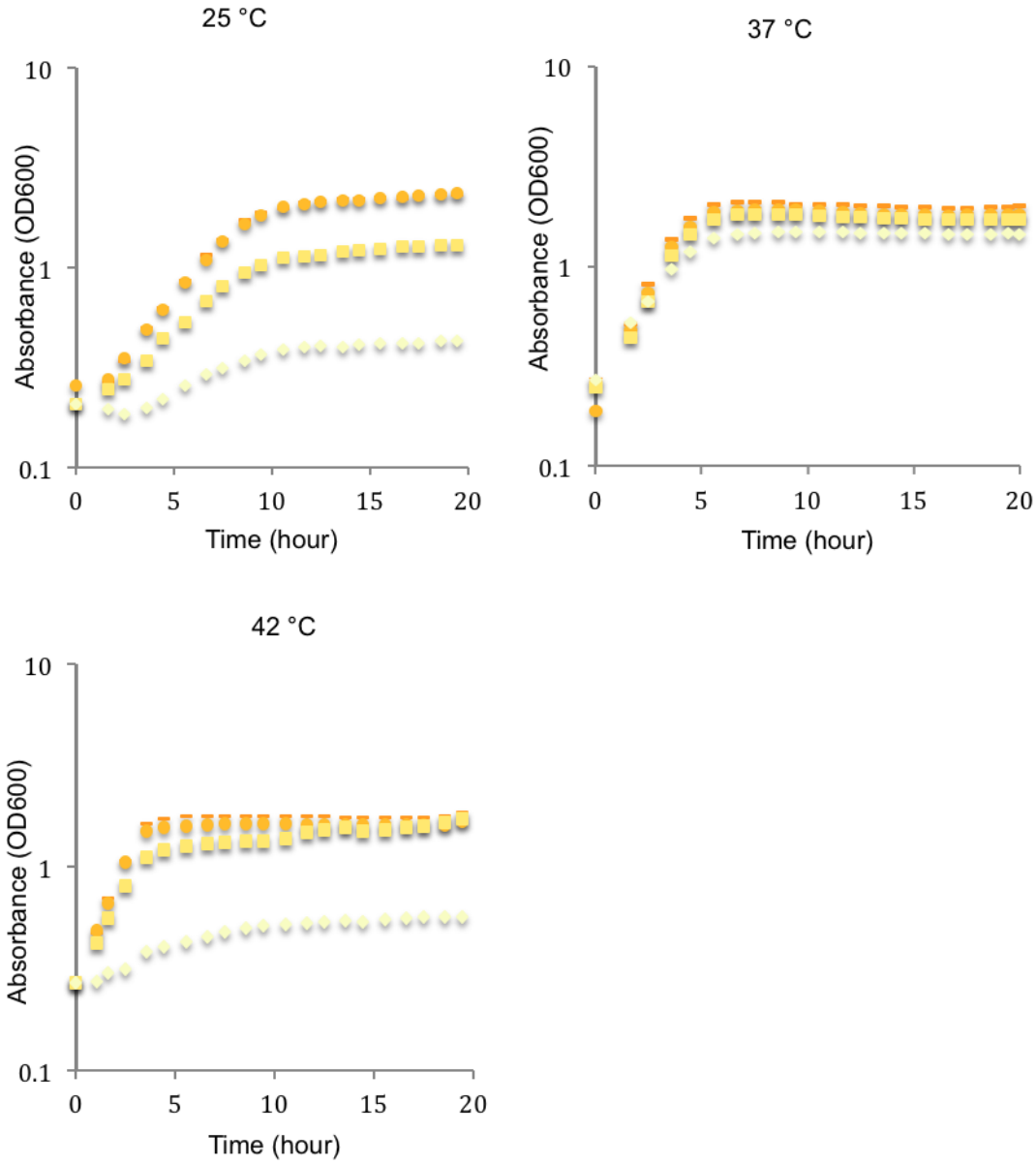


Figure 4.1 B. Growth of *S. aureus* Newman at different temperatures in the presence or absence of ethanol-solubilised cholesterol. Growth was determined at temperatures 25 °C, 37 °C and 42 °C. Wild type bacteria no ethanol (orange; line), bacteria with ethanol control (light orange; circle), bacteria with cholesterol 0.3 mM (light yellow; square), and bacteria with cholesterol 3 mM (cream yellow; diamond). This experiment is representative of three experiments.

4.3.2 The effects of ethanol-solubilised cholesterol on hydrogen peroxide resistance

A well-described role of staphyloxanthin in *S. aureus* is the protection it provides from reactive oxygen species, such as peroxides (Clauditz et al., 2006). Since the previous observations in Chapter 3 were that ethanol-solubilised cholesterol reduced the levels of staphyloxanthin that are methanol extractable, an experiment was designed to explore whether these reductions in the pigment present in the membrane had a phenotypic effect on hydrogen peroxide resistance. The cells of both Newman and SH1000 strains were grown to exponential phase in the absence and presence of ethanol-solubilised cholesterol (0.3 mM) and were washed prior to the addition of H_2O_2. Viable counting of samples across a 60 min time period was used to determine the survival of each strain from challenge with 7.5 millimolar hydrogen peroxide. At sampling time points, H_2O_2 was neutralised by immediate dilution in PBS buffer containing catalase. For the challenge experiment an ethanol-solubilised cholesterol concentration of 0.3 mM was chosen. This concentration was chosen specifically for its range of effects on growth and pigment production outlined in Chapter 3. At the same time it has a minimal effect on the growth rate and yield of strains Newman and SH1000 at 37 °C (Fig 4.1). In contrast, ethanol-solubilised cholesterol at 3 mM was demonstrated to have pronounced effects on these phenotypes, whereby reduced growth rate and yield were considered too great to unambiguously examine its role physiologically.

Growth in ethanol-solubilised cholesterol, when included at 0.3 mM in the culture medium, reduced the H_2O_2 survival of *S. aureus* strains Newman (Figure 4.2 A) (*P=0.0331*) and SH1000 (Figure 4.2 B) (*P =0.0008*), following challenge with 7.5 mM H_2O_2. This was a statistically significant reduction in survival of both strain Newman and SH1000 compared with controls cultured in the absence of cholesterol and presence of ethanol solvent. The reduction in survival was modest at around 20-30 % for strain Newman at 60 min and 30-40 % for strain SH1000 at 60 min. It is notable, however, that despite the presence of an active catalase (Horsburgh et al., 2002), there was a measurable loss of viability due to the presence of ethanol-solubilised cholesterol in the culture medium.

A

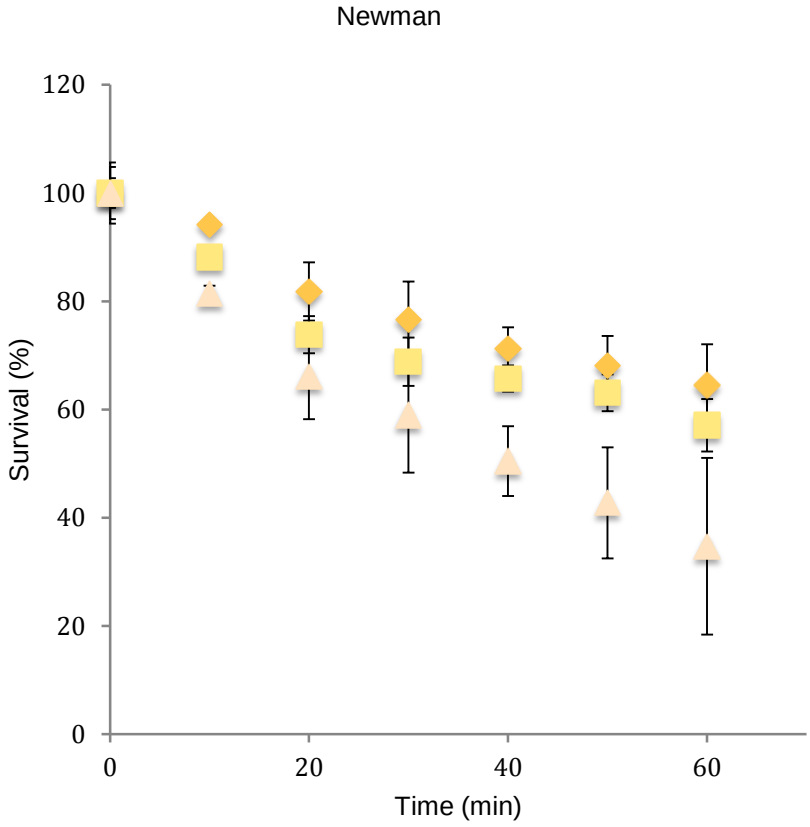


Figure 4.2 A. The effect of ethanol-solubilised cholesterol on *S. aureus* Newman survival after challenge with hydrogen peroxide. Washed cells of strain Newman were grown to exponential phase in the presence or absence of ethanol-solubilised cholesterol and challenged with 7.5 mM H_2O_2. Symbols represent no cholesterol (orange; diamond), ethanol control (yellow; square) (0.29 %), and ethanol-solubilised cholesterol 0.3 mM (0.29 % ethanol) (beige; triangle) *P* = 0.0331, Student t-test was used. This experiment is representative of three experiments. Error bars represent standard error of the mean calculated from technical replicates.

B

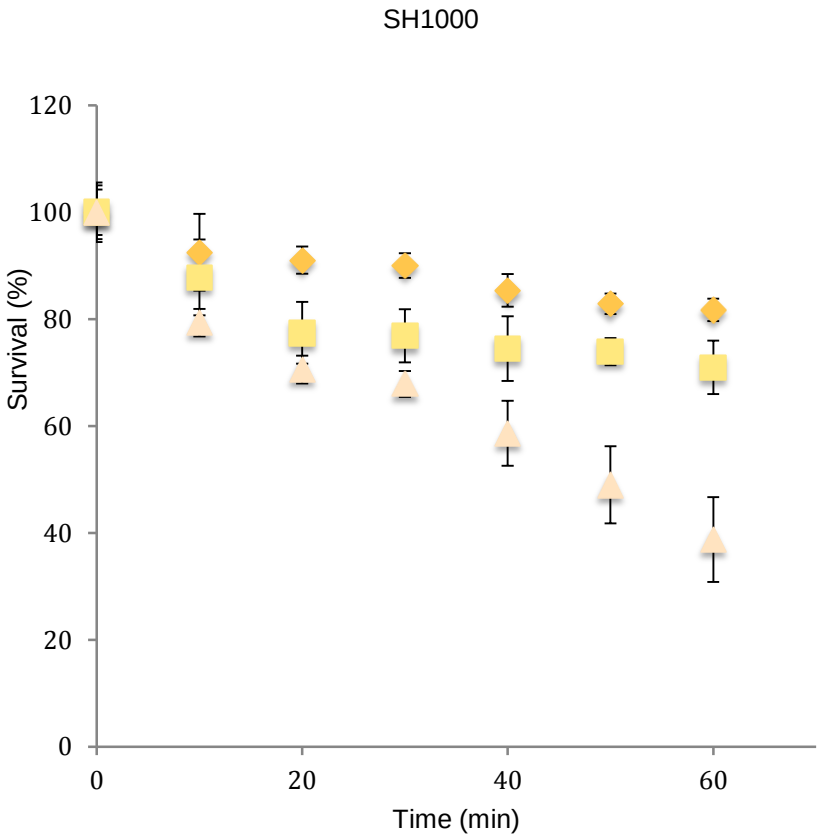


Figure 4.2 B. The effect of ethanol-solubilised cholesterol on *S. aureus* SH1000 survival after challenge with hydrogen peroxide. Washed cells of strain SH1000 were grown to exponential phase in the presence or absence of ethanol-solubilised cholesterol and challenged with 7.5 mM H_2O_2. Symbols represent no cholesterol (orange diamond), ethanol control (yellow square) (0.29 %), and cholesterol 0.3 mM (0.29 % ethanol) (beige triangle) *P* value=0.0008 in SH1000. This experiment is representative of three experiments. Error bars represent standard error of the mean calculated from technical replicates.

4.3.3 The effects of ethanol-solubilised cholesterol on linoleic acid resistance

In light of the experiments presented previously, revealing the effects of ethanol-solubilised cholesterol on cell growth and the reduction of linoleic acid toxicity, the impact of cholesterol on linoleic acid resistance was examined further. An experiment was designed to determine whether ethanol-solubilised cholesterol affected survival by altering resistance to linoleic acid without cholesterol being present simultaneously in the cell culture medium. Similar to the hydrogen peroxide resistance experiment described above (Section 4.3.2), *S. aureus* was cultured in the presence of 0.3 mM cholesterol, and then washed prior to challenge with 1 mM linoleic acid. It was determined that cholesterol inclusion during growth of strain Newman reduced subsequent survival of the cells upon challenge with linoleic acid (Figure 4.3). When compared with both control cultures (solvent control and cholesterol free) the difference in survival was statistically significant ($P = 0.00353$).

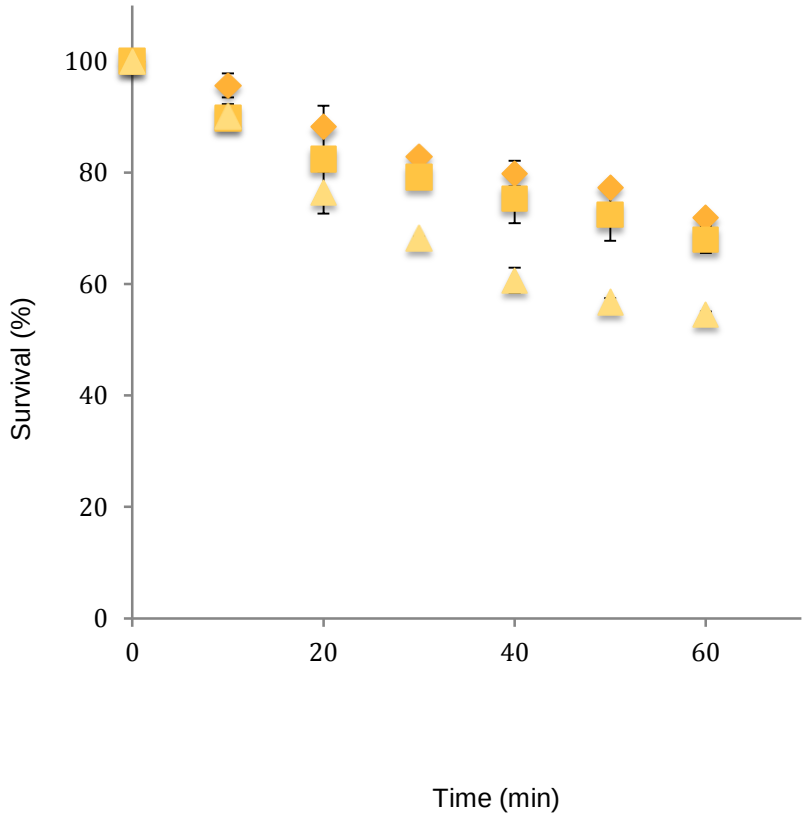


Figure 4.3. Linoleic acid challenge of cells cultured in ethanol-solubilised cholesterol on *S. aureus* Newman. *S. aureus* Newman was cultured in the absence of ethanol-solubilised cholesterol (orange), presence of ethanol (solvent control 0.37 % ethanol) (yellow), or 0.3 mM cholesterol (beige) (0.37 % ethanol) and approximately 10^8 cfu ml^{-1} were challenged with 1 mM of linoleic acid. Survival was determined by viable counting. This experiment is representative of three experiments. Error bars represent standard error of the mean calculated from biological replicated experiments. The survival difference between cholesterol presence and absence cultures was statistically significant (P = 0.00353); Student t-test was used.

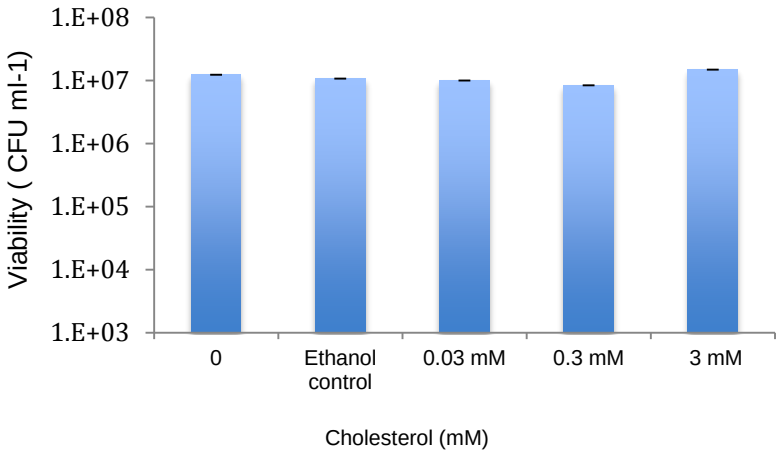
4.3.4 Cell surface hydrophobicity of ethanol-solubilised cholesterol treated cells

To gain insight into the physiological effects of growth in cholesterol-containing growth medium, cell surface hydrophobicity was investigated using the microbial adhesion to hydrocarbon (MATH) test (Kotzamanidis et al., 2010). Cholesterol may interact with the bacterial membrane via hydrophobic interactions and this might consequently alter the cell surface hydrophobicity, which could in turn modify membrane permeability. Hydrophobic membrane interactions have been reported to result in daptomycin-resistant bacteria whereby the hydrophobic part of the antibiotic alters cell surface properties that become genetically fixed through selection (Sauermann et al., 2007, Hachmann et al., 2011, Silverman et al., 2003).

An experiment was designed to examine the concentration-dependent effect of ethanol-solubilised cholesterol on strain Newman to explore whether culture in the presence of the lipid alters cell surface hydrophobicity. Cultures were grown for 18 hours at 37 °C, washed with sterile distilled water three times and cells were suspended to OD_{440} =0.5. The cultures (3 ml) were then mixed with 500 µl of n-hexadecane for 1 min prior to incubation at room temperature for 3 min. The OD_{440} was recorded for 1 ml of the aqueous (top) layer, and another 50 µl was taken to determine cell numbers by viable counting. The results revealed that across different concentrations of ethanol-solubilised cholesterol there was no clear impact on the cell hydrophobicity (Fig 4.4 A & B). This experiment requires further analysis, since the standard assay proved more difficult due to the properties of

cholesterol, meaning that an increase in surface hydrophobicity could not be definitively ruled out.

A



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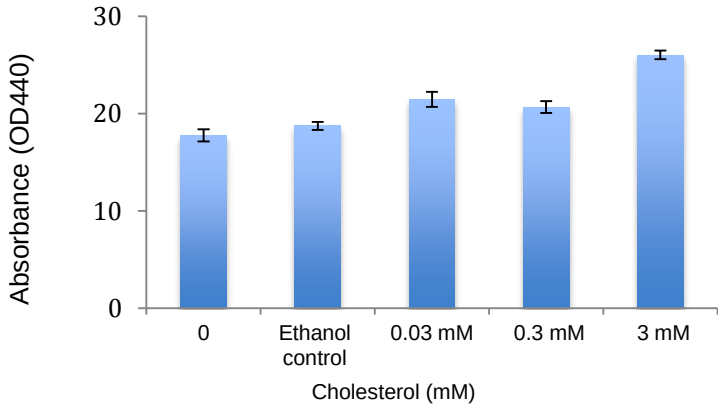

Figure 4.4. Cell surface hydrophobicity of cells grown in the absence and presence of ethanol-solubilised cholesterol. This was measured by using the microbial adhesion to hydrocarbon (MATH test). (A) Viability (CFU ml^{-1}) and (B) cell surface hydrophobicity (absorbance OD_{440}) for Newman strain with different concentrations of cholesterol (0.03 mM (0.029 % ethanol), 0.3 mM (0.29 % ethanol) and 3 mM (2.98 % ethanol)) absence of cholesterol and ethanol solvent (2.98 % ethanol). Error bars represent standard error of the mean.

4.4 Genetic characterisation of ethanol solubilised cholesterol-dependent expression of staphyloxanthin

The data presented in chapter 3 revealed that staphyloxanthin expression could be modulated by ethanol-solubilised cholesterol in a concentration-dependent manner. At present there is no described mechanism that explains this response, although possible reasons were discussed in section 3.3.6. These mechanisms include a regulatory pathway whereby changes in the membrane are sensed, leading to regulated changes in gene transcription and/or a cell surface receptor binds to cholesterol (or ethanol) and modulates gene expression. Correspondingly, mechanisms were explored experimentally using two different approaches: 1) screening known regulatory mutants to identify changes in staphyloxanthin expression with respect to addition of ethanol-solubilised cholesterol in the cell culture medium; 2) utilising transposon mutant library screens to identify genes contributing to cholesterol-dependent staphyloxanthin expression and survival.

4.4.1 Staphyloxanthin expression in Two Component Signal Transduction System (TCSTS) mutants

Investigations of the ethanol-solubilised cholesterol-dependent expression of staphyloxanthin were previously performed using the *S. aureus* strains Newman and SH1000 (section 3.3.6). The availability of a set of gene inactivation mutants in 11 TCSTS systems in a distinct strain, WCUH29 (described in Table 2.2), however, provided an easy route into testing the

role of signal transduction systems in staphyloxanthin expression. Use of strain WCUH29, a pre-antibiotic era clinical isolate from the Glaxo-Smithkline strain collection, meant that this strain background needed to be verified first for the same ethanol-solubilised cholesterol regulatory phenomenon. *S. aureus* strain WCUH29 was confirmed to display a very similar response to solvent/cholesterol as that observed in strains Newman and SH1000 (Fig.3.10). This finding enabled the set of WCUH29 TCSTS mutant strains to be screened for their contribution as potential sensing systems that might contribute to phenotypic changes upon addition of ethanol-solubilised cholesterol.

The TCSTS mutants and their isogenic wild-type parent strain (WCUH29) were cultured in BHI broth in the presence or absence of ethanol-solubilised cholesterol as described previously (Fig 4.5). The mutants *desR, nsaR, vraS, saeR, arlS, graR, kdpD, srhS, phoR, saeS* and *hssR* were tested. As presented in Fig 4.6A, adding 0.3 mM of ethanol-solubilised cholesterol reduced pigmentation in the *desR, nsaR, vraS*, *saeR, kdpD*, *graR* and *arlS* mutants, similar to levels observed for the wild type parent strain (WCUH29). Adding ethanol-solubilised cholesterol (0.3 mM) to *srhS, phoR, saeS* and *hssR* revealed a distinctly altered response, as judged by levels of extracted staphyloxanthin (Fig4.6 B). In each of these mutants there were reduced pigment levels in the presence of cholesterol, but also notably in its absence too.

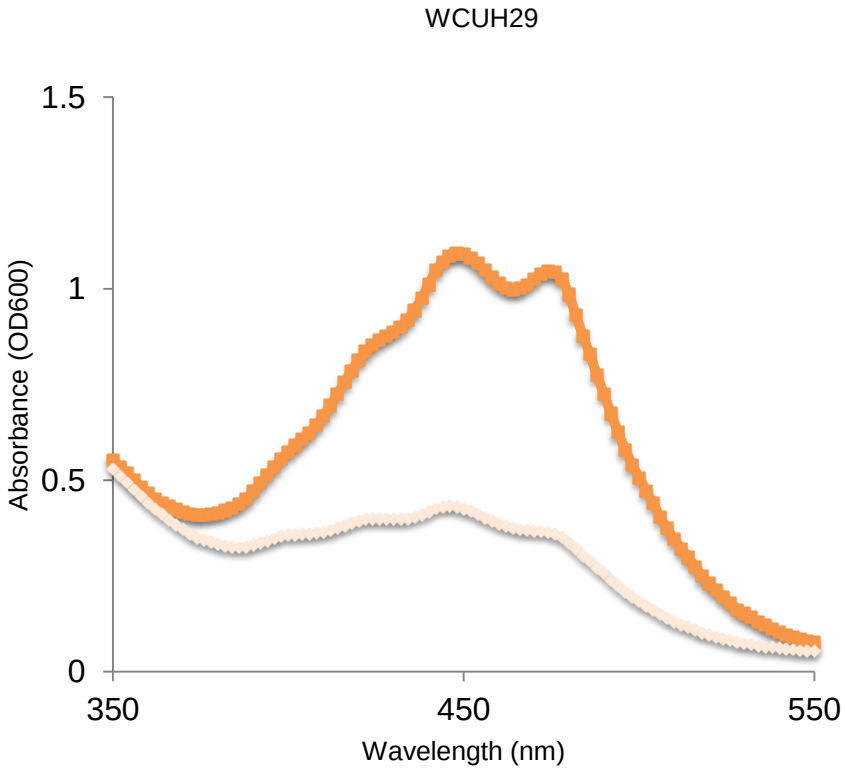

Figure 4.5. Staphyloxanthin expression in ethanol-solubilised cholesterol-treated *S. aureus* strain WCUH29 (wild type). Absorbance spectrum of methanol-extracted staphyloxanthin of cells treated with ethanol-solubilised cholesterol (0.3 mM) 0.29 % ethanol, (cream orange) compared with cells grown in the absence of ethanol-solubilised cholesterol and solvent (orange). This experiment is representative of three experiments biologically replicated.

