The Silver Cation (Ag^+): Antibacterial Mode of Action and Mechanisms of Resistance

Christopher Paul Randall

Submitted in accordance with the requirements for the degree of
Doctor of Philosophy

The University of Leeds
School of Molecular and Cellular Biology
Faculty of Biological Sciences
September 2013

Intellectual property and publication statements

The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Chapters 3 and 4 contain work based on jointly-authored publications. The publications are referenced below, together with a description of the contribution made by each author to the publication.

Publication one:
Randall CP, Oyama LB, Bostock JM, Chopra I & O'Neill AJ (2013) The silver cation (Ag^{+}): antistaphylococcal activity, mode of action and resistance studies. *Journal of Antimicrobial Chemotherapy* **68**: 131-138.
CPR concieved the study, designed and carried out experiments and wrote the manuscript; LBO assisted with some experiments; JMB provided intellectual input; IC and AJO assisted in concieving the study, designing experiments and in writing the manuscript.

Publication two:
Randall CP, Mariner KR, Chopra I & O'Neill AJ (2013) The target of daptomycin is absent from *Escherichia coli* and other Gram-negative pathogens. *Antimicrobial Agents and Chemotherapy* **57**: 637-639
CPR concieved the study, designed and carried out experiments and wrote the manuscript; KRM carried out preliminary experiments; IC and AJO assisted in concieving the study, designing experiments and writing the manuscript.

Acknowledgements

I am extremely grateful to both Dr Alex O'Neill and Professor Ian Chopra for giving me the opportunity to work in their respective laboratories for the past four years. Thank you also for the support, advice and guidance you have provided me during this time. I would also like to acknowledge both the MRC and Smith and Nephew plc. for providing the funding for this project. At Smith and Nephew I would like to thank Kiersten Vaughan, Emma Woodmansey, and all of the members of the wound care team for your assistance with this project, and for being so welcoming during my time spent working at York.

Thank you to all members (past and present) of the O'Neill and Chopra groups for making the last four years such a memorable and enjoyable experience, and also for providing me with the technical advice and guidance to make it through my PhD studies. In addition, I am immensely grateful to Kirsty Owen, Bjorn Mohl and John Heritage for their friendship during this time. Thank you also to Catherine Bladen, Laura Wetherill and Sally Harrison for their assistance with qPCR, liposome creation, and next-generation sequencing, respectively.

Finally, I would like to thank and dedicate this thesis to my family and to my partner Katherine. The work presented in this thesis would not have been possible without your constant and unwavering support.

Abstract

The increasing prevalence of infections attributed to antibiotic-resistant bacteria has prompted renewed interest in exploiting the antibacterial properties of Ag^+ to treat such infections. However, the antibacterial mode of action (MOA) and bactericidal activity of Ag^+ are poorly understood, and there are concerns that the prolific and unrestricted use of Ag^+ in consumer products will select bacterial Ag^+ resistance, thus limiting the clinical utility of Ag^+. Ag^+ resistance already exists, although aspects of the molecular basis of Ag^+ resistance, and the current prevalence of Ag^+-resistant pathogens are unclear. This thesis sought to address these issues.

Ag^+ was found to be bacteriostatic in culture media and bactericidal in buffer, and was unable to eradicate *Staphylococcus aureus* biofilms *in vitro*. MOA studies indicated that the primary antibacterial target of Ag^+ is the cell membrane. Evidence was obtained suggesting that Ag^+ does not interfere with the phospholipid component of the membrane, but instead probably damages integral membrane proteins to produce an antibacterial effect.

A survey of hospital staphylococcal isolates (*n*=1006) found universal susceptibility to Ag^+, and Ag^+ resistance could not be selected in *S. aureus* and several other pathogens *in vitro*. However, in *Escherichia coli*, high-level Ag^+ resistance arose rapidly and was not associated with a fitness cost likely to prevent its emergence in the clinical setting. Ag^+-resistant strains contained mutations in genes regulating expression of an Ag^+ efflux mechanism and outer membrane porins. A detailed characterisation of a known Ag^+-resistance determinant (the *sil* operon), was also conducted to provide further insights into the mechanism of Ag^+ resistance conferred by this determinant.

Collectively, these studies provide further insights into the MOA of Ag^+ and the mechanisms of Ag^+ resistance, which could potentially be applied to optimising the future uses of Ag^+ as an antibacterial agent.

Table of contents

List of Tables

List of Figures

Abbreviations

ABC	Adenosine triphosphate binding cassette
Ag^+	Silver cation
$AgNO_3$	Silver nitrate
Ala	Alanine
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
B.C.E	Before Common Era
bMIC	Biofilm minimum inhibitory concentration
BSA	Bovine serum albumin
BSAC	British society for antimicrobial chemotherapy
CA	Catalytic/ATP-binding domain
Ca^{2+}	Calcium cation
CCCP	Carbonyl cyanide *m*-chlorophenyl hydrazone
cDNA	Complementary DNA
CF	Carboxyfluorescein
CFU	Colony forming units
CL	Cardiolipin
Cl^-	Chloride anion
CLSI	Clinical laboratory standards institute
CMR	Comprehensive microbial resource
CoNS	Coagulase-negative staphylococci
CTAB	Cetyltrimethylammonium bromide
Cu^+/Cu^{2+}	Copper cation
DHp	Dimerization and histidine phosphotransfer domain
$DiSC_3(5)$	3,3'-dipropylthiadicarbocyanine iodide
DMSO	Dimethyl sulphoxide
DMT	Drug/metabolite transporter
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
EDTA	Ethylenediaminetetra acetic acid
EMRSA	Epidemic meticillin resistant *Staphylococcus aureus*
EPS	Extracellular polymeric substances
FNT	Formate/nitrate transporter
ESBL	Extended-spectrum β-lactamase
GFP	Green fluorescent protein
GNAT	Gcn5-related N-acetyltransferase
H_2O	Water
HAMP	Present in histidine kinases, adenylate cyclases, methyl accepting proteins and phosphatases
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HPF	Hydroxyphenyl fluorescein
HTH	Helix-turn-helix
HTS	High-throughput screening
I-TASSER	Iterative threading assembly refinement
IC_{50}	Half maximal inhibitory concentration
IS	Isosensitest
JCVI	J. Craig Venter Institute
KPO_4	Potassium phosphate
LBA	Luria-Bertani agar
LBB	Luria-Bertani broth
LPS	Lipopolysaccharide

Lsa	Lincosamide/streptogramin A
MBC	Minimum bactericidal concentration
MBEC	Minimum biofilm eradication concentration
MFS	Majory facilitator superfamily
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimum inhibitory concentration
MOA	Mode of action
MRSA	Meticillin-resistant *Staphylococcus aureus*
MSSA	Meticillin-susceptible *Staphylococcus aureus*
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
NADH	Reduced nicotinamide adenine dinucleotide
NaH_2PO_4	Sodium hydrogen phosphate
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NCTC	National collection of type cultures
NDM-1	New-Delhi metallo β-lactamase
NHS	National Health Service
O_2	Molecular oxygen
O_2^-	Superoxide
OD	Optical density
$OH^·$	Hydroxyl
ORF	Open reading frame
PA	L-α-phosphatidic acid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	1,2-dioleoyl-*sn*-phosphatidylglycerol
PIA	Polysaccharide intercellular adhesin
PTS	Phosphotransferase system
qPCR	Quantitative polymerase chain reaction
RAST	Rapid annotation and subsystem technology
RBS	Ribosome binding site
RCT	Randomised controlled trial
RNA	Ribonucleic acid
RNAP	RNA polymerase
RND	Resistance, nodulation, division
ROS	Reactive oxygen species
RPE	lissamine rhodamine B-labelled L-α-phosphatidylethanolamine
SAMMD	*Staphylococcus aureus* microarray meta-database
SBDD	Structure-based drug design
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
TCA	Trichloroacetic acid
Vga	Virginiamycin a
VISA	Vancomycin-intermediate *Staphylococcus aureus*

Chapter one: Introduction

1.1. The use of antibacterial chemotherapy in the treatment of infectious disease

1.1.1. Infectious disease in the pre-antibiotic era

The debilitating and deadly impact of bacterial infection on human health has been noted from the start of written history. Accounts from ancient Greece and Egypt describe diseases that are indicative of leprosy, tuberculosis and diphtheria (Watts, 1999). The causative agent of bubonic plague, *Yersinia pestis*, is believed to have caused the death of approximately 90% of the population of Europe during the 12th Century, to have killed a further 40 million people worldwide in the 14th Century, and remained the cause of high-mortality epidemics until the early 19th Century (Rosen, 1993; Nelson *et al.*, 2007). Other diseases attributed to infectious processes were also prevalent and made a substantial contribution to human morbidity and mortality at the time; these included syphilis, leprosy, cholera, typhoid fever and dysentery (caused by *Treponema pallidum, Mycobacterium leprae, Vibrio cholerae, Salmonella* Typhi and *Shigella* spp./*Entamoeba histolytica*, respectively) (Rosen, 1993; Watts, 1999; Nelson *et al.*, 2007). Syphilis was considered endemic across Europe during the 18th and 19th Century and is proposed to have been responsible for the deaths of numerous famous philosophers, painters and musicians at the time, including Nietzsche, Manet and Mozart (Franzen, 2008). Prior to the 20th Century, any condition that required medical or surgical intervention carried the risk of severe infection, for example puerperal fever following childbirth, or streptococcal, staphylococcal, and clostridial infections in surgical wounds (Finch *et al.*, 2010).

Following the introduction of experimental evidence supporting the germ-theory of disease by Pasteur, and the work of individuals such as Semmelweis, Snow, Jenner and

Lister in preventing infectious disease through the use of improved sanitation, education, vaccines and antiseptics, fewer people acquired the infections that had been so prevalent in the past (Mandell *et al.*, 2009). This led to the percentage of deaths attributed to infectious disease decreasing from an estimated 75% in the 17th Century to ~40% by the end of the 19th Century (Omran, 1971). Nevertheless, although transmission of infectious disease could be prevented to an extent, there was still little that could be done to cure individuals of infection once it had been acquired. Indeed, in the early 20th Century childhood diseases such as diphtheria, pneumonia and rheumatic fever were still associated with death rates approaching 40% (Armstrong *et al.*, 1999). In the case of systemic *Staphylococcus aureus* infections, mortality rates were >80% prior to the 1940's (Fuda *et al.*, 2005).

1.1.2. A brief history of antibacterial chemotherapy

From the late 19th Century onwards, antiseptics used for the sterilisation of surgical equipment (*e.g.* carbolic acid, boric acid, hydrogen peroxide and perchlorite solutions) were also used in an attempt to sterilize infected wounds (Fleming, 1919). Although reducing bacterial burden at the site of the wound, this approach could not be used to treat systemic infection owing to the toxicity of the compounds used (Fleming, 1919, Manring *et al.*, 2009). The concept of using a compound as a systemic agent in patients that could kill bacteria without causing significant damage to the host (*i.e.* the "magic bullet") is attributed to Erlich, whose research group subsequently discovered that the synthetic compound arsphenamine could be used systemically for the treatment of syphilis (Schwartz, 2004, Lloyd *et al.*, 2005). This was followed with the introduction of the first sulphonamide, Prontosil (sulphanilamide) in 1932, which was less toxic than arsphenamine and could treat infections caused by a wider range of

bacteria, such as streptococci and *Neisseria* species (Mandell *et al.*, 2009; Finch *et al.*, 2010).

In addition to the synthetic compounds described above, a number of organisms were found to produce antibacterial compounds as secondary metabolites (*i.e.* compounds not essential for basic metabolic function of an organism) that had the potential to be employed in the treatment of infectious disease (Duckett, 1999). An early example of this is the development of penicillin. Although published studies in the late 19th and early 20th Century had commented on the antibacterial properties of the genus *Penicillium* (Tyndall, 1876; Duckett, 1999), it was not until the early 1940's that the potential medical application of this discovery began to be truly exploited (Drews, 2000). Named penicillin by Fleming (1929), the secondary metabolite of *Penicillium chrysogenum* was successfully extracted and purified by Florey and colleagues, and could effectively treat systemic infection caused by a range of microorganisms including *T. pallidum*, *S. aureus* and *Streptococcus* spp. with relatively few side-effects (Drews, 2000).

The realisation that compounds such as arsphenamine, penicillin and sulphanilamide could cure infections that were previously fatal prompted efforts to identify further compounds displaying selective antibacterial activity. This ultimately led to the discovery of a range of antibacterial compounds between the 1940's and 1970's, of which a number were subsequently introduced into clinical practice for the treatment of infectious disease (Table 1.1). These compounds consist of secondary metabolites (also known as 'natural products', for example streptomycin and vancomycin), semi-synthetic derivatives of natural products (*e.g.* rifampicin and flucloxacillin), or entirely synthetic compounds (*e.g.* ciprofloxacin and linezolid), and are collectively referred to as 'antibiotics' (although this term was originally used to refer solely to

natural products with antibacterial activity, its definition has since been extended) (Fischbach & Walsh, 2009).

The introduction of antibiotics into clinical practice revolutionised healthcare. For example, following the introduction of the sulphonamides, mortality attributed to puerperal fever decreased from ~4 deaths per 1000 births to <1 death per 1000 births by the 1970's (Finch *et al.*, 2010). In the USA, mortality attributed to systemic staphylococcal infection is presently ~20%, having decreased from >80% in the pre-antibiotic era (Fuda *et al.*, 2005; van Hal *et al.*, 2012). Furthermore, the introduction of antibiotics is believed to have contributed to an increase in the average life expectancy of approximately 30 years in developed countries; either as a direct consequence of treating infection, or indirectly in their use as a prophylactic to reduce the risk of infection from invasive surgical procedures (Walsh, 2003).

1.1.3. Mode of action (MOA) of antibiotics

Antibiotics are compounds that, at a low concentration, selectively inhibit (bacteriostatic) or kill (bactericidal) bacteria, by interfering with the function of one or more macromolecules within the target organism (Walsh, 2003). The selective toxicity of antibiotics against bacteria is usually a consequence of the fact that their macromolecular 'targets' are either not found in eukaryotes (*e.g.* enzymes involved in peptidoglycan synthesis), are of a sufficiently different structure to an equivalent eukaryotic homologue (*e.g.* the ribosome), or are protected by a component of the eukaryotic cell (*e.g.* the mitochondrial membrane(s) acting as a barrier to prevent ribosomal inhibitors from reaching the mitochondrial ribosome) (Walsh, 2003). This property allows for the use of antibiotics in treating infectious disease without (in most cases) harming the patient. Antibiotics in clinical use typically disrupt processes

involved in DNA replication, transcription, translation and peptidoglycan synthesis; alternatively, compounds such as the polymyxins and daptomycin elicit an antibacterial effect by damaging the bacterial cell membrane(s) (Finch *et al.*, 2010). A list of the antibacterial targets for each antibiotic class in clinical use can be found in Table 1.1.

Table 1.1. Antibiotic classes in clinical use (Adapted from Walsh, 2003; Silver, 2011, Mandell *et al*., 2009; Conly & Johnston, 2005)

Class	Year of first reported discovery/patent	Year of first regulatory approval/introduction to clinical practice	Antibacterial target
β-lactams	1929[1]	1941	Transpeptidases
Sulphonamides	1932	1935	Dihydropterate synthase
Aminoglycosides	1943	1946	30S ribosomal subunit
Bacitracin[2]	1945	1948	Undecaprenyl pyrophosphate
Nitrofurans	1946	1952	Multiple targets[3]
Phenicols	1947	1949	50S ribosomal subunit
Polymyxins	1947	1959	Cell membranes
Tetracyclines	1945	1948	30S ribosomal subunit
Pleuromutilins	1950	2007	50S ribosomal subunit
Macrolides	1949	1952	50S ribosomal subunit
Isoniazid[2]	1952	1952	Multiple targets[3]
Glycopeptides	1953	1955	D-ala-D-ala terminus of peptidoglycan precursors
Streptogramins	1953	1955	50S ribosomal subunit
D-cycloserine[2]	1955	1956	Alanine racemase and D-ala-D-ala ligase
Aminocoumarins	1951	1960	DNA gyrase
Rifamycins	1957	1959	RNA polymerase
Nitroimidazoles	1959	1959	Multiple targets[3]
Fusidic acid[2]	1961	1962	Elongation factor G
Lincosamides	1955	1961	50S ribosomal subunit
Trimethoprim[2]	1961	1965	Dihydrofolate reductase
Quinolones	1961	1962	DNA gyrase/topoisomerase
Fosfomycin[2]	1969	1971	MurA/MurZ
Mupirocin[2]	1971	1985	Isoleucyl-tRNA synthetase
Oxazolidinones	1978	2000	50S ribosomal subunit
Lipopeptides	1987	2003	Cytoplasmic membrane
Fidaxomicin[2]	1975	2011	RNA polymerase

[1]Date of publication noting the antibacterial activity of filtrates from *P. chrysogenum* cultures (Fleming, 1929). [2]Names correspond to compounds, as they are the only members of their class. [3]Nitrofurans, nitroimidazoles and isoniazid are all metabolised by the host organism into reactive intermediates that inhibit multiple cellular macromolecules.

1.1.4. Antibiotic resistance

The continued and widespread use of antibiotics in the treatment of infectious disease provides the selection pressure necessary for the emergence of antibiotic resistance; a phenotype that enables a bacterium to survive and proliferate in the presence of an antibiotic at concentrations that are inhibitory against other bacterial populations. Infections caused by antibiotic-resistant bacteria cannot be treated effectively with antibiotics that the causative bacteria are resistant to (Walsh, 2003; Hawkey, 2008).

Bacterial resistance to antibiotics can arise by endogenous (mutational) or exogenous (horizontally-acquired) mechanisms (Finch *et al.*, 2010), with endogenous resistance resulting from the selection of bacterial strains harbouring mutations in genes or non-coding elements of DNA already present in the cell (Mayers *et al.*, 2009). These mutations typically lead to resistance by altering the target of the antibacterial such that the binding affinity is reduced (*e.g. rpoB* and rifampicin resistance), by increasing expression of the antibacterial target (*e.g. alrA* and D-cycloserine resistance), or increasing expression of antibacterial detoxification mechanisms, such as efflux pumps (*e.g. norA* and ciprofloxacin resistance) (Nikaido, 2009; Wright, 2011). In contrast, exogenous resistance arises when the host bacterium acquires foreign DNA via horizontal gene transfer (*i.e.* conjugation, transformation, or transduction) that encodes an antibiotic resistance determinant. These determinants can encode efflux pumps (*e.g. tet*(A) and tetracycline resistance), antibiotic-inactivating enzymes (*e.g. cat* and chloramphenicol resistance), and mechanisms to modify the target (*e.g. ermC* and erythromycin resistance), or produce a functional homologue of the antibiotic target that is resistant to antibiotic inhibition (*e.g. mupA* and mupirocin resistance) (Chopra & Roberts, 2001; Wright, 2011). For every antibiotic class in clinical use, bacteria have

evolved one or more mechanisms to resist their action; these mechanisms are summarised in Table 1.2.

Table 1.2. Mechanisms of bacterial resistance to antibiotic classes in clinical use

Class	Example(s) of mechanisms conferring resistance	Reference
Aminocoumarins	Point mutation(s) in gene (*gyrA*) encoding target, reducing antibiotic binding affinity; MexAB-/AcrAB-mediated efflux	(Confreres & Maxwell, 1992; Nikaido, 2009)
Aminoglycosides	MexAB-/AcrAD-mediated efflux; enzymatic inactivation of antibiotic by adenylation, acetylation or phosphorylation	(Mayers *et al.*, 2009, Nikaido, 2009)
Bacitracin	Increased production of target; BcrABC-mediated efflux (putative)	(Manson *et al.*, 2004)
D-cycloserine	Overexpression of gene encoding target (alanine racemase)	(Caceres *et al.*, 1997)
Fidaxomicin	Point mutation(s) in genes (*rpoB, rpoC*) encoding target, reducing antibiotic binding affinity[1]	(Babakhani *et al.*, 2004)
Fosfomycin	Point mutation(s) in gene (*murA/Z*) encoding target; enzymatic inactivation by glyoxylase-family enzymes	(Rigsby *et al.*, 2005; Takahata *et al.*, 2010)
Fusidic acid	Point mutation(s) in gene (*fusA*) encoding target, reducing fusidic acid binding affinity; acquisition of genes (*fusB, fusC*) encoding target protection mechanisms	(Besier *et al.*, 2003; Cox *et al.*, 2012)
Glycopeptides	Modification of target (lipid II) to reduce affinity of glycopeptide binding (mediated by VanHAX)	(Bugg *et al.*, 1991)
Isoniazid	Point mutation(s) in gene encoding catalase (*katG*), resulting in reduced conversion of isoniazid into reactive intermediates	(Heym *et al.*, 1995)
Lincosamides	Lsa-mediated resistance (unknown mechanism); enzymatic modification (methylation) of target (23S rRNA) to reduce antibiotic binding affinity	(Reynolds *et al.*, 2003; Mayers *et al.*, 2009)
Lipopeptides	Modification of target (cell membrane) via point mutation(s) in *mprF*, *yycG* and *rpoB/C*, to reduce binding affinity of daptomycin	(Friedman *et al.*, 2006)
Macrolides	MexAB-/AcrAB-mediated efflux; enzymatic modification (methylation) of target (23S rRNA) to reduce antibiotic binding affinity	(Mayers *et al.*, 2009; Nikaido, 2009)
Mupirocin	Point mutation(s) in gene (*ileS*) encoding target, reducing mupirocin-binding affinity; acquisition of isoleucyl-tRNA synthetase (MupA, MupB) resistant to inhibition	(Hurdle *et al.*, 2004; Hurdle *et al.*, 2005)

Table 1.2. (Continued)

Nitrofurans	Point mutation(s) in genes encoding nitroreductases, preventing conversion of nitrofurans into reactive intermediates	(Sandegren *et al.*, 2008)
Nitroimidazoles	Enzymatic inactivation of antibiotic by *nim*-encoded proteins; point mutation(s) in gene (*rdxA*) encoding a nitroreductase, presumably preventing conversion of nitroimidazoles into reactive intermediates	(Dhand & Snydman, 2009)
Oxazolidinones	Enzymatic modification (methylation) of target (23S rRNA) to reduce antibiotic binding affinity	(Mayers *et al.*, 2009)
Phenicols	MexAB-/AcrAB-mediated efflux; enzymatic inactivation of antibiotic by acetylation	(Mayers *et al.*, 2009; Nikaido, 2009)
Pleuromutilins	Enzymatic modification (methylation) of target (23S rRNA) to reduce antibiotic binding affinity	(Long *et al.*, 2006)
Polymyxins	Enzymatic modification of target (Lipid A) to reduce polymyxin binding affinity	(Falagas *et al.*, 2010)
Quinolones	NorA/AcrAB-mediated efflux; point mutation(s) in genes (*gyrA*, *parC*) encoding targets, reducing antibiotic binding affinity	(Kaatz *et al.*, 1993; Hooper, 1999)
Rifamycins	Point mutation(s) in gene (*rpoB*) encoding target, reducing rifamycin binding affinity	(O'Neill *et al.*, 2001)
Streptogramins	Vga/Lsa-mediated resistance (unknown mechanism); enzymatic modification of target (23S rRNA) to reduce antibiotic binding affinity	(Reynolds *et al.*, 2003; Mayers *et al.*, 2009)
Sulphonamides	MexAB-mediated efflux; point mutation(s) in gene encoding dihydropterate synthase; acquisition and production of dihydropterate synthase resistant to inhibition	(Sköld, 2000, Nikaido, 2009)
Tetracyclines	Efflux (mediated by Tet(A)-(L)); protection of target (mediated by Tet(M), (O), (S) or (W)); Tet(X)-mediated inactivation of antibiotic	(Chopra & Roberts, 2001)
Trimethoprim	Point mutation(s) in gene encoding target (dihydrofolate reductase), reducing trimethoprim binding affinity; acquisition and production of dihydrofolate reductase resistant to inhibition; MexAB-/AcrAB-mediated efflux	(Huovinen *et al.*, 1995; Nikaido, 2009)
β-lactams	Inactivation by β-lactamases; production of penicillin-binding protein with reduced affinity for β-lactam binding (*e.g.* MecA)	(Davies & Davies, 2010)

[1]Mechanism of resistance identified from resistant isolates selected under laboratory conditions. Clinically-relevant fidaxomicin resistance has been identified in *Clostridium difficile*, but the mechanism of resistance is unknown (Babakhani *et al.*, 2004).

1.1.5. History and impact of antibiotic resistance

Bacterial resistance to penicillin was encountered soon after its clinical introduction, with the first report of staphylococcal penicillin resistance in 1942 (Fuda *et al.*, 2005). This soon became widespread, with >50% of all *S. aureus* isolates resistant to penicillin by the 1950's (compared with 6% in the early 1940's) (Fuda *et al.*, 2005). A similar trend was observed following the introduction of other antibiotic classes, such as the tetracyclines and the macrolides (Finch *et al.*, 2010). However, due to the rapid discovery and introduction of new antibiotics between 1940 and 1970 (termed the 'golden age' of antibiotic discovery) (Silver, 2011), and the structural modification of existing antibiotic classes to overcome resistance mechanisms (*e.g.* the penicillinase-stable β-lactams oxacillin and meticillin) (Fischbach & Walsh, 2009), it was believed by many in the 1960's to 1970's that antibiotic resistance would not be a barrier to the continued success of antibiotics in treating infectious disease (Fauci, 2001; Spellberg, 2008) . However, due to a number of reasons (described later in this chapter), the rate of introducing new antibiotics into clinical practice diminished, whilst the prevalence of antibiotic-resistant bacteria has continued to rise (Fischbach & Walsh, 2009).

In the present day, antibiotic resistance has been described as representing a 'catastrophic threat' to the prevention and treatment of infectious disease (Davies, 2013). For example, *S. aureus* resistant to the penicillinase-stable β-lactams (termed meticillin-resistant *S. aureus* [MRSA]) comprise up to 60% of all total *S. aureus* isolated in the USA, which has increased from ~25% in 1995 (Boucher & Corey, 2008). Infections caused by MRSA are estimated to kill between 18,000-25,000 people per year in the USA and Europe, and are associated with higher rates of mortality compared

with those caused by meticillin-susceptible *S. aureus* (MSSA) (Whitby *et al.*, 2001, Cosgrove *et al.*, 2003). This has in part been attributed to the need to use antibiotics such as linezolid, vancomycin and daptomycin, which are inferior to β-lactams (the treatment of choice for MSSA infections) in treating staphylococcal infection (Whitby *et al.*, 2001; Stryjewski *et al.*, 2007). Furthermore, strains resistant to one or more of these antibiotics have been isolated from patients, suggesting that their future utility may be limited.

Amongst Gram-negative bacteria, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Acinetobacter baumannii* and *Enterobacter* spp. are routinely responsible for hospital-acquired urinary tract infections (UTIs), surgical site infections (SSIs), bloodstream infections (BSIs) and pneumonia (Weinstein *et al.*, 2005). Infections attributed to these bacteria are typically treated with β-lactams (*e.g.* extended-spectrum cephalosporins, monobactams and carbapenems), aminoglycosides or fluoroquinolones; however the prevalence of isolates resistant to one or more of these classes is increasing (Chopra *et al.*, 2008). For example, in the UK the number of Enterobacteriaceae reported to be carbapenem-resistant has increased from <20 in 2003 to ~550 in 2011 (Davies, 2013). Resistance to β-lactams, aminoglycosides and fluoroquinolnes are typically the consequence of the expression/overexpression of efflux pumps with a broad substrate range, such as those of the resistance-nodulation-division (RND) superfamily (*e.g.* MexAB in *P. aeurginosa*, AdeAB in *A. baumannii*) (Nikaido, 2009), and (in the case of β-lactams) the acquisition of β-lactamases with activity against 3rd-generation cephalosporins and carbapenems (*e.g.* AmpC, IMP, VIM and NDM-1) (Thomson & Bonomo, 2005). Alarmingly, strains of *A. baumanni*, *P. aeruginosa* and *K. pneumoniae* have been isolated from patients that are resistant to all clinically-used antibiotic classes, rendering any chemotherapeutic

intervention ineffective against infections caused by such strains (Falagas & Bliziotis, 2007).

Given the lack of new compounds active against multi-drug resistant (MDR) and pan-drug resistant (PDR) bacteria, it has been suggested that we are entering a 'post-antibiotic era' wherein mortality rates associated with infectious disease and surgical procedures with a high risk of infection are likely to return to the levels seen prior to the introduction of penicillin (Alanis, 2005; Falagas & Bliziotis, 2007; Davies 2013). It is therefore apparent that new antibiotics, or alternative approaches to the treatment and prevention of infection, are urgently needed to prevent this scenario from occurring (Fischbach & Walsh, 2009; Silver, 2011).

1.1.6. The antibiotic discovery 'void' and the need for alternative strategies to treat infectious disease

Between 2000 and 2011, only four new antibiotic classes were introduced into clinical practice (the oxazolidinones, lipopeptides, pleuromutilins and the macrocycle, fidaxomicin), which is in stark contrast to the myriad of compounds introduced between the 1940's and 1960's (Fischbach & Walsh, 2009). In addition, only two of these classes (oxazolidinones and lipopeptides) are approved for the systemic treatment of bacterial infection, and none of these classes is effective in treating infection caused by Gram-negative bacteria (Silver, 2011). The lack of new classes has been described as an antibiotic discovery 'void' and is attributed to two main factors (Silver, 2011). The first is the failure of modern antibiotic discovery methods to identify potential antibiotic compounds. Methods such as high-throughput screening (HTS) of chemical libraries and structure-based drug design (SBDD) focus on identifying compounds with *in vitro* activity against a single enzyme target. However, the resultant 'hits' frequently have no

antibacterial activity *in vivo* (as they cannot pass through the bacterial membrane), or display 'off-target' antibacterial activity, which typically correlates with the compound being toxic to a patient if used systemically (Silver, 2011). This is exemplified by a publication in 2006, which revealed that GlaxoSmithKline identified only 5 compounds with the potential to be developed as an antibiotic, following HTS of 260,000-530,000 compounds against 70 antibacterial targets (Payne *et al.*, 2006). Further HTS campaigns by 34 different pharmaceutical companies failed to yield any potential antibiotic candidates (Chan *et al.*, 2004). In addition, the estimated time to identify, develop and gain regulatory approval for the clinical use of an antibiotic is approximately 12-14 years, at a cost of >$800 million (Conly & Johnston, 2005). As antibiotics are only prescribed for short periods (when compared with medications for chronic conditions), and have a limited therapeutic lifespan (due to the inevitable emergence of resistance), the revenue obtained from sales of antibiotics is relatively low (Payne *et al.*, 2006; Fischbach & Walsh, 2009). As a consequence of the above, a number of the large pharmaceutical companies are withdrawing from the field of antibiotic discovery and development, thus further reducing the rate at which new antibiotics are likely to be discovered (Silver, 2011).

Alternative strategies for the treatment of infectious disease have been proposed in an attempt to mitigate the decline in antibiotic discovery. These include the use of 'phage-based systems to deliver toxic compounds (or genes encoding their production) into pathogenic bacteria (Fairhead, 2009); anti-virulence compounds to prevent bacterial colonisation at a potential site of infection, or prevent a pathogen from expressing other virulence factors (Rasko & Sperandio, 2010); and the use of antibiotic 'adjuvants', compounds that disable or disrupt intrinsic antibiotic resistance mechanisms of Gram-negative bacteria (*e.g.* efflux pumps and the outer membrane), allowing for

previously ineffective antibiotics (*i.e.* Gram-positive-specific antibiotics such as vancomycin and telavancin) to reach their antibacterial target and display clinically-relevant levels of activity (Kalan & Wright, 2011; Hornsey *et al.*, 2012). However, each of these strategies have the same barrier to their clinical introduction, in that regulatory approval for their use is first required, which is typically an expensive and long (~6-8 year) process (Payne *et al.*, 2006).

Another strategy without this disadvantage is to reevaluate the use of underexploited antibacterial agents; such agents have received regulatory approval, but are rarely used or have been neglected due to limitations in the range of their potential applications (*e.g.* a topical antibacterial agent opposed to one that could be used systemically), or due to their relatively high toxicity when compared with current antibiotics (Falagas *et al.*, 2008; Fabbretti *et al.*, 2011). One example is colistin (polymyxin E), which was originally approved for clinical use in the 1950's, however as a consequence of its reported nephrotoxicity, its use was abandoned in the 1970's (Falagas *et al.*, 2008). Due to the increasing prevalence of MDR Gram-negative bacteria, the use of colistin has increased as a 'last resort' compound for treating infections caused by these organisms. Another example is the use of compounds containing silver. As described later in this chapter, silver has been used for centuries as an antibacterial agent and, although its use decreased with the introduction of antibiotics, the increased prevalence of antibiotic-resistant bacteria has led to a resurgence in its use. The remainder of this chapter will discuss the history of antibacterial silver, its current and potential applications in the treatment of infectious disease, and then describe the antibacterial MOA of silver and the potential for bacteria to resist its antibacterial effect.

1.2. Silver as an antibacterial agent

1.2.1. History of silver

Silver (Ag) is an element with the atomic number 42 and a relative atomic mass of 107.8682 g/mol (Silver, 2003). The electron configuration of silver classifies it as a transition metal element (McNaught & Wilkinson, 1997), and it can also be described as a 'heavy' metal (as it has a specific gravity >5) (Duxbury, 1986) or, when it is in its ionised state (Ag^+), a soft (Lewis) acid (Gadd, 1992). The antibacterial properties of silver have been exploited for millennia; there are reports suggesting that silver containers were used by the ancient Greeks, Romans and Egyptians to prevent the spoilage of food and water when stored or transported (Alexander, 2009). Furthermore, a Roman pharmacopeia, believed to have been published in 69 B.C.E., noted the use of silver nitrate ($AgNO_3$) presumably as a treatment for medical conditions (Klasen, 2000a).

Between the start of the Common Era and the 18th Century, silver was used to treat a number of medical conditions, including those that are now known to be a consequence of infectious disease (Klasen, 2000a; Weber & Rutala, 2001; Alexander, 2009). For example, $AgNO_3$ was applied to wounds in the 14th Century in a likely attempt to prevent the onset of infection (Klasen, 2000a), and in the 15th Century 'maladies of the brain', including epilepsy, were thought to be curable by ingesting a silver coin (Grier, 1968; Alexander, 2009). The use of hardened $AgNO_3$ (referred to as *lapis infernalis*) was recommended as an alternative to using a scalpel for opening abscesses in an 18th Century surgical text book (Klasen, 2000a), and in the same Century, $AgNO_3$ was used for the treatment of venereal diseases such as condylomata (genital warts; now known to be caused by human papilloma virus, or as a symptom of secondary syphilis)

and chancre, an eroding ulcer that is typically attributed to primary syphilis (Klasen, 2000a).

Following the proposal of the germ theory of infectious disease in the 19th Century, it was soon discovered that silver compounds possessed antibacterial activity against organisms such as *S. aureus* and *Bacillus anthracis* (Schneider, 1984). In 1880, a 2% (w/v) solution of $AgNO_3$ was found to prevent ophthalmia neonatorum, a gonococcal or chlamydial infection of the conjunctiva in new-borns that frequently leads to blindness. The use of $AgNO_3$ in this manner caused a noticeable decrease in the prevalence of this infection, from 13.6 to 0.5% of all new-born children in Germany (Alexander, 2009). Further success at employing silver as prophylaxis against infection was noted throughout the remainder of the 18th-, 19th- and the early 20th Century (Schneider, 1984; Klasen, 2000a); for example, silver compounds and silver-containing dressings were noted to prevent infection in burns and other wounds, and silver-containing sutures were routinely used for the repair of hernias and fistulas. Silver was also used to directly treat infection at this time; for example silver suspensions were used to treat streptococcal sepsis and various infections of the eye (Roe, 1915, Sanderson-Wells, 1918). A derivative of Salvarsan (arsphenamine) was created using silver (silver Salvarsan), which was found to be more soluble than the original compound, and less toxic when used systemically for the treatment of syphilis (Vecki & Ottinger, 1921).

Following the introduction of antibiotics into clinical practice, the use of silver as an antibacterial agent declined (Klasen, 2000b). However, in the 1960's $AgNO_3$ was recommended as a cheap and effective alternative to antibiotics in the prevention of infection in burns (Moyer *et al.*, 1965). Following a burn, the body attempts to maintain homeostasis by promoting coagulation at the site of injury and hyperaemia in the

surrounding, undamaged skin (Edwards-Jones & Greenwood, 2003). This action produces an immunosuppressed, moist environment that promotes colonisation and subsequent infection by bacteria such as *S. aureus*, *Streptococcus pyogenes* and *P. aeruginosa*, which can rapidly lead to further tissue destruction, sepsis and death. At a concentration of 0.5% (w/v), $AgNO_3$ was found to prevent growth of the above organisms whilst having no impact on the successful grafting of donor skin (Moyer *et al.*, 1965). A retrospective study indicated that using $AgNO_3$ in the treatment of burns covering >40% of the body resulted in a 20% reduction in mortality and a reduced length of hospitalisation (from ~180 days to ~70) when compared with using other burn wound treatments (Ziffren, 1968). However, there were disadvantages in using $AgNO_3$ in this manner. For example, to remain effective at preventing infection, $AgNO_3$ had to be reapplied to the wound up to 12 times a day, in a process that was found to be painful for the patient (Klasen, 2000b). In attempts to overcome these shortcomings, silver was combined with the sulphonamide sulphadiazine to produce silver sulphadiazine (AgSu) (Stanford *et al.*, 1969; Klasen, 2000b). Used at a concentration of 1% (w/v) in a hydrophilic cream, AgSu prevented wound colonisation with one application per day in an animal model, and caused no pain to patients upon its application (Stanford *et al.*, 1969). Indeed, AgSu was found to be superior to both $AgNO_3$ and gentamicin in preventing burn wound infection in a retrospective study of 454 patients (Hummel *et al.*, 1970), and AgSu-treated burn wounds were less likely to be infected compared with wounds treated with mafenide acetate; a sulphonamide derivative frequently used in the prevention of wound infection (Pegg *et al.*, 1979).

1.2.2. Current applications of antibacterial silver

Today, $AgNO_3$ is still used as prophylactic agent in some countries to prevent ophthalmia neonatorum (Mullick *et al.*, 2005), and AgSu is considered to be the

treatment of choice to prevent burn wound infection, with one recent review describing it as “the gold standard in topical burn treatment” (Atiyeh *et al.*, 2007). Nevertheless, AgSu still has limitations; for example, reapplication of AgSu may first require removal of a pseuedoeschar that can form when using AgSu, which is known to be painful to the patient (Dunn & Edwards-Jones, 2004) and, as AgSu requires daily reapplication, pseudoeschar removal can be time consuming for medical staff. Another potential issue with AgSu is that its use may prolong wound healing, as it delays epithelialisation in three separate animal burn models (Muller *et al.*, 2003; Cho Lee *et al.*, 2005; Maghsoudi *et al.*, 2011). These issues have led to the development of dressings that are impregnated with various formulations of silver, including AgSu, silver phosphate and silver nanoparticles (metallic silver contained in clusters <100 nm in diameter) (Atiyeh *et al.*, 2007). Examples of commercially available silver dressings are presented in Table 1.3. These dressings are all purported to release concentrations of silver over a prolonged period (between 3-7 days, depending on the dressing), and therefore do not require removal and reapplication as frequently as previous silver formulations. In addition, silver dressings are claimed to be easier to apply and remove, whilst causing only minimal pain to the patient (Lansdown, 2006). Given the increasing prevalence of antibiotic-resistant bacteria, the use of silver-containing medical products has expanded from the prevention of burn wound infection to treatment of a variety of heavily colonised and infected wounds, including acute and chronic wounds, decubitus (pressure) ulcers, or ulcers arising as a consequence of diabetic neuropathy and peripheral vascular disease (Lansdown, 2006; Edwards-Jones, 2009).

Although there is an array of studies demonstrating the antibacterial activity of silver-containing dressings *in vitro* (reviewed by Mooney *et al.* [2006]), the efficacy of these dressings *in vivo* are disputed. For example, one randomised controlled trial

(RCT) between AgSu and a dressing containing silver nanoparticles (Acticoat) for the treatment of burn wounds revealed that wounds treated with Acticoat were less likely to become infected and healed faster compared with those treated with AgSu (Gravante *et al.*, 2009). A meta-analysis of 4 RCTs evaluating healing of infected venous ulcers exposed to another silver dressing (Contreet) or dressings without an antibacterial additive indicated that ulcers treated with Contreet healed quicker (Leaper *et al.*, 2013). In contrast, a separate RCT found no significant difference between an additive-free dressing and Aquacel, Acticoat, Contreet and Urgotul silver-containing dressings in healing of venous ulcers (Michaels *et al.*, 2009), whereas two Cochrane meta-analyses noted that there was insufficient evidence to recommend silver dressings in the treatment of diabetic foot ulcers, and that there was no appreciable difference between silver dressings and additive-free dressings in resolution of infection in chronic or acute wounds (Bergin & Wraight, 2006; Vermeulen *et al.*, 2007). Despite the contention regarding the utility of silver-containing dressings, their use in the prevention and treatment of wound infection is obvious and widespread (Silver *et al.*, 2006; Atiyeh *et al.*, 2007; Edwards-Jones, 2009; NHS National Prescribing Centre, 2010).

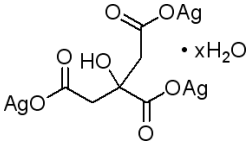
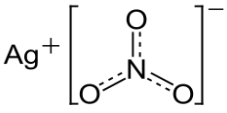
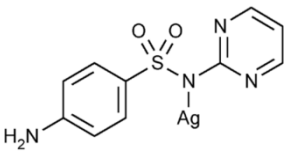
Table 1.3. Examples of commercially available silver-containing dressings

Dressing	Composition	Manufacturer	Reference
Acticoat	Three layers of polyethylene gauze coated with nanocrystalline silver (silver nanoparticles <20nm diameter)	Smith and Nephew	(Silver *et al.*, 2006)
Actisorb	Nylon dressing containing activated charcoal bound to silver	Johnson and Johnson	(Silver *et al.*, 2006)
Aquacel Ag	Silver impregnated within a carboxymethylcellulose fibre dressing	Convatec	(Silver *et al.*, 2006)
Arglaes	Polymer film containing silver phosphate	Medline	(Mooney *et al.*, 2006)
Calgitrol Ag	Calcium alginate dressing containing silver	Magnus Bio-medical	(Silver *et al.*, 2006)
Contreet (Biatain Ag)	Hydrocolloid dressing containing silver	Coloplast	(Ip *et al.*, 2006)
PolyMem Silver	Polyurethane membrane with a starch copolymer containing silver	Ferris	(Ip *et al.*, 2006)
SilvaSorb	Silver halides suspended within a polyacrylate dressing	Acrymed	(Castellano *et al.*, 2007)
Silvercel	Carboxymethylcellulose fibre dressing blended with a metallic silver-coated nylon fibre	Johnson and Johnson	(Castellano *et al.*, 2007)
Silverlon	Polymeric fabric coated with metallic silver	Argentum Medical	(Mooney *et al.*, 2006)
Urgotul SSD	Hydrocolloid dressing impregnated with Vaseline and silver sulphadiazine	Urgo	(Ip *et al.*, 2006)

In addition to the treatment of wounds, silver is frequently incorporated into implanted medical devices such as central venous catheters, urinary catheters and endotracheal tubes, with the intention to prevent bacterial colonisation of the device and subsequent infection in the patient (Weber & Rutala, 2001; Silver *et al.*, 2006). In contrast to silver

dressings, a number of RCTs and subsequent meta-analyses have confirmed the utility of such devices in preventing bacterial colonisation and infection when compared with medical devices coated with other antibacterial agents, or additive-free devices (Saint *et al.*, 1998; Veenstra *et al.*, 1999; Li *et al.*, 2012). Outside of the clinical setting, an increasing number of products are available that also contain various formulations of silver (examples of such formulations are presented in Table 1.4) to exploit its antibacterial effect. Examples of such products include clothing, water purifiers, deodorants, paint, kitchen appliances and children's toys (Silver, 2003; Luoma, 2008).

Table 1.4. Examples of antibacterial silver formulations

Name	Composition	Active species	Uses	Reference
Silver chloride	AgCl, $AgCl_2^-$, $AgCl_3^{2-}$ (depending on concentration in solution)	Ag^+	Antibacterial additive in certain deodorants	(Edwards-Jones, 2009)
Silver citrate		Ag^+	Antibacterial additive in certain deodorants	(Edwards-Jones, 2009)
Silver nanoparticles	Metallic silver contained in clusters <100 nm diameter	Ag^+ (possibly Ag^0)	Prevention and treatment of burns and chronic wound infections. Incorporated into a variety of consumer products	(Rai *et al.*, 2009)
Silver nitrate		Ag^+	Prevention and treatment of burns, chronic wound infections and opthalmia neonatorum	(Klasen, 2000b)
Silver sulphadiazine		Ag^+	Prevention and treatment of burns and chronic wound infections	(Klasen, 2000b)
Silver zeolite	Silver ions contained in an immunosillicate matrix	Ag^+, reactive oxygen species	Incorporated into plastics and water filters	(Matsumura *et al.*, 2003)

Exploitation of the antibacterial properties of silver has increased dramatically over the past decade. In 2004, the NHS spent £858,000 on silver dressings (Chambers *et al.*, 2007); this increased to ~£25 million in 2010 (NHS National Prescribing Centre, 2010). Furthermore, the number of consumer products containing antibacterial silver increased from ~30 in 2006 to ~300 by 2011 (Project on Emerging Nanotechnologies, http://www.nanotechproject.org/inventories/consumer/analysis_draft/, last accessed 17th September 2013). The number of products containing antibacterial silver is expected to increase further over the next few years, with one publication suggesting that by 2015 ~90 tonnes of silver per year will be required for their manufacture, compared with 15 tonnes per year in 2010 (GFMS, 2011). However, there are concerns that the increasing and unrestricted use of antibacterial silver will lead to the emergence of bacterial silver resistance, thus impacting the utility of silver-containing products in the prevention and treatment of infectious disease (Chopra, 2007). This will be discussed later in this chapter.

1.2.3. Antibacterial activity of silver

1.2.3.1. Inhibitory activity of silver

The silver cation (Ag^+) is responsible for the antibacterial activity of silver-containing compounds (Grier, 1968; Clement & Jarrett, 1994; Nies, 1999). Ag^+ has been shown to inhibit the growth of a range of Gram-positive and Gram-negative bacteria, including human pathogens such as *S. aureus*, *Streptococcus* spp., *E. coli*, *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*, as well as antibiotic-resistant populations of these species (Ip *et al.*, 2006a; Ip *et al.*, 2006b). However, although Ag^+ can inhibit the growth of these organisms, the minimum concentration of Ag^+ required to achieve this (termed the minimum inhibitory concentration [MIC]) is unclear (Chopra, 2007).

For example, depending upon the study, the MIC of silver compounds against strains of *S. aureus* is 8, 32, 80, or 128 μg/ml (Carr *et al.*, 1973; Maple *et al.*, 1992; Hamilton-Miller *et al.*, 1993; Ug & Ceylan, 2003). A similar variation is seen for Gram-negative bacteria (Carr *et al.*, 1973; Vasishta *et al.*, 1989; Hamilton-Miller *et al.*, 1993; Ip *et al.*, 2006b). These disparities in MIC data are likely to result from a number of factors. The first is that there is no apparent standardisation in MIC methodology between studies; for example the inoculum density of bacterial culture and the incubation temperature/time differ between studies, all variables known to affect the MIC of antibiotics (Andrews, 2001; Wayne, 2012). In addition, studies also vary in the choice of bacteriological culture media when determining Ag^+ MICs. Both the chloride anion (Cl^-) and the thiol-containing amino acid cysteine are usually present in culture media and can bind with a strong affinity to Ag^+, producing a product that has no antibacterial activity (Liau *et al.*, 1997; Gupta *et al.*, 1998). The relative concentrations of Cl^- and thiol-containing compounds vary between different culture media formulations (Atlas, 2010), thus the amount of active Ag^+ (and therefore the MIC against any one bacterial strain) will also likely differ depending on the media it is used in. A further issue is that published studies utilise different silver formulations (*e.g.* $AgNO_3$, AgSu, silver nanoparticles) for MIC determinations. The results from such studies cannot be directly compared, as the relative amounts of Ag^+ released into solution by each of these formulations (and therefore the antibacterial activity) varies (Atiyeh *et al.*, 2007). This lack of standardisation (either in MIC methodology, or choice of silver formulation) between studies is an important reason why there are no estimates into the current prevalence of bacterial silver resistance amongst clinical isolates; this will be discussed later in this chapter.

1.2.3.2. Bactericidal activity of silver

The *in vitro* definition of a bactericidal antibacterial agent is a compound that can reduce the number of viable cells in a bacterial population (of a density $\geq 5x10^5$ cfu/ml) by ≥99.9% (*i.e.* a 3-$\log_{10}$ reduction) following 24 h exposure (Pankey & Sabath, 2004; Wayne, 2012). In addition, some studies suggest that the concentration of antibacterial agent that produces a ≥99.9% reduction in viability needs to be ≤4 times the MIC (Wayne, 2012).

When using bacteriostatic agents to manage infection (*e.g.* tetracyclines, sulphonamides and fusidic acid) treatment success is reliant upon clearance of growth-inhibited bacteria by the host immune system, as upon cessastion of therapy, bacterial growth (and any infectious process) would resume (Peterson & Shanholtzer, 1992). In comparison, bactericidal agents (*e.g.* β-lactams, aminoglycosides and fluoroquinolones) are less reliant upon contribution from the host immune system and therefore prove advantageous in treating infections where the efficacy of the immune system is compromised (such as in cases of bacterial endocarditis or meningitis) (Pankey & Sabath, 2004). Nevertheless, there are situations in which the use of bacteriostatic agents is favoured. For example, in the treatment of haemolytic uremic syndrome a bactericidal agent that induces cell lysis (*e.g.* β-lactams) would promote the release of additional endotoxin as the target bacterium died, thus potentially causing further harm to the patient (Dundas *et al.*, 2005). Because of the different properties afforded by bacteriostatic and bactericidal agents, it is vital to assess the cidal properties of all antibacterial compounds so that their advantages can be effectively exploited whilst mitigating any disadvantages.

It is unclear if Ag^+ is a bactericidal compound when using the above definition. Whilst some studies suggest that the activity of Ag^+ is cidal (*i.e.* $\geq$3-$\log_{10}$ drop after 24 h) (Matsumura *et al.*, 2003), others indicate that Ag^+ is bacteriostatic (Gordon *et al.*, 2010). It is likely that this difference is once again a consequence of methodological variations between studies (*e.g.* inoculum density, choice of media), although this has yet to be confirmed.

1.2.4. Toxicity of silver

Heavy metals such as mercury, cadmium, arsenic and lead are toxic to humans at relatively low concentrations (Ibrahim *et al.*, 2006); however, silver appears to be well tolerated when used topically, with adverse reactions typically limited to skin irritation (Weber & Rutala, 2001). In addition, there have been no reports of toxicity in patients with implanted medical devices containing silver (*e.g.* catheters, endotracheal tubes) that are currently in clinical use (Veenstra *et al.*, 1999). However, when repeatedly ingested or used systemically (for example the use of silver Salvarsan), silver can become deposited in a range of tissues, including the skin, liver, kidneys, spleen, lungs and the brain (Lansdown, 2007). Deposition of silver in the skin and mucous membranes can lead to a visible blue-grey discolouration of these tissues, which is termed argyria, a condition that is not known to be life threatening and typically resolves upon cessation of silver therapy (Lansdown, 2007). Deposition of silver into the other tissues described above does not appear to affect their function, except in the case of silver deposition the brain and nervous system, which has been associated with the development of neurological conditions such as peripheral neuropathy, seizures and myoclonic status epilepticus (Vik *et al.*, 1985; Ohbo *et al.*, 1996; Mirsattari *et al.*, 2004). Nevertheless, given its current medical applications, the toxicity associated with

silver does not currently appear to be a barrier to its continued use as an antibacterial agent (Lansdown, 2007).

1.2.5. Antibacterial MOA of Ag^+

As alluded to earlier, Ag^+ has a strong binding affinity for a number of nucleophiles, including those containing sulphur (*e.g.* S^{2-}, S^-, SCN^-, $S_2O_3^{2-}$), or halides (*e.g.* Cl^-, I^- and Br^-) (Grier, 1968). As sulphur-containing compounds (*e.g.* cysteine) are prevalent in biological systems, it seems likely that the affinity of Ag^+ to these compounds is related to the antibacterial activity of Ag^+. Indeed, Nies *et al.* (1999) demonstrated that the inhibitory activity of metal cations against bacteria is directly proportional to their binding affinity to sulphur-containing nucleophiles (as determined by the dissociation constant K_d). Nevertheless, the mechanism by which Ag^+ exerts an antibacterial effect is not currently understood. Several targets which Ag^+ is known to interact with in some manner (and therefore could potentially contribute to the MOA of Ag^+) have however been identified and are discussed below.

1.2.5.1. DNA

Initial studies into the antibacterial MOA of Ag^+ discovered that DNA from cells treated with $AgNO_3$ or AgSu had a higher sedimentation rate than untreated cells, signifying that silver compounds bind to DNA in some manner (Rosenkranz & Rosenkranz, 1972). It was revealed later that Ag^+ could inhibit the incorporation of radiolabelled thymidine into DNA of *P. aeruginosa*, suggesting that Ag^+ inhibited DNA replication (Rosenkranz & Carr, 1972). Given that the bases within DNA contain nitrogen (a known binding partner of Ag^+), Fox (1969) proposed that Ag^+ binds to nitrogen atoms involved in the hydrogen bonding of bases between opposite DNA strands (*i.e.* N1 of adenine and guanine, N3 of cytosine and thymine), thus altering the Watson-Crick structure of DNA

sufficiently to prevent its replication. Using Fourier transform infrared spectroscopy, it was later confirmed that Ag^+ can form complexes with DNA (and RNA) *in vitro*; however, unlike Fox's hypothesis, Ag^+ preferentially bound N7 of guanine and adenine (Arakawa *et al.*, 2001). This binding appeared to affect the ultrastructure of DNA *in vivo*; as using transmission electron microscopy and X-ray microanalysis, DNA of *S. aureus* and *E. coli* exposed to Ag^+ was found to be in a condensed form (Feng *et al.*, 2000). The authors proposed that in this state, DNA could not replicate. However, as this study only visualised cells after a protracted (4 h) exposure to Ag^+, it is unclear if this effect is secondary to other forms of cell damage mediated by Ag^+.

1.2.5.2. Protein

It has long been known that silver binds and forms complexes with amino acids; for example, silver is used to stain proteins in polyacrylamide gels (Clement & Jarrett, 1994). In addition to binding to thiols present on cysteine residues, Ag^+ can form co-ordination complexes with residues harbouring sulphur or nitrogen-containing side chains (*e.g.* arginine, lysine, histidine and methionine) (Jover *et al.*, 2008). It is likely that the binding of Ag^+ to these residues would disrupt the tertiary structure of some proteins, or inhibit the catalytic function of certain enzymes. Indeed, *in vitro* studies have identified enzymes that are inhibited by Ag^+, including succinate dehydrogenase, aconitase, NADH dehydrogenase, NADH quinone reductase and phosphoribosyl 1-pyrophosphatase (Martin, 1963; Bragg & Rainnie, 1974; Semeykina & Skulachev, 1990; Gordon *et al.*, 2010; Xu & Imlay, 2012). However, the ability of Ag^+ to inhibit these enzymes *in vitro* does not necessarily imply this will occur *in vivo*, or that this property contributes to the antibacterial MOA of Ag^+. Nevertheless, it is known that Ag^+ can inhibit dehydratase-family enzymes of *E. coli in vivo* (Xu & Imlay, 2012), thus these enzymes may represent an antibacterial target of Ag^+.

Dehydratase-family enzymes contain iron-sulphur clusters (4Fe-S) used for catalysis, and Ag^+ inhibits this function by binding the sulphur in these clusters and releasing Fe^{2+} into solution (Xu & Imlay, 2012). This release of Fe^{2+} was proposed to be a potential mechanism by which Ag^+ causes cell death, as Fe^{2+} can generate toxic reactive oxygen species (ROS) via the Fenton reaction (Xu & Imlay, 2012). ROS as a mechanism of Ag^+-induced cell death will be discussed in section 1.2.5.4.

1.2.5.3. Cell membrane

Exposure of *P. aeruginosa* to radiolabelled Ag^+ (in the form of ^{110}AgSu) for 1 h indicated that 80% of total Ag^+ bound to the membrane (with the remaining 19 and 1% binding the cell wall and DNA/RNA, respectively) (Rosenkranz & Carr, 1972), and subsequent studies using TEM revealed that AgSu exposure caused the cytoplasmic membrane to detach from the cell wall in *S. aureus*, and led to distortion of *P. aeurginosa* cell membranes (Coward *et al.*, 1973a; Coward *et al.*, 1973b). However the relevance of these results with respect to the antibacterial MOA of Ag^+ is unclear. Nevertheless, further studies have shown that Ag^+ can cause rapid ($\leq$ 10 min) dissipation of the proton motive force in *E. coli* and *Vibrio cholerae*, which is generated at the membrane by the export of protons from oxidative phosphorylation (Dibrov *et al.*, 2002; Holt & Bard, 2005). This result would therefore imply that the structure and/or the function of the membrane is somehow perturbed by Ag^+. Evidence to support this has been provided by Lok *et al.* (2006), with the observation that Ag^+ exposure led to a rapid and substantial leakage of K^+ and ATP from cells of *E. coli*, which is indicative of generalised damage to the membrane. In addition, Ag^+ caused electron transfer to stall at complex I (NADH dehydrogenase) of the oxidative phosphorylation pathway, suggesting that Ag^+ inhibited this complex (Holt & Bard, 2005). It is likely that both of these mechanisms would impact cell viability and could therefore contribute the

antibacterial MOA of Ag^+. In addition, the stalling of electron transport may lead to the generation of ROS, an additional mechanism by which Ag^+ could exert an antibacterial effect (Holt & Bard, 2005). This is discussed below.

1.2.5.4. Generation of reactive oxygen species

During normal cellular metabolism, molecular oxygen (O_2) can be reduced into a variety of reactive states dependent upon the number of electrons received (Figure 1.1). These states are superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^\cdot$) and are collectively known as reactive oxygen species (ROS).

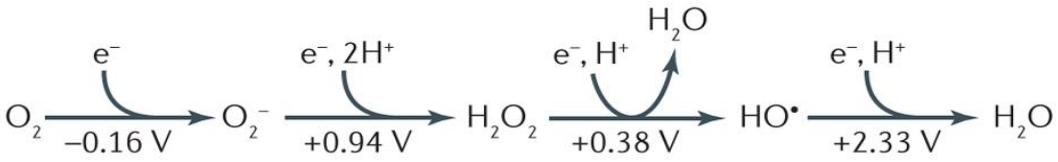


Figure 1.1. The reduction series of oxygen. Adapted from (Imlay, 2013)

The interaction of molecular oxygen with flavin- or quinone-containing components of the electron transport chain, or with non-respiratory flavoproteins can lead to the formation of O_2^- or H_2O_2, which are relatively short-lived species with minimal toxic effects (Imlay, 2013). However, these species can damage iron-sulphur (Fe-S) clusters found in a variety of enzymes, such as respiratory and non-respiratory dehydratases, ferredoxins and dehydrogenases, leading to the release of free iron in the form of Fe^{2+} which itself can react with H_2O_2 to produce $HO^\cdot$ via the Fenton reaction (Imlay, 2013):

$$Fe^{2+} + H_2O_2 \rightarrow [FeO]^{2+} + H_2O$$
$$[FeO]^{2+} + H^+ \rightarrow Fe^{3+} + HO^\bullet$$

Unlike O_2^- and H_2O_2, $HO^\cdot$ has a strong oxidation potential and can lead to damage of DNA (by base-pair oxidation), proteins (by amino-acid carbonylation) and cell membranes (by lipid peroxidation) and can ultimately cause to cell death (Kohanski *et al.*, 2007). The toxic effects of ROS are normally mitigated in the bacterial cell by the presence of scavenging enzymes, with catalases and peroxidases scavenging H_2O_2 and superoxide dismutases scavenging O_2^-. However, under certain environmental conditions (*e.g.* hyperbaria or the presence of exogenous oxidants), these protection mechanisms can become overwhelmed (Seaver & Imlay, 2001). Indeed, this process is exploited by the innate immune system in a variety of eukaryotes to kill invading pathogens (Miller & Britigan, 1995). Metal ions other than Fe^{2+}, such as Cu^{2+}, Ni^{2+} and Co^{2+} (Lloyd & Phillips, 1999) can also be utilised in the Fenton reaction to produce $HO^\cdot$ and may provide some insight into their antibacterial activity. Although silver can exist in a variety of oxidation states, there is no evidence to suggest it can be substituted into the Fenton reaction; however, there are studies indicating that Ag^+ can induce ROS formation, this is discussed below.