A

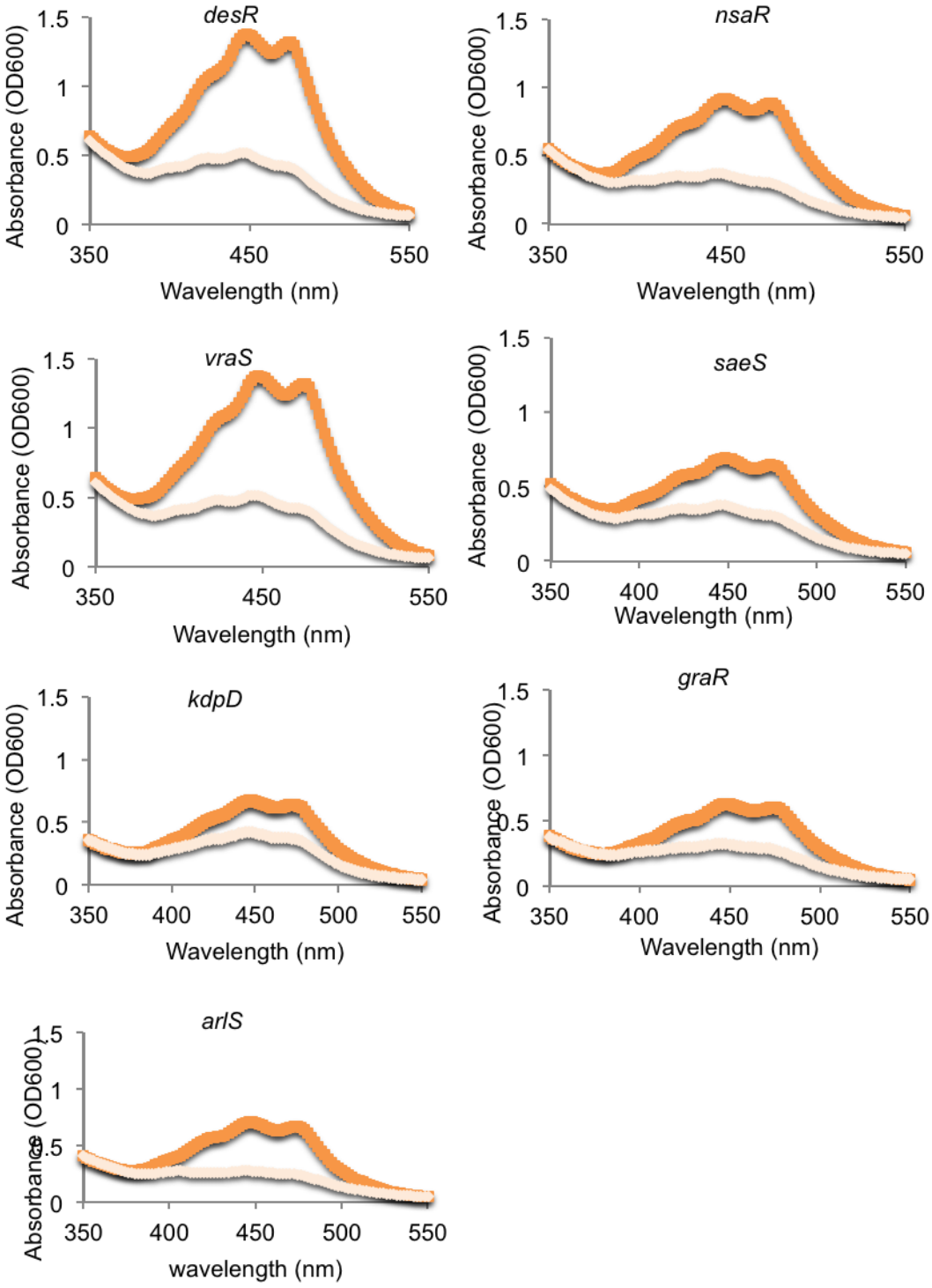

Figure 4.6A. Carotenoid expression in ethanol-solubilised cholesterol-treated *S. aureus* strain WCUH29 TCSTS mutant strains. (A) WCUH29 TCSTS mutant strains that show similar levels and/or regulation of pigment as wild-type WCUH29. Absorbance spectrum of methanol-extracted staphyloxanthin of cells treated with ethanol-solubilised cholesterol (0.3 mM) 0.29 % ethanol, (beige) compared with cells grown in the absence of ethanol-solubilised cholesterol and solvent (orange). This experiment is representative of three experiments biologically replicated.

B

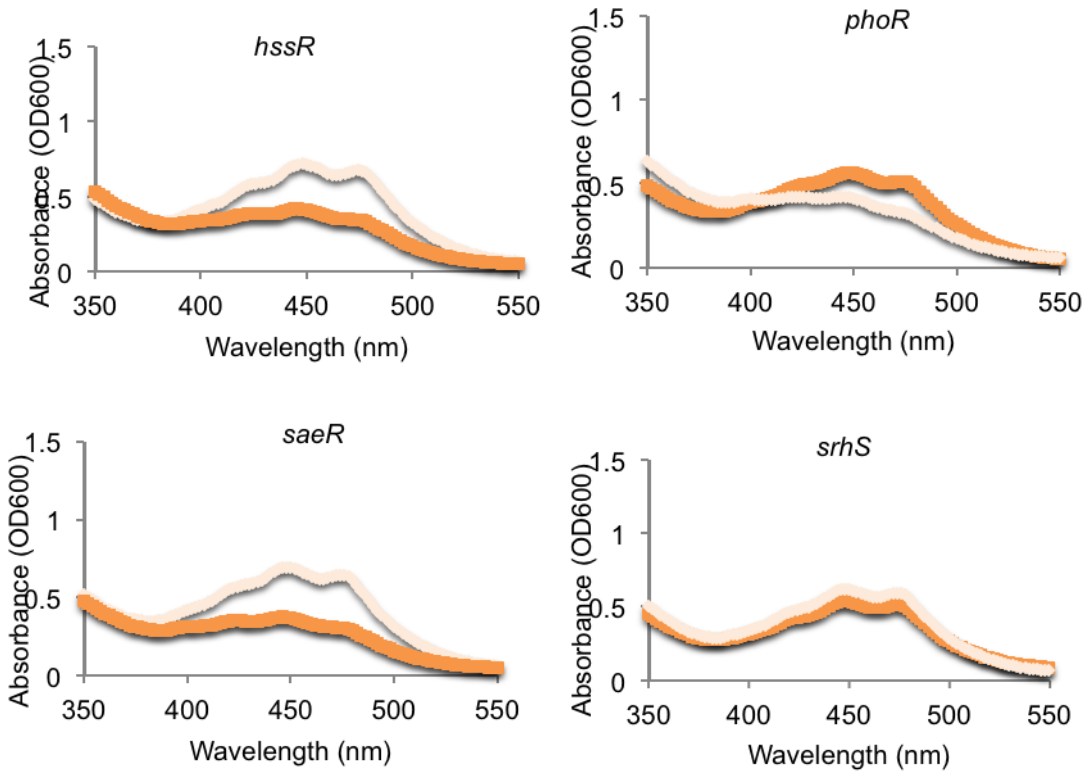

Figure 4.6 B. Carotenoid expression in ethanol-solubilised cholesterol-treated *S. aureus* strain WCUH29 TCSTS mutant strains. (B) WCUH29 TCSTS mutant strains with altered ethanol-solubilised cholesterol response and staphyloxanthin expression. Absorbance spectrum of methanol-extracted staphyloxanthin of cells treated with ethanol-solubilised cholesterol (0.3 mM) 0.29 % ethanol, (beige; diamond) compared with cells grown in the absence of cholesterol and solvent (orange; square). This experiment is representative of three experiments biologically replicated.

4.3. 5. Carotenoid expression in AFA survival mutants

Identifying *S. aureus* TCSTS mutants displaying altered staphyloxanthin expression gives the possibility that several mutants have a clearly altered response to ethanol-solubilised cholesterol. Signal transduction systems that could be regulating the response to cholesterol or solvent were thus potentially identified through the mutants being unresponsive. Identifying altered staphyloxanthin expression responses in this subset of TCSTS mutants led to wider testing to identify further mutants associated with a survival response. Previous studies by Kenny *et al.* (2009) identified a set of mutants of the SH1000 strain with reduced survival in the presence of millimolar levels of linoleic acid (Kenny et al., 2009a). Linoleic acid is an antimicrobial fatty acid (AFA) present on human skin, together with cholesterol in the epidermal (stratum corneum) lipid pool. The AFA survival mutants *sigB*, *sasF*, *crtM*, *SAR2632 (mmpL), mtlD, rsbU, arcA, vraE* and *vraS* were assayed to determine whether they exhibited altered carotenoid expression in the presence/absence of ethanol-solubilised cholesterol (0.3 mM) (Fig 4.7 A & B). These strains were cultured as described previously.

A

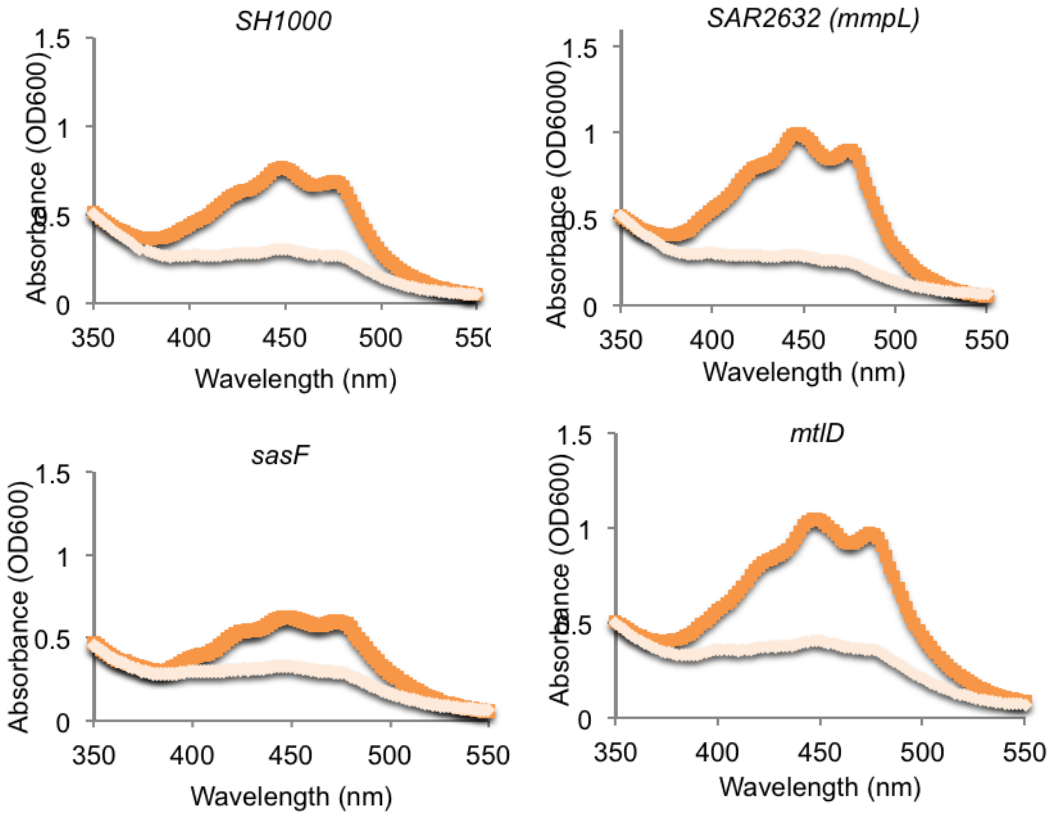

Figure 4.7 A. Carotenoid expression in ethanol-solubilised cholesterol-treated *S. aureus* linoleic acid survival mutants. Absorbance spectrum showing reduced carotenoid in cells treated with ethanol-solubilised cholesterol (0.3 mM) (beige) 0.29 % ethanol, compared with cells grown in the absence of cholesterol and solvent (orange), the same behaviour as SH1000 wild type. This experiment is representative of three experiments.

B

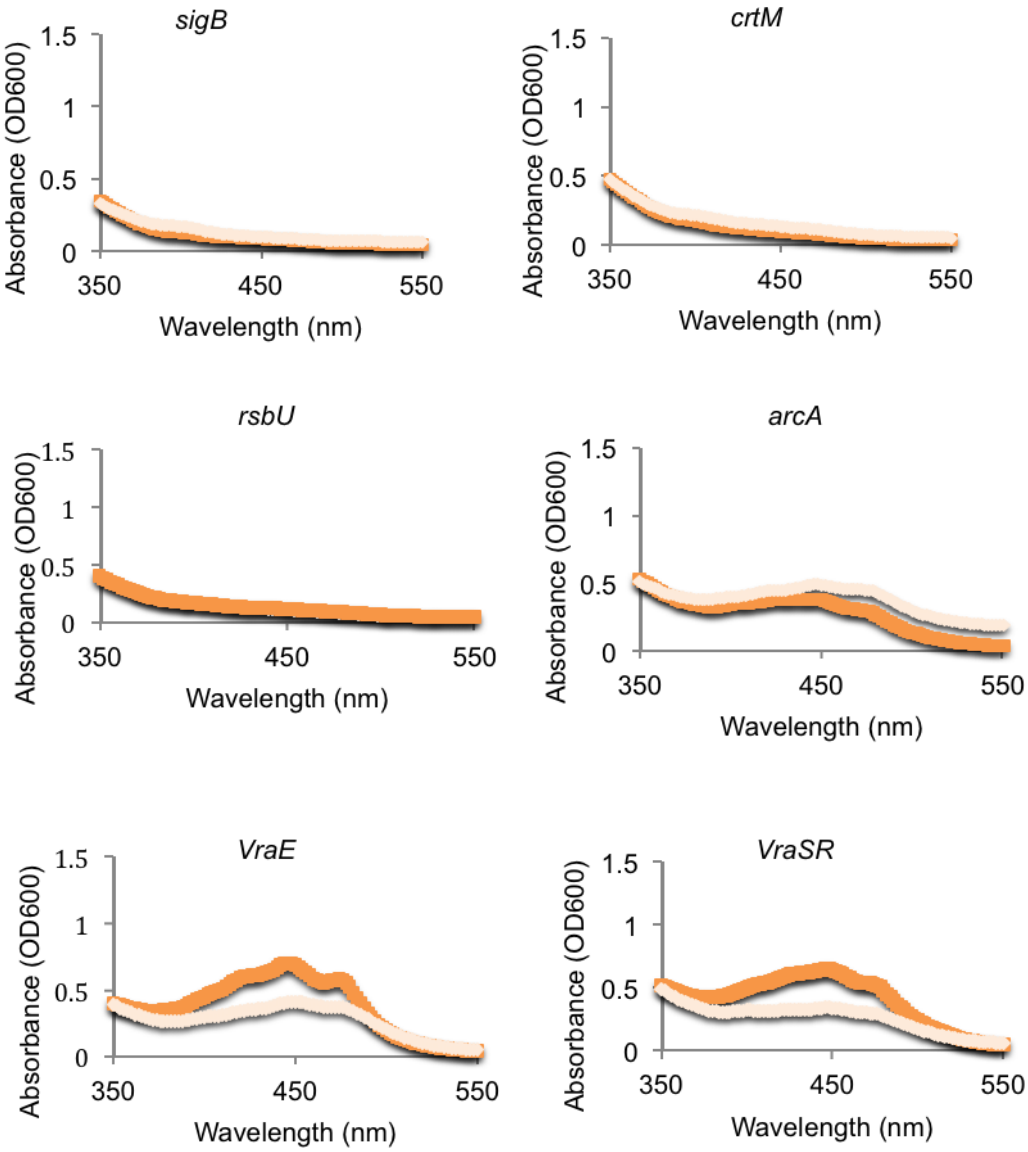

Figure 4.7 B. Carotenoid expression in ethanol-solubilised cholesterol-treated *S. aureus* linoleic acid survival mutants. Absorbance spectrum showing different expression compared with wild-type SH1000 for SH1000 in cells treated with cholesterol (0.3 mM) (beige) 0.29 % ethanol, compared with cells grown in the absence of cholesterol and solvent (orange). These data are representative of three experiments.

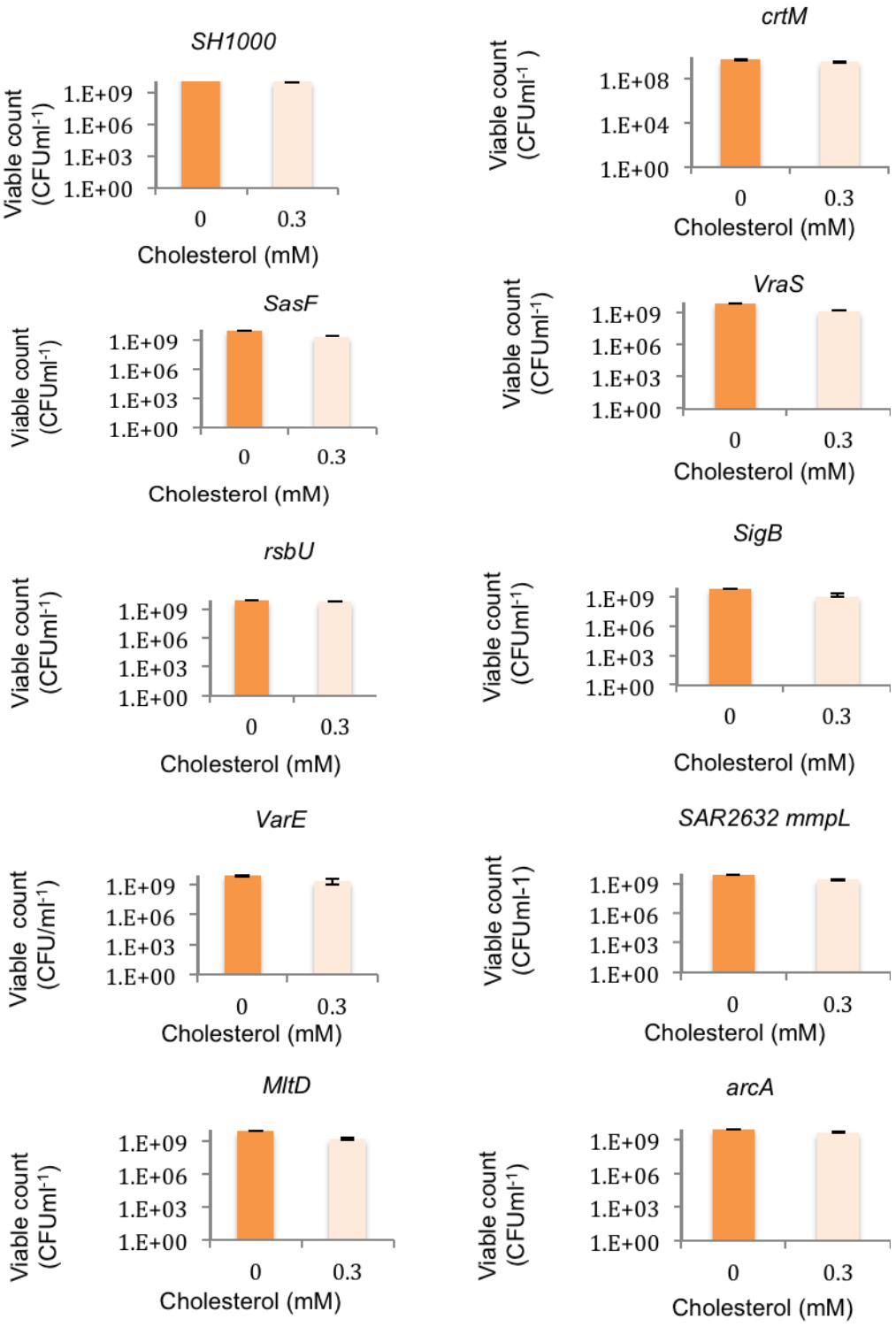

Figure 4.7 C. Viable counts of ethanol-solubilised cholesterol treated *S. aureus* mutant strains. Colony forming units were determined after overnight growth in the absence of cholesterol (orange), or in the presence of 0.3 mM cholesterol (beige) 0.29 % ethanol. These data are representative of three separate experiments.

Due to their central roles in expression and biosynthesis of staphyloxanthin the SH1000 *sigB, rsbU* and *crtM* mutants showed an expected loss of staphyloxanthin expression, having no extractable pigment (Fig 4.7 B). The SH1000 *mmpL, sasF, mtlD* showed similar levels of expression of staphyloxanthin in the presence and absence of cholesterol compared with wild-type (SH1000) (Fig 4.7 A). In contrast a SH1000 *arcA* mutant showed clearly altered expression of staphyloxanthin in the presence and absence of ethanol-solubilised cholesterol. The *vraE and vraSR* mutants exhibited similar staphyloxanthin levels to each other in the presence and absence of cholesterol (Fig 4.7 B), however, in the absence of ethanol-solubilised cholesterol their expression of staphyloxanthin consistently appeared lower compared with the wild-type control strain. Viable count data of cell growth shown with or without ethanol-solubilised cholesterol (Figure 4.7 C) demonstrated that growth yield was equivalent. However any differences in growth were used to adjust absorbance values to enable a reasonable comparison of the levels of staphyloxanthin between strains.

4.3.6 Construction of a *desR* mutant

It was identified that one of the TCSTS of *S. aureus* could be a homologue of DesK/DesR of *B. subtilis* which regulates membrane lipid desaturase and fluidity (Ibarra et al., 2013) and initial data from staphyloxanthin expression analysis of a WCUH29 mutant suggested in *S. aureus* it might have altered cholesterol/solvent responsiveness. Consequently, the construction of a *desR* mutant in Newman and SH1000 was performed to test if the observed phenotype in strain WCUH29 would also be observed in the SH1000 strain background. It was important to identify if this observation of the *desR* mutant effect on pigment was strain or species-specific. Downstream of the putative *desRK* TCSTS operon is the gene encoding cardiolipin synthetase. The importance of cardiolipin to membrane integrity and long-term adaptation of membranes with respect to composition changes (Shiba et al., 2004) further encouraged the decision to inactivate the gene in the SH1000 and Newman strains. The *desR* gene was PCR amplified as two flanking segments for insertion of a tetracycline resistance gene cassette to create an allelic replacement vector. The *tet* gene was amplified and each of the purified and digested DNA fragments were co-ligated into the suicide plasmid pMUTIN4 (Vagner et al., 1998) (section 2.22). The ligated products were used to transform *E. coli* TOP10 cells and, thereafter, clones resistant to tetracycline were selected (Fig 4.9).

The plasmid pMUTIN4des was purified from a clone of transformed *E. coli* and was confirmed by restriction digest, prior to transformation of *S. aureus* RN4220. Selection of a tet^R erm^R clone established integration of the suicide vector and a phage lysate was prepared of the transformed RN4220 strain

using Φ11. Transduction of *S. aureus* strain SH1000 was performed to enable outcross of the wild-type and mutant alleles and the clones obtained were screened to identify *erm*^{R} *tet*^{R} clones indicating the mutant allele (Fig 4.8). Two independent clones, Des1 and Des2 were selected and used to characterise their staphyloxanthin expression.

Staphyloxanthin expression was measured using the previously described protocols to compare clones Des1 and Des2 with their isogenic parent strain SH1000. A consistent, but minor, difference in staphyloxanthin levels was observed (Fig 4.10) for clones Des 1 and Des 2 in relation to cholesterol concentration. Although repeated experiments demonstrated there were potential differences in the regulation of the carotenoid expression, the small difference from wild-type, coupled with the original data in WCUH29 that directed the investigation not being supported with further repeats, meant that this line of investigation was discontinued.

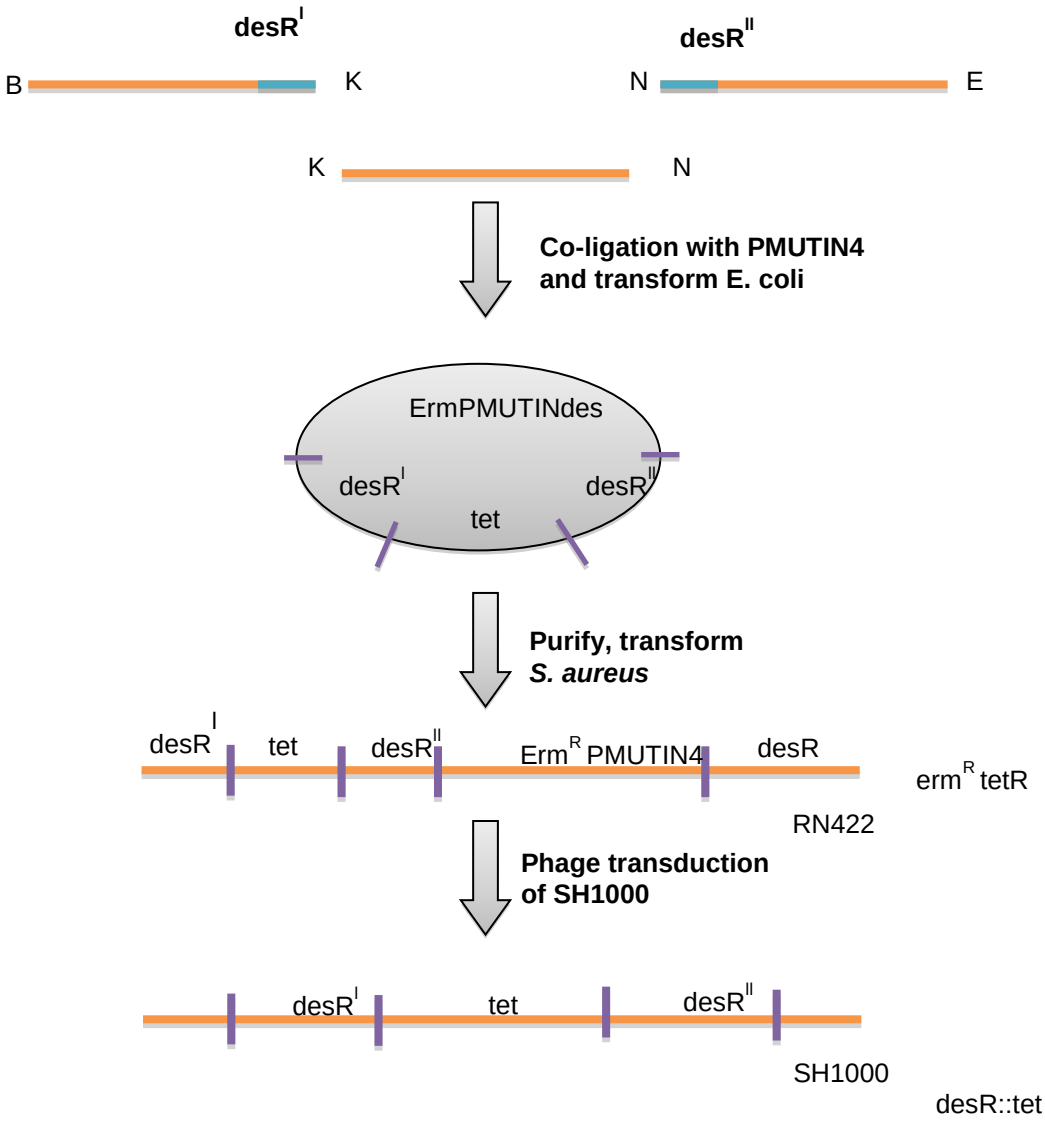

Figure 4.8 Schematic diagram of *desR::tet* construction in *S. aureus* SH1000.

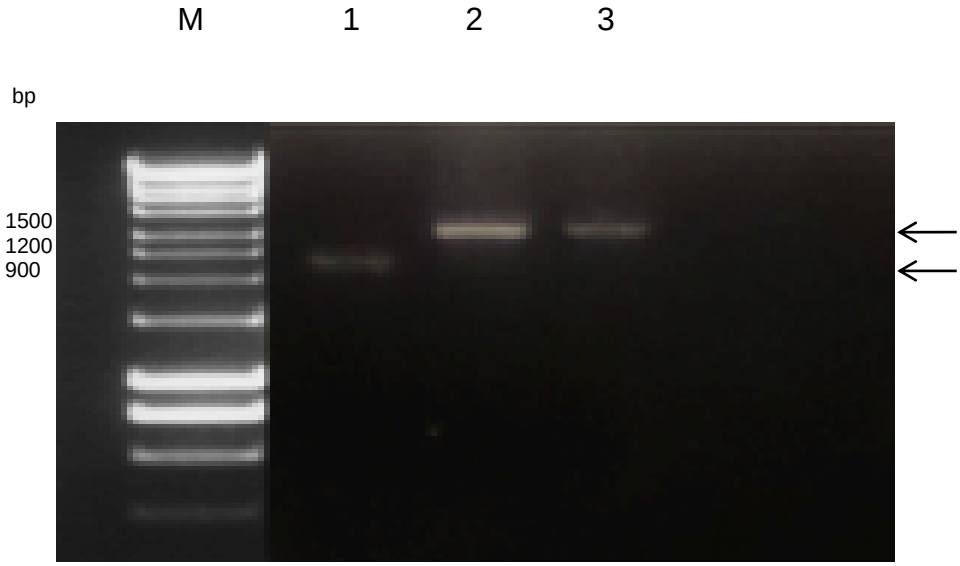

Figure 4.9. Allelic replacement confirmation of *desR::tet*. Agarose electrophoresis gel of PCR products to identify the size differences of *desR* between (1) SH1000 [size 1200 bp], (2) *Des1* and (3) *Des2* [size 1500 bp] after allelic replacement with a tetracycline resistance cassette. M is molecular size markers.

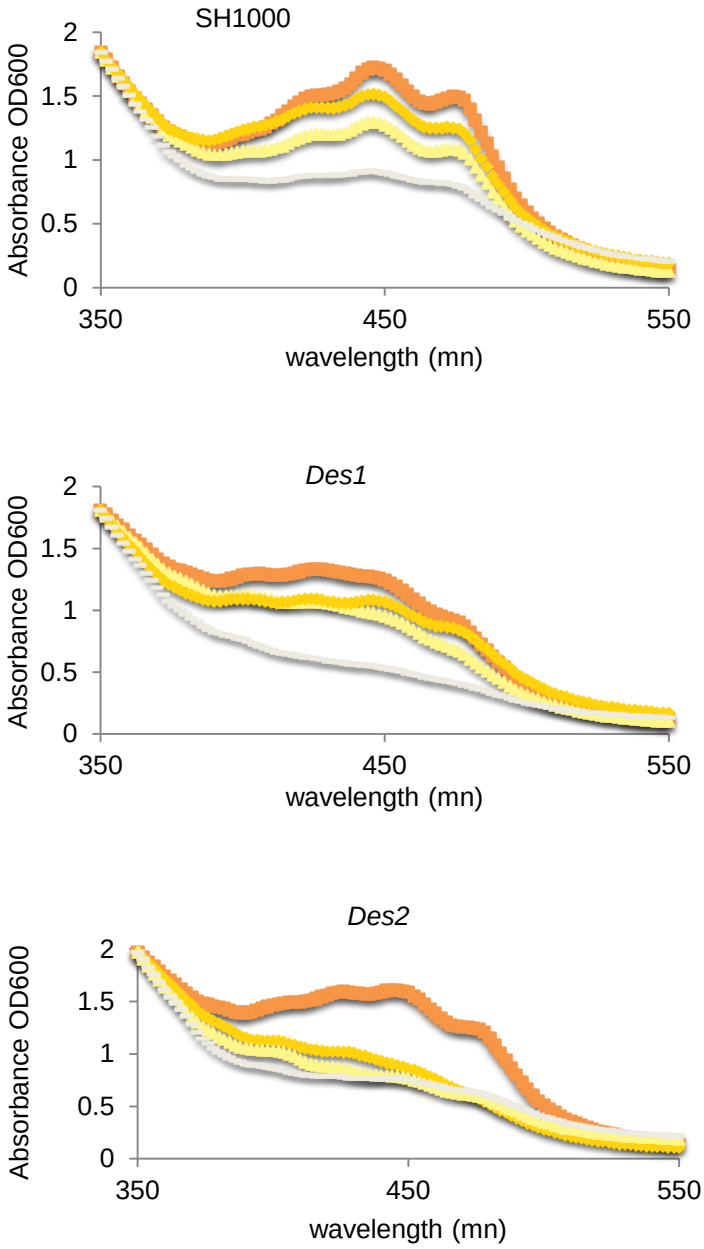

Figure 4.10. Carotenoid expression after 48 h of culture ith ethanol-solubilised cholesterol treatment of *Des1*, *Des2* and SH1000 strains. Absence of cholesterol (orange; square), 0.03 mM of cholesterol (yellow; diamond) (0.029 % ethanol), cholesterol 0.3 mM (light yellow; triangle) (0.29 % ethanol) and cholesterol 3 mM (beige; line) (2.89 % ethanol). This experiment is representative of three experiments biologically replicated.

4.3.7 Use of a transposon mutant screen to identify response networks.

4.3.7.1 Screen based on carotenoid expression levels

The identification of key regulators of *S. aureus* virulence factor expression have frequently been elucidated by transposon mutagenesis and screening, with inactivation of individual loci by transposon insertions contributing to clear effects on virulence factor production (McNamara et al., 2000). Since ethanol-solubilised cholesterol can reduce staphyloxanthin expression, a screen was designed to identify transposon mutants with increased expression of staphyloxanthin in the presence of cholesterol.

To screen for the impact of ethanol/cholesterol on *S.aureus,* 5 µl of each Tn mutant clone was inoculated into BHI (250 µl) with or without 0.3 mM ethanol-solubilised cholesterol. Pigmentation screening required 1 µl of transposon mutant overnight cultures to be pipetted into 96-deep well plates (Grenier Bio One) containing 0.9 ml BHI with the presence or absence of ethanol/cholesterol (0.3 mM). Both plates were incubated at 37 ℃ for 48 h in aerobic conditions. The plates were then centrifuged at 4000 rpm for 15 min. Following this, the supernatant was discarded and methanol was added to all samples and incubated at 37 ℃ for 15 min. The methanol extract was centrifuged again and 96-well polystyrene microtitre plates were loaded with 250 µl of extract in triplicate (Fig 4.11 A, B, C) to determine growth yield from viable counts (Fig 4.12 A, B, C).

Approximately one thousand transposon library mutants were screened and differences between 96 well plate cultures grown with BHI and with the

presence of 0.3 mM of ethanol/cholesterol were observed and assessed as a first screen. Clones of interest expressing pigment, where conditions mean this should be repressed, were identified visually by comparing the pigment produced in BHI with pigment produced when supplemented with ethanol solubilised cholesterol.

Next, a secondary screen was performed as per the first screen, with the selected mutants being assayed to confirm pigment production phenotypes compared with SH1000. Mutants that were confirmed as having altered pigment production in the presence of ethanol-solubilised cholesterol in the repeat screen were selected for further analysis. A tertiary screen based upon methanol extraction of cell pellets from 10 ml BHI cultures was performed as a final check for pigmentation differences from the parent strain. Relative growth of the mutants was also compared with the wild type strain in the presence and absence of ethanol-solubilised cholesterol to adjust for growth differences, as discussed previously, and to account for the potential of changes to pigment via growth differences. Of the mutant clones that were screened, 100 were selected in the first screening, which were reduced to 47 clones in the secondary screen. The tertiary screen supported 29 clones as exhibiting altered pigmentation in the presence of ethanol/cholesterol.

To identify the Tn*917* transposon insertion sites responsible for altered pigment production, the selected clones were lysed and processed to extract chromosomal DNA. Arbitrary-primed PCR was used to amplify the genome insertion site of the transposon for all of the selected mutants (Fig 4.18). The arbitrary primers ARB1B, ARB3 or ARB5 were used to prime amplification and this resulted in successful amplification of 17 PCR products from the 29

and these were sequenced. BLASTn was used to analyse these sequence data from transposon junction sequencing (Table 4.1) to define the insertion site and the cognate gene disruption leading to pigment expression differences.

Identification of the transposon insertion site enabled the position of the transposon to be established relative to gene boundaries and to assess the overall pattern of insertions across the genome. From the 17 insertions, it was observed that the majority were located close together on the chromosome. Twelve of the insertion sites were determined to be between genes SA1046-SA1360, which corresponds to a transposon insertion hot spot described previously (Beenken et al., 2004). Transposition into this region results, via an unidentified mechanism, in the altered expression of σ^B and thus carotenoid expression (Shaw et al., 2006). However, this previous study revealed insertions in this region were associated with reduced activity and reduced pigmentation, which is opposite to the effect identified here of increased pigmentation.

Of the disrupted genes identified in this increased pigmentation screen, *dapB* forms part of the *dap* operon (Wiltshire and Foster, 2001) which encodes enzymes for the biosynthesis of lysine. The gene *trpC* lies within the operon encoding enzymes for tryptophan biosynthesis (Jang et al., 2008). The *pyrAB* genes encode non-identical subunits of carbamoylphosphate synthetase (Ghim and Neuhard, 1994). The *nasD* gene encodes nitrite reductase that is part of the switch between oxygen, nitrate, or nitrite as electron acceptors in the electron transfer chain, associated with anaerobiosis. Other disrupted genes include *potC*, which forms part of an

ABC transport system for polyamine import (Mounsey K. E et al., 2006). The *braB* gene encodes an enzyme for branched chain amino acid biosynthesis in *S. aureus* (Wang et al., 2008a) The *SA2061* gene encodes a transporter of the major facilitator family with unknown specificity. *SA0318* encodes functions associated with phosphotransferase system transport of substrates. The genes *SA0445, SA1209, SA1240* and *SA1369* each encode hypothetical proteins. The gene *SA1064* (*msrA)* encodes methionine sulphoxide reductase with roles in oxidative stress resistance. The gene reference IDs are all taken from the *S. aureus* N315 genome (Table 4.1). The *S. aureus* genome showing all genes with transposon insertions that were identified in this screen are depicted in Fig. 4.19 and highlights the grouping of insertions in the previously described 'hotspot' (including *SA1046-SA1364*) described by Shaw et al (2006). The results indicate that cholesterol-associated staphyloxanthin expression may encompass a greater number of genes in the previously described *SA1194-SA1240* region reported, however since the converse association with pigment was identified, this represents only a similar association.

A

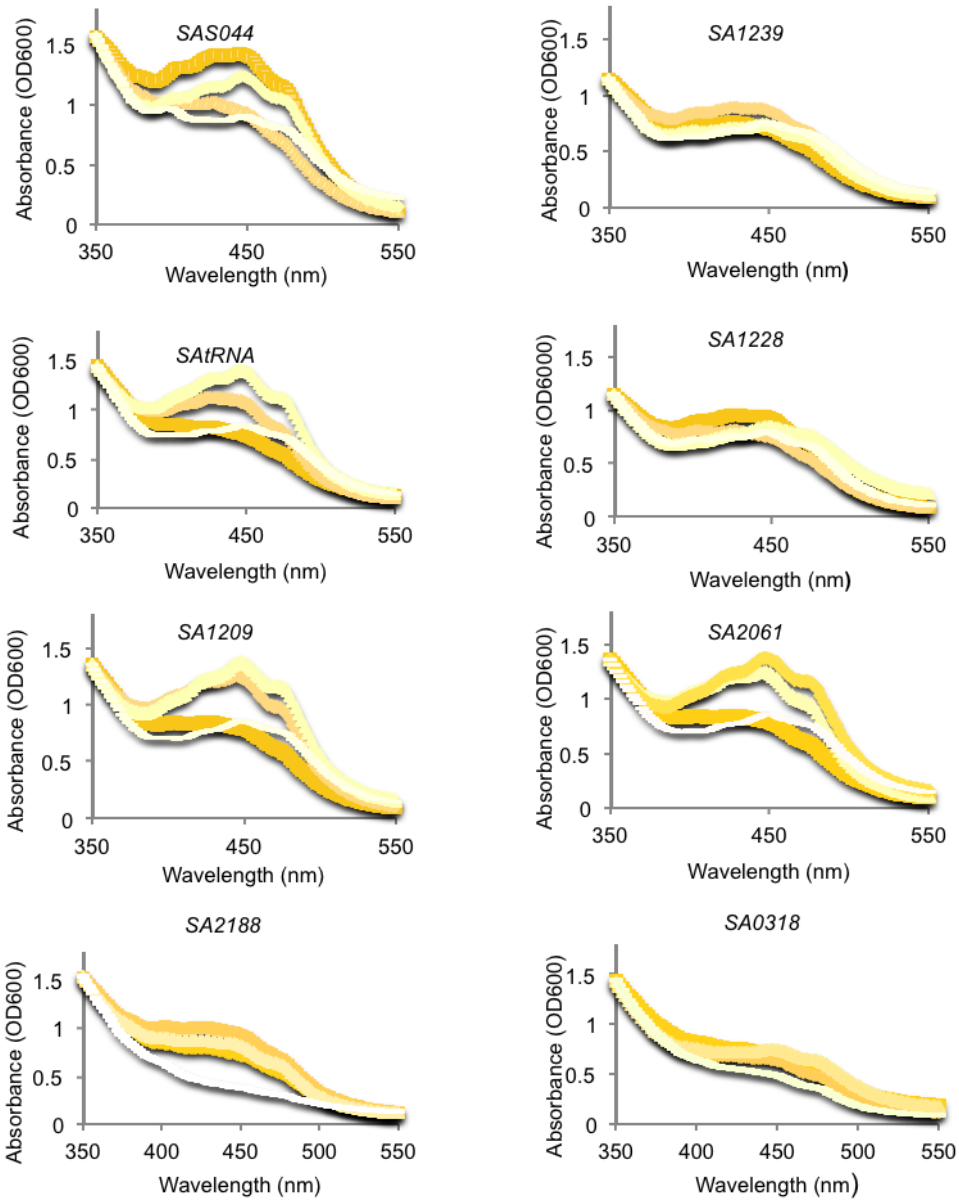

Figure 4.11 A. Carotenoid expression in ethanol-solubilised cholesterol-treated Tn*917* mutant strains, (A) Absorbance spectrum cells cultured in BHI in the of methanol extracts from absence of ethanol/cholesterol (orange; square), at the presence 0.03 mM cholesterol (light orange; diamond) 0.029 % ethanol, cholesterol 0.3 mM (light yellow; triangle) 0.29 % ethanol and cholesterol 3 mM (beige; line) 2.89 % ethanol. These data from the Tn*917* mutants are from the tertiary screen. This experiment is representative of three experiments biologically replicated.

B

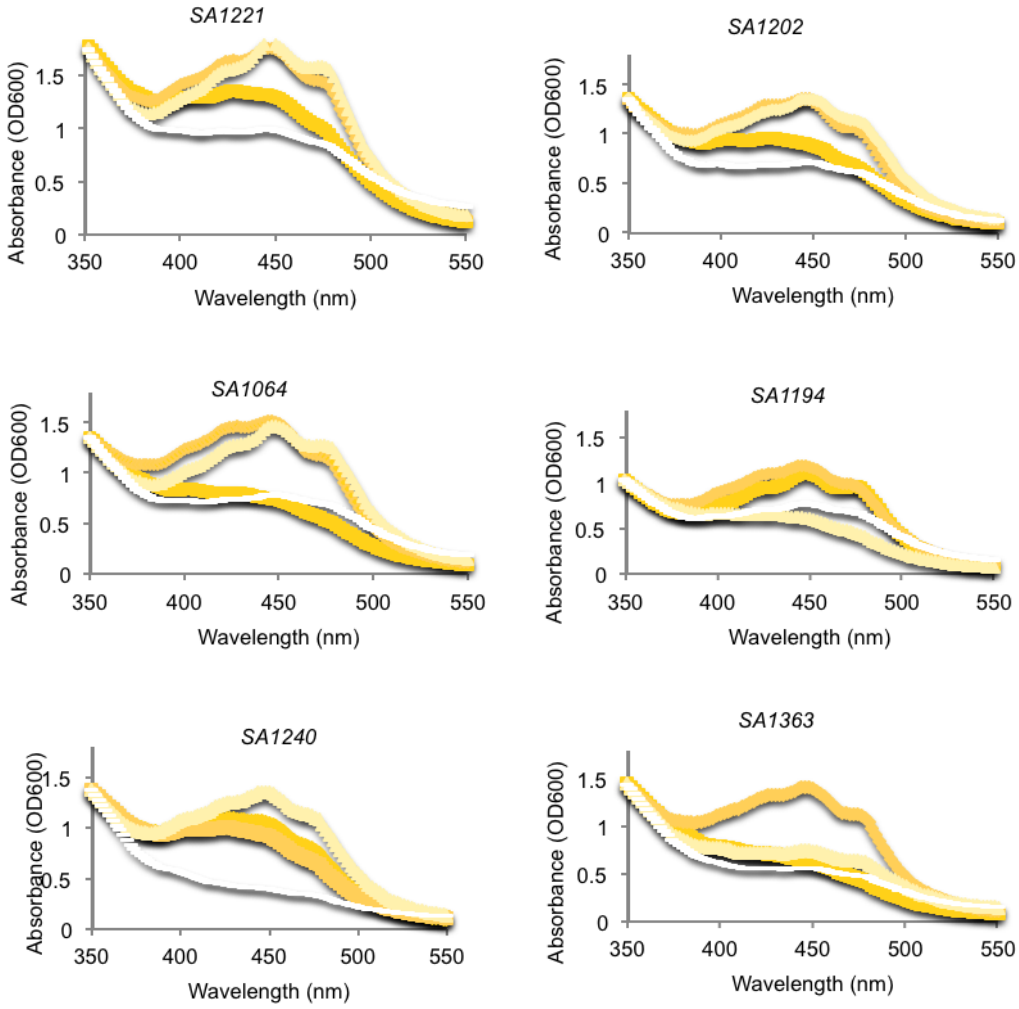

Figure 4.11 B. Carotenoid expression in ethanol-solubilised cholesterol-treated Tn*917* mutant strains, (B) Absorbance spectrum cells cultured in BHI in the of methanol extracts from absence of ethanol/cholesterol (orange; square), at the presence of 0.03 mM cholesterol (light orange; diamond) 0.029 % ethanol, cholesterol 0.3 mM (light yellow; triangle) 0.29 % ethanol and cholesterol 3 mM (beige; line) 2.89 % ethanol. These data from the Tn*917* mutants are from the tertiary screen. This experiment is representative of three experiments biologically replicated.

C

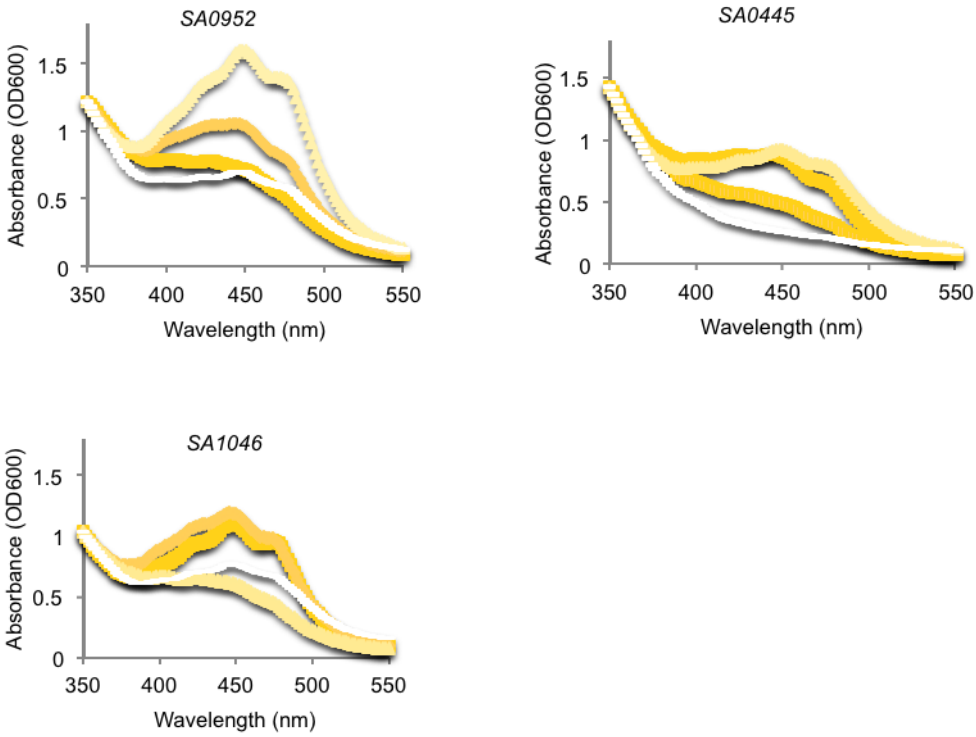

Figure 4.11 C. Carotenoid expression in ethanol-solubilised cholesterol-treated Tn*917* mutant strains, (C) Absorbance spectrum cells cultured in BHI in the of methanol extracts from absence of ethanol/cholesterol (orange; square), at the presence of 0.03 mM cholesterol (light orange; diamond) 0.029 % ethanol, cholesterol 0.3 mM (light yellow; triangle) 0.29 % ethanol and cholesterol 3 mM (beige; line) 2.89 % ethanol. These data from the Tn*917* mutants are from the tertiary screen. This experiment is representative of three experiments biologically replicated.