Given the propensity of Ag^+ to bind to thiol groups, it has been proposed that Ag^+ inhibition of thiol-containing enzymes of the electron transport chain such as NADH and succinate dehydrogenase would stall electron transfer and generate favourable conditions for O_2^- generation (Bragg & Rainnie, 1974; Messner & Imlay, 1999). Indeed, up-regulation of *soxS*, encoding a transcription factor activated by superoxide stress, has been observed in cultures of *E. coli* exposed to Ag^+ (Holt & Bard, 2005; Park *et al.*, 2009) and a strain of *E. coli* deficient in catalase was more susceptible to killing by Ag^+ than its parental strain (Matsumura *et al.*, 2003). In *S. epidermidis*, it was shown that exposure to Ag^+ leads to the formation of $HO^\cdot$ (as detected by the $HO^\cdot$-responsive dye, hydroxyphenyl fluorescein [HPF]), and in the presence of thiourea (a quencher of ROS)

the antibacterial activity of Ag^+ decreased. This not only suggests that ROS are produced, but that they contribute to the activity of Ag^+ (Gordon *et al.*, 2010). However, it was recently demonstrated that HPF does not specifically detect $HO^{\cdot}$ alone, as in *E. coli* grown under anaerobic conditions (where $HO^{\cdot}$ would not be present), activated HPF can still be detected (Keren *et al.*, 2013). Furthermore thiourea can exist in two tautomers, one of which contains a thiol group. As it is known that thiols can bind and inactivate Ag^+ (Liau *et al*., 1997), it is unclear if the loss of Ag^+ activity observed by Gordon *et al.* is a consequence of the thiol-binding or quenching properties of thiourea. In addition, Ag^+ still maintains antibacterial activity (albeit at a reduced level) against *E. coli* and *S. aureus* under anaerobic conditions where ROS generation would not occur (Park *et al*., 2009), and it is therefore clear that the generation of ROS and any subsequent cell damage cannot be the sole mechanism by which Ag^+ exerts an antibacterial effect. Nevertheless, given the limited information regarding the role of ROS in contributing to the antibacterial MOA of Ag^+, further studies are required to define their role.

In summary, although the studies described above have revealed that Ag^+ can bind and disrupt a number of cellular targets, it is unclear which of these events are the primary causes of Ag^+-mediated cell death and which events are secondary to this action.

1.2.6. Bacterial resistance to Ag^+

With a myriad of potential targets, it is unlikely that the antibacterial MOA of Ag^+ relies upon the inhibition of one target alone. This is in contrast to most antibiotics, which typically achieve antibacterial activity through binding and inhibition of a single enzyme (Walsh, 2003). The possibility that Ag^+ targets multiple cellular processes would imply that spontaneous mutation to Ag^+ resistance is unlikely to occur, as

bacteria would potentially need to accrue mutations in each target to render them insusceptible to Ag^+. Evidence to support this hypothesis has been provided by Maple *et al*. (1992), with the demonstration that spontaneous mutation to Ag^+ resistance in *S. aureus* did not arise at a limit of detection of $1x10^{-9}$. In comparison, resistance to antibiotics with a single target (*e.g.* mupirocin and rifampicin) typically arise at frequencies between 10^{-7} and 10^{-8} (O'Neill & Chopra, 2004). Nevertheless, since the re-introduction of Ag^+ as an antibacterial agent, Ag^+ resistance has been encountered in clinical and environmental bacterial isolates (Table 1.5), which in a number of cases has been attributed to the horizontal acquisition of an exogenous Ag^+ resistance element. For example, Ag^+ resistance in *Salmonella enterica* serovar Typhimurium isolated from a burns unit was associated with a 180 kb conjugative plasmid that also encoded resistance to mercuric chloride, ampicillin, chloramphenicol, tetracycline, streptomycin and sulphonamide antibiotics (McHugh *et al.*, 1975). This plasmid was named pMG101 and was later found to confer Ag^+ resistance by encoding mechanisms to sequester and efflux Ag^+ from the periplasmic space. These mechanisms will be described in more detail later in this chapter. *Psuedomonas stutzeri* isolated from a silver mine contained a non-conjugative 75 kb plasmid (pKK1) conferring Ag^+ resistance (Haefeli *et al.*, 1984). Unlike pMG101, pKK1 did not contain any other resistance determinants and was proposed to confer resistance to Ag^+ by encoding mechanisms leading to the intracellular detoxification of Ag^+, presumably by the increased expression of sulphur-rich compounds that sequester Ag^+. However, there are only limited experimental data to support this. A similar mechanism of Ag^+ detoxification is believed to be conferred by the 54 kb conjugative plasmid pUPI199, and plasmid pJT1, which were originally identified in an environmental isolate of *A. baumannii* and a burn wound isolate of *E. coli*, respectively (Starodub & Trevors,

1989; Deshpande & Chopade, 1994). However, this again has not been conclusively demonstrated.

Table 1.5. Examples of clinical and environmental bacterial isolates resistant to Ag^+

Organism (year of isolation/publication of result)	Source of isolate	Source of resistance mechanism	Reference
Enterobacter cloacae (1974)	Burn wound	Unknown (unstable resistance)	(Rosenkranz *et al.*, 1974)
Salmonella Typhimurium (1975)	Burn wound	pMG101	(McHugh *et al.*, 1975)
Enterobacter cloacae (1976)	Burn wound	Unknown (unstable resistance)	(Annear *et al.*, 1976)
Enterobacter cloacae (1978)	Burn wound	Unknown	(Gayle *et al.*, 1978)
Pseudomonas aerugionosa (1979)	Burn wound	Unknown (unstable resistance)	(Bridges *et al.*, 1979)
Enterobacteriaceae (1979)	Burn wounds and patients with silver-impregnated tracheostomy tubes	Unknown	(Hendry & Stewart, 1979)
Pseudomonas stutzeri (1984)	Silver mine	pKK1	(Haefeli *et al.*, 1984)
Pseudomonas aeruginosa (1989)	Clinical culture collection	Unknown	(Vasishta *et al.*, 1989)
Escherichia coli (1989)	Burn wound	pJT1	(Starodub & Trevors, 1989)
Pseudomonas aeruginosa (2003)	Burn wound	Unknown (unstable resistance)	(Pirnay *et al.*, 2003)
Acinetobacter baumannii (1994)	Environmental isolate	pUPI199	(Deshpande & Chopade, 1994)
Enterobacter cloacae (2006)	Burn wound	Unknown	(Ip *et al.*, 2006)
Enterobacter cloacae (2007)	Leg ulcer	pMG101	(Lansdown & Williams, 2007)

Although Ag^+ resistance has been encountered in environments where it is employed therapeutically, it does not appear to be prevalent; only 10 cases of Ag^+ resistance have been reported in the past 49 years. However, the Ag^+-susceptibility of wound isolates from patients about to receive, or undergoing Ag^+ therapy, is not routinely tested (Warriner & Burrell, 2005), nor are there any routine large scale surveys into the prevalence of Ag^+ resistance amongst clinical bacterial isolates. Indeed, in the past seven years, there has only been one such study, with a relatively small number of isolates tested (n=117) from a single hospital (Ip *et al.*, 2006). The true prevalence of bacterial Ag^+ resistance may therefore be greater than currently believed, and it is clear that a sufficiently large survey of bacterial Ag^+ susceptibility of isolates from a range of hospitals is required to assess this. However, as discussed earlier, a standardised methodology is first required for assessing bacterial Ag^+ susceptibility. This will prevent variables that are known to affect the inhibitory activity of Ag^+ (*e.g.* choice of culture media and Ag^+ formulation) from influencing the interpretation of results, and to allow data from future Ag^+ susceptibility surveys to be directly compared with each other.

The impact of Ag^+ resistance on the continued use of Ag^+ in the treatment of infection is also not clear, with recent publications noting that Ag^+ resistance is unstable in a number of studies, implying that the resistance phenotype confers a metabolic cost to the host strain that could potentially limit its emergence and spread in a patients (Lansdown & Williams, 2007). Nevertheless, this hypothesis has not been confirmed experimentally for any Ag^+-resistant strain. Furthermore, there is conflicting evidence over the role of Ag^+ resistance in the failure of Ag^+ therapy to treat infection. For example, two Ag^+-containing dressings were able to reduce the bioburden of a chronic wound, irrespective of the fact that an Ag^+-resistant *E. cloacae was* present (Lansdown

& Williams, 2007). In contrast, Ag^+-resistant *E. cloacae* was responsible for the failure of AgSu in preventing burn wound infections that resulted in the death of 13 patients at a burn treatment centre (Gayle *et al.*, 1978), whilst an Ag^+-resistant *S.* Typhimurium had a similar effect in patients treated with $AgNO_3$ (McHugh *et al.*, 1975). Given the popularity of Ag^+-containing products in preventing and treating infection, and the likelihood that the use of such products will increase, it is apparent that a thorough investigation into the potential impact of Ag^+ resistance is required, particularly to establish if Ag^+ resistance will act as a barrier to the continued use of Ag^+ therapy. Such an investigation should include studies to examine the potential for Ag^+ resistance to arise by endogenous and exogenous mechanisms and determine the propensity for such phenotypes to spread, persist and cause infection in patients. In addition, elucidating the molecular basis of Ag^+ resistance phenotypes could identify potential targets for the rational design of inhibitors against the resistance mehcanism, allowing for the continued use of Ag^+ against resistant strains. The remainder of this chapter will explore what is currently known about these aspects and highlight where additional studies are required.

1.2.6.1. Exogenous Ag^+ resistance

Four plasmids have been identified that confer Ag^+ resistance (Table 1.5); of these, pMG101 is the only one to have been characterised at the molecular level. Analysis of pMG101 revealed a 14.2 kb region harbouring the Ag^+ resistance determinant (Gupta *et al.*, 1999). Subsequent DNA sequence determination revealed that this determinant contained nine open reading frames in a divergently-transcribed operon, which has been named the *sil* operon (Figure 1.2) (Gupta *et al.*, 1999). With the exception of the protein encoded by *ORF105*, the putative functions of the proteins encoded by this operon have been hypothesised (Figure 1.2) (Gupta *et al.*, 1999; Silver, 2003).

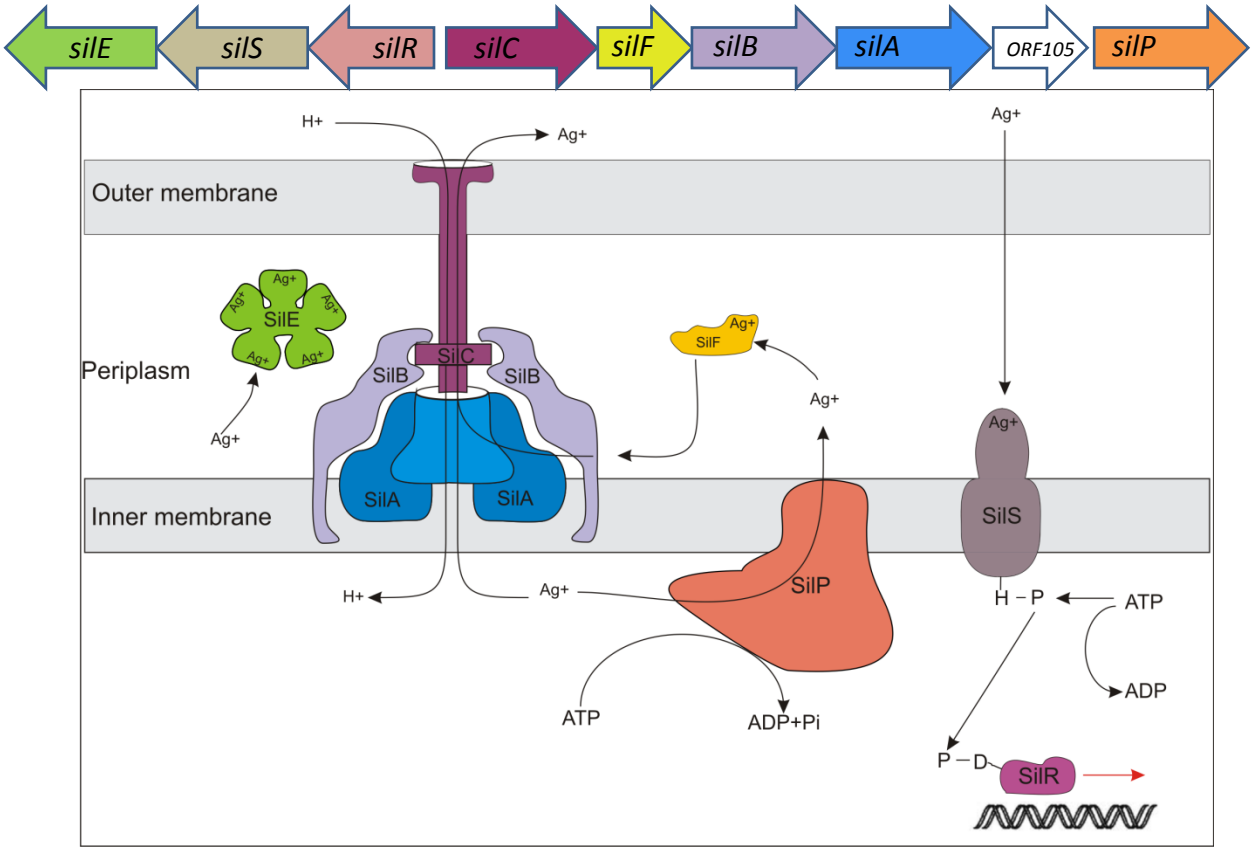


Figure 1.2. Proposed role of the Sil proteins in Ag^+ resistance of Gram-negative bacteria. SilE binds Ag^+, preventing its entry into the cell. SilP is a P-type ATPase that transports Ag^+ from the cell into the periplasm. Ag^+ is then exported from the cell by the efflux pump SilCBA in a process chaperoned by SilF. SilRS, a two-component system, mediates transcription of the *sil* genes. Adapted from Gupta *et al.*, (1999) and Silver, (2003)

1.2.6.1.1. SilE

The *silE* gene encodes a periplasmic metal-binding protein (Gupta *et al.*, 1999). Binding studies of SilE with heavy metal ions (Cd^{2+}, Cu^{2+}, Ag^+) have revealed that SilE specifically binds Ag^+ (Gupta *et al.*, 1999). SilE can bind up to 5 Ag^+ ions, with each ion co-ordinated by two histidine residues (Gupta *et al.*, 1999). It is likely that SilE acts to sequester Ag^+ as it enters the cytoplasm, thus preventing Ag^+ from reaching its antibacterial target(s).

1.2.6.1.2. SilRS

The protein products of *silS* and *silR* are believed to encode a sensor kinase and a cognate response regulator, respectively, to form a two-component sensor-response system. This is based on amino-acid sequence identity with PcoRS (~42% average identity), a two-component regulator of copper resistance, and CzcRS, a system regulating cobalt, zinc and cadmium resistance (~40% average identity) (Gupta *et al.*, 1999). Although the structure and function of SilRS has not been characterised *in vitro*, or *in vivo*, it is believed that this system acts as a positive regulator for the expression of the entire *sil* operon if Ag^+ is present (Gupta *et al.*, 1999). Following sensing of a substrate (presumably Ag^+), SilS subsequently phosphorylates SilR and changes its structure to enable it to bind DNA (likely the intergenic region between *silC* and *silR*, as this is where the *sil* operon diverges) and recruits RNA polymerase for transcription of downstream genes (Silver, 2003).

1.2.6.1.3 SilCBA

Transcribed divergently from *silERS*, the product of s*ilCBA* is hypothesised to be a cation anti-porter of the resistance nodulation division (RND) superfamily (Gupta *et al.*, 1999). SilA is the inner membrane component of this system and shares partial sequence identity with AcrB from *E. coli* (Gupta *et al.*, 1999). The structure of AcrB has been solved and forms a trimer in the inner membrane, transporting a wide spectrum of antibiotics from the intracellular milieu into the periplasm (Nikaido, 2009; Silver, 2003). It is believed that SilA performs the same action, with the exception that it transports Ag^+ instead. An outer membrane porin, SilC, is fused to SilA by the adaptor protein SilB. This tripartite system transports Ag^+ either from the cytoplasm, or the periplasm, to the outside of the cell (Gupta *et al.*, 1999). The structure of CusCBA, a

known Cu^+- and Ag^+-efflux pump in *E. coli* has recently been solved and was found to transport Ag^+/Cu^+ via a methionine shuttle (Su *et al.*, 2011). As CusCBA and SilCBA share ~60% sequence identity, it is likely that SilCBA transports Ag^+ from the cell in a similar manner.

1.2.6.1.4. SilF

The function of SilF remained undefined in the initial characterisation of the Sil system (Gupta *et al.*, 1999). However, later work revealed similarities between SilF and CusF (amino acid identity of ~50%), the latter a known periplasmic Ag^+- and Cu^+-binding protein of *E. coli* (Silver, 2003, Silver *et al.*, 2006). CusF binds a single Ag^+/Cu^+ resulting from co-ordination with histidine, methionine and tryptophan residues (Xue *et al.*, 2008). These residues are conserved in SilF (Xue *et al.*, 2008), thus it is likely that SilF also binds Ag^+ in this manner, and contributes to Ag^+ resistance by sequestering Ag^+ entering the periplasm (as with SilE) (Silver *et al.*, 2006), although this has not been established experimentally.

1.2.6.1.5. SilP

SilP is a putative member of the P-type ATPase family of heavy metal efflux pumps (Gupta *et al.*, 1999). This has been deduced based on amino acid sequence similarities between SilP and cadmium, copper and zinc ATPases (Gupta *et al.*, 1999). SilP does differ from other P-type ATPases in that it possesses an N-terminal polyhistidine region, which replaces a usually conserved cysteine-X-X-cysteine motif. It is believed that this substitution confers specificity of SilP for Ag^+ (Silver *et al.*, 2006). P-type ATPases are known to efflux metal ions from the cytoplasm into the periplasm (Solioz & Vulpe, 1996) and it is likely that SilP has the same mechanism to transport Ag^+. As with other Sil system components, this remains to be confirmed.

Even though the putative functions of Sil system components have been assigned, the relative contribution made by each component to the Ag^+ resistance phenotype has not been determined. This would be of benefit as it may reveal that certain Sil components are essential to the resistance phenotype, and might represent a promising target for developing inhibitors of Ag^+ resistance. In addition, the fitness cost imposed on strains harbouring a known exogenous Ag^+-resistance determinant (*i.e.* pMG101, pKK1, pUPI199, pJT1) has not been established, thus the potential for these determinants to survive and spread in a clinical setting (*e.g.* a hospital ward) is unclear.

Unlike in Gram-negative bacteria, there is no evidence of Ag^+ resistance arising by exogenous mechanisms in Gram-positive bacteria.

1.2.6.2. Endogenous Ag^+ resistance

Although endogenous Ag^+ resistance does not arise in *S. aureus* by a single mutational event (Maple *et al*., 1992), continuous exposure of *S. aureus, E. coli* and *K. pneumoniae* to sub-inhibitory concentrations of Ag^+ in the laboratory resulted in the selection of isolates with a reduced susceptibility to Ag^+, presumably as a consequence of multiple mutational events (Kaur *et al.*, 1987; Gupta *et al.*, 1992; Li *et al.*, 1997). In *S. aureus*, an isolate resistant to 70 μg/ml $AgNO_3$ could be selected (compared with the parental strain, for which $AgNO_3$ had an MIC of 8 μg/ml) (Kaur *et al.*, 1987), whereas a *K. pneumoniae* isolate resistant to 150 μg/ml $AgNO_3$ (MIC against parental strain: 15 μg $AgNO_3$/ml) was selected by Gupta *et al*. (1992). Although both of these studies revealed that the virulence of resistant isolates was reduced compared with their respective parental strain, the time taken for resistance to emerge, the fitness cost, or the molecular basis of the resistance phenotype were not established. As discussed earlier,

each of these aspects are important in understanding the relevance of such strains from the perspective of the continued clinical use of Ag^+.

In contrast to Ag^+-resistant *S. aureus* and *K. pneumoniae*, the mechanisms of endogenous Ag^+ resistance in *E. coli* have been explored in relative detail. The MIC of $AgNO_3$ against strains of Ag^+-resistant *E. coli* was >1024 μg/ml ($AgNO_3$ MIC of parental strain: 8 μg/ml) (Li *et al.*, 1997). Such strains were deficient in OmpF, or both OmpF and OmpC outer membrane porins (although it was not established if porin loss was essential for the maintenance of the resistance phenotype), and could efflux Ag^+ from the cell by an energy-dependent mechanism (Li *et al.*, 1997). A later study provided evidence to suggest that Ag^+ efflux was mediated by overexpression of the CusCBA efflux pump (described in section1.2.6.1.3), as the expression of a component of this pump (CusB) and another protein encoded by the same operon (CusF) were found to be up-regulated in resistant strains, and a missense mutation was identified in *cusS*, which encodes the sensor kinase that regulates expression of this operon (Lok *et al.*, 2008). However, the genetic changes necessary to confer endogenous Ag^+ resistance in *E. coli* have yet to be elucidated, and, as with endogenous Ag^+ resistance in *S. aureus* and *K. pneumoniae*, key experiments vital to understanding the threat this phenotype poses to the clinical utility of antibacterial Ag^+ have not been performed (*e.g.* fitness studies and establishing the time taken for resistance to arise).

1.3. Introduction to the research in this study; aims and objectives

The antibacterial properties of the silver cation have been exploited for centuries, and with the rising prevalence of bacterial pathogens that are resistant to multiple classes of antibiotics, the use of Ag^+ as an alternative for the treatment of infectious disease is increasing (Atiyeh *et al.*, 2007). In addition, Ag^+ is now frequently incorporated into consumer products, such as deodorants, clothing and work surfaces, as a biocide (Silver, 2003). There are concerns that such prolific use of Ag^+ will select for the emergence of Ag^+ resistance, which may limit the utility of Ag^+ in treating infectious disease (Chopra, 2007). However, the current prevalence of Ag^+ resistance amongst bacterial pathogens is unclear. In addition, the ease in which Ag^+ resistance can arise, persist and spread in an environment where Ag^+ is employed therapeutically, or the mechanisms employed by bacteria to resist Ag^+ are either not currently known, or are poorly understood. One aim of the research presented in this thesis was to address these issues, by establishing a standardised methodology for testing bacterial susceptibility to Ag^+, and using it to establish the frequency of Ag^+ resistance in a large and geographically diverse range of clinical bacterial isolates. Using established techniques, the potential for endogenous Ag^+ to emerge in a number of relevant pathogens was explored and the fitness costs associated with any arising Ag^+ resistance phenotype determined. The genetic basis and mechanism of Ag^+ resistance resulting from endogenous and exogenous means was also explored.

Although a number of potential antibacterial targets of Ag^+ have been identified, the exact mechanism by which Ag^+ exerts an antibacterial effect has not been elucidated. Furthermore, there is conflicting evidence in the scientific literature regarding the killing kinetics of Ag^+. The second aim of the work presented in this thesis was to provide further insight into both of these aspects of Ag^+, by using assays that are

routinely employed in the pre-clinical evaluation of antibiotic drug candidates (O'Neill & Chopra, 2004).

Sections of the work included in Chapters 3-5 have been published. A list of these publications is presented in Appendix 3.

Chapter two: Materials and Methods

2.1 Bacterial strains and plasmids

All strains used in this study (Tables 2.1-2.2) were stored in 8% (v/v) glycerol at -80^{o}C.

Table 2.1. Laboratory strains used in this study

Strain	Description/Application	Reference/Source
***Staphylococcus aureus* strains:**		
SH1000	Derivative of strain 8325-4, containing functional *rsbU*	(Horsburgh *et al.*, 2002)
RN4220	Restriction-deficient cloning host	(Kreiswirth *et al.*, 1983)
KS100	SH1000 deficient in catalase- KatA (*katA*::Tn*917*)	(Cosgrove *et al.*, 2007)
MHKM	SH1000 deficient in superoxide dismutase - SodM (*sodM*::*tetL*)	(Karavolos *et al.*, 2003)
MHKA	SH1000 deficient in superoxide dismutase - SodA (*sodA*::Tn*917*)	(Karavolos *et al.*, 2003)
MHKAM	SH1000 deficient in superoxide dismutases – SodA and SodM (*sodA*::Tn*917 sodM*::*tetL*)	(Karavolos *et al.*, 2003)
KCO43	SH1000 deficient in alkyl hydroperoxide reductase- AhpC and superoxide dismutase- SodA (*ahpC*::tet *sodA*::*Tn*917)	(Cosgrove *et al.*, 2007)
SH1000β	SH1000 containing pJIM2246 with *lacZ* insert under control of staphylococcal promoter *cap1A*	(O'Neill *et al.*, 2004)
CB1618	Daptomycin-resistant derivative of *S. aureus* MW2	Friedman *et al.*, 2006)
NR111	Nisin/daptomycin-resistant derivative of *S.aureus* SH1000	Blake *et al.*, 2011)
***Bacillus subtilis* strains:**		
1S34	Parental strain of reporter constructs	(Urban *et al.*, 2007)
1S34 (pS63)	RNA biosynthesis inhibition reporter	(Urban *et al.*, 2007)
1S34 (pS72)	Protein biosynthesis inhibition reporter	(Urban *et al.*, 2007)

Table 2.1 (continued)

1S34 (pS77)	DNA biosynthesis inhibition reporter	(Urban *et al.*, 2007)
1S34 (pS107)	Cell envelope biosynthesis inhibition reporter	(Urban *et al.*, 2007)
1S34 (pNS14)	Fatty acid biosynthesis inhibition reporter	(Urban *et al.*, 2007)
***Enterococcus hirae* strains:**		
9790	Source of copper-efflux pump CopB	ATCC
***Pseudomonas aeruginosa* strains**		
PAO1		ATCC (number 47085)
***Enterobacter cloacae* strains**		
13047	Harbours Sil system	ATCC
***Citrobacter freundii* strains**		
8090	Harbours Cus system	ATCC
***Escherichia coli* strains:**		
DH5α	*fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Life Technologies
BW25113	Derivative of *E. coli* K12 strain BD792 (*lacIq* $rrnB_{T14}$ Δ$lacZ_{WJ16}$ *hsdR514* Δ$araBAD_{AH33}$ Δ$rhaBAD_{LD78}$) and parental strain of Keio collection	(Datsenko & Wanner, 2000; Baba *et al.*, 2006)
BW25113-Δ*ompR*	Derivative of BW25113 deficient in OmpR (*ompR*::*neoR*)	(Baba *et al.*, 2006)
BW25113-Δ*ompF*	Derivative of BW25113 deficient in OmpF (*ompF*::*neoR*)	(Baba *et al.*, 2006)
BW25113-Δ*ompA*	Derivative of BW25113 deficient in OmpA (*ompA*::*neoR*)	(Baba *et al.*, 2006)
BW25113-Δ*cusC*	Derivative of BW25113 deficient in CusC (*cusC*::*neoR*)	(Baba *et al.*, 2006)
BW25113-Δ*cusF*	Derivative of BW25113 deficient in CusF (*cusF*::*neoR*)	(Baba *et al.*, 2006)

Table 2.1 (continued)

BW25113-Δ*cusB*	Derivative of BW25113 deficient in CusB (*cusB*::*neoR*)	(Baba *et al.*, 2006)
BW25113-Δ*cusA*	Derivative of BW25113 deficient in CusA (*cusA*::*neoR*)	(Baba *et al.*, 2006)
50110	*E. coli* J53 harbouring silver-resistance plasmid pMG101	NCTC
DY441	*E. coli* harbouring *cat-sacB* allelic replacement cassette	Donald Court, National Cancer Institute, USA

Table 2.2. Clinical isolates used in this study

Strain	Description	Reference/Source
***Staphylococcus aureus* strains:**		
29213, 25923	*Staphylococcus aureus* subsp. aureus Rosenbach	ATCC
G1- G100, O1-O100, Y1-Y100, W1-100, C1-C47, K1-K23, KT1-KT11 (n= 483)	Clinical isolates (isolated between 1997-2001)	Department of Microbiology, Leeds General Infirmary (O'Neill *et al.*, 2002)
IC1-IC54 (n=54)	Clinical isolates from patients with burns (isolated in 2010):	Department of Microbiology, Chelsea and Westminster Hospital, UK
A2-A84 (n=77)	Fusidic acid-resistant clinical isolates (isolated in 1999)	Departments of Dermatology and Microbiology, Harrogate district Hospital, UK (Ravenscroft *et al*., 2000)
N1-N100 (n=95)	Clinical isolates (isolated in 2010)	Department of Microbiology, Freeman Hospital, Newcastle, UK
SN 1-65 (n=65)	Clinical isolates from patients with diabetic ulcers (isolated in 2010)	Smith and Nephew, York, UK
F1-F70 (n=70)	Clinical MRSA isolates (isolated in 2007)	(Larsen *et al.*, 2008)
Coagulase-negative staphylococci strains:		
SN 66-80 (n=14)	Clinical isolates from patients with diabetic ulcers (isolated in 2010)	Smith and Nephew, York, UK
IT1-IT29 (n=29)	Clinical isolates (isolated in 2010)	Department of Experimental Medicine and Biochemical Sciences, University of Perugia, Italy
GRL05001- GRL05020 (n=20)	Meticillin-susceptible (n=10) and methicilin-resistant (n=10) *S. epidermidis* (isolated in 2006)	G.R. Micro Limited
L1-L27 (n=27)	Clinical isolates (isolated between 1997-2001)	Department of Microbiology, Leeds General Infirmary
IC55 and 56 (n=2)	*S. epidermidis* (isolated in 2010)	Department of Microbiology, Chelsea and Westminster Hospital, UK

Table 2.2. (continued)

S. lugdenensis (n=8)	Clinical isolates (isolated in 2005)	(O'Neill *et al.*, 2007)
CL1 and CL2 (n=2)	*S. pseudintermedius* isolates (isolated in 2004)	(Guardabassi *et al.*, 2004)
7292,11041-11046, 11048 and 15306 (n=9)	Various coagulase-negative staphylococci	NCTC
CA1-39 (n=39)	Various coagulase-negative staphylococci (isolated in 2010)	London Health Sciences Centre, Victoria Hospital, Ontario, Canada
RST 1-10 (n=10)	*Staphylococcus rostri* isolates (isolated in 2010)	Vincent Perreten, University of Berne, Berne, Switzerland
***Klebsiella pneumoniae* strains:**		
KP1-3 (n=3)	Clinical isolates (isolated in 2011)	Department of Microbiology, Leeds General Infirmary
***Shigella sonnei* strains:**		
SS1	Clinical isolate (isolated in 2011)	Department of Microbiology, Leeds General Infirmary
***Escherichia coli* strains:**		
EC18	Clinical isolate deficient in OmpC/F (isolated in 2010)	Health Protection Agency Antibiotic Resistance Monitoring & Reference Laboratory, Colindale, (Beceiro *et al.*, 2011)

Table 2.3. Plasmids used in this study

Plasmid	Description	Reference/Source
pSIM8	Contains temperature inducible λ exo-β-γ for DNA recombineering. Confers ampicillin resistance (*blaZ*)	(Datta *et al.*, 2006)
pSIM18	Contains temperature inducible λ exo-β-γ for DNA recombineering. Confers hygromycin resistance (*hph*)	(Datta *et al.*, 2006)
pCP20	Encodes yeast FLP recombinase for excision of FRT-flanked resistance cassettes. Confers ampicillin and chloramphenicol resistance (*blaZ*, *cat*)	(Cherepanov & Wackernagel, 1995; Datsenko & Wanner, 2000)
pKD4	Contains FRT-*neoR*-FRT cassette	(Datsenko & Wanner, 2000)

2.2 Antibacterial agents and chemicals

Unless otherwise stated, all antibacterial agents and chemicals were from Sigma-Aldrich (Poole, UK). Vancomycin, cetyltrimethylammonium bromide (CTAB), nisin and triclosan were from Duchefa Biochemie (Haarlem, Netherlands), BDH Laboratory Supplies (Poole, UK) NBS Biologicals Ltd. (Huntingdon, UK) and LG Life Sciences (South Korea) respectively. Daptomycin and XF-73 were gifts from Cubist Pharmaceuticals (Lexington, MA, USA) and Destiny Pharmaceuticals (Brighton, UK) respectively. Stock solutions were prepared by dissolving the compound in an appropriate solvent (see Table 2.4) before dilution in sterile distilled water.

Table 2.4. Antibacterial agents used in this study

Antibiotic	Solvent
Silver nitrate	H_2O
Erythromycin	H_2O
Fusidic acid	50% EtOH
Vancomycin	H_2O
Tetracycline	H_2O
Triclosan	50% EtOH
Rifampicin	50% DMSO
Ciprofloxacin	20mM HCl
CTAB	H_2O
Daptomycin	H_2O
Nisin	20mM HCl
Mupirocin	50% EtOH
Ampicillin	H_2O
Chloramphenicol	50% EtOH
Penicillin G (benzyl penicillin)	H_2O
Gentamicin	H_2O
Kanamycin	H_2O
XF-73	H_2O
Hygromycin	H_2O

2.3. Bacteriological media and culture conditions

All bacteriological media were purchased from Oxoid Ltd (Basingstoke, UK) and prepared according to the manufacturer's instructions. Aerobic culture and determination of antibacterial susceptibilities of the *S. aureus*, coagulase-negative staphylococci, and Gram-negative strains were routinely carried out with cultures grown using Mueller-Hinton agar (MHA) and broth (MHB). For maintenance of constructs, strains were grown on MHB and MHA under appropriate antibiotic selection. Plate cultures were grown aerobically at 37^{o}C for 18 hours, with the exception of strains harbouring pSIM8, pSIM18 or pCP20, which were grown at 30^{o}C due to the temperature-sensitive origin of replication present on these plamids. Liquid cultures were incubated under the same conditions in a rotary shaker at 300 rpm. Experiments

under anaerobic conditions utilised MHB that was de-oxygenated for at least 16 hours in an anaerobic cabinet. For studies with daptomycin, MHB was supplemented with Ca^{2+} (in the form of $CaCl_2.6H_2O$) to a final concentration of 50 μg/ml. Silver nitrate ($AgNO_3$) was used as a source of Ag^+, unless otherwise stated.

2.4. General microbiology techniques

2.4.1. Determination of susceptibilities to antibacterial agents

MICs of antibacterial agents were routinely determined by 2-fold serial dilution in MHB according to the CLSI broth microdilution method (Wayne, 2012). However, susceptibility of clinical staphylococcal isolates to Ag^+ was determined using the CLSI agar dilution method. Preliminary studies also utilised the BSAC standardised broth microdilution and agar dilution methods of antimicrobial susceptibility testing, as described elsewhere (Andrews, 2001). Biofilm MICs (bMICs) and minimum biofilm eradication concentrations (MBECs) were determined in MHB using the Calgary Biofilm Device as described previously (Miller *et al.*, 2005).

2.4.2. Time-kill analysis

Time-kill assays were carried out as described previously (Oliva *et al.*, 2003), with modifications. Briefly, a 1:100 dilution of overnight culture of either *S. aureus* SH1000, MHKAM or KCO43 in MHB was grown to an OD_{600} of 0.2 units and then exposed to antibacterial compounds at 4X MIC. Sample volumes of culture were taken at time points over a 24-hour period, diluted in phosphate buffered saline and spread onto MHA. Viable counts were determined following an overnight incubation at 37°C and the number of colony forming units (CFU)/ml calculated. Variations of this method were also carried out as described below.

Time-kill assays under anaerobic conditions were carried out using *S. aureus* SH1000 as described above, with the exception that all work was undertaken in an MG1000 anaerobic workstation (Don Whitley Scientific, Shipley, UK) with MHB and PBS that had been de-oxygenated for at least 16 h.

Time-kill assays in HEPES glucose buffer were carried out under aerobic conditions as described above, with the exception that once cultures reached an OD_{600} of 0.2 units, cells were harvested by centrifugation, washed twice and resuspended in an equal volume of 5 mM HEPES and 5 mM glucose buffer (pH 7.2) before challenge with antibacterial agents at 4X MIC.

2.5. Resistance studies

2.5.1. Selection of spontaneous antibacterial-resistant mutants

Spontaneous antibacterial-resistant mutants were selected by plating sample volumes of overnight culture of the strain of interest onto MHA containing 4X MIC of antibacterial agent. Plates were then incubated for 48 hours at 37^{o}C. If colonial growth was observed, colonies were selected at random and MICs determined to confirm that they did indeed show reduced susceptibility compared with the parental strain. The frequency of spontaneous mutation (mutation frequency) was calculated as the number of resistant mutants per number of viable bacteria.

2.5.2. Selection of endogenous Ag^{+} resistance

2.5.2.1. Repeated exposure method

Resistance selection was carried out as an adaptation of a method described previously (Miller *et al.*, 2008). Briefly, overnight cultures in MHB were exposed to 100 μg $AgNO_3$/ml for six hours. Following exposure, cells were harvested by centrifugation at

5000 x *g* and washed twice in sterile deionised water before being resuspended in an equal volume of MHB. Sample volumes (90 µl) of the washed culture were then transferred into fresh, drug-free MHB and the cycle repeated for 42 days. The $AgNO_3$ MIC was determined every five cycles from a sample of culture.

2.5.2.2. Continuous exposure method

Bacrteria were continuously exposed to $AgNO_3$ in a manner analogous to the method described by Friedman *et al*. for selecting for daptomycin resistance (Friedman *et al.*, 2006). On day one a microdilution broth MIC was performed for cultures as described in section 2.4.1., using three doubling dilution series of $AgNO_3$. This extended gradient provided the following silver nitrate concentrations (µg/ml):

128 96 80 64 48 40 32 24 20 16 12 10 8 6 5 4 3 2.5 2 1.5 1.25

Following incubation and determination of the MIC, the well that contained the highest concentration of $AgNO_3$ that permitted growth was diluted 1:100 in MHB and used as the inoculum for the next MIC assay. This process was repeated for a maximum of 42 days.

2.5.3. Determination of bacterial fitness

2.5.3.1. Growth rate determination

Growth rates of cells in the presence of $AgNO_3$ were carried out in microtitre plates. Briefly, overnight cultures of test strains were diluted 1:100 in MHB and sample volumes (180 µl) transferred to the wells of a 96-well plate containing 20 µl of $AgNO_3$ at 20 µg/ml (final concentration of $AgNO_3$: 2 µg/ml). Plates were then transferred to a Fluostar Optima plate reader (BMG-Labtech Ltd, Aylesbury, UK)

pre-warmed to 37°C and incubated for 18 h with shaking (200 rpm) for 30 sec every 5 min. During incubation OD_{600} readings were taken for each well every 10 min.

2.5.3.2. Determination of competitive fitness

Sample volumes (9 µl and 90 µl respectively) of overnight cultures of Ag^+-resistant and parental strains (in MHB) were combined and used to inoculate 9 ml of MHB. A sample volume of culture was diluted and 100 µl volumes of appropriate dilutions were plated onto both MHB and MHB containing 64 µg $AgNO_3$/ml. Sample volumes (100 µl) of parental cultures were also plated onto MHB containing 64 µg $AgNO_3$/ml to determine the number of spontaneous mutants to Ag^+ resistance. The remaining mixed culture was incubated for 24 h and the plating procedure described above was repeated. All plates were incubated overnight at 37°C with aeration and the number of colonies counted. Relative fitness was then calculated using the following equation:

$$Relative\ fitness = \frac{\ln\left(\frac{Nr(24)}{Nr(0)}\right)}{\ln\left(\frac{Ns(24)}{Ns(0)}\right)}$$

Where *Nr*(0) and *Nr*(24) represent the population density of the resistant strain at time 0 h or 24 h respectively, following subtraction of the number of spontaneous mutants to Ag^+ resistance. *Ns*(0) and *Ns*(24) represent the population density of the parental strain at time 0 or 24 hr respectively (O'Neill, *et al*., 2001)

2.5.6. Assessment of porin expression in E. coli outer membranes

Outer membrane proteins were extracted and purified according to methods previously described (Stapleton *et al.*, 1999). Overnight cultures of *E. coli* BW25113, BW25113 Δ*ompR* and BW25113 AgR grown in LBB were diluted 1:100 in 200 ml

LBB and grown at 37^{o}C with aeration and shaking (200 rpm) to an OD_{600} of 0.8 units. At this point cells were collected by centrifugation (5000 x *g*, 15 min, 4^{o}C), resuspended in 9 ml PBS containing 100 μg lysozyme/ml, and incubated on ice for 20 min. Cells were then sonicated in an MSE Soniprep 150 sonicator (MSE scientific instruments, Crawley, UK) for 5 min on ice. Sonication consisted of cycles of 30 sec bursts at amplitude of 20 microns followed by 30 sec rest periods. Membranes were collected by first centrifuging at 5000 x *g* for 15 min to remove cell debris and unlysed cells, then subjecting the supernatant to a second centrifugation (18,000 x *g*, 90 min, 4^{o}C) to collect the membranes, which were resuspended in 10 ml of Tris buffer (50 mM, pH 7.6). Cytoplasmic membranes were solubilised by adding sodium lauryl-sarcosinate to a final concentration of 1.7% (w/v) and incubating without shaking at room temperature for 20 min. This was followed with a further centrifugation step (18,000 x *g*, 90 min, 4^{o}C) to pellet the outer membranes. The pellet was resuspended in 50 ml of sterile H_2O and porin expression assessed by SDS-PAGE, as described in section 2.5.7.

2.5.7. SDS-PAGE

Sample volumes (10 μl) of protein suspension diluted 1:1 with loading buffer (Sambrook, 2001) were incubated at 95^{o}C for 5 min prior to separation by SDS-PAGE. Samples were run on a pre-cast 4-20% gradient polyacylamide gel (Expedeon Ltd, Harston, Cambridgeshire, UK) according to guidelines provided by the manufacturer. Gels were stained overnight in Comassie blue stain and de-stained for 4 h in 50% (v/v) methanol and 10% (v/v) acetic acid, with the de-stain replaced every hour.

2.6. Mode of action studies

2.6.1. BacLight™ assay of membrane integrity

Bacterial membrane integrity following exposure to antibacterial agents was assessed using the Live/Dead *Bac*Light™ bacterial viability kit (Life Technologies, Paisley, UK) as described previously (Hilliard *et al.*, 1999). Briefly, *S. aureus* SH1000 was grown in MHB to an OD_{600} of 0.5 units, and volumes of culture (500 μl) were washed twice and resuspended in 900 μl of H_2O. Volumes (100 μl) of antibacterial compounds at a concentration of 40x MIC were then added and samples mixed for 10 minutes at 37^{o}C. Following incubation, samples were washed twice and resuspended in 1 ml of H_2O. In a dark room, 50 μl of each sample were added to the wells of a blacked out 96-well microtitre plate containing 150 μl of *Bac*Light™ reagent (consisting of propidium iodide and SYTO 9 stock solutions diluted 300-fold in H_2O) and incubated in the dark at room temperature for 15 min. Fluorescence was measured in a Fluostar Optima plate reader with excitation set at 485 nm and emission at 645 nm (propidium iodide fluorescence) or 530 nm (SYTO 9 fluorescence). Fluorescence values were normalised against drug-free controls and percentage membrane integrity was then expressed as the ratio of SYTO 9:Propidium iodide fluorescence relative to cultures treated with 0.5% (w/v) SDS.

Bacterial membrane integrity following exposure to antibacterial agents under anaerobic conditions was carried out as described above with the exception that all work was carried out in an MG1000 anaerobic workstation using MHB and sterile H_2O that had been de-oxygenated for at least 16 h.

2.6.2. Membrane potential

Bacterial membrane potential following exposure to $AgNO_3$ and comparator agents was measured over a 3 h time course using the fluorescent dye 3,3'-dipropylthiadicarbocyanine iodide ($DiSC_3(5)$) (Life Technologies, Paisley, UK) according to methods described previously (Hobbs *et al.*, 2008), with adaptations. Overnight cultures of *S. aureus* SH1000 in MHB were diluted 1:100 into 20 ml of MHB and incubated at 37°C with aeration to an OD_{600} of 0.2 units. Cells were then pelleted by centrifugation (5000 x *g*, 15 min, 37°C), washed twice and resuspended in 20 ml of 5 mM HEPES, 5 mM glucose buffer (pH 7.0) containing 100 mM KCl and 2 μM $DiSC_3(5)$ reagent. Cultures were then incubated as described above for an additional 30 min in the dark before addition of test compounds to a final concentration of 4X MIC. Cultures continued to be incubated for 120 min with samples taken at 0, 10, 20, 30, 40, 50, 60, 90 and 120 min.

Samples removed at time-points were centrifuged at 13,000 x *g* for 30 sec and the supernatant (containing extracellular dye) transferred into an equal volume of DMSO. Cell pellets were re-suspended in DMSO and incubated at room temperature for 10 min to release intracellular dye prior to the addition of an equal volume of 5 mM HEPES, 5 mM glucose buffer (pH 7.2). The fluorescence of both extracellular and intracellular samples was measured in a Perkin Elmer LS 45 luminescence spectrometer with excitation set at 622 nm and emission at 670 nm. The membrane potential at each time point was then calculated using the Nernst equation (see below) and then expressed as a percentage of the time 0 value.

$$Membrane\ potential = -\left(\frac{RT}{F}\right)\ln\left(\frac{If}{Ef}\right)$$

Where R is the gas constant (8.3144621), T is the temperature in degrees Kelvin, F is the Faraday constant and *If* and *Ef* are the intracellular fluorescence and extracellular fluorescence respectively.

2.6.3. Construction of liposomes

Carboxyfluorescein-filled liposomes were constructed as described previously (StGelais *et al.*, 2007), with modifications. Stock solutions (10 mg/ml in chloroform) of L-α-phosphatidic acid (PA), L-α-phosphatidylcholine and lissamine rhodamine B-labelled L-α-phosphatidylethanolamine (RPE) were mixed in a 49:49:2 ratio to a final volume of 100 µl in a glass test tube. All lipids were purchased from Avanti polar lipids (Alabaster, Alabama, USA). All chloroform was removed by first drying the lipids under a stream of argon then placing the tubes under vacuum for 2 h prior to adding 1 ml of CF buffer (50 mM carboxyfluorescein, 10 mM NaCl, 10 mM HEPES, 134 mMNaOH, pH 7.4) and incubating overnight at 30^{o}C with shaking (200 rpm). Liposomes were produced by extrusion through a 0.4 µm filter in an Avanti mini-extruder with glass syringes and purified by ultracentrifugation (100,000 x *g*, 20 min) and washing three times in assay buffer (1 mM HEPES, 107 mM NaCl, pH 7.4). Liposomes were resuspended in 500 µl of assay buffer and the liposome concentration calculated by measuring rhodamine absorbance at OD_{570}. To create "staphylococcal" liposomes, this method was repeated, but the lipids described above were substituted for 1,2-dioleoyl-*sn*-phosphatidylglycerol (PG), cardiolipin (CL) and RPE in a 59:39:2 ratio (Epand, *et al.*, 2007). Liposomes of *E. coli* membrane phospholipid were created using *E. coli* polar lipid extract (consisting 67% [w/w] phosphatidylenanolamine, 23.2% [w/w] PG and 9.8 % [w/w] CL) mixed with RPE in a 48:2 ratio.

2.6.4. Liposome damage assay

To the wells of a blacked-out 96-well plate, 50 mM of liposomes were mixed with the antibacterial agent to be tested at 4x MIC and incubated in the dark at 37^{o}C for 2 h. Fluorescence measurements were then taken in a Fluostar Optima plate reader with excitation at 485 nm and emission at 520 nm. Fluorescence values were normalised to drug-free controls and the percentage liposome integrity expressed as the fluorescence intensity relative to that of liposomes treated with 0.1% (w/v) Triton X-100.

2.6.5. β-galactosidase leakage

Assessment of β-galactosidase leakage from *S. aureus* was carried out as described previously (O'Neill *et al.*, 2004), with the exception that the assays were performed using strain SH1000β. Briefly, cultures of SH1000β were grown in MHB to an OD_{600} of 0.6 units before the addition of $AgNO_3$ and comparator compounds at a final concentration of 4X MIC. Following a 10 min incubation at 37 ^{o}C, cells were removed by centrifugation and 4-methylumbelliferyl-β-D-galactoside was added to samples (180 μl) of culture supernatant (final concentration 1 mg/ml) prior to incubation for 2 h at 25 ^{o}C. Sample fluorescence was measured (excitation 365 nm, emission 460 nm) in a Fluostar Optima plate reader plate reader and values converted into fluorophore (4-methylumbelliferone) concentration using a calibration curve consisting of known fluorophore concentrations.

2.6.6. Bacillus subtilis reporters

Five *B. subtilis* antibiotic biosensors containing specific promoters that are induced by inhibition of DNA, RNA, fatty acid, cell wall and protein biosynthesis were utilised as described previously (Urban *et al.*, 2007), with modifications. Strains were grown in

LBB to an OD_{600} of 0.2 units, then samples (90 μl) of culture were treated with $AgNO_3$ or an appropriate control antibiotic in a 96 well plate and incubated at 37°C for 1-3 h (depending on the construct). Volumes (60 μl) of 0.8M luciferin in 0.1M citrate buffer (pH 5.0) were then added and the luminescence of the sample recorded by a Fluostar Optima plate reader. Luminescence values were compared relative to a non-induced control and a >2-fold increase in luminescence was considered a positive result.

2.6.7. Macromolecular synthesis assays

The inhibition of DNA, RNA, protein and peptidoglycan synthesis by $AgNO_3$ and control compounds was monitored by determining the incorporation of radiolabelled precursors [methyl-^{3}H] thymidine (DNA), [5, 6-^{3}H] uridine (RNA), L-[G-^{3}H] glutamine (protein) and [1-^{14}C] glycine (peptidoglycan), as described previously (Cherrington *et al.*, 1990). All radiolabelled compounds were purchased from Perkin-Elmer (Cambridge, UK). Overnight cultures of SH1000 in MHB were diluted 1:100 in 9 ml MHB and incubated at 37°C with aeration and shaking (200 rpm) to an OD_{600} of 0.2 units. Isotopes were added at a final concentration of 1 μCi/ml for ^{3}H compounds and 0.1 μCi/ml for ^{14}C-glycine 10 min prior to addition of antibacterial agent at a final concentration of 4X MIC. Following a further 10 min incubation, 500 μl of culture was added to 4.5 ml of 10 % (w/v) ice-cold trichloroacetic acid (TCA) and incubated on ice for 40 min to precipitate macromolecules. Samples were then processed as previously described (Wilson *et al.*, 1995) and radioactivity measured using a TriCarb 2100TR liquid scintillation counter (Packard Bioscience Ltd, Berkshire, UK).

2.6.8. Enzyme specificity assays

2.6.8.1. RNA polymerase

The ability of Ag^+ and comparator compounds (at 4X MIC) to inhibit *E. coli* RNA polymerase (RNAP) was determined by measuring transcription using SYBR green as described previously (Mariner *et al.*, 2010). Briefly, test compounds (or compound diluents) were mixed with 0.2 U of *E. coli* core RNAP (Epicentre, Madison, Wisconsin, USA) and incubated at 37°C for 10 min prior to the addition of Kool™ NC-45™ Universal RNA polymerase template (Epicentre) and rNTPs to a final concentration of 125 ng/ml and 0.5 mM respectively. Following incubation at 37°C for 2 h, the reaction was stopped by the addition of EDTA to a final concentration of 20 mM EDTA (pH 8) and the amount of transcript produced was measured by adding 0.07% (v/v) SYBR-Green I (Life Technologies) and determining fluorescence intensity at 520 nm, using an excitation wavelength of 497 nm. Fluorescence readings were determined using an EnVision 2103 Multilabel Reader (Perkin-Elmer). Percentage inhibition of RNAP by test compounds was calculated as the fluorescence intensity relative to reactions treated with compound diluents, following normalisation to template-free controls.

2.6.8.2. Malate dehydrogenase

The ability of Ag^+ and comparator agents (at 4X MIC) to inhibit the conversion of oxaloacetic acid + β-NADH to L-malic acid and β-NAD by malate dehydrogenase was determined spectrophotometrically as described previously (Seidler *et al.*, 2003). Briefly, test compound was added to a cuvette containing 50 mM KPO_4 buffer (pH 7.35), followed by addition of malate dehydrogenase (final concentration 0.002 μM), NADH (Boehringer Mannheim; final concentration 0.2 mM) and

oxaloacetic acid (final concentration 0.2 mM). The contents of the cuvette were mixed and OD_{340} readings were taken every 30 sec for 6.5 min in a SPECTRAmax PLUS^{384} Microplate Spectrophotometer (Molecular Devices Ltd, Sunnyvale, California, USA). Readings were plotted on a scatter graph in Microsoft Excel software and the rate of reaction calculated as the gradient of a trendline fitted to the data. Percentage inhibition was calculated using the following equation:

$$Percentage\ inhibition = 100 - \left(\left(\frac{Rc - Rb}{Rd - Rb}\right) * 100\right)$$

Where *Rc*= Rate of reaction when exposed to antibacterial agent, *Rb*= Rate of reaction with no malate dehydrogenase present and *Rd*=Rate of reaction when exposed to diluent only.

2.6.8.3. Chymotrypsin

The ability of Ag^+ and comparator agents (at 4X MIC) to inhibit the conversion of the substrate Suc-AAPF-pNA to 4-nitroaniline by chymotrypsin was determined spectrophotometrically as described previously (Seidler *et al.*, 2003). Briefly, test compound was added to a cuvette containing 50 mM KPO_4 buffer (pH7.35), followed by addition of chymotrypsin (final concentration 0.05 μM) and Suc-AAPF-pNA (final concentration 0.15 mM). The contents of the cuvette were mixed and OD_{410} readings were taken every 30 sec for 6.5 min in a SPECTRAmax PLUS^{384} Microplate Spectrophotometer (Molecular Devices). Readings were plotted on a scatter graph in Microsoft Excel software and the rate of reaction calculated as the gradient of a trendline fitted to the data. Percentage inhibition was calculated using the equation described in section 2.6.8.2.

2.6.8.4. β-galactosidase

The effect of Ag^+ and comparator compounds (at 4X MIC) to inhibit the conversion of 4-methylumbelliferyl-β-D-galactopyranoside to 4-methylumbelliferone by β-galacotsidase was determined by fluorescence intensity. Test compound was added to the wells of a blacked-out 96-well plate containing AB buffer (100mM NaCl, 60mM K_2PO_4 and 40mM KH_2PO_4), followed by the addition of 1 U *E. coli* β-galactosidase and 4-methylumbelliferyl-β-D-galactopyranoside to a final concentration of 1 μg/ml. Reactions were incubated for 90 min at 25°C and the reaction stopped by addition of Na_2CO_3 to a final concentration of 0.2 M. Fluorescence intensity was measured in a Fluostar Optima plate reader at 460 nm, using an excitation wavelength of 365 nm. Percentage inhibition of β-galactosidase by test compounds was calculated as the fluorescence intensity relative to reactions treated with compound diluents, following subtraction of background fluorescence from each sample.

2.6.9. Transcriptome analysis

Overnight cultures of *S. aureus* SH1000 were diluted 1:100 into 27 ml of MHB and incubated at 37°C with aeration and shaking to an OD_{600} of 0.2 units. At this point 3 ml of $AgNO_3$ was added at a concentration that would inhibit growth of cultures by 25% (relative to a drug-free controls, into which 3 ml of sterile H_2O was added) over a 40 min period (Freiberg, *et al*., 2005). Following incubation, 10 ml volumes were transferred into 50 ml falcon tubes containing 20 ml of RNA protect bacterial reagent (Qiagen, Manchester, UK), vortexed for 10 sec, and then incubated without shaking at room temperature for 10 min. Following centrifugation of samples (at 5000 x *g*) and removal of supernatant, cell pellets were washed twice in TE buffer (10 mM tris (hydroxymethyl) aminomethane, 1 mM ethelynediaminetetra-acetic acid [pH 8.0]),

re-suspended in TE buffer containing 200 μg RNAse-free lysostaphin/ml, and then incubated at 37°C for 90 min with gentle mixing every 15 min. RNAse-free Proteinase K was then added to a final concentration of 40 μg/ml and the cell suspension incubated at room temperature for a further 10 min. Total RNA was extracted from cell suspensions using an RNAeasy midi kit (Qiagen) according to manufacturer's instructions, and eluted in 500 μl of nuclease-free H_2O.

Determination of RNA quality, synthesis and hybridisation of cDNA to microarrays and subsequent analysis (*i.e.* fluorescence measurements and normalisation) was performed by Roche Nimblegen (Madison, Wisconsin, USA). Microarray probe sequences were derived from the chromosomal DNA sequence of *S. aureus* 8325 and fluorescence values were normalised using quantile normalisation.

Results from microarray experiments were subsequently analysed using ArrayStar 4 software (DNAstar, Madison, Wisconsin, USA) and a ≥2-fold difference in gene expression between control and test samples (at a 99% confidence limit) was considered significant.

2.6.10. Screening of a near-saturation transposon library of S. aureus

A library of *S. aureus* SH1000 comprising ~20,500 transposants was screened for Ag^+ hypersusceptibility according to a method described previously (Blake & O'Neill, 2013). The concentration of $AgNO_3$ selected for screening transposants was determined as the concentration that inhibited the growth of 1-2 library strains per 96-well plate, without inhibiting growth of the parental strain under identical conditions.

2.7. Molecular biology techniques

2.7.1. Isolation of plasmid DNA

Plasmid DNA from *E. coli* was isolated from 3 ml of overnight culture in LBB using the QIAprep miniprep kit from Qiagen according to manufacturer's instructions. Plasmid DNA extraction from *S. aureus* followed the method above with the exception that 100 μg lysostaphin/ml was added to cells following their resuspension in buffer P1 and cells were incubated at 37^{o}C for 30 min prior to continuation of the manufacturer's protocol. In cases where high concentrations of plasmid were required, multiple minipreps were performed and the eluted plasmid DNA was concentrated by ethanol precipitation according to the method described previously (Sambrook, 2001). Plasmid DNA was stored at -20^{o}C

2.7.2. Isolation of chromosomal DNA

Chromosomal DNA from Gram-negative species and *S. aureus* was isolated using the bacterial genomic DNA purification kit from Edge biosystems (Maryland, VA, USA) following the manufacturer's instructions, with the exception that for *S. aureus* cultures 100 μg lysostaphin/ml was added to cells following their resuspension in spheroplast buffer. Cells were then incubated for 30 minutes at 37^{o}C prior to continuation of the manufacturer's protocol. All chromosomal DNA was stored at -20^{o}C

2.7.3. Polymerase chain reaction

Oligonucleotide primers for PCR were designed using Oligo software (Molecular Biology Insights inc., West Cascade, Colorado, USA) or primer 3 (Untergasser, *et al*., 2012) and ordered from Eurofins MWG operon (Ebersberg, Germany). PCR was carried out using a Techne TC-3000 thermal cycler (Bibby Scientific Ltd, Staffordshire,

UK) using Phusion high-fidelity DNA polymerase (New England Biolabs, Ipswich, Massachusetts, USA) in 25 µl volume reactions. PCR reactions consisted of 5 µl 5x Phusion HF buffer, 0.5 µl 10 mM dNTPs (Promega– consisting 2.5 mM of each dNTP), 1.25 µl of each primer (stock concentration 100 pmol/µl), 1 µl of template DNA (approx. 50-100 ng) and 15.75 µl of nuclease-free H_2O. Thermal cycling conditions consisted of initial denaturation at 98^{o}C for 1 min followed by 30 cycles of denaturation at 98^{o}C for 10 sec, annealing for 30 sec and extension at 72^{o}C for 30 sec/kb and a final extension cycle at 72^{o}C for 7 minutes. Reactions were then held at 4^{o}C until required. The annealing temperature was optimised for each reaction and was based on the melting temperature (Tm) for each primer used. Primer Tm was calculated using Phusion Tm calculator at https://www.neb.com/tools-and-resources/interactive-tools/tm-calculator (last accessed 29/06/2013)

2.7.4. Determination of DNA concentration

DNA was quantified spectrophotometrically at 260 nm using a P300 nanophotometer (Implen, Munich, Germany). The purity of the DNA obtained was determined by measuring the ratios of wavelengths 260 nm/280nm and 260nm/230nm (Sambrook, 2001)

2.7.5. Colony PCR

Colonies were picked into 20 µl of nuclease-free H_2O and 3 µl of this suspension was transferred onto an index plate of LBA containing an appropriate selection antibiotic. Colony PCR was performed using GoTaq PCR mastermix (Promega). Reactions consisted of 1.5 µl of cell suspension, 12.5 µl of GoTaq mastermix, 1.25 µl of each

primer (100 pmol/μl stock concentration) and 8.5 μl of nuclease-free water. Thermocycler cycling conditions consisted of initial denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 1 min/kb. This was followed by a final extension at 72°C for 7 min. Reactions were held at 4°C until required.

2.7.6. Agarose gel electrophoresis and DNA fragment purification

Gels comprised of 0.8% (w/v) agarose in TAE buffer containing SYBR safe DNA stain (Life Technologies). Prior to loading, samples were mixed with 6x loading dye (Promega) in a 6:1 ratio. Samples of DNA from colony PCR already contained loading buffer and so no additional buffer was added. Electrophoresis was performed in TAE buffer at 90 V for 30 min. DNA fragments required for further applications were excised from gels and purified using the MinElute gel extraction kit from Qiagen according to manufacturer's instructions.

2.7.7. DNA sequence determination (Sanger)

The nucleotide sequences of PCR products and plasmid constructs were determined by Beckman Coulter Genomics (Takeley, Essex, UK) with appropriate oligonucleotide primers. Results were analysed using Sequencher software by GeneCodes (Ann Arbor, Michigan, USA).

2.7.8. Next generation sequence determination

Samples (1 μg) of total (*i.e.* chromosomal and plasmid) DNA from *E. coli* J53 pMG101 and purified plasmid pMG101 were sent to the Leeds Clinical Molecular Genetics centre (St. James' Hosptial, University of Leeds) and nucleotide sequences determined using the Illumina sequencing-by-synthesis method (2x250 paired-end

reactions) on a MiSeq platform. *De novo* assembly of the J53 chromosome and pMG101 was performed using CLC genomics workbench, version 6 (CLC Bio, Cambridge, Massachusetts, USA).

2.7.9. Quantitative PCR (qPCR)

Total RNA was extracted from cultures using Trizol reagent (Life Technologies), following manufacturer's instructions and was resuspended in nuclease-free H_2O. RNA concentration was determined by measuring absorbance at 260 nm using a P300 nanophotometer and quality of samples were assessed by visual inspection of bands corresponding to the 16S and 23S rRNA subunits following gel electrophoresis.

First-strand cDNA synthesis was performed using Omniscript RT kit (Qiagen) with oligo-dT(15) primers and RNAse inhibitor (Promega) at final concentrations of 1 μM and 10 U respectively. qPCR was carried out in an MxPro Mx3005P thermal cycler (Agilent, Wokingham, UK) using Quantifast SYBR Green PCR kit (Qiagen) in 25 μl reactions containing 100 ng of cDNA using oligonucleotide primers specific to the gene of interest or towards 16S cDNA, which acted as an internal control. Thermocycler conditions consisted of an initial denaturation step at 95^{o}C for 5 min, followed by 35 cycles of denaturation (95^{o}C, 10 sec) and a combined annealing/extension step (60^{o}C, 30 sec). SYBR green fluorescence was measured at the end of each annealing/extension step. Relative expression from the gene of interest was calculated using the ΔΔCt method, with the abundance of 16S cDNA used as the normalising control. Calculations were performed using MxPro Mx3005P software.

2.7.10. Lambda red recombineering

Genes of interest were modified using recombineering (recombination-based genetic engineering) methods, as described previously (Sawitzke *et al.*, 2007). First, plasmid pSIM9 or pSIM18 (containing the lambda red system) were transformed into the strain of interest using the method described in section 2.7.13, with the exception that strains were recovered at 30°C to prevent loss of plasmid. Overnight cultures of transformants were diluted 1:100 in 30 ml of LBB and incubated at 30°C with shaking to an OD_{600} of 0.5 units. Lambda red functions were then induced by incubating cultures at 42°C for 15 min. Lamda red-induced cells were then made electrocompetent by washing 3 times in ice-cold H_2O and resuspending in a final volume of 200 µl H_2O. Sample volumes (50 µl) of cells were then mixed with 100-200 ng of modification cassette (see below) in a 0.1 cm-gap electroporation cuvette and pulsed in a Genepulser XCell electroporator (Biorad Ltd, Hemel Hempstead, Hertfordshire, UK) at 1.35 kV, 600 Ω and 10 µFD. Following electroporation, 1 ml of LB was added to the cuvette. The contents of the cuvette were transferred to a 50 ml Falcon tube and incubated for 2 h at 30°C with aeration and shaking (200 rpm). Cells were plated onto LBA containing 30 µg kanamycin/ml (FRT-*neoR*-FRT cassette), 10 µg chloramphenicol/ml (*cat-sacB* cassette), or 6% (w/v) sucrose (allelic replacement cassette) and incubated overnight at 30°C. Colonies were screened for the presence of gene modification by PCR amplification and sequence determination using appropriate oligonucleotide primers. Strains were cured of pSIM plasmids by of overnight culture at 37°C and checking for loss of resistance to the plasmid selection antibiotic.

2.7.10.1. Construction of disruption cassette

To disrupt a gene of interest, bipartite oligonucleotide primers were designed. Each primer consisted of 50 bases homologous to the regions flanking the gene of interest on the chromosome and an additional 20 bases, which primed to the FRT-*neoR*-FRT cassette on plasmid pKD4. Following amplification of FRT-*neoR*-FRT, the PCR product was gel purified as described in section 2.7.6 and introduced into lambda red-induced cells by electroporation.

2.7.10.2. Removal of disruption cassette

To remove the disruption cassette (and thus leave a markerless deletion), strains were transformed with plasmid pCP20 (containing FLP recombinase) as described in section 2.7.13, with the exception that strains were recovered at 30^{o}C to prevent loss of plasmid. Following transformation, strains were incubated in LBB overnight at 30^{o}C with aeration and shaking, then checked for the loss of disruption cassette by looking for absence of growth on LBA containing 30 μg kanamycin/ml. Strains were cured of pCP20 by 3 cycles of overnight culture at 37^{o}C and checking for loss of pCP20-mediated ampicillin resistance.

2.7.10.3. Allelic replacement

Genes containing mutations of interest were transferred into a recipient strain by *cat-sacB* counterselection, as previously described (Thomason *et al.,* 2007). Bipartite primers were designed as in 2.7.10.1 with the exception that the amplification target was *cat-sacB* from strain DY441, or the replacement allele from the strain of interest. Following correct recombination of the *cat-sacB* cassette into the recipient strain, sucrose hypersensitivity was confirmed by subculture onto LBA containing 6% (w/v) sucrose. Recombineering functions were again induced in the recipient strain prior to

transformation with the allelic replacement cassette, which in positive recombinants replaces the *cat-sacB* cassette and restores sucrose resistance.

Chapter three: Development of standardised methodology to assess the antibacterial activity of Ag^+, and its use in examining the prevalence of Ag^+ resistance amongst the staphylococci

Abstract

The growing prevalence of antibiotic resistance in bacteria has led to an increased interest in exploiting the antibacterial properties of Ag^+. However, there are several poorly understood aspects of Ag^+, including its activity against bacteria in both planktonic and biofilm growth states, the prevalence of Ag^+ resistance amongst bacteria isolated from patients, and the propensity for Ag^+ resistance to arise. This chapter sought to investigate these issues by using methodologies routinely used in the assessment of antibiotics. The CLSI standard method for assessing bacterial susceptibility to antibiotics was found to be suitable for use with Ag^+ in the form of $AgNO_3$. The time-kill method used for assessing antibiotic kill-kinetics revealed that at 4X MIC, Ag^+ was bactericidal against *S. aureus* in buffered solution, but bacteriostatic in growth medium. Ag^+ was unable to eradicate *S. aureus* biofilms. There was no evidence of Ag^+ resistance amongst a large collection of staphylococcal hospital isolates (*n*=1006), and continuous exposure of *S. aureus* to sub-inhibitory concentrations of Ag^+ for 42 days failed to select for endogenous Ag^+ resistance. The universal susceptibility of staphylococci to Ag^+ and the inability to select for Ag^+ resistance in *S. aureus* suggests that Ag^+ remains a viable option for the prevention and treatment of staphylococcal infections.

3.1. Introduction

3.1.1 Aspects of the antibacterial activity of Ag^+ are unclear

When bacterial infection is suspected in a patient, empirical antibacterial therapy is usually initiated, and samples (*e.g.* swabs, blood cultures, *etc.*) may be taken so that the infecting organism can be identified and the susceptibility of the organism to a range of antibacterial agents can be determined (Mandell *et al.*, 2009). This informs the clinician if the empirical therapy is likely to prove effective in treating the infection, and if not, enables selection of an antibacterial agent that provides the best clinical outcome for their patient. To assess bacterial susceptibility to antibiotics, clinical laboratories typically follow a standardised method, such as the disk-diffusion or minimum inhibitory concentration (MIC) methods published by the Clinical Laboratory Standards Institute (CLSI) and the British Society for Antibacterial Chemotherapy (BSAC) (Andrews, 2001, Wayne, 2012). This standardisation allows for susceptibility data to be compared between clinical laboratories, providing an additional advantage in that the spread of antibiotic-resistant bacteria can be followed on a national and international scale. These data can then be utilised to provide guidance to clinicians, and prevent an antibiotic being used against infections where it is likely to be ineffective.

In contrast to the situation with antibiotics, when a patient presents with an infection that may benefit from treatment with an Ag^+-containing product (*e.g.* a burn or chronic wound), determining the susceptibility of isolates to Ag^+ does not routinely take place. (Warriner & Burrell, 2005; Ip *et al.*, 2006a). A major reason for this appears to be that there is currently no standardised method for determining bacterial susceptibility to Ag^+; therefore, of the limited number of Ag^+ susceptibility surveys that have been published, each have used methodologies that differ in the use of culture media,

Ag^+ formulations, bacterial inoculum densities, and incubation conditions. As a consequence results between studies differ and are therefore not directly comparable (Carr *et al.*, 1973; Vasishta *et al.*, 1989; Maple *et al.*, 1992; Hamilton-Miller *et al.*, 1993; Ug & Ceylan, 2003). Ultimately, the lack of routine Ag^+ susceptibility testing means that the current prevalence of Ag^+ resistance amongst common wound pathogens is unclear. This omission in testing could considerably impact the morbidity or mortality of patients treated with Ag^+-containing products if they are infected with an Ag^+-resistant isolate. It is therefore apparent that if the use of Ag^+-containing products for the treatment of bacterial infection is to continue, the issues described above should be rectified and a standardised method for assessing and interpreting bacterial susceptibility to Ag^+ should be introduced.

As with determining bacterial susceptibility to Ag^+, there is currently no standardised method for assessing the killing kinetics of Ag^+-containing compounds; therefore the killing (bactericidal) activity of Ag^+ is also unclear. Indeed, the lack of a standardised methodology has led to reports that Ag^+ is bactericidal (*i.e.* capable of reducing bacterial cell viability by $\geq$3-$\log_{10}$ cfu/ml over 24 h exposure) in some studies (Matsumura *et al.*, 2003), and bacteriostatic (<3-$\log_{10}$ reduction) in others (Gordon *et al.*, 2010). Burns and chronic wounds are typically poorly vascularised, resulting in a limited presence of immune system components at the site of infection once it occurs (Warriner & Burrell, 2005). As a consequence it would be beneficial for Ag^+-containing compounds to be bactericidal, as treatment would then not be as reliant upon the patient's immune response to clear the infection (as would be the case if using a bacteriostatic agent).

In addition to establishing the killing activity of Ag^+ against bacteria growing in a planktonic (free-living) state, it would also be of use to determine the ability of Ag^+ to

eradicate biofilm cultures of bacteria. Biofilms are sessile communities of bacteria encased in a matrix of extracellular polymeric substances (EPS) consisting of proteins, polysaccharides and DNA (Costerton *et al.*, 1999). In addition to forming on plastics, abiotic surfaces coated in plasma, and environments exposed to high levels of hydrodynamic shear (Costerton *et al.*, 1999), it is becoming increasingly apparent that chronic wound infections contain bacteria in a biofilm state (James *et al.*, 2008). Once formed, biofilms are highly recalcitrant to clearance by the host immune system and antibacterial chemotherapy, a phenomenon attributable to several key properties of the biofilm. For example, EPS can act as a permeability barrier to immune system effectors, and can bind aminoglycosides by charge interactions, in both cases preventing them reaching their microbial target (Shigeta *et al.*, 1997; Costerton *et al.*, 1999). Furthermore, high populations of non-growing and 'persister' cells are encountered within a biofilm (Eng *et al.*, 1991; Kint *et al.*, 2012), which, as a consequence of their attenuated metabolic activity, are tolerant to the killing activities of agents such as the β-lactams and fluoroquinolones (Eng *et al.*, 1991; Kint *et al.*, 2012). A number of studies have demonstrated that Ag^+ can inhibit biofilm formation, or prevent the further proliferation of an existing biofilm (De Prijck *et al.*, 2007; Stobie *et al.*, 2008). However, as biofilms are recalcitrant to clearance by the host immune system, the simple inhibition of biofilm growth by Ag^+ would be insufficient to achieve clinical cure, as upon cessation of Ag^+ therapy, the biofilm would once again proliferate (Jensen *et al.*, 2010). Against biofilms of *P. aeruginosa* it was suggested that silver sulphadiazine at concentrations ≥ 5 μg/ml could cause complete eradication (Bjarnsholt *et al.*, 2007). This study used a *P. aeruginosa* strain constitutively expressing GFP to identify "live" cells and the stain propidium iodide to identify "dead" cells when visualised by confocal microscopy. However, it is unclear if metabolically inert cells of

P. aeruginosa within a biofilm would express GFP, or if propidium iodide could successfully penetrate the entirety of a biofilm, thus it is not apparent from this study if complete biofilm eradication can be achieved with Ag^+, which necessitates further studies using an appropriate testing methodology.

3.1.2. The propensity for bacterial Ag^+ resistance to arise is unknown

As discussed above, the lack of a standardised method for evaluating bacterial susceptibility to Ag^+ has prevented the prevalence of bacterial Ag^+ resistance amongst clinical isolates from being established. In addition, the propensity for bacterial Ag^+ resistance to arise and spread is not known. Endogenous (mutational) resistance to Ag^+ has reportedly been selected in the laboratory by continuous exposure of *E. coli* and *S. aureus* to sub-inhibitory Ag^+, with the MIC of $AgNO_3$ increasing from 4 and 8 μg/ml against parental strains of *E. coli* and *S. aureus*, respectively, to >256 and 70 μg/ml against resistant strains (Li *et al*., 1997; Kaur *et al*., 1987). However, in neither study was the time taken for resistance to arise reported, a factor which is important to establish the rapidity with which endogenous Ag^+ resistance might arise in a patient treated with Ag^+. Exogenous (horizontally-acquired) Ag^+ resistance has been found in strains of *Salmonella* Typhimurium and *Enterobacter cloacae* isolated from patients (McHugh *et al*., 1975; Lansdown & Williams, 2007); however, the propensity for this mechanism to spread has not been established, and it is not known if exogenous Ag^+ resistance currently exists, or can arise in Gram-positive bacteria commonly isolated from wounds, such as *S. aureus*. Furthermore, in all cases of Ag^+ resistance discovered thus far, the metabolic (fitness) cost associated with the resistance phenotype has not been assessed. All this information is vital to provide an indication for an Ag^+ resistance phenotype to emerge, persist and spread in any given environment.

3.1.3. Use of the staphylococci as a model genus for this study

Members of the genus *Staphylococcus* are Gram-positive, catalase-positive cocci that are facultative anaerobes, and can be transient or permanent commensals of the microbiota on the skin and mucous membranes of a variety of mammals, including humans (Mandell *et al*., 2009). *Staphylococcus epidermidis* is the predominant species isolated from humans; however other species, such as *S. aureus*, *S. capitis* and *S. hominis* are also frequently isolated. Although all these species can cause opportunistic infection in the human host, *S. aureus* is the most pathogenic, as a consequence of harbouring an array of virulence factors that enable it to cause infections ranging from the relatively benign (*e.g.* boils and impetigo) to the severe and life-threatening (*e.g.* abscesses, endocarditis, pneumonia and bacteraemia) (Mandell *et al.*, 2009). Of interest to this study, however, is that members of the genus *Staphylococcus* (*S. aureus* in particular) can frequently colonise and cause wound infections that are resistant to antibiotics (Rezaei *et al.*, 2011; Anderson & Kaye, 2009). *Staphylococcus* therefore represents a genus of bacteria against which Ag^+ would need to display antibacterial activity in order to retain clinical utility. For the same reason, it would also be important to ascertain the susceptibility of clinical isolates of this genus to Ag^+, and establish the propensity for Ag^+ resistance to emerge, persist and spread by endogenous and exogenous mechanisms. As a consequence staphylococci were used as model organisms in the studies presented in this chapter.

3.2 Objectives

The objectives of the work presented in this chapter were:

- to develop a standardised methodology for assessing bacterial susceptibility to Ag^{+}
- to establish the bactericidal activity of Ag^{+} against planktonic and biofilm cultures of *S. aureus*
- to elucidate the prevalence of Ag^{+} resistance amongst patient isolates of staphylococci using a standardised methodology
- to establish the propensity for Ag^{+} resistance to emerge in *S. aureus*

3.3 Results and discussion

3.3.1. Antibacterial activity of Ag^+

3.3.1.1 Validation of methodology for Ag^+ susceptibility testing

In testing of bacterial susceptibility to antibiotics, clinicians and researchers typically follow the agar dilution or broth microdilution methods described by BSAC or CLSI (Andrews, 2001, Wayne, 2012). These methodologies are almost identical, although BSAC recommends the use of Iso-Sensitest (IS) media, whilst CLSI recommends Mueller-Hinton (MH) media. Although originally developed as a medium for growing *Neisseria* spp. (Mueller & Hinton, 1941), MH medium was found to be suitable for performing antibacterial susceptibility testing, as it contains low concentrations of thymine and thymidine (which are antagonists of sulphonamides and trimethoprim) and includes sufficient Mg^{2+} and Ca^{2+} to maintain the activity of aminoglycosides and tetracyclines, respectively (Bauer *et al.*, 1966, Wayne, 2012). Nevertheless, there have been reports of batch-to-batch variability with MH medium, leading to variations in its performance (Barry & Effinger, 1974). Although this has been rectified by the CLSI with the standardisation of MH composition (CLSI standard M6-A), IS has been adopted by some groups, due to it being a semi-defined media that enables better standardisation of composition between commercial suppliers (Andrews, 2001).

If either CLSI or BSAC methodologies are suitable for use in determining bacterial susceptibility to Ag^+, then they could be quickly adopted by microbiology reference centres, given that these methods are already in use to monitor antibiotic resistance. Therefore, $AgNO_3$ MICs were determined against *S. aureus* strains SH1000, MRSA252, ATCC 29213 and ATCC 25923 and *E. coli* strains BW25113 and J53-pMG101 (an Ag^+-resistant strain), using both agar dilution and broth microdilution methods in IS or

MH media according to CLSI/BSAC guidelines. As a comparison, bacterial susceptibility to Ag^+ was also determined in Luria-Bertani (LB) broth and agar, due to its use in previous studies on the antibacterial activity and mode of action of Ag^+ (Li *et al.*, 1997; Gupta *et al.*, 1999; Feng *et al.*, 2000) (Table 3.1). Silver nitrate ($AgNO_3$) was chosen as the source of Ag^+ for this study and the remaining studies throughout this thesis, as unlike most other antibacterial Ag^+ formulations (*i.e.* silver sulphadiazine, silver nanoparticles) it can be readily dissolved in water and culture media.

Table 3.1. Determination of bacterial susceptibility to Ag^+ on various culture media using agar dilution or broth microdilution

Strain	$AgNO_3$ MIC (μg/ml)					
	ISA	**ISB**	**LBA**	**LBB**	**MHA**	**MHB**
SH1000	32	32	8	8	16	16
MRSA252	32	32	8	4	16	8
ATCC 29213	32	16	8	8	8	8
ATCC 25923	32	32	4	4	16	8
BW25113	8	8	4	4	4	4
J53-pMG101	>256	>256	>256	>256	>256	>256

ISA/ISB: Iso-Sensitest agar/broth; LBA/LBB: Luria-Bertani agar/broth; MHA/MHB: Mueller-Hinton agar/broth.

IS media inhibited Ag^+ activity to a greater extent than MH and LB media as demonstrated by the higher MIC values obtained. This difference in Ag^+ activity is likely due to differing amounts of inhibitory components in each culture medium. LB media utilises a pancreatic digest of casein (*i.e.* tryptone) as a source of nitrogen and amino acids for bacterial metabolism, whereas IS and MH media utilise an acid hydrosylate of casein (Atlas, 2010). Pancreatic digests lead to an incomplete breakdown of casein compared with acid hydrolysis, thus in tryptone (and therefore LB), there may be fewer exposed amino acid side-chains capable of binding and inactivating Ag^+ (such

as thiol groups), meaning there is more active Ag^+ present to inhibit bacterial growth. Although IS and MH media both contain casein hydrosylate, IS media is supplemented with additional cysteine (Atlas, 2010), which is likely responsible for the reduced activity observed. As MH medium is standardised (unlike LB) and inhibited Ag^+ activity to a lesser extent than IS (thus providing greater sensitivity when comparing Ag^+ susceptibility between isolates), the CLSI method for antibiotic susceptibility testing (using MH medium) was employed for all remaining studies investigating the antibacterial activity of Ag^+.

3.3.1.2. Bactericidal activity of Ag^+ against S. aureus

As with determining bacterial susceptibility to Ag^+, there is currently no standardised method for assessing the killing kinetics of Ag^+-containing compounds, which prevents direct comparison of results between studies. In establishing the bactericidal activity of antibiotics, the 'time-kill' method is routinely used; in this method exponential-phase bacterial cultures of a defined density (usually $5x10^8$ cfu/ml) are exposed to a test compound at 4X MIC (Oliva *et al.*, 2003). At regular time points, the number of viable bacteria remaining within a culture are determined and subsequently used to establish the kill-kinetics of the test compound (Oliva *et al.*, 2003). To establish if this method would give reproducible results with Ag^+, exponential-phase cultures of *S. aureus* SH1000 were exposed to $AgNO_3$ and comparator compounds at 4X MIC, and viable cell counts determined every 30 min for 6 hours (Figure 3.1). Bacterial cultures were suspended in MHB, to maintain consistency with the media used in susceptibility testing.

Against *S. aureus* cultures in MHB, Ag^+ exhibited only weak killing activity, causing a <1 $\log_{10}$ drop in cell viability over the time course. This result was reproducible and was

found to be comparable to that obtained for fusidic acid, a bacteriostatic antibiotic frequently used in the treatment of topical staphylococcal infections (Brown & Thomas, 2002). After 24 h exposure to $AgNO_3$, a 2.2 ± 0.09 log_{10}-drop in cell viability was observed and would therefore define Ag^+ as a bacteriostatic compound. Cultures exposed to daptomycin or nisin underwent a >7-log_{10}-drop in cell viability after 24 h, defining these compounds as bactericidal, which is in agreement with previous studies (Hobbs *et al.*, 2008).

Prior studies that have assessed the bactericidal activity of Ag^+ have produced conflicting data, with some studies indicating that $AgNO_3$ is bacteriostatic, and others suggesting that it is bactericidal (Matsumura *et al.*, 2003; Gordon *et al.*, 2010). As components of culture media (*i.e.* protein and sodium chloride) affect the inhibitory activity of Ag^+ (Liau *et al*., 1997; Gupta *et al*., 1998), it seems likely that a similar effect will be observed when evaluating the killing activity of Ag^+. To assess this, the above experiment was repeated, with the exception that *S. aureus* SH1000 cultures were re-suspended in HEPES-glucose buffer (which lacks culture media components) (Figure 3.1). Whilst only a slight increase in bactericidal activity was observed for comparator agents nisin and daptomycin relative to that seen against cultures in MHB, Ag^+ was rapidly bactericidal in buffer, with a >7 log_{10}-drop in cell viability observed after 10 min exposure.

The fact that culture media components have a dramatic impact on the killing activity of Ag^+ not only provides further indication of the need of a standardised method to assess the antibacterial activity of Ag^+ products, but also raises questions as to the activity of Ag^+ when it is employed therapeutically in wounds. Both acute and chronic wounds can secrete fluid (exudate) with a high protein and salt content that could bind (and therefore inhibit the antibacterial activity of) Ag^+ released from Ag^+-containing products

(Cutting, 2003). However, although there have been numerous studies into the bactericidal activity of silver preparations *in vitro*, none appear to have taken into account the high protein content of the wound environment. In consequence, these studies could be over-estimating the true bactericidal activity of these dressings *in vivo*. It is therefore apparent that future studies should seek to employ a method of assessing the bactericidal activity of Ag^{+}-containing products in an environment more analogous to that found in wounds to address this issue.

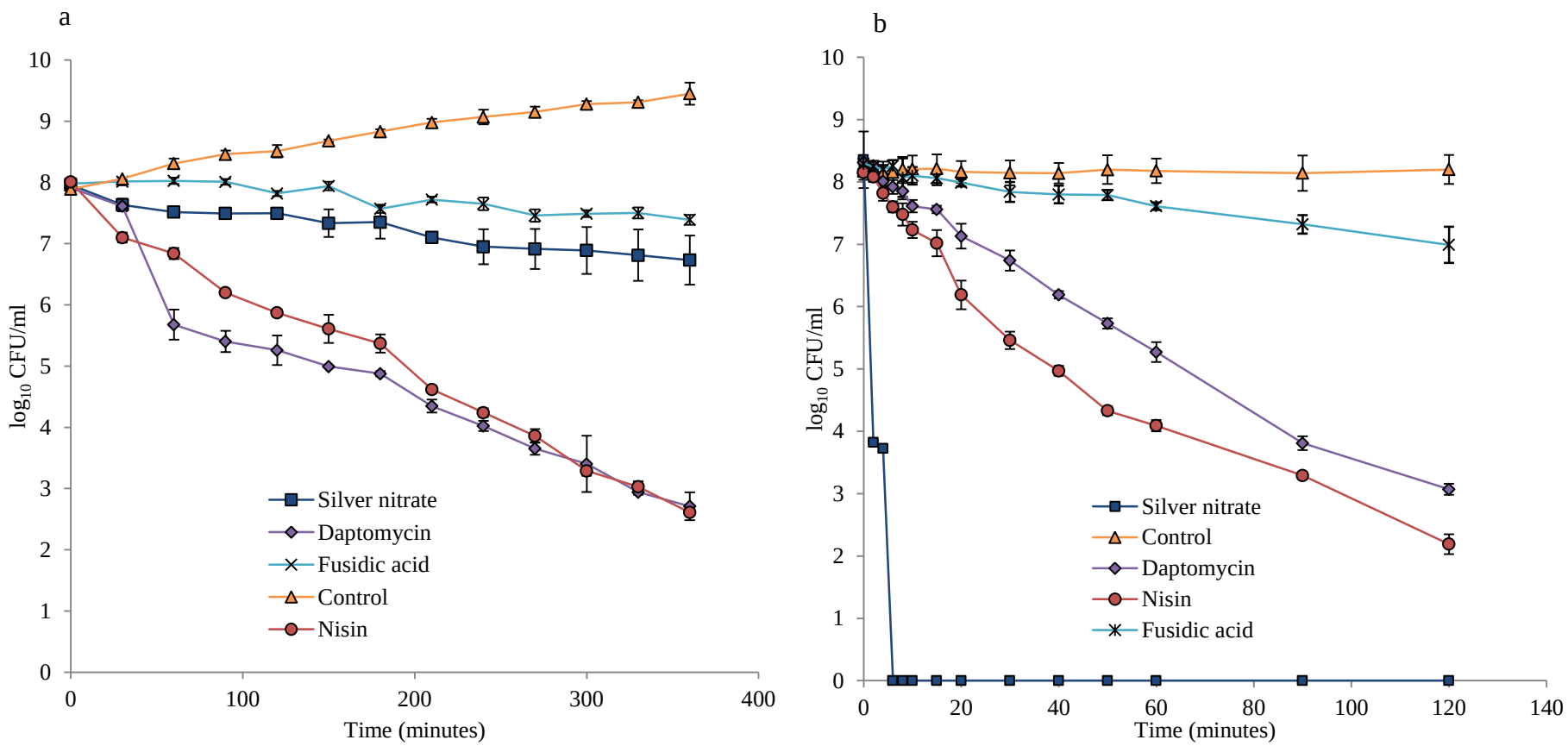


Figure 3.1: Effects of $AgNO_3$ and comparator agents on the viability of exponential cultures of *S. aureus* SH1000 over a 6 h period in MHB (a) and over a 2 h period in cultures re-suspended in 5mM HEPES-glucose buffer (b). Values shown are the means of three replicates from three independent experiments. Error bars represent the standard deviation from the mean.

3.3.1.3. Activity of Ag^+ against biofilm cultures of S. aureus

Bacteria growing as a biofilm can cause persistent infections that are highly refractory to antibacterial therapy and may present in environments where Ag^+ is used, such as in chronic wounds (James *et al.*, 2008). Therefore the ability of Ag^+ to inhibit and eradicate biofilms of SH1000 and MRSA252 grown on the Calgary biofilm device was assessed, as determined by the biofilm MIC (bMIC) and minimum biofilm eradication concentration (MBEC) methods, respectively (Ceri *et al.*, 1999) (Table 3.2). These methods have previously been utilised for the rapid screening of compounds for anti-biofilm activity (Ceri *et al.*, 1999; Ooi *et al*., 2010). At a concentration of 16 μg/ml, $AgNO_3$ could prevent shedding of planktonic cells from established biofilms *in vitro* (Table 3.2). This may indicate that, *in vivo*, Ag^+ could prevent further biofilm growth and dissemination to other sites. However, as this bMIC is equivalent to the MIC of Ag^+, it seems likely that Ag^+ inhibits the growth of planktonic cells following their release from the biofilm and has no direct effect on the viability of the biofilm itself. Indeed, Ag^+ was unable to eradicate established biofilms (MBEC >256 μg/ml) (Table 3.2). This may be a consequence of Ag^+ binding to negatively charged extracellular polymeric substances within the biofilm, thereby preventing Ag^+ from reaching cells in sufficient concentration to exert an antibacterial effect (Chaw *et al.*, 2005).

With the exception of XF-73, all control compounds were also unable to eradicate biofilm cultures at the concentrations tested (MBEC= >256 μg/ml, or >64 μg/ml for nisin) (Table 3.2), which is in agreement with previous studies (Ooi *et al.*, 2010). In contrast, XF-73, as previously reported (Ooi *et al.*, 2010), displayed potent activity against both SH1000 and MRSA252 biofilms, with an MBEC of 2 μg/ml (Table 3.2).

Table 3.2: Activity of $AgNO_3$ and comparator compounds against biofilms

Compound	bMIC (μg/ml)		MBEC (μg/ml)	
	SH1000	**MRSA252**	**SH1000**	**MRSA252**
Silver nitrate	16	16	>256	>256
Vancomycin	2	1	>256	>256
Tetracycline	0.5	1	>256	>256
XF-73	1	2	2	2
Fusidic acid	0.25	0.125	>256	>256
Daptomycin	2	2	>256	>256
Nisin	16	16	>64	>64

bMIC: biofilm minimum inhibitory concentration; MBEC: Minimum biofilm eradication concentration. Results are representative of at least three independent experiments

As Ag^+-containing dressings typically release Ag^+ at concentrations ranging from 70-100 μg/ml (Warriner & Burrell, 2005), the results obtained from this study would suggest that such dressings would be unsuitable for the treatment of chronic wounds with a biofilm component. Indeed, during the course of this study, a separate investigation revealed that a range of commercial Ag^+-containing dressings were unable completely to eradicate biofilms of *S. aureus, P. aeuriginosa* or *E. coli* grown in a chronic wound model (Kostenko *et al.*, 2010). However, given this result, it is unclear what could be used in place of Ag^+ to treat chronic wound infections with a biofilm component. As demonstrated in this and in prior studies, antibiotics currently in clinical use are also ineffective at eradicating biofilms. There is evidence to suggest that biocides such as hydrogen peroxide, *N*-propanol and a commercial mixture of propanol, ethanol and chlorhexidine can eradicate biofilms of *S. epidermidis* (Presterl *et al*., 2007); however, these compounds are also known to reduce the healing of wounds drastically. The novel porphyrin compound, XF-73 was found to be able to eradicate

biofilms at a concentration close to its MIC and would therefore be of benefit in treating biofilm-associated infections. This compound is currently in stage-one clinical trials as a topical antibacterial agent (I. Chopra, personal communication). Nevertheless, this study highlights the urgent need to develop new compounds with anti-biofilm activity for use in antibacterial chemotherapy.

3.3.2. Staphylococcal resistance to Ag^+

3.3.2.1. Survey of staphylococcal isolates for Ag^+ resistance

In order to provide a comprehensive assessment of current Ag^+ susceptibility amongst the staphylococci, a large collection of isolates obtained from a range of locations should be surveyed (Chopra, 2007). To fulfil these criteria, a collection of 1006 staphylococcal isolates was assembled for this study, primarily consisting of isolates obtained from patients in a number of hospitals from the UK, Denmark, Italy, France and Canada. Of the 1006 isolates, 876 were *S. aureus* (including meticillin-resistant and vancomycin-intermediate strains) and 130 were coagulase-negative staphylococci (CoNS), representing 18 different species/subspecies (Table 3.3). At least 230 isolates in the collection came from patients with burns and diabetic foot ulcers in clinics where therapeutic Ag^+ was known to be in use at the time of isolation. Silver nitrate MICs were determined against each isolate using the CLSI agar dilution method (*i.e.* using MH medium).

Table 3.3. Staphylococcal species present in culture collection

Species	Number of isolates
S. aureus	876 (comprising 706 MSSA, 166 MRSA and 4 VISA isolates)
S. epidermidis	38
S. rostri	10
S. lugdenensis	8
S. haemolyticus	7
S. intermedius	7
S. saprophyticus	7
S. sciuri	6
S. simulans	6
S. xylosus	6
S. caprae	5
S. hominis	5
S. schleiferi	5
S. capitis	4
S. cohnii (subspecies *cohnii*)	4
S. cohnii (subspecies *urealyticum*)	3
S. hominis (subspecies *novobiosepticum*)	3
S. pseudintermedius	3
S. warneri	3

All isolates from the collection were found to be susceptible to $AgNO_3$ at concentrations between 8 and 16 μg/ml, with the exception of a single isolate of *S. intermedius* for which $AgNO_3$ had an MIC of 4 μg/ml. As there are no breakpoints for Ag^+, the $AgNO_3$ MIC that corresponds to clinically significant Ag^+ resistance in staphylococci is unknown (Chopra, 2007). However, as the $AgNO_3$ MICs obtained against isolates in this study are equal to that of SH1000, a laboratory strain of *S. aureus*, and are significantly lower than that obtained for a known Ag^+-resistant strain (J53-pMG101) it can be assumed that all isolates in this study would be susceptible to therapeutic concentrations of Ag^+. The universal susceptibility of staphylococci to Ag^+, as suggested by this study, would indicate that Ag^+ resistance is unlikely to act as a barrier to the continued use of Ag^+ as an alternative for the treatment of topical staphylococcal skin infections, including those that are resistant to commonly used topical antibiotics.

However, surveillance of bacterial susceptibility to Ag^+ needs to continue, particularly in clinical settings where Ag^+ is frequently used. As the method described in this study produced consistent, reproducible results, it could be utilised by clinicians to determine susceptibilities of wound isolates to Ag^+. Indeed, as this method is based on CLSI guidelines that clinical laboratories routinely follow for antibiotic susceptibility testing, it would be straightforward for this method to be included in their testing regimens. By employing this method, clinicians could be alerted to emergence of Ag^+ resistance and consequently change their prescribing practise accordingly.

3.3.2.2. Evaluating the propensity for Ag^+ resistance to emerge in S. aureus via endogenous mechanisms

This study has demonstrated that staphylococci isolated from hospital patients appear universally susceptible to Ag^+. However, the increasing exploitation of Ag^+ for its antibacterial properties will likely provide sufficient selection pressure for the emergence of Ag^+-resistant staphylococci at some point in the future (Chopra, 2007). It would therefore be of benefit to ascertain the ease with which Ag^+ resistance can emerge in a laboratory setting, as this could be used to inform clinicians and appropriate regulatory bodies of the potential risks in using therapeutic Ag^+ without appropriate stewardship. Although a previous study revealed that endogenous resistance to Ag^+ could be selected in *S. aureus*, the time taken for resistance to arise, the mechanism of resistance, or the fitness cost associated with the resistance phenotype was not assessed (Kaur *et al.*, 1987). The present study therefore sought to select endogenous Ag^+ resistance in *S. aureus*, and characterise the mechanism and fitness cost associated with any Ag^+-resistance phenotype that arises.

To select for endogenous Ag^+ resistance, *S. aureus* strains SH1000 and MRSA 252 (a strain representative of the EMRSA-16 lineage endemic to hospitals in the UK), were continuously exposed to sub-inhibitory concentrations of Ag^+ in the form of repeated microdilution broth MICs, using an extended concentration gradient of $AgNO_3$. The extended range of $AgNO_3$ concentrations would in principle allow for incremental increases in Ag^+ resistance to occur, and has been previously used to select for endogenous resistance to daptomycin, a lipopeptide antibiotic reserved for treating infections caused by multidrug-resistant *S. aureus* and vancomycin-resistant enterococci (VRE) (Friedman *et al.*, 2006). Continuous Ag^+ exposure was carried out for six weeks (42 days), as this is the typical amount of time in which a patient with a burn or chronic wound is treated with an Ag^+-containing product (Dr Chris Roberts, Smith and Nephew, personal communication). Parallel experiments were also performed using daptomycin instead of $AgNO_3$ (Figure 3.2).

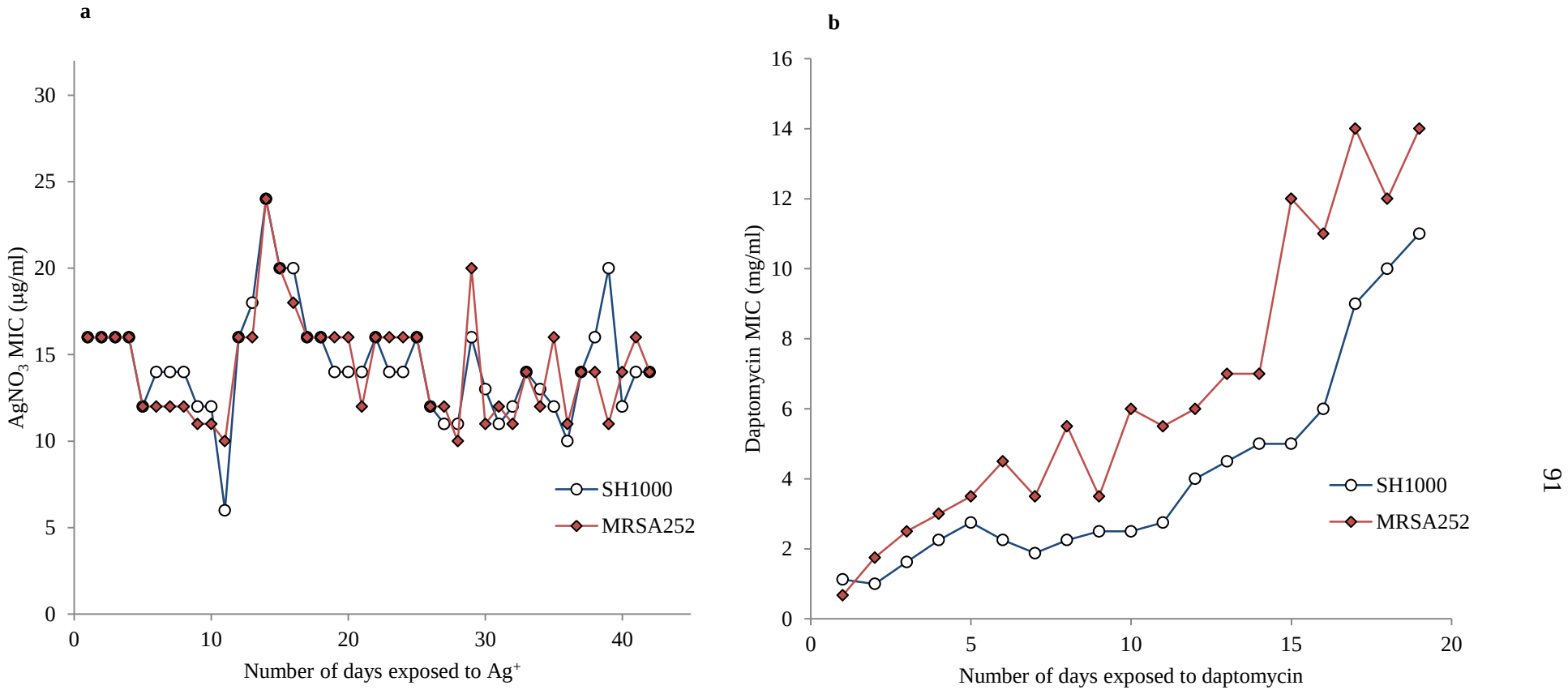


Figure 3.2: Susceptibility of *S. aureus* SH1000 and MRSA252 continuously passaged in sub-inhibitory concentrations of $AgNO_3$ (a) or daptomycin (b).

After 42 days of continuous exposure to Ag^+, selection of *S. aureus* strains with reduced susceptibility to Ag^+ did not occur. This result appears to contrast with that obtained by Kaur *et al*. (1987); however as this prior study did not reveal the length of selection necessary for the emergence of Ag^+ resistance, it is possible that the length of selection used in the present study was insufficient to select an Ag^+ resistance phenotype. Alternatively, the strain of *S. aureus* used by Kaur *et al*. may have possessed genetic factors that predisposed it to developing Ag^+-resistance. Nevertheless, the inability to select for Ag^+-resistance in *S. aureus* strains over such a prolonged period indicates that the emergence of Ag^+ resistant *S. aureus* by endogenous mechanisms in a clinical setting is likely to be rare.

In contrast to Ag^+ resistance, continuous exposure of strains to daptomycin resulted in rapid selection of isolates with a reduced daptomycin susceptibility. Daptomycin had an MIC of 3 and 2.25 μg/ml against isolates of MRSA252 and SH1000 respectively following continuous exposure for four days, which is in both cases above the resistance breakpoint considered by the CLSI for clinically-relevant daptomycin resistance (≥2 μg/ml) (Wayne, 2012). After further exposure (20 days), isolates were selected to which daptomycin had an MIC of >10 μg/ml.

3.4 Conclusions

This study has demonstrated that the susceptibility of bacterial isolates to Ag^+ can be determined by the CLSI agar dilution method used for antibiotic susceptibility testing. By employing this method, it was determined that staphylococci isolated from hospital patients are universally susceptible to Ag^+. Given the substantial sample size and geographical diversity of isolates used in this study, the results obtained indicate that the prevalence of staphylococcal Ag^+ resistance in the clinical environment is minimal or non-existent. In addition, the inability to select endogenous Ag^+ resistance in *S. aureus* over a period of 6-weeks would suggest that the propensity for staphylococcal Ag^+ resistance to arise in the future is limited. These results would therefore indicate that Ag^+-containing compounds remain a promising alternative for the treatment of topical staphylococcal infections that are resistant to commonly used antibacterials. Nevertheless, although the probability of staphylococcal Ag^+ resistance emerging is low, clinicians would be advised to utilise the standardised method presented in this study to monitor Ag^+ susceptibility amongst clinical isolates and contain the spread of Ag^+ resistance if it emerges.

While the above results favour the continued use of Ag^+, staphylococcal biofilms were found to be refractory to eradication by Ag^+ at concentrations higher than that found in commercially available Ag^+ dressings. In addition, Ag^+ was shown to be bacteriostatic against planktonic cultures of *S. aureus* at 4X MIC. This would suggest that the utility of Ag^+ is likely to be limited for treating wound infections that are poorly vascularised (where the host immune response would be compromised), or contain bacteria in a biofilm mode of growth. As there is little information as to the true efficacy of Ag^+-containing dressings in the treatment of chronic wound infections (Bergin &

Wraight, 2006; Vermeulen *et al.*, 2007), it is clear that further *in vivo* studies are required to justify the continued use of Ag^{+} in this manner.

Chapter four: Antibacterial mode of action of Ag^+

Abstract

The mechanism by which Ag^+ exerts its antibacterial effect is unclear. Although Ag^+ can bind to a range of cellular macromolecules, the link between such binding and any subsequent impact on cell viability has yet to be established. Furthermore, Ag^+ exposure has also been proposed to induce the formation of reactive oxygen species that are known to cause oxidative damage to intracellular components and may therefore also contribute to the antibacterial mode of action of Ag^+. Using mode of action studies frequently used in the pre-clinical evaluation of new antibacterial agents these aspects were explored in *S. aureus*.

Exposure of *S. aureus* to Ag^+ at 4X MIC resulted in a rapid (10 min) and simultaneous inhibition of the the incorporation of radiolabelled precursors into DNA, RNA, protein and peptidoglycan, which is a hallmark of a nonspecific antibacterial effect typically associated with membrane damage. Membrane damage was confirmed by the *Bac*Light™, K^+-leakage and $DiSC_3(5)$ assays. Against carboxyfluorescein-filled liposomes with a phospholipid content analogous to that of *S. aureus* membranes, Ag^+ caused no overt damage, suggesting Ag^+ does not cause membrane damage by interfering with the phospholipid bi-layer. DNA microarray analysis of Ag^+-exposed cultures revealed a substantial number of genes encoding membrane proteins were up regulated, together with genes encoding anaerobic mechanisms of energy utilisation. Taken together, these results suggest that Ag^+-mediated membrane damage arises from Ag^+ binding to and disrupting the structure of integral membrane proteins, which is sufficient to permit leakage of intracellular components.

Exposure of *S. aureus* to Ag^+ resulted in the generation of ROS, as evidenced by increased activity of Ag^+ against strains lacking ROS-protection mechanisms. The source of ROS is likely from Ag^+ damage to the electron transport chain components as evidenced by the up-regulation of genes involved in anaerobic mechanisms of energy generation. However, as the killing and membrane-damaging activity of Ag^+ were not perturbed under anaerobic conditions, the generation of ROS does not appear to contribute directly to the antibacterial MOA of Ag^+.

4.1. Introduction

Silver has been used for centuries to treat infection (Klasen, 2000a). Its broad spectrum of activity and relatively minimal toxicity make it an ideal topical antibacterial agent. However, the mechanism by which the silver cation (Ag^+) exerts an antibacterial effect is poorly understood. Given the clinical success of Ag^+, defining its antibacterial mode-of-action (MOA) would be beneficial as these properties could, for example, help identify compounds that may have synergistic activity when used in combination with Ag^+, allow for the development of Ag^+-containing compounds with improved activity or reduced toxicity to enable systemic usage, or be used to guide the rational design of future antibacterial agents.

As described in Chapter 1, a myriad of cellular targets are purportedly affected by Ag^+, including DNA, enzymatic processes and the cell membrane. However, the ability of Ag^+ to inhibit enzymatic processes *in vitro* does not necessarily imply that this contributes to the antibacterial MOA of Ag^+ *in vivo*. Furthermore, studies that have reported effects of Ag^+ on DNA have utilised prolonged (≥4 h) incubation times, and the possibility that Ag^+-mediated DNA damage is secondary to other effects of Ag^+ cannot therefore be excluded (Feng *et al.*, 2000). An imaging study using TEM revealed that Ag^+ exposure resulted in detachment of the cell membrane from the cell wall and prompted the formation of structural defects within the bacterial membrane, which is indicative of membrane damage (Jung *et al.*, 2008). However, this study again exposed strains to Ag^+ for a prolonged period (>2 h); thus this effect may too be secondary to the primary action of Ag^+. Nevertheless, Lok *et al.* (2006) demonstrated that against *E. coli*, Ag^+ caused a rapid (5 min) loss of membrane potential and leakage of K^+ and ATP from cells (Lok *et al.*, 2006), which would seem to suggest a role for the membrane in the antibacterial MOA of Ag^+ that warrants further investigation.

In addition, Ag^+-mediated inhibition of components of the electron transport chain (such as NADH dehydrogenase) is known to stall electron transfer and generate favourable conditions for O_2^- generation (Bragg & Rainnie, 1974, Messner & Imlay, 1999), and subsequent production of the toxic hydroxyl radical ($HO^{\cdot}$) via the Fenton reaction (Gordon *et al*., 2010). However, it is unclear to what extent $HO^{\cdot}$ contributes to the antibacterial activity of Ag^+, as methods previously used to detect $HO^{\cdot}$ were recently shown to be unreliable (Keren *et al.*, 2013). Furthermore, results from studies demonstrating the antibacterial activity of Ag^+ under anaerobic conditions (where $HO^{\cdot}$ would not be generated), are contradictory, with one study indicating that the antibacterial activity of Ag^+ is perturbed under anaerobic conditions (Park *et al*., 2009), and another finding no difference in Ag^+ activity between aerobic and anaerobic environments (Xiu *et al*., 2011). It is therefore clear that further studies are required to define the role of $HO^{\cdot}$ (and ROS in general) in the antibacterial MOA of Ag^+.

4.2. Objectives

The objectives of the work presented in this chapter were

- to investigate the antibacterial MOA of Ag^+ against *S. aureus*, using assays routinely employed as part of the pre-clinical evaluation of new antibacterial drugs.
- to determine if *S. aureus* generates ROS upon exposure to Ag^+ and to establish if sufficient ROS are generated to overcome intrinsic protection mechanisms and contribute to the antibacterial activity of Ag^+.

4.3. Results and Discussion

Monitoring the ability of an antibacterial compound to inhibit biosynthesis of cellular macromolecules can provide insights into its mode of action (O'Neill & Chopra, 2004). As most antibacterial agents inhibit DNA, RNA, protein or peptidoglycan biosynthesis, prior studies have measured the rate at which radiolabelled precursors are integrated into these macromolecules (Oliva *et al.*, 2001; Oliva *et al.*, 2004; Hobbs *et al.*, 2008; Ooi *et al.*, 2009). By comparing the rate of incorporation between drug-exposed and drug-free cultures, inhibition of macromolecular synthesis can be detected and quantified. Thus, the ability of $AgNO_3$ to inhibit incorporation of radiolabelled precursors into DNA, RNA, protein and peptidoglycan was assessed and compared with the activity of antibacterial agents known specifically to inhibit these biosynthetic pathways (ciprofloxacin, rifampicin, tetracycline and vancomycin, respectively) (Figure 4.1). In contrast to the control compounds, $AgNO_3$ caused substantial and comparable inhibition of all four biosynthetic pathways after 10 min. This non-specific inhibition is indicative of disruption to energy producing metabolic pathways, or membrane damage sufficient to cause leakage of intracellular components essential for cell viability. Indeed, this inhibition profile has been described before for the membrane-damaging agents XF-73, daptomycin and clofazimine (Oliva *et al.*, 2004; Hobbs *et al.*, 2008; Ooi *et al.*, 2009).

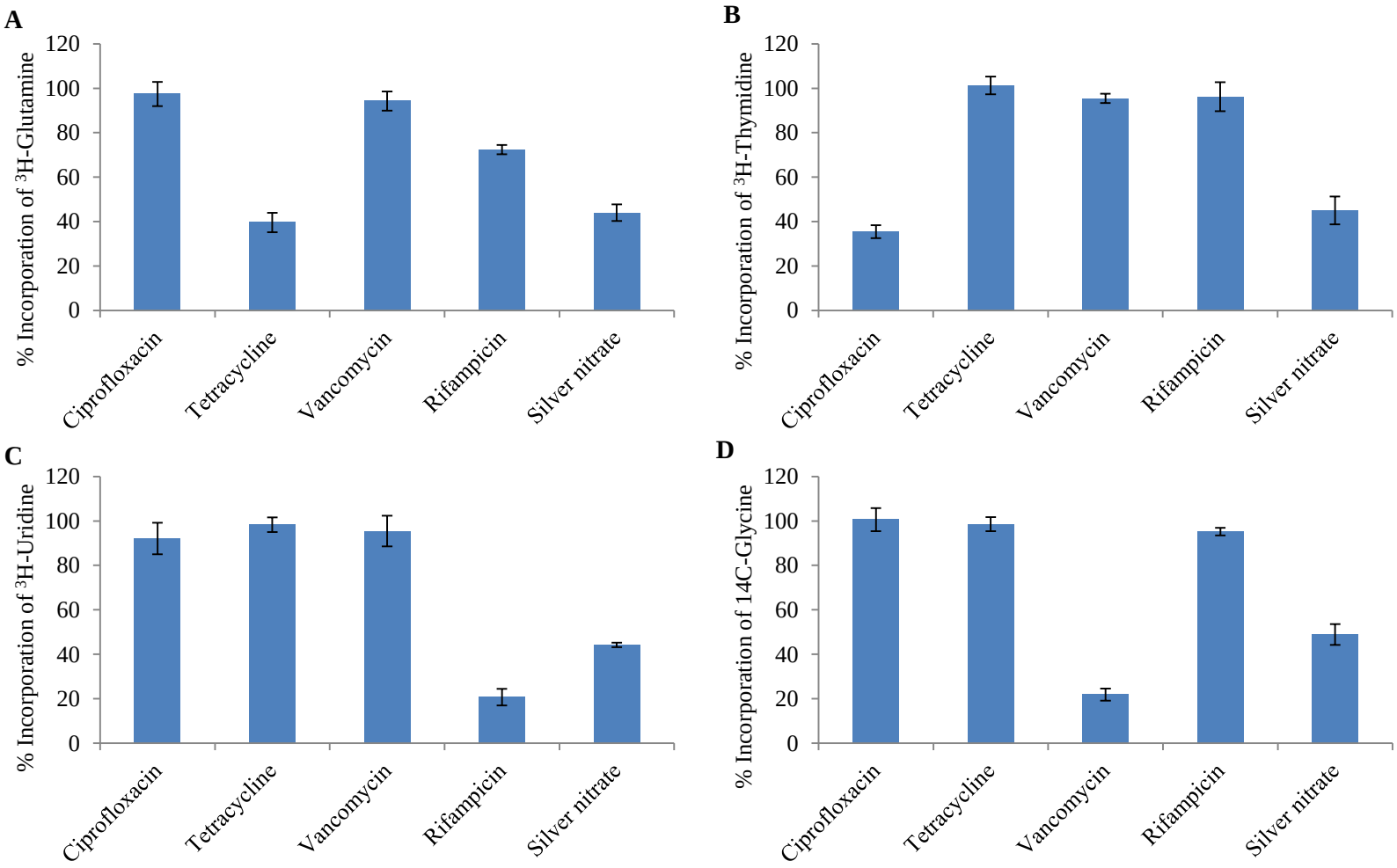


Figure 4.1. Inhibitory activity of $AgNO_3$ and comparator compounds against incorporation of radiolabelled precursors into protein (A), DNA (B), RNA (C) and peptidoglycan (D). Error bars represent the standard deviation from at least three independent replicates

To further explore the antibacterial target of Ag^{+}, five *B. subtilis* whole-cell biosensors each containing a luciferase reporter gene fused to promoters induced by inhibiton of fatty acid, protein, DNA, RNA and cell envelope biosynthesis were employed. These constructs have previously been shown to be of benefit in mode of action studies of novel antibacterial compounds such as ferrimycin A1, 8-hydroxyquinoline and juglone (Urban *et al.*, 2007; Mariner *et al.*, 2011). Through gene expression profiling, inhibitors of fatty acid, protein, DNA, RNA and cell-envelope biosynthesis were shown to induce the genes *fabHB*, *yheI*, *yorB*, *yvgS* and *ypuA* respectively (Urban *et al.*, 2007). While *fabHB* is known to encode an acyl carrier protein synthase, the function of the other genes remains unknown. The promoters of these genes were fused to a luciferase gene by Urban *et al*., allowing for induction of expression to be quantified by luminescence (Urban *et al.*, 2007).

Cultures of each biosensor were exposed to a range of concentrations of $AgNO_3$ and control agents and the luminescence of each sample recorded following an incubation period (Table 4.1). Exposure of the biosensor strains to their positive control agents resulted in a greater than two-fold increase in luminescence at concentrations of 0.5 μg/ml (vancomycin), 0.1 μg/ml (triclosan), 0.02 μg/ml (ciprofloxacin), 0.001 μg/ml (rifampicin) and 0.02 μg/ml (fusidic acid). Exposure of the strains to $AgNO_3$ failed to elicit a significant increase in luminescence. The inability of $AgNO_3$ to induce any of the biosensors again suggests that Ag^{+} likely has a non-specific antibacterial MOA such as membrane damage. This is reinforced by a previous study showing that compounds known to perturb the bacterial cell membrane (valinomycin, CTAB and chlorhexidine) also fail to induce any of the *B. subtilis* biosensors (Mariner *et al.*, 2011). Although $AgNO_3$ failed to induce any of the biosensor strains, care must be taken in interpreting results from studies utilising inducible reporters when exposed to an antibacterial agent

with an unknown MOA. The compound may directly inhibit production of the reporter molecule (or the reaction catalysed by the reporter molecule), or quench the output of the reporter. To confirm that this did not occur with $AgNO_3$, cultures of the DNA biosensor were exposed to 0.02 μg ciprofloxacin/ml (*i.e.* the concentration that produced maximal induction). Immediately prior to addition of luciferin, volumes of $AgNO_3$ stock solution were added to cultures to achieve final concentrations in the range of 0.125 to 64 μg/ml and luminescence values obtained to calculate fold increase in induction (data not shown). At concentrations up to 16 μg/ml, $AgNO_3$ had no effect on the output of the DNA reporter, with levels of induction comparable to that seen with cultures exposed to ciprofloxacin alone. However, at concentrations greater than 16 μg/ml, a substantial reduction in output could be observed (>50%), suggesting that at these concentrations $AgNO_3$ directly inhibits the reaction mediated by luciferase. Nevertheless, given that induction of the *B. subtilis* biosensors is typically seen at concentrations at or below the MIC of the compound tested (Urban *et al.*, 2007; Mariner *et al.*, 2011) and the MIC of $AgNO_3$ against *B. subtilis* (8 μg/ml) is below the concentration at which an inhibitory effect is seen against luciferase, the results obtained in this study truly represent the inability of $AgNO_3$ to induce any of the biosensors, indicating that the MOA of Ag^+ is distinct from the specific inhibition of the pathways these biosensors detect.

Table 4.1. Relative expression of *B. subtilis* biosensors by $AgNO_3$ and control compounds

Antibacterial agent	Maximal induction ratio of *B. subtilis* biosensors (±SD)				
	Cell-envelope	*Protein*	*DNA*	*RNA*	*Fatty-acid*
Silver nitrate	1.1 (±0.1)	1.0 (±0.1)	1.3 (±0.1)	1.1 (±0.4)	0.9 (±0.2)
Vancomycin	**3.6 (±0.4)**	0.6 (±0.1)	0.9 (±0.2)	0.5 (±0.1)	1.0 (±0.01)
Fusidic acid	0.1 (±0.2)	**3.1 (±0.1)**	0.8 (±0.02)	1.1 (±0.2)	0.8 (±0.1)
Rifampicin	0.3 (±0.1)	1.6 (±0.1)	1.0 (±0.1)	**2.7 (±0.2)**	0.4 (±0.1)
Ciprofloxacin	1.6 (±0.2)	1.0 (±0.2)	**62.5 (±12.1)**	0.9 (±0.1)	1.0 (±0.2)
Triclosan	0.9 (±0.2)	0.9 (±0.2)	1.6 (±0.4)	0.8 (±0.04)	**5.0 (±0.1)**

The maximal induction ratio represents the maximum reporter signal of the biosensor when exposed to a test compound as a ratio of the signal from drug-free biosensor cultures. Results represent means and standard deviations arising from at least three independent experiments. Values in bold are above the published threshold for induction and represent induction of the reporter.