A

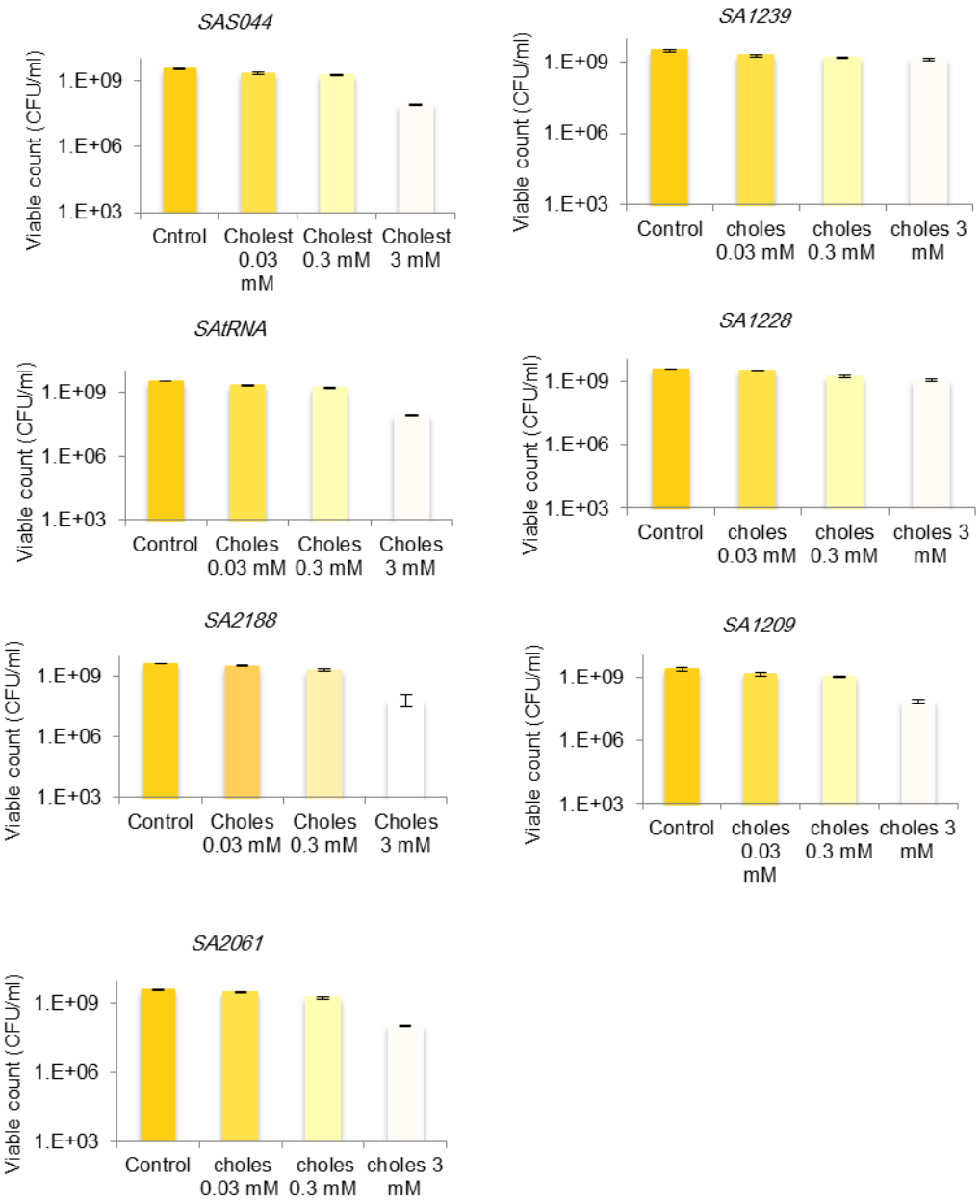

Figure 4.12 A. Carotenoid expression in ethanol-solubilised cholesterol treated Tn*917* mutant strains, (A) Viable counts of cells grown with or without ethanol/cholesterol. This is an example of the Tn*917* mutant and is from the tertiary screen. This experiment is representative of three experiments biologically replicated. The error bars represent standard error of the mean.

B

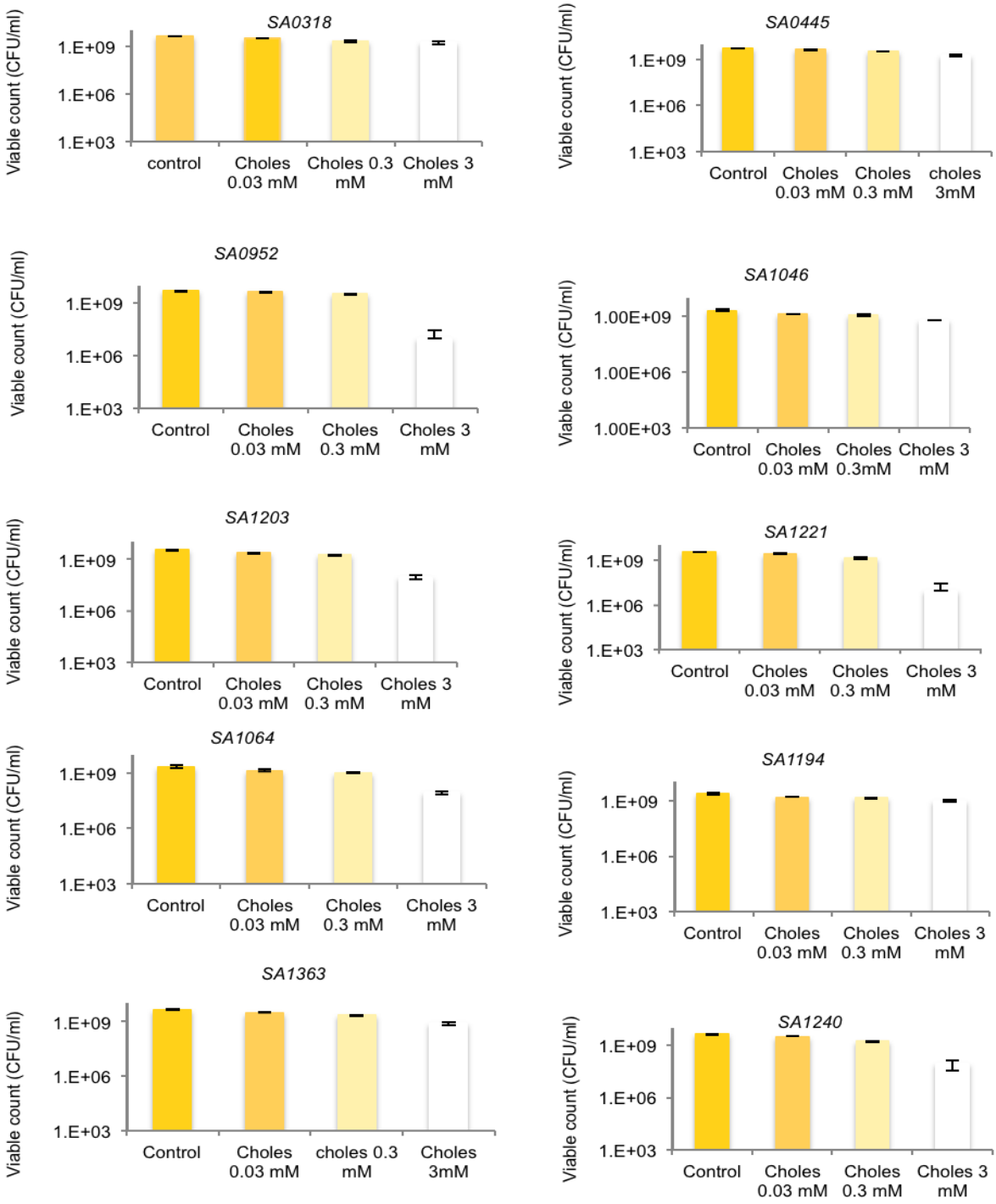

Figure 4.12 B. Carotenoid expression in ethanol/cholesterol treated Tn*917* mutant strains, (B) Viable counts of cells grown with or without ethanol/cholesterol. This is an example of the Tn*917* mutant and is from the tertiary screen. This experiment is representative of three experiments biologically replicated. The error bars represent standard error of the mean.

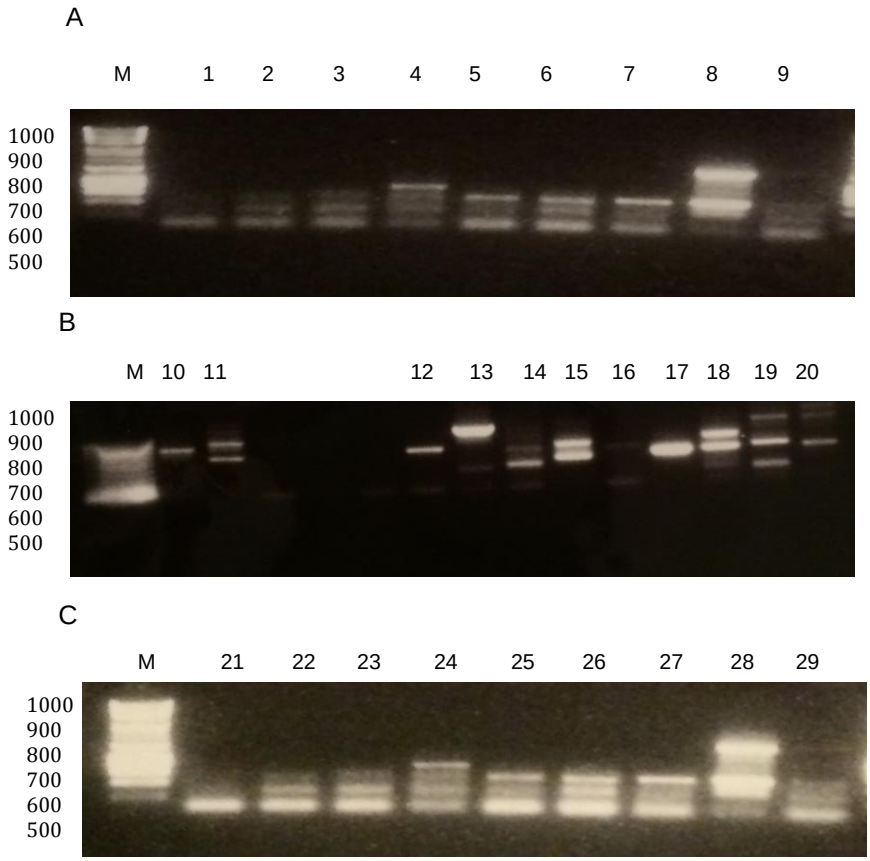

Figure 4.13. Electrophoresis gel image of transposon insertion PCR products. Arbitrary PCR of Tn*917* library mutants with ethanol-solubilise cholesterol (0.3 mM) using (A) ARB1B primer, (B) ARB3 primer and (C) ARB5 primer. The product numbers correspond to Table 4. M is molecular size markers.

Table 4.1. Tn*917* mutants demonstrating altered carotenoid expression in the presence of ethanol-solubilise cholesterol

Colour orange to white	Number as per Fig (4.12)	Plate well	Category Functional	Accession Name	Gene disrupted	Gene Size (bp)	Gene Description	Blastn E-value	Gene coordinates	Insertion site
Orange	29	L/A3	Transport/binding proteins and lipoproteins	SA0318	*SA0318*	1347	hypothetical protein, similar to transport protein SgaT (sugar permease)	2e-70	375713-377059	535/1347
Yellow	27	CH/F5	Similar to unknown proteins	SA0445	*SA0445*	726	methyltransferase	e 0.15	517096-517821	581/726
Slightly yellow	9	L/H8	tRNA	SAtRNA	*SAtRNA16*	89	tRNA-Ser	0.0	1002.631-1002.719	6/89
Dark yellow	5	F/G8	Transport/binding proteins and lipoproteins	SA0952	*potC*	810	Spermidine putrescine ABC transporter homolog	0.0	1078757-1079566	488/810
Yellow	8	D/B2	Metabolism of nucleotides and nucleic acids	SA1046	*pyrAB*	3174	carbamoyl-phosphate synthase large chain	0.0	1183131-1186304	3042/3174
Dark yellow	5	O/G2	Protein modification	SA1064	*SA1064*	1995	protein kinase	5e-48	1204606-1205481	669/1995
Light yellow	24	C/B8	Protein modification	SA1194	*msrA*	510	Peptide methionine sulfoxide reductase homolog	8e-37	1366337-1366846	242/510
Yellow	3	T/C8	Metabolism of lipids	SAS044	*SAS044*	189	4-Oxalocrotonate tautomerase	5e-15	1368054-1368242	81/189
Dark yellow	22	AA/F1	Metabolism of amino acids and related molecules	SA1202	*trpC*	783	indole-3-glycerol phosphate synthase	9e-50	1375540-1376322	423/783
Dark yellow	4	L/A7	Similar to unknown proteins	SA1209	*SA1209*	768	conserved hypothetical protein	4e-33	1383009-1383776	709/768

Table 4.1. contd

Colour	Number as per Fig 4.12	Plate/well	Category Functional	Accession Name	Gene disrupted	Gene Size (bp)	Gene Description	Blastn value	Gene coordinates	Insertion site
Dark yellow	23	L/A3	Membrane bioenergetics (electron transport chain and ATP synthase	SA1221	*SA1221*	984	thioredoxin reductase	0.0	1394073-1395056	275/984
Orange	6	CH/F1	Metabolism of amino acids and related molecules	SA1228	*dapB*	723	Dihydrodipicolinate reductase	2e-44	1402887-1403609	-70/723
Yellow	3	CH/B5	Transposon/binding protein and lipoproteins	SA1239	*braB*	1344	Branched-chain amino acid carrier protein	8e-15	1411832-1413175	269/1344
Dark yellow	26	E/D10	Similar to unknown proteins	SA1240	*SA1240*	1887	conserved hypothetical protein	0.0	1416273-1416476	194/1887
Orange	15	AB/A7	Similar to unknown proteins	SA1360	*SA1360*	831	Amino acid metabolism	6e-59	1571130-1571960	345/831
Yellow	19	K/B12	Transport/binding proteins and lipoproteins	SA2061	*acpS*	1212	Major facilitator family transporter	0.0	2324412- 2325623	-339/1212
Yellow	25	H/E11	Metabolism of amino acids and related molecules	SA2188	*nasD*	2406	nitrite reductase	0.0	2461429-2461965	2122/2406
Dark yellow	28	CH/C1	Transport/binding proteins and lipoproteins	SA1213	*Opp-2C*	831	oligopeptide transporter membrane permease domain	0.0	1385575-1386705	452/831

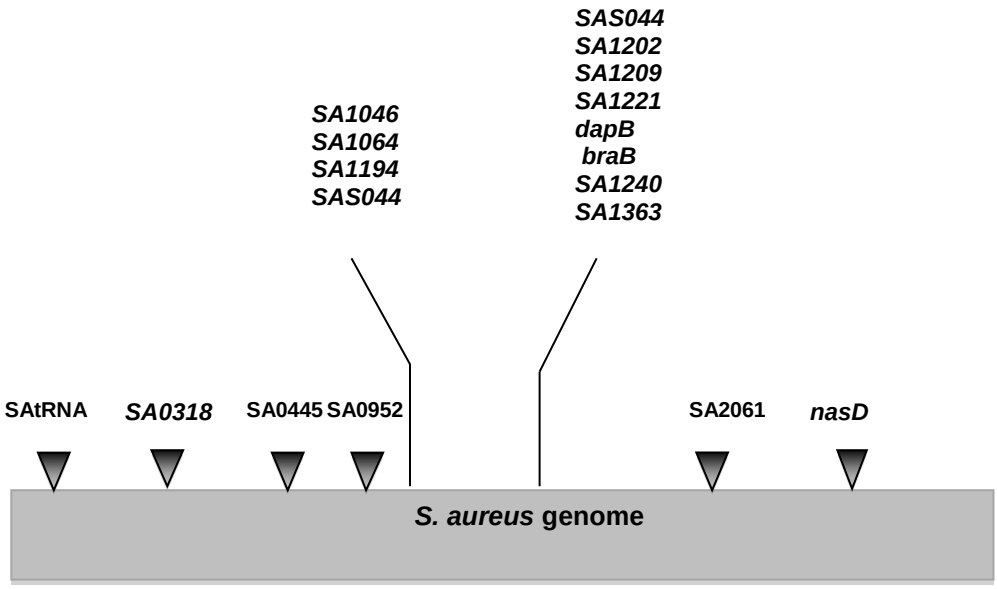

Figure 4.14 Diagrammatic representation of insertion sites of Tn*917* mutants producing increased expression of pigment. Gene IDs are labeled according to the *S. aureus N315* genome

4.3.7.2 Screen for ethanol-solubilised cholesterol recovery mutants

Previous work established that cholesterol addition rescued the antimicrobial effects of linoleic acid, while ethanol/cholesterol-treated cells showed reduced resistance from subsequent exposure to linoleic acid. However, none of the data generated was able to establish if cholesterol interacts with linoleic acid at the cell surface of *S. aureus.* Rather, there remains a possibility that cholesterol interacts with linoleic acid in the culture medium. To investigate using a molecular approach the hypothesis that cholesterol has a mode of action at the cell surface, a further transposon screen was designed. This screen sought to identify growth mutants that were not recovered by ethanol/cholesterol in the presence of antimicrobial levels of linoleic acid. This assay was devised to seek transposon mutants that might provide insights into possible interactions at the cell surface. The assay might identify mutants with reduced survival that reports on the physiological effects on the cell.

One thousand Tn*917* transposon library mutants were assayed by comparing growth in BHI to that in BHI containing 0.3 mM of ethanol-solubilised cholesterol plus 0.1 mM of ethanol-solubilised linoleic acid (Fig 4.14). In the first screen the clones which had poor growth in the presence of cholesterol and linoleic acid were identified. Selected clones were confirmed to have altered recovery in repeat growth experiments, similar to the process described in the previous screen. Arbitrary-primed PCR was performed on genomic DNA extracted from individual clones determined to have reduced

growth in the presence of cholesterol and linoleic acid. These PCR reactions successfully amplified 24 DNA products (Fig 4.15), and following Exo-Sapit-treatment they were sent for Sanger sequencing (GATC Biotech). BLASTn was used to analyse the data obtained (Table 4.3) for 8 mutants with successful sequence data.

The sequenced products identified transposon insertion of genes *SA1209, SA1221* and *SA1240* all encoded as hypothetical protein. The relative fold reduction in cell growth measured by optical density, relative to wild type, was 4.8, 12.6 and 10 respectively for these gene mutants. These genes are located in the previously described 'hotspot' as is the gene *SA1243* encoding an ABC transmembrane transporter that uses ATP to transfer an unknown substrate through the membrane. The mutant of this gene showed a 2.9-fold reduction in OD relative to wild type.

The DNA III polymerase gamma and tau subunits are encoded by the SA0436 gene, and growth of this mutant was 4.52-fold reduced in OD relative to wild type. The two-component signal transduction system ArlSR modifies the activity of extracellular serine proteases and contributes to virulence regulation (Fournier et al., 2001) The *arlS* gene encodes a protein kinase which acts as the sensor (signal transduction) as part of the *arl* operon. This reduces and inactivates the expression of virulence factors by downregulating their transcription. The growth of the *arlS* mutant was reduced 11.3-fold relative to wild type indicating that this mutant had a pronounced defect for growth in ethanol solubilised cholesterol. The *SA1247* (*ebhA)* gene encodes a virulence factor (toxin and colonization factor) and

the mutant for this gene had the greatest reduction in growth relative to wild type.

For the transposon insertion identified at locus *SA2061,* the hypothetical encoded product is a transport protein belonging to the major facilitator superfamily that has been predicted to have 12 transmembrane regions (Krogh et al., 2001). The mutant of this gene had a fold reduction in OD relative to wild type of 6.56 (Table 4.2).

The aim of this investigation was to identify proteins that might contribute to the survival of *S. aureus* in the presence of ethanol-solubilised cholesterol recovery of linoleic acid toxicity, or potential signalling pathways that may regulate responses to extracellular cholesterol. In this regard the genes at locus *SA1243, ebhA*, *arlS* and *SA2061* have potential roles in interaction with the solvent and lipids since each encode surface-associated proteins, which are inactivated by Tn*917* (Table 4.3). In addition, transcriptional repressor genes co-located with the *SA2061*-encoded transporter were identified as bordering the *SA2061* gene, which was identified in both of these screens. This may indicate the existence of pathway involving surface-associated lipid interactions, which leads to the regulation of the response to cholesterol/lipid or the solvent.

Table 4.2. Fold-level reduction in optical density of the selected transposon library mutants (Tn*917*) relative to wild type in the ethanol-solubilised cholesterol recovery screen

Number on plates	Relative OD	Number on plates	Relative OD
A1	1.62	G2	12.6
A3	2.75	G3	6.56
A6	4.52	G6	1.8
A8	2.74	G11	1.09
A10	3	G12	2
A12	3.02	H4	3.8
B1	3.8	H7	2.9
B2	3.5	H10	3
B3	3.12	H11	4.2
B4	1.18	H12	4.8
B5	1.57	E5	10
B7	3.7	E6	4.3
B8	2.9	E7	1.5
B10	2.8	E9	11.3
C1	2.32	F1	1.35
C8	1.97	F4	2.2
C9	2.2	F6	4.2
C12	4.2	F9	1.7
D2	2.33	F10	1.8
E1	4.11		

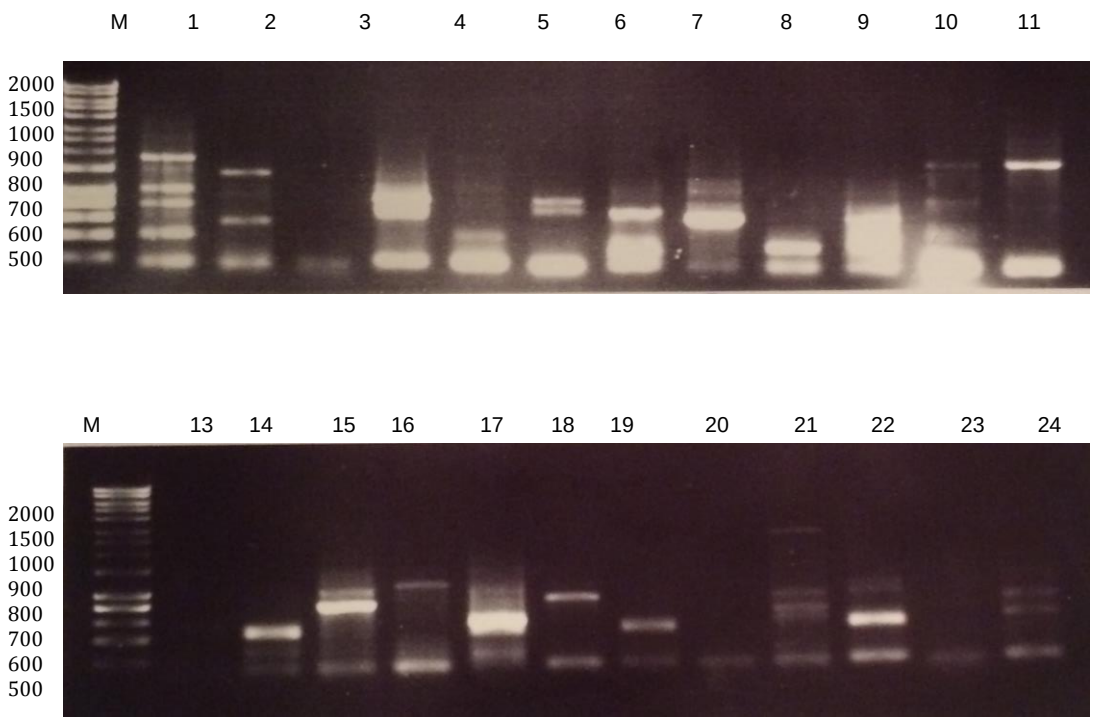

Figure 4.15. Agarose gel electrophoresis of transposon-chromosome, junction PCR products. Arbitrary primed PCR of Tn*917* library mutants with defective recovery growth in linoleic acid (0.1 mM) and ethanol-solubilise cholesterol (0.3 mM) using ARB1B primer. The numbers correspond to those in Table 4.3. M is molecular size markers.

Table 4.3. Features of Tn 917 library mutant genes with defective growth in the presence of linoleic acid (0.1 mM) and ethanol-solubilise cholesterol (0.3 mM)

Number as shown in Figure (4.15)	Plate/ Well	Relative OD	Group Functional	Accession Name	Gene disrupted by insertion	Gene Size (Base Pair)	Gene Description	Blastn E-value	Transposon insertion coordinate	Insertion site
15	AA/A6	4.52	DNA replication	*SA0436*	*dnaX*	1698	DNA polymerase III gamma and tau subunits	2e-5	502594-504291	166/1698
16	L/H12	4.8	Similar to unknown proteins	*SA1209*	*SA1209*	768	unknown	4e-33	1383009-1383776	324/768
6	CH/G 2	12.6	Similar to unknown proteins	*SA1221*	*SA1221*	984	thioredoxine reductase	0.0	1394073- 1395056	275/984
2	CH/D5	10	Similar to unknown proteins	*SA1240*	*SA1240*	1887	conserved hypothetical protein	0.0	1413401-1415287	954/1887
24	X/H7	2.9	Transport/binding protein and lipoproteins	*SA1243*	*SA1243*	810	ABC transporter homolog	5e-22	1416505- 1417314	0/810
22	L/D9	11.3	Sensors (signal transduction)	*SA1246*	*arlS*	1356	putative protein histidine kinase ArlS	0.0	1422192- 1423547	797/1356
21	CH/F1	1.35	Pathogenic factors (toxins and colonization factors	*SA1267*	*ebhA*	20142	hypothetical protein, similar to streptococcal adhesin emb	3e-12	1437928-1458069	16837/20142
1	E/G3	6.56	Transport/binding proteins and lipoproteins	*SA2061*	*acpS*	1212	Major facilitator family transporter	0.0	2324412- 2325623	-339/1212

4.4 Discussion

4.4.1 *S. aureus* response to extracellular changes

Bacteria have the ability to adapt to changes in the external environment such as temperature that impact on their membrane properties and functionality. These adaptations involve adjustments to the distribution of bilayer and non-bilayer lipids, fluidity of the membrane and changing lipid-protein interactions (Mrozik, 2004).