4.3.1. Activity of Ag^+ against the S. aureus membrane

The non-specific inhibition of biosynthetic processes by $AgNO_3$ could be the result of damage to the cell membrane and consequent loss of intracellular components. Multiple assays have been developed to assess the effect of antibacterial agents on the bacterial membrane; for example, following exposure to an antibacterial agent for 10 min, the *Bac*Light™ assay can be used to evaluate % membrane integrity relative to drug-free (100 % integrity) and SDS-treated (0 % integrity) controls based on the uptake of membrane-permeable and membrane-impermeable dyes (SYTO-9 and propidium iodide), respectively (O'Neill *et al.*, 2004). The kinetics of membrane damage can be determined by measuring the leakage of small ions (such as K^+), and the fluorescent reporter $DiSC_3(5)$ can be used to establish the effect of a compound on bacterial membrane potential (Hobbs *et al.*, 2008; Ooi *et al.*, 2009). As each assay provides a different insight into the effect of a compound on the bacterial membrane, all three assays were used to determine if membrane damage contributes to the MOA of Ag^+. However, as the *Bac*Light™ and $DiSC_3(5)$ assays both rely upon the measuring the fluorescence of a fluorophore, confirmation was first required that $AgNO_3$ did not

fluoresce at the excitation wavelengths used in these assays, or quench the fluorescence of any of the fluorophores, as this would lead to an incorrect interpretation of the results. Thus, the emission spectra for a solution of Ag^+ at 4X MIC was determined between 400 and 700 nm using excitation wavelengths equivalent to those used in each assay (485 and 622nm for *Bac*Light™ and $DiSC_3(5)$ respectively). No significant fluorescence emission could be detected at either of the excitation wavelengths used (data not shown). Furthermore, Ag^+ did not quench the fluorescence of $DiSC_3(5)$, SYTO-9, or propidium iodide (data not shown), suggesting that both *Bac*Light™ and $DiSC_3(5)$ assays are suitable for use with $AgNO_3$.

Following exposure of *S. aureus* SH1000 to $AgNO_3$ and comparator antibiotics at 4X MIC, membrane integrity decreased by 97%, as measured by the *Bac*Light™ assay (Table 4.2). This decrease was comparable to that seen with bacteria exposed to nisin (99 %), clofazimine, XF-70, XF-73 and CTAB (all 100 %); compounds that are known to damage the bacterial membrane (Ruhr & Sahl, 1985, O'Neill *et al.*, 2004; Oliva *et al.*, 2004; Hobbs *et al.*, 2008; Ooi *et al.*, 2009). The membrane potential (as measured by $DiSC_3(5)$ fluorescence) was also affected in cells exposed to Ag^+, with a >50% decrease observed over 1 h (Figure 4.2b). The kinetics of membrane damage following Ag^+ exposure was further explored by measuring the leakage of intracellular K^+ from *S. aureus* SH1000 over 2 h (Figure 4.2a). In less than 10 min, >90 % of intracellular K^+ was lost from cells exposed to Ag^+; again, this effect was comparable with that observed for bacteria exposed to nisin. The rapid and substantial loss of bacterial membrane integrity following Ag^+ exposure strongly suggests that the antibacterial activity of Ag^+ results directly from damage to the cell membrane.

Table 4.2. Effect of $AgNO_3$ and comparator compounds on *S. aureus* SH1000 membrane integrity as determined by *Bac*Light™ assay

Compound	% Membrane integrity after 10 min (±SD)
$AgNO_3$	2.9 (0.3)
Nisin	0.8 (0.3)
CTAB	0 (0)
Clofazimine	0 (0)
XF-70	0 (0)
XF-73	0 (0)
Tetracycline	98.9 (2.2)
Rifampicin	97.1 (3.6)
Vancomycin	93.9 (2.7)
Ciprofloxacin	88.5 (5.7)

Results represent means and standard deviations from at least three independent experiments.

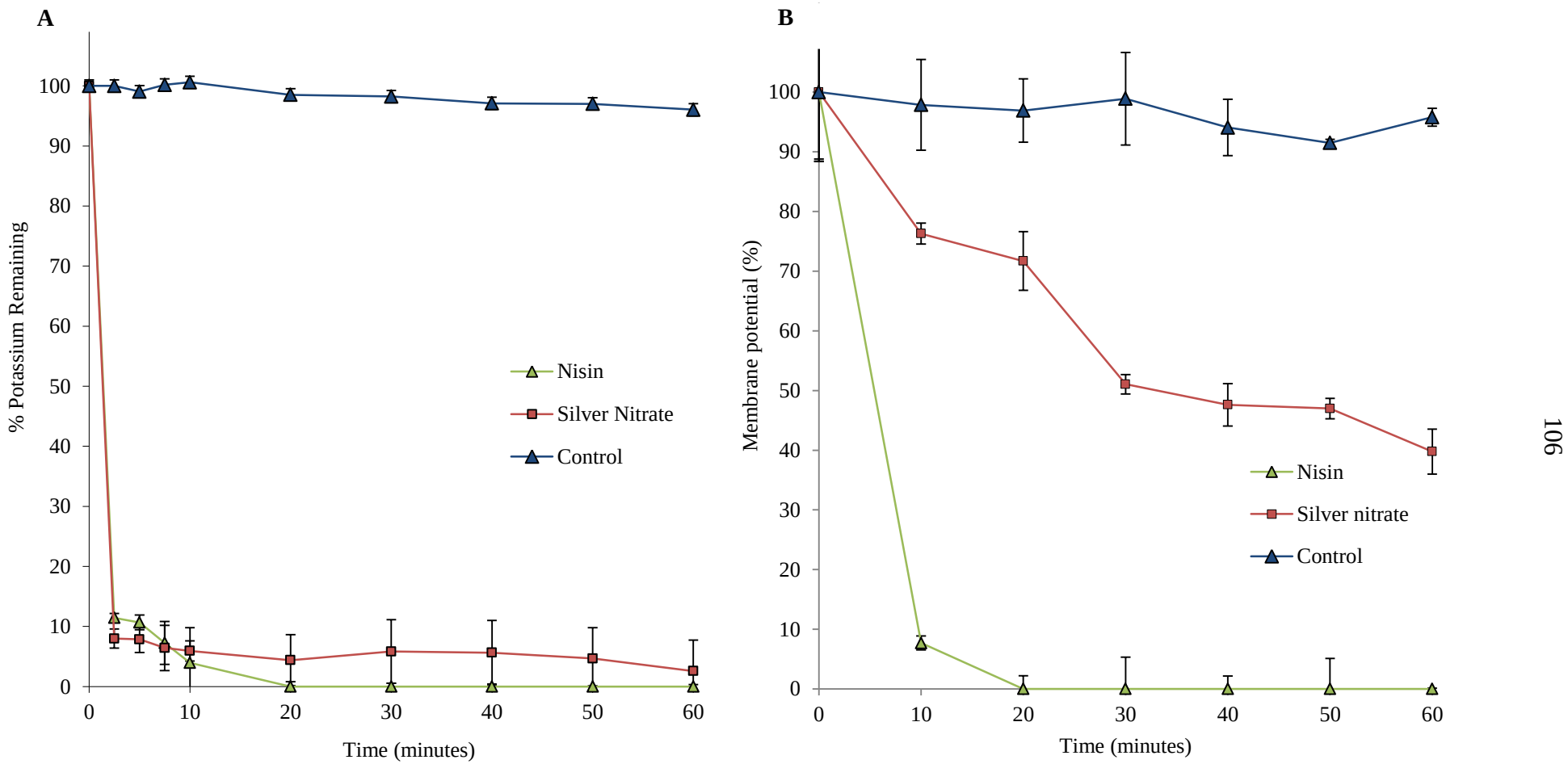


Figure 4.2. Effect of $AgNO_3$ and comparator compounds on intracellular K^+-levels (A) and membrane potential (B) of *S. aureus* SH1000. Error bars represent the standard deviation from at least three independent replicates. Data for Figure 4.3(A) were collected by L. Oyama, University of Leeds.

Cells treated with certain membrane-damaging compounds (for example nisin and CTAB) eventually undergo lysis as a consequence of damage to the membrane (O'Neill *et al.*, 2004). As Ag^+ exposure caused membrane damage, the ability for Ag^+ to prompt cell lysis was examined. *S. aureus* SH1000 cells re-suspended in 5mM HEPES, 5mM glucose buffer (pH 7.2) were exposed to $AgNO_3$ at 4X MIC and monitored for lysis over a period of 2 h by optical density measurements (Figure 4.3). Results were compared with that of nisin-exposed cultures. Over 2 h, the optical density of cultures exposed to nisin decreased by approximately 37%, indicating that lysis was occurring. There was no change in the optical density of cultures exposed to $AgNO_3$, suggesting that under these conditions Ag^+ did not cause lysis. To confirm the absence of lysis and to detect any changes in the morphology of cells exposed to Ag^+, cells were visualised using scanning electron microscopy (SEM) following exposure to Ag^+ or nisin for 5 and 120 min. Cells visualised after 120 min exposure to nisin showed evidence of lysis, but no lysis or other changes in cell morphology were seen for cells treated with $AgNO_3$ (Figure 4.3).

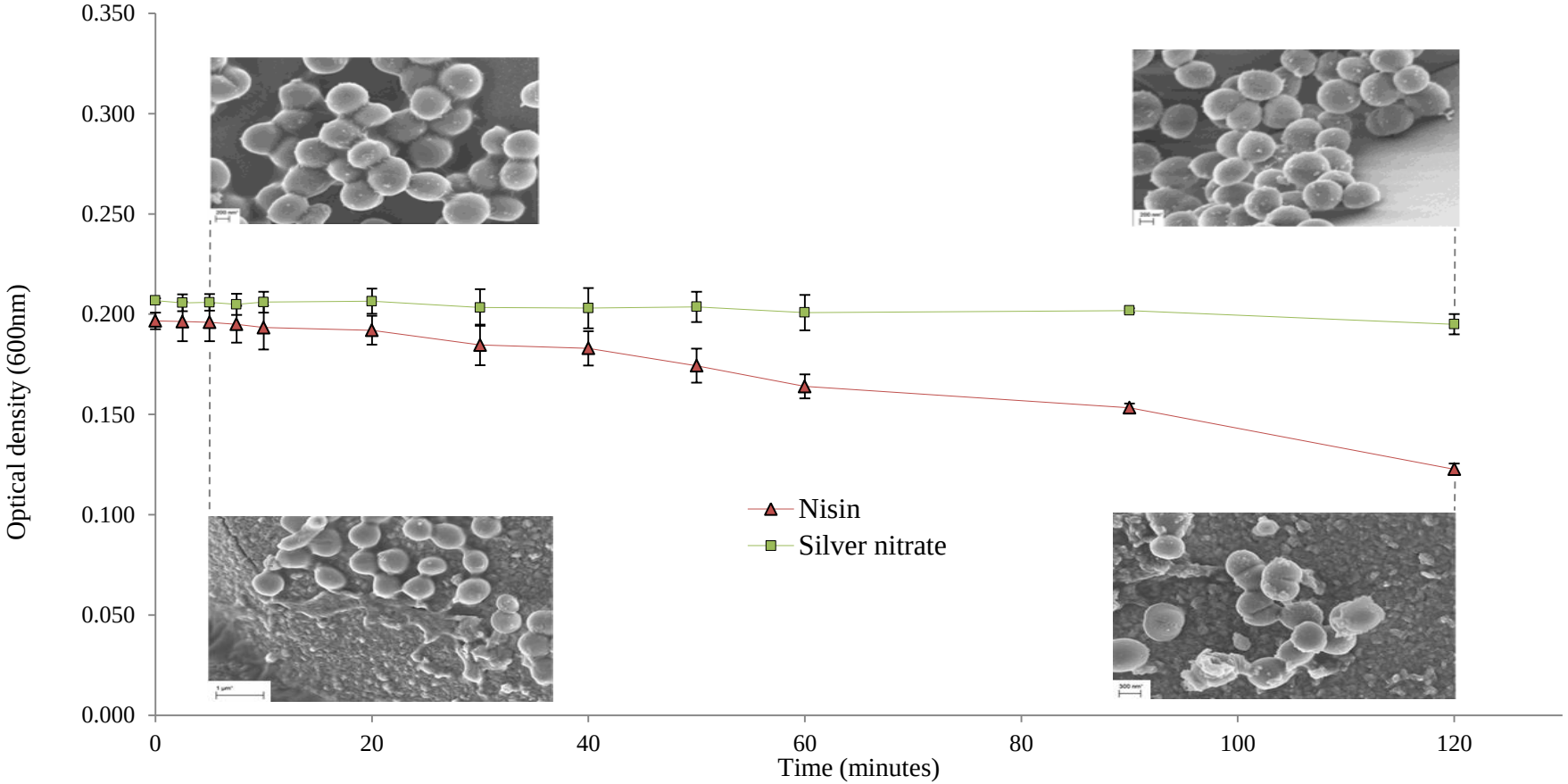


Figure 4.3. Effects of Ag^{+} and nisin on the integrity of *S. aureus* SH1000 re-suspended in HEPES/glucose buffer as determined by optical density measurements at 600nm and SEM. Values shown are the means of three replicates from three independent experiments. Broken lines point to scanning electron micrographs of cells taken from cultures at indicated time-points. Error bars represent standard deviations from the mean. Electron micrographs were taken by L. Oyama, Univeristy of Leeds.

Although Ag^+ can cause membrane damage that is sufficient to permit leakage of K^+ and allow for ingress of propidium iodide (molecular mass of 668 Da), the inability of Ag^+ to prompt cell lysis indicates that the extent of membrane damage caused by Ag^+ is unclear. To provide further insight into the extent of Ag^+-mediated membrane damage, the ability of larger molecules to leak out of the cell was assessed following Ag^+ exposure. Previously, the 464 kDa protein β-galactosidase has been used for this purpose, as it can catalyze the cleavage of 4-methylumbelliferyl-β-D-galactoside into the fluorophore 4-methylumbelliferone, thus allowing for its detection and quantitation (O'Neill *et al.*, 2004) in the extracellular milieu. Against *S. aureus* SH1000β (SH1000 constitutively expressing β-galactosidase), $AgNO_3$ caused no apparent leakage of β-galactosidase when used at concentrations of 4X MIC, suggesting that the extent of membrane damage was insufficient to permit leakage of this molecule (Table 4.3). This is in contrast with exposure to nisin and CTAB which in both cases resulted in >3-fold increase in β-galactosidase release from cells, in agreement with a previous study (O'Neill *et al.*, 2004). However, as discussed above, a compound with an unknown MOA may directly inhibit the catalytic activity of a reporter molecule. Unfortunately, this occurred with β-galactosidase exposed to Ag^+, as exposure to concentrations of $AgNO_3$ greater than 1 μg/ml directly inhibited the ability of β-galactosidase to produce 4-methylumbelliferone (Figure 4.4). As a consequence, it remains unclear if Ag^+-mediated damage to *S. aureus* membrane is sufficient to permit leakage of β-galactosidase. This result nevertheless emphasizes the necessity of appropriate control experiments when working with reporter molecules, to prevent incorrect interpretation of results obtained from such assays.

Table 4.3. Ability of $AgNO_3$ and comparator agents to induce β-galactosidase leakage in *S. aureus* SH1000β

Compound	Relative β-galactosidase release (±SD)
$AgNO_3$	0.4 (0.1)
Tetracycline	1.1 (0.1)
Vancomycin	0.9 (0.1)
CTAB	4.0 (0.4)
Nisin	4.4 (0.3)

Values and standard deviations shown are the mean of three independent experiments. Values of ≥2-fold compared with drug-free controls are considered significant for release of β-galactosidase.

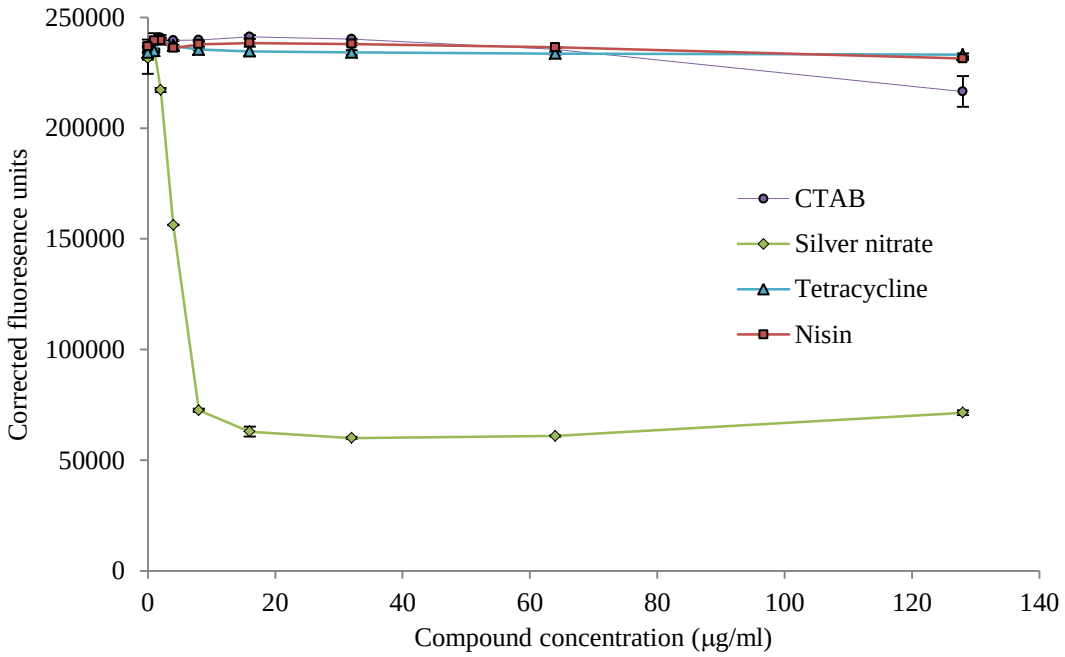


Figure 4.4. Activity of β-galactosidase in the presence of $AgNO_3$ and comparator compounds. Values shown are the mean of at least three independent experiments. Error bars represent the standard deviation from the mean.

Although Ag^+ has been shown to bind multiple targets within the cell, their role in the antibacterial MOA of Ag^+ is unknown. However, to bind to these intracellular targets,

Ag^+ must first pass through the cell membrane. As it has been demonstrated that Ag^+ causes rapid damage to cell membranes, the leakage of intracellular components will result in cell death irrespective of the ability Ag^+ to bind and inhibit intracellular targets. Indeed, prior studies demonstrating the interaction of Ag^+ with cellular macromolecules used prolonged incubation times (Feng *et al.*, 2000; Jung *et al.*, 2008), suggesting that these effects would only occur long after membrane damage. Thus, it seems likely that the bacterial membrane is the primary target of Ag^+, although the subsequent binding of Ag^+ to intracellular components and their consequent inhibition may accelerate bacterial killing.

Various intracellular enzymes are known to be inhibited by Ag^+, thus it seems reasonable to assume that the ability of Ag^+ to bind and inhibit intracellular targets is non-specific. To test this hypothesis, the catalytic activities of three unrelated enzymes (chymotrypsin, malate dehydrogenase and RNA polymerase) were determined in the presence of $AgNO_3$ and comparator agents. In each case, the half maximal inhibitory concentration (IC_{50}) was determined (Table 4.4). As expected, tetracycline, vancomycin and ciprofloxacin demonstrated no inhibitory activity against all three enzymes at the concentrations tested, whereas rifampicin had an IC_{50} of 0.021 μg/ml against RNA polymerase, which is consistent with its known mode of action (Konno *et al.*, 1973). In contrast, $AgNO_3$ was found to be inhibitory against all three enzymes, with IC_{50} values at concentrations equivalent to ≤4 x MIC of $AgNO_3$ against *S. aureus*. The promiscuous inhibitory activity of Ag^+ against enzymes at biologically relevant concentrations would appear to suggest that Ag^+ does not have a specific intracellular target. Therefore once Ag^+ has reached the intracellular milieu, it could inhibit multiple biological processes simultaneously. As inhibition of certain cellular processes are known to result in cell death rather than cessation of growth (*i.e.* transcription,

DNA synthesis), this property of Ag^+ may contribute to the rate of cell killing following disruption of the membrane. Nevertheless, as the cell membrane is the primary target of Ag^+, the remainder of this study attempted to elucidate the component of the membrane that is targeted by Ag^+ to elicit an antibacterial effect.

Table 4.4. Inhibitory activity of $AgNO_3$ and comparator agents against malate dehydrogenase, chymotrypsin and RNA polymerase

Compound	IC_{50} against (μg/ml)(±SD)		
	Malate dehydrogenase	**Chymotrypsin**	**RNA polymerase**
$AgNO_3$	19.37 (0.09)	26.56 (0.03)	4.94 (0.052)
Tetracycline	>128	>128	>128
Ciprofloxacin	>128	>128	>128
Rifampicin	>128	>128	0.021 (0.008)
Vancomycin	>128	>128	>128

Values and standard deviations shown are the mean of three independent experiments

4.3.2. Identification of the target(s) of Ag^+ at the S. aureus cell membrane

The bacterial cell membrane predominantly consists of a phospholipid bilayer interspersed with integral membrane proteins (*i.e.* containing one or more domains inserted into, or spanning the membrane) and peripheral membrane proteins (proteins tethered to the membrane by a lipid tail, or via binding to an integral membrane protein). Unlike eukaryotes, bacterial membranes do not contain sterols (which serve to stabilise eukaryotic membranes), but instead contain hopanoids, which are believed to serve a similar purpose (Madigan *et al.*, 2010). Damage to either the phospholipid bilayer or to integral membrane proteins could potentially permit leakage of intracellular components and cause cell death; thus, Ag^+ presumably achieves its antibacterial activity via one or both of these mechanisms. To investigate the ability of Ag^+ to disrupt the phospholipid component of membranes, phospholipid vesicles

(liposomes) encapsulating the fluorescent dye carboxyfluorescein at a self-quenching concentration were used. This system has been employed previously to ascertain the effects of agents on phospholipid bilayer integrity, as following sufficient damage to the bilayer, carboxyfluorescein will leak from the liposome, reaching an extracellular concentration that is no longer self-quenching; consequently, leakage (and therefore damage) can be detected and quantified by measuring carboxyfluorescein fluorescence (Jung *et al.*, 2004; StGelais *et al.*, 2007). As the phospholipid content of cell membranes differs between species and organisms (Epand *et al*., 2007), three separate liposome formulations were created analogous to staphylococcal, *E. coli* and mammalian membranes respectively, for which the compositions are detailed in section 2.6.3. Furthermore, to ensure that identical concentrations of liposomes were used in each experiment, 0.5 % (w/w) of rhodamine-labelled phosphatidylethanolamine was incorporated into each liposome preparation; the concentration of liposomes in any given solution could then be calculated based on rhodamine absorbance at 570 nm. Following exposure of liposomes to compounds for 10, 60 and 180 minutes, carboxyfluorescein fluorescence was measured and % liposome integrity calculated relative to Triton X-100 (0% integrity) and solvent-only (100% integrity) treated controls (Table 4.5). Prior to carrying out this assay, it was confirmed that none of the compounds tested fluoresced at 520 nm when excited at 485 nm, or quenched the fluorescence of carboxyfluorescein (data not shown).

Table 4.5. Activity of $AgNO_3$ and comparator compounds against liposomes analogous to the phospholipid bilayers of *S. aureus*, *E. coli* and mammalian cell membranes

Compound	**% Liposome integrity**								
	***S. aureus* liposomes**			***E. coli* liposomes**			**Mammalian liposomes**		
	10 min	**60 min**	**180 min**	**10 min**	**60 min**	**180 min**	**10 min**	**60 min**	**180 min**
$AgNO_3$	101.0	99.3	104.4	96.9	102.4	98.4	93.1	98.5	99.1
	(1.22)	(1.57)	(0.73)	(0.82)	(1.33)	(4.51)	(5.01)	(4.14)	(0.52)
CTAB	99.2	94.3	98.3	91.4	82.0	76.9	104.2	97.3	97.8
	(0.51)	(0.80)	(0.38)	(3.01)	(2.71)	(2.66)	(3.51)	(0.88)	(0.40)
SDS	**42.7**	**0**	**0**	**51.6**	**0**	**0**	**31.0**	**0**	**0**
	(0.77)	(0)	(0)	(1.68)	(0)	(0)	(0.66)	(0)	(0)
Tetracycline	90.3	92.2	88.6	102.1	98.1	99.2	93.4	95.0	99.7
	(1.41)	(0.53)	(2.72)	(3.22)	(1.17)	(1.37)	(1.66)	(0.11)	(0.29)
Vancomycin	99.8	100.1	101.5	90.8	99.6	101.1	98.4	99.5	99.6
	(1.10)	(0.19)	(0.70)	(5.38)	(1.22)	(0.20)	(0.29)	(0.44)	(0.07)
Gentamicin	95.2	93.3	96.7	96.8	95.5	100.5	102.8	95.2	98.6
	(0.93)	(1.08)	(0.38)	(3.71)	(0.17)	(0.02)	(1.90)	(2.98)	(0.20)
Rifampicin	100.4	99.8	104.6	97.2	97.0	97.9	99.1	102.5	103.4
	(1.37)	(1.78)	(3.46)	(0.81)	(1.21)	(0.53)	(0.71)	(3.90)	(4.73)
Ampicillin	102.0	100.9	98.5	95.4	97.4	100.5	91.3	98.2	99.5
	(1.08)	(0.88)	(1.99)	(2.24)	(2.01)	(1.58)	(0.56)	(1.00)	(0.29)
XF-73	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
Clofazimine	**22.3**	**0**	**0**	**38.9**	**0**	**0**	**33.4**	**0**	**0**
	(0.55)	(0)	(0)	(0.94)	(0)	(0)	(1.26)	(0)	(0)
Daptomycin	**62.5**	**21.7**	**5.21**	97.3	94.5	86.3	90.4	93.3	91.5
	(0.06)	(0.15)	(0.07)	(2.11)	(1.92)	(3.51)	(0.42)	(0.28)	(0.66)

Values and standard deviations shown are the mean of at least three independent experiments. Values demonstrative of a substantial loss of membrane integrity (≤70%) are highlighted in bold

Antibacterial agents that do not target the bacterial membrane (*e.g.* tetracycline, vancomycin, gentamicin, rifampicin and ampicillin) had no effect upon liposome integrity after 3 h exposure. In contrast, compounds previously shown damage bacterial membranes (SDS, clofazimine and XF-73) had a significant effect on the integrity of all three types of liposome, albeit with different kinetics of damage. In the *Bac*Light™ assay, SDS is used as the 0% membrane integrity control as the damage it causes to bacterial membranes is extensive and rapid (O'Neill *et al.*, 2004). However against all three liposome formulations, the damage mediated by SDS was not as rapid, as after 10 min exposure liposome integrities remained at >30%. This result may suggest that the rapid damage to bacterial membranes by SDS is a consequence of combined effects against both the phospholipid bilayer and integral membrane proteins. Indeed, the ability of SDS to denature proteins is well established (Bhuyan, 2010). The lack of integral membrane proteins also appeared to affect the rapidity of damage mediated by clofazimine, when comparing the results obtained here against the *Bac*Light™ data in Table 4.2. Unlike SDS, clofazimine is used as a systemic antibacterial agent in the treatment of leprosy (caused by *Mycobacterium leprae*) (Oliva *et al.*, 2004); it is therefore unusual that clofazimine causes equivalent damage to liposomes with a mammalian phospholipid composition, as this would imply that clofazimine would demonstrate equivalent toxicity to mammalian and bacterial cells. Nevertheless, although clofazimine can cause fatal enteropathy in patients taking high doses over a prolonged period, it has successfully been used clinically for over 40 years (Parizhskaya *et al.*, 2001). This would therefore indicate that either the membrane-damaging activity of clofazimine is not present when used *in vivo*, or that other components of the mammalian cell membrane protect the phospholipid bilayer from the activity of clofazimine. In either case it is apparent that mammalian liposomes should not be

considered as a reliable surrogate for mammalian toxicity studies, and appropriately validated studies should instead be utilised in evaluating the bacterial specificity of any new antibacterial agent.

As with SDS and Triton X-100, CTAB is a surfactant that causes damage to bacterial membranes, yet it caused no significant reduction in liposome integrity after 3 h exposure. This would therefore suggest that it cannot directly disrupt the phospholipid bilayer and must damage bacterial membranes via an alternate mechanism, for example by denaturing integral membrane proteins, or via the induction of reactive oxygen species. Indeed, analysis of spontaneous mutants of *E. coli* displaying resistance to CTAB revealed mutations in *soxR*, which resulted in constitutive expression of the *soxRS* regulon that provides protection against O_2^- (Nakata *et al.*, 2010). Thus, ROS (particularly O_2^-) generation appears critical to the antibacterial MOA (and therefore the membrane-damaging activity) of CTAB.

Daptomycin is a member of the lipopeptide family of antibacterial agents used for the treatment of antibiotic-resistant Gram-positive skin and skin-structure infections and *S. aureus* bacteraemia and right-sided endocarditis (Carpenter & Chambers, 2004). The antibacterial mode of action of daptomycin against *S. aureus* results from Ca^{2+}-mediated insertion into the phospholipid bilayer of the cell membrane that causes damage sufficient to permit the leakage of Mg^{2+}, K^+ and ATP, leading to the de-energisation of cellular processes and cell death (Jung *et al.*, 2004; Hobbs *et al.*, 2008). At 4X MIC, daptomycin caused substantial damage to staphylococcal liposomes, with liposome integrity decreasing to 5 % after 3 h exposure. The calcium cation is believed to be essential for the antibacterial action of daptomycin as it allows daptomycin to interact with the negatively-charged head groups of the staphylococcal phospholipid bilayer (Jung *et al.*, 2004). This requirement also provides an indication as to why daptomycin

displays selective toxicity towards bacteria, as mammalian cell membranes consist of neutral phospholipids, which likely prevents insertion of the Ca^{2+}-daptomycin complex as a consequence of a reduced charge interaction. The inability of daptomycin to insert into mammalian membranes has been demonstrated previously (Jung *et al.*, 2004) and again in this study, as no damage to liposomes with a mammalian phospholipid composition could be observed over 3 h exposure to daptomycin. Daptomycin was also unable to damage liposomes consisting of phospholipid analogous to *E. coli* membranes. Although the membranes of Gram-negative bacteria do contain negatively charged phospholipid, the overall proportion is substantially less than that found in Gram-positive bacteria (~20% vs 100% respectively) (Epand *et al.*, 2007; Epand *et al.*, 2010). It therefore seems that there is an insufficient quantity of negatively-charged phospholipid to permit insertion of daptomycin at concentrations necessary to cause overt damage. Daptomycin does not display any antibacterial activity against Gram-negative bacteria and it was believed that this was a sole consequence of impermeability afforded by the outer membrane; however, the data presented here suggest that the target of daptomycin is not present in Gram-negative bacteria. Recent attempts have been made to potentiate Gram-positive-specific antibacterial agents (*e.g.* vancomycin, telavancin) for use in treating Gram-negative infections, under the assumption that the antibacterial target is present but is protected by intrinsic resistance mechanisms (such as outer membrane impermeability or efflux) (Gordon *et al.*, 2010; Hornsey *et al.*, 2012). The data presented here suggest that daptomycin would not be a suitable candidate for such a potentiation. This was confirmed in further experiments that are beyond the scope of this thesis to be presented here (Randall *et al.*, 2013).

As with CTAB, Ag^+ failed to damage any of the three types of liposomes. It was confirmed that this was not a consequence of Ag^+ quenching the fluorescence of carboxyfluorescein, as Ag^+ did not impact fluorescence measurements of Triton X-100-treated liposomes (data not shown). The inability of Ag^+ to damage phospholipid bilayers implies that Ag^+ targets another component of the cell membrane (*e.g.* integral membrane proteins), or as hypothesized above with CTAB, induces the formation of ROS which in turn causes oxidative damage to the membrane. There have been previous suggestions that the Ag^+ may induce the formation of excessive ROS that could contribute to the antibacterial activity of Ag^+ by causing oxidative damage to intracellular components (including the membrane), and evidence to support this has been provided by studies in both *E. coli* and *S. epidermidis* (Park *et al.*, 2009; Gordon *et al.*, 2010). However the extent of the contribution ROS provides to the antibacterial activity of Ag^+ remains unclear. This area therefore warrants further investigation.

4.3.3 Contribution of reactive oxygen species to the antibacterial MOA of Ag^+

S. aureus employs a number of mechanisms to mitigate the effect of ROS. To protect against O_2^-, *S. aureus* utilises superoxide dismutases SodA and SodM, which convert O_2^- into H_2O_2 and O_2 (Karavolos *et al.*, 2003), whilst protection from H_2O_2 results from its conversion into H_2O and O_2 by catalase (encoded by *katA*) and H_2O alone by alkyl hydroperoxide reductase (encoded by *ahpC*) (Cosgrove *et al.*, 2007). To confirm that Ag^+ can induce ROS formation in *S. aureus*, $AgNO_3$ MICs were determined against strains deficient in one or more of the above mechanisms, as any reduction in MIC against these strains would be indicative of excessive ROS production (Table 4.6). Silver nitrate MICs did not differ between the parental strain and strains deficient in one ROS protection mechanism, with the exception of strain MHKA (Δ*sodA*) which was

susceptible to $AgNO_3$ at half the concentration required to inhibit SH1000. Strain KC043 (deficient in AhpC and KatA) also showed a two-fold reduction in MIC, whereas strain MHKAM, which lacked SodA and SodM was the most susceptible to $AgNO_3$, with a four-fold decrease in the MIC compared with the parental strain. As strain MHKAM was the most susceptible to Ag^+, it seems likely that O_2^- is the primary ROS produced by Ag^+ exposure. Given that Ag^+ is unable to produce ROS directly as part of a Fenton-like reaction, excess O_2^- presumably arises from a cellular process that has been affected by Ag^+. This is supported by the fact that strains deficient in SodA alone also demonstrate reduced susceptibility to Ag^+, as it has been shown that SodA provides protection primarily against endogenous sources of O_2^- (SodM protects from exogenous sources) (Karavolos *et al.*, 2003).

Table 4.6. Inhibitory activity of $AgNO_3$ and comparator agents against *S. aureus* strains deficient in one or more ROS detoxification mechanisms

Compound	MIC (μg/ml)					
	SH1000	KS100 (Δ*katA*)	MHKM (Δ*sodM*)	MHKA (Δ*sodA*)	MHKAM (Δ*sodA*, Δ*sodM*)	KC043 (Δ*ahpC*, Δ*katA*)
Silver nitrate	16	16	16	8	4	8
Rifampicin	0.016	0.016	0.016	0.016	0.016	0.016
CTAB	2	2	2	2	2	2
Gentamicin	0.125	0.125	0.125	0.125	0.125	0.125
Vancomycin	2	2	2	2	2	2
Tetracycline	0.5	0.5	0.5	0.5	0.5	0.5
Fusidic acid	0.5	0.5	0.5	0.5	0.5	0.5
Penicillin G	0.25	0.25	0.25	0.25	0.25	0.25
Ciprofloxacin	4	4	4	4	1	1

In contrast to the results observed with Ag^+, the MIC of control antibiotics (with the exception of ciprofloxacin) did not differ between strains. This would suggest that ROS are not part of the antibacterial effect of these compounds. Against *E. coli* Kohanski *et al*. (2007) have suggested that the aminoglycoside, β-lactam and fluoroquinolone

antibiotic classes prompt a common mechanism of bacterial cell death, involving the production of O_2^- from hyper-stimulation of the electron transport chain and subsequent conversion of excessive O_2^- into $OH^\cdot$ via the Fenton reaction (Kohanski *et al.*, 2007; Kohanski *et al.*, 2008). The same study also provided limited evidence to suggest that the same phenomenon could be observed in *S. aureus* following exposure to norfloxacin or vancomycin, as increased levels of $OH^\cdot$ could be detected in cells (Kohanski *et al.*, 2007). This 'ROS hypothesis' is currently an issue of contention within the scientific literature, as multiple papers have refuted the findings of Kohanski *et al.* (Ricci *et al.*, 2012; Keren *et al.*, 2013; Liu & Imlay, 2013). However these articles all utilised *E. coli* or *Salmonella* spp. as their model organisms, thus it is unclear if the ROS hypothesis remains correct for *S. aureus*. The results in Table 4.6 suggest that out of the bactericidal antibiotics tested only ciprofloxacin induced ROS formation; however, this experiment only measured the contribution of ROS to the inhibitory effect of a compound and not its bactericidal activity. Thus, to provide further insight into this issue, the bactericidal activity of compounds against strains deficient in peroxide- or O_2^--protection mechanisms was assessed (Figure 4.5). Compared with the parental strain, no significant increase in killing activity could be observed for the bactericidal antibiotics ciprofloxacin, penicillin G, gentamicin, rifampicin, or vancomycin against KC043 or MHKAM. This result would therefore suggest that ROS are not responsible for the killing activity of bactericidal antibiotics against *S. aureus* and thus provide further evidence to refute the hypothesis suggested by Kohanski *et al.*, (2007). Unlike the results obtained for bactericidal antibiotics, $AgNO_3$ was significantly more effective at killing KC043 and MHKAM, with decreases in cell viability of 3.4 and 5.5 $\log_{10}$ respectively over 24 h (compared with 2.51 $\log_{10}$ against SH1000). This result provides further indication that Ag^+ can induce the formation of ROS. CTAB also

demonstrated greater killing activity against MHKAM, with a 7-$\log_{10}$ reduction in cell number compared with a 5.5-$\log_{10}$ reduction against SH1000. Earlier, it was described that previous studies indicate a role for ROS (particularly O_2^-) generation in the MOA of CTAB in *E. coli*. The results obtained in this study provide further evidence to the possibility that a similar mechanism may also occur in *S. aureus*.

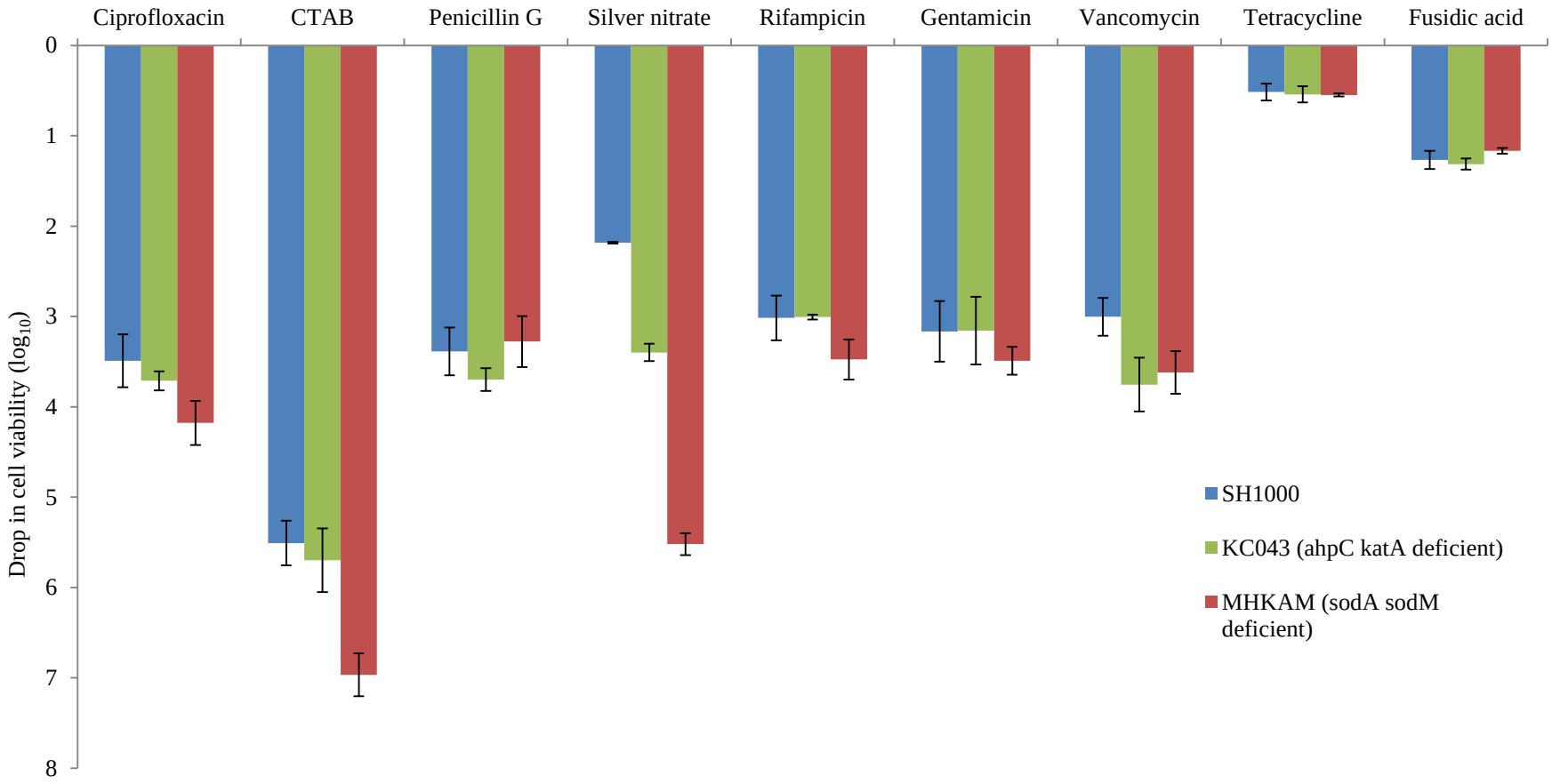


Figure 4.5. Bactericidal activity of $AgNO_3$ and comparator compounds against *S. aureus* strains deficient in ROS detoxification mechanisms. Values are means of at least three independent experiments. Error bars represent the standard deviation from the mean.

Against strains lacking ROS-protection mechanisms, Ag^+ displayed greater inhibitory and killing activity, which would suggest that Ag^+ induces increased ROS formation. To provide further validation for this hypothesis, the inhibitory and killing activity of Ag^+ against *S. aureus* was determined in an anaerobic environment. As there will be no O_2 available for the production of ROS, the killing activity of Ag^+ should decrease if ROS contributes to Ag^+ activity. As demonstrated in Figure 4.6, this was not the case, as the reduction in cell viability of SH1000 cultures exposed to $AgNO_3$ in the presence or absence of O_2 was comparable. This suggests that although Ag^+ exposure generates increased levels of ROS, the levels produced are insufficient to affect cell viability when ROS protection mechanisms are intact. These results contradict the findings of Park *et al*. (2009), who found that under anaerobic conditions the cidality of Ag^+ decreased against both *S. aureus* and *E. coli* (Park *et al.*, 2009). However in their study, Park *et al*. performed killing experiments against cells suspended in phosphate buffer, instead of MHB used in this study. In Chapter 3 it was demonstrated that Ag^+ is potently bactericidal against cultures suspended in buffer; thus, it is possible at this level of activity, sufficient ROS are generated to contribute to the antibacterial effect. Furthermore, unlike culture media, buffer may lack components that can contribute to mitigating the effects of ROS; for example, the manganese ion (Mn^{2+}) has been shown to provide significant levels of protection against O_2^- (Karavolos *et al.*, 2003). Finally, Park *et al*. measured reduction in cell viability after 40 and 60 min exposure to Ag^+, whereas in this study viable cell counts were performed only after 24 h exposure, thus under anaerobic conditions the kinetics of Ag^+-mediated cell death could differ without affecting the loss of cell viability over a prolonged period.

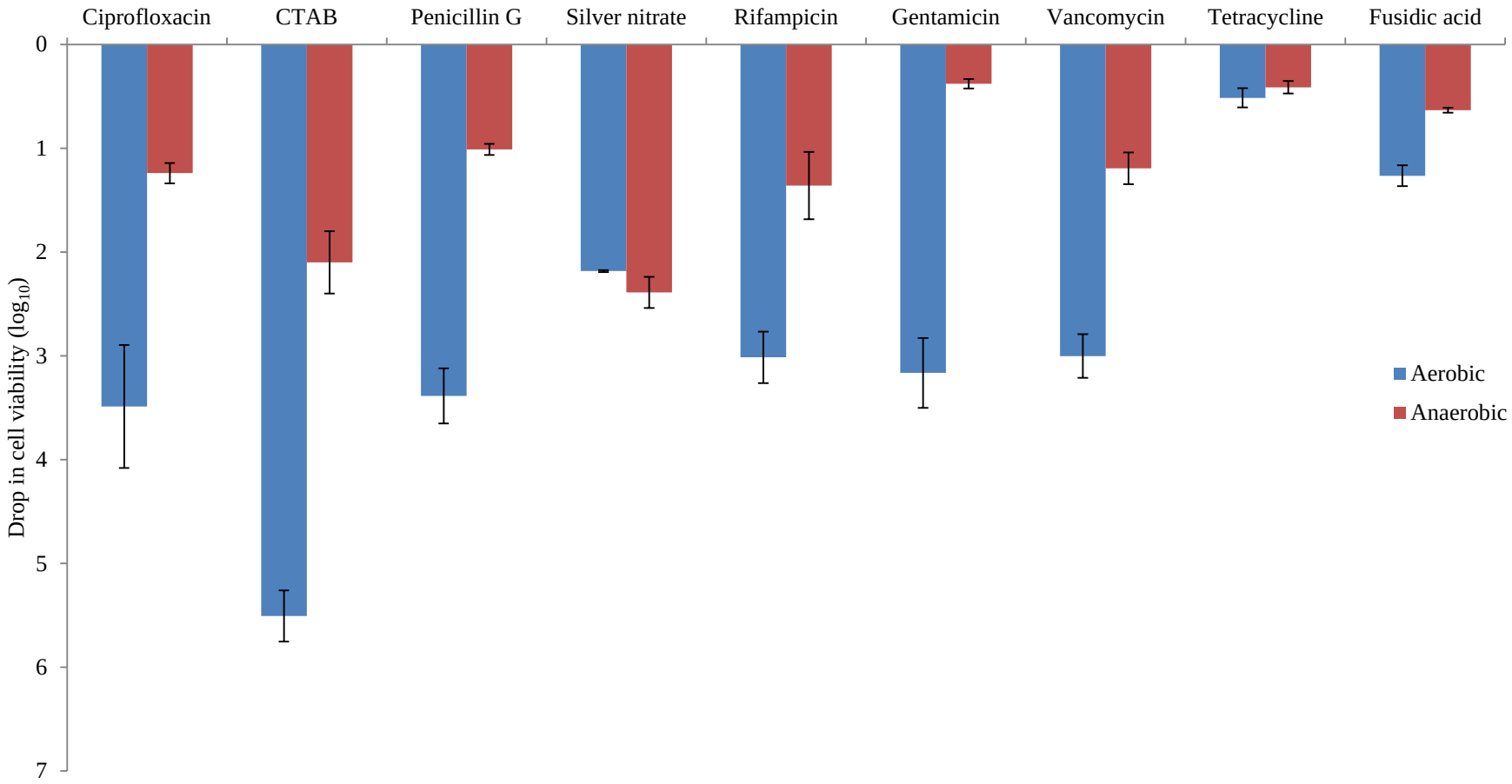


Figure 4.6. Bactericidal activity of $AgNO_3$ and comparator agents against *S. aureus* under aerobic and anaerobic conditions. Values are means of three independent experiments. Error bars represent the standard deviation from the mean.

In contrast to $AgNO_3$, other antibacterial agents exhibited reduced bactericidal activity in an anaerobic environment. This may suggest that these compounds partially rely on ROS generation. However, under anaerobic conditions cultures of SH1000 took longer (5 h compared with 2.5 h under aerobic conditions) to reach the correct optical density for this experiment (OD_{600}= 0.2 units). A reduced growth rate impacts the efficacy of killing by bactericidal agents, as most compounds require active metabolic processes to exert an optimal antibacterial effect (Kolter *et al.*, 1993; Mascio *et al.*, 2007). Indeed, this has been demonstrated for β-lactams, rifampicin, ciprofloxacin and vancomycin (Mascio *et al.*, 2007; Ooi *et al.*, 2010). It is therefore unclear if the inability to generate ROS directly affects the bactericidal activity of these compounds. Aminoglycosides (*e.g.* gentamicin) require an active membrane potential to permit uptake into target cells (Vakulenko & Mobashery, 2003); this membrane potential cannot be generated under anaerobic conditions, explaining the substantial decrease in killing activity observed for gentamicin against SH1000. However, the antibacterial target of CTAB is the bacterial membrane, which is present irrespective of growth phase. Consequently, the reduced activity of CTAB under anaerobic conditions provides further corroboration of the hypothesis that the antibacterial activity of this compound is partially reliant upon the generation of ROS.

Although the generation of ROS does not appear to affect the inhibitory activity of Ag^+, it is still unclear if ROS impacts the kinetics of Ag^+-mediated cell death. ROS could accelerate Ag^+ killing by contributing to bacterial membrane damage, or by causing oxidative damage to other cellular macromolecules. To determine if ROS contributes to bacterial membrane damage, the effect of Ag^+ on bacterial membrane integrity under anaerobic conditions was determined using the *Bac*Light™ assay (Table 4.7). With the exception of CTAB, all compounds known to cause membrane damage (including Ag^+)

retained this ability under anaerobic conditions with no apparent decrease in potency compared with that observed in an aerobic environment.

Table 4.7. Effect of $AgNO_3$ and comparator compounds on *S. aureus* SH1000 membrane integrity under anaerobic conditions, as determined by *Bac*Light™ assay

Compound	% Membrane integrity after 10 min (±SD)	
	Aerobic	Anaerobic
$AgNO_3$	2.9 (0.3)	3.5 (0.2)
Nisin	0.8 (0.3)	0 (0)
CTAB	0 (0)	38.9 (1.3)
Clofazimine	0 (0)	0 (0)
Tetracycline	98.9 (2.2)	99.0 (4.8)
Rifampicin	97.1 (3.6)	95.4 (2.9)
Vancomycin	93.9 (2.7)	96.6 (3.4)
Ciprofloxacin	88.5 (5.7)	94.6 (4.0)

Values and standard deviations shown are the mean of at least three independent experiments

The decrease in the membrane-damaging activity of CTAB against *S. aureus* membranes under anaerobic conditions seems to indicate that ROS are at least partially responsible for the membrane-damaging properties of this compound in an aerobic environment. This process is likely a consequence of ROS-mediated peroxidation of unsaturated lipids contained in the phospholipid bilayer and/or carbonylation of integral membrane proteins, leading to their denaturation (Imlay, 2013). Confirmation that these mechanisms are involved could be achieved using previously established assays (Placer *et al.*, 1966; Luo & Wehr, 2009). Nevertheless, as CTAB retains some activity against *S. aureus* membranes under anaerobic conditions it seems that this compound can also cause direct damage to the membrane. As CTAB does not affect the integrity of liposomes, this direct damage must rely upon the interaction of CTAB with integral membrane proteins.

In contrast to CTAB, Ag^+ was equally effective at damaging *S. aureus* membranes under aerobic and anaerobic conditions. This observation confirms that Ag^+ causes

direct damage to the membrane and is not due to the action of ROS. As described with CTAB above, Ag^+ did not damage liposomes, which implies that the phospholipid bilayer is not the target for Ag^+ in bacterial membranes. Membrane damage by Ag^+ presumably therefore relies on the denaturation of one or more integral membrane proteins, which allows for the leakage of intracellular components. Although this study has confirmed that Ag^+-mediated membrane damage occurs in this manner by a process of elimination, final confirmation of this MOA could be achieved by determining the ability of Ag^+ to damage liposomes into which integral membrane proteins have been inserted (also known as proteoliposomes), or vesicles comprising bacterial membrane extract (*i.e.* phospholipid and protein) that are extruded in a buffer containing carboxyfluorescein at a self-quenching concentration. Although attempts were made to create both during the course of this study, numerous technical difficulties were encountered which resulted in vesicles that could not retain carboxyfluorescein.

4.3.4. Genetic techniques to identify integral membrane proteins responsible for Ag^+ activity

Since Ag^+ is non-specific in its ability to inhibit the activity of bacterial enzymes, it is likely that Ag^+ can bind indiscriminately to integral membrane proteins to inhibit their function. However, certain integral membrane proteins may be more susceptible to damage by Ag^+ (*i.e.* possess more functional groups that can co-ordinate Ag^+), or be essential in maintaining bacterial membrane integrity. If this were the case, then preventing or altering the expression of such membrane proteins would likely affect the susceptibility of the host to Ag^+.

In previous studies of bacterial gene function, transposon mutagenesis has been utilised, to generate derivatives that contain a transposable element integrated into a random

location within the chromosome (Bordi *et al.*, 2008). If transposon integration occurs within the coding region, or between the coding region and the promoter of an extant gene, then the protein product of this gene will no longer be expressed/no longer function (also known as insertional inactivation). If the protein product of this gene is involved in regulating expression of other proteins, then these too will be affected. Other studies have engineered transposable elements with outward-facing promoters of varying strengths, thus enabling expression of antisense mRNA (resulting in knockdown of expression of extant genes), or altering expression levels of downstream genes, depending on the orientation which it inserts into the chromosome (Bertram *et al.*, 2005; Meredith *et al.*, 2012). Indeed, such a system was used to aid in identifying the target of CDFI, a novel antibacterial agent, as an essential membrane protein encoded by SAV1754 in *S. aureus* COL (Wang *et al.*, 2011; Meredith *et al.*, 2012). Recently a near-saturation transposon library (comprising approximately 20,500 strains) was constructed in *S. aureus* SH1000 using the modified mariner transposon $InsTet^{G+}2^{Cm}$, which contains a chloramphenicol resistance marker to allow selection of transposants, and a tetracycline-inducible outward-facing promoter (P*xyl*/*tet*) (Blake & O'Neill, 2013). Using this library, genes affecting the susceptibility of *S. aureus* to vancomycin, nisin and daptomycin were identified by determining the ability of each transposant to grow in the presence of each antibiotic at concentrations that were sub- or supra-inhibitory against non-mutagenised SH1000 (Blake & O'Neill, 2013). As this library has been previously used successfully with antibacterial agents, it was used to screen against Ag^+ in an attempt to identify integral membrane proteins that are essential to the ability of Ag^+ to damage the cell membrane. It was decided to screen for mutants that were hyper-susceptible to Ag^+, as together with potentially identifying genes essential to the membrane-damaging activity of Ag^+, other targets could be

identified that may lead to the rational design of compounds to potentiate the activity of Ag^+.

Prior to screening the library, the concentration of $AgNO_3$ allowing for selection of transposants that were hyper-susceptible to Ag^+ required optimization. To achieve this, a small collection (*n*=576) of transposants from the library were inoculated onto petri dishes containing a range of sub-inhibitory concentrations of $AgNO_3$. The concentration that led to inhibition of at least one transposant per 96 transposants screened, while permitting growth of non-mutagenised SH1000 was found to be 12 μg $AgNO_3$/ml. Using this concentration, 13,056 transposants from the library were screened, from which 34 strains were hyper-susceptible to the concentration of $AgNO_3$ used. Upon re-test, 14 strains were found to be false-positive results (*i.e.* could grow in the presence of 12 μg $AgNO_3$/ml) and were excluded from further testing. MICs of ciprofloxacin and rifampicin were determined against the remaining hyper-susceptible transposants to establish if they showed a generalised growth defect. All transposants tested were hyper-susceptible to both compounds, suggesting a growth defect was present and that hyper-susceptibility in these strains was not specific to Ag^+. As a consequence, identification of the genetic locus disrupted by the transposon in each isolate was not carried out. Given the inability to identify putative targets of Ag^+ within the cell membrane using transposon library screening, Ag^+-mediated membrane damage likely arises from denaturation of multiple integral membrane proteins, or from damage against an integral membrane protein that is essential for cell survival. Therefore, in order to explore the mechanism of Ag^+-mediated membrane damage further an alternative technique (DNA microarray) was employed.

The use of DNA microarrays to analyse bacterial transcriptomes (*i.e.* levels of global gene expression) following exposure to an antibacterial agent have been previously employed to provide insights into the antibacterial MOA for a variety of compounds; for example transcriptional analysis of *S. aureus* following exposure to daptomycin revealed a similar expression profile to cells exposed to inhibitors of peptidoglycan synthesis (vancomycin, oxacillin) and compounds that affect the membrane (nisin, CCCP), suggesting that daptomycin may have a dual mechanism of action (Muthaiyan *et al.*, 2008). Furthermore, the targets of two novel antibacterial agents; moiramide B and a phenyl-thiazolylurea derivative were identified in *B. subtilis* following transcriptome analysis of cultures exposed to these agents (Freiberg *et al.*, 2005). In the case of Ag^+ exposure, transcriptome analysis could reveal putative protein targets for Ag^+ at the membrane (which would be indicated by increased expression of genes encoding such proteins as the organism attempts to replace protein damaged by Ag^+). Furthermore, as described with daptomycin above, the transcriptional profile of cells exposed to Ag^+ could be compared with profiles arising from exposure to antibacterial agents with a known MOA, or from growth under different conditions (*e.g.* anaerobic, or biofilm-promoting environments) to identify any similarities that could provide additional insight into the effects of Ag^+ exposure. Indeed, in the case of *S. aureus*, a database of transcriptional responses to a variety of conditions arising from published studies is available (the *Staphylococcus aureus* microarray meta-database [SAMMD], accessible at http://www.bioinformatics.org/sammd/ last accessed 23rd July 2013), thus allowing for a comprehensive comparison between these conditions and Ag^+ exposure.

Analysis of gene expression was carried out in *S. aureus* SH1000 by DNA microarray. Previous studies into bacterial gene expression following antibiotic exposure recommended using a sub-inhibitory concentration of the antibacterial to be tested, as a

supra-inhibitory concentration may result in gene expression that is not specific to the antibacterial being used, but instead related to cell death (Freiberg *et al.*, 2005; O'Neill *et al.*, 2009). A number of expression studies have used a sub-inhibitory concentration of antibacterial agent sufficient to inhibit growth of exponential phase cultures by 25% (relative to drug-free controls) after 40 min exposure (Freiberg *et al.*, 2005; O'Neill *et al.*, 2009); therefore it was decided to do the same with Ag^{+} exposure. The concentration of $AgNO_3$ that produced this result was found to be 1.125 μg/ml. Total RNA was extracted from Ag^{+}-exposed and control cultures (three biological replicates of each) and subjected to transcriptional profiling by DNA microarray. Transcriptomes from Ag^{+}-exposed and drug-free cultures were then compared using LaserGene ArrayStar 4 software to identify differentially expressed genes (*i.e.* genes up- or down-regulated by at least 2-fold). This is summarised graphically in Figure 4.7.

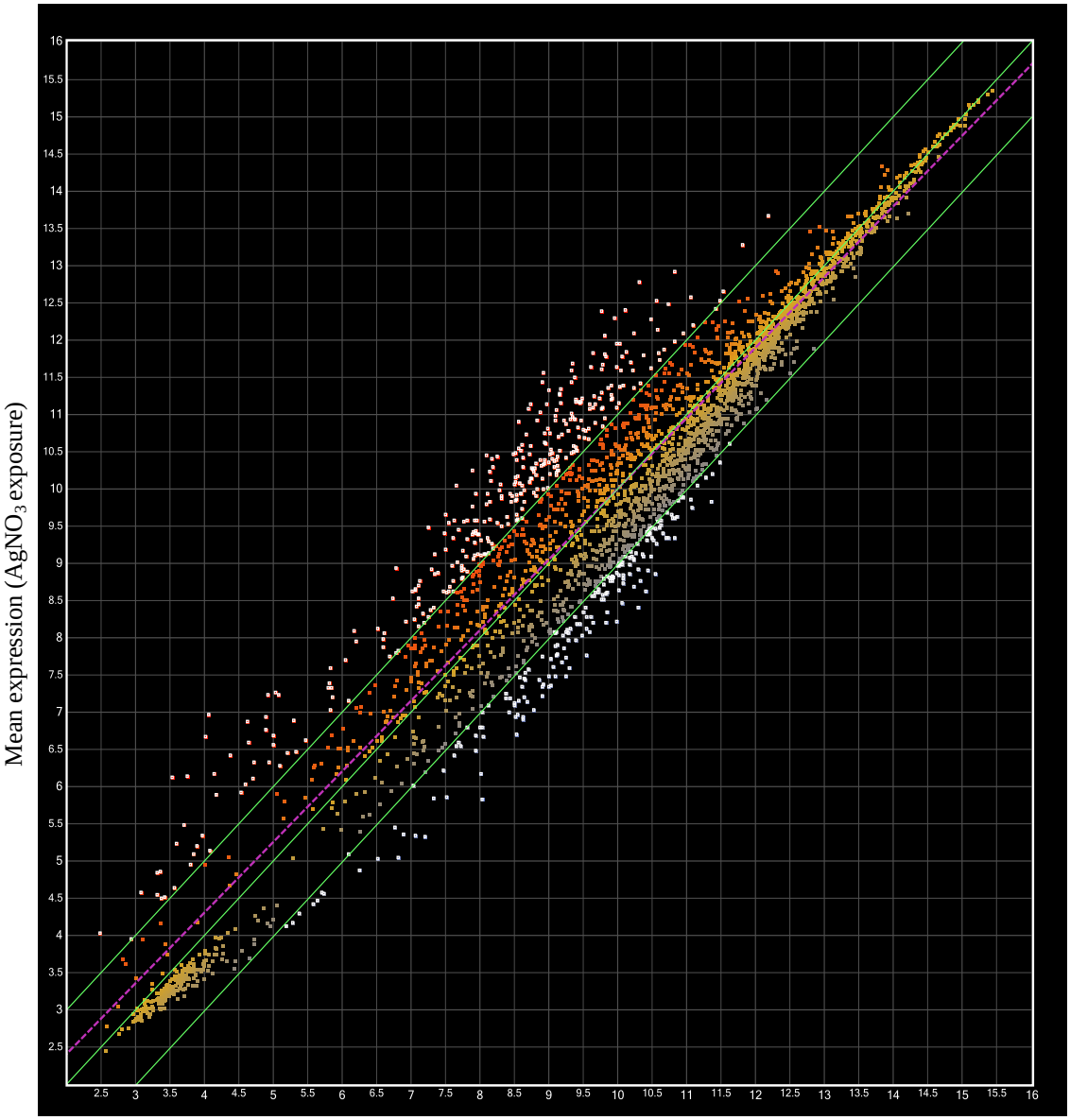


Figure 4.7. Scatter plot of differentially expressed genes following exposure to $AgNO_3$. Outer green lines represent the threshold (2-fold) considered significant for differential gene expression between $AgNO_3$-treated and drug-free control cultures. Genes with a ≥2-fold change in expression are shown in white.

In cells exposed to Ag^{+}, 526 genes were differentially regulated (339 up, 187 down) by at least 2-fold when compared with drug-free controls. This represents 18% of the total gene content of the *S. aureus* SH1000 genome. A description of the function for each gene that was differentially regulated was downloaded from the NCBI database; however, a substantial number (*n*=332) did not have an assigned function. As a consequence, BLASTp searches (at http://blast.ncbi.nlm.nih.gov/Blast.cgi, last accessed 23rd July 2013) were employed to identify conserved homologues with a known function in other bacterial species. A list of the differentially expressed genes together with a description of their function can be found in Appendix 1 (Table A1.1 and A1.2. To assist with analysis, all genes were grouped into cellular role categories using the J. Craig Venter Institute (JCVI) comprehensive microbial resource (CMR) database (http://cmr.jcvi.org/cgi-bin/CMR/CmrHomePage.cgi, last accessed 23rd July 2013), which utilises a number of homology searches and prediction algorithms to assign each gene a proposed functional role within one of seventeen categories (each containing their own sub-categories) (Tanenbaum *et al.*, 2010) (Figure 4.8).

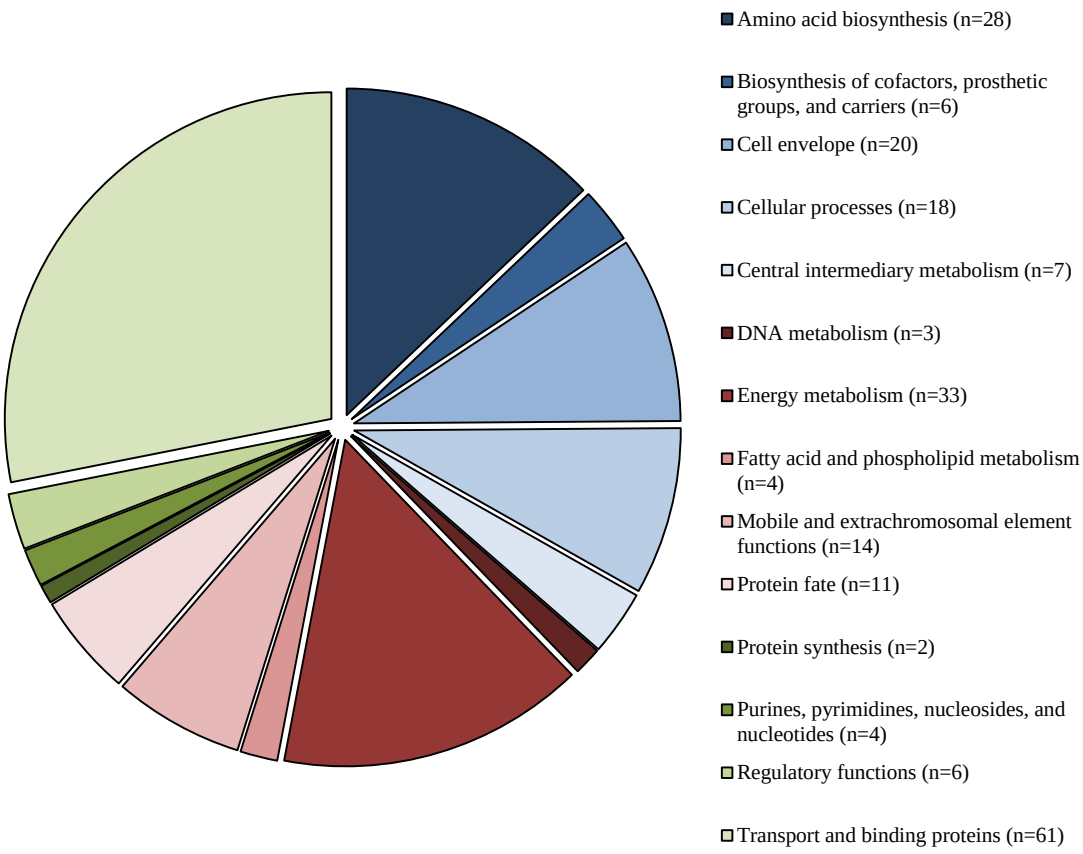


Figure 4.8. Assignment of genes up-regulated under Ag^+ exposure to JCVI cellular role categories

Of the up-regulated genes with a known function (*n*=217), a relatively large proportion (37%) were associated with processes involving the cell membrane (*i.e.* genes grouped into the "Cell envelope" and "Transport and binding proteins" categories). This result is in agreement with the finding that Ag^+ causes membrane damage, as gene up-regulation of membrane-associated components will likely occur as a secondary effect resulting from Ag^+-mediated membrane stress, or as a consequence of direct binding (and inhibition) of Ag^+ to the protein products of these genes. As the CMR database does not provide information on if the protein product of the gene is an integral membrane protein (and can thus be a direct antibacterial target of Ag^+), putative integral membrane

proteins were identified using the program SOSUI, which uses the physicochemical properties of the amino acids present in a sequence to identify an integral membrane protein based on three parameters; the presence of α-helices, and the hydrophobicity and amphiphilicity of any α-helices detected (Hirokawa *et al.*, 1998). This analysis is based on the fact that transmembrane proteins typically contain large transmembrane helices with a hydrophobic core, with amphiphilic residues at the ends of the helix to stabilise the structure (Hirokawa *et al.*, 1998). Screening of all up-regulated genes following Ag^+ challenge revealed that 93 encoded predicted integral membrane proteins (Table A1.3).

Of the genes identified, there appeared to be little functional or structural similarity between their protein products (*i.e.* number of transmembrane domains, presence of cysteine residues) that might indicate a shared site of interaction with Ag^+, suggesting that the interaction between Ag^+ and integral membrane proteins is likely non-specific. Furthermore, it is unclear which of these proteins, when inhibited by Ag^+, would be damaged sufficiently to perturb the bacterial membrane. This could be investigated by expressing each of these proteins individually and inserting them into carboxyfluorescein-filled proteoliposomes prior to challenge with Ag^+ and assessment of proteoliposome damage. However, due to the difficulties already described in constructing proteoliposomes, combined with the need to express 93 functionally active proteins, this experiment was not performed, but could be considered for future work to specify the proteins responsible for the primary MOA of Ag^+.

Together with membrane-associated proteins, a number of genes associated with intracellular processes were also up-regulated following Ag^+ exposure (such as amino acid and energy metabolism) (Table 4.7). It is unknown if Ag^+ can pass through the

membrane and into the cytoplasm of bacteria when used at sub-inhibitory concentrations; however, it would seem likely that at sub-inhibitory concentrations any Ag^+ present would bind to the membrane and not pass into the intracellular milieu. If this is the case, then genes associated with intracellular processes are likely up-regulated as an indirect effect of Ag^+ exposure. Indeed, most up-regulated genes involved in intracellular processes can be linked to the inhibition of specific transport proteins by Ag^+ (Tables 4.8-4.10). For example, a number of integral membrane proteins involved in the transport of free or peptide-associated amino acids were up regulated, probably as a consequence of direct inhibition by Ag^+. As this would reduce the concentration of intracellular amino acids, up-regulation of genes involved in amino acid biosynthesis occurs as a homeostatic mechanism in an attmpt to restore the amino acid concentration to levels necessary for cellular maintenance and growth (Table 4.8). A similar affect can be observed with the inhibition of sugar import and the up-regulation of genes involved in alternative metabolic pathways, which may also include use of amino acids as an energy source (thus acting as an additional stimulus to up-regulation of genes involved in amino acid biosynthesis) (Table 4.9) and the inhibition of iron uptake and up-regulation of genes involved in siderophore synthesis (Table 4.10).

Table 4.8. Association between inhibition of amino acid transport following Ag^{+}exposure and up-regulation of processes involved in amino acid biosynthesis

Gene	Fold Change	Function
Amino acid transport		
SAOUHSC_00168	2.1 up	Peptide ABC transporter, permease protein, putative
SAOUHSC_00169	3.7 up	Peptide ABC transporter, permease protein, putative
SAOUHSC_00932	2.4 up	Oligopeptide ABC transporter, permease protein, putative
SAOUHSC_01450	2.7 up	Putative amino acid permease/transporter protein
SAOUHSC_02967	2.3 up	Arginine/ornithine antiporter, putative
Amino acid biosynthesis		
SAOUHSC_00008	2.1 up	Histidine ammonia-lyase
SAOUHSC_00076	4.6 up	Ornithine cyclodeaminase, putative
SAOUHSC_00150	3.0 up	Ornithine aminotransferase
SAOUHSC_00340	2.9 up	Trans-sulfuration enzyme family protein
SAOUHSC_00341	3.0 up	Cystathionine gamma-synthase, putative
SAOUHSC_00435	2.1 up	Glutamate synthase, large subunit, putative
SAOUHSC_01319	2.1 up	Aspartate kinase
SAOUHSC_01367	2.0 up	Anthranilate synthase component II
SAOUHSC_01371	2.7 up	Tryptophan synthase subunit beta
SAOUHSC_01372	2.1 up	Tryptophan synthase subunit alpha
SAOUHSC_01394	2.3 up	Aspartate kinase
SAOUHSC_01451	3.3 up	Threonine dehydratase
SAOUHSC_01452	3.2 up	Alanine dehydrogenase
SAOUHSC_02281	3.5 up	Dihydroxy-acid dehydratase
SAOUHSC_02282	2.6 up	Acetolactate synthase large subunit
SAOUHSC_02284	2.5up	Ketol-acid reductoisomerase
SAOUHSC_02285	2.4 up	2-isopropylmalate synthase
SAOUHSC_02286	2.6 up	3-isopropylmalate dehydrogenase
SAOUHSC_02287	2.5 up	Isopropylmalate isomerase large subunit
SAOUHSC_02288	2.5 up	Isopropylmalate isomerase small subunit
SAOUHSC_02606	2.8 up	Imidazolonepropionase
SAOUHSC_02607	3.3 up	Urocanate hydratase
SAOUHSC_02965	2.4 up	Carbamate kinase
SAOUHSC_02968	3.6 up	Ornithine carbamoyltransferase
SAOUHSC_03008	3.8 up	Imidazole glycerol phosphate synthase subunit *hisF*, putative
SAOUHSC_03009	4.4 up	Imidazole-4-carboxamide isomerase
SAOUHSC_03013	3.3 up	Histidinol dehydrogenase

Table 4.9. Association between inhibition of sugar transport following Ag^+ exposure and up-regulation of processes involved in sugar utilisation and alternate metabolic pathways

Gene	Fold Change	Function
Sugar transport		
SAOUHSC_00158	2.9 up	Sucrose-specific PTS system component
SAOUHSC_00177	2.9 up	Maltose ABC transporter, permease protein, putative
SAOUHSC_00183	4.1 up	Hexose phosphate antiporter uhpt
SAOUHSC_00209	3.0 up	Glucose-specific PTS system component
SAOUHSC_00216	3.2 up	Galacticol-specific PTS system component
SAOUHSC_00310	2.5 up	Ascorbate-specific PTS system component
SAOUHSC_02597	2.3 up	Maltose-specific PTS system component
SAOUHSC_02806	2.4 up	Gluconate permease, putative
Sugar utilisation and alternate metabolic pathways		
SAOUHSC_00088	3.7 up	UDP-glucose 4-epimerase
SAOUHSC_00113	3.2 up	Bifunctional acetaldehyde-coa/alcohol dehydrogenase
SAOUHSC_00142	4.0 up	Formate dehydrogenase
SAOUHSC_00187	2.4 up	Formate acetyltransferase
SAOUHSC_00188	3.3 up	Pyruvate formate-lyase 1 activating enzyme, putative
SAOUHSC_00217	3.2 up	Sorbitol dehydrogenase, putative
SAOUHSC_00219	2.8 up	Sorbitol dehydrogenase, putative
SAOUHSC_00291	4.8 up	Phospho-fructokinase family carbohydrate kinase
SAOUHSC_00298	3.4 up	N-acetylmannosamine-6-phosphat e 2-epimerase
SAOUHSC_00608	2.3 up	Alcohol dehydrogenase
SAOUHSC_01846	2.5 up	Acetyl-coa synthetase
SAOUHSC_01849	2.7 up	Acetoin utilization protein
SAOUHSC_02451	2.8 up	PTS system, lactose-specific component
SAOUHSC_02452	2.3 up	Tagatose 1,6-diphosphate aldolase
SAOUHSC_02453	2.2 up	Tagatose-6-phosphate kinase
SAOUHSC_02454	2.1 up	Galactose-6-phosphate isomerase subunit LacB
SAOUHSC_02455	2.5 up	Galactose-6-phosphate isomerase subunit
SAOUHSC_02921	3.3 up	Alpha-acetolactate decarboxylase

Table 4.10. Association between inhibition of iron following Ag^+ exposure and up-regulation of iron sequestration/transport mechanisms

Gene	Fold change	Function
Iron transport		
SAOUHSC_00071	2.2 up	Permease component of ABC-type Fe^{3+}-siderophore transport system
SAOUHSC_00072	3.3 up	Permease component of ABC-type Fe^{3+} -siderophore transport system
SAOUHSC_00078	3.7 up	Siderophore staphylobactin biosynthesis protein SbnD
SAOUHSC_00327	2.3 up	Ferrous iron transport permease EfeU
SAOUHSC_02864	2.6 up	FeoB family ferrous iron (Fe^{2+}) uptake protein
SAOUHSC_02902	2.7 up	FeoB family ferrous iron (Fe^{2+}) uptake protein
Iron sequestration mechanisms		
SAOUHSC_00077	4.5 up	Siderophore staphylobactin biosynthesis protein SbnC (IucA/IucC family siderophore biosynthesis protein)
SAOUHSC_00079	3.1 up	Siderophore staphylobactin biosynthesis protein SbnE (IucA/IucC family siderophore biosynthesis protein)
SAOUHSC_00080	2.7 up	Siderophore biosynthesis protein SbnF (IucA/IucC family siderophore biosynthesis protein)
SAOUHSC_00081	2.9 up	Siderophore staphylobactin biosynthesis protein SbnG
SAOUHSC_00082	2.9 up	Siderophore staphylobactin biosynthesis protein SbnH/
SAOUHSC_00083	3.3 up	Siderophore staphylobactin biosynthesis protein SbnI
SAOUHSC_01843	2.5 up	Haptoglobin-binding heme uptake protein HarA
SAOUHSC_00130	4.5 up	Heme-degrading monooxygenase IsdI

The inhibition of transport proteins by Ag^+ may suggest that, at sub-inhibitory concentrations, Ag^+ exposure restricts uptake of sufficient quantities of the intracellular substrates required for cell growth and survival. As the concentration of Ag^+ increases, this effect is likely to become more pronounced until a concentration of Ag^+ sufficient to cause lethal damage to the membrane is reached. In pathogenic bacteria, nutrient limitation typically leads to the expression of genes involved in virulence and the switch to an alternate method of growth (for example biofilm formation) in an attempt by the organism to mitigate these effects (Anderson *et al.*, 2006). Indeed, this can be observed in the *S. aureus* transcriptome following sub-inhibitory Ag^+ exposure, with the up-regulation of secreted proteases and mechanisms to protect from host immune responses (Table 4.11). Furthermore, components of the *ica* operon were found to be differentially regulated following Ag^+ exposure. The *ica* operon encodes components responsible for synthesis of polysaccharide intercellular adhesin (PIA) which enables attachment of *S. aureus* to a variety of surfaces, and is the first step in biofilm formation (Brady *et al.*, 2006). The propensity of *S. aureus* to increase the expression of virulence factors when exposed to sub-inhibitory Ag^+ suggests that care should be taken when employing therapeutic Ag^+ for the treatment of bacterial infection, as sub-inhibitory concentrations of Ag^+ could negatively impact on patient morbidity by prompting an increase in bacterial virulence, or by triggering a switch to a biofilm mode of growth that is highly recalcitrant to eradication by Ag^+ and other antibacterial agents (as described in Chapter three).

Table 4.11. Up-regulation of known and putative virulence factors following exposure to Ag^+

Gene	Fold Change	Description	Reference
SAOUHSC_00069	2.7 up	Staphylococcal protein A (Spa). Inhibits phagocytosis	(Palmqvist *et al.*, 2002)
SAOUHSC_00089	4.3 up	Capsular polysaccharide biosynthesis glycosyltransferase, putative. Extracellular capsule production to protect from phagocytosis	(O'Riordan & Lee, 2004)
SAOUHSC_00256	2.9 up	Staphyloxanthin biosynthesis protein, putative. Provides protection aginst external sources of oxidative stress such as neutrophil respiratory bursts	(Liu *et al.*, 2005)
SAOUHSC_00259	2.3 up	Virulence protein EssA. Component of the ESAT-6 secretion system used to secrete virulence factors	(Russell *et al.*, 2011)
SAOUHSC_00264	2.2 up	Virulence protein EsaC. Function same as SAOUHSC_00259	(Russell *et al.*, 2011)
SAOUHSC_00292	4.4 up	Indigoidine synthase A-like protein. Indigoidine is a pigment implicated in pathogenicity and protection from oxidative stress.	(Revercho n *et al.*, 2002)
SAOUHSC_00383	3.3 up	Putative superantigen-like protein. Proposed to limit neutrophil recruitment and general modulation of immune response	(Itoh *et al.*, 2010)
SAOUHSC_00384	2.4 up	Putative superantigen-like protein. Function same as SAOUHSC_00383	(Itoh *et al.*, 2010)
SAOUHSC_00389	5.7 up	Putative superantigen-like protein. Function same as SAOUHSC_00383	(Itoh *et al.*, 2010)
SAOUHSC_00390	3.6 up	Putative superantigen-like protein. Function same as SAOUHSC_00383	(Itoh *et al.*, 2010)
SAOUHSC_00391	3.6 up	Putative superantigen-like protein. Function same as SAOUHSC_00383	(Itoh *et al.*, 2010)
SAOUHSC_00393	2.4 up	Putative superantigen-like protein. Function same as SAOUHSC_00383	(Itoh *et al.*, 2010)
SAOUHSC_00399	3.6 up	Putative superantigen-like protein. Function same as SAOUHSC_00383	(Itoh *et al.*, 2010)
SAOUHSC_00674	2.6 down	Staphylococcal accessory protein X (SarX) - Negative regulator of agr expression	(Manna & Cheung, 2006)
SAOUHSC_00694	2.3 down	Transcriptional regulator MgrA. Regulator of *sarX* and *norB* expression	(Manna & Cheung, 2006)
SAOUHSC_01873	2.4 up	Cell-wall-anchored protein SasC. Proposed role in intercellular adhesion and biofilm accumulation	(Schroeder *et al.*, 2009)
SAOUHSC_01935	2.2 up	Serine protease SplF. Secreted protease	(Popowicz *et al.*, 2006)
SAOUHSC_01939	2.5 up	Serine protease SplC. Secreted protease	(Popowicz *et al.*, 2006)
SAOUHSC_01941	3.7 up	Serine protease SplB. Secreted protease	(Popowicz *et al.*, 2006)

Table 4.11 (continued)

SAOUHSC_01942	4.1 up	Serine protease SplA. Secreted protease	(Popowicz *et al.*, 2006)
SAOUHSC_01954	2.1 up	Leukotoxin LukD. In combination with LukE forms pores in leukocyte cell membranes leading to their death	(Gravet *et al.*, 1998)
SAOUHSC_01955	2.3 up	Leukotoxin LukE. In combination with LukD forms pores in leukocyte cell membranes leading to their death	(Gravet *et al.*, 1998)
SAOUHSC_02971	4.2 up	Zinc metalloproteinase aureolysin, putative. Cleaves compliment C3 to assist in *S. aureus* immune evasion	(Laarman *et al.*, 2011)
SAOUHSC_02998	3.4 up	Capsular polysaccharide biosynthesis protein, Cap5C. Function same as SAOUHSC_00089	(O'Riordan & Lee, 2004)
SAOUHSC_02999	3.8 up	Capsular polysaccharide biosynthesis protein Cap5B. Function same as SAOUHSC_00089	(O'Riordan & Lee, 2004)
SAOUHSC_03000	2.7 up	Capsular polysaccharide biosynthesis, CapA, putative. Function same as SAOUHSC_00089	(O'Riordan & Lee, 2004)
SAOUHSC_03001	2.8 down	Ica operon transcriptional regulator IcaR, putative. Negative regulator of *ica* operon	(Cramton *et al.*, 1999)
SAOUHSC_03002	3.4 up	N-glycosyltransferase IcaA. Catalyses first step of polysaccharide intercellular adhesin synthesis	(Cramton *et al.*, 1999)
SAOUHSC_03003	3.0 up	Intercellular adhesion protein D (IcaD). Catalyses second step of polysaccharide intercellular adhesin synthesis	(Cramton *et al.*, 1999)

Comparing transcriptomes between *S. aureus* exposed to Ag^+ and *S. aureus* exposure to other antibacterial agents or environmental conditions could provide further insight into the cellular events occurring following Ag^+ exposure. Using SAMMD, gene expression data from a variety of conditions was compared with that following Ag^+ exposure. Most conditions shared little similarity in gene-expression profiles with Ag^+ exposure; however, comparison between cultures exposed to Ag^+ and those exposed to the proton ionophore CCCP revealed a similar profile in differentially expressed genes, particularly those involved in the transport of amino acids and sugars, and subsequent processes of amino acid synthesis and utilisation of alternative substrates for energy production (Muthaiyan *et al.*, 2008). Furthermore, the expression of genes *lgrAB* were up-regulated under both conditions, as well as in cells exposed to daptomycin, another

membrane-damaging compound. The genes *lrgAB* both encode putative antiholin proteins that have previously been found to be overexpressed when the membrane potential is disrupted (Patton *et al.*, 2006). This is to be expected for CCCP as it is a proton ionophore that dissipates membrane potential. It has previously been demonstrated that Ag^+ exposure dissipates the membrane potential when used at 4X MIC, however this result suggests that the membrane potential is also impacted when used at sub-inhibitory concentrations. The mechanism by which Ag^+ dissipates the membrane potential is likely to be different from that of CCCP, in that it will bind to and inhibit membrane proteins involved in the efflux of protons, as opposed to CCCP, which forms pores in the membrane to allow effluxed protons to diffuse back into the cell. Although there are similarities in gene expression between Ag^+ and CCCP-exposed cultures, a number of differences can also be observed. For example, exposure to CCCP leads to up-regulation of a number of genes involved in degradation of proteins, peptides and glycopeptides and protein folding and stabilisation (Muthaiyan *et al.*, 2008). This difference may be a consequence of the different mechanisms by which CCCP and Ag^+ cause membrane damage, or, as the concentration of CCCP used in transcriptional analysis was above its MIC against *S. aureus*, the up-regulation of these genes may be a non-specific result of cell death. Nevertheless, other genes that are commonly found to be up-regulated by membrane damaging agents are not up-regulated following Ag^+ exposure. The K^+-import ATPase, encoded by *kdpCBA* is up-regulated in the presence of daptomycin, CCCP, clofazimine and the putative membrane-damaging agent sepracor 155342 (Muthaiyan *et al.*, 2008; Mariner, 2011). Again, this may indicate a different mechanism by which Ag^+ causes membrane damage. Indeed, unlike Ag^+, both clofazimine and daptomycin cause membrane damage via disruption of the phospholipid bilayer, therefore this mechanism of damage may be more efficient at

promoting K^+ release, leading to the up-regulation of mechanisms to mitigate such effects.

The transcriptomes of Ag^+-exposed cultures also shared similarities with cultures grown under anaerobic conditions. When *S. aureus* is exposed to anaerobic conditions, its transcriptome changes to reflect the reduced output of ATP from the electron transport chain (due to the lack of oxygen to act as a terminal electron acceptor) (Fuchs *et al.*, 2007). However, if a source of nitrate/nitrite is present, these can act as terminal electron acceptors, which allow the continued functioning of the electron transport chain (Fuchs *et al.*, 2007). To facilitate this, under anaerobic conditions genes encoding nitrate reductases and nitrite transporters are all up-regulated (irrespective of whether a source of nitrate/nitrite is present) (Fuchs *et al.*, 2007). This effect can also be observed in cultures exposed to Ag^+, with the up-regulation of genes encoding nitrate reductase (alpha and beta subunits) (SAOUHSC_02680/02681), assimilatory nitrite reductase (SAOUHSC_02684), transcriptional regulator NirR (SAOUHSC_02685), nitrite extrusion protein (SAOUHSC_02671) and a nitrite transporter SAOUHSC_00281). If a source of nitrate/nitrite is unavailable then *S. aureus* derives energy from alternate sources. This can include mixed acid and butanediol fermentation and the conversion of the amino acids arginine, alanine, glycine, and serine into formate by formate acetyltransferase and pyruvate-formate lyase. Formate can then be used to generate ATP by formate dehydrogenase (Fuchs *et al.*, 2007). Following Ag^+ exposure, all these components were found to be up-regulated (SAOUHSC_00608, 00113, 02282, 02283, 02921, 01849 00187, 00188 and 00142). This switch to anaerobic mechanisms of energy generation would suggest that the electron transport chain is damaged by Ag^+ exposure, thus preventing the generation of ATP by this pathway. Indeed, it has been shown that when the electron transport chain is disrupted under aerobic conditions,

expression of mixed acid and butanediol fermentation enzymes is up-regulated (Kohler *et al.*, 2003; Seggewiss *et al.*, 2006). As all of the components of the electron transport chain in *S. aureus* are membrane-associated or integral membrane proteins, it is likely that Ag^+ directly inhibits one or more of these components. It has been suggested that the inhibition of enzymes involved in the initial steps of the electron transport chain by Ag^+ would lead to electron stalling, allowing premature interaction with O_2 to produce O_2^- (Park *et al.*, 2009). The results obtained in this current study seem to support this hypothesis. However, at sub-inhibitory concentrations of Ag^+, it appears that damage to the electron transport chain is insufficient to generate ROS, as up-regulation of genes encoding ROS protection mechanisms (*i.e.* superoxide dismutases, catalase and alkyl hydroperoxide reductase) did not occur.

During the course of this work, a study into the effect of Ag^+ on gene expression in *S. epidermidis* was published (Gordon *et al.*, 2010). As expected, a comparison of results between studies revealed a number of similarities in gene-expression profiles. In particular, genes involved in fermentative processes were up-regulated, whereas those encoding TCA cycle components were down regulated, suggesting that under both conditions damage to the electron transport chain is occurring. Furthermore, processes related to amino acid synthesis and the initial stages of biofilm formation were up-regulated, which is indicative of substrate starvation and a switch to a protective mode of growth to mitigate this effect. However, a number of discrepancies in gene expression also exist between the studies. For example, the iron transport systems of *S. epidermidis* were found to be down-regulated under Ag^+ exposure, in contrast to the results obtained in this study, where equivalent systems were up-regulated.

Furthermore, in *S. epidermidis* genes encoding the anti-holin enzymes LgrAB are down-regulated, and there was no evidence of inhibition of amino acid or sugar

transport proteins. Taken together, this difference may suggest that the mechanism by which Ag^+ damages the *S. epidermidis* cell membrane differs from *S. aureus*, or that Ag^+ relies upon an alternate antibacterial target to inhibit *S. epidermidis*. However, in their study Gordon *et al*. analysed the *S. epidermidis* transcriptome following exposure to a supra-inhibitory concentration of Ag^+ (20 μg $AgNO_3$/ml) (Gordon *et al.*, 2010). It is therefore probable that a number of differentially expressed genes are a consequence of non-specific effects relating to cell death. Alternatively, at a supra-inhibitory concentration of Ag^+, damage to the bacterial membrane will be more extensive, thus the expression of genes encoding membrane-associated and integral membrane proteins may differ as a result.

Conclusions

Although Ag^+ has the potential to inhibit multiple enzymatic processes, the rapid and substantial loss of bacterial membrane integrity following exposure to Ag^+ suggests that the primary target of Ag^+ in *S. aureus* is the membrane. The observation that Ag^+ does not damage phospholipid bilayers (in the form of liposomes), but causes significant up-regulation of a substantial number of integral membrane proteins would suggest that Ag^+ causes membrane damage by non-specific binding to membrane proteins, resulting in damage sufficient to permit the leakage of intracellular contents, and ultimately leading to cell death (Figure 4.9). Following membrane damage, any remaining bioavailable Ag^+ can enter the cell and bind intracellular components, causing their inhibition and accelerating Ag^+-mediated killing (Figure 4.9). Most antibacterial agents target metabolic processes and are therefore ineffective against treating growth-arrested cells when these processes are inactive (Mascio *et al.*, 2007; Ooi *et al.*, 2010). This makes such compounds ineffective against treating persistent infections where growth-arrested cells predominate, such as in chronic wounds (Hurdle *et al.*, 2011). As Ag^+ causes membrane damage, it should retain activity against cells in such a state and thus emphasises an advantage of Ag^+ over other topical antibacterial agents such as mupirocin and fusidic acid in the treatment of persistent wounds. However, as demonstrated in Chapter 3, this activity does not translate into the ability of Ag^+ to kill growth-arrested cells present within a biofilm.

In studies using *E. coli* and *S. epidermidis*, Ag^+ has been shown to generate ROS, which are believed to contribute to the antibacterial MOA of Ag^+ by causing oxidative damage to essential cellular components; however, the extent of the contribution provided by ROS was unclear. The increased inhibitory and killing activity of Ag^+ observed against *S. aureus* strains deficient in ROS protection mechanisms indicate that Ag^+ does induce

ROS formation, and transcriptional profiling of *S. aureus* exposed to Ag^+ revealed that Ag^+ inhibited components of the electron transport chain, which suggests a likely source of ROS generation (Figure 4.9). However, although ROS is generated by Ag^+, this study revealed that it was at a concentration insufficient to contribute to the killing activity of Ag^+ or Ag^+-mediated membrane damage, as under anaerobic conditions the killing activity of Ag^+ and the ability of Ag^+ to damage *S. aureus* membranes was not affected. Nevertheless, as Ag^+ induces the production of ROS, it could be combined with other ROS-producing compounds to potentiate the antibacterial activity of Ag^+. Indeed, the combination of Ag^+ with the ROS-producing methyl viologen (Paraquat) was found to be synergistic against *E. coli* (Park *et al*., 2009).

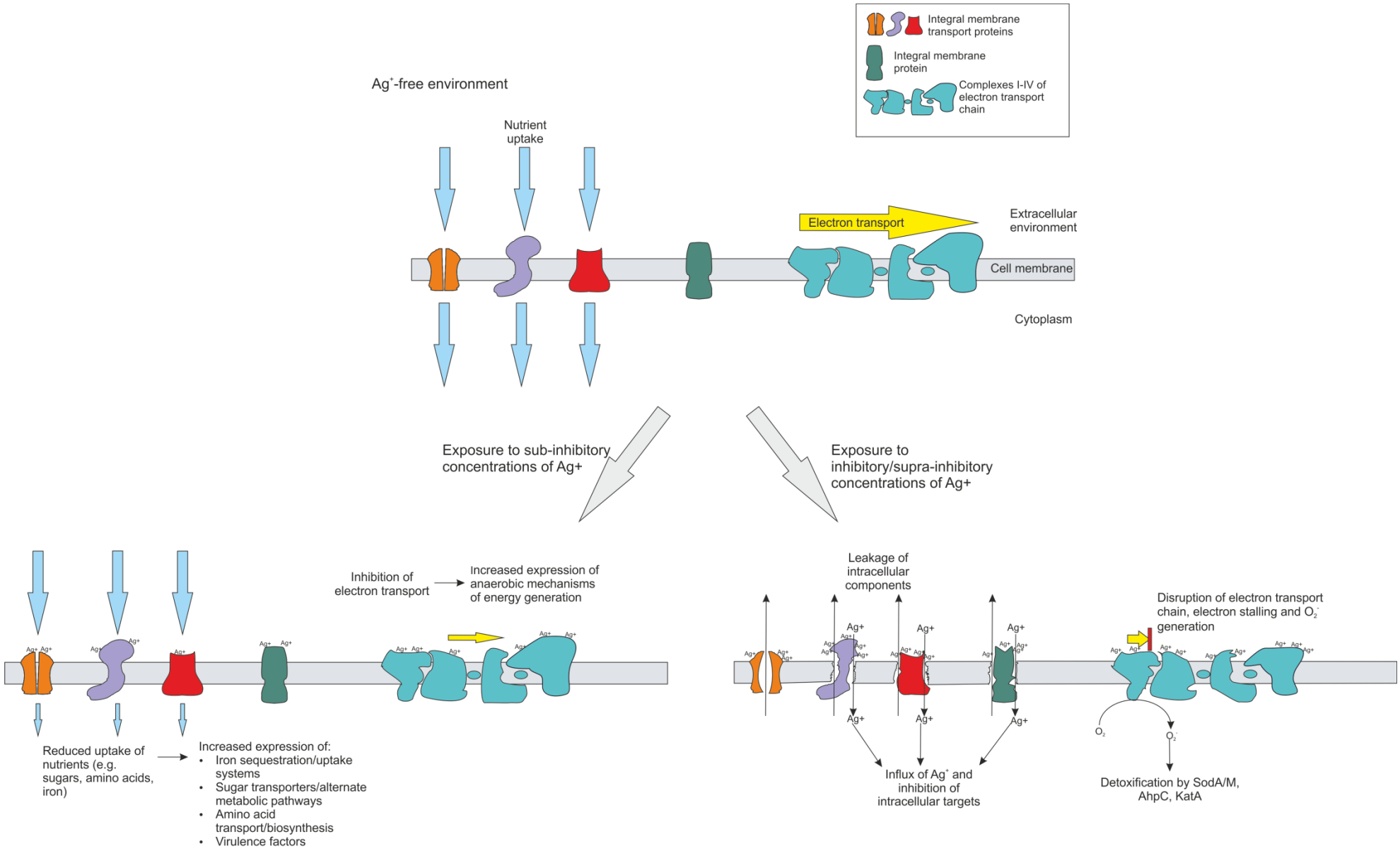


Figure 4.9. Proposed antibacterial mode of action of Ag^{+} against *S. aureus*

As well as defining the antibacterial MOA of Ag^{+}, insights into the antibacterial mechanisms of other compounds were provided by this study. The inability of daptomycin to disrupt bilayers comprising *E. coli* phospholipid provided evidence to suggest that the antibacterial activity of daptomycin is dependent upon the concentration of anionic phospholipids at the cell membrane (Randall *et al*., 2013). Furthermore, this study revealed that the anti-staphylococcal activity of CTAB is reliant upon generation of ROS, as its ability to damage membranes and its killing activity was significantly reduced under anaerobic conditions.

Chapter five: Resistance to Ag^+ in Gram-negative bacteria: a dissection of endogenous and exogenous mechanisms

Abstract

In contrast to the situation in Gram-positive bacteria, high-level Ag^+ resistance has been identified in Gram-negative bacteria and can arise by two alternate mechanisms. In *E. coli*, endogenous (mutational) Ag^+ resistance can be selected in the laboratory by continuous exposure to Ag^+. Such resistant mutants show a porin-deficient phenotype and can efflux Ag^+, however the genetic changes underlying this phenotype, and the propensity for resistance by this mechanism to emerge, persist, and spread in an environment where therapeutic Ag^+ is used remains unclear. An alternative mechanism of Ag^+ resistance is conferred by the nine-component Sil-system found on the *Salmonella* Typhimurium conjugative plasmid pMG101, which is proposed to confer Ag^+ resistance by a combination of Ag^+ sequestration and efflux; however, the relative contribution of each component to resistance is unknown. This study found that endogenous Ag^+ resistance can arise under six days of continuous exposure to sub-inhibitory Ag^+, and results from a loss of function mutation in OmpR (preventing expression of OmpC/F), and a second mutation in the sensor kinase CusS, enabling constitutive expression of the Ag^+ efflux pump CusCFBA. Using a next-generation sequencing approach, the *sil* operon on plasmid pMG101 was found to be part of a Tn*7*-like transposon that can integrate into the host chromosome. A systematic deletion strategy revealed that only the periplasmic binding protein SilE and efflux pump SilCFBA were essential for an Ag^+ resistance phenotype. In addition, the *sil* operon was found to be a native component of strains of *Enterobacter cloacae* and *Klebsiella pneumoniae*; however, these species were not intrinsically resistant to Ag^+ unless gain-of-function mutations were selected in the sensor kinase SilS. The relative

ease with which endogenous Ag^+ resistance can be selected, combined with the risk associated with horizontal transmission of the Sil-system, highlights the need to control the proliferation of Ag^+-containing products so that the clinical utility of antibacterial Ag^+ is not lost.

5.1. Introduction

In Chapter 3 it was demonstrated that Ag^+ resistance is not prevalent amongst clinical isolates of *S. aureus*, nor does resistance arise easily via endogenous mechanisms during continuous exposure to Ag^+ in a laboratory environment. However, in Gram-negative bacteria Ag^+ resistance is already known to exist. Under laboratory conditions Li *et al*., (1997) were able to isolate strains of *E. coli* resistant to an Ag^+ concentration >64 times that of the parental strain (Li *et al.*, 1997). Resistant strains were found to be deficient in the porin OmpF alone, or in combination with OmpC, and demonstrated the ability to efflux Ag^+ (Li *et al.*, 1997). Further analysis suggested that Ag^+ efflux in these strains was carried out by the Ag^+/Cu^+ transporter CusCFBA, as proteomic analysis identified components of this system (CusF and CusB) to be overexpressed in a resistant strain, and deletion of c*usF* restored Ag^+ susceptibility to that of the parental strain (Lok *et al*., 2008). Although this study identified a mutation in *cusS*, which encodes an amino acid substitution in the sensor kinase that regulates CusCFBA expression, the genetic events leading to endogenous Ag^+ resistance have not been explored in detail. In addition, the time taken for resistance by this mechanism to arise was not reported, thus the potential for emergence of this phenotype in an environment where Ag^+ is used to prevent and treat infection is unclear.

Unlike endogenous (mutational) Ag^+ resistance described above, exogenous (horizontally-acquired) Ag^+ resistance has been encountered clinically. In 1975, an Ag^+-resistant strain of *Salmonella enterica* serovar Typhimurium caused an outbreak on a burns ward resulting in the death of three people (McHugh *et al.*, 1975). The Ag^+-resistance determinant was found to be located on a 14.2 kb region of the plasmid pMG101 and was named the Sil system (Gupta *et al.*, 1999). The *sil* operon comprises nine open reading frames (*silS, R, E, C, F, B, A, ORF105* and *P)*, and, with the

exception of SilE, which was shown to be a periplasmic Ag^+ binding protein, the function of the remaining components have yet to be confirmed experimentally. However, based on homology searches, the Sil operon is believed to confer Ag^+ resistance by a combination of Ag^+ sequestration in the periplasm (via SilE and SilF binding) and efflux (via the RND pump homologue SilCBA and P-type ATPase homologue SilP). Nevertheless, the relative contribution provided by each component to the resistance phenotype is unknown.

5.2. Objectives

The objectives of the work presented in this chapter are as follows:

- determine the time taken for endogenous Ag^+ resistance to arise in *E. coli*, and define the genetic and phenotypic events that confer the Ag^+resistance phenotype.
- determine the relative contribution to Ag^+ resistance provided by each component of the Sil system.

5.3. Results and discussion

5.3.1. Selection and characterisation of endogenous silver resistance in E. coli

5.3.1.1. Endogenous Ag^+ resistance can arise in E. coli after six days of continuous exposure to Ag^+, as a consequence of two mutational events

Although it has been demonstrated that endogenous resistance to Ag^+ can be selected in the laboratory, the time taken for resistance to emerge has not been reported (Li *et al.*, 1997). To determine if endogenous Ag^+ resistance can arise from a single mutational event, overnight cultures of *E. coli* strain BW25113 were plated onto MHA containing $AgNO_3$ at 4X MIC (16 μg of $AgNO_3$/ml). Following a 24 h incubation, no spontaneous mutants could be obtained (limit of detection $<1\times10^{-10}$), implying that endogenous Ag^+ resistance in *E. coli* cannot arise from a single mutational event (O'Neill & Chopra, 2004). As a consequence, strain BW25113 was subjected to continuous exposure of sub-inhibitory Ag^+ in an identical manner to that described for *S. aureus* in Chapter 3. Following continuous exposure for six days, a strain resistant to >256 μg/ml $AgNO_3$ was selected (parental strain MIC: 4 μg/ml). The ability to select for resistance in this manner, and over this time, was reproducible (n=3).

Endogenous Ag^+ resistance in *E. coli* results from down-regulation or loss of outer membrane porins, and up-regulation of Ag^+ efflux (likely mediated by CusCFBA) (Li *et al.*, 1997; Lok *et al.*, 2008); however, the genetic changes that confer this phenotype have not been explored in detail. To address this, the DNA sequence of the following genes were determined in the Ag^+-resistant strain: *ompF, ompC, ompA* (encoding the three major porins of *E. coli*), *ompR, envZ* (encoding regulators of porin expression), *cusR* and *cusS* (encoding regulators of expression of the CusCFBA efflux transporter). DNA sequence determination identified missense mutations $G_{596}A$ in *ompR* and $G_{1130}A$ in *cusS* (leading to amino acid substitutions $R_{199}H$ and $R_{377}H$ in OmpR and CusS, respectively). To confirm that these two mutations were necessary and sufficient for the

Ag^+ resistance phenotype, they were introduced, alone and in combination, into the parental strain (*E. coli* BW25113) by recombineering. Individually, neither mutation conferred any reduction in susceptibility to Ag^+ compared with the parental strain (MIC of 4 μg $AgNO_3$/ml). However, when both mutations were introduced into the same strain, Ag^+ resistance (MIC >256 μg of $AgNO_3$/ml) was observed, indicating that high-level endogenous Ag^+ resistance can arise as a consequence of only two mutational events. Furthermore, in strains carrying only one of these mutations, spontaneous Ag^+-resistant mutants could be selected by plating onto agar containing $AgNO_3$ at 4X MIC, with mutants obtained at a frequency of $6.7 \pm 1.4 \times 10^{-8}$ and $9.2 \pm 1.2 \times 10^{-8}$ for strains BW25113 *cusS*G_{1130}A and BW25113 *ompR*G_{596}A, respectively. These frequencies are consistent with that of a single mutational event (O'Neill & Chopra, 2004). Sequence determination of Ag^+-resistant BW25113 *cusS*G_{1130}A revealed mutations in *ompR*, whereas mutations in *cusS* were found in Ag^+-resistant BW25113 *ompR*G_{596}A, thus giving further indication that mutations in both *ompR* and *cusS* are required to confer an Ag^+ resistance phenotype.

By determining the sequence of *ompR* and *cusS* from multiple independent mutants, the diversity of mutations that contribute to Ag^+ resistance could be determined, which may assist in elucidating the functional changes that occur in OmpR and CusS to bestow Ag^+ resistance. Therefore, *ompR* or *cusS* was sequenced from five independent Ag^+-resistant mutants derived from BW25113 *cusS*G_{1130}A and BW25113 *ompR*G_{596}A, respectively (Table 5.1). These results are discussed in the following sections of this chapter.

Table 5.1. Mutations in *ompR* and *cusS* associated with endogenous Ag^+ resistance in *E. coli* BW25113

Nucleotide change	Amino acid substitution
Mutations in *cusS*	
$A_{49}C$	$T_{17}P$
$T_{638}G$	$I_{213}S$
$C_{935}A$	$A_{312}E$
$C_{1052}A$	$A_{351}E$
$G_{1130}A$	$R_{377}H$
Mutations in *ompR*	
$A_{611}C$	$Q_{204}P$
$C_{88}T$	$Q_{30}STOP$
$G_{596}A$	$R_{199}H$

5.3.1.2. Mutational changes in the ompR gene of Ag^+-resistant mutants prevent the expression of outer membrane porins OmpC and OmpF

The EnvZ-OmpR two-component system is responsible for sensing and regulating cellular changes to extracellular osmolarity (Cai & Inouye, 2002). In environments of low osmolarity the kinase activity of EnvZ is repressed, limiting the phosphorylation of its cognate response regulator OmpR, and resulting in low intracellular concentrations of phosphorylated OmpR (pOmpR). Under these conditions, pOmpR preferentially binds to regions of DNA upstream of *ompF*, recruits RNA polymerase and enables *ompF* transcription (Cai & Inouye, 2002). The protein product, OmpF, is an integral outer membrane protein with a relatively large pore diameter (1.16 nm), which thereby allows for an increased passive diffusion of solutes into the cell (Cowan *et al.*, 1995). If the extracellular osmolarity increases, the kinase activity of EnvZ is activated, leading to increased phosphorylation of OmpR. At high concentrations, pOmpR can interact with additional regions of DNA, such as upstream of *ompC* (enabling its transcription) and an additional site upstream of the *ompF* promoter, altering the tertiary structure of DNA in this region such that RNA polymerase recruitment is prevented and *ompF* transcription cannot occur (Cai & Inouye, 2002). In combination, this has the effect of

reducing expression of OmpF and increasing expression of OmpC, which has a smaller pore diameter than OmpF (1.08 nm) and limits passive diffusion of water from the cell as a consequence (Basle *et al.*, 2006).

During the course of this study three independent mutations were identified in *ompR* that contributed to the endogenous Ag^+ resistance phenotype. The mutation $C_{88}T$ introduces a premature stop codon into *ompR*, which would lead to the expression of a truncated protein product. Truncated OmpR, irrespective of its phosphorylation status, is unlikely to be able to bind DNA and mediate transcription of *ompC/F*; as a consequence, cells harbouring this mutation will be unable to express OmpC or OmpF. It seems likely that the mutations in *ompR* identified from other Ag^+-resistant strains ($A_{611}C$ and $G_{596}A$) similarly disrupt the function of OmpR. To confirm that the mutations identified in *ompR* prevented expression of OmpC/F, outer membranes were extracted and purified from strain BW25113 *ompR*$G_{596}A$ *cusS*$G_{1130}A$ and the parental strain. Analysis of porin expression using SDS-PAGE revealed that bands corresponding to OmpC/F were not present in strains containing these mutations (Figure 5.1).

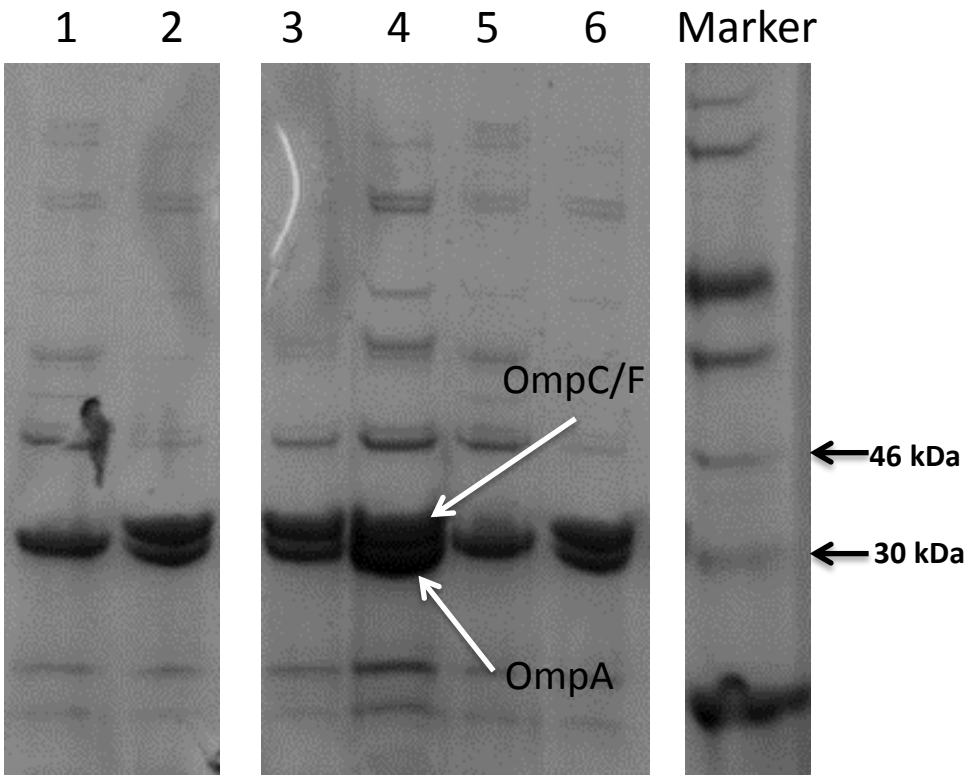


Figure 5.1. SDS-PAGE of outer membrane proteins from BW25113 *ompR*G_{596}A *cusS*G_{1130}A and comparator strains. Lane 1, BW25113 *ompR*G_{596}A *cusS*G_{1130}A; Lane 2, BW25113 Δ*ompF*, Lane 3, BW25113 Δ*ompC*; Lane 4, BW25113; Lane 5, BW25113 Δ*ompR*; Lane 6, NCTC 50110. N.B. OmpC and OmpF co-migrate under standard SDS-PAGE conditions

As the structure of the C-terminal domain of OmpR has been determined (PDB accession: 10DD), the amino acid substitutions encoded by $A_{611}C$ and $G_{596}A$ were mapped onto this structure in order to provide insight into how they may disrupt OmpR function (Figure 5.2).

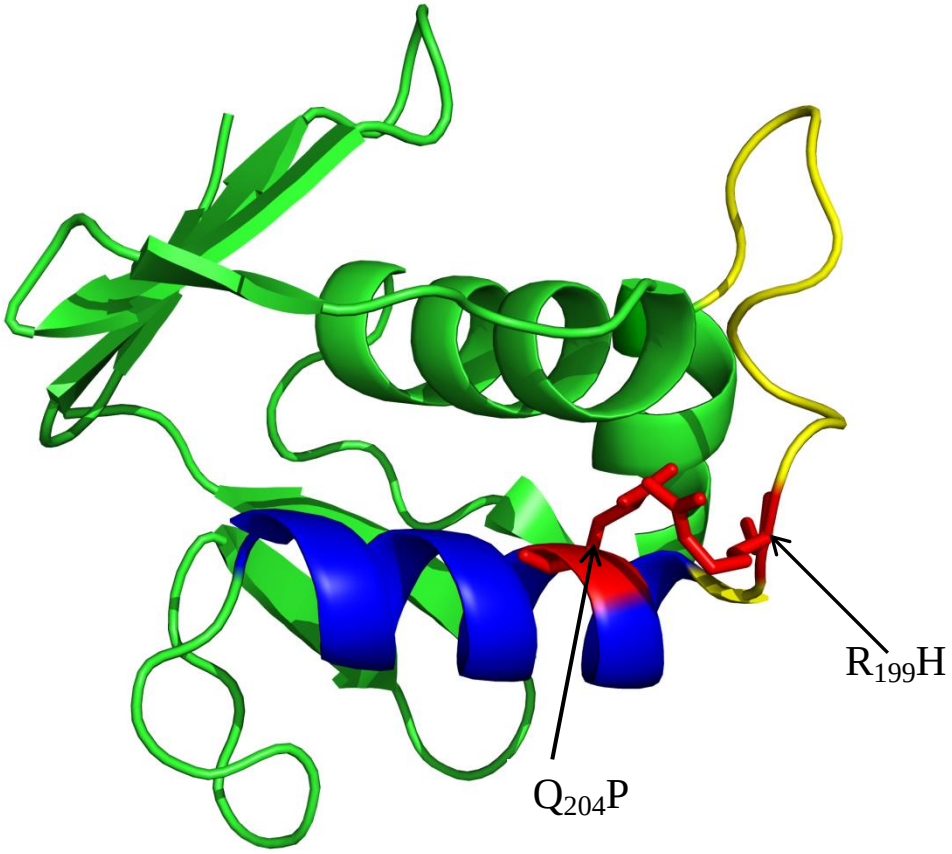


Figure 5.2. Location of amino acid substitutions found in OmpR in Ag^+-resistant mutants of *E. coli.* The residues comprising the α3 DNA-binding helix are highlighted blue, whereas those highlighted yellow represent the "turn" (loop) region essential for recruitment of RNA polymerase. Residues in red are those found to be altered in Ag^+-resistant strains.

The residues 138 to 233 of OmpR comprise a winged helix-turn-helix (wHTH) domain (Kondo *et al.*, 1997). This domain is capable of binding DNA and variations of it exist amongst a number of transcriptional regulators and other DNA-binding proteins (Santos *et al.*, 2009). In OmpR, the α3 helix (residues 200-213) and the loop extending from this helix (residues 214-220) are responsible for DNA recognition and binding, whereas

the α2 helix (residues 182-190) stabilises the OmpR-DNA interaction (Kondo *et al.*, 1997; Martinez-Hackert & Stock, 1997; Rhee *et al.*, 2008). In addition to the wHTH domain binding DNA, the "turn" region contained in this domain (residues 191-199) recruits RNA polymerase to the site of OmpR-DNA interaction, allowing for transcription of downstream genes (*i.e. ompC* or *ompF*) (Kondo *et al.*, 1997; Pratt & Silhavy, 1994). The amino acid substitutions $R_{199}H$ and $Q_{204}P$ found in OmpR of Ag^+-resistant *E. coli* are both located in the wHTH domain; with $Q_{204}P$ located within the α3 helix and $R_{199}H$ located within the "turn" region. It therefore seems likely that these substitutions disrupt the ability of OmpR to bind DNA or recruit RNA polymerase, respectively. Disruption of either of these functions would presumably prevent the expression of OmpF and OmpC, thus giving the porin-deficient phenotype observed in Ag^+-resistant strains.

In contrast to the work presented here, where all Ag^+-resistant strains harboured *ompR* mutations that prevents both OmpC and OmpF expression, a selection of Ag^+-resistant isolates obtained by Li *et al*. (2007) lacked OmpF alone. To ascertain if strains deficient in OmpF require only a single additional mutational event (*e.g* in *cusS*) to confer Ag^+ resistance (as occurs with strains deficient in OmpR), the frequency of spontaneous mutation to Ag^+ resistance was determined for BW25113 deleted for *ompF* or *ompR*. Whereas strains deleted for *ompR* yielded Ag^+-resistant mutants at a frequency of $7.5\pm1.5x10^{-8}$, a frequency consistent with a single mutational event (O'Neill *et al*., 2004), no spontaneous Ag^+-resistant mutants could be selected for strains deleted for *ompF*. This result would suggest that the loss of OmpF alone is insufficient for Ag^+ resistance. Although this result appears to contradict that obtained by Li *et al*. (2007), the duration of the resistance selection carried in their study was not disclosed, and it is possible that disruption of OmpF alone is sufficient to contribute to Ag^+ resistance when

mutational changes in addition to those in *cusS* are present. Indeed, further characterisation of an Ag^+-resistant strain that was deficient in OmpF revealed down-regulation of a number of periplasmic proteins (for example periplasmic maltose-binding protein and periplasmic histidine-binding protein) and an additional outer membrane channel (TolC), all changes that may provide additional protection against Ag^+ (Lok *et al.*, 2008).

Porin loss is likely to contribute to an Ag^+ resistance phenotype by limiting the diffusion of Ag^+ into the periplasm to a concentration that can be successfully transported out of the cell before it causes damage to the cytoplasmic membrane. It therefore seems reasonable to suggest that the primary mechanism by which Ag^+ traverses the outer membrane is through OmpC/F. Indeed, it has been established that OmpC/F preferentially transport cations (*e.g.* Ag^+) as opposed to anions and compounds with a net neutral charge (Benz *et al.*, 1985; Danelon *et al.*, 2003). However, as OmpC/F allow for the diffusion of a range of important solutes into the cell (*e.g.* sugars, ions and amino acids), the loss of these porins under Ag^+ resistance suggests that cells would be compromised in their ability to acquire nutrients; this would likely reduce their growth rate and affect their ability to compete with other organisms in an Ag^+-free environment. The impact of this phenotype on the clinical significance of endogenous Ag^+ resistance will be explored later in this chapter.

5.3.1.3 Mutations in CusS lead to uncoupling of Ag^+ sensing from expression of CusCFBA, resulting in constitutive expression of the latter

The CusRS two-component system is a positive regulator of the c*usCFBA* operon (Munson *et al.*, 2000). Upon sensing extracellular Cu^+ or Ag^+, the sensor kinase (CusS) phosphorylates its cognate response regulator (CusR), which in turn binds to a region of

DNA (termed the "CusR box") upstream of *cusCFBA* and activates *cusCFBA* transcription (Munson *et al.*, 2000). Increased expression of *cusCFBA* has been observed following both Ag^+ and Cu^+ challenge in transcriptional profiling experiments (Yamamoto & Ishihama, 2005, Gudipaty *et al.*, 2012). The components CusCBA comprise an efflux pump of the resistance nodulation division (RND) family that has been shown to efflux both Cu^+ and Ag^+ (Su *et al.*, 2011), whilst CusF is a periplasmic Cu^+ and Ag^+ binding protein that is believed to sequester ions in the periplasm and transport them to CusB for subsequent efflux (Xue *et al.*, 2008). Deletion of any component of the Cus system results in hyper-susceptibility to Cu^+ in anaerobic environments, suggesting that the primary role of the Cus system is to enable copper homeostasis during anaerobiosis (Munson *et al.*, 2000).

Given that the CusRS two-component system is a positive regulator of CusCFBA, it seems likely that mutations in *cusS* that contribute to Ag^+ resistance do so by promoting gain of function in CusS, thereby increasing the expression of CusCFBA and consequently the rate of Ag^+ efflux. To establish the effect of such a mutation on the expression of CusCFBA, qPCR was used to determine relative expression of CusC in the parental strain and Ag^+-resistant strain BW25113 *ompR*G_{596}A *cusS*G_{1130}A, in the presence and absence of $AgNO_3$ at 1 μg/ml, using a previously validated oligonucleotide primer set (Gudipaty *et al.*, 2012) (Figure 5.3). Whilst the parental strain exhibited Ag^+-dependent expression of CusCFBA, the Ag^+-resistant derivative exhibited high-level and constitutive expression of this operon even in the absence of Ag^+ stimulus, suggesting that Ag^+ sensing is uncoupled from CusCFBA expression in Ag^+-resistant strains.

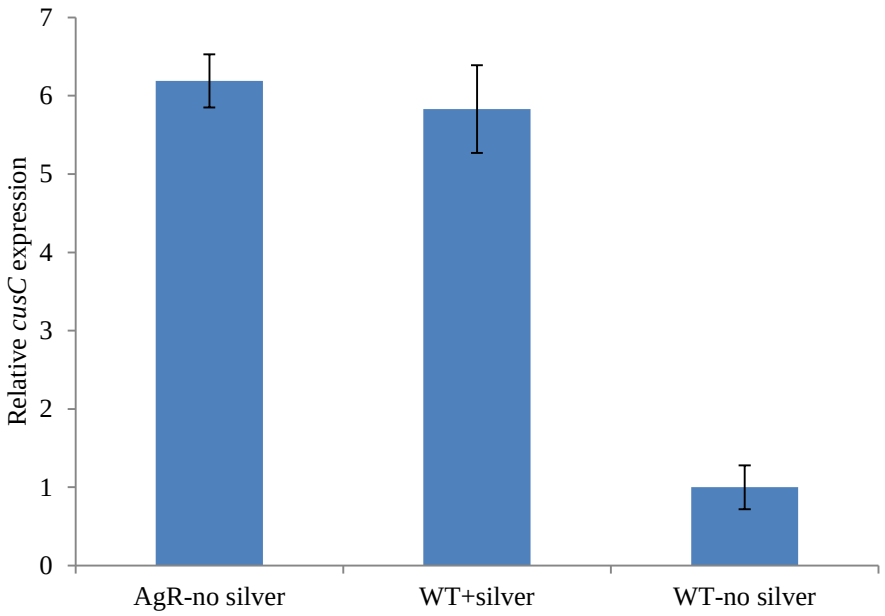


Figure 5.3 Relative expression of CusC in Ag^+ susceptible (WT) and resistant (AgR) strains of BW25113 in the presence and absence of 1 μg $AgNO_3$/ml. Error bars represent the standard deviation from three independent experiments.