The biology of pathogenic Gram-positive bacteria with respect to extracellular cholesterol is poorly understood. Cholesterol may impact on the expression profile, cell physiology and survival against skin antimicrobials of these organisms; therefore these were investigated with respect to *S. aureus*.

In prokaryotes it has been observed that polar carotenoids regulate membrane properties in the same manner as cholesterol in eukaryotes (Rohmer et al., 1979). In addition, it has been described that staphyloxanthin synthesis in *S. aureus* is linked to resistance from phagocyte-mediated killing (Liu et al., 2008, Liu et al., 2005a). Many diverse mechanisms facilitate the survival of *S. aureus* in changeable environments, and provide support for its resistance to external pressures. This versatility also allows the bacteria to adapt to the innate immune defences found on host surfaces (Mishra et al., 2011c).

To clarify the impact of ethanol-solubilised cholesterol upon the physiology and metabolism of *S. aureus*, an experiment was designed to culture the bacterium in a range of conditions; specifically, differing concentrations of

ethanol-solubilised cholesterol at different temperatures. Two *S. aureus* strains, SH1000 and Newman, were grown in media containing different concentrations of cholesterol (0.3 mM and 3 mM) solubilised with ethanol prior to addition, at three different temperatures (25 °C, 37 °C and 42 °C). The results showed that the growth rate and yield was lower in the presence of 3 mM ethanol-solubilised cholesterol, when incubated at 42 °C or 25 °C; 2 and 4 fold less respectively when compared with growth in the absence of ethanol-solubilised cholesterol (Figure 4.1A and 4.1B).

Certain bacterial species such as *E. coli* and *B. subtilis* alter their cytoplasmic membrane fatty acid composition and fluidity in response to fluctuations of temperature (Mrozik, 2004). Bacterial growth at high temperatures in these species is accompanied by an increase in the proportion of long-chain and saturated fatty acids in the membrane, which function to restrict the fluidity of the membrane. In contrast, short-chain branched and saturated short chain fatty acids are increased at lower temperature, which decreases the rigidity of the membrane (Sinensky, 1974, Cronan and Vagelos, 1972, Keweloh et al., 1991). Changes to the external temperature have been found to be accompanied by alterations in the composition of phospholipids in the membrane, so that the glycerophospholipids with higher melting points and greater packing thicknesses have been observed to be incorporated in the membrane as temperature rises (Denich et al., 2003).

Similar lipid modifications in response to growth temperature have not been found in investigations of *S. aureus*. For instance, *S. aureus* presented no

major alterations in fatty acid composition when grown at 25 °C when compared with growth at 37 °C (Denich et al., 2003).

Data for the growth of *S. aureus* at different temperatures is limited in this study, and further investigations should be undertaken to examine the effect of sterols in order to identify effects specific to cholesterol, distinct from the effects of the solvent. In addition, the mutant strains identified in the transposon screens that have altered pigmentation or recovery in the presence of cholesterol could be tested to more fully characterise the interplay between growth, cholesterol and its solvent ethanol with respect to membrane properties.

Data herein from the study reveal that ethanol-solubilised cholesterol decreased the quantity of staphyloxanthin that was methanol extractable from *S. aureus* (Fig 3.10). Staphyloxanthin in *S. aureus,* contributes to resistance to reactive oxygen species, such as hydrogen peroxide, O^{2-}, and HOCl (Liu et al., 2008). The addition of hydrogen peroxide decreased the viability of ethanol-solubilised cholesterol-treated cells approximately 20-30 % for the Newman strain after 60 minutes, and 30-40 % for strain SH1000 after 60 minutes when compared with cultures where cholesterol was absent, or control cultures in ethanol solvent (Fig 4.2A and Fig 4.2B). This reduction in survival correlated with reduced pigmentation. The alternative sigma factor σ^B in *S.aureus* is a vital regulator of stress-induced protein synthesis (Boylan *et al*., 1993b, Varon *et al*., 1993, Volker *et al*., 1994). Increased *sigB* activity after stress in the stationary phase correlates with the increased synthesis of σ^B-dependent stress proteins (Benson and Haldenwang, 1993, Boylan et al., 1993a, Boylan et al., 1993b, Boylan et al., 1992, Volker et al., 1994).

Catalase (*katA*) is the major enzyme in *S. aureus* peroxide defence (Harris et al., 2002a). Previous studies have investigated the impact of different stress conditions on the expression of *katA*, that is controlled through the vegetative sigma factor A, and further regulated by *sigB* (Bol and Yasbin, 1994). It was shown that *katA* transcription significantly increased, by 140-fold, after the addition of H_2O_2 to growing cells (Engelmann *et al*., 1995). Two genes of the *PerR* regulon, *ahpC* (encoding alkylhydroperoxide reductase) and *katA* demonstrate peroxide regulation, with both associated and independent functions (Cosgrove et al., 2007). An *ahpC* mutation improved resistance to H_2O_2, owing to increased expression of *katA*. Both *ahpC* and *katA* are required for persistence in the environment and nasal colonisation (Cosgrove et al., 2007). Therefore, peroxide stress resistance is a significant factor in the ability of *S. aureus* to survive in the environment (Cosgrove et al., 2007).

4.4.2 Effects of cholesterol upon the plasma membrane of *S. aureus*

The effects of cholesterol outlined in this thesis could be due to its influence on cell membrane functionality. There are however several potential scenarios which are not mutually exclusive: cholesterol may cover the cell surface; it may bind at entry points to linoleic acid/AFAs; or it could stimulate some change in cell properties, such that gene expression is activated that makes the cell more resistant to environmental stress.

Alternatively it may be that the effect observed when cholesterol was present, was partly or wholly due to ethanol since as outlined in the previous chapter

the failure to effectively control for the increasing ethanol when raising the cholesterol concentration is a major limitation of all the work presented here. The accompanied increase in ethanol concentration in the experiments testing growth rescue of *S. aureus* and staphyloxanthin expression meant a fundamental experimental control was absent; and therefore the results obtained cannot be used to demonstrate any effects from addition of cholesterol to the media.

The effects could be due to ethanol, however there is also the potential for synergy between the cholesterol and ethanol solvent which is problematic for studies like these, but adequate controls should have been designed from the outset. It may be that both cholesterol and ethanol cause changes in gene expression that lead to a decrease in bacterial pigmentation. Importantly, there was no expectation that the level of pigmentation is directly changed by ethanol, since previous studies have demonstrated that ethanol has no effect on σ^B expression (Conlon et al., 2002). Thus the current study has found results which do not agree, i.e. are opposite to the literature. This makes it difficult to have clear conclusions from the presented work, as it is difficult to separate the effects found when cholesterol is solubilised in ethanol; thus it cannot be shown what effect each component in the cultures was having even though very pronounced phenotypic changes were documented.

A cell hydrophobicity measurement was carried out using the MATH test (Rosenberg, 2006) to compare untreated cultures (Newman) and cultures grown in different concentrations of ethanol-solubilised cholesterol (0.03 mM, 0.3 mM and 3 mM). This assay detected that there were no significant

differences in membrane hydrophobicity with respect to the control. However the results did show a reduction in methanol extractable staphyloxathin when the strain was grown in cholesterol in comparison to the absence of cholesterol (Figure 3.13). Mishra et al. (2011) have shown that strains of *S. aureus* having the greatest production of staphyloxanthin also had the most rigid cell membrane suggesting that staphyloxanthin contributes to membrane rigidity, which is known for cholesterol (Cooper, 1978). Therefore these results could indicate that as cholesterol in the environment is increased, expression of staphyloxanthin may be decreased, thus helping to maintain the relative rigidity or fluidity of the membrane.

While the results detailed in chapter 3 show that a reduction in staphyloxanthin expression occurs when *S. aureus* is cultured with ethanol-solubilised cholesterol, the agent and mechanism behind this remains unknown. However this transition could be controlled by a sensory mechanism, likely to be located in the membrane and capable of modulating transcription of the *crtOPQMN* operon encoding the genes for staphyloxanthin biosynthesis. This mechanism may be intimately linked with membrane order and fluidity/rigidity.

4.4.3 *S. aureus* sensing of membrane fluidity

Several two-component systems (TCS) have previously been identified as having an influence on cell membrane fluidity, namely *NsaRS* (Kolar et al.,

2011) and *GraRS* (Li et al., 2007). To investigate whether these or another TCS may act through cholesterol sensing or membrane rigidity/fluidity sensing, a series of TCS mutants were studied. These were available in mutants of WCUH29, a *S. aureus* strain confirmed to show similar ethanol-solubilised cholesterol responsiveness with regard to staphyloxanthin expression as the Newman strain. A second TCS mutant of SrrAB (also known as SrhSR) was shown to be completely insensitive to ethanol/cholesterol; so could act as a regulator in this response. SrrAB downregulates expression of pigment in anaerobic conditions, and plays a vital role in the regulation of energy generation in response to oxygen availability (Throup et al., 2001). *S. aureus srrAB* inactivation leads to alterations in cell morphology, the expression of proteins participating in energy metabolism, and other metabolic processes involving arginine catabolism (Kohler et al., 2003). SrrAB is important for the increase in synthesis of many fermentation enzymes, and repression of the enzymes involved in the tricarboxylic acid cycle in anaerobic conditions. SrrAB has also been described as being a comprehensive virulence factor regulator (Pragman et al. 2004). It responds to nitric oxide induced stress, and mutants lacking this TCS are hypersensitive to nitric oxide stress (Richardson, Dunman and Fang, 2006).

SrrAB could be further investigated by studying cell membrane polarisation and lipid composition of the *srrAB* mutants. The transcriptome of *srrAB* in conditions of nitrosative stress (Kinkel et al., 2013) has been studied; and this could be further investigated in the presence or absence of cholesterol to

identify the interplay of genes involved in ethanol/cholesterol sensing and rescue of growth.

In *B. subtilis* ResDE is a TCSTS and represents one of the main regulatory systems involved in the regulation of genes in anaerobic conditions (Nakano et al., 1997, Nakano and Hulett, 1997, Hoffmann et al., 1998, Nakano et al., 1996, Sun et al., 1996). The membrane-bound histidine kinase, ResE, and the cytoplasmic regulators, ResD, are involved in oxygen sensing (Hartig and Jahn, 2012). The TCS therefore acts to positively regulate genes for survival in anaerobic conditions. This is similar to the TCS SrrAB, involved in transformation of the metabolism of *S. aureus* from aerobic to anaerobic (Pragman et al., 2004, Throup et al., 2001, Yarwood et al., 2001).

The current study identified that mutants for *phoRS, SaeR* and *HssRS* expressed lower levels of pigment in the presence of ethanol-solubilised cholesterol; therefore these may be regulators of pigment production in the presence of ethanol/cholesterol. These regulators might respond to the effect of these components directly or indirectly. Therefore further investigations of the transcriptome may also prove useful to confirm whether these signal transduction systems regulate the response to ethanol/cholesterol.

Early data from a *desR* mutant of *S. aureus* WCUH29 suggested its involvement in the expression of carotenoid, however these results could not be consistently shown to be significantly different from the levels of staphyloxanthin expression found in the parental WCUH29 strain. The small changes observed in a mutant generated in *S.aureus* SH1000 were, however, reproducible. The current results indicate a potential role for the

*desR*K operon in the expression of staphyloxanthin that might differ depending on the strain background. However, further investigations are needed to provide further insights.

The role of the putative *desRK* operon in *S. sureus* is unknown. However in *B. subtilis* the two genes are members of the TCSTS regulating an adjacent gene, *desA*. The product of *desK* is a transmembrane histidine kinase (Albanesi et al., 2004), which phosphorylates itself and the *desR* gene product. The phosphorylated DesR protein binds a region of DNA upstream from the *desA* gene, activating transcription of the *desA* gene product, a Δ5-phospholipid desaturase enzyme (Zhang and Rock, 2009). The DesK protein acts as a sensor of membrane fluidity (Zhang and Rock, 2009) and its activation leads to increased membrane fluidity due to the upregulation of the Δ5-phospholipid desaturase.

The gene for cardiolipin synthase is downstream of the *desRK* operon. The *S. aureus desR* mutant generated in this study was designed so that the operon was disrupted by the insertion of the tetracycline resistance gene. The homolog of this gene in *B. subtilis* is also thought to be involved in regulating the expression of genes in response to temperature changes (Ibarra et al., 2013), therefore the product of this gene may have a similar function in *S. aureus*. Cardiolipin synthase is integral to the bacterial membrane and catalyses the transfer of a phosphatidyl group from a phosphatidylglycerol to a second phosphatidylglycerol molecule; in the process cardiolipin is formed (diphosphatidylglycerol). This enzyme is important for helping to maintain the long-term adaptation of the plasma membrane and membrane integrity in *E. coli* (Shiba et al., 2004). It may have a similar role in *S. aureus.* Studies have

also shown that cardiolipin is required for the growth of *S. aureus* in conditions of high salinity (Tsai et al., 2011), acting as a protector from environmental stress. It is possible that disruption of *desR* in the mutant may disrupt the expression of cardiolipin synthase and hence reduce expression of cardiolipin, or alternatively disrupt the mechanism of transcription regulation by DesR.

An *S. aureus arcA* mutant revealed very similar expression of pigment to the *srrAB* mutant; *arcA* encode arginine deiminase. Previous studies (Makhlin et al., 2007, Fuchs et al., 2007) have shown the *arcABDC* genes and *arcR* does ArcR transcriptionally control both. This system enables *S. aureus* to use arginine as an energy source in the absence of glucose and nitrate. The *srrAB* gene is also regulated by *arcR*.

The *srrA* and *arcA* mutants responded to the presence of ethanol/cholesterol with slightly altered expression of staphyloxanthin compared with the wild type (Fig 4.6B & Fig 4.7B, respectively). The similarity in the expression of the pigment reinforces the notion that each impact on staphyloxanthin biosynthesis. Evidence suggests that *arcA* is involved in the control of *srrA* (Makhlin et al., 2007) and the TCSTS might have become activated in the presence of ethanol/cholesterol. The results of the current study indicate *nasD, srrA* and *arcA* are multiple components of a network, which regulates staphyloxanthin expression.

A *nasD* (nitrite reductase) mutant (Table 4.1) was identified in the transposon screen for staphyloxanthin expression in the presence of ethanol/cholesterol, as it had increased staphyloxanthin expression. This gene and its operon are

known to be controlled by the NreBC TCSTS, which regulates the oxygen-responsive switch for use of nitrate/nitrite system in *S. aureus*, which is activated when oxygen concentrations are limiting (Schlag et al., 2008). Within this operon *nreB* encodes a histidine kinase that acts as an oxygen sensor. A cysteine residue on the enzyme is required for its oxygen sensing function (Fuchs et al., 2007). NreB is half of the TCSTS with NreC, which is the transcription regulator. Put together, these studies indicate a TCSTS that alters expression in response to the levels of environmental oxygen may be involved in the expression of staphyloxanthin.

The transposon mutant of *SA2061* had increased staphyloxanthin expression (table 4.3) in the presence of ethanol/cholesterol. *SA2061* encodes an uncharacterised transmembrane transport protein (UniProt, 2015). Genes that border *SA2061*, the transcriptional regulators encoded by *SA2060* and *SA2062,* may have been affected by this insertion (Fig 4.16). The first of these genes encodes sarZ, a transcription factor, while the second encodes SarV (Manna et al., 2004). The functions of these regulators may give clues to the regulation of staphyloxanthin expression. In *S. aureus* the global regulators SarA and MgrA both act to suppress expression of SarV, which itself potentially regulates 19 virulence genes, and is thought to be involved in increasing the autolysis of *S. aureus.* It is known that MRSA has a higher rate of autolysis (Gustafson et al., 1992) and SarV also plays a vital role in regulating multiple resistances to antibiotics (Cohen et al., 1993). There does not appear to be a clear explanation for the increased staphyloxanthin expression of the *SA2061* mutant, therefore further studies are required to identify the mechanism and determine if it acts alone or whether the Sar

homologs are implicated. Decreased growth of the *SA2061* mutant was noted from the cholesterol/linoleic acid transposon screen, providing strong support for its involvement in the response to the ethanol/cholesterol supplementation.

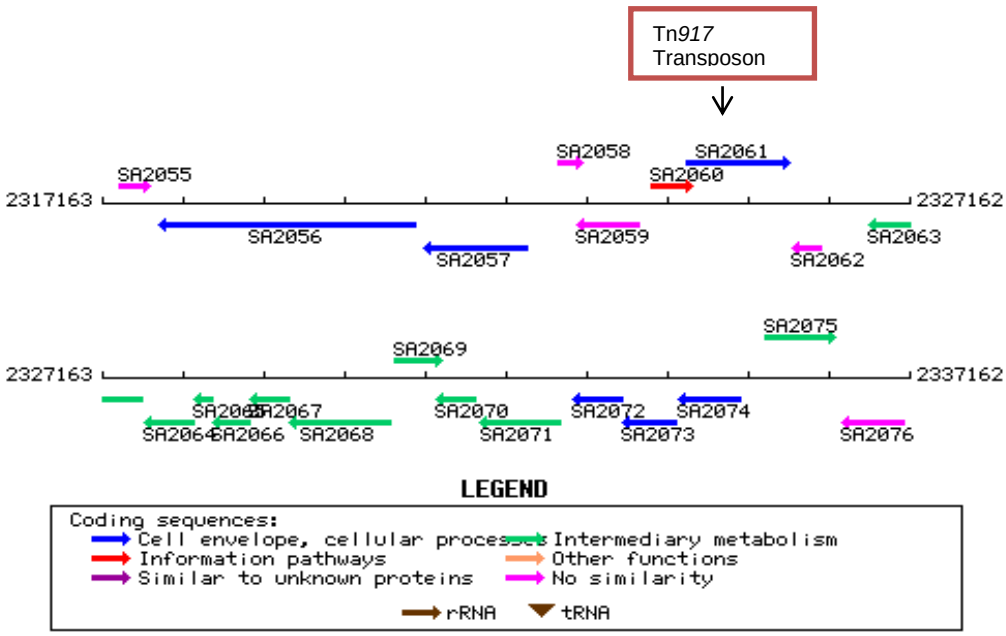

Figure 4.16. Diagram shows the transposon insertion and gene structure of the gene SA2061 (from: http://genolist.pasteur.fr/AureoList/genome.cgi?external_query+SA2061

It was established that ethanol-solubilised cholesterol promotes survival in the presence of linoleic acid, while also reducing bacterial viability at high concentrations (chapter 3). Therefore, transposon mutants that exhibited a poor survival rate in the presence of cholesterol were examined. The aim

was to identify the components that contribute to response to cholesterol in the media by selecting mutants with an altered phenotype or change in pigment expression. Two different screens were designed, firstly to find genes relating to pigmentation and secondly genes relating to the protection offered by cholesterol to linoleic acid toxicity.

The weaknesses with the transposon screening investigations were that the mode of action of the ethanol/cholesterol is unknown and this molecular approach has shed little light on this, which is probably not surprising. The screens have revealed some puzzles, particularly the inverse association of the transposon hotspot with pigmentation changes and the potential suite of ethanol/cholesterol associated surface proteins that might be associated with sensing and/or survival of *S. aureus*. While the effects of transposon insertions, with respect to polarity of downstream genes in the mutants, are unclear, there is a set of gene loci that can be studied in greater detail with further analyses. Further studies will also be needed to confirm genetic linkage, preferably with clean, allelic replacement mutations.

CHAPTER 5 –Characterisation of the *S. aureus* response to solubilised cholesterol and solvent

5.1 Introduction

Human skin serves a range of antimicrobial functions, forming a strong barrier against invasion by bacteria. Thus, as a successful human pathogen, *S. aureus* must possess numerous mechanisms that evade and resist antimicrobials produced by skin (Coates et al., 2014).

The accessory sigma factor σ^B has been described as a stress sigma factor of *S. aureus,* due to its various regulon member genes associated with adaptation. Sigma factors combine with the RNA polymerase holoenzyme, enabling binding to the gene promoter site with the cognate consensus sequence, in order initiate transcription. Transcription is tightly regulated to ensure that the expression profile changes according to the growth phase requirements. Recent studies demonstrated that *S. aureus* has three key expression regulators; these being the *agr* quorum sensing system, σ^B and SarA*;* together they repectively respond to cell densities, stress and the phase of the cell cycle (Arciola et al., 2015).

Several virulence factors of *S. aureus* are proteases, including *sspA* (a serine protease) and *sspB* (a cysteine protease), which are transcribed together; as well as *aur* (a metalloprotease). It is notable that only *sigA*, the housekeeping sigma factor binds their gene promoters; however σ^B is able repress transcription from the *aur* and *scp* operons (Shaw et al., 2004). It was demonstrated that the repressor of toxins (Rot) transcription factor is

also a global regulator in *S. aureus* that functions to repress expression of virulence factors including protease and alpha-hemolysin. The activitiy of Rot itself is regulated by σ^B (Hsieh et al., 2008). Rot translation is negatively regulated by cleavage of its mRNA, using an antisense mechanism, by RNAIII of the *agr* locus. SarA modulates protease expression via activation of transcription at the *agr* locus (Chien Y, 1998) and SarU positively regulates *agr* expression (Manna and Cheung, 2003). Thus σ^B, Rot, the *agr* locus and the Sar regulator family all have an impact on protease expression of *S. aureus*. SarA acts as a protease activity repressor (Cheung et al., 2008). The protease activity regulation in response to external stimuli may allow the organism to respond quickly to changes in its environment. Thus, SarA may not only regulate virulence determinant expression directly, but may also be involved in maintaining surface and exoprotein levels through protease activity. This feature would help the organism to rapidly change its virulence determinant surface array, and its innate ability to be a successful pathogen (Chan and Foster, 1998).

Aureolysin is a metalloprotease expressed by *S. aureus* that is responsible for cleavage of antimicrobial peptides, and thus is a colonisation and virulence factor. The serine protease SspA also has a possible role in bacterial adhesion (Rice et al., 2001). SarA and Rot are the key regulators, repressing transcription of these proteases. σ^B plays a role in the transcriptional regulation of several virulence loci, such as *sarH, sarA* and *coa* (Bayer et al., 1996, Deora et al., 1997, Tegmark et al., 2000). The transcription of *sigB* is controlled by environmental signals in *S. aureus* and it

is expressed throughout growth, not just as a stationary phase transition regulator. It helps the cell to recover from heat shock and survive in conditions where acid or hydrogen peroxide is present (Chan et al., 1998). σ^B expression is upregulated by *sarA*, and as *sarA* regulates virulence genes, including the Agr locus, the survival of the cell under stress conditions is therefore intimately linked with virulence regulation. Therefore, it seems that σ^B is a stress and stationary phase specific global regulator, which is directly and indirectly linked with the expression of virulence genes (Kullik I, et al., 1998).

σ^B is activated directly via the RsbU regulator (Pane-Farre et al., 2009). The genes for three Rsb proteins of *S. aureus* are located upstream within the *sigB* operon (Palma and Cheung, 2001) RsbU is a histidine kinase involved in the response to environmental stress and becomes phosphorylated and hence activated in stress conditions. Under normal conditions *sigB* is bound to the RsbW anti-sigma factor protein in a complex. RsbU phosphorylates RsbV is an anti-anti sigma factor and under stress conditions, it competes with *sigB* for RsbW, forming an anti-anti sigma factor partnership, releasing σ^B which transcribes its regulon of stress genes as its levels increase in the cell (Pane-Farre et al., 2009). RsbU has also been shown to be involved in the up regulation of staphyloxanthin expression and down-regulation of *agr* via s*igB* (Olivier et al., 2009).

In several other Gram-positive bacteria, σ^B is up regulated by an extended Rsb protein pathway due to the reduction of intracellular ATP, such as

nutritionally limiting conditions; this extended pathway has not been found in *S. aureus*.

Quantitative reverse-transcribed polymerase chain reaction (qRT-PCR) is currently the chosen method for studying the transcription of differential mRNA of small numbers of genes (McMillan and Pereg, 2014). qRT-PCR is a very sensitive technique for monitoring the alterations in transcription throughout processes such as growth, phase transition and cellular differentiation (Boisset et al., 2007, Korem et al., 2005). qRT-PCR can be utilised as an independent method for expression analysis and to verify differential gene/protein studies found via transcriptomics or proteomics analysis (Roberts et al., 2011, Bradford et al., 2010).