Sensor kinases exist in the cell as homodimers, and regulate gene expression by altering the phosphorylation state of their cognate response regulator (CusR in this case) (Stewart, 2010). This is achieved through modulation of the relative kinase or phosphatase activities within the sensor kinase (Stewart, 2010). As CusRS is a positive regulator of CusCFBA, the concentration of phosphorylated CusR in the cytoplasm will be proportional to the level of CusCFBA expression. Therefore the CusS mutations identified in Ag^+ resistant *E. coli* could enable continuous kinase activity, or inactivate the phosphatase properties of this protein. In either case, levels of phosphorylated CusR would remain high and allow for the constitutive expression of CusCFBA that has been observed in this study. Further insight into how the observed mutations might affect the function of CusS could be obtained by mapping the amino acid substitutions onto a

structure of CusS, in a similar manner to that carried out earlier in this study with OmpR. However, unlike OmpR, the structure of CusS has yet to be determined. The structure and functional domains of CusS were therefore predicted using the iterative threading assembly refinement (I-TASSER) server and the protein families (Pfam) database, respectively.

The I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/ last accessed 6th August 2013) produces a predicted three-dimensional protein structure based on a submitted amino acid sequence. This is achieved via a four-stage process; threading, structural assembly, model selection and refinement, and structure-based functional annotation (Roy *et al.*, 2010). To summarise, I-TASSER uses the submitted amino acid sequence to search protein structure databases for homologous regions of sequence for which there is structural data. These data are then amalgamated into a predicted structure, which is further refined by a comparison with structural homologues of the whole protein (Roy *et al.*, 2010). In a similar manner to I-TASSER, Pfam (http://pfam.sanger.ac.uk/ last accessed 6th August 2013) uses the submitted amino acid sequence to search a database for regions of sequence that share homology. Unlike I-TASSER, Pfam utilises a database of proteins for which a functional role has been determined (Punta *et al.*, 2012). As CusS is a putative sensor kinase, it will contain an extra-cytoplasmic sensing domain of a unique structure (as this is required for substrate specificity in sensing). As I-TASSER predicts protein structure based on homology searches, it is likely that the sensing domain of CusS will negatively impact the predictive ability of I-TASSER (Roy *et al.*, 2010). As a consequence, the sequence corresponding to the extracellular sensing- and membrane-spanning domains of CusS were identified by SOSUI (as described in Chapter 4) and omitted from the sequence

submitted to I-TASSER. The predicted structure for the cytoplasmic region of CusS was highlighted with functional domains identified by Pfam and is presented in Figure 5.4.

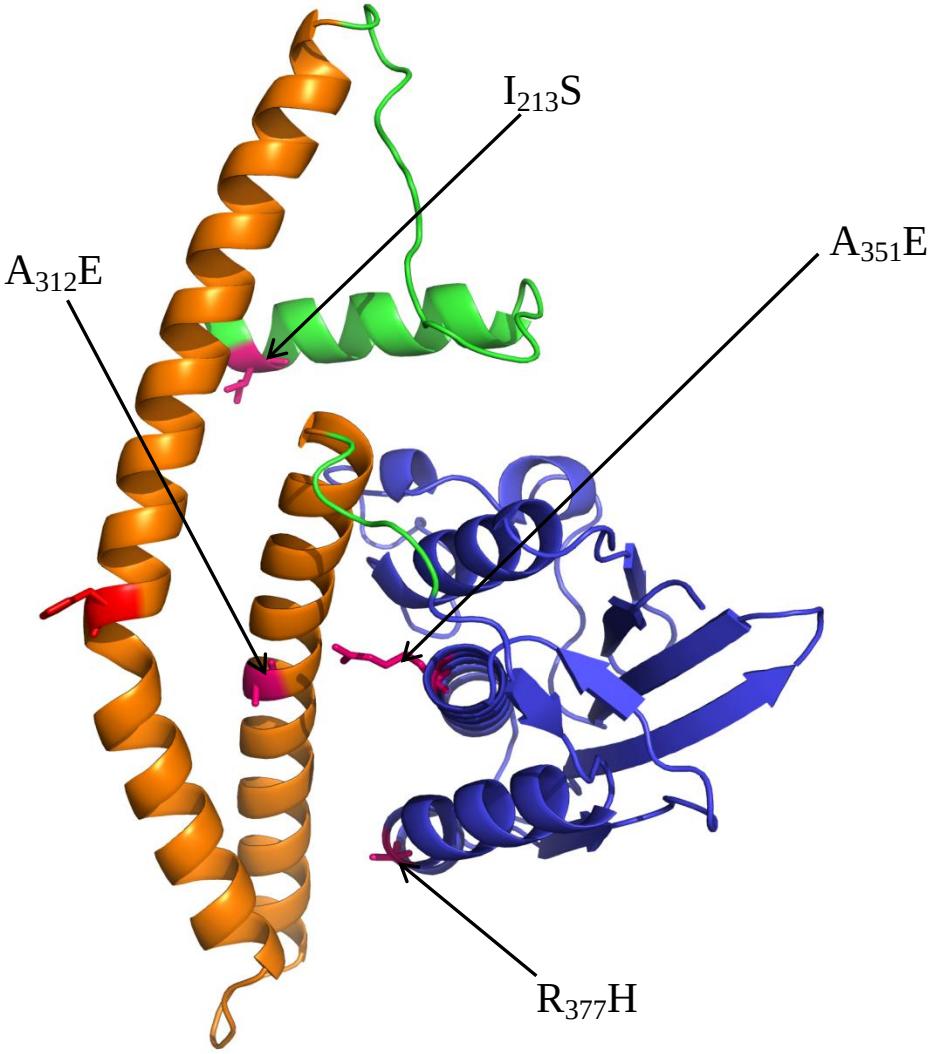


Figure 5.4. Predicted structure of the cytoplasmic domain of CusS. Dimerization and catalytic/ATP-binding domains are shaded orange and purple respectively. The conserved histidine residue required for phosphorylation of CusR is shaded red and sites of amino acid substitutions associated with Ag^{+} resistance are shaded pink.

As expected, the overall structure of CusS is similar to that of a typical sensor histidine kinase. The amino acid sequence from residues 209 to 237 is consistent with a HAMP domain that is responsible for transducing signals from the sensing domain to the cytoplasmic domains (Hulko *et al.*, 2006). The region containing residues 242 to 322 is

a putative dimerization and histidine phosphotransfer (DHp) domain (Ferris *et al.*, 2012), containing a conserved histidine at position 271 that likely transfers phosphate to CusR. The remaining amino acid sequence (332 to 480) corresponds to a catalytic and ATP-binding (CA) domain which, as indicated, binds ATP and transfers a phosphate group to the conserved histidine residue within the DHp domain when the kinase is activated (Stewart, 2010).

Inserting the amino acid changes required for Ag^+ resistance onto the predicted structure of CusS reveals that the changes are distributed throughout the structure (Figure 5.4). The amino acid substitution $I_{213}S$ occurs within the proposed HAMP domain, whilst substitution $A_{312}E$ is located within the DHp domain. The remaining substitutions, $A_{351}E$ and $R_{377}H$, are located in the CA domain. The kinase and phosphatase activities of a sensor kinase are activated by a conformational change in the cytoplasmic domains of the protein, which moves the conserved histidine residue in the DHp domain into the CA domain (Ferris *et al.*, 2012). It is possible that the mutations observed in Ag^+ resistance affect the structure of CusS so that the latter conformation is locked in place, leading to continuous kinase activity (or blocking phosphatase activity), which in turn leads to maintenance of high intracellular concentrations of phosphorylated CusR and constitutive expression of CusCFBA as a consequence.

5.3.1.4. All components of the CusCFBA system are essential for endogenous Ag^+ resistance

As discussed above, CusCBA is a tri-partite efflux pump of the RND family, with CusC functioning as an outer membrane channel, CusB as a membrane fusion protein and CusA as the Cu^+/Ag^+ pump, which effluxes Cu^+/Ag^+ via a methionine shuttle (Su *et al.*, 2011). CusF is a periplasmic protein which can bind a single Cu^+ or Ag^+ by methionine

and cation-π interactions with tryptophan (Xue *et al.*, 2008). Using a yeast two-hybrid approach and chemical crosslinking, CusF has been shown to interact with CusB at a specific site; thus, it has been suggested that CusF acts as a chaperone to transport Ag^+ or Cu^+ to CusCBA for subsequent efflux (Mealman *et al.*, 2011). Deletion of either CusC, F, B, or A confers hyper-susceptibility to Cu^+ under anaerobic conditions (Franke *et al.*, 2003); however, although strains lacking CusS or CusR are hyper-susceptible to Ag^+, and deletion of *cusF* in Ag^+-resistant strains restores Ag^+ susceptibility to that of the parental strain, it is unclear if the deletion of genes encoding any component of the CusCBA efflux pump in Ag^+-resistant strains will disrupt the phenotype. Therefore, using a recombineering approach, markerless deletions of *cusC, F, B,* or *A* were created in strain BW25113 *ompR*G_{596}A *cusS*G_{1130}A and the subsequent effect on the Ag^+ resistance phenotype established by determining the $AgNO_3$ MIC for each strain. Irrespective of the component that was deleted, $AgNO_3$ MICs decreased from >256 μg/ml to 4 μg/ml, indicating that the CusCBA efflux pump, in addition to CusF, is essential to the Ag^+ resistance phenotype. Furthermore, the confirmation that loss of Ag^+ resistance occurs following disruption of CusF indicates that that the ability of CusCBA to efflux Ag^+ is dependent upon CusF. This result may indicate that, unlike other pumps of the RND family, CusCBA cannot sequester its substrate directly from the periplasm and is reliant upon CusF for this purpose.

In strains containing native CusS (*i.e.* not harbouring mutations found in Ag^+ resistance), Ag^+ challenge will activate CusS and increase the level of phosphorylated CusR, which in turn will mediate transcription of *cusCFBA* (Munson *et al.*, 2000). However, by the time this has happened and the expressed Cus proteins have inserted into the periplasm, Ag^+ will have already caused substantial damage to the inner membrane. Indeed, it was demonstrated in Chapter four that Ag^+ causes lethal

bacterial membrane damage after only 10 min exposure. Thus, the constitutive and high-level expression of CusCFBA in Ag^+-resistant strains allows for Ag^+-protection mechanisms to be present prior to challenge with Ag^+, thereby allowing for Ag^+ detoxification without a time delay. Nevertheless, the continual expression of periplasmic proteins is likely to put a substantial metabolic strain upon the cell, which may impact its ability to survive when in competition with other organisms in an Ag^+-free environment.

5.3.1.5. Emergence and maintenance of endogenous Ag^+ resistance

This study has established that two mutational events are required for Ag^+ resistance in *E. coli*; however, the order in which these mutations arose is unknown. As this information may provide insights into the evolution of this resistance phenotype, DNA sequence determination of *ompR* and *cusS* from strains isolated on each day of resistance selection was carried out to identify at which locus the first mutation arose. In all three independent selection experiments, mutations in *ompR* arose first (after 4-5 days), with the mutation in *cusS* arising shortly after (day 6). As the MIC of $AgNO_3$ did not change against strains harbouring *ompR* mutations (data not shown), it was hypothesised that such mutations confer a growth advantage over parental strains in sub-inhibitory concentrations of Ag^+. Therefore, growth of the parental strain, BW25113 *ompR*G_{596}A, and BW25113 Δ*ompR* in the presence of 2 μg $AgNO_3$/ml was assessed by means of optical density measurements at 600 nm over an 18 h period (Figure 5.5). Under these conditions, strains harbouring mutagenised *ompR* had an increased growth rate compared with the parental strain. In addition, the ability of strains harbouring *cusS* mutations to confer a growth advantage in sub-inhibitory Ag^+ was also assessed; although a growth advantage could be observed, it was to a lesser

extent than that observed with mutagenised *ompR* strains (Figure 5.5). This result suggests that at sub-inhibitory concentrations of Ag^+, porin loss provides a greater degree of protection against Ag^+ than CusCFBA-mediated efflux. This increased level of protection would translate into a stronger selection pressure in favour of strains harbouring *ompR* mutations, thus allowing for this strain to predominate over the parental strain and other spontaneous mutants during continuous exposure to Ag^+. As the population of *ompR* mutants increases, the probability that a spontaneous mutation in *cusS* occurs to complete an Ag^+ resistance phenotype will also increase.

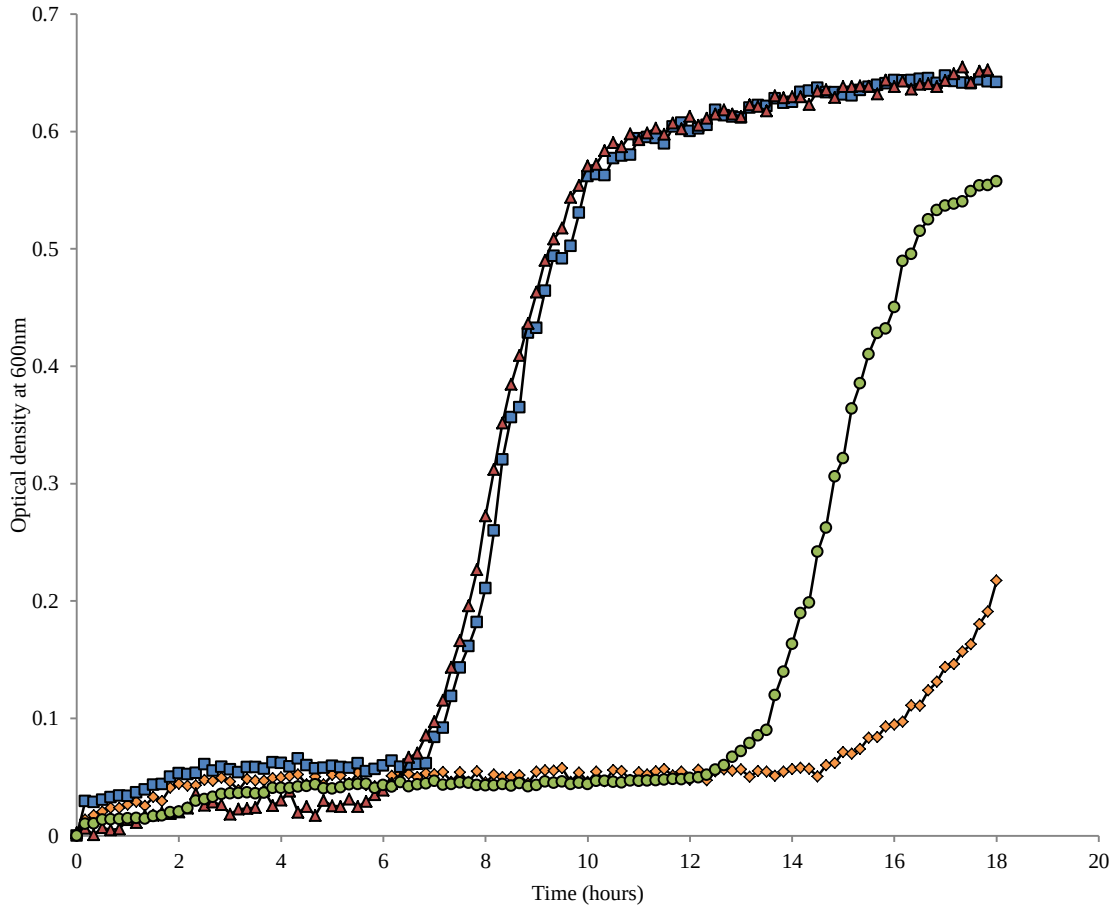


Figure 5.5 Growth curves of BW25113 (orange diamonds), *ompR*G_{596}A (blue squares), Δ*ompR* (red triangles) and *cusS* (green circles) in 2 μg $AgNO_3$/ml. Results are representative of at least three independent experiments.

Although endogenous Ag^+ resistance could be selected in *E. coli* by continuous exposure to sub-inhibitory Ag^+, this type of selection pressure would not typically be encountered in a clinical setting. In the management of infection, silver formulations are used that employ Ag^+ concentrations well in excess of the MIC (Yin *et al.*, 1999). Therefore, in order to determine if Ag^+ resistance could be selected under conditions more analogous to those that might be encountered in the clinical setting, cultures of *E. coli* BW25113 were repeatedly exposed to supra-inhibitory concentrations of $AgNO_3$ (40, 80 or 100 μg/ml), using a previously established method (Miller *et al.*, 2008). Using this method Ag^+ resistance could not be selected over a period of 42 days. As this method contains periods during which cultures are allowed to recover following Ag^+ exposure, it is likely that there is insufficient selection pressure to permit the proliferation of the OmpC/F-deficient phenotype. As hypothesised earlier, loss of OmpC/F expression would impact the ability of the cell to take up nutrients, which in turn will allow it to be out-competed by the parental strain during periods when Ag^+ is absent. Indeed, the impact of porin loss on bacterial growth has been established previously (Adler *et al.*, 2013), with cultures of *E. coli* found to grow 20% slower when porins are not expressed.

Unfortunately, porin-deficient phenotypes of *E. coli* already exist in the clinical setting, as a mechanism that can confer resistance to an extended spectrum of β-lactams in strains already expressing a β-lactamase. For example porin-deficient strains containing an extended-spectrum or AmpC β-lactamase have a reduced susceptibility to carbapenems, as porin loss limits the ingress of the carbapenem into the periplasm to concentrations that can be successfully degraded by the β-lactamase (Martinez-Martinez, 2008; Adler *et al.*, 2013). As a consequence, endogenous Ag^+ resistance could in principle arise in such clinical isolates via a single mutational event,

thus by-passing the apparent inability to select for *ompR* mutations with supra-inhibitory concentrations of Ag^+. To test this, the mutation frequency to spontaneous Ag^+ resistance was determined for the clinical *E. coli* isolate EC18, a strain originally detected owing to a "false" ESBL phenotype resulting from a combination of expression of the TEM-1 β-lactamase and loss of OmpC/F expression (Beceiro *et al.*, 2011). A mutation frequency to Ag^+ resistance of $7.5\pm0.9x10^{-8}$ was recorded, with mutants resistant to >256 μg $AgNO_3$/ml. This frequency is consistent with a single mutational event, and would therefore seem to confirm the possibility that spontaneous Ag^+ resistance could arise in porin-deficient strains of *E. coli* known to cause infection.

Although the above results indicate that Ag^+-resistant *E. coli* could emerge in an environment where high concentrations of Ag^+ are encountered (*e.g.* in wound care), the metabolic impact (also known as the fitness cost) of porin loss combined with overexpression of CusCFBA may be sufficient to limit the spread and survival of this phenotype, due to it being out-competed by other microorganisms, and/or be unable to survive in nutrient-poor environments. To give an indication of the fitness cost associated with endogenous Ag^+ resistance, the ability of strain BW25113 *ompR*G_{596}A *cusS*G_{1130}A to compete with the parental strain BW25113 was evaluated by pair-wise competition assays. A relative fitness value (*W*) of 0.71 (±0.02) was obtained. Although this value is indicative of a considerable fitness cost, strains harbouring other endogenous antibiotic resistance phenotypes with a similar or greater fitness burden have been encountered in the clinic. For example, clinical isolates of rifampicin-resistant *S. aureus* have been encountered with *W* values as low as 0.49 (O'Neill *et al.*, 2006). This would suggest that the fitness cost observed in endogenous Ag^+ resistance would not necessarily act as a barrier to this resistance phenotype arising and persisting in a clinical setting. However, the fitness evaluation

used in this study was over a 24 h period and may not be representative of the Ag^+ resistance phenotype in competition over a longer period. Furthermore, this study does not take into consideration the impact of the resistance phenotype on pathogenicity or on the ability of the organism to evade immune response mechanisms. Expanding fitness studies using an animal model of infection would therefore be beneficial in future studies.

E. coli is rarely encountered (relative to other bacteria) in environments where Ag^+ is employed therapeutically (*e.g.* burn and chronic wounds) (Posluszny *et al.*, 2011; Rezaei *et al.*, 2011), thus the clinical significance of endogenous Ag^+ resistance in *E. coli* is unclear. However, there is the possibility that endogenous Ag^+ resistance can arise in other Gram-negative bacteria that have greater relevance to the continued usage of Ag^+ in wound care. To identify other Gram-negative bacteria that harbour the Cus system (and therefore the potential for endogenous resistance to emerge), a BLASTp search was carried out using the amino acid sequence of CusF from *E. coli* MG1655 (a parental strain of BW25113) as the search input. Results were then refined by confirming the presence of the remaining Cus components.

BLASTp searches identified an intact Cus system in a substantial number of *E. coli* strains (data not shown). In addition, intact Cus systems were identified in *Shigella* and *Citrobacter* spp. with an overall amino acid identity of >60%. Both of these genera have been previously implicated in wound infection (Gregory *et al.*, 1974; Arens & Verbist, 1997). Attempts were therefore made to select for Ag^+ resistance in *Citrobacter freundii* (strain ATCC 8090) and a clinical isolate of *Shigella sonnei*, by continuous exposure to sub-inhibitory Ag^+. Prior to selection, the presence of *cusS* was confirmed by PCR using primers specific to this gene in each organism, in order to provide a putative indication that the Cus system was present (data not shown). In addition to the above

strains, selection of endogenous Ag^+ resistance was also attempted in strains of *Pseudomonas aeruginosa* (strain PA01) and *Acinetobacter baumannii* (strain ATCC 19606). Although these species are frequently isolated from wounds and are a significant cause of morbidity and mortality in patients with burn wound infections (Rezaei *et al.*, 2011), they lack a Cus system and therefore acted as negative controls in this study.

After 42 days of continuous exposure, mutants with a reduced susceptibility to Ag^+ could not be isolated in any of the strains tested. Although the Cus system and homologues of OmpC/F are present in both *C. freundi* and *S. sonnei*, it is possible the loss of OmpC/F in these strains conveys too great a fitness cost to be compatible with survival, or that loss of OmpC/F is unable to reduce levels of Ag^+ entering the periplasm to a concentration that can be negated by CusCFBA overexpression. The inability to select for endogenous resistance in a range of wound pathogens would suggest that Ag^+-containing products still have utility in the prevention and treatment of bacterial infection; however, the possibility that these organisms can acquire Ag^+-resistance determinants by horizontal gene transfer has yet to be explored. Indeed, in addition to the strains above, the Cus system was also found to be present in strains of *Klebsiella pneumoniae* and *Enterobacter cloacae*, however these strains were also found to harbour an intact Sil system. The implication of this finding will be explored later in this chapter.

5.3.2. Characterisation of the Sil system: an exogenous Ag^+-resistance determinant

5.3.2.1 Analysis of the Ag^+ resistance plasmid pMG101 using a next-generation sequence determination approach.

Plasmid pMG101 from *S. enterica* serovar Typhimurium was the original source of the *sil* operon characterised by Gupta *et al*. (1999). This plasmid is a member of the IncH incompatibility group, and together with Ag^+ resistance was found to confer resistance against ampicillin, chloramphenicol, streptomycin, tetracycline and mercury (McHugh *et al.*, 1975; Gupta *et al.*, 2001). The nucleotide sequence of the region of pMG101 harbouring the *sil* operon was determined and deposited in GenBank (accession number AF067954).

Given that there is limited information on the structure and function of the components of the Sil-system, a study related to this thesis sought to overexpress and purify each Sil-component for future structural studies. However during the process of cloning genes encoding Sil components from pMG101 onto suitable expression vectors, a number of nucleotide differences (*i.e.* substitutions, deletions and insertions) were noticed when comparing the sequence of s*il* genes obtained in this study with that on GenBank (data not shown). As some of these nucleotide changes affected the resultant amino acid sequence, a next-generation approach was employed to determine the nucleotide sequence of the entirety of *E. coli* strain NCTC 50110 (which contains pMG101) as this would identify all sequence errors in the *sil* operon. In addition, determining the entire genome sequence of NCTC 50110 would allow the molecular basis of other antibacterial resistance determinants present on pMG101 to be identified and could provide additional insights into the mechanisms involved in the horizontal transfer of the *sil* operon.

Next-generation DNA sequence determination represents a substantial improvement over the Sanger (dye-terminator) method in terms of sample throughput (Shendure & Ji, 2008). By running thousands of sequencing reactions simultaneously, next-generation technologies can determine the sequence of an entire human genome (3.2 Gb) in

24-72 hours (depending on the technology used) (Shendure & Ji, 2008). In comparison, the first attempt to determine the sequence of a human genome using the Sanger method took over 10 years to complete (Lander *et al.*, 2001; Venter *et al.*, 2001). For the present study, Illumina technology was used to determine the NCTC 50110 genome sequence.

Genomic DNA from NCTC 50110 was extracted and purified, then sent to the Next Generation Genomics facility, University of Leeds for library preparation (*i.e.* fragmentation of DNA and cluster generation) and sequence determination on an Illumina MiSeq platform using a 2x250 bp paired-end method. Using this method, 26 million fragment reads were obtained, which corresponds to 6.5 Gb of nucleotide sequence. To assemble these reads into the NCTC 50110 genome by *de novo* assembly, the genome assembler provided as part of the CLC Genomics Workbench software (version 6.0) was used. This assembler uses de Bruijn graphs to identify and merge regions of overlap between reads into contiguous sequences (referred to as contigs) (Compeau *et al.*, 2011). Following *de novo* assembly, 157 contigs were obtained which equated to ~4.8 Mb of unique nucleotide sequence at a mean coverage of ~200x. Additional data regarding the quality of this assembly can be found in Table 5.2. The sequence of each contig was then used as the input for BLASTn searches against the *E. coli* J53 genome (NCBI accession: PRJNA180157), so that contigs corresponding to the *E. coli* chromosome could be removed. This process found 8 contigs (corresponding to 151,329 bases) that shared no homology with the *E. coli* J53 genome, with further homology searches revealing these contigs to share similarity with a number of *Salmonella* spp. plasmids (data not shown). It is therefore highly likely that these sequences correspond to pMG101. To provide a comprehensive annotation of the genes present on the contigs corresponding to pMG101, nucleotide sequences were submitted to the Rapid Annotation and Subsystem Technology (RAST) server

(http://rast.nmpdr.org/ last accessed 3rd August 2013). Using a combination of algorithms, RAST first identifies regions of sequence that are likely to encode tRNA-, rRNA- and protein-encoding genes, then uses a derivative of BLASTx to assign a functional role to the gene products based on sequence homology (Aziz *et al.*, 2008). The genes identified on each contig can be found in Appendix 2.

Table 5.2. Quality measurements from *de novo* assembly of *E. coli* NCTC 50110 genome

Total number of reads	26,512,772
Number of reads assembled into contigs	21,845,309
Number of reads discarded from assembly[1]	4,667,463
Total number of contigs arising from assembly	157
N50 of contigs arising from assembly[2]	103,583

[1]Reads of poor quality (*e.g.* containing ambiguous bases) were discarded by the assembly program. [2]N50 corresponds to the minimum nucleotide length of 50% of all contigs arising from the assembly

Of the genes identified on pMG101, a relatively large proportion (29/79) were members of the *tra* and *trb* operons that encode proteins necessary to facilitate conjugation (Li *et al.*, 1999). Other genes involved in plasmid replication/maintenance (*rep* and *par* genes) (Sia *et al.*, 1995; Venkova-Canova *et al.*, 2003) and survival (toxin/antitoxin genes *ccdAB*, *vagCD* and *pemIK*) (Hayes, 2003) were also present, as were genes involved in nutrient uptake; these would likely enhance the ability of the host organism to survive in a range of environments. In addition, genes encoding a class D (OXA) β-lactamase, chloramphenicol acetyltransferase, streptomycin 3"-O-adenylyltransferase and a tetracycline-efflux pump (TetA) were also identified. Resistance to mercury is likely conferred by the Mer system; given the presence of an intact *mer* operon on pMG101. The Mer system is a relatively unusual resistance mechanism by which a toxic moiety (in this case Hg^{2+}) is actively transported into the cell for detoxification. This process is

mediated by the periplasmic Hg^{2+}-binding protein MerP and the membrane-bound Hg^{2+}-transporter MerT. Once inside the cell, Hg^{2+} is reduced to elemental Hg (Hg^0) by the mercuric reductase MerA. Due to its volatile state, Hg^0 is non-toxic and rapidly diffuses out of the cell (Mathema *et al.*, 2011). Although it has been known since its discovery that pMG101 can confer resistance to a range of antibacterial agents apart from Ag^+, the results of this study nevertheless re-emphasises that horizontally-acquired genetic elements frequently carry more than one antibacterial resistance determinant and therefore the use of therapeutic Ag^+ can potentially select for exogenous resistance towards a range of antibacterial agents.

Although a substantial portion of pMG101 sequence was recovered and annotated, genes corresponding to the *sil* operon could not be located. A potential reason for the lack of the *sil* operon despite this strain displaying resistance to Ag^+ could be that the operon had integrated into the *E. coli* chromosome. Indeed, this was found to be the case, as a BLAST search of contigs other than those already associated with pMG101 using the nucleotide sequence of *silE* revealed that the *sil* operon had inserted into the host chromosome. Further analysis of the contig harbouring the *sil* operon revealed that it was part of a 32.2 kb integrated element. As the *sil* operon is only 14.2 kb, the entirety of the contig harbouring the integrated element was annotated using RAST, to provide an insight into the other components that had integrated with the *sil* operon (Appendix 2). In addition to the *sil* operon, a region with high sequence similarity to *pcoABCDRSE* was identified. The *pco* operon encodes proteins that confer resistance to copper (in the form of Cu^+ and Cu^{2+}) via a mechanism of sequestration (PcoE, PcoC), oxidation to Cu^{2+} (PcoA) and/or efflux (PcoB and PcoD), in a process regulated by the PcoRS two-component system (Lee *et al.*, 2002). Of primary interest however, was the presence on the integrated element of genes encoding proteins

homologous to the Tn*7* transposition system. The archetypical Tn*7* system contains 5 proteins; TnsA, B, C, D and E (Peters & Craig, 2001). TnsAB form a transposase that recognises inverted repeat (IR) elements at either end of the transposon (termed Tn*7*-L and Tn*7*-R), and creates double-strand DNA breaks at these sites, thus excising the transposon from the donor site. TnsC binds to recipient DNA at the site of transposon insertion (identified by TnsD or TnsE) and, in an ATP-driven process with TnsAB, integrates the transposon into this site. The target site on the recipient DNA for transposon integration is mediated by TnsD or TnsE. TnsD recognises a specific site on the recipient DNA, termed the *attTn7* sequence. This sequence is ubiquitous in bacterial species as it is found upstream of the coding sequence of *glmS* (encoding glutamine synthetase), a highly conserved gene due to its essential role. In contrast, TnsE does not recognise a specific sequence of DNA, but rather directs transposon integration into conjugative plasmids during their replication by lagging-strand DNA synthesis (Peters & Craig, 2001). Analysis of the Tn*7* system from the integrated element containing the *sil* and *pco* operons revealed the presence of TnsA, B, C and D homologues, although a homologue of TnsE was not detected. Additionally the IR elements Tn*7*-L and Tn*7*-R could not be located within, or at the terminal ends of the integrated element (data not shown). This suggests that although Tn*7*-homologues are present and are likely responsible for transposition of this integrated element from pMG101 into the *E. coli* chromosome, the mechanism by which this occurs is likely to be different from that mediated by the archetypical Tn*7* system. Nevertheless, the fact that the *sil* operon is contained within an apparently functional transposon suggests that exogenous Ag^+ resistance could easily disseminate between plasmids and bacterial species and thus represent a substantial threat if introduced into an environment where therapeutic Ag^+ was employed clinically. This implication will be explored later in this chapter.

Comparison of the *sil* operon sequence obtained from this study with that of the sequence originally submitted to GenBank (accession number AF067954) revealed a number of errors in the original sequence. To summarise, 51 nucleotide differences were identified, which corresponded to 10 base changes, 13 insertions and 28 deletions. These nucleotide differences equated to numerous changes in the amino acid sequence of Sil system components (Table 5.3 and Figure 5.6). In addition, the start codon of *silF*, *P*, and *ORF105* identified by Gupta *et al*. was found to be incorrect, thus affecting the final length of these proteins (Figure 5.6).

Table 5.3. Errors in Sil-system components based on original GenBank submission (accession number AF067954).

Sil operon component	Number of changes to amino acid sequence
SilE	None
SilS	14
SilR	7
SilC	None
SilF	1*
SilB	None
SilA	None
ORF105 (SilG)	None*
SilP	7*

* represents proteins of different length than originally identified due to identification of incorrect start codon

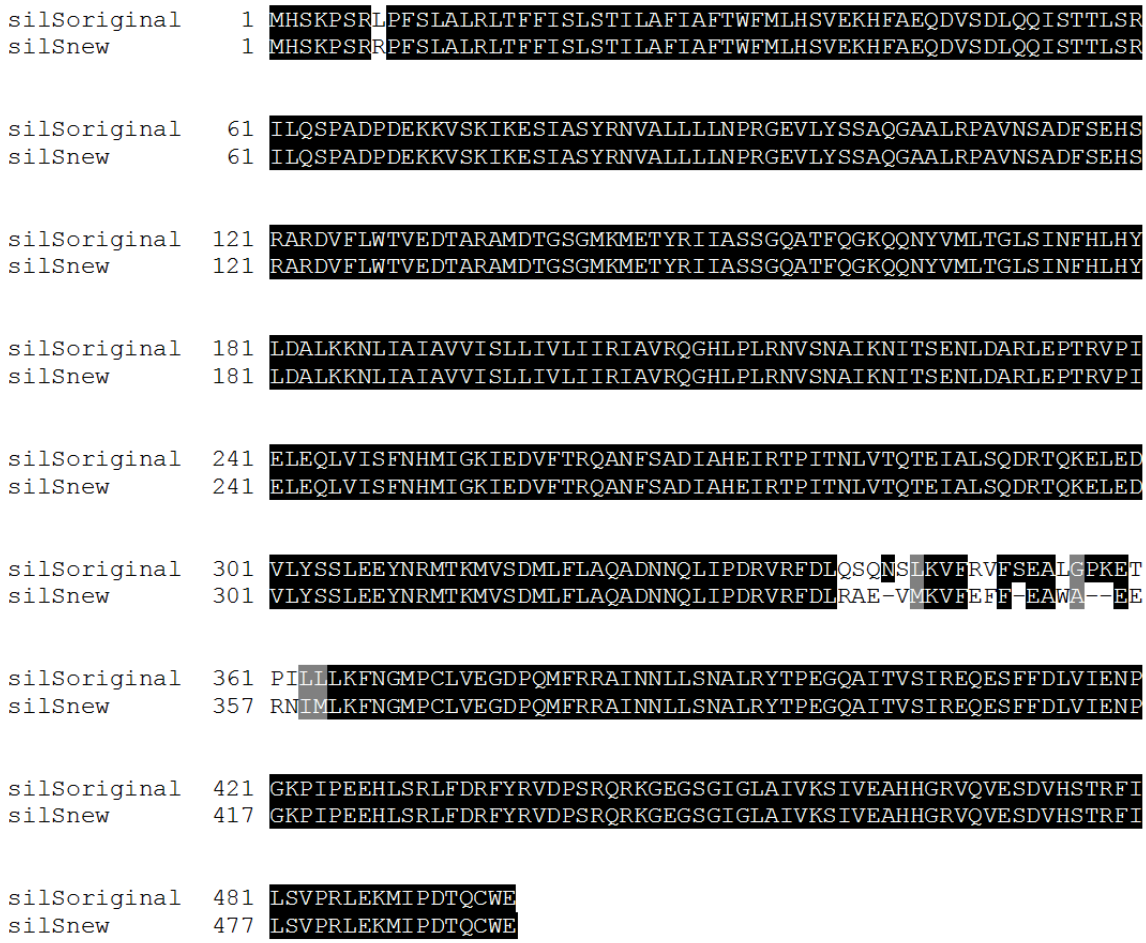


Figure 5.6a Alignment of amino acid sequences for SilS, derived from the original sequence of pMG101 and that arising from this study

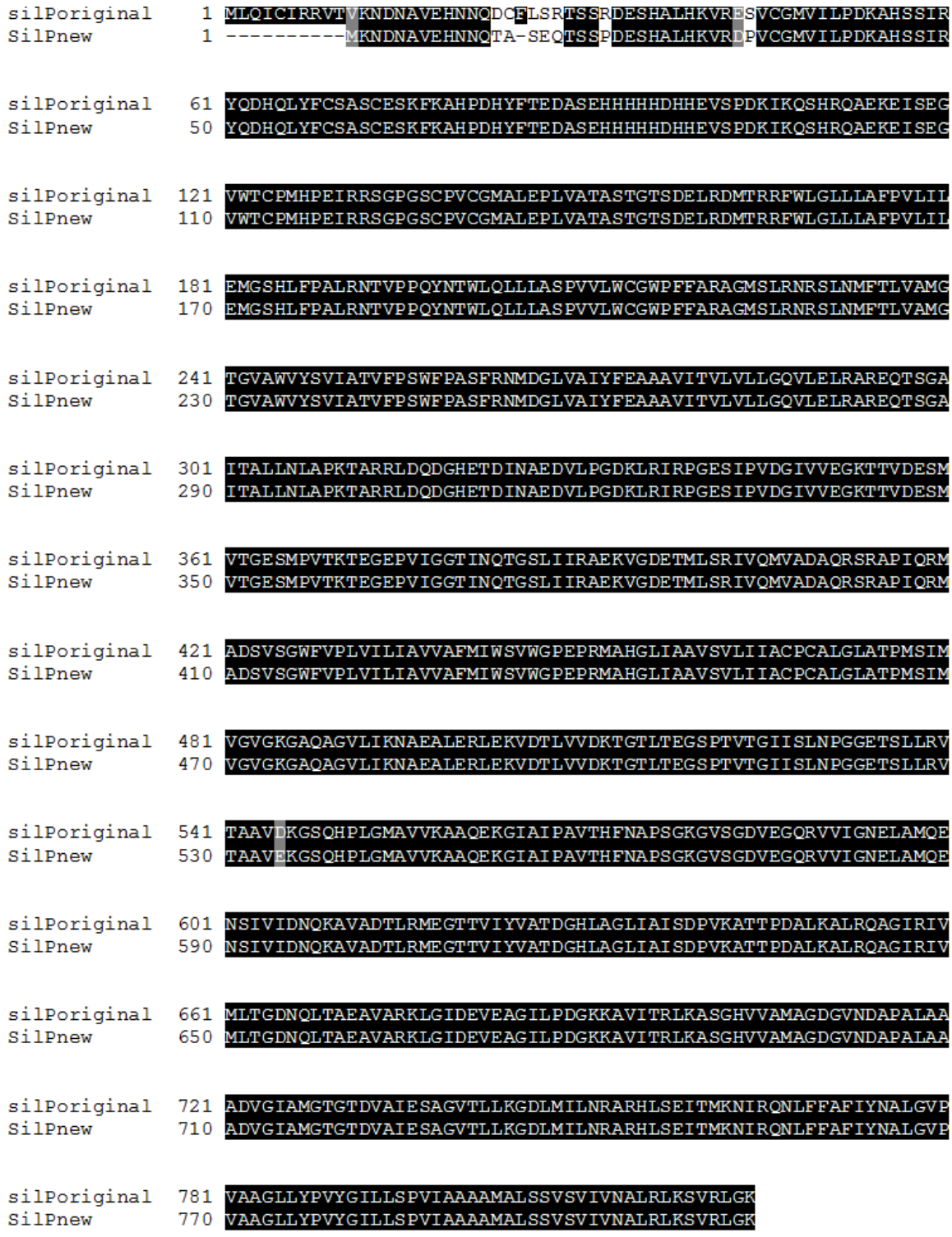


Figure 5.6b Alignment of amino acid sequences for SilP, derived from the original sequence of pMG101 and that arising from this study

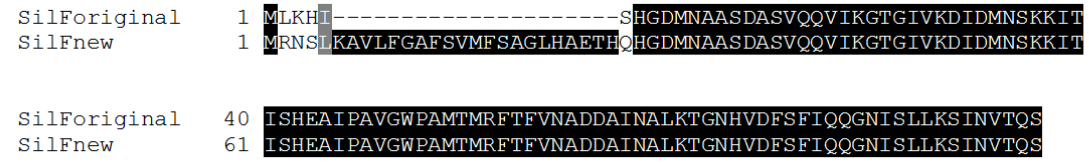


Figure 5.6c Alignment of amino acid sequences for SilF, derived from the original sequence of pMG101 and that arising from this study

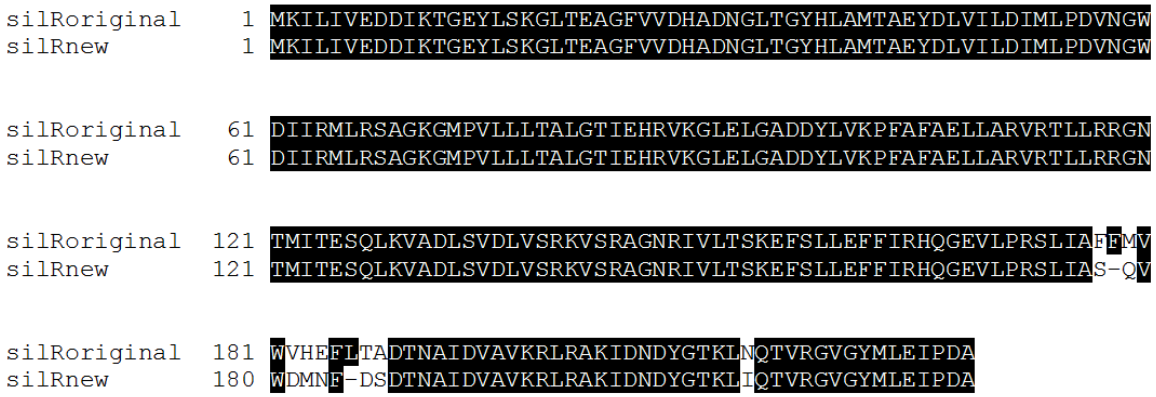


Figure 5.6d Alignment of amino acid sequences for SilR, derived from the original sequence of pMG101 and that arising from this study

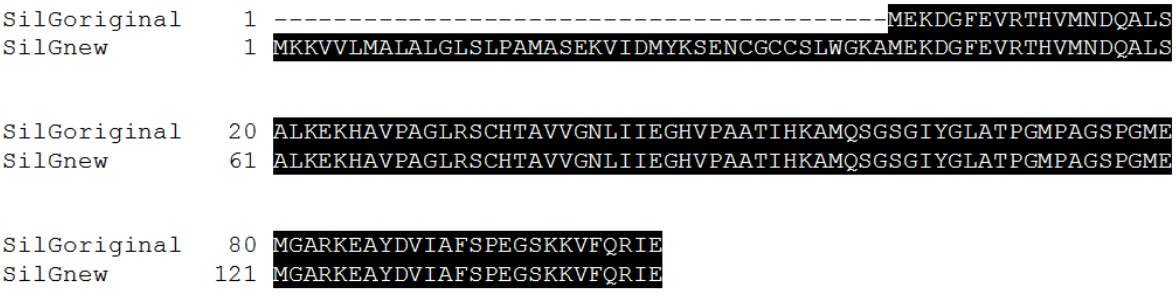


Figure 5.6e Alignment of amino acid sequences for SilG, derived from the original sequence of pMG101 and that arising from this study

The original DNA sequence determination of the *sil* operon revealed an open reading frame located between *silA* and *silP* that encoded a protein of unknown function (*ORF105*) (Gupta *et al.*, 1999). Homologues of the protein product encoded by this ORF were identified using BLASTp with the amino acid sequence of ORF105 from this study. This process revealed a number of homologous proteins predicted to be capable of binding metals, including the protein CopG from *Cupriavidus metallidurans*. The gene encoding CopG in this organism is part of a 19-gene operon which has been shown to confer high-level resistance to Cu^{+} and Cu^{2+} by a combination of cytoplasmic and periplasmic detoxification (Monchy *et al.*, 2006). The role of CopG has yet to be confirmed, however it has been hypothesised that it can sequester Cu^{+}/Cu^{2+} (based on the presence of a CxxC metal-binding motif) and transport it for efflux via CopF, a P1-type ATPase (Monchy *et al.*, 2006). Alignment of BLASTp hits revealed that the CxxC metal-binding motif is conserved between these proteins (Figure 5.7). Given the similarity of this protein to CopG, it seemed appropriate to designate it “SilG” to maintain the nomenclature of the Sil operon, and will be referred to as such for the remainder of this thesis.

Figure 5.7. Alignments of SilG from NCTC 50110 with CopG proteins from various species. Conserved CxxC metal-binding motif highlighted in red.

As the remainder of the *sil* operon encodes periplasmic- or membrane-bound proteins, it seems likely that SilG also fits one of these categories. Analysis of the amino acid sequence of SilG by SOSUI determined that this protein does not contain transmembrane domains. However, repeating this analysis using the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/ last accessed 6th August 2013) (Petersen *et al.*, 2011), revealed that the first 19 amino acids of SilG comprise an N-terminal signal peptide. In bacteria, proteins with an N-terminal signal peptide enables the translocation of the protein into the membrane or the periplasm (Petersen *et al.*, 2011). As SilG does not appear to contain any transmembrane domains, which suggests that SilG is a periplasmic protein. Although the functional roles of SilG homologues are poorly defined, it could be that SilG acts as a periplasmic Ag^{+} chaperone to shuttle Ag^{+} between the P1-type ATPase SilP and the RND-type efflux pump SilCBA; however further experiments will be required to define this role.

5.3.2.2. Role of Sil-system components in Ag^+ resistance

Although the *sil* operon is known to confer Ag^+ resistance, the relative contribution provided to Ag^+ resistance by each protein product produced by the *sil* operon is unclear. Therefore, using a recombineering approach, strains of NCTC 50110 harbouring a markerless deletion of each *sil* gene were created. The susceptibility of these strains to $AgNO_3$ were then determined. Deletion of either *silC, B, A,* or *E* led to a complete loss of Ag^+ resistance, with the MIC of $AgNO_3$ decreasing from >256 μg/ml to 4-8 μg/ml against these strains. Disruption of SilF, P, or G had no observable effect on the resistance phenotype. As NCTC 50110 also harbours a Cus operon, it may be that components of the Cus system complement the Sil system when SilF, P or G are deleted. Indeed, sequence alignments of CusB with SilB reveal that the site of CusF binding to CusB is conserved in SilB, thus suggesting that CusF could potentially complement SilF when it is not present, and therefore maintain the Ag^+ resistance phenotype (data not shown). To examine this, the entire *cus* operon was deleted in strains lacking *silF, G* or *P*, and $AgNO_3$ susceptibility testing repeated. Deletion of the Cus system had no effect on the Ag^+ resistance phenotype when an intact *sil* operon was present. However, deletion of *silF* in a Cus-negative background reduced the MIC of $AgNO_3$ from >256 to 8 μg/ml, confirming that SilF is essential to Ag^+ resistance when the Cus system is not present. In contrast, deletion of s*ilP* or *silG* individually or combined in a Cus-negative background had no effect on the Ag^+ resistance phenotype, with the MIC of $AgNO_3$ against such strains remaining at >256 μg/ml.

As SilP is a predicted efflux pump of the P-type ATPase family, it is anticipated to reside within the cytoplasmic membrane and transport substrates (*e.g.* Ag^+) from the cytoplasm into the periplasm. However, as described in Chapter 4, the antibacterial

mode of action of Ag^+ results from direct damage to the cytoplasmic membrane. Thus, the ability for SilP to transport Ag^+ out of the cytoplasm is not of direct relevance to the Ag^+ resistance phenotype, as any Ag^+ in the cytoplasm will presumably have already passed through (and damaged) the cytoplasmic membrane. Due to the fact that SilP and SilG appear not to be essential for conferring Ag^+ resistance, it would seem likely that the primary function of the Sil system is not related to Ag^+ resistance. As discussed previously, components of Cu^+ homeostasis systems (*e.g.* Cus and Pco systems) can bind and transport Ag^+, due to the structural similarities between Cu^+ and Ag^+ (Zimmermann *et al.*, 2012). It is therefore plausible that the primary function of the Sil system is to act as a Cu^+ homeostasis mechanism. As the *sil* operon is found on a transposable element with the *pco* operon (encoding a known Cu^+/Cu^{2+} homeostasis system), the Sil system likely compliments the Pco system, thus allowing the host organism to survive in environments containing high concentrations of copper. Future studies could use a similar systematic deletion strategy to the one used here to determine how Sil-system components contribute to Cu^+ homeostasis.

The endogenous Ag^+ resistance phenotype in *E. coli* results from reduced ingress of Ag^+ into the periplasm (arising from loss of OmpC/F expression) to a concentration that can be successfully transported out of the cell by CusCFBA. The mechanism of Ag^+ resistance conferred by SilCFBA and SilE appears to be comparable to that of endogenous Ag^+ resistance, in that SilE reduces the concentration of active Ag^+ in the periplasm (albeit by sequestration), and SilCFBA transports the remaining Ag^+ out of the cell before Ag^+ can damage the cytoplasmic membrane. Although SilE is fulfilling the same function as the loss of OmpC/F in Ag^+ resistance, it is likely to do so in a manner that would not confer the fitness burden observed with porin loss. This is due to the fact that porin loss also limits the uptake of important solutes, which presumably

does not result from expression of SilE. However, in Sil-mediated Ag^+ resistance it seems likely that SilE would soon become saturated with Ag^+, leading to Ag^+ concentrations in the periplasm reaching toxic levels. It is therefore probable that SilE acts in a similar manner to SilF/CusF and chaperones Ag^+ to SilCBA, either directly or via transfer to SilF, whereupon Ag^+ is transported from the periplasm into the extracellular milieu.

5.3.2.3. Sil-based Ag^+ resistance in other bacterial genera

Earlier in this study it was demonstrated that the *sil* operon is contained within a Tn*7*-like transposon. As a consequence, the *sil* operon may have spread and integrated into the chromosomes of a range of bacterial species. To ascertain this, a BLASTn search was performed using the nucleotide sequence of the entire Tn*7*-like transposon obtained from this study (*i.e.* the *tns, sil* and *pco* operons). Analysis of the results revealed that a number of Gram-negative genera contained the entire transposon, the *sil* and *pco* operons, or just the *sil* operon, including *Salmonella*, *Klebsiella*, *Enterobacter, Serratia* and *Coronobacter* spp. In addition, the transposon was located in a number of shiga toxin-positive *E. coli* isolated from an outbreak in 2011 (Ahmed *et al.*, 2012). In the isolates containing an intact *sil* operon, *silR, S, C, F, B* and *A* are all incorrectly annotated as *cus* genes, which is likely to provide a false indication of the true levels of *sil* operon prevelance. To confirm that the presence of the *sil* operon confers Ag^+ resistance in these strains, $AgNO_3$ MICs were determined for *En. cloacae* ATCC 13047 and a clinical isolate of *K. pneumoniae* obtained from Leeds General Infirmary. These strains were selected as they are both members of the ESKAPE group of pathogens that frequently cause nosocomial infections (Boucher *et al.*, 2009) and could therefore be present in burn and chronic wound infections treated with Ag^+-containing products. In addition, high-level Ag^+ resistance has previously been encountered in

En. cloacae isolates (Ip *et al.*, 2006; Lansdown & Williams, 2007; Woods *et al.*, 2009). Neither strain demonstrated Ag^+ resistance, as $AgNO_3$ had an MIC of 4 μg/ml against both strains. However, as with the Cus system, constitutive expression of Sil components may be required to confer resistance in a process that requires a mutational event in the sequence encoding SilS. As such, the frequency of spontaneous mutation to Ag^+ resistance was assessed for both strains and was found to be $1.9 \pm 0.9 \times 10^{-8}$ and $3.4 \pm 1.7 \times 10^{-8}$, respectively for *En. cloacae* and *K. pneumoniae.* These frequencies are consistent with a single mutational event (O'Neill *et al*., 2004). Sequence determination of *silS* in both strains revealed the presence of mutations encoding amino acid substitutions $P_{213}H$ or $A_{102}T$ for *En. cloacae* and *K. pneumoniae*, respectively. It seems likely that as with CusS, the mutational events in *silS* result in the constitutive expression of downstream components. Indeed, it has been noted that expression of Sil components from pMG101 is constitutive and not dependent upon the presence of Ag^+ (Gupta *et al.*, 2001). In order to ascertain the diversity of mutations in *silS* that can confer Ag^+ resistance, *silS* was sequenced from a further five independent Ag^+-resistant mutants of *En. cloacae* and the subsequent mutations mapped to an I-TASSER-predicted structure of the cytoplasmic domain of SilS (as described above for CusS) (Figure 5.8). Unlike CusS, mutations in *silS* that confer Ag^+ resistance all resulted in amino acid substitutions localised around the HAMP/signal transduction domain of SilS. As hypothesised earlier for CusS, it is likely that HAMP domain mutations in SilS allow for the active site of the CA domain to be brought into close vicinity of the conserved histidine present on the DHp domain. This would lead to continuous phosphorylation of the cognate response regulator (SilR in this case) and therefore constitutive expression of the *sil* operon. However as with CusS, further

structural studies will be required to confirm that this conformational change is occurring and that the kinase activity of SilS is affected as a consequence.

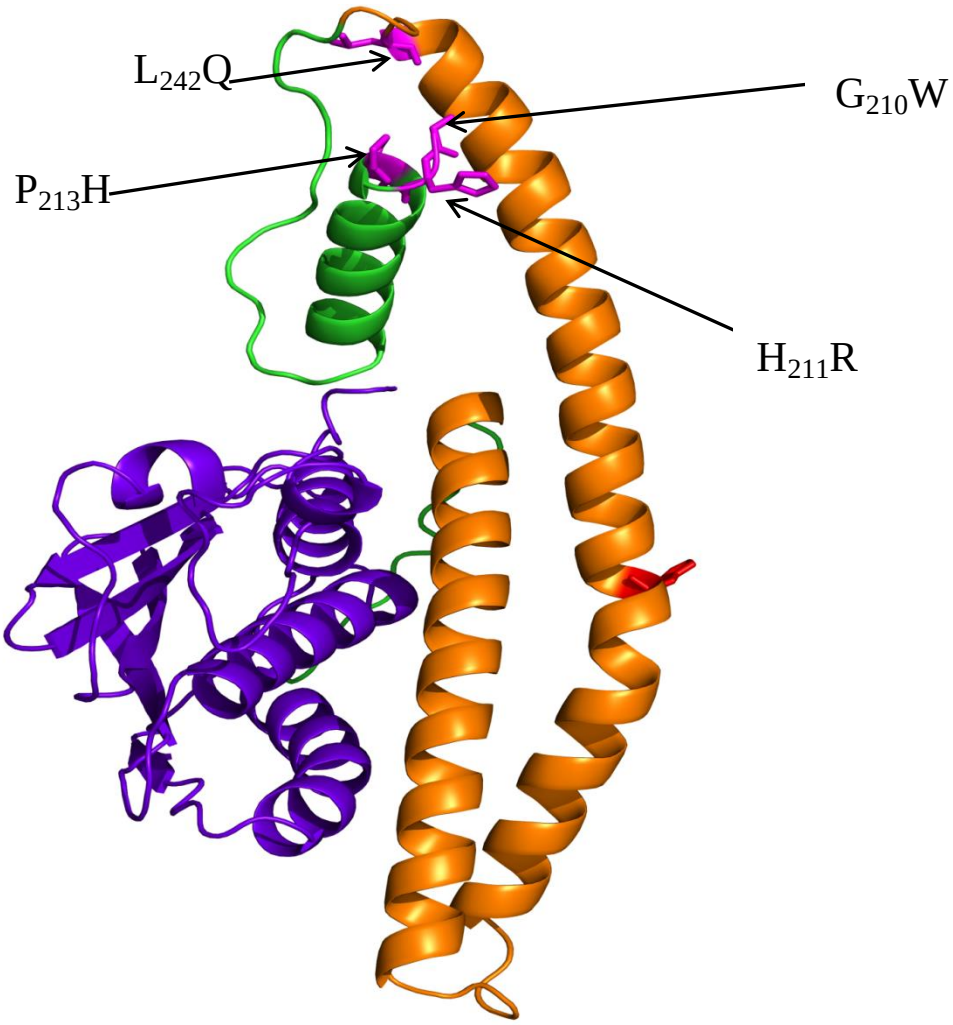


Figure 5.8. Predicted structure of the cytoplasmic domain of SilS in *En. cloacae.* Dimerization and catalytic/ATP-binding domains are shaded orange and purple respectively. The conserved histidine residue required for phosphorylation of SilR is shaded red and sites of amino acid substitutions associated with Ag^{+} resistance are shaded pink.

Unlike endogenous Ag^+ resistance, Ag^+ resistance conferred by the Sil system does not require porin loss to sustain an Ag^+ resistance phenotype. For this reason it is likely that Sil-based Ag^+ resistance confers a relatively small fitness cost to the cell, as strains harbouring the *sil* operon can still utilise porins to acquire nutrients from the extracellular environment. To determine if this is the case, pair-wise competition assays were carried out for both Ag^+-resistant *En. cloacae* and *K. pneumoniae* with their respective parental strains over 24 h. This resulted in relative fitness values of 0.91 (±0.04) and 0.98 (±0.02) obtained for *En. cloacae* and *K. pneumoniae*, respectively. This result indicates that there is only a minimal fitness burden associated with Sil-based Ag^+ resistance and thus provides evidence to suggest that SilE is used as an alternative to porin loss to negate the fitness cost that would be imposed by the latter phenotype.

5.4. Conclusions

This study has identified the genetic events that underpin endogenous Ag^+ resistance in *E. coli*. As a consequence of mutations in *cusS* and *ompR*, constitutive expression of CusCFBA and loss of OmpC/F expression confers an Ag^+ resistance phenotype with a susceptibility to $AgNO_3$ concentrations ≥64-times less than the parental strain. The fact that endogenous Ag^+ resistance arises from disruption to two separate two-component systems is an unusual phenomenon, as endogenous resistance to antibacterial agents typically arises as a consequence of mutation or overexpression of the antibacterial target. The only reported cases of antibacterial resistance arising from mutations to two-component systems are nisin resistance in *S. aureus*, whereby mutations in the sensor kinase NsaS result in constitutive expression of VraDE and a homologue of VraFG that is essential for the resistance phenotype (Blake *et al*., 2012; Randall, CP; Cruz, M and O'Neill, AJ., unpublished observations) and polymyxin B resistance in *P. aeruginosa*, which results from constitutive expression of *arnBCADTEF* (leading to a reduction in net negative charge of LPS) as a consequence of mutations in the sensor kinase PhoQ (Gutu *et al.*, 2013). However, in all of these studies the mechanistic changes conferred by sensor kinase mutations, or the relative kinase/phosphatase activities have yet to be elucidated and should be the subject of future studies.

Although endogenous Ag^+ resistance in *E. coli* can arise in a short time frame (six days) in the laboratory, its significance from a clinical perspective is unclear. *E. coli* is rarely encountered at sites where Ag^+ is employed therapeutically when compared with other bacterial species, and the endogenous Ag^+ phenotype harbours a fitness cost which may affect its ability to arise and persist in patients. On the other hand, additional prolonged exposure to Ag^+ may select for compensatory mutations that may partially mitigate the

fitness cost associated with this phenotype. Again, further studies would be required to assess this possibility.

In contrast to endogenous resistance, horizontally acquired Ag^+ resistance in the form of the Sil system confers no significant fitness cost and is present on a Tn*7*-like transposon, thus potentially allowing for its dissemination into a range of Gram-negative pathogens. However, it appears that the presence of the Sil system is not synonymous with Ag^+ resistance, as this study revealed that Sil-positive strains of *En. cloacae* and *K. pneumoniae* remained susceptible to Ag^+ until mutations in *silS* were selected. Previous studies have attempted to determine the prevalence of Ag^+ resistance by searching for the presence of *sil* operon components using a genetic approach (Gupta *et al.*, 2001; Silver, 2003; Loh *et al.*, 2009). However, as the data from this study indicate, this could lead to an overestimation in the number of strains harbouring an Ag^+ resistance phenotype. Future studies should instead focus on screening for phenotypic Ag^+ resistance by determination of $AgNO_3$ MICs, as this will give a true estimate of resistance prevalence.

Although this study has demonstrated that Ag^+ resistance can arise in some cases via a single mutational step, the frequency of spontaneous mutation to Ag^+ resistance is at a similar level to that seen for most antibiotics. This indicates that Ag^+-containing products still have potential utility for the prevention and treatment of bacterial infection. However, clinicians should seek to develop surveillance programs to monitor the emergence and spread of Ag^+-resistant isolates to mitigate any effects of resistance on patient morbidity and mortality. Finally, this work has revealed that Ag^+ efflux is essential for both the endogenous and exogenous Ag^+ resistance phenotypes. This discovery could lead to the rational design of efflux pump inhibitors that would negate Ag^+ resistance and extend the utility of Ag^+ in treating future bacterial infection.

Chapter six: General conclusions and future work

6.1. Bacterial resistance to Ag^+

The increasing trend of infections caused by bacteria resistant to one or more antibiotics has led to concerns that we are soon to enter a 'post-antibiotic era', wherein mortality resulting from infectious disease will reach rates similar to that observed prior to the introduction of sulphanilamide and penicillin (Alanis, 2005, Falagas & Bliziotis, 2007). It is vital that alternative methods to treat infectious disease are explored to prevent this from happening. One approach is to make more substantial use of antibacterial compounds that are currently underexploited, such as those containing silver (Ag^+). Originally introduced into modern clinical practice as a cheap alternative to antibiotics for preventing burn wound infection (Moyer *et al.*, 1965), the use of antibacterial Ag^+ has become widespread over the past decade, with applications ranging from the treatment of wound infection, to its incorporation into consumer goods to prevent microbial contamination (Silver, 2003, Edwards-Jones, 2009). However, there are concerns that such extensive and unrestricted use of antibacterial Ag^+ will select for the proliferation of Ag^+ resistance amongst bacterial pathogens, thus impacting the utility of Ag^+ in treating bacterial infection (Chopra, 2007). Bacterial resistance to Ag^+ is known to exist (Silver, 2003), although several aspects of the molecular basis of Ag^+ resistance are unclear. In addition, the current prevalence of Ag^+-resistant pathogens is unknown, nor is the propensity for such isolates to arise, persist and spread in a clinical environment. The research presented in this thesis sought to address these issues.

In Chapter 3, the CLSI agar dilution and broth microdilution MIC methodologies were found suitable for determining susceptibility to Ag^+ (in the form of $AgNO_3$) against a range of bacteria. Using this method, 1006 isolates of staphylococci collected from

hospitals across Europe and Canada were found to be universally susceptible to Ag^+; providing strong evidence to suggest that Ag^+ resistance amongst this genus is currently minimal, or non-existent. However, since a range of other bacterial species also cause wound infections that are typically treated with Ag^+ (*e.g. Streptococcus* and *Klebsiella* spp., *P aeruginosa* and *A. baumannii*) (Rezaei *et al.*, 2011), it would also be important to establish the current prevalence of Ag^+ resistance amongst isolates of these species, to justify the continued use of Ag^+ in treating wound infection. Future studies should attempt to address this aspect, and should strive to utilise the standardised method outlined in this thesis to enable a direct comparison of susceptibility data to be made between such studies.

In addition to establishing the current prevalence of Ag^+ resistance amongst staphylococci, the results presented in Chapter 3 revealed that endogenous resistance could not be selected in two separate strains of *S. aureus in vitro*, following continuous exposure to sub-inhibitory concentrations of Ag^+ for 42 days. The universal susceptibility of staphylococci to Ag^+, and failure to select for endogenous resistance to Ag^+, would suggest that Ag^+-containing compounds remain a viable option for the prevention and treatment of topical staphylococcal infections.

Unlike *S. aureus*, endogenous Ag^+ resistance arose relatively quickly (6 days) in *E. coli* following continuous exposure to sub-inhibitory concentrations of Ag^+ (Chapter 5). This phenotype resulted from a missense mutation in *ompR* (which prevented expression of the porins OmpC and OmpF), and a second missense mutation in *cusS* (which enabled constitutive expression of the Ag^+ efflux pump, CusCFBA). Using pair-wise competition assays, this phenotype was found to confer a significant fitness cost (W=0.71); however, as antibiotic-resistant strains of bacteria have been isolated from patients with W values less than this (O'Neill *et al.*, 2006), it appears unlikely that the

fitness cost associated with endogenous Ag^+ resistance in *E. coli* will act as a barrier to the emergence and survival of this phenotype in patients. Nevertheless, it would be beneficial to ascertain if selection of this phenotype could occur *in vivo*, using an appropriate animal model and a commercial Ag^+-containing product.

Although endogenous Ag^+ resistance could be selected in *E. coli*, resistance did not arise in other Gram-negative bacteria (*A. baumannii*, *P. aeruginosa*, *C. freundii* and *S. sonnei*), after continuous exposure to Ag^+ for 42 days. This suggests that in the emergence of endogenous Ag^+ resistance in these strains would be unlikely *in vivo*. However, this result does not exclude the possibility that these strains (or others) could acquire an Ag^+-resistance determinant by horizontal gene transfer. Indeed, exogenous Ag^+ resistance has previously been encountered in Gram-negative bacteria (*see* Table 1.5.), and may therefore represent a threat to the continued use of therapeutic Ag^+. One such exogenous Ag^+-resistance determinant is the *sil* operon, which was first identified on the *S.* Typhimurium plasmid pMG101 (Gupta *et al.*, 1999). To provide further insight into this determinant, the whole genome sequence of *E. coli* strain NCTC 50110 (containing pMG101) was determined (Chapter 5). Analysis revealed that the *sil* operon was located on a Tn*7*-like transposable element that had integrated into the *E. coli* chromosome (termed Tn*7sil*). The archetypical Tn*7* system can integrate into a specific site on bacterial chromosomes, termed the *attTn7* sequence, in a process mediated by the transposition proteins TnsABCD (Peters & Craig, 2001). Non-specific integration of Tn*7* into conjugative plasmids can also occur, resulting from TnsE directing TnsABC-mediated transposition of such plasmids during their replication (Peters & Craig, 2001). The presence of a clinically-relevant Ag^+-resistance determinant on a Tn*7*-like transposon is therefore a cause for concern, as this determinant could disseminate with relative ease into a range of bacterial species; including those which

cause infections routinely treated with Ag^+. However, Tn*7sil* does not contain TnsE and lacks IR elements (Tn*7*-L and Tn*7*-R) that typically flank Tn*7*-like transposons, thus the functionality of Tn*7sil* relative to the archetypical Tn*7* system is unclear; particularly with respect to the ability of Tn*7sil* to integrate into conjugative plasmids (given the lack of TnsE in this system). To establish if Tn*7sil* can transpose from the chromosome onto a conjugative plasmid, NCTC 50110 could be cured of pMG1 01, then transformed with another conjugative plasmid. Transposition of Tn*7sil* onto this new plasmid could be confirmed by establishing if Ag^+ resistance is transmissible to other strains by conjugation.

Although the functionality of Tn*7sil* is unclear, a BLASTn search using the nucleotide sequence of Tn*7sil* revealed it to be present in strains of *E. coli*, *S.* Heidelberg, *Se. marcescens* and *Coronobacter sakazakii* with >99% sequence identity. This would seem to suggest that Tn*7sil* is indeed capable of disseminating Ag^+ resistance. In addition, the *sil* operon (*i.e.* not contained within Tn*7sil*) was identified in a number of *En. cloacae* and *K. pneumoniae* strains. However, as demonstrated in Chapter 5, representative isolates of these species did not display intrinsic Ag^+ resistance until missense mutations were selected in *silS*, which encodes the putative sensor kinase believed to regulate expression of the Sil system (Gupta *et al.*, 1999). This result indicates that, as with CusCFBA, it is likely that the constitutive, high-level, expression of Sil system components is required to confer Ag^+ resistance; this could be confirmed by similar qPCR experiments to those performed in Chapter 3. This study has revealed that the presence of *sil* is not synonymous with Ag^+ resistance, thus highlighting that the genotypic screens for *sil* components used in prior studies (Silver, 2003; Percival *et al.*, 2008) may not reliably predict the true prevalence of phenotypic Ag^+ resistance.

In summary, Ag^+ resistance can arise with relative ease in *E. coli*, *En. cloacae* and *K. pneumoniae in vitro*, and an exogenous Ag^+-resistance determinant has been identified on a transposable element that has the potential to disseminate Ag^+ resistance amongst a range of Gram-negative bacteria. As Ag^+ resistance has resulted in the failure of Ag^+ therapy to prevent infection in the past (McHugh *et al.*, 1975; Gayle *et al.*, 1978), it would seem prudent to restrict the use of Ag^+ in consumer goods; the current prolific use of Ag^+ in such products likely provides a selective pressure that could accelerate the emergence of widespread Ag^+ resistance amongst clinically relevant pathogens, which in turn will limit the use of Ag^+ in treating infectious disease.

Inhibitors of the Ag^+ resistance phenotype conferred by Sil- and Cus-based mechanisms could be of benefit in limiting the clinical impact of Ag^+ resistance. Using recombineering, deletion of any component of the RND-type efflux pumps of the Cus (CusCBA) and Sil (SilCBA) systems resulted in a complete loss of Ag^+ resistance (Chapter 5). This finding suggests that inhibitors of Ag^+ resistance could be developed by identifying a compound that can bind and disrupt the function of one (or more) of these proteins. Inhibitors of RND-type efflux pumps (*e.g.* PAβN and chlorpromazine) have already been identified (Renau *et al.*, 1999; Viveiros *et al.*, 2008) and a future study could assess the utility of these compounds in disrupting Ag^+ resistance. In addition, as the structures of CusC, B and A have been solved (Kulathila *et al.*, 2011; Su *et al.*, 2011), a SBDD approach could be used to identify putative inhibitors of these proteins (Simmons *et al.*, 2010).

Although this study has furthered our understanding of Cus- and Sil-based Ag^+ resistance, other exogenous Ag^+-resistance determinants have been identified (present on plasmids pKK1, pJT1 and pUPI199) that are proposed to confer resistance by an alternative mechanism to efflux (*see* section 1.2.6). Unsuccessful attempts were made to

obtain these plasmids. Therefore the molecular basis of resistance, and the fitness cost associated with the maintenance of these elements, remain unclear.

6.2. Antibacterial MOA of Ag^+

Another objective of the work presented in this thesis was to elucidate the antibacterial MOA of Ag^+. Although a number of studies have demonstrated that Ag^+ can bind and impact the integrity of DNA, proteins and the cell membrane, it is unclear if these effects are the cause of Ag^+-mediated cell inhibition/death, or are secondary to the primary antibacterial action of Ag^+. The results presented in Chapter 4 indicate that the cell membrane is the likely antibacterial target of Ag^+, as Ag^+ exposure resulted in a rapid (10 min) and substantial loss of integrity. The inability of Ag^+ to damage liposomes implies that Ag^+-mediated membrane damage likely arises from disruption of integral membrane proteins. Indeed, evidence from DNA microarray analysis seems to support this hypothesis, as the expression of a number of integral membrane proteins increased in cells exposed to Ag^+. This mechanism of membrane damage could be confirmed in future studies by assessing the ability of Ag^+ to damage carboxyfluorescein-filled vesicles comprised of reconstituted bacterial membrane (*i.e.* comprising phospholipid and protein), performed in a similar manner to the liposome assay described in this thesis. In addition, the effect of Ag^+ on membrane proteins could be directly visualised *in vivo* using atomic force microscopy (AFM). Indeed, AFM has previously been used to image the outer membrane of live *Magnetospirillum magnetotacticum*, at a resolution sufficient to visualise porin channels (Yamashita *et al.*, 2012).

One rationale for elucidating the MOA of Ag^+ was to enable the identification of ways to improve antibacterial activity of Ag^+. In Chapter 4, it was shown that exposure of

S. aureus to Ag^+ induced the formation of ROS, albeit at levels that did not impact cell viability. Nevertheless, if Ag^+ could be combined with another compound that also induces ROS formation, their combined action might produce sufficient ROS to overwhelm host detoxification mechanisms and cause oxidative damage to the cell. A previous study has demonstrated that the combination of Ag^+ and Paraquat (which generates O_2^- via redox cycling) is synergistic against *E. coli* (Park *et al.,* 2009). Furthermore, Ag^+-mediated membrane damage may enable increased uptake of antibiotics with an intracellular target and therefore be synergistic in combination. This phenomenon has been described previously for known membrane-damaging compounds used in combination with β-lactams, macrolides, tetracyclines and aminoglycosides (Hemaiswarya & Doble, 2009, Choi & Lee, 2012). Indeed, a publication arising after the completion of the work in this thesis revealed that Ag^+ is synergistic with ampicillin and ofloxacin against *E. coli in vitro* and with gentamicin and vancomycin in an *in vivo* mouse UTI and peritoneal infection model, respectively (Morones-Ramirez *et al.*, 2013). Although this latter study did not assess the long-term toxicity associated with using Ag^+ in this manner, this result demonstrates a potential future application of Ag^+ as a systemic antibacterial agent. This prospect provides further impetus to restrict the use of antibacterial Ag^+ in consumer products, so that the clinical lifespan of Ag^+ is not prematurely cut short by the rapid proliferation of Ag^+ resistance.

As discussed in Chapters 1 and 3, Ag^+ can be bound and inactivated by protein and halide anions. As these components are present in environments where Ag^+ is employed, it is unclear how much Ag^+ remains available to exert an antibacterial effect (Liau *et al.*, 1997; Gupta *et al.*, 1998). As a consequence the antibacterial properties of Ag^+-containing products *in vitro* may be attenuated in environments such as the wound.

One way to address this problem experimentally is to develop bacterial Ag^+ biosensors, which link Ag^+ sensing to the production of a quantifiable output (*e.g.* the production of fluorescence from GFP or luminescence from luciferase). Such a system could then be used to give a true measure of the active Ag^+ concentration in any given environment (as only free Ag^+ would be detected by the sensor). This concept has been employed previously in the creation of biosensors that can detect and quantify arsenic (Diesel *et al.*, 2009). To create an Ag^+ biosensor, the CusRS two-component system could be exploited for the sensing of extracellular Ag^+, as this system was shown to be a positive regulator of gene expression upon Ag^+ exposure in Chapter 5. A gene encoding a reporter protein (*e.g.* GFP of luciferase) could then be fused downstream of the *cusR-cusC* intergenic region (*i.e.* the region that phosphorylated CusR binds and recruits RNA polymerase), to enable its expression following Ag^+ stimulus.

Collectively, this work has produced detailed insights into the MOA of Ag^+ and the prevalence and mechanisms of bacterial Ag^+ resistance. The results presented in this thesis, in combination with the additional studies recommended in this chapter should provide further insight into optimising the future use of Ag^+ in treating infectious disease.

References

Adler M, Anjum M, Andersson DI & Sandegren L (2013) Influence of acquired β-lactamases on the evolution of spontaneous carbapenem resistance in *Escherichia coli*. *J Antimicrob Chemother* **68**: 51-59.

Ahmed SA, Awosika J, Baldwin C, *et al.* (2012) Genomic comparison of *Escherichia coli* O104:H4 isolates from 2009 and 2011 reveals plasmid, and prophage heterogeneity, including shiga toxin encoding phage *stx2*. *PLoS One* **7**: e48228.

Alanis AJ (2005) Resistance to antibiotics: are we in the post-antibiotic era? *Arch Med Res* **36**: 697-705.

Alexander JW (2009) History of the medical use of silver. *Surg Infect* **10**: 289-292.

Anderson KL, Roberts C, Disz T, *et al.* (2006) Characterization of the *Staphylococcus aureus* heat shock, cold shock, stringent, and SOS responses and their effects on log-phase mRNA turnover. *J Bacteriol* **188**: 6739-6756.

Anderson DJ & Kaye KS (2009) Staphylococcal surgical site infections. *Infect Dis Clin North Am* **23**: 53-72.

Andrews JM (2001) Determination of minimum inhibitory concentrations. *J Antimicrob Chemother* **48 Suppl 1**: 5-16.

Annear DI, Mee BJ & Bailey M (1976) Instability and linkage of silver resistance, lactose fermentation, and colony structure in *Enterobacter cloacae* from burn wounds. *J Clin Pathol* **29**: 441-443.

Arakawa H, Neault J & Tajmir-Riahi H (2001) Silver (I) complexes with DNA and RNA studied by Fourier transform infrared spectroscopy and capillary electrophoresis. *Biophys J* **81**: 1580-1587.

Arens S & Verbist L (1997) Differentiation and susceptibility of *Citrobacter* isolates from patients in a university hospital. *Clin Microbiol Infect* **3**: 53-57.

Armstrong GL, Conn LA & Pinner RW (1999) Trends in infectious disease mortality in the United States during the 20th Century. *JAMA-J Am Med Assoc* **281**: 61-66.

Atiyeh BS, Costagliola M, Hayek SN & Dibo SA (2007) Effect of silver on burn wound infection control and healing: review of the literature. *Burns* **33**: 139-148.

Atlas RM (2010) *Handbook of Microbiological Media*. CRC Press.

Aziz RK, Bartels D, Best AA, *et al.* (2008) The RAST Server: rapid annotations using subsystems technology. *BMC Genomics* **9**: 75.

Baba T, Ara T, Hasegawa M, *et al.* (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2**: 2006 0008.

Babakhani F, Shangle S & Robert N (2004) Resistance development, cross-resistance, and synergy studies of OPT-80 [abstract E-2047]. Presented at the 44th Inter-science Conference on Antibacterial Agents and Chemotherapy, Washington, DC, USA.

Barry AL & Effinger LJ (1974) Performances of Mueller-Hinton agars prepared by three manufacturers. *Am J Clin Pathol* **62**: 113-117.

Basle A, Rummel G, Storici P, Rosenbusch JP & Schirmer T (2006) Crystal structure of osmoporin OmpC from *E. coli* at 2.0 Å. *J Mol Biol* **362**: 933-942.

Bauer AW, Kirby WM, Sherris JC & Turck M (1966) Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* **45**: 493-496.

Beceiro A, Maharjan S, Gaulton T, *et al.* (2011) False extended-spectrum β-lactamase phenotype in clinical isolates of *Escherichia coli* associated with increased expression of OXA-1 or TEM-1 penicillinases and loss of porins. *J Antimicrob Chemother* **66**: 2006-2010.

Benz R, Schmid A & Hancock RE (1985) Ion selectivity of Gram-negative bacterial porins. *J Bacteriol* **162**: 722-727.

Bergin SM & Wraight P (2006) Silver based wound dressings and topical agents for treating diabetic foot ulcers. *Cochrane Database Syst Rev* CD005082.

Bertram R, Kostner M, Muller J, Vazquez Ramos J & Hillen W (2005) Integrative elements for *Bacillus subtilis* yielding tetracycline-dependent growth phenotypes. *Nucleic Acids Res* **33**: e153.

Besier S, Ludwig A, Brade V & Wichelhaus TA (2003) Molecular analysis of fusidic acid resistance in *Staphylococcus aureus*. *Mol Microbiol* **47**: 463-469.

Bhuyan AK (2010) On the mechanism of SDS-induced protein denaturation. *Biopolymers* **93**: 186-199.

Bjarnsholt T, Kirketerp-Moller K, Kristiansen S, *et al.* (2007) Silver against *Pseudomonas aeruginosa* biofilms. *APMIS* **115**: 921-928.

Blake KL & O'Neill AJ (2013) Transposon library screening for identification of genetic loci participating in intrinsic susceptibility and acquired resistance to antistaphylococcal agents. *J Antimicrob Chemother* **68**: 12-16.

Bordi C, Butcher BG, Shi Q, Hachmann AB, Peters JE & Helmann JD (2008) *In vitro* mutagenesis of *Bacillus subtilis* by using a modified Tn*7* transposon with an outward-facing inducible promoter. *Appl Environ Microbiol* **74**: 3419-3425.

Boucher HW, Talbot GH, Bradley JS, *et al.* (2009) Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin Infect Dis* **48**: 1-12.

Brady RA, Leid JG, Camper AK, Costerton JW & Shirtliff ME (2006) Identification of *Staphylococcus aureus* proteins recognized by the antibody-mediated immune response to a biofilm infection. *Infect Immun* **74**: 3415-3426.

Bragg PD & Rainnie DJ (1974) The effect of silver ions on the respiratory chain of *Escherichia coli*. *Can J Microbiol* **20**: 883-889.

Brown EM & Thomas P (2002) Fusidic acid resistance in *Staphylococcus aureus* isolates. *Lancet* **359**: 803.

Bridges K, Kidson A, Lowbury EJ & Wilkins MD (1979) Gentamicin- and silver-resistant *Pseudomonas* in a burns unit. *Br Med J* **1**: 446-449.

Bugg TD, Dutka-Malen S, Arthur M, Courvalin P & Walsh CT (1991) Identification of vancomycin resistance protein VanA as a D-alanine: D-alanine ligase of altered substrate specificity. *Biochemistry* **30**: 2017-2021.

Caceres NE, Harris NB, Wellehan JF, Feng Z, Kapur V & Barletta RG (1997) Overexpression of the D-alanine racemase gene confers resistance to D-cycloserine in *Mycobacterium smegmatis*. *J Bacteriol* **179**: 5046-5055.

Cai SJ & Inouye M (2002) EnvZ-OmpR interaction and osmoregulation in *Escherichia coli*. *J Biol Chem* **277**: 24155-24161.

Carpenter CF & Chambers HF (2004) Daptomycin: another novel agent for treating infections due to drug-resistant Gram-positive pathogens. *Clin Infect Dis* **38**: 994-1000.

Carr HS, Wlodkowski TJ & Rosenkranz HS (1973) Silver sulfadiazine: *in vitro* antibacterial activity. *Antimicrob Agents Chemother* **4**: 585-587.

Castellano JJ, Shafii SM, Ko F *et al.* (2007) Comparative evaluation of silver-containing antibacterial dressings and drugs. *Int Wound J* **4**: 114-122.

Ceri H, Olson ME, Stremick C, Read RR, Morck D & Buret A (1999) The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J Clin Microbiol* **37**: 1771-1776.

Chambers H, Dumville JC & Cullum N (2007) Silver treatments for leg ulcers: a systematic review. *Wound Repair Regen* **15**: 165-173.

Chan PF, Holmes DJ & Payne DJ (2004) Finding the gems using genomic discovery: antibacterial drug discovery strategies–the successes and the challenges. *Drug Discov Today Ther Strateg* **1**: 519-527.

Chaw KC, Manimaran M & Tay FE (2005) Role of silver ions in destabilization of intermolecular adhesion forces measured by atomic force microscopy in *Staphylococcus epidermidis* biofilms. *Antimicrob Agents Chemother* **49**: 4853-4859.

Cherepanov PP & Wackernagel W (1995) Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* **158**: 9-14.

Cherrington CA, Hinton M & Chopra I (1990) Effect of short-chain organic acids on macromolecular synthesis in *Escherichia coli*. *J Appl Bacteriol* **68**: 69-74.

Cho Lee A-R, Leem H, Lee J & Park KC (2005) Reversal of silver sulfadiazine-impaired wound healing by epidermal growth factor. *Biomaterials* **26**: 4670-4676.

Choi H & Lee DG (2012) Antibacterial peptide pleurocidin synergizes with antibiotics through hydroxyl radical formation and membrane damage, and exerts antibiofilm activity. *Biochim Biophys Acta* **1820**: 1831-1838.

Chopra I & Roberts M (2001) Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Bio R* **65**: 232-260.

Chopra I (2007) The increasing use of silver-based products as antibacterial agents: a useful development or a cause for concern? *J Antimicrob Chemother* **59**: 587-590.

Chopra I, Schofield C, Everett M *et al.* (2008) Treatment of health-care-associated infections caused by Gram-negative bacteria: a consensus statement. *Lancet Infect Dis* **8**: 133-139.

Chu L, Mukhopadhyay D, Yu H, Kim KS & Misra TK (1992) Regulation of the *Staphylococcus aureus* plasmid pI258 mercury resistance operon. *J Bacteriol* **174**: 7044-7047.

Chung CT, Niemela SL & Miller RH (1989) One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. *Proc Natl Acad Sci U S A* **86**: 2172-2175.

Citron DM, Goldstein EJ, Merriam CV, Lipsky BA & Abramson MA (2007) Bacteriology of moderate-to-severe diabetic foot infections and in vitro activity of antibacterial agents. *J Clin Microbiol* **45**: 2819-2828.

Clement JL & Jarrett PS (1994) Antibacterial silver. *Met Based Drugs* **1**: 467-482.

Compeau PE, Pevzner PA & Tesler G (2011) How to apply de Bruijn graphs to genome assembly. *Nat Biotechnol* **29**: 987-991.

Confreres A & Maxwell A (1992) *gyrB* mutations which confer coumarin resistance also affect DNA supercoiling and ATP hydrolysis by *Escherichia coli* DNA gyrase. *Mol Microbiol* **6**: 1617-1624.

Conly J & Johnston B (2005) Where are all the new antibiotics? The new antibiotic paradox. *Can J Infect Dis* **16**: 159-160.

Cosgrove K, Coutts G, Jonsson IM, Tarkowski A, Kokai-Kun JF, Mond JJ & Foster SJ (2007) Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) have compensatory roles in peroxide stress resistance and are required for survival, persistence, and nasal colonization in *Staphylococcus aureus*. *J Bacteriol* **189**: 1025-1035.

Cosgrove SE, Sakoulas G, Perencevich EN, Schwaber MJ, Karchmer AW & Carmeli Y (2003) Comparison of mortality associated with methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* bacteremia: a meta-analysis. *Clin Infect Dis* **36**: 53-59.

Costerton JW, Stewart PS & Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. *Science* **284**: 1318-1322.

Cowan SW, Garavito RM, Jansonius JN, *et al.* (1995) The structure of OmpF porin in a tetragonal crystal form. *Structure* **3**: 1041-1050.

Coward JE, Carr HS & Rosenkranz HS (1973a) Silver sulfadiazine: effect on the ultrastructure of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **3**: 621-624.

Coward JE, Carr HS & Rosenkranz HS (1973b) Silver sulfadiazine: effect on the growth and ultrastructure of staphylococci. *Chemotherapy* **19**: 348-353.

Cox G, Thompson GS, Jenkins HT *et al*. (2012) Ribosome clearance by FusB-type proteins mediates resistance to the antibiotic fusidic acid. *Proc Natl Acad Sci* **109**: 2102-2107.

Cramton SE, Gerke C, Schnell NF, Nichols WW & Gotz F (1999) The intercellular adhesion (*ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infect Immun* **67**: 5427-5433.

Cutting KF (2003) Wound exudate: composition and functions. *Br J Community Nurs* **8**: suppl 4-9.

Danelon C, Suenaga A, Winterhalter M & Yamato I (2003) Molecular origin of the cation selectivity in OmpF porin: single channel conductances vs. free energy calculation. *Biophys Chem* **104**: 591-603.

Datsenko KA & Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**: 6640-6645.

Datta S, Costantino N & Court DL (2006) A set of recombineering plasmids for Gram-negative bacteria. *Gene* **379**: 109-115.

Davies J & Davies D (2010) Origins and evolution of antibiotic resistance. *Microbiol Mol Biol R* **74**: 417-433.

Davies SC (2013) Annual Report of the Chief Medical Officer, Volume Two, 2011, Infections and the rise of antibacterial resistance. *London: Department of Health* (2013)

de Prijck K, Nelis H & Coenye T (2007) Efficacy of silver-releasing rubber for the prevention of *Pseudomonas aeruginosa* biofilm formation in water. *Biofouling* **23**: 405-411.

Deshpande LM & Chopade BA (1994) Plasmid mediated silver resistance in *Acinetobacter baumannii* . *Biometals* **7**: 49-56.

Dhand A & Snydman DR (2009) Mechanism of resistance in metronidazole. In: *Antibacterial Drug Resistance*. 223-227. Springer.

Dibrov P, Dzioba J, Gosink KK & Hase CC (2002) Chemiosmotic mechanism of antibacterial activity of Ag^{+} in *Vibrio cholerae*. *Antimicrob Agents Chemother* **46**: 2668-2670.

Diesel E, Schreiber M & van der Meer JR (2009) Development of bacteria-based bioassays for arsenic detection in natural waters. *Anal Bioanal Chem* **394**: 687-693.

Drews J (2000) Drug discovery: a historical perspective. *Science* **287**: 1960-1964.

Duckett S (1999) Ernest Duchesne and the concept of fungal antibiotic therapy. *Lancet* **354**: 2068-2071.

Dundas S, Todd WA, Neill MA & Tarr PI (2005) Using antibiotics in suspected haemolytic-uraemic syndrome: Antibiotics should not be used in *Escherichia coli* O157: H7 infection. *Brit Med J* **330**: 1209.

Dunn K & Edwards-Jones V (2004) The role of Acticoat with nanocrystalline silver in the management of burns. *Burns* **30 Suppl 1**: S1-9.

Duxbury T (1986) Microbes and heavy metals: an ecological overview. *Microbiol Sci* **3**: 330.

Edwards-Jones V & Greenwood JE (2003) What's new in burn microbiology? James Laing Memorial Prize Essay 2000. *Burns* **29**: 15-24.

Edwards-Jones V (2009) The benefits of silver in hygiene, personal care and healthcare. *Lett Appl Microbiol* **49**: 147-152.

Eng RH, Padberg FT, Smith SM, Tan EN & Cherubin CE (1991) Bactericidal effects of antibiotics on slowly growing and nongrowing bacteria. *Antimicrob Agents Chemother* **35**: 1824-1828.

Epand RF, Savage PB & Epand RM (2007) Bacterial lipid composition and the antibacterial efficacy of cationic steroid compounds (Ceragenins). *Biochim Biophys Acta* **1768**: 2500-2509.

Epand RF, Pollard JE, Wright JO, Savage PB & Epand RM (2010) Depolarization, bacterial membrane composition, and the antibacterial action of ceragenins. *Antimicrob Agents Chemother* **54**: 3708-3713.

Fabbretti A, Gualerzi CO & Brandi L (2011) How to cope with the quest for new antibiotics. *FEBS lett* **585**: 1673-1681.

Fairhead H (2009) SASP gene delivery: a novel antibacterial approach. *Drug News Perspect* **22**: 197.

Falagas ME & Bliziotis IA (2007) Pandrug-resistant Gram-negative bacteria: the dawn of the post-antibiotic era? *Int Journal Antimicrob Agents* **29**: 630-636.

Falagas ME, Grammatikos AP & Michalopoulos A (2008) Potential of old-generation antibiotics to address current need for new antibiotics. *Expert Rev Ant Infect Ther* **6**: 593-600.

Falagas ME, Rafailidis PI & Matthaiou DK (2010) Resistance to polymyxins: mechanisms, frequency and treatment options. *Drug Resist Update* **13**: 132-138.

Fauci AS (2001) Infectious diseases: considerations for the 21st Century. *Clin Infect Dis* **32**: 675-685.

Feng QL, Wu J, Chen GQ, Cui FZ, Kim TN & Kim JO (2000) A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*. *J Biomed Mater Res* **52**: 662-668.

Ferris HU, Dunin-Horkawicz S, Hornig N, *et al.* (2012) Mechanism of regulation of receptor histidine kinases. *Structure* **20**: 56-66.

Finch RG, Greenwood D, Whitley RJ & Norrby SR (2010) *Antibiotic and chemotherapy*. Elsevier Health Sciences. pp1-9

Fischbach MA & Walsh CT (2009) Antibiotics for emerging pathogens. *Science* **325**: 1089-1093.

Fleming A (1919) The action of chemical and physiological antiseptics in a septic wound. *Brit J Surg* **7**: 99-129.

Fleming A (1929) On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of *B. influenzae*. *Brit J Exp Pathol* **10**: 226-236.

Franke S, Grass G, Rensing C & Nies DH (2003) Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. *J Bacteriol* **185**: 3804-3812.

Franzen C (2008) Syphilis in composers and musicians—Mozart, Beethoven, Paganini, Schubert, Schumann, Smetana. *Eur J Clin Microbiol* **27**: 1151-1157.

Freiberg C, Fischer HP & Brunner NA (2005) Discovering the mechanism of action of novel antibacterial agents through transcriptional profiling of conditional mutants. *Antimicrob Agents Chemother* **49**: 749-759.

Friedman L, Alder JD & Silverman JA (2006) Genetic changes that correlate with reduced susceptibility to daptomycin in *Staphylococcus aureus*. *Antimicrob Agents Chemother* **50**: 2137-2145.

Fuda CCS, Fisher JF & Mobashery S (2005) β-Lactam resistance in *Staphylococcus aureus*: the adaptive resistance of a plastic genome. *Cell Mol Life Sci* **62**: 2617-2633.

Fuchs S, Pane-Farre J, Kohler C, Hecker M & Engelmann S (2007) Anaerobic gene expression in *Staphylococcus aureus*. *J Bacteriol* **189**: 4275-4289.

Gadd GM (1992) Metals and microorganisms: a problem of definition. *FEMS Microbiol Lett* **100**: 197-203.

Gayle WE, Mayhall CG, Lamb VA, Apollo E & Haynes BW, Jr. (1978) Resistant *Enterobacter cloacae* in a burn center: the ineffectiveness of silver sulfadiazine. *J Trauma* **18**: 317-323.

GFMS (2011) The future of silver industrial demand. http://www.silverinstitute.org/images/stories/silver/PDF/futuresilverindustrialdemand.pdf (Date last accessed, 8 September 2013).

Gordon NC, Png K & Wareham DW (2010) Potent synergy and sustained bactericidal activity of a vancomycin-colistin combination versus multidrug-resistant strains of *Acinetobacter baumannii* . *Antimicrob Agents Chemother* **54**: 5316-5322.

Gordon O, Vig Slenters T, Brunetto PS, *et al.* (2010) Silver coordination polymers for prevention of implant infection: thiol interaction, impact on respiratory chain enzymes, and hydroxyl radical induction. *Antimicrob Agents Chemother* **54**: 4208-4218.

Gravante G, Caruso R, Sorge R, Nicoli F, Gentile P & Cervelli V (2009) Nanocrystalline silver: a systematic review of randomized trials conducted on burned patients and an evidence-based assessment of potential advantages over older silver formulations. *Ann Plas Surg* **63**: 201-205.

Gravet A, Colin DA, Keller D, Girardot R, Monteil H & Prevost G (1998) Characterization of a novel structural member, LukE-LukD, of the bi-component staphylococcal leucotoxins family. *FEBS Lett* **436**: 202-208.

Gregory JE, Starr SP & Omdal C (1974) Wound infection with *Shigella flexneri*. *J Infect Dis* **129**: 602-604.

Grier N (1968) Silver and its compounds. In: *Disinfection, sterilization, and preservation. Philadelphia: Lippincott Williams & Wilkins* pp415-430

Guardabassi L, Loeber ME & Jacobson A (2004) Transmission of multiple antibacterial-resistant *Staphylococcus intermedius* between dogs affected by deep pyoderma and their owners. *Vet Microbiol* **98**: 23-27.

Gudipaty SA, Larsen AS, Rensing C & McEvoy MM (2012) Regulation of Cu(I)/Ag(I) efflux genes in *Escherichia coli* by the sensor kinase CusS. *FEMS Microbiol Lett* **330**: 30-37.

Gupta L, Jindal R, Beri H & Chhibber S (1992) Virulence of silver-resistant mutant of *Klebsiella pneumoniae* in burn wound model. *Folia microbiol* **37**: 245-248.

Gupta A, Maynes M & Silver S (1998) Effects of halides on plasmid-mediated silver resistance in *Escherichia coli*. *Appl Environ Microbiol* **64**: 5042-5045.

Gupta A, Matsui K, Lo JF & Silver S (1999) Molecular basis for resistance to silver cations in *Salmonella*. *Nat Med* **5**: 183-188.

Gupta A, Phung LT, Taylor DE & Silver S (2001) Diversity of silver resistance genes in IncH incompatibility group plasmids. *Microbiology* **147**: 3393-3402.

Gutu AD, Sgambati N, Strasbourger P, *et al.* (2013) Polymyxin resistance of *Pseudomonas aeruginosa phoQ* mutants is dependent on additional two-component regulatory systems. *Antimicrob Agents Chemother* **57**: 2204-2215.

Haefeli C, Franklin C & Hardy K (1984) Plasmid-determined silver resistance in *Pseudomonas stutzeri* isolated from a silver mine. *J Bacteriol* **158**: 389-392.

Hamilton-Miller JM, Shah S & Smith C (1993) Silver sulphadiazine: a comprehensive *in vitro* reassessment. *Chemotherapy* **39**: 405-409.

Hawkey P (2008) The growing burden of antibacterial resistance. *J Antimicrob Chemother* **62**: i1-i9.

Hayes F (2003) Toxins-antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. *Science* **301**: 1496-1499.

Heath RJ, Li J, Roland GE & Rock CO (2000) Inhibition of the *Staphylococcus aureus* NADPH-dependent enoyl-acyl carrier protein reductase by triclosan and hexachlorophene. *J Biol Chem* **275**: 4654-4659.

Hemaiswarya S & Doble M (2009) Synergistic interaction of eugenol with antibiotics against Gram negative bacteria. *Phytomedicine* **16**: 997-1005.

Hendry AT & Stewart IO (1979) Silver-resistant Enterobacteriaceae from hospital patients. *Can J Microbiol* **25**: 915-921.

Heym B, Alzari PM, Honore N & Cole ST (1995) Missense mutations in the catalase-peroxidase gene, *katG*, are associated with isoniazid resistance in *Mycobacterium tuberculosis*. *Mol Microbiol* **15**: 235-245.

Hilliard JJ, Goldschmidt RM, Licata L, Baum EZ & Bush K (1999) Multiple mechanisms of action for inhibitors of histidine protein kinases from bacterial two-component systems. *Antimicrob Agents Chemother* **43**: 1693-1699.

Hirokawa T, Boon-Chieng S & Mitaku S (1998) SOSUI: classification and secondary structure prediction system for membrane proteins. *Bioinformatics* **14**: 378-379.

Hobbs JK, Miller K, O'Neill AJ & Chopra I (2008) Consequences of daptomycin-mediated membrane damage in *Staphylococcus aureus*. *J Antimicrob Chemother* **62**: 1003-1008.

Hodgson JE, Curnock SP, Dyke KG, Morris R, Sylvester DR & Gross MS (1994) Molecular characterization of the gene encoding high-level mupirocin resistance in *Staphylococcus aureus* J2870. *Antimicrob Agents Chemother* **38**: 1205-1208.

Holt kb & Bard AJ (2005) Interaction of silver(I) ions with the respiratory chain of *Escherichia coli*: an electrochemical and scanning electrochemical microscopy study of the antibacterial mechanism of micromolar Ag^{+}. *Biochemistry* **44**: 13214-13223.

Hooper DC (1999) Mechanisms of fluoroquinolone resistance. *Drug Resist Update* **2**: 38-55.

Hornsey M, Longshaw C, Phee L & Wareham DW (2012) *In vitro* activity of telavancin in combination with colistin versus Gram-negative bacterial pathogens. *Antimicrob Agents Chemother* **56**: 3080-3085.

Horsburgh MJ, Aish JL, White IJ, Shaw L, Lithgow JK & Foster SJ (2002) sigmaB modulates virulence determinant expression and stress resistance: characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325-4. *J Bacteriol* **184**: 5457-5467.

Hulko M, Berndt F, Gruber M, *et al.* (2006) The HAMP domain structure implies helix rotation in transmembrane signaling. *Cell* **126**: 929-940.

Hummel RP, MacMillan BG & Altemeier WA (1970) Topical and systemic antibacterial agents in the treatment of burns. *Ann Surg* **172**: 370.

Huovinen P, Sundström L, Swedberg G & Sköld O (1995) Trimethoprim and sulfonamide resistance. *Antimicrob Agents Chemother* **39**: 279.

Hurdle JG, O'Neill AJ, Ingham E, Fishwick C & Chopra I (2004) Analysis of mupirocin resistance and fitness in *Staphylococcus aureus* by molecular genetic and structural modeling techniques. *Antimicrob Agents Chemother* **48**: 4366-4376.

Hurdle JG, O'Neill AJ, Mody L, Chopra I & Bradley SF (2005) *In vivo* transfer of high-level mupirocin resistance from *Staphylococcus epidermidis* to methicillin-resistant *Staphylococcus aureus* associated with failure of mupirocin prophylaxis. *J Antimicrob Chemother* **56**: 1166-1168.

Hurdle JG, O'Neill AJ, Chopra I & Lee RE (2011) Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. *Nat Rev Microbiol* **9**: 62-75.

Imlay JA (2013) The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. *Nat Rev Microbiol* **11**: 443-454.

Ibrahim D, Froberg B, Wolf A & Rusyniak DE (2006) Heavy metal poisoning: clinical presentations and pathophysiology. *Clin Lab Med* **26**: 67-97.

Ip M, Lui SL, Chau SS, Lung I & Burd A (2006a) The prevalence of resistance to silver in a burns unit. *J Hosp Infect* **63**: 342-344.

Ip M, Lui SL, Poon VK, Lung I & Burd A (2006b) Antibacterial activities of silver dressings: an *in vitro* comparison. *J Med Microbiol* **55**: 59-63.

Itoh S, Hamada E, Kamoshida G, Takeshita K, Oku T & Tsuji T (2010) Staphylococcal superantigen-like protein 5 inhibits matrix metalloproteinase 9 from human neutrophils. *Infection and Immunity* **78**: 3298-3305.

James GA, Swogger E, Wolcott R, *et al.* (2008) Biofilms in chronic wounds. *Wound Repair Regen* **16**: 37-44.

Jensen PO, Givskov M, Bjarnsholt T & Moser C (2010) The immune system vs. *Pseudomonas aeruginosa* biofilms. *FEMS Immunol Med Microbiol* **59**: 292-305.

Ji G & Silver S (1992) Reduction of arsenate to arsenite by the ArsC protein of the arsenic resistance operon of *Staphylococcus aureus* plasmid pI258. *Proc Natl Acad Sci U S A* **89**: 9474-9478.

Jover J, Bosque R & Sales J (2008) A comparison of the binding affinity of the common amino acids with different metal cations. *Dalton T* 6441-6453.

Jung D, Rozek A, Okon M & Hancock RE (2004) Structural transitions as determinants of the action of the calcium-dependent antibiotic daptomycin. *Chem Biol* **11**: 949-957.

Jung WK, Koo HC, Kim KW, Shin S, Kim SH & Park YH (2008) Antibacterial activity and mechanism of action of the silver ion in *Staphylococcus aureus* and *Escherichia coli*. *Appl Environ Microbiol* **74**: 2171-2178.

Kaatz GW, Seo SM & Ruble CA (1993) Efflux-mediated fluoroquinolone resistance in *Staphylococcus aureus*. *Antimicrob Agents Chemother* **37**: 1086-1094.

Karavolos MH, Horsburgh MJ, Ingham E & Foster SJ (2003) Role and regulation of the superoxide dismutases of *Staphylococcus aureus*. *Microbiology* **149**: 2749-2758.

Kalan L & Wright GD (2011) Antibiotic adjuvants: multicomponent anti-infective strategies. *Expert Rev. Mol. Med* **13**: e5.

Kaur P, Saxena M, Sareen M & Vadehra DV (1987) Effect of silver ion resistance in *Staphylococcus aureus* on production of extracellular enzymes & toxins. *Indian J Med Res* **86**: 716-721.

Keren I, Wu Y, Inocencio J, Mulcahy LR & Lewis K (2013) Killing by bactericidal antibiotics does not depend on reactive oxygen species. *Science* **339**: 1213-1216.

Kint CI, Verstraeten N, Fauvart M & Michiels J (2012) New-found fundamentals of bacterial persistence. *Trends Microbiol* **20**: 577-585.

Klasen HJ (2000a) Historical review of the use of silver in the treatment of burns. I. Early uses. *Burns* **26**: 117-130.

Klasen HJ (2000b) A historical review of the use of silver in the treatment of burns. II. Renewed interest for silver. *Burns* **26**: 131-138.

Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA & Collins JJ (2007) A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* **130**: 797-810.

Kohanski MA, Dwyer DJ, Wierzbowski J, Cottarel G & Collins JJ (2008) Mistranslation of membrane proteins and two-component system activation trigger antibiotic-mediated cell death. *Cell* **135**: 679-690.

Kohler C, von Eiff C, Peters G, Proctor RA, Hecker M & Engelmann S (2003) Physiological characterization of a heme-deficient mutant of *Staphylococcus aureus* by a proteomic approach. *J. Bacteriol* **185**: 6928-6937.

Kolter R, Siegele DA & Tormo A (1993) The stationary phase of the bacterial life cycle. *Annu Rev Microbiol* **47**: 855-874.

Kondo H, Nakagawa A, Nishihira J, Nishimura Y, Mizuno T & Tanaka I (1997) *Escherichia coli* positive regulator OmpR has a large loop structure at the putative RNA polymerase interaction site. *Nat Struct Biol* **4**: 28-31.

Konno K, Oizumi K & Oka S (1973) Mode of action of rifampin on mycobacteria. II. Biosynthetic studies on the inhibition of ribonucleic acid polymerase of *Mycobacterium bovis* BCG by rifampin and uptake of rifampin-^{14}C by *Mycobacterium phlei*. *Am Rev Respir Dis* **107**: 1006-1012.

Kostenko V, Lyczak J, Turner K & Martinuzzi RJ (2010) Impact of silver-containing wound dressings on bacterial biofilm viability and susceptibility to antibiotics during prolonged treatment. *Antimicrob Agents Chemother* **54**: 5120-5131.

Kreiswirth BN, Lofdahl S, Betley MJ, O'Reilly M, Schlievert PM, Bergdoll MS, & Novick RP (1983). The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage. *Nature*, **305:** 709-712.

Kulathila R, Kulathila R, Indic M & van den Berg B (2011) Crystal structure of *Escherichia coli* CusC, the outer membrane component of a heavy metal efflux pump. *PLoS One* **6**: e15610.

Laarman AJ, Ruyken M, Malone CL, van Strijp JA, Horswill AR & Rooijakkers SH (2011) *Staphylococcus aureus* metalloprotease aureolysin cleaves complement C3 to mediate immune evasion. *J Immunol* **186**: 6445-6453.

Lander ES & Linton LM & Birren B, *et al.* (2001) Initial sequencing and analysis of the human genome. *Nature* **409**: 860-921.

Lansdown AB (2006) Silver in health care: antibacterial effects and safety in use. *Curr Probl Dermatol* **33**: 17-34.

Lansdown AB (2007) Critical observations on the neurotoxicity of silver. *Crit Rev Toxicol* **37**: 237-250.

Lansdown A & Williams A (2007) Bacterial resistance to silver-based antibiotics. *Nurs Times* **103**: 48-49.

Larsen AR, Bocher S, Stegger M, Goering R, Pallesen LV & Skov R (2008) Epidemiology of European community-associated methicillin-resistant *Staphylococcus aureus* clonal complex 80 type IV strains isolated in Denmark from 1993 to 2004. *J Clin Microbiol* **46**: 62-68.

Lee SM, Grass G, Rensing C, Barrett SR, Yates CJ, Stoyanov JV & Brown NL (2002) The Pco proteins are involved in periplasmic copper handling in *Escherichia coli*. *Biochem Biophys Res Commun* **295**: 616-620.

Leaper D, Münter C, Meaume S, Scalise A, Mompó NB, Jakobsen BP & Gottrup F (2013) The use of biatain Ag in hard-to-heal venous leg ulcers: meta-analysis of randomised controlled trials. *PLoS One* **8**: e67083.

Li PL, Hwang I, Miyagi H, True H & Farrand SK (1999) Essential components of the Ti plasmid *trb* system, a type IV macromolecular transporter. *J Bacteriol* **181**: 5033-5041.

Li XZ, Nikaido H & Williams KE (1997) Silver-resistant mutants of *Escherichia coli* display active efflux of Ag^{+} and are deficient in porins. *J Bacteriol* **179**: 6127-6132.

Li X, Yuan Q, Wang L, Du L & Deng L (2012) Silver-coated endotracheal tube versus non-coated endotracheal tube for preventing ventilator-associated pneumonia among adults: a systematic review of randomized controlled trials. *J Evid Based Med* **5**: 25-30.

Liau SY, Read DC, Pugh WJ, Furr JR & Russell AD (1997) Interaction of silver nitrate with readily identifiable groups: relationship to the antibacterial action of silver ions. *Lett Appl Microbiol* **25**: 279-283.

Liu GY, Essex A, Buchanan JT, *et al.* (2005) *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *J Exp Med* **202**: 209-215.

Liu Y & Imlay JA (2013) Cell death from antibiotics without the involvement of reactive oxygen species. *Science* **339**: 1210-1213.

Lloyd DR & Phillips DH (1999) Oxidative DNA damage mediated by copper (II), iron (II) and nickel (II) Fenton reactions: evidence for site-specific mechanisms in the formation of double-strand breaks, 8-hydroxydeoxyguanosine and putative intrastrand cross-links. *Mutat Res-Fund Mol M* **424**: 23-36.

Lloyd NC, Morgan HW, Nicholson BK & Ronimus RS (2005) The composition of Ehrlich's salvarsan: resolution of a century-old debate. *Angew Chem Int Edit* **44**: 941-944.

Loh JV, Percival SL, Woods EJ, Williams NJ & Cochrane CA (2009) Silver resistance in MRSA isolated from wound and nasal sources in humans and animals. *Int Wound J* **6**: 32-38.

Lok CN, Ho CM, Chen R, *et al.* (2006) Proteomic analysis of the mode of antibacterial action of silver nanoparticles. *J Proteome Res* **5**: 916-924.

Lok CN, Ho CM, Chen R, Tam PK, Chiu JF & Che CM (2008) Proteomic identification of the Cus system as a major determinant of constitutive *Escherichia coli* silver resistance of chromosomal origin. *J Proteome Res* **7**: 2351-2356.

Long KS, Poehlsgaard J, Kehrenberg C, Schwarz S & Vester B (2006) The Cfr rRNA methyltransferase confers resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A antibiotics. *Antimicrob Agents Chemother* **50**: 2500-2505.

Luo S & Wehr NB (2009) Protein carbonylation: avoiding pitfalls in the 2,4-dinitrophenylhydrazine assay. *Redox Rep* **14**: 159-166.

Luoma SN (2008) Silver nanotechnologies and the environment. *Woodrow Wilson International Center for Scholars. Washington, DC, USA* 72.

Madigan MT, Martinko JM, Stahl D & Clark DP (2010) *Brock Biology of Microorganisms*. Benjamin Cummings.

Maghsoudi H, Monshizadeh S & Mesgari M (2011) A comparative study of the burn wound healing properties of saline-soaked dressing and silver sulfadiazine in rats. *Indian J Surg* **73**: 24-27.

Mandell GL, Bennett JE & Dolin R (2009) *Principles and Practice of Infectious Diseases*. Churchill Livingstone.

Manring M, Hawk A, Calhoun JH & Andersen RC (2009) Treatment of war wounds: a historical review. *Clin Orthop Relat R* **467**: 2168-2191.

Manna AC & Cheung AL (2006) Expression of SarX, a negative regulator of *agr* and exoprotein synthesis, is activated by MgrA in *Staphylococcus aureus*. *J Bacteriol* **188**: 4288-4299.

Manson JM, Keis S, Smith JM & Cook GM (2004) Acquired bacitracin resistance in *Enterococcus faecalis* is mediated by an ABC transporter and a novel regulatory protein, BcrR. *Antimicrob Agents Chemother* **48**: 3743-3748.

Maple PA, Hamilton-Miller JM & Brumfitt W (1992) Comparison of the *in vitro* activities of the topical antibacterials azelaic acid, nitrofurazone, silver sulphadiazine and mupirocin against methicillin-resistant *Staphylococcus aureus*. *J Antimicrob Chemother* **29**: 661-668.

Mariner KR (2011) The activity, mode of action and generation of resistance to novel antibacterial agents. PhD Thesis, University of Leeds.

Mariner KR, Ooi N, Roebuck D, O'Neill AJ & Chopra I (2011) Further characterization of *Bacillus subtilis* antibiotic biosensors and their use for antibacterial mode-of-action studies. *Antimicrob Agents Chemother* **55**: 1784-1786.

Mariner KR, Trowbridge R, Agarwal AK, Miller K, O'Neill AJ, Fishwick CW & Chopra I (2010) Furanyl-rhodanines are unattractive drug candidates for development as inhibitors of bacterial RNA polymerase. *Antimicrob Agents Chemother* **54**: 4506-4509.

Martin RG (1963) The first enzyme in histidine biosynthesis: the nature of feedback inhibition by histidine. *J Biol Chem* **238**: 257-268.

Martinez-Hackert E & Stock AM (1997) The DNA-binding domain of OmpR: crystal structures of a winged helix transcription factor. *Structure* **5**: 109-124.

Martinez-Martinez L (2008) Extended-spectrum β-lactamases and the permeability barrier. *Clin Microbiol Infect* **14 Suppl 1**: 82-89.

Mascio CT, Alder JD & Silverman JA (2007) Bactericidal action of daptomycin against stationary-phase and nondividing *Staphylococcus aureus* cells. *Antimicrob Agents Chemother* **51**: 4255-4260.

Mathema VB, Thakuri BC & Sillanpaa M (2011) Bacterial *mer* operon-mediated detoxification of mercurial compounds: a short review. *Arch Microbiol* **193**: 837-844.

Matsumura Y, Yoshikata K, Kunisaki S & Tsuchido T (2003) Mode of bactericidal action of silver zeolite and its comparison with that of silver nitrate. *Appl Environ Microbiol* **69**: 4278-4281.

Mayers DL, Lerner SA, Ouellette M & Sobel JD (2009) *Antibacterial drug resistance*. Springer.

McHugh GL, Moellering RC, Hopkins CC & Swartz MN (1975) *Salmonella typhimurium* resistant to silver nitrate, chloramphenicol, and ampicillin. *Lancet* **1**: 235-240.

McNaught AD & Wilkinson A (1997) *Compendium of chemical terminology*. Blackwell Science Oxford.

Mealman TD, Bagai I, Singh P, *et al.* (2011) Interactions between CusF and CusB identified by NMR spectroscopy and chemical cross-linking coupled to mass spectrometry. *Biochemistry* **50**: 2559-2566.

Meredith TC, Wang H, Beaulieu P, Grundling A & Roemer T (2012) Harnessing the power of transposon mutagenesis for antibacterial target identification and evaluation. *Mob Genet Elements* **2**: 171-178.

Messner KR & Imlay JA (1999) The identification of primary sites of superoxide and hydrogen peroxide formation in the aerobic respiratory chain and sulfite reductase complex of *Escherichia coli*. *J Biol Chem* **274**: 10119-10128.

Michaels J, Campbell B, King B, Palfreyman S, Shackley P & Stevenson M (2009) Randomized controlled trial and cost-effectiveness analysis of silver-donating antibacterial dressings for venous leg ulcers (VULCAN trial). *Brit J Surg* **96**: 1147-1156.

Miller RA & Britigan BE (1995) The formation and biologic significance of phagocyte-derived oxidants. *J Investig Med* **43**: 39-49.

Miller K, O'Neill AJ, Wilcox MH, Ingham E & Chopra I (2008) Delayed development of linezolid resistance in *Staphylococcus aureus* following exposure to low levels of antibacterial agents. *Antimicrob Agents Chemother* **52**: 1940-1944.

Miller K, Storey C, Stubbings WJ, Hoyle AM, Hobbs JK & Chopra I (2005) Antistaphylococcal activity of the novel cephalosporin CB-181963 (CAB-175). *J Antimicrob Chemother* **55**: 579-582.

Mirsattari S, Hammond R, Sharpe M, Leung F & Young G (2004) Myoclonic status epilepticus following repeated oral ingestion of colloidal silver. *Neurology* **62**: 1408-1410.

Monchy S, Benotmane MA, Wattiez R, *et al.* (2006) Transcriptomic and proteomic analyses of the pMOL30-encoded copper resistance in *Cupriavidus metallidurans* strain CH34. *Microbiology* **152**: 1765-1776.

Mooney EK, Lippitt C & Friedman J (2006) Silver dressings. *Plast Reconstr Surg* **117**: 666-669.

Morones-Ramirez JR, Winkler JA, Spina CS & Collins JJ (2013) Silver enhances antibiotic activity against Gram-negative bacteria. *Sci Transl Med* **5**: 190ra181.

Moyer CA, Brentano L, Gravens DL, Margraf HW & Monafo WW, Jr. (1965) Treatment of large human burns with 0.5 per cent silver nitrate solution. *Arch Surg* **90**: 812-867.

Mueller JH & Hinton J (1941) A protein-free medium for primary isolation of the gonococcus and meningococcus. *Experimental Biology and Medicine* **48**: 330-333.

Müller M, Hollyoak M, Moaveni Z, Brown TLH, Herndon D & Heggers J (2003) Retardation of wound healing by silver sulfadiazine is reversed by *Aloe vera* and nystatin. *Burns* **29**: 834-836.

Mullick S, Watson-Jones D, Beksinska M & Mabey D (2005) Sexually transmitted infections in pregnancy: prevalence, impact on pregnancy outcomes, and approach to treatment in developing countries. *Sex Transm Infect* **81**: 294-302.

Munson GP, Lam DL, Outten FW & O'Halloran TV (2000) Identification of a copper-responsive two-component system on the chromosome of *Escherichia coli* K-12. *J Bacteriol* **182**: 5864-5871.

Muthaiyan A, Silverman JA, Jayaswal RK & Wilkinson BJ (2008) Transcriptional profiling reveals that daptomycin induces the *Staphylococcus aureus* cell wall stress stimulon and genes responsive to membrane depolarization. *Antimicrob Agents Chemother* **52**: 980-990.

Nakata K, Koh MM, Tsuchido T & Matsumura Y (2010) All genomic mutations in the antibacterial surfactant-resistant mutant, *Escherichia coli* OW66, are involved in cell resistance to surfactant. *Appl Microbiol Biotechnol* **87**: 1895-1905.

Nelson KE, Williams CFM & Masters Williams C (2007) Early history of infectious disease: epidemiology and control of infectious diseases. In: *Infectious Disease Epidemiology: Theory and Practice. 2nd ed. Burlington, MA: Jones and Bartlett Publishers.*

Ng EY, Trucksis M & Hooper DC (1994) Quinolone resistance mediated by *norA*: physiologic characterization and relationship to *flqB*, a quinolone resistance locus on the *Staphylococcus aureus* chromosome. *Antimicrob Agents Chemother* **38**: 1345-1355.

NHS National Prescribing Centre (2010) Evidence-based prescribing of advanced wound dressings for chronic wounds in primary care. *MeReC Bulletin* **21**.

Nies DH (1999) Microbial heavy-metal resistance. *Appl Microbiol Biotechnol* **51**: 730-750.

Nikaido H (2009) Multidrug resistance in bacteria. *Annu Rev Biochem* **78**: 119-146.

Nucifora G, Chu L, Misra TK & Silver S (1989) Cadmium resistance from *Staphylococcus aureus* plasmid pI258 cadA gene results from a cadmium-efflux ATPase. *Proc Natl Acad Sci U S A* **86**: 3544-3548.

Ohbo Y, Fukuzako H, Takeuchi K & Takigawa M (1996) Argyria and convulsive seizures caused by ingestion of silver in a patient with schizophrenia. *Psychiat Clin Neuros* **50**: 89-90.

Omran AR (1971) The epidemiologic transition: a theory of the epidemiology of population change. *The Milbank Memorial Fund Quarterly* **49**: 509-538.

O'Neill AJ, Cove JH & Chopra I (2001) Mutation frequencies for resistance to fusidic acid and rifampicin in *Staphylococcus aureus*. *J Antimicrob Chemother* **47**: 647-650.

O'Neill AJ & Chopra I (2004) Preclinical evaluation of novel antibacterial agents by microbiological and molecular techniques. *Expert Opin Investig Drugs* **13**: 1045-1063.

O'Neill AJ, Huovinen T, Fishwick CW & Chopra I (2006) Molecular genetic and structural modeling studies of *Staphylococcus aureus* RNA polymerase and the fitness of rifampin resistance genotypes in relation to clinical prevalence. *Antimicrob Agents Chemother* **50**: 298-309.

O'Neill AJ, Lindsay JA, Gould K, Hinds J & Chopra I (2009) Transcriptional signature following inhibition of early-stage cell wall biosynthesis in *Staphylococcus aureus*. *Antimicrob Agents Chemother* **53**: 1701-1704.

O'Neill AJ, McLaws F, Kahlmeter G, Henriksen AS & Chopra I (2007) Genetic basis of resistance to fusidic acid in staphylococci. *Antimicrob Agents Chemother* **51**: 1737-1740.

O'Neill AJ, Miller K, Oliva B & Chopra I (2004) Comparison of assays for detection of agents causing membrane damage in *Staphylococcus aureus*. *J Antimicrob Chemother* **54**: 1127-1129.

Oku Y, Kurokawa K, Ichihashi N & Sekimizu K (2004) Characterization of the *Staphylococcus aureus mprF* gene, involved in lysinylation of phosphatidylglycerol. *Microbiology* **150**: 45-51.

Oliva B, Miller K, Caggiano N, *et al.* (2003) Biological properties of novel antistaphylococcal quinoline-indole agents. *Antimicrob Agents Chemother* **47**: 458-466.

Oliva B, O'Neill A, Wilson JM, O'Hanlon PJ & Chopra I (2001) Antibacterial properties and mode of action of the pyrrothine holomycin. *Antimicrob Agents Chemother* **45**: 532-539.