5.2 Specific aims

The previous chapters revealed pronounced changes to cell survival in the presence of AFAs and pigmentation after supplementation with cholesterol that was solubilised-using ethanol as a vehicle. An unfortunate design error with the use of the solvent majorly limited interrogation of the data obtained from the studies, but their remains the fact that hitherto un-described effects of ethanol-solubilised cholesterol were identified. Should these effects be due to ethanol alone, and then this would clearly contrast with published data on regulation of staphyloxanthin, which is solely regulated by σ^B-mediated transcription. The effects of cholesterol were the original aims of this study and effective controls with the use of required solvent would enable clearer interrogation.

Consequently, the aims for the experiments detailed in this chapter were to gain insight into the relative contributions of cholesterol and ethanol. This would be achieved by investigating the inter-regulated transcription of *sigB, crtM, aur* and *sspA* using qRT-PCR to determine whether gene transcription is altered by cholesterol, or if the ethanol solvent is alone modulating transcription, or alternately if the modulation of transcription occurs due to synergy of the ethanol with cholesterol.

5.3 Results

5.3.1 Comparative *sigB and crtM* transcription after ethanol-solubilised cholesterol challenge

The aim of this experiment was to identify whether cholesterol and ethanol affects the transcription of the *sigB* and *crtM* gene during culture of *S. aureus*. This experiment was designed to test the effects of cholesterol and ethanol, with ethanol also tested alone as an effective control. Cultures of both SH1000 and Newman strains were initiated by inoculation of 50 ml BHI to OD_{600}=0.05, in a 250 ml flask incubated in shaking water bath at 37 °C. Cultures were grown until the OD_{600} reached 0.6. Thereafter ethanol-solubilised cholesterol was added to achieve cholesterol concentrations of 0.03 mM, 0.3 mM or 3 mM, with an additional ethanol control matching the ethanol concentration of the 3 mM cholesterol condition. After 30 minutes samples were withdrawn for RNA and staphyloxanthin extraction.

The effects of cholesterol and ethanol on the Newman strain tested at the mid-log phase of growth using a range of cholesterol concentrations revealed clear effects as shown in Figure 5 this confirmed changes matched those documented previously, despite previous work not involving challenge with cholesterol solutions. Growth of *S. aureus* reduced as the cholesterol concentration increased, with a pronounced reduction at the highest concentration of cholesterol (3 mM) (Fig 5.1). This relationship was also confirmed for culture of the SH1000 strain with the same conditions (Fig 5.8).

Staphyloxanthin expression reduced in this experiment, as observed in the cell extracts at 8 h and with a pronounced difference after 24 h and 48 h. It was also demonstrated that the highest cholesterol concentration in the medium reduced the staphyloxanthin to minimal levels (Fig 5.2), thus confirming that the test reproduced the reduction of staphyloxanthin expression noted in the experiments described in chapter 3 with continuous exposure during growth (Fig 3.13 SH1000 & Fig 3.15 Newman). This experiment confirmed that there was a reduction in pigment with the addition of different concentrations of cholesterol. To investigate this change in staphyloxanthin expression relative to ethanol-solubilised cholesterol and ethanol alone, RNA was extracted 30 minutes post-addition with both the Newman and SH1000 strains (Fig 5.3). Purified RNA from the samples was tested by qRT-PCR to determine whether *crtM* and *sigB* transcription was affected (Fig 5.3).

Reference genes were chosen for the qRT-PCR and included *16S rRNA* (Takle et al., 2007) and *ftsZ* (Duquenne et al., 2010). Their effective amplification was checked using a standard curve prepared by utilising a dilution series to generate standardised concentrations of the cDNA. These were used to quantify cDNA of *16S rRNA, ftsZ, sigB* and *crtM* transcripts in both Newman (Fig 5.4) and SH1000 strains. Melt-curve analysis of the qRT-PCR output was used to determine whether the amplification was produced at the melting temperature (Tm). The results were used to determine whether the expected cDNA transcripts for Newman (Fig 5.5) and SH1000 strains were produced. The presence of single peaks on the melt-curve for

each sample demonstrated high specificity of the PCR products produced (Fig 5.11), which were also visualised on a gel by agarose electrophoresis (Fig 5.6).

Adding either ethanol-solubilised cholesterol or ethanol resulted in the *sigB* and *crtM* transcript levels being strongly reduced indicating their downregulation in the presence of 0.3 mM cholesterol. This was calculated from ΔΔct values of *sigB* in 0.3 mM of cholesterol (-43.89 fold) and for *crtM* in 0.3 mM of cholesterol (-3.25 fold), relative to the no ethanol/cholesterol control and compared with the expression in ethanol control conditions of the Newman strain, calculated from ΔΔct *sigB* (-12.92 fold) and *crtM* (-1.12 fold) (Figure 5.7). The *sigB* and *crtM* transcript levels in SH1000 were similarly reduced in the presence of 0.3 mM ethanol-solubilised cholesterol relative to the no cholesterol/ethanol control conditions and from the calculated ΔΔct values of *sigB* in 0.3 mM of cholesterol (-22.8 fold) and of *crtM* in 0.3 mM of cholesterol (-12.99 fold). These values were compared with expression in ethanol control conditions, calculated from ΔΔct *sigB* for the ethanol control (-13.7 fold) and *crtM* ethanol control; (-9.2 fold) (Fig 5.12). These results indicated that the effect of reduced expression after ethanol-solubilised cholesterol is majorly affected by ethanol in the media.

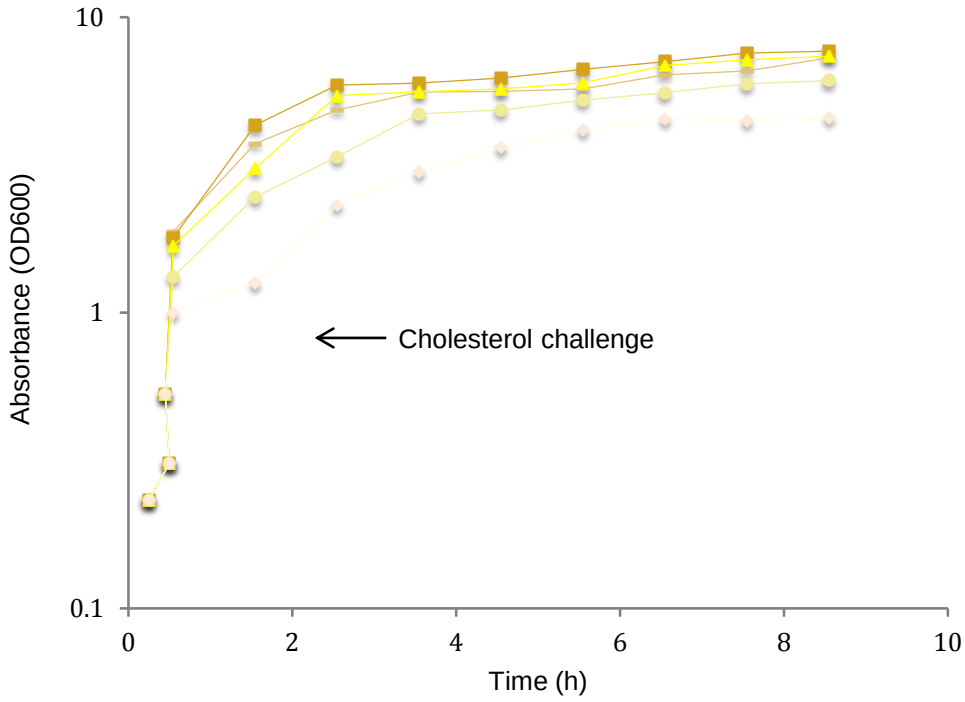

Figure 5.1. Ethanol-solubilised cholesterol challenge of *S. aureus* Newman. Comparison of the growth of the strain Newman in different concentrations of ethanol-solubilised cholesterol (no cholesterol: orange, square; ethanol solvent control: light orange, line (2.89 % ethanol); 0.03 mM of cholesterol: yellow, triangle (0.029 % ethanol), 0.3 mM of cholesterol: light yellow, diamond (0.29 % ethanol); and 3 mM of cholesterol: beige, circle (2.89 % ethanol)) in BHI at 37 °C with shaking.

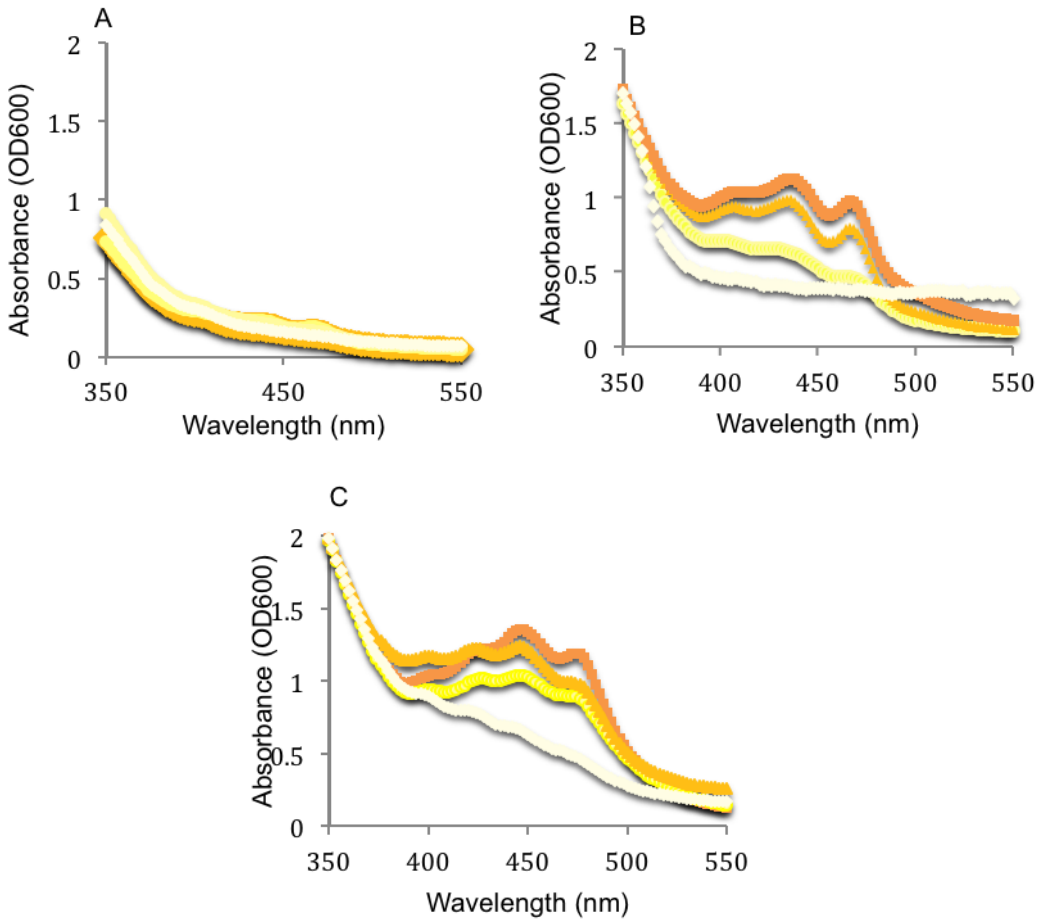

Figure 5.2. Staphyloxanthin expression following ethanol-solubilised cholesterol challenge. *S. aureus* Newman. Absorbance spectrum of cells treated with cholesterol (0.03 mM, yellow; triangle (0.029 % ethanol); 0.3 mM, light yellow; circle (0.29 % ethanol); 3 mM, beige; diamond (2.89 % ethanol); ethanol control, light orange; line (2.89% ethanol) compared to cells grown in the absence of cholesterol (orange) (square). (A) shows absorbance spectra after 8 hours (B) after 24 hours and (C) after 48 hours.

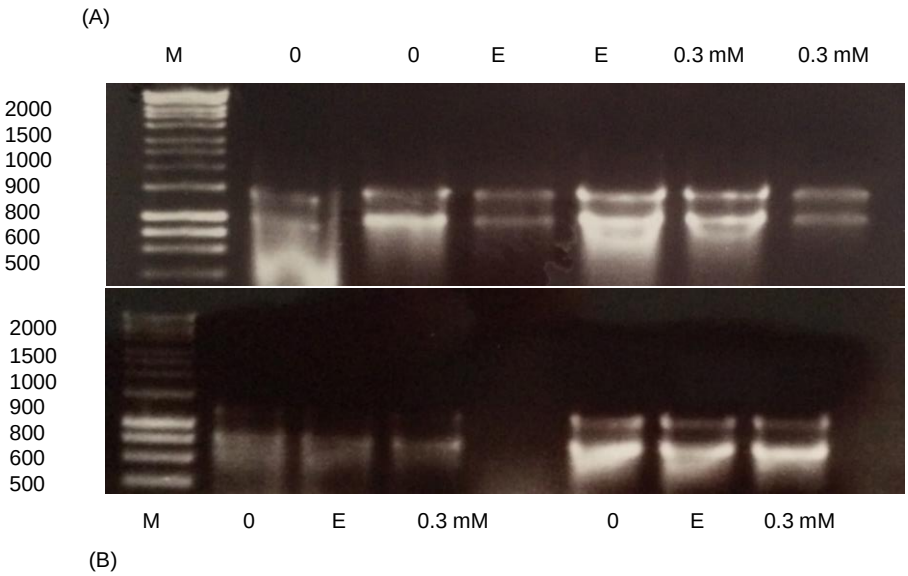

Figure 5.3. Gel electrophoresis of RNA extracted from cholesterol-challenged *S. aureus*. Newman (A) and SH1000 (B) strains 30 min post-challenge to use RNA for testing qRT-PCR (ethanol solvent control (E) and 0.3 mM of cholesterol). M is molecular size markers.

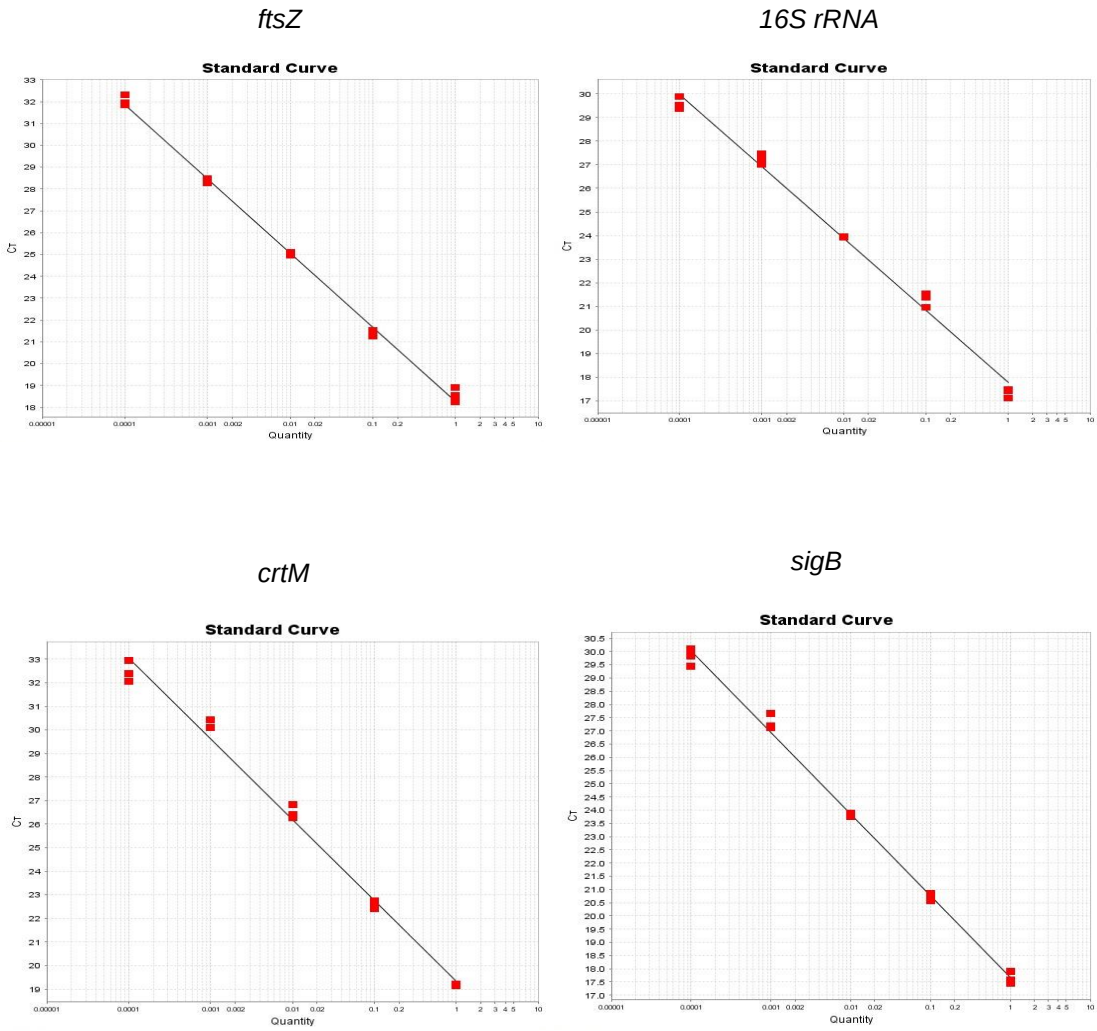

Figure 5.4. Complementary DNA (cDNA) amplification of target and control mRNAs. Real-time PCR with SYBR Green discovery was performed on the LightCycler using the QuantiFast SYBR Green PCR Kit. The standard curves show that the QuantiFast SYBR Green PCR Kit enabled high PCR efficiencies of 99 % (for *ftsZ*), 95 % (for *16s rRNA*) and 94 % (for *crtM* and *sigB*).

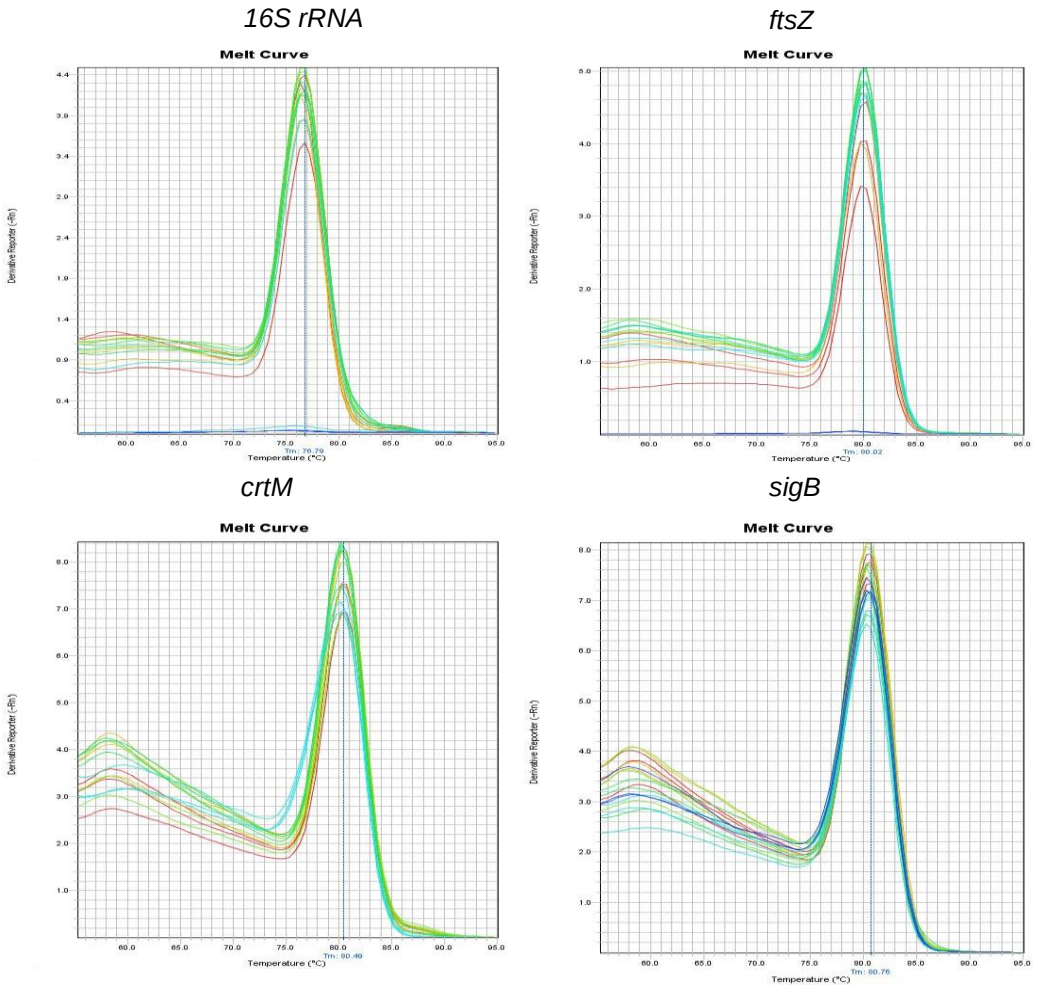

Figure 5.5. Melting curve analysis of PCR amplicons. Melt curves were performed at the end of SYBR Green qPCRs to verify specificity. In this study, *sigB* and *crtM* analysis was conducted with *16S rRNA* and *ftsZ* as reference genes for *Newman* strain. This experiment was performed.

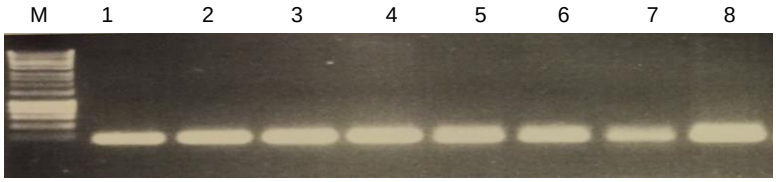

Figure 5.6. cDNA RT-PCR products of the Newman strain *sigB* and *crtM* genes, visualised following agarose gel electrophoresis (cultured in the absence of cholesterol (0), ethanol control conditions (E), and with 0.3 mM of cholesterol). 1 *(sigB* 0), 2 (*sigB* E), 3 (*sigB* 0.3 mM), 4 (DNA *sigB*), 5 (*crtM* 0), 6 (*crtM* E), 7 (*crtM* 0.3 mM), 8 (DNA *crtM*).

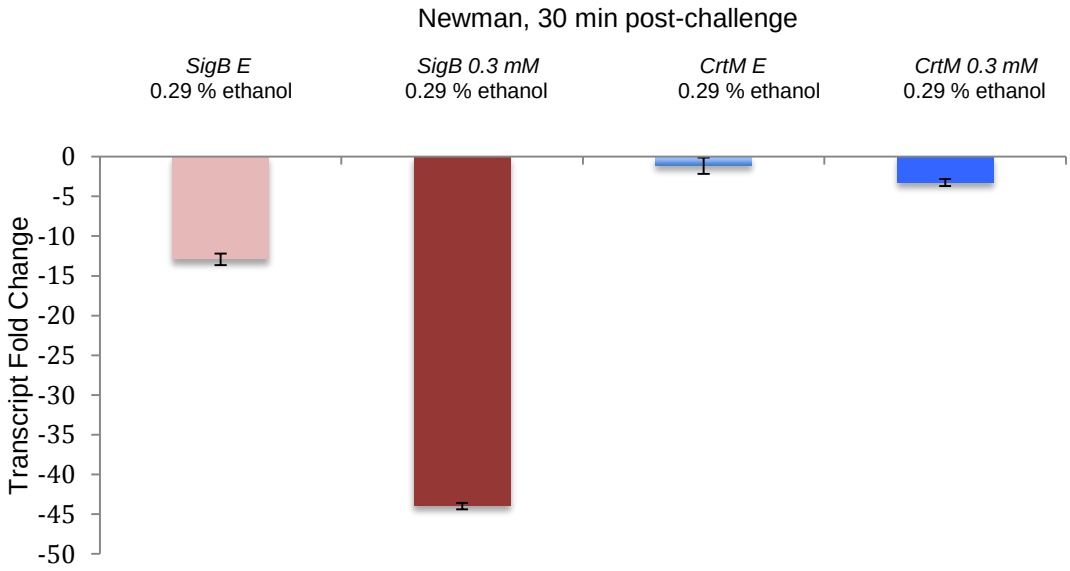

Figure 5.7. qRT-PCR to determine differential gene expression in the *S. aureus* Newman strain. Gene expression of *sigB* and *crtM* in 0.3 mM cholesterol and ethanol control (E) calibrated against expression of *16S* rRNA and *ftsZ* as reference genes 30 minutes post challenge. The ΔΔct was used to calculate transcript fold changes (*sigB* ethanol control; -12.92 (0.29 % ethanol), *sigB* 0.3 mM of cholesterol (0.29 % ethanol); -43.89, *crtM* ethanol control (0.29 % ethanol); -1.12 and *crtM* 0.3 mM of cholesterol (0.29 % ethanol); -3.25). The error bars show the standard deviation for each point of triplicates

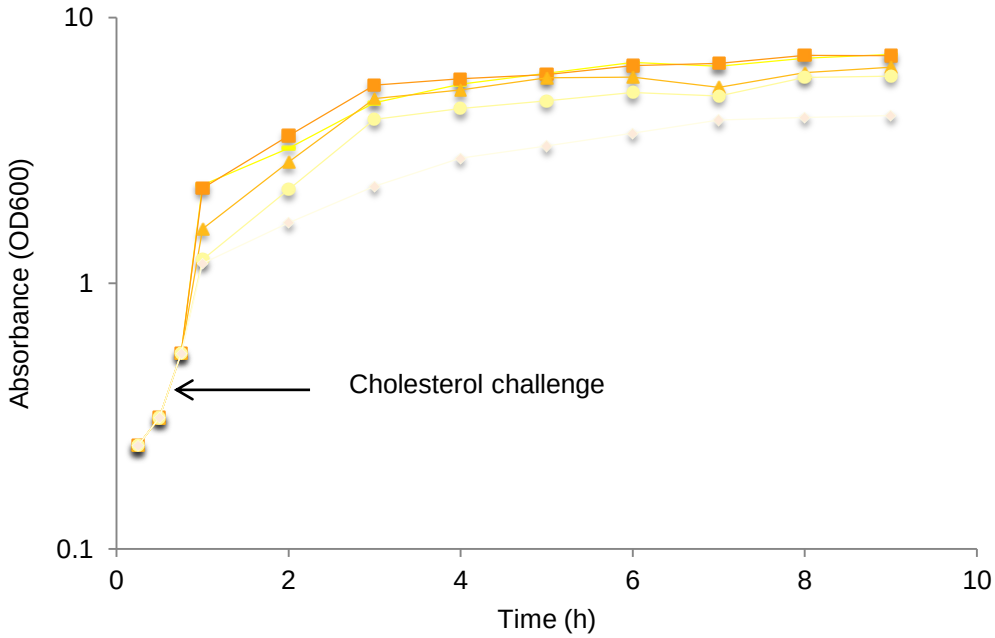

Figure 5.8. Ethanol-solubilsed cholesterol challenge of *S. aureus* SH1000. Comparison of the growth of the SH1000 strain challenged in the absence or presence of cholesterol; three varying ethanol and cholesterol conditions were used (no cholesterol: orange, square; ethanol solvent control, line: light orange (2.89 %), triangle; 0.03 mM of cholesterol: yellow, triangle (0.029 % ethanol); 0.3 mM of cholesterol: light yellow, circle (0.29 % ethanol), and 3 mM of cholesterol: beige, diamond (2.89 % ethanol) in BHI at 37 °C with shaking.

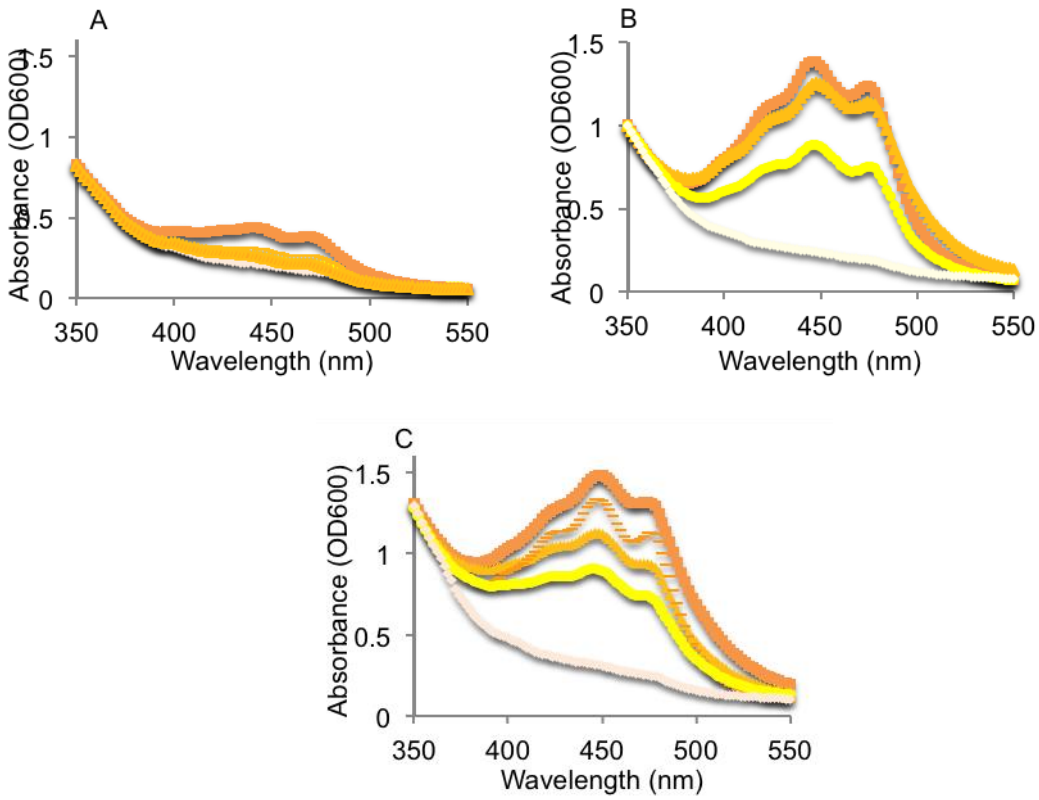

Figure 5.9. Staphyloxanthin expression in ethanol-solubilised cholesterol challenged *S. aureus* SH1000 strain. The absorbance spectrum of methanol extracted staphyloxanthin obtained from the bacterium under different growth conditions (0.3 mM, light yellow, triangle; 3 mM, beige, line; compared to cells grown in the absence of cholesterol (orange) and ethanol solvent light orange; diamond. (A) shows absorbance spectrum after 1 hour (B) after 24 hours and (C) after 48 hours.

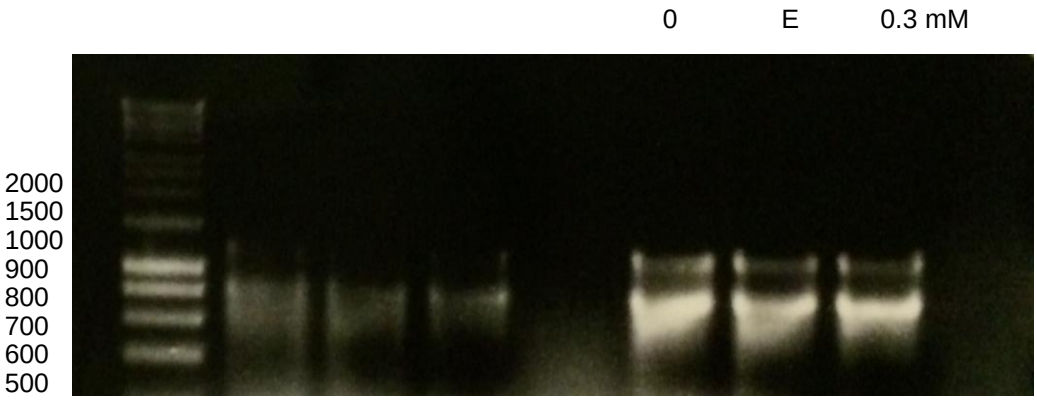

Figure 5.10. Gel electrophoresis of RNA extracted from *S. aureus* SH1000 strain. The RNA was extracted 30 minutes post-challenge for use in qRT-PCR (ethanol solvent control (E) and 0.3 mM of cholesterol). M is molecular size markers.

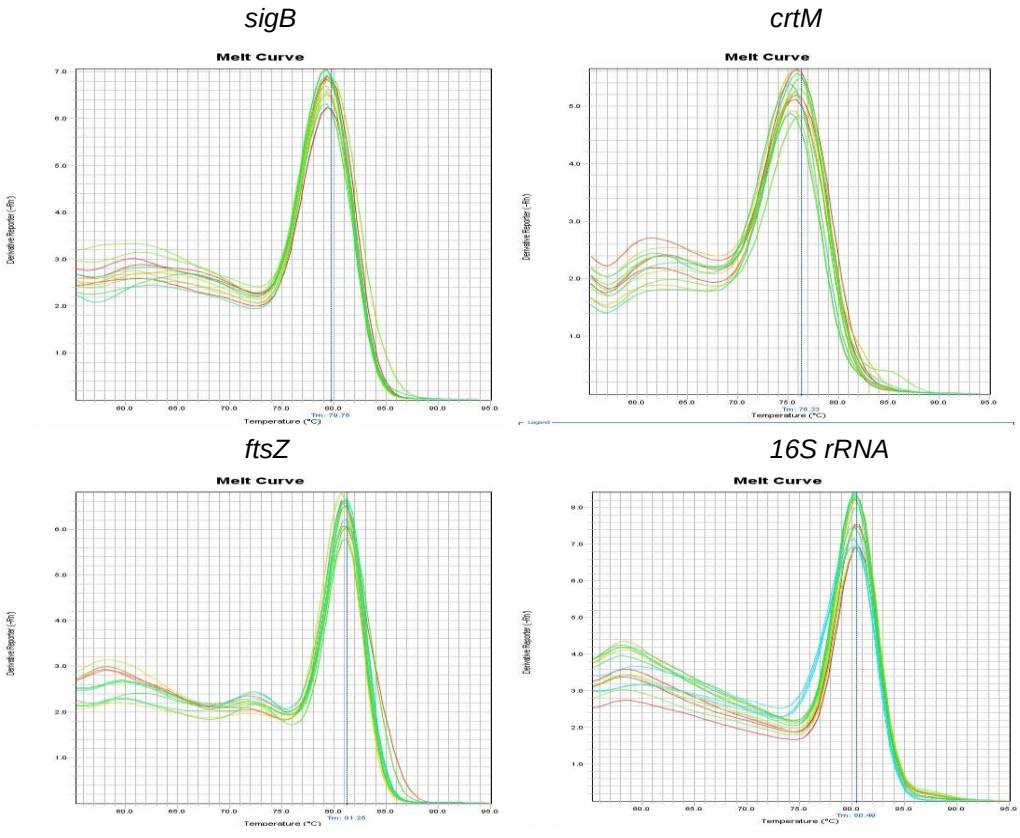

Figure 5.11. Melting curve analysis of PCR amplicons. Melt-curves were performed at the end of SYBR Green qPCRs to verify specificity. In this study, *sigB* and *crtM* analysis was conducted using *16S rRNA* and *ftsZ* as reference genes for the *SH1000* strain.

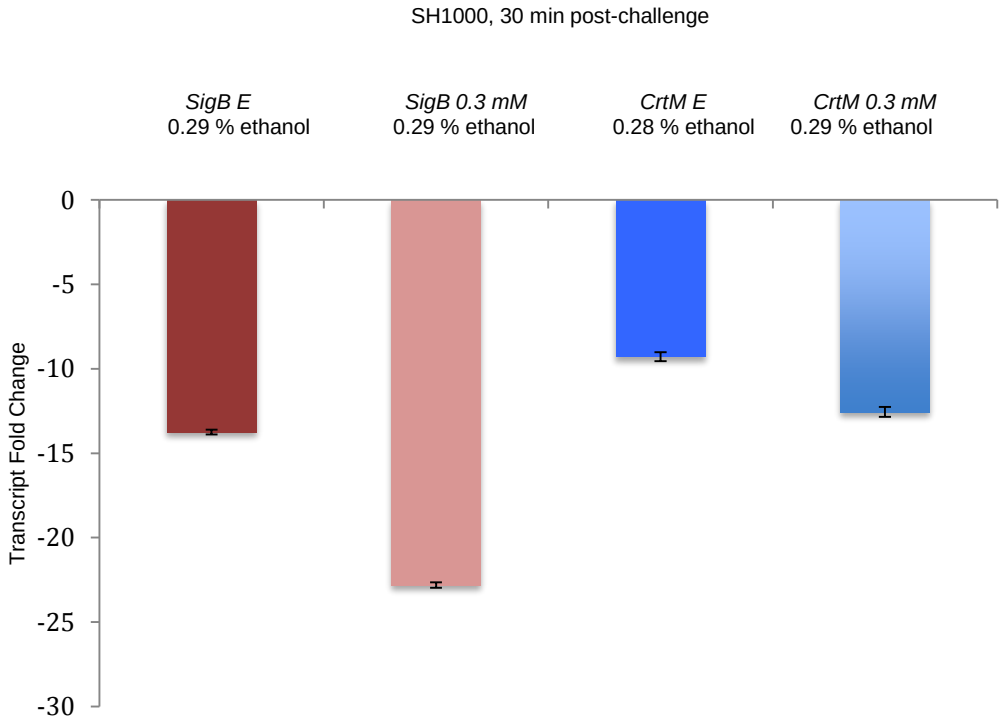

Figure 5.12. qRT-PCR used to determine differential gene expression in the *S. aureus* SH1000 strain. The expression of *sigB* and *crtM* was determined in 0.3 mM cholesterol and in ethanol control (E) using *16S* rRNA and *ftsZ* as reference genes. ΔΔct was used to calculated transcript fold changes (*sigB* ethanol control (0.29 %); -13.7, *sigB* 0.3 mM of cholesterol (0.29 % ethanol); -22.8, *crtM* ethanol control (0.29 % ethanol); -9.2 and *crtM* 0.3 mM of cholesterol; -12.99 (0.29 % ethanol). The error bars show the standard deviation for each point of triplicates.

5.3.2 investigation of σ^B on exoprotein gene expression

S. aureus expresses several extracellular proteases that contribute to virulence, such as aureolysin (Aur) and *Staphylococcus* serine protease (SspA) (Shaw et al., 2004). A reduction in σ^B activity results in higher expression of protease expression in *S. aureus* (Horsburgh *et al*., 2002). This experiment aimed to investigate the effect of ethanol-solubilised cholesterol and ethanol solvent on the transcription of extracellular protease, whereby the previously indicated qRT-PCR reduction in σ^B activity should consequently lead to increased protease gene transcription. As previously described, 0.3 mM of cholesterol and ethanol control were added to the media during culture of SH1000 and RNA was extracted (section 5.3.1). This RNA was used for qRT-PCR of both the *aur* and *sspA* genes to determine whether the protease transcription was changed (Fig 5.13).

The *16S rRNA* (Takle et al., 2007) or *ftsZ* (Duquenne et al., 2010) were used as reference genes to a produce a standard curve by utilising a dilution series of 5 concentrations of the standard (cDNA). This was used to check the slope for *16S rRNA, ftsZ, aur* and *sspA* expressed by the *S. aureus* SH1000 strain (Fig 5.15). Melt-curve analysis of the products of qRT-PCR was performed to determine whether the amplicon melting temperature (Tm) described the expected cDNA transcript (Fig 5.15). Single peaks were obtained for each sample demonstrating high specificity (Fig 5.14) and PCR products were visualised on a gel (Fig 5.16).

The *aur* and *sspA* transcript levels increased in the presence of 0.3 mM cholesterol and with ethanol solvent. This was established by calculating ΔΔct values of *aur* in 0.3 mM of cholesterol to determine increase in gene expression (7.7 fold) relative to the ΔΔct values of *aur* in the ethanol control (4.9 fold transcript increase), *sspA in* the ethanol control (4.7 fold transcript increase) and *sspA* in 0.3 mM of cholesterol (8.6 fold transcript increase) (Figure 5.16). Addition of ethanol-solubilised cholesterol resulted in the transcription of extracellular protease genes *sspA* and *aur* being increased; however, it was determined that ethanol had a major effect on the transcription of *aur* and *sspA*.

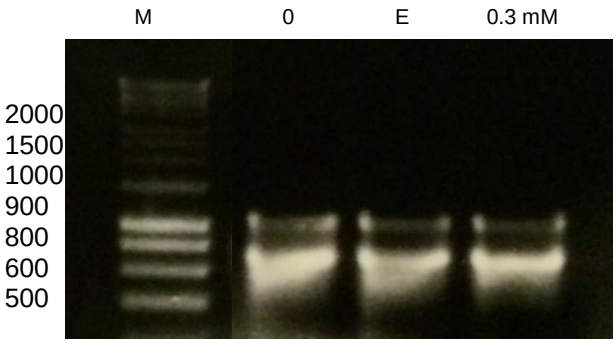

Figure 5.13. Gel electrophoresis of RNA extracted from the SH1000 strain to identify *sigB*-regulated exoprotein genes. The RNA utilised for the testing of gene expression via qRT-PCR (absence of cholesterol (0), ethanol solvent control (E) and 0.3 mM of cholesterol) was extracted from the SH1000 strain 30 minutes post cholesterol challenge. M is molecular size markers.

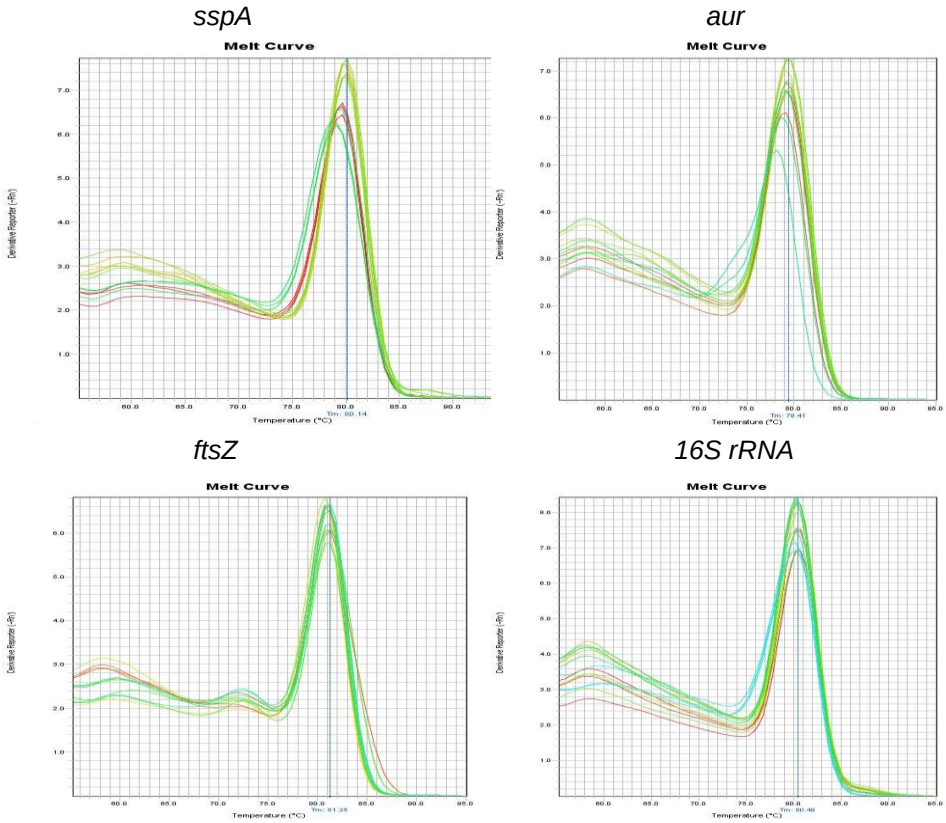

Figure 5.14. Melting curve analysis of PCR amplicons. Melt-curves were obtained at the end of SYBR Green qPCRs to verify specificity. In this study, *sspA* and *aur* analysis was conducted against the reference genes *16S rRNA* and *ftsZ* for the *SH1000* strain.

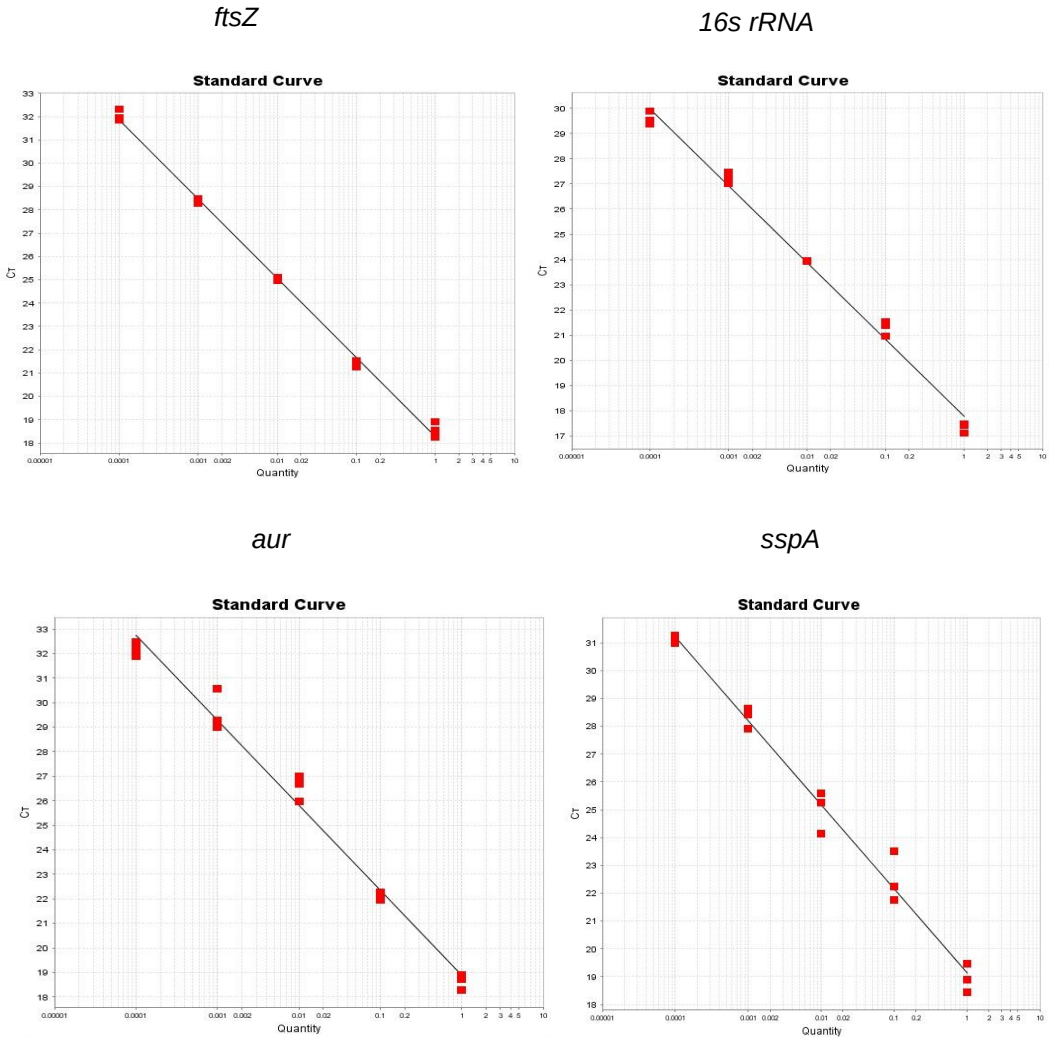

Figure 5.15. Complementary DNA (cDNA) amplification of target and control mRNAs. Fast real-time PCR with SYBR Green discovery was performed using the LightCycler and QuantiFast SYBR Green PCR Kit. The standard curves show that the QuantiFast SYBR Green PCR Kit enabled high PCR efficiencies of 97 % (for *ftsZ*), 96 % (for *16s rRNA*) and 94 % (for *aur* and *sspA*).

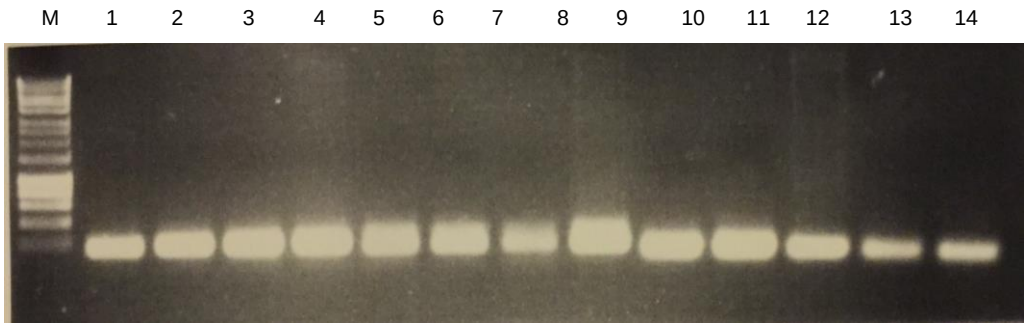

Figure 5.16. Gel electrophoresis results of cDNA PCR products. The expression of the SH1000 *S. aureus* strain for *sigB, crtM, aur* and *sspA* was tested in a variety of conditions (absence of cholesterol (0), ethanol control (E), 0.3 mM of cholesterol, DNA). M is molecular size markers. 1 (*sigB* 0), 2 (*sigB E*), 3 (*sigB* 0.3 mM), 4 (DNA *sigB)*, 5 (*crtM* 0), 6 (*crtM* E), 7 (*crtM* 0.3 mM), 8 (DNA *crtM),* 9 (*aur* 0), 10 (*aur* E), 11 (*aur* 0.3 mM), 12 (DNA *aur*), 13 (sspA 0), 14 (sspA DNA). M is molecular size markers.

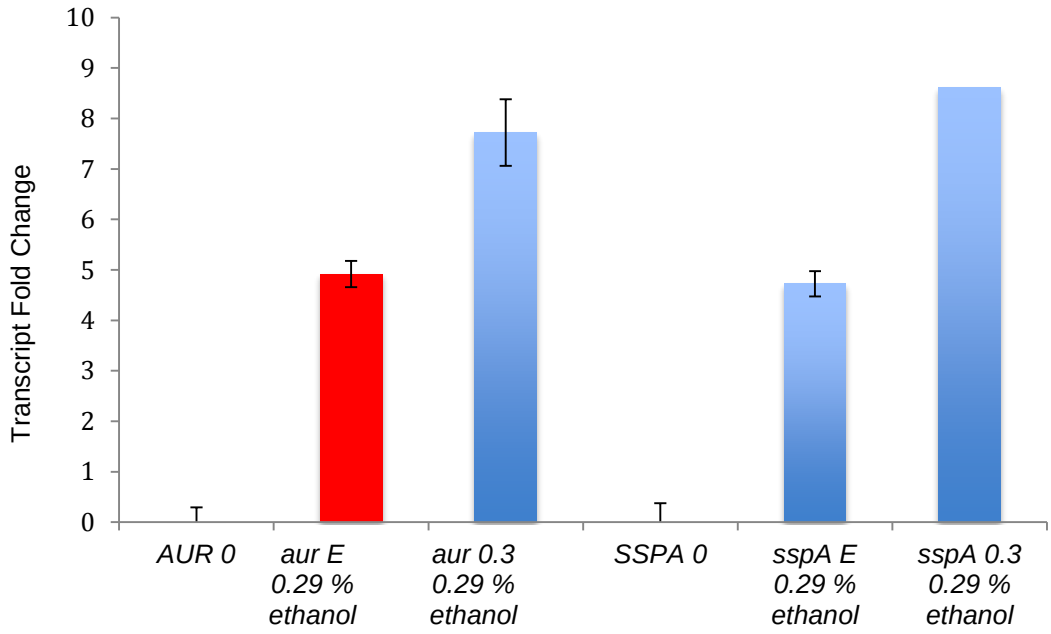

Figure 5.17. QRT-PCR to determine the differential gene expression in the *S. aureus SH1000* strain. The gene expression of *aur* and *sspA* was determined in the absence of cholesterol (0), 0.3 mM cholesterol (0.29 % ethanol) and ethanol control conditions (E, 0.29 % ethanol) using *16S* rRNA and *ftsZ* as reference genes. The ΔΔct was calculated for various conditions (*aur* ethanol control; 4.9, *aur* 0.3 mM of cholesterol; 7.7, *sspA* ethanol control; 4.7 and *sspA* 0.3 mM of cholesterol; 8.6).

5.3.3 The influence of σ^B on secreted protein expression

S. aureus depends on many extracellular factors, including secreted and surface proteins, for its virulence. These factors are accessory gene products and assist the organism in adapting to environmental conditions such as stress and the presence of specific nutritional substrates (Herbert et al., 2001).

To study the impacts of ethanol-solubilised cholesterol and ethanol on the exoprotein fraction of *S. aureus*, two strains were used; SH1000 and SH1000 *sigB*. The aim was to provide further confirmation that addition of ethanol-solubilised cholesterol increased protease expression via effects on downregulation of *sigB* transcription. Exoproteins were purified from culture supernatants. Cultures were inoculated to OD 0.05, and placed in a water bath at 37 °C with shaking, until the culture reached OD 0.6. Different concentrations of ethanol-solubilised cholesterol were added to the media (0, 0.3 mM, 3 mM) and an ethanol control was included. Samples were then taken from the culture after overnight growth following addition of cholesterol and analysed for viable count, staphyloxanthin spectra and exoprotein expression using SDS-PAGE.

Viable counts revealed there were no cell growth differences between the SH1000 and *SigB* mutant (Fig 5.18). During the experiments conducted with ethanol-solubilised cholesterol, it was confirmed that the carotenoid pigmentation reduced as the cholesterol concentration was increased. This matched the results of the experiment detailed in chapter 3 (Fig 3.16), while

showing that growth of the *sigB* mutant was equal for all concentrations of cholesterol and ethanol solvent tested (Fig 5.19).

After 1 hour post-addition the cell cultures were harvested to examine the exoprotein expressed using TCA to precipitate exoproteins from culture supernatant (Section 2.1) and the proteins were separated by SDS-PAGE (Section 2.3, Fig 5.20). Expression of the putative sspA protease band (based on size and matches to previous studies e.g. Horsburgh et al., 2002; Shaw et al., 2006) was observed to be increased in the *sigB* mutant strain at all concentrations of cholesterol as expected for the dysregulation (figure 5.20). In contrast expression in the SH1000 strain was increased only at the highest ethanol-solubilised cholesterol concentration.

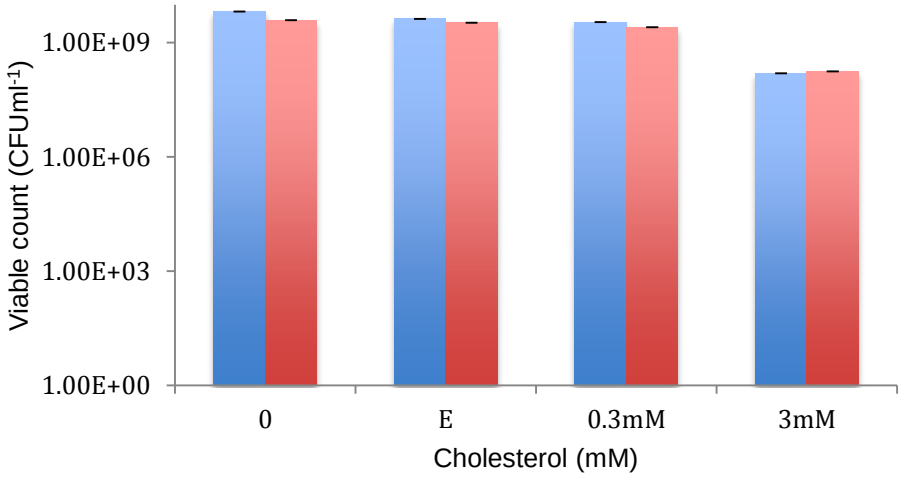

Figure 5.18. Comparison of the growth of the SH1000 (blue) and *SigB* (red) strains using viable count measurements. The samples were taken from cultures with different concentrations of cholesterol (0,ethanol control, 0.3 mM, 3 mM) in BHI at 37 °C, 1 hour of adding cholesterol. The error bars display the standard error of the mean from triplicate experiments. Exoproteins were collected for analysis by 1D SDS-PAGE at 1 hour of growth after addition of cholesterol.

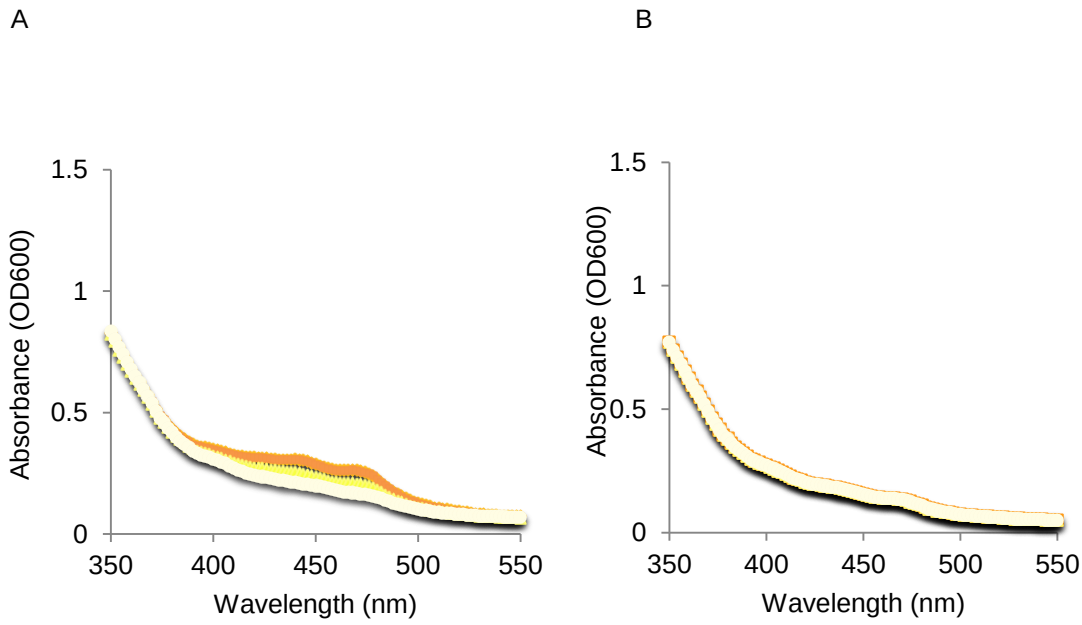

Figure 5.19. Carotenoid expressin of cholesterol treated strains. Different concentrations of cholesterol were used to treat (0, ethanol control, 0.3 mM, 3 mM) the SH1000 and *sigB* mutant strains. Differences in colour and impact of cholesterol are displayed. (B) shows *sigB* mutant strain extract absorbance spectrum after 1 hour of treatment

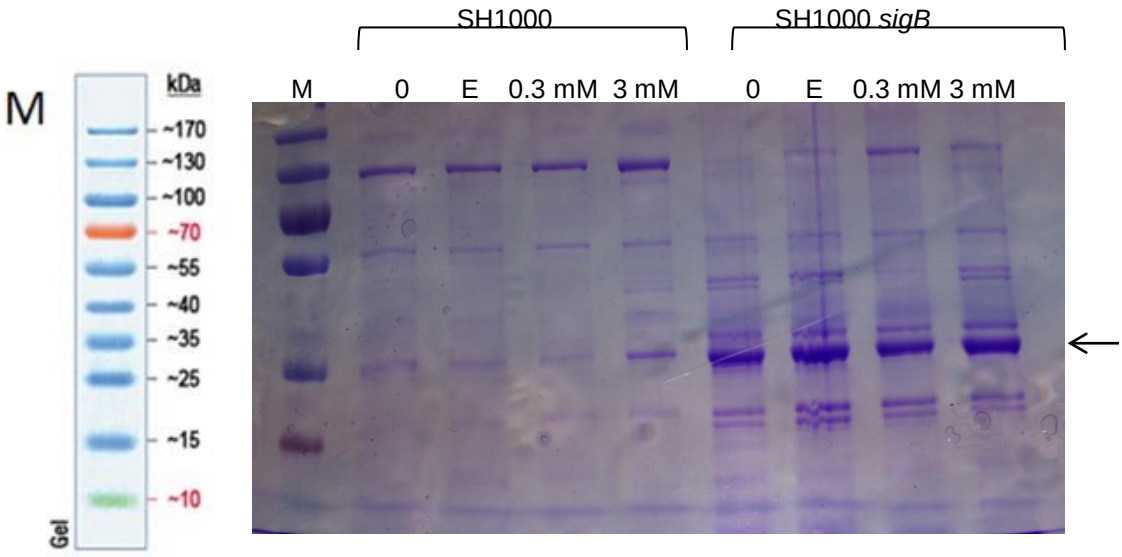

Figure 5.20. The effect of ethanol-solubilised cholesterol on secreted protein expression. The samples were collected for analysis by SDS-PAGE at 1 hour after the addition of different concentrations (0, ethanol control, 0.03 mM, 0.3 mM and 3 mM) of solubilised cholesterol. M indicates the molecular size marker. Comparison of the exoprotein profiles recovered from the cell culture supernatant of *SH1000* and *sigB* mutant at 1 hour growth after addition of cholesterol. The displays the results shown from experiments made in triplicate. The arrows illustrated the positions of protease on the protein gel. It was noted that in high concentration of cholesterol (3 mM) there was increased levels of a band expected to be SspA.

5.3 Discussion

The experiments in this chapter sought to test the effects of ethanol-solubilised cholesterol and ethanol on the growth and expression profile of *S. aureus*. The challenge conditions used for the study were designed to be able to show that the effects on staphyloxanthin expression matched the data of the experiments detailed in the previous chapters.

The data reported herein of reduced growth of *S. aureus* Newman strain (Fig 5.1) and SH1000 strain (Fig 5.8) when challenged by cholesterol, as well as reduced expression of staphyloxanthin in strain Newman (Fig 5.2) and strain SH1000 (Fig 5.9). This concordance with the continuous exposure enabled challenge conditions to be joined with earlier data to investigate the responses to cholesterol and its solvent. The reduced expression of staphyloxanthin resulted from down-regulation of *sigB* and *crtM* as noted from the qRT-PCR experiments (Fig 5.7 and 5.12). The transcription of *sigB* and *crtM* was greatly reduced when cultures were challenged with 0.3 mM of ethanol-solubilised cholesterol (Fig 5.7 with strain Newman and 5.12 with strain SH1000) and the presence of ethanol hugely reduced the expression of *sigB*; 12.9 fold reduced in strain Newman and 13.7 fold reduced in strain SH1000. QRT-PCR indicates that ethanol has a major contribution to *sigB* transcription reduction, revealing that the effect of cholesterol is very unclear.

In contrast, *aur* and *sspA* expression were increased in SH1000 strain (Fig 5.17), as an expected consequence of reduced *sigB* expression, which acts to repress the expression of these two proteases. This theory agrees with the

current knowledge or global regulation in *S. aureus*. The evidence is confusing, however, and demonstrates that while solubilised cholesterol impacts on extracellular protease expression, ethanol by itself also has a major effect; with a 13.9 fold decrease and 22.8 fold decrease in *sigB* and *crtM* respectively (Fig 5.12) In addition, the presence of ethanol by itself results in a 4.9 fold increase in *aur* and 4.7 increase in *sspA* expression, respectively (Fig 5.17). There is of course the potential for some synergy in the action of cholesterol and ethanol, resulting in the altered expression data obtained in this investigation.

Therefore, the results of the current investigation when taken together with previous evidence suggests that extracellular ethanol directly affects expression of staphyloxanthin via σ^B activity. The specific mechanism that leads to reduced σ^B activity requires further investigation. However, noting the intimate links between the global regulators of *S. aureus* expression including *sigB*, *Agr*, *sarA* and *Rot*, this may explain the results obtained. Using another solvent such as dimethyl sulfoxide (DMSO) to dissolve cholesterol might distinguish effects from cholesterol and its solvent ethanol and this need further investigation.

Many bacteria such as *B. subtilis* can be induced to the stress response by the presence of ethanol (Pane-Farre et al., 2009). Low concentrations of ethanol can affect post-stationary-phase recovery of *S. aureus* (Chatterjee et al., 2006), with scanning electron microscopy showing membrane alterations. There is evidence that many genes are upregulated following ethanol

exposure (Korem et al., 2010). These genes, including *icaAD, sdrDE, pyr*, and *ure,* are associated with biofilm production and the response to oxidative stress and increased expression of alcohol dehydrogenases. *S. aureus* appears to counteract the effect of ethanol by upregulating fatty acid metabolism. This may help to counteract the damaging effects of ethanol, on the membrane in *S. aureus* (Korem et al., 2010). This study, however, unfortunately used the *rsbU* mutant strain *S. aureus* 8325-4, which renders any interpretation of σ^B activity in response to ethanol as meaningless.

Little is known about the effect of extracellular cholesterol on *S. aureus.* However it had previously been proposed to rescue the growth reduction caused by antimicrobial fatty acids (Shine et al., 1993a). The presence of cholesterol either bound to lipoprotein or combined in lipid molecules (Craven, 1976), can induce the transcription of genes responsible for the synthesis of pigment in *S. aureus*.

S.aureus produces multiple extracellular proteases which act as virulence factors, such as serine protease (*sspA*) metalloprotease (*aur*), two cysteine proteases (*scpA* and *sspB*) (Gustafsson and Oscarsson, 2008) elastase, exotoxin A, and haemolysins (Ulusoy and Bosgelmez-Tinaz, 2013). These help pathogens adapt to the environment and survive in the host through various mechanisms, either via superior adhesion to host tissues or through an increase in its ability to invade.

The *crtM* gene encodes the enzyme dehydrosqualene synthase, which is involved in one of the early steps of staphyloxanthin biosynthesis. It is structurally similar to the human squalene synthase, which is involved in the biosynthesis of cholesterol (Liu et al., 2008). It is known that *SigB* binds the upstream promoter prior to *crtO*, and is thus required for staphyloxanthin expression (Pelz et al., 2005).

Several two-component regulatory systems (TCS) couple the stress stimulus at the cytoplasmic membrane, detected by trans membrane proteins; with the expression response in *S. aureus* via auto phosphorylating histidine kinases. These enzymes then activate phosphorylation of response regulators, which in turn regulate the expression of specific genes (Cegelski et al., 2008). Therefore external environment changes can modulate intracellular expression.

The investigation also sought to determine the effect of cholesterol and ethanol on the exoprotein fraction of *S. aureus*. The data showed reduced growth (by CFU) of the SH1000 and *SigB* mutant strains (Fig 5.18), while there was no difference between the two. This indicates that *sigB* does not impact on growth. However when comparing the carotenoid expression of the cholesterol treated strains (Fig 5.19) with each other, it was notably different as expected due to the requirement of *sigB* for staphyloxanthin expression. Further, the SH1000 strain had reduced expression of the pigment as the cholesterol concentration increased. Put together, these results indicate that ethanol and cholesterol are involved in either direct or

indirect interaction with *sigB*, as the *sigB* mutant had equal expression of pigment in all concentrations of cholesterol and ethanol solvent.

Lastly, it was noted that the expression of proteases was observed in high concentrations of cholesterol and increased expression of protease was apparent as indicated in (Fig 5.20) for the *sigB* mutant. Again this is to be expected as a functional *sigB* is linked to negative regulation of protease expression, whereas the mutant is not able to repress transcription from the *aur* and *scp* operons.

The transcription of the global regulator *sarA* is also at least partially controlled by *sigB* (Kullik et al., 1998). However an analysis of production of *sarA* in SH1000 showed that its levels remained consistent at both the transcriptional and protein level, with no difference noted for the *sigB* mutant strain. This data is in agreement with the results of previous research (Blevins et al., 1999), which determined the level of *sarA* transcription whereas other studies have demonstrated that *sarA* transcription mediated via *sigB* is dependent on the P3 promoter (Bischoff et al., 2001).

Many housekeeping genes have been utilised for the study of *S. aureus* gene expression using single internal reference genes, such as *ftsZ* (Schwan et al., 2006), *pta* (Pereira et al., 2007), *hu* (Chien et al., 1999, Fournier et al., 2001, Schmidt et al., 2003), and *gyrB* (Goerke et al., 2001a, Goerke et al., 2000, Valle et al., 2003, Wolz et al., 2002). The use of more than one reference gene results in more accurate gene expression data (Vandesompele et al., 2002) and many studies use three.

Chapter 6: General discussion and future studies

6.1 Characterisation of cholesterol rescue of *S. aureus* from Antimicrobial Fatty Acids

The aim of the investigation detailed in chapter 3 was to identify whether cholesterol could counteract the activity of antimicrobial fatty acids, which are present on host skin. The results of the study were that cholesterol decreased the growth inhibition caused by linoleic acid and this was extended to D-sphingosine, another skin lipid. This recovery was noted by increased viable cell counts, in a concentration dependent manner. In addition the Newman *crtM* mutant strain, which was more sensitive to linoleic acid, was also tested for cholesterol rescue and it was found that there was a 1,000-fold improvement in the yield of the mutant in the presence of 0.03 mM cholesterol. This strain was also challenged with D-sphingosine, and also rescued by cholesterol with a 130 fold increased rate of survival.

It was identified partway through the study that cholesterol solubilised in ethanol was being included without a comparative ethanol control. Given the literature and knowledge of σ^B expression and activity it was entirely unexpected to identify a potential solvent effect of ethanol and could not have been foreseen. The poor experimental design was quite unfortunate. It is clear, however, that in this study ethanol could be the main effector of the recovery and subsequently identified changes in pigmentation. Indeed, one critical review study by the major research group in Germany (Pane-Farre et al., 2009) with a long track record of studying σ^B identified that ethanol was

not an inducer. While this fits with the observations it does not tally with the observation of the inverse, i.e. markedly reduced σ^B expression and activity or may also contribute to the rescue of the *S. aureus.*

Subsequent independent experiments in the laboratory have identified that the cholesterol recovery of antimicrobial lipid growth reduction noted in Chapter 3 is repeatable with appropriate solvent controls (Josephine Moran, personal communication), indicating that cholesterol, not ethanol, is the mediator of this effect. There are several possible explanations for the observations of cholesterol-dependent recovery. Firstly, it may be that cholesterol in the media is directly interacting with the AFAs sequestering these away so that they do not reach the cell surface to cause an inhibition of *S. aureus* growth. Alternatively, the cholesterol enters the cell membrane and increases order (rigidity), or cholesterol could bind to the exterior of the cell to interact upon the surface with AFA. These scenarios require further experimentation and raise questions that were not answered in this study.

Several mechanisms of free fatty acid activities have been studied elsewhere (Desbois and Smith, 2010). Free fatty acids have a wide-range of activities because of their lipophilic properties, thus are able to cooperate with most organism's behaviour due to the presence of lipid bilayer structures (Coates et al., 2014) in the plasma membrane. A large number of hypothesised antimicrobial mechanisms of free fatty acids arise from their ability to be inserted into membranes. Accordingly, unsaturated long chain fatty acids cause enhanced membrane fluidity due to their shape, as presence in the plasma membrane decreases packing density. In *S. aureus,* linoleic acid

(18:2Δ9,Δ12) inhibited the concentrations of early protein leakage and inhibited the metabolic action of the electron transport chain for example (Galbraith and Miller, 1973, Greenway and Dyke, 1979). A new and interesting observation established that, although oleic acid (18:1Δ9) was a substrate for phospholipid biosynthesis, the fatty acid sapienic acid (16:1Δ9) needed elongation for its incorporation into cellular pathways (Parsons et al., 2012). According to Arsic *et al.,* in some *S. aureus* strains, fatty acids as well as other long chain unsaturated fatty acids tested, changeably produced protease expression (Arsic et al., 2012).

Previous reports have illustrated that the esters of fatty acids are unable to kill many staphylococcal species (Mortensen et al., 1992a). Alternately, when cholesterol is present *S. aureus* adjusts to changes in the structure of its cytoplasmic membrane (Shine et al., 1993b) in order to contain cholesterol, but the mechanism of cholesterol entry needs to be determined, and the membrane lipid levels tested to identify the presence of cholesterol. Although the growth of *S. aureus* is ameliorated by cholesterol in the presence of minimum levels of fatty acids, it may serve as an antimicrobial when used in excessive concentrations (Shine et al., 1993b). The current results presented emphasize that the survival of *S. aureus* is strongly ameliorated owing to the presence of cholesterol due to its protective effects against the action of linoleic acid.

When another species, *S. epidermidis,* was challenged with linoleic acid in the presence of different concentrations of cholesterol (Fig 3.5) it was observed that cholesterol increased the survival of *S. epidermidis* in a similar

manner to *S. aureus*. Further investigations confirmed the influence of cholesterol rescue on *S. aureus* across growth cultures over 48 hours in the presence of cholesterol (Fig 3.10). This may be due to cholesterol entering in the membrane as some research has assumed that cholesterol incorporates into the cytoplasmic membrane. Since cholesterol easily incorporates into phospholipid bilayers and monolayers, the presence of it in membranes may result from specific interactions with phospholipids, instead of membrane proteins (Haberland and Reynolds, 1973) or cholesterol may interact in the culture broth with the AFAs, reducing the amount reaching the cell. The c*rtM* mutant was shown to be more sensitive to linoleic acid. When cholesterol was added to the media in the presence of linoleic acid, there was clear evidence that cholesterol ameliorates the effect of the fatty acid, as noted by the growth of the *crtM* mutant being restored to that of the wild type. Also, it was noted that in the presence of cholesterol, *agr* and *sar* mutants were rescued from fatty acid challenge. It is known that the enzyme FAME esterifies lipids with cholesterol, reducing their bacterial cellular toxicity. Thus it may be that FAME could be acting when cholesterol is present, and therefore this may be the mode of cholesterol growth rescue.

It was identified that solubilised cholesterol at higher concentrations decreased pigmentation and reduced bacterial growth. While this could be because cholesterol enters the cell membrane, subsequent studies in the laboratory have identified that with appropriate controls ethanol does have a major effect upon reducing pigmentation. Cholesterol might also have an effect, but ethanol has the major effect.

There was no expectation of such an effect on pigmentation and previous reports have determined that ethanol does not have an effect on *sigB* gene expression (Conlon et al., 2002) and it is reported to not be an inducer of σ^B (Pane-Farre et al., 2009).

There are potential benefits of determining how cholesterol affects *S. aureus* given the possibility that this lipid could generate key data on effects regarding the control of membrane function and fluidity maintenance. Given the spectre of untreatable infections in the future due to antibiotic resistance there is a need for new data to provide research leads and novel targets. The membrane, together with peptidoglycan, represent major antimicrobial targets and the likely source of ne inhibitors. Continuation of the study presented here will build a more complete picture of the mechanics of *S. aureus* and its environmental flexibility and adaptability.

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