Oliva B, O'Neill AJ, Miller K, Stubbings W & Chopra I (2004) Anti-staphylococcal activity and mode of action of clofazimine. *J Antimicrob Chemother* **53**: 435-440.

Ooi N, Miller K, Hobbs J, Rhys-Williams W, Love W & Chopra I (2009) XF-73, a novel antistaphylococcal membrane-active agent with rapid bactericidal activity. *J Antimicrob Chemother* **64**: 735-740.

Ooi N, Miller K, Randall C, Rhys-Williams W, Love W & Chopra I (2010) XF-70 and XF-73, novel antibacterial agents active against slow-growing and non-dividing cultures of *Staphylococcus aureus* including biofilms. *J Antimicrob Chemother* **65**: 72-78.

O'Riordan K & Lee JC (2004) *Staphylococcus aureus* capsular polysaccharides. *Clin Microbiol Rev* **17**: 218-234.

Palmqvist N, Foster T, Tarkowski A & Josefsson E (2002) Protein A is a virulence factor in *Staphylococcus aureus* arthritis and septic death. *Microb Pathog* **33**: 239-249.

Pankey GA & Sabath LD (2004) Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. *Clin Infect Dis* **38**: 864-870.

Parizhskaya M, Youssef NN, Di Lorenzo C & Goyal RK (2001) Clofazimine enteropathy in a pediatric bone marrow transplant recipient. *J Pediatr* **138**: 574-576.

Park HJ, Kim JY, Kim J, Lee JH, Hahn JS, Gu MB & Yoon J (2009) Silver-ion-mediated reactive oxygen species generation affecting bactericidal activity. *Water Res* **43**: 1027-1032.

Payne DJ, Gwynn MN, Holmes DJ & Pompliano DL (2006) Drugs for bad bugs: confronting the challenges of antibacterial discovery. *Nat Rev Drug Disc* **6**: 29-40.

Patton TG, Yang SJ & Bayles KW (2006) The role of proton motive force in expression of the *Staphylococcus aureus cid* and *lrg* operons. *Mol Microbiol* **59**: 1395-1404.

Pegg SP, Ramsay K, Meldrum L & Laundy M (1979) Clinical comparison of maphenide and silver sulphadiazine. *Scand J Plast Recons* **13**: 95-101.

Percival SL, Woods E, Nutekpor M, Bowler P, Radford A & Cochrane C (2008) Prevalence of silver resistance in bacteria isolated from diabetic foot ulcers and efficacy of silver-containing wound dressings. *Ostomy Wound Manage* **54**: 30-40.

Presterl E, Suchomel M, Eder M, Reichmann S, Lassnigg A, Graninger W & Rotter M (2007) Effects of alcohols, povidone-iodine and hydrogen peroxide on biofilms of *Staphylococcus epidermidis*. *J Antimicrob Chemother* **60**: 417-420.

Peters JE & Craig NL (2001) Tn*7*: smarter than we thought. *Nat Rev Mol Cell Biol* **2**: 806-814.

Petersen TN, Brunak S, von Heijne G & Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat Methods* **8**: 785-786.

Peterson L & Shanholtzer C (1992) Tests for bactericidal effects of antibacterial agents: technical performance and clinical relevance. *Clinical Microbiol Rev* **5**: 420-432.

Placer ZA, Cushman LL & Johnson BC (1966) Estimation of product of lipid peroxidation (malonyl dialdehyde) in biochemical systems. *Anal Biochem* **16**: 359-364.

Pirnay J-P, De Vos D, Cochez C *et al.* (2003) Molecular epidemiology of *Pseudomonas aeruginosa* colonization in a burn unit: persistence of a multidrug-resistant clone and a silver sulfadiazine-resistant clone. *J Clin Microbiol* **41**: 1192-1202.

Popowicz GM, Dubin G, Stec-Niemczyk J, Czarny A, Dubin A, Potempa J & Holak TA (2006) Functional and structural characterization of Spl proteases from *Staphylococcus aureus*. *J Mol Biol* **358**: 270-279.

Posluszny JA, Jr., Conrad P, Halerz M, Shankar R & Gamelli RL (2011) Surgical burn wound infections and their clinical implications. *J Burn Care Res* **32**: 324-333.

Presterl E, Suchomel M, Eder M, Reichmann S, Lassnigg A, Graninger W & Rotter M (2007) Effects of alcohols, povidone-iodine and hydrogen peroxide on biofilms of *Staphylococcus epidermidis*. *J Antimicrob Chemother* **60**: 417-420.

Prunier AL & Leclercq R (2005) Role of *mutS* and *mutL* genes in hypermutability and recombination in *Staphylococcus aureus*. *J Bacteriol* **187**: 3455-3464.

Punta M, Coggill PC, Eberhardt RY, *et al.* (2012) The Pfam protein families database. *Nucleic Acids Res* **40**: D290-301.

Rai M, Yadav A & Gade A (2009) Silver nanoparticles as a new generation of antibacterials. *Biotechnol Adv* **27**: 76-83.

Randall CP, Mariner KR, Chopra I & O'Neill AJ (2013) The target of daptomycin is absent from *Escherichia coli* and other Gram-negative pathogens. *Antimicrob Agents Chemother* **57**: 637-639.

Rasko DA & Sperandio V (2010) Anti-virulence strategies to combat bacteria-mediated disease. *Nat Rev Drug Disc* **9**: 117-128.

Ravenscroft JC, Layton A & Barnham M (2000) Observations on high levels of fusidic acid resistant *Staphylococcus aureus* in Harrogate, North Yorkshire, UK. *Clin Exp Dermatol* **25**: 327-330

Renau TE, Leger R, Flamme EM, *et al.* (1999) Inhibitors of efflux pumps in *Pseudomonas aeruginosa* potentiate the activity of the fluoroquinolone antibacterial levofloxacin. *J Med Chem* **42**: 4928-4931.

Rensing C, Sun Y, Mitra B & Rosen BP (1998) Pb(II)-translocating P-type ATPases. *J Biol Chem* **273**: 32614-32617.

Reverchon S, Rouanet C, Expert D & Nasser W (2002) Characterization of indigoidine biosynthetic genes in *Erwinia chrysanthemi* and role of this blue pigment in pathogenicity. *J Bacteriol* **184**: 654-665.

Reynolds E, Ross JI & Cove JH (2003) Msr(A) and related macrolide/streptogramin resistance determinants: incomplete transporters? *Int J Antimicrob Ag* **22**: 228-236.

Rezaei E, Safari H, Naderinasab M & Aliakbarian H (2011) Common pathogens in burn wound and changes in their drug sensitivity. *Burns* **37**: 804-806.

Rhee JE, Sheng W, Morgan LK, Nolet R, Liao X & Kenney LJ (2008) Amino acids important for DNA recognition by the response regulator OmpR. *J Biol Chem* **283**: 8664-8677.

Ricci V, Loman N, Pallen M, *et al.* (2012) The TCA cycle is not required for selection or survival of multidrug-resistant *Salmonella*. *J Antimicrob Chemother* **67**: 589-599.

Rigsby RE, Fillgrove KL, Beihoffer LA & Armstrong RN (2005) Fosfomycin resistance proteins: a nexus of glutathione transferases and epoxide hydrolases in a metalloenzyme superfamily. *Method Enzymol* **401**: 367-379.

Roe AL (1915) Collosol argentum and its ophthalmic uses. *Brit Med J* **1**: 104.

Rosen G (1993) *A history of public health*. JHU Press.

Rosenkranz HS & Carr HS (1972a) Silver sulfadiazine: effect on the growth and metabolism of bacteria. *Antimicrob Agents Chemother* **2**: 367-372.

Rosenkranz HS & Rosenkranz S (1972b) Silver sulfadiazine: interaction with isolated deoxyribonucleic acid. *Antimicrob Agents Chemother* **2**: 373-383.

Rosenkranz HS, Coward JE, Wlodkowski TJ & Carr HS (1974) Properties of silver sulfadiazine-resistant *Enterobacter cloacae* . *Antimicrob Agents Chemother* **5**: 199-201.

Roy A, Kucukural A & Zhang Y (2010) I-TASSER: a unified platform for automated protein structure and function prediction. *Nat Protoc* **5**: 725-738.

Ruhr E & Sahl HG (1985) Mode of action of the peptide antibiotic nisin and influence on the membrane potential of whole cells and on cytoplasmic and artificial membrane vesicles. *Antimicrob Agents Chemother* **27**: 841-845.

Russell AB, Hood RD, Bui NK, LeRoux M, Vollmer W & Mougous JD (2011) Type VI secretion delivers bacteriolytic effectors to target cells. *Nature* **475**: 343-U392.

Saha B, Singh AK, Ghosh A & Bal M (2008) Identification and characterization of a vancomycin-resistant *Staphylococcus aureus* isolated from Kolkata (South Asia). *J Med Microbiol* **57**: 72-79.

Saint S, Elmore JG, Sullivan SD, Emerson SS & Koepsell TD (1998) The efficacy of silver alloy-coated urinary catheters in preventing urinary tract infection: a meta-analysis. *Am J Med* **105**: 236-241.

Sambrook J, Fritsch, E. F., Maniatis, T. (2001) *Molecular Cloning: A laboratory manual*. Cold Spring Harbor Laboratory Press.

Sandegren L, Lindqvist A, Kahlmeter G & Andersson DI (2008) Nitrofurantoin resistance mechanism and fitness cost in *Escherichia coli*. *J Antimicrob Chemother* **62**: 495-503.

Sanderson-Wells T (1918) A case of puerperal septicæmia successfully treated with intravenous injections of collosol argentum. *Lancet* **191**: 258-259.

Santos CL, Tavares F, Thioulouse J & Normand P (2009) A phylogenomic analysis of bacterial helix-turn-helix transcription factors. *FEMS Microbiol Rev* **33**: 411-429.

Sawitzke JA, Thomason LC, Costantino N, Bubunenko M, Datta S & Court DL (2007) Recombineering: in vivo genetic engineering in *E. coli*, *S. enterica*, and beyond. *Methods Enzymol* **421**: 171-199.

Schenk S & Laddaga RA (1992) Improved method for electroporation of *Staphylococcus aureus*. *FEMS Microbiol Lett* **73**: 133-138.

Schneider G (1984) Silver nitrate prophylaxis. *Can Med Assoc J* **131**: 193.

Schroeder K, Jularic M, Horsburgh SM, *et al.* (2009) Molecular characterization of a novel *Staphylococcus aureus* surface protein (SasC) involved in cell aggregation and biofilm accumulation. *PLoS One* **4**: e7567.

Schwartz RS (2004) Paul Ehrlich's magic bullets. *New Engl J Med* **350**: 1079.

Seaver LC & Imlay JA (2001) Hydrogen peroxide fluxes and compartmentalization inside growing *Escherichia coli*. *J Bacteriol* **183**: 7182-7189.

Seggewiss J, Becker K, Kotte O, *et al.* (2006) Reporter metabolite analysis of transcriptional profiles of a *Staphylococcus aureus* strain with normal phenotype and its isogenic *hemB* mutant displaying the small-colony-variant phenotype. *J Bacteriol* **188**: 7765-7777.

Seidler J, McGovern SL, Doman TN & Shoichet BK (2003) Identification and prediction of promiscuous aggregating inhibitors among known drugs. *J Med Chem* **46**: 4477-4486.

Semeykina AL & Skulachev VP (1990) Submicromolar Ag^+ increases passive Na^+ permeability and inhibits the respiration-supported formation of Na^+ gradient in *Bacillus* FTU vesicles. *FEBS Lett* **269**: 69-72.

Shendure J & Ji H (2008) Next-generation DNA sequencing. *Nat Biotechnol* **26**: 1135-1145.

Shigeta M, Tanaka G, Komatsuzawa H, Sugai M, Suginaka H & Usui T (1997) Permeation of antibacterial agents through *Pseudomonas aeruginosa* biofilms: a simple method. *Chemotherapy* **43**: 340-345.

Sia EA, Roberts RC, Easter C, Helinski DR & Figurski DH (1995) Different relative importances of the *par* operons and the effect of conjugal transfer on the maintenance of intact promiscuous plasmid RK2. *J Bacteriol* **177**: 2789-2797.

Silver LL (2011) Challenges of antibacterial discovery. *Clin Microbiol Rev* **24**: 71-109.

Silver S (2003) Bacterial silver resistance: molecular biology and uses and misuses of silver compounds. *FEMS Microbiol Rev* **27**: 341-353.

Silver S & Phung LT (1996) Bacterial heavy metal resistance: new surprises. *Annu Rev Microbiol* **50**: 753-789.

Silver S, Phung LT & Silver G (2006) Silver as biocides in burn and wound dressings and bacterial resistance to silver compounds. *J Ind Microbiol Biotechnol* **33**: 627-634.

Simmons KJ, Chopra I & Fishwick CW (2010) Structure-based discovery of antibacterial drugs. *Nat Rev Microbiol* **8**: 501-510.

Sköld O (2000) Sulfonamide resistance: mechanisms and trends. *Drug Resist Update* **3**: 155-160.

Solioz M & Odermatt A (1995) Copper and silver transport by CopB-ATPase in membrane vesicles of *Enterococcus hirae*. *J Biol Chem* **270**: 9217-9221.

Solioz M & Vulpe C (1996) CPx-type ATPases: a class of P-type ATPases that pump heavy metals. *Trends Biochem Sci* **21**: 237-241.

Solioz M & Stoyanov JV (2003) Copper homeostasis in *Enterococcus hirae*. *FEMS Microbiol Rev* **27**: 183-195.

Spellberg B (2008) Dr William H. Stewart: mistaken or maligned? *Clin Infect Dis* **47**: 294-294.

Stapleton PD, Shannon KP & French GL (1999) Carbapenem resistance in *Escherichia coli* associated with plasmid-determined CMY-4 β-lactamase production and loss of an outer membrane protein. *Antimicrob Agents Chemother* **43**: 1206-1210.

Stanford W, Bert WR & Charles LF (1969) Clinical experience with silver sulfadiazine, a new topical agent for control of *Pseudomonas* infections in burns. *J Trauma Acute Care Surg* **9**: 377-388.

Starodub ME & Trevors JT (1989) Silver resistance in *Escherichia coli* R1. *J Med Microbiol* **29**: 101-110.

Stewart RC (2010) Protein histidine kinases: assembly of active sites and their regulation in signaling pathways. *Curr Opin Microbiol* **13**: 133-141.

StGelais C, Tuthill TJ, Clarke DS, Rowlands DJ, Harris M & Griffin S (2007) Inhibition of hepatitis C virus p7 membrane channels in a liposome-based assay system. *Antiviral Res* **76**: 48-58.

Stobie N, Duffy B, McCormack DE, Colreavy J, Hidalgo M, McHale P & Hinder SJ (2008) Prevention of *Staphylococcus epidermidis* biofilm formation using a low-temperature processed silver-doped phenyltriethoxysilane sol-gel coating. *Biomaterials* **29**: 963-969.

Stryjewski ME, Szczech LA, Benjamin DK *et al.* (2007) Use of vancomycin or first-generation cephalosporins for the treatment of hemodialysis-dependent patients with methicillin-susceptible *Staphylococcus aureus* bacteremia. *Clin Infect Dis* **44**: 190-196.

Su CC, Long F & Yu EW (2011) The Cus efflux system removes toxic ions via a methionine shuttle. *Protein Sci* **20**: 6-18.

Tanenbaum DM, Goll J, Murphy S, *et al.* (2010) The JCVI standard operating procedure for annotating prokaryotic metagenomic shotgun sequencing data. *Stand Genomic Sci* **2**: 229-237.

Takahata S, Ida T, Hiraishi T *et al.* (2010) Molecular mechanisms of fosfomycin resistance in clinical isolates of *Escherichia coli*. *Int J Antimicrob Ag* **35**: 333-337.

Thomason L, Court DL, Bubunenko M, Costantino N, Wilson H, Datta S & Oppenheim A (2007) Recombineering: genetic engineering in bacteria using homologous recombination. *Curr Protoc Mol Biol* **Chapter 1**: Unit 1 16.

Thomson JM & Bonomo RA (2005) The threat of antibiotic resistance in Gram-negative pathogenic bacteria: β-lactams in peril! *Curr Opin Microbiol* **8**: 518-524.

Tyndall J (1876) The optical deportment of the atmosphere in relation to the phenomena of putrefaction and infection. *Philos Tr R Soc* **166**: 27-74.

Ubukata K, Nonoguchi R, Matsuhashi M & Konno M (1989) Expression and inducibility in *Staphylococcus aureus* of the *mecA* gene, which encodes a methicillin-resistant *S. aureus*-specific penicillin-binding protein. *J Bacteriol* **171**: 2882-2885.

Ug A & Ceylan O (2003) Occurrence of resistance to antibiotics, metals, and plasmids in clinical strains of *Staphylococcus* spp. *Arch Med Res* **34**: 130-136.

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M & Rozen SG (2012) Primer3-new capabilities and interfaces. *Nucleic Acids Res* **40**.

Urban A, Eckermann S, Fast B, *et al.* (2007) Novel whole-cell antibiotic biosensors for compound discovery. *Appl Environ Microbiol* **73**: 6436-6443.

Vakulenko SB & Mobashery S (2003) Versatility of aminoglycosides and prospects for their future. *Clin Microbiol Rev* **16**: 430-450.

van Hal SJ, Jensen SO, Vaska VL, Espedido BA, Paterson DL & Gosbell IB (2012) Predictors of mortality in *Staphylococcus aureus* bacteremia. *Clin Microbiol Rev* **25**: 362-386.

Vasishta R, Chhibber S & Saxena M (1989) Heavy metal resistance in clinical isolates of *Pseudomonas aeruginosa*. *Folia Microbiol (Praha)* **34**: 448-452.

Vecki VG & Ottinger MR (1921) Treatment of syphilis with silver-Salvarsan. *Cal State J Med* **19**: 438.

Veenstra DL, Saint S, Saha S, Lumley T & Sullivan SD (1999) Efficacy of antiseptic-impregnated central venous catheters in preventing catheter-related bloodstream infection. *JAMA-J Am Med Assoc* **281**: 261-267.

Venkova-Canova T, Patek M & Nesvera J (2003) Control of *rep* gene expression in plasmid pGA1 from *Corynebacterium glutamicum*. *J Bacteriol* **185**: 2402-2409.

Venter JC & Adams MD & Myers EW, *et al.* (2001) The sequence of the human genome. *Science* **291**: 1304-1351.

Vermeulen H, van Hattem JM, Storm-Versloot MN & Ubbink DT (2007) Topical silver for treating infected wounds. *Cochrane Database Syst Rev* CD005486.

Vickers AA, Chopra I & O'Neill AJ (2007) Intrinsic novobiocin resistance in *Staphylococcus saprophyticus*. *Antimicrob Agents Chemother* **51**: 4484-4485.

Vik H, Andersen K, Julshamn K & Todnem K (1985) Neuropathy caused by silver absorption from arthroplasty cement. *Lancet* **325**: 872.

Viveiros M, Martins A, Paixao L, *et al.* (2008) Demonstration of intrinsic efflux activity of *Escherichia coli* K-12 AG100 by an automated ethidium bromide method. *Int J Antimicrob Agents* **31**: 458-462.

Walsh C (2003) *Antibiotics: actions, origins, resistance*. American Society for Microbiology (ASM).

Wang H, Claveau D, Vaillancourt JP, Roemer T & Meredith TC (2011) High-frequency transposition for determining antibacterial mode of action. *Nat Chem Biol* **7**: 720-729.

Warriner R & Burrell R (2005) Infection and the chronic wound: a focus on silver. *Adv Skin Wound Care* **18 Suppl 1**: 2-12.

Watts SJ (1999) *Epidemics and history: disease, power, and imperialism*. Yale University Press.

Wayne PA (2012) Methods for dilution antibacterial susceptibility tests for bacteria that grow aerobically; approved standard-Ninth Edition. *Clinical and Laboratory Standards Institute* **CLSI Document M07-A9**.

Weber DJ & Rutala WA (2001) Use of metals as microbicides in preventing infections in healthcare. *Disinfection, sterilization, and preservation. Philadelphia: Lippincott Williams & Wilkins* 415-430.

Weinstein RA, Gaynes R & Edwards JR (2005) Overview of nosocomial infections caused by Gram-negative bacilli. *Clin Infect Dis* **41**: 848-854.

Wertheim HF, Melles DC, Vos MC, van Leeuwen W, van Belkum A, Verbrugh HA & Nouwen JL (2005) The role of nasal carriage in *Staphylococcus aureus* infections. *Lancet Infect Dis* **5**: 751-762.

Whitby M, McLaws M-L & Berry G (2001) Risk of death from methicillin-resistant *Staphylococcus aureus* bacteraemia: a meta-analysis. *Med J Australia* **175**: 264-267.

Wilson JM, Oliva B, Cassels R, O'Hanlon PJ & Chopra I (1995) SB 205952, a novel semisynthetic monic acid analog with at least two modes of action. *Antimicrob Agents Chemother* **39**: 1925-1933.

Woods EJ, Cochrane CA & Percival SL (2009) Prevalence of silver resistance genes in bacteria isolated from human and horse wounds. *Vet Microbiol* **138**: 325-329.

Wright GD (2011) Molecular mechanisms of antibiotic resistance. *Chem Commun* **47**: 4055-4061.

Xiong A & Jayaswal RK (1998) Molecular characterization of a chromosomal determinant conferring resistance to zinc and cobalt ions in *Staphylococcus aureus*. *J Bacteriol* **180**: 4024-4029.

Xu FF & Imlay JA (2012) Silver (I), mercury (II), cadmium (II), and zinc (II) target exposed enzymic iron-sulfur clusters when they toxify *Escherichia coli*. *Appl Environ Microb* **78**: 3614-3621.

Xue Y, Davis AV, Balakrishnan G, *et al.* (2008) Cu(I) recognition via cation-π and methionine interactions in CusF. *Nat Chem Biol* **4**: 107-109.

Yamamoto K & Ishihama A (2005) Transcriptional response of *Escherichia coli* to external copper. *Mol Microbiol* **56**: 215-227.

Yamashita H, Taoka A, Uchihashi T, Asano T, Ando T & Fukumori Y (2012) Single-molecule imaging on living bacterial cell surface by high-speed AFM. *J Mol Biol* **422**: 300-309.

Yazdankhah SP, Scheie AA, Hoiby EA, *et al.* (2006) Triclosan and antibacterial resistance in bacteria: an overview. *Microb Drug Resist* **12**: 83-90.

Yin HQ, Langford R & Burrell RE (1999) Comparative evaluation of the antibacterial activity of Acticoat antibacterial barrier dressing. *J Burn Care Rehabil* **20**: 195-200.

Ziffren SE (1968) Results of the treatment of burns with silver nitrate. *Ann NY Acad Sci* **150**: 946-949.

Zimmermann M, Udagedara SR, Sze CM, Ryan TM, Howlett GJ, Xiao Z & Wedd AG (2012) PcoE-a metal sponge expressed to the periplasm of copper resistance *Escherichia coli*. Implication of its function role in copper resistance. *J Inorg Biochem* **115**: 186-197.

Appendix 1. Transcriptional profile of *S. aureus* SH1000 exposed to Ag^+

Table A1.1. Up-regulated genes in *S. aureus* SH1000 in response to Ag^+ exposure (sorted according to JCVI cellular role categories)

Gene	Fold change in expression	Protein/Function	JCVI Cellular Role SubCategory
Amino acid biosynthesis			
SAOUHSC_01367	2.0 up	Anthranilate synthase component II	Aromatic amino acid family
SAOUHSC_01371	2.7 up	Tryptophan synthase subunit beta	Aromatic amino acid family
SAOUHSC_01372	2.2 up	Tryptophan synthase subunit alpha, TrpA	Aromatic amino acid family
SAOUHSC_00340	2.8 up	Cystathionine beta-lyase	Aspartate family
SAOUHSC_00341	3.0 up	Cystathionine gamma-synthase	Aspartate family
SAOUHSC_01319	2.1 up	Aspartate kinase	Aspartate family
SAOUHSC_01394	2.2 up	Aspartate kinase	Aspartate family
SAOUHSC_02770	2.0 up	Diaminopimelate epimerase family protein	Aspartate family
SAOUHSC_00150	2.9 up	Ornithine aminotransferase	Glutamate family
SAOUHSC_00435	2.0 up	Glutamate synthase, large subunit, putative	Glutamate family
SAOUHSC_02968	3.5 up	Ornithine carbamoyltransferase	Glutamate family
SAOUHSC_02970	3.0 up	Transcriptional regulator of arginine synthesis	Glutamate family
SAOUHSC_03008	3.7 up	Imidazole glycerol phosphate synthase subunit H*isF*, putative	Histidine family
SAOUHSC_03009	4.3 up	1-(5-phosphoribosyl)-5-[(5- phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase	Histidine family

Table A1.1 (continued)

SAOUHSC_03010	4.0 up	Imidazole glycerol phosphate synthase subunit HisH	Histidine family
SAOUHSC_03011	3.4 up	Imidazoleglycerol-phosphate dehydratase. HisB	Histidine family
SAOUHSC_03013	3.3 up	Histidinol dehydrogenase	Histidine family
SAOUHSC_03014	3.8 up	ATP phosphoribosyltransferase catalytic subunit HisG	Histidine family
SAOUHSC_00264	2.2 up	Virulence protein EsaC (component of the ESAT-6 secretion system)	Pyruvate family
SAOUHSC_01451	3.2 up	Threonine dehydratase	Pyruvate family
SAOUHSC_02281	3.5 up	Dihydroxy-acid dehydratase	Pyruvate family
SAOUHSC_02282	2.5 up	Acetolactate synthase large subunit	Pyruvate family
SAOUHSC_02284	2.4 up	Ketol-acid reductoisomerase	Pyruvate family
SAOUHSC_02285	2.3 up	2-isopropylmalate synthase	Pyruvate family
SAOUHSC_02286	2.5 up	3-isopropylmalate dehydrogenase	Pyruvate family
SAOUHSC_02287	2.5 up	Isopropylmalate isomerase large subunit	Pyruvate family
SAOUHSC_02288	2.4 up	Isopropylmalate isomerase small subunit, LeuD	Pyruvate family
SAOUHSC_00075	3.7 up	Cysteine synthase/cystathionine beta-synthase family protein	Serine family
Biosynthesis of cofactors, prosthetic groups, and carriers			
SAOUHSC_02714	2.2 up	Biotin synthase	Biotin
SAOUHSC_02715	2.2 up	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase	Biotin
SAOUHSC_02716	2.3 up	Dethiobiotin synthase, putative	Biotin
SAOUHSC_00171	2.6 up	Gamma-glutamyltranspeptidase, putative	Glutathione and analogs
SAOUHSC_02682	2.1 up	Uroporphyrin-III C-methyltransferase, SirB, putative	Heme, porphyrin, and cobalamin
SAOUHSC_02901	2.5 up	Cobalamin (vitamin B12) biosynthesis protein	Heme, porphyrin, and cobalamin
Cell envelope			
SAOUHSC_00052	2.5 up	Tandem lipoprotein	Other

Table A1.1 (continued)

SAOUHSC_00137	2.1 up	Sulfonate/nitrate/taurine transport system substrate-binding protein	Other
SAOUHSC_01843	2.4 up	Haptoglobin-binding heme uptake protein, HarA	Other
SAOUHSC_01873	2.4 up	Cell-wall-anchored protein, SasC	Other
SAOUHSC_01917	2.6 up	Putative lipoprotein	Other
SAOUHSC_00181	2.2 up	Sugar phosphate isomerase/epimerase	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
SAOUHSC_00089	4.3 up	Capsular polysaccharide biosynthesis glycosyltransferase (sugar transferase)	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
SAOUHSC_00091	2.4 up	Hypothetical capsular biosynthesis protein/O-antigen polymerase	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
SAOUHSC_03000	2.6 up	Capsular polysaccharide biosynthesis, capA, putative	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
SAOUHSC_03002	3.3 up	N-glycosyltransferase PgaC	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
SAOUHSC_00053	2.0 up	Tandem lipoprotein	Other
SAOUHSC_00146	2.1 up	Integral membrane protein	Other
SAOUHSC_00182	2.1 up	Putative isoprenylcysteine carboxyl methyltransferase	Other
SAOUHSC_00200	2.3 up		Other
SAOUHSC_00255	2.4 up	Membrane protein, putative	Other
SAOUHSC_00327	2.3 up	Ferrous iron transport permease EfeU	Other
SAOUHSC_02888	4.5 up	Hypothetical phosphotransferase system IIC component	Other
SAOUHSC_03020	2.5 up	Membrane protein of unknown function	Other
SAOUHSC_03035	2.1 up	Conserved hypothetical protein of unknown function	Other
SAOUHSC_01764	2.0 up	A24 type IV prepilin peptidase family protein	Surface structures

Table A1.1 (continued)

Cellular processes			
SAOUHSC_02932	2.5 up	Choline dehydrogenase	Adaptations to atypical conditions
SAOUHSC_02933	2.1 up	Betaine aldehyde dehydrogenase	Adaptations to atypical conditions
SAOUHSC_00229	2.3 up	Cell wall biosynthesis protein ScdA	Cell division
SAOUHSC_00766	2.0 up	Hypothetical protein	DNA transformation
SAOUHSC_01939	2.4 up	Serine protease SplC	Pathogenesis
SAOUHSC_01942	4.1 up	Serine protease SplA	Pathogenesis
SAOUHSC_01954	2.0 up	Leukotoxin, LukD	Pathogenesis
SAOUHSC_01955	2.3 up	Leukotoxin, LukE, putative	Pathogenesis
SAOUHSC_00130	4.4 up	Heme-degrading monooxygenase IsdI	Toxin production and resistance
SAOUHSC_00383	3.3 up	Superantigen-like protein	Toxin production and resistance
SAOUHSC_00384	2.4 up	Superantigen-like protein	Toxin production and resistance
SAOUHSC_00386	5.0 up	Superantigen-like protein	Toxin production and resistance
SAOUHSC_00389	5.6 up	Superantigen-like protein	Toxin production and resistance
SAOUHSC_00390	3.6 up	Superantigen-like protein 5	Toxin production and resistance
SAOUHSC_00391	3.6 up	Superantigen-like protein	Toxin production and resistance
SAOUHSC_00393	2.4 up	Superantigen-like protein	Toxin production and resistance
SAOUHSC_00399	3.5 up	Superantigen-like protein	Toxin production and resistance
SAOUHSC_03023	3.4 up	Calcium-dependent lactonase Drp35	Toxin production and resistance
Central intermediary metabolism			
SAOUHSC_00081	2.8 up	Siderophore staphylobactin biosynthesis protein SbnG	Other
SAOUHSC_02555	2.1 up	Acyl-CoA dehydrogenase, C-terminal domain protein	Other
SAOUHSC_02924	5.5 up	4-aminobutyrate aminotransferase	Other

Table A1.1 (continued)

SAOUHSC_02958	3.1 up	Alkaline phosphatase III precursor, putative	Other
SAOUHSC_02997	2.2 up	Acetyltransferase, GNAT family	Other
SAOUHSC_02998	3.4 up	Capsular polysaccharide biosynthesis protein, Cap5C	Other
SAOUHSC_03012	3.1 up	Histidinol-phosphate aminotransferase, putative	Other
DNA metabolism			
SAOUHSC_00765	2.3 up	Competence protein ComFA	DNA replication, recombination, and repair
SAOUHSC_01763	3.1 up	DNA repair protein RadC	DNA replication, recombination, and repair
SAOUHSC_01933	2.2 up	Type I restriction-modification system, M subunit	Restriction/modification
Energy metabolism			
SAOUHSC_00008	2.0 up	Histidine ammonia-lyase, HutH	Amino acids and amines
SAOUHSC_00076	4.5 up	Ornithine cyclodeaminase, putative	Amino acids and amines
SAOUHSC_01452	3.1 up	Alanine dehydrogenase	Amino acids and amines
SAOUHSC_02606	2.8 up	Imidazolonepropionase	Amino acids and amines
SAOUHSC_02607	3.2 up	Urocanate hydratase	Amino acids and amines
SAOUHSC_02965	2.4 up	Carbamate kinase	Amino acids and amines
SAOUHSC_02969	2.9 up	Arginine deiminase	Amino acids and amines
SAOUHSC_00142	3.9 up	Formate dehydrogenase	Anaerobic
SAOUHSC_00188	3.2 up	Pyruvate formate-lyase 1 activating enzyme, putative	Anaerobic
SAOUHSC_02680	2.3 up	Nitrate reductase, beta subunit	Anaerobic
SAOUHSC_02681	3.5 up	Nitrate reductase, alpha subunit	Anaerobic
SAOUHSC_02685	4.0 up	Transcriptional regulator, NirR	Anaerobic

Table A1.1 (continued)

SAOUHSC_02452	2.2 up	Tagatose 1,6-diphosphate aldolase, LacD	Biosynthesis and degradation of polysaccharides
SAOUHSC_02453	2.2 up	Tagatose-6-phosphate kinase, LacC	Biosynthesis and degradation of polysaccharides
SAOUHSC_02454	2.0 up	Galactose-6-phosphate isomerase subunit LacB	Biosynthesis and degradation of polysaccharides
SAOUHSC_02455	2.5 up	Galactose-6-phosphate isomerase subunit, LacA	Biosynthesis and degradation of polysaccharides
SAOUHSC_02904	2.6 up	Hypothetical thioredoxin reductase	Electron transport
SAOUHSC_02911	2.7 up	Conserved hypothetical protein	Electron transport
SAOUHSC_00113	3.1 up	Bifunctional acetaldehyde-CoA/alcohol dehydrogenase	Fermentation
SAOUHSC_00170	2.8 up	RGD-containing lipoprotein	Fermentation
SAOUHSC_00187	2.3 up	Formate acetyltransferase	Fermentation
SAOUHSC_00217	3.2 up	Sorbitol dehydrogenase, putative	Fermentation
SAOUHSC_00608	2.2 up	Alcohol dehydrogenase	Fermentation
SAOUHSC_02921	3.2 up	Alpha-acetolactate decarboxylase	Fermentation
SAOUHSC_01846	2.4 up	Acetyl-CoA synthetase	Other
SAOUHSC_01849	2.6 up	Acetoin utilization protein, AcuC	Other
SAOUHSC_02825	3.0 up	Putative glyoxylase-family protein	Other
SAOUHSC_02894	3.2 up	Putative glyoxylase-family protein	Other
SAOUHSC_00088	3.7 up	UDP-glucose 4-epimerase	Sugars
SAOUHSC_00219	2.7 up	Sorbitol dehydrogenase, putative	Sugars
SAOUHSC_00291	4.8 up	PfkB-family carbohydrate kinase	Sugars
SAOUHSC_00298	3.4 up	N-acetylmannosamine-6-phosphate 2-epimerase	Sugars
SAOUHSC_02821	2.0 up	Transmembrane protein SmpB	Electron transport
Fatty acid and phospholipid metabolism			
SAOUHSC_00196	2.4 up	3-hydroxyacyl-CoA dehydrogenase, FadB	Degradation
SAOUHSC_00197	4.1 up	Acyl-CoA dehydrogenase, FadD	Degradation
SAOUHSC_00195	3.8 up	Acetyl-CoA acetyltransferase, FadA	Other

Table A1.1 (continued)

SAOUHSC_00051	3.2 up	1-phosphatidylinositol phosphodiesterase precursor, putative	Degradation
Mobile and extrachromosomal element functions			
SAOUHSC_00112	2.0 up	IS200 family transposase	Transposon functions
SAOUHSC_00352	2.2 up	Integrase-like protein	Transposon functions
SAOUHSC_00353	2.1 up	Putative integrase	Transposon functions
SAOUHSC_01390	2.8 up	Truncated IS1272 transposase	Transposon functions
SAOUHSC_01410	2.4 up	Putative transposase	Transposon functions
SAOUHSC_01804	2.6 up	Transposase, putative	Transposon functions
SAOUHSC_01805	2.2 up	Putative transposase	Transposon functions
SAOUHSC_01905	2.6 up	Truncated transposase, putative	Transposon functions
SAOUHSC_01906	2.9 up	Truncated transposase, putative	Transposon functions
SAOUHSC_01911	2.2 up	IS200 family transposase	Transposon functions
SAOUHSC_02437	2.4 up	ISSau3 transposase	Transposon functions
SAOUHSC_02438	2.4 up	ISSau3 transposase	Transposon functions
SAOUHSC_02440	2.9 up	ISSau3 transposase	Transposon functions
SAOUHSC_02639	2.2 up	ISSau3 transposase	Transposon functions
Protein fate			
SAOUHSC_00326	3.1 up	Dyp-type peroxidase family protein	Degradation of proteins, peptides, and glycopeptides
SAOUHSC_02605	2.9 up	Putative amino acid amidohydrolase/peptidase	Degradation of proteins, peptides, and glycopeptides
SAOUHSC_02755	2.1 up	Putative cellulase/endo-1,4-beta-glucanase	Degradation of proteins, peptides, and glycopeptides
SAOUHSC_02915	2.3 up	CocE/NonD family hydrolase/peptidase	Degradation of proteins, peptides, and glycopeptides
SAOUHSC_02971	4.2 up	Zinc metalloproteinase aureolysin	Degradation of proteins, peptides, and glycopeptides
SAOUHSC_03025	3.3 up	Pyrrolidone-carboxylate peptidase	Degradation of proteins, peptides, and glycopeptides

Table A1.1 (continued)

SAOUHSC_01935	2.1 up	Serine protease SplF, putative	Degradation of proteins, peptides, and glycopeptides
SAOUHSC_01941	3.6 up	Serine protease SplB	Degradation of proteins, peptides, and glycopeptides
SAOUHSC_00299	3.0 up	Putative membrane protein	Protein and peptide secretion and trafficking
SAOUHSC_01641	2.1 up	Competence protein, ComGB	Protein and peptide secretion and trafficking
SAOUHSC_02902	2.7 up	FeoB family ferrous iron (Fe^{2+}) uptake protein	Protein and peptide secretion and trafficking
Protein synthesis			
SAOUHSC_00198	3.9 up	Conserved hypothetical protein	tRNA aminoacylation
SAOUHSC_00237	3.1 up	SAM dependent methyltransferase	tRNA and rRNA base modification
Purines, pyrimidines, nucleosides and nucleotides			
SAOUHSC_02941	2.8 up	Anaerobic ribonucleotide reductase activating protein	2'-Deoxyribonucleotide metabolism
SAOUHSC_00107	2.0 up	5' nucleotidase family protein	Nucleotide and nucleoside interconversions
SAOUHSC_00097	2.0 up	Purine nucleoside phosphorylase	Salvage of nucleosides and nucleotides
SAOUHSC_03015	4.3 up	ATP phosphoribosyltransferase regulatory subunit	Salvage of nucleosides and nucleotides
Regulatory functions			
SAOUHSC_02726	2.2 up	Positive transcriptional activator, putative	DNA interactions
SAOUHSC_02809	2.5 up	Gluconate operon transcriptional repressor	DNA interactions
SAOUHSC_00157	2.4 up	N-acetylmuramic acid-6-phosphate etherase	Other
SAOUHSC_02826	2.6 up	MarR-family transcription regulator	Other
SAOUHSC_02451	2.8 up	PTS system, lactose-specific IIA component, LacE	Protein interactions
SAOUHSC_00233	5.2 up	Antiholin-like protein LrgB	Other

Table A1.1 (continued)

Transport and binding proteins

SAOUHSC_00136	4.1 up	Sulfonate/nitrate/taurine transport system ATP-binding protein	Amino acids, peptides and amines
SAOUHSC_00167	2.2 up	Glutathione import ATP-binding protein GsiA	Amino acids, peptides and amines
SAOUHSC_00176	3.4 up	Maltose/maltodextrin transporter	Amino acids, peptides and amines
SAOUHSC_00928	2.9 up	Oligopeptide ABC transporter, substrate-binding protein, putative	Amino acids, peptides and amines
SAOUHSC_00929	3.4 up	Oligopeptide ABC transporter, ATP-binding protein, putative	Amino acids, peptides and amines
SAOUHSC_02283	3.0 up	Acetolactate synthase 1 regulatory subunit	Amino acids, peptides and amines
SAOUHSC_03019	2.4 up	Putative cobalt ABC transporter, ATP-binding protein	Amino acids, peptides and amines
SAOUHSC_00010	2.4 up	Putative azaleucine resistance protein AzlC	Amino acids, peptides and amines
SAOUHSC_00012	2.3 up	Putative azaleucine resistance protein AzlD	Amino acids, peptides and amines
SAOUHSC_00078	3.7 up	Siderophore staphylobactin biosynthesis protein SbnD	Amino acids, peptides and amines
SAOUHSC_00168	2.1 up	Nickel ABC transporter, permease subunit NikB	Amino acids, peptides and amines
SAOUHSC_00169	3.7 up	Peptide ABC transporter, permease protein, putative	Amino acids, peptides and amines
SAOUHSC_00241	2.0 up	Ribose transporter RbsU	Amino acids, peptides and amines
SAOUHSC_00932	2.3 up	Oligopeptide ABC transporter, permease protein, putative	Amino acids, peptides and amines
SAOUHSC_01450	2.7 up	Amino acid permease/transporter protein	Amino acids, peptides and amines
SAOUHSC_02967	2.2 up	Arginine/ornithine antiporter, putative	Amino acids, peptides and amines
SAOUHSC_00104	2.4 up	Phosphonate ABC transporter, ATP-binding protein	Anions
SAOUHSC_00102	2.9 up	Phosphonate ABC transporter, permease protein PhnE	Anions
SAOUHSC_00103	2.8 up	Phosphonate ABC transporter, permease PhnE	Anions

Table A1.1 (continued)

SAOUHSC_00138	4.0 up	Putative ABC-type nitrate sulfonate taurine bicarbonate transport system permease component	Anions
SAOUHSC_00281	2.5 up	FNT family formate-nitrite transporter	Anions
SAOUHSC_01387	2.7 up	Phosphate ABC superfamily ATP binding cassette transporter PstC	Anions
SAOUHSC_02671	2.4 up	Nitrite extrusion protein	Anions
SAOUHSC_00175	3.7 up	Multiple sugar-binding transport ATP-binding protein, putative	Carbohydrates, organic alcohols, and acids
SAOUHSC_00213	2.2 up	Putative phosphotransferase system regulator. Contains BlgG-family transcriptional anti-terminator	Carbohydrates, organic alcohols, and acids
SAOUHSC_00214	2.0 up	Phosphoenolpyruvate-dependent sugar phosphotransferase system, EIIA 2, putative	Carbohydrates, organic alcohols, and acids
SAOUHSC_00215	3.2 up	PTS system, galactitol-specific enzyme II B component	Carbohydrates, organic alcohols, and acids
SAOUHSC_00311	3.3 up	PTS system, ascorbate-specific IIB component	Carbohydrates, organic alcohols, and acids
SAOUHSC_00312	3.2 up	PTS family porter component IIA	Carbohydrates, organic alcohols, and acids
SAOUHSC_00313	2.0 up	Putative phosphotransferase system regulator. Contains BlgG-family transcriptional anti-terminator	Carbohydrates, organic alcohols, and acids
SAOUHSC_02708	2.2 up	Gamma-hemolysin h-gamma-II subunit, putative	Carbohydrates, organic alcohols, and acids
SAOUHSC_00158	2.9 up	PTS system component, sucrose specific	Carbohydrates, organic alcohols, and acids
SAOUHSC_00209	3.0 up	PTS system, glucose-specific IIBC component, putative	Carbohydrates, organic alcohols, and acids
SAOUHSC_00216	3.2 up	PTS system component	Carbohydrates, organic alcohols, and acids
SAOUHSC_00310	2.5 up	Ascorbate-specific PTS system enzyme, UlaA	Carbohydrates, organic alcohols, and acids
SAOUHSC_02597	2.3 up	PTS system component, putative	Carbohydrates, organic alcohols, and acids
SAOUHSC_02806	2.4 up	Gluconate permease, putative	Carbohydrates, organic alcohols, and acids
SAOUHSC_00077	4.5 up	Siderophore staphylobactin biosynthesis protein SbnC (IucA/IucC family siderophore biosynthesis protein)	Cations and iron carrying compounds

Table A1.1 (continued)

SAOUHSC_00079	3.1 up	Siderophore staphylobactin biosynthesis protein SbnE (IucA/IucC family Siderophore biosynthesis protein)	Cations and iron carrying compounds
SAOUHSC_00080	2.6 up	Siderophore biosynthesis protein SbnF (IucA/IucC family siderophore biosynthesis protein)	Cations and iron carrying compounds
SAOUHSC_02767	2.1 up	Nickel ABC transporter, periplasmic nickel-binding protein	Cations and iron carrying compounds
SAOUHSC_00071	2.1 up	Lipoprotein, SirC, putative	Cations and iron carrying compounds
SAOUHSC_00072	3.2 up	Lipoprotein, SirB, putative	Cations and iron carrying compounds
SAOUHSC_00420	3.8 up	NSS family amino acid:sodium (Na+) symporter	Cations and iron carrying compounds
SAOUHSC_02270	2.7 up	Ammonium transporter	Cations and iron carrying compounds
SAOUHSC_02864	2.6 up	Ferrous iron transport protein B	Cations and iron carrying compounds
SAOUHSC_00293	3.7 up	Nucleoside permease NupC, putative	Nucleosides, purines and pyrimidines
SAOUHSC_00315	2.1 up	Multidrug export protein MepA	Other
SAOUHSC_00317	3.0 up	glycerol-3-phosphate transporter	Other
SAOUHSC_00423	2.0 up	Methionine import ATP-binding protein MetN	Unknown substrate
SAOUHSC_02820	2.0 up	ABC transporter ATP-binding protein	Unknown substrate
SAOUHSC_00099	2.1 up	MFS family transporter: tetracycline:cation symporter	Unknown substrate
SAOUHSC_00177	2.8 up	Maltose ABC transporter, permease protein, putative	Unknown substrate
SAOUHSC_00183	4.1 up	Sugar phosphate antiporter, UhpT	Unknown substrate
SAOUHSC_00246	3.4 up	Quinolone-resistance protein NorB, putative	Unknown substrate
SAOUHSC_00294	4.3 up	SSS family solute:sodium (Na+) symporter	Unknown substrate
SAOUHSC_02658	2.2 up	Membrane protein, putative	Unknown substrate
SAOUHSC_02719	2.1 up	ABC transporter ATP-binding protein	Unknown substrate
SAOUHSC_02797	2.1 up	Major facilitator superfamily transporter	Unknown substrate
SAOUHSC_02815	2.1 up	MFS family major facilitator transporter	Unknown substrate
SAOUHSC_03037	2.0 up	ATP permease, VraD, putative	Unknown substrate

Table A1.1 (continued)

SAOUHSC_00087	5.5 up	Hypothetical protein of unknown function	Unknown substrate
SAOUHSC_00108	3.4 up	Putative transcription regulator	Unknown substrate
SAOUHSC_00316	2.5 up	MepB - component of MepRAB multidrug export operon	Unknown substrate
Unknown/unassigned function			
SAOUHSC_00323	2.6 up	Hypothetical protein of unknown function	
SAOUHSC_00387	4.2 up	Hypothetical protein of unknown function	
SAOUHSC_00418	4.4 up	Hypothetical protein of unknown function	
SAOUHSC_00962	2.1 up	Hypothetical protein of unknown function	
SAOUHSC_00977	2.8 up	Hypothetical protein of unknown function	
SAOUHSC_01300	2.1 up	Conserved hypothetical protein of unknown function	
SAOUHSC_01465	2.1 up	Hypothetical protein of unknown function	
SAOUHSC_02596	3.1 up	Conserved hypothetical protein of unknown function	
SAOUHSC_02655	2.0 up	Conserved hypothetical protein of unknown function	
SAOUHSC_02769	2.7 up	rRNA methyltransferase FmrO	
SAOUHSC_02805	4.3 up	Conserved hypothetical protein of unknown function	
SAOUHSC_02893	2.7 up	Conserved hypothetical protein of unknown function	
SAOUHSC_02896	2.4 up	Conserved hypothetical protein of unknown function	
SAOUHSC_00243	2.4 up	NADH dehydrogenase subunit, putative	
SAOUHSC_01409	2.3 up	Putative transposase	
SAOUHSC_00069	2.6 up	Staphylococcal protein A, Spa	
SAOUHSC_00256	2.9 up	Staphyloxanthin biosynthesis protein	
SAOUHSC_00154	4.5 up	Hypothetical protein of unknown function	
SAOUHSC_00189	2.9 up	Hypothetical protein of unknown function	
SAOUHSC_00194	2.6 up	Hypothetical protein of unknown function	
SAOUHSC_00212	2.0 up	Hypothetical protein of unknown function	
SAOUHSC_00238	3.5 up	Hypothetical protein of unknown function	
SAOUHSC_00303	2.2 up	Hypothetical protein of unknown function	

Table A1.1 (continued)

SAOUHSC_00396	5.3 up	Hypothetical protein of unknown function
SAOUHSC_00609	2.3 up	Hypothetical protein of unknown function
SAOUHSC_00745	4.3 up	Hypothetical protein of unknown function
SAOUHSC_00967	3.4 up	Hypothetical protein of unknown function
SAOUHSC_01003	2.4 up	Hypothetical protein of unknown function
SAOUHSC_01212	2.2 up	Hypothetical protein of unknown function - no known homologues
SAOUHSC_01293	2.0 up	Hypothetical protein of unknown function
SAOUHSC_01298	2.3 up	Hypothetical protein of unknown function
SAOUHSC_01299	2.8 up	Phage protein F-like protein
SAOUHSC_01339	2.1 up	Hypothetical protein of unknown function - no known homologues
SAOUHSC_01765	2.8 up	Hypothetical protein
SAOUHSC_01834	2.1 up	Hydrolase, haloacid dehalogenase-like family protein
SAOUHSC_01844	2.7 up	Hypothetical protein of unknown function
SAOUHSC_01934	2.3 up	Hypothetical protein of unknown function
SAOUHSC_01937	2.3 up	Hypothetical protein of unknown function - no known homologues
SAOUHSC_02105	2.4 up	Hypothetical protein of unknown function
SAOUHSC_02252	2.6 up	Conserved hypothetical protein
SAOUHSC_02290	6.2 up	Conserved hypothetical protein of unknown function
SAOUHSC_02325	2.8 up	Hypothetical protein of unknown function
SAOUHSC_02326	2.9 up	Hypothetical protein of unknown function
SAOUHSC_02339	2.1 up	Hypothetical protein of unknown function
SAOUHSC_02411	3.2 up	Hypothetical protein of unknown function
SAOUHSC_02616	2.6 up	Hypothetical protein of unknown function
SAOUHSC_02634	2.3 up	Conserved hypothetical protein of unknown function
SAOUHSC_02657	3.0 up	Conserved hypothetical protein of unknown function
SAOUHSC_02693	2.4 up	Hypothetical protein of unknown function
SAOUHSC_02717	2.9 up	Conserved hypothetical protein of unknown function

Table A1.1 (continued)

SAOUHSC_02720	3.3 up	Conserved hypothetical protein of unknown function
SAOUHSC_02746	2.9 up	Conserved hypothetical protein of unknown function
SAOUHSC_02817	2.4 up	MFS family major facilitator transporter
SAOUHSC_02868	2.0 up	Conserved hypothetical protein of unknown function
SAOUHSC_02889	5.1 up	Conserved hypothetical protein of unknown function
SAOUHSC_02890	2.7 up	Conserved hypothetical protein of unknown function
SAOUHSC_02914	2.4 up	Hypothetical protein of unknown function
SAOUHSC_02934	2.3 up	Hypothetical protein of unknown function
SAOUHSC_02939	3.6 up	Conserved hypothetical protein of unknown function
SAOUHSC_02940	3.1 up	Hypothetical protein of unknown function
SAOUHSC_00025	2.6 up	5'-nucleotidase family protein
SAOUHSC_00036	2.3 up	Metallo-beta-lactamase family protein
SAOUHSC_00082	2.8 up	Siderophore staphylobactin biosynthesis protein SbnH
SAOUHSC_00179	2.9 up	Oxidoreductase
SAOUHSC_00180	2.1 up	Oxidoreductase
SAOUHSC_00339	2.6 up	Bifunctional homocysteine S-methyltransferase/5,10-methylenetetrahydrofolate reductase protein
SAOUHSC_02728	2.6 up	Metallophosphoesterase
SAOUHSC_02824	3.3 up	Phospholipase/carboxylesterase family protein
SAOUHSC_02828	2.8 up	Glyoxalase
SAOUHSC_00106	3.8 up	Hypothetical protein
SAOUHSC_00109	2.0 up	Replication initiation protein, putative
SAOUHSC_00110	2.0 up	Truncated replication initiation protein, putative
SAOUHSC_00160	2.2 up	RpiR family transcriptional regulator
SAOUHSC_00292	4.4 up	Indigoidine synthase A-like protein
SAOUHSC_00295	3.8 up	N-acetylneuraminate lyase
SAOUHSC_00296	4.1 up	ROK-family protein

Table A1.1 (continued)

SAOUHSC_02964	2.0 up	HTH-type transcriptional regulator ArcR
SAOUHSC_03021	2.4 up	Conserved hypothetical protein of unknown function
SAOUHSC_03022	2.5 up	Conserved hypothetical protein
SAOUHSC_02711	2.8 up	Biotin biosynthesis protein BioX, putative
SAOUHSC_02872	2.6 up	Conserved hypothetical protein
SAOUHSC_03047	4.0 up	Conserved hypothetical protein
SAOUHSC_03048	4.3 up	Conserved hypothetical protein
SAOUHSC_00259	2.2 up	Virulence protein EssA
SAOUHSC_02863	2.2 up	Conserved hypothetical protein of unknown function
SAOUHSC_00303	2.9 up	Conserved hypothetical protein
SAOUHSC_00062	3.0 up	DMT superfamily drug/metabolite transporter
SAOUHSC_00232	4.0 up	Murein hydrolase regulator LrgA

Table A1.2. Down-regulated genes in *S. aureus* SH1000 in response to Ag^+ exposure (sorted according to JCVI cellular role categories)

Gene	Fold change in expression	Function	JCVI Cellular Role SubCategory
Amino acid biosynthesis			
SAOUHSC_01635	2.7 down	Shikimate kinase, putative	Aromatic amino acid family
Biosynthesis of cofactors, prosthetic groups, and carriers			
SAOUHSC_01434	2.2 down	Dihydrofolate reductase	Folic acid
SAOUHSC_01874	2.2 down	Rhodanese domain protein	Molybdopterin
Cell envelope			
SAOUHSC_00973	2.5 down	Glycosyltransferase	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
SAOUHSC_00991	2.1 down	Acetyltransferase/acyltransferase, putative	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
SAOUHSC_01114	2.8 down	Fibrinogen-binding protein, putative	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
SAOUHSC_00332	2.0 down	Putative membrane protein	Other
SAOUHSC_00672	2.4 down	Hypothetical protein	Other
SAOUHSC_00717	3.2 down	Putative lipoprotein	Other
SAOUHSC_00754	2.1 down	Putatative lipoprotein	Other
SAOUHSC_00808	2.0 down	Putatative lipoprotein	Other
SAOUHSC_01131	2.1 down	Putative membrane protein	Other
SAOUHSC_01627	2.5 down	Lipoprotein, putative	Other
SAOUHSC_02694	2.1 down		Other
SAOUHSC_02695	2.2 down	Lipoprotein, putative	Other
Cellular processes			
SAOUHSC_02381	2.4 down	DNA-binding protein DpsA, putative	Adaptations to atypical conditions
SAOUHSC_02665	3.0 down	Conserved hypothetical protein	Adaptations to atypical conditions
SAOUHSC_00585	2.2 down	Putative membrane protein	Cell adhesion
SAOUHSC_00591	2.1 down	Putative membrane protein	Cell adhesion
SAOUHSC_00598	2.4 down	Hypothetical protein of unknown function	Cell adhesion
SAOUHSC_00602	2.0 down	Hypothetical protein of unknown function	Cell adhesion
SAOUHSC_00835	2.6 down	Glutaredoxin, putative	Detoxification

Table A1.2 (continued)

Central intermediary metabolism			
SAOUHSC_02565	2.1 down	Urease accessory protein UreD	Nitrogen metabolism
SAOUHSC_01882	2.0 down	Lysophospholipase, putative	Other
DNA metabolism			
SAOUHSC_01342	2.0 down	Exonuclease SbcCD, C subunit	DNA replication, recombination and repair
SAOUHSC_00840	2.7 down	TOPRIM domain topiosmerase/primase	Other
Energy metabolism			
SAOUHSC_00834	2.1 down	Thioredoxin	Electron transport
SAOUHSC_00841	2.1 down	Thioredoxin	Electron transport
SAOUHSC_01100	2.0 down	Thioredoxin	Electron transport
Fatty acid and phospholipid metabolism			
SAOUHSC_01201	2.3 down	Acyl carrier protein, AcpP	Biosynthesis
SAOUHSC_01260	2.7 down	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	Biosynthesis
SAOUHSC_02163	6.2 down	Truncated beta-hemolysin	Degradation
Mobile and extrachromosomal element functions			
SAOUHSC_00624	2.7 down	Phage recombinase/integrase	Prophage functions
SAOUHSC_00830	2.3 down	Acetyltransferase, GNAT family	Prophage functions
SAOUHSC_02038	2.2 down	HK97 family phage protein	Prophage functions
SAOUHSC_01928	2.4 down	Transposase family protein, putative	Transposon functions
SAOUHSC_02061	2.1 down	Phi PVL orf 50-like protein	Transposon functions
SAOUHSC_02211	2.1 down	Phi PVL orf 50-like protein	Transposon functions
Protein fate			
SAOUHSC_01162	2.0 down	Lipoprotein signal peptidase, putative	Protein and peptide secretion and trafficking
SAOUHSC_02255	2.5 down	Co-chaperonin GroES	Protein folding and stabilization
Protein synthesis			
SAOUHSC_00521	2.2 down	50S ribosomal protein L7/L12	Ribosomal proteins: synthesis and modification
SAOUHSC_01191	2.5 down	50S ribosomal protein L28, RpmB	Ribosomal proteins: synthesis and modification
SAOUHSC_01208	0.430642	30S ribosomal protein S16	Ribosomal proteins: synthesis and modification
SAOUHSC_01211	2.3 down	50S ribosomal protein L19, RplS	Ribosomal proteins: synthesis and modification

Table A1.2 (continued)

SAOUHSC_01651	2.3 down	50S ribosomal protein L33	Ribosomal proteins: synthesis and modification
SAOUHSC_01678	2.3 down	30S ribosomal protein S21	Ribosomal proteins: synthesis and modification
SAOUHSC_01784	2.7 down	50S ribosomal protein L20	Ribosomal proteins: synthesis and modification
SAOUHSC_02361	2.1 down	50S ribosomal protein L31 type B	Ribosomal proteins: synthesis and modification
SAOUHSC_01625	2.2 down	Elongation factor P	Translation factors
Regulatory functions			
SAOUHSC_00070	3.8 down	Staphylococcal accessory regulator-like protein, SarS	DNA interactions
SAOUHSC_00620	3.1 down	Staphylococcal accessory regulator T, putative	DNA interactions
SAOUHSC_00673	2.4 down	AraC/XylS-family transcriptional regulator	DNA interactions
SAOUHSC_01685	2.8 down	Heat-inducible transcription repressor HrcA	DNA interactions
SAOUHSC_01850	2.2 down	Catabolite control protein A, CcpA	DNA interactions
SAOUHSC_02155	9.2 down	GntR family transcriptional regulator	DNA interactions
SAOUHSC_02566	3.6 down	Staphylococcal accessory regulator R, SarR	DNA interactions
SAOUHSC_00674	2.5 down	HTH-type transcriptional regulator SarX	Other
SAOUHSC_00694	2.2 down	HTH-type transcriptional regulator MgrA - regulator of SarX and NorB	Other
SAOUHSC_01361	2.3 down	Transcriptional regulator, putative	Other
SAOUHSC_01362	2.9 down	4-oxalocrotonate tautomerase, putative	Other
SAOUHSC_01997	2.7 down	Ferric uptake regulator-like protein, putative	Other
SAOUHSC_02669	2.2 down	HTH-type transcriptional regulator SarZ	Other
SAOUHSC_00540	2.0 down	Deoxynucleoside kinase/deoxypurine kinase, putative	Protein interactions
SAOUHSC_01419	2.7 down	Hypothetical protein	Protein interactions
Transcription			
SAOUHSC_01247	2.2 down	Ribosome-binding factor A	RNA processing
SAOUHSC_00767	3.5 down	Ribosomal subunit interface protein, putative	Transcription factors
Transport and binding proteins			
SAOUHSC_00892	2.2 down	Putative S1 RNA binding domain/general stress protein 13	Amino acids, peptides and amines
SAOUHSC_02151	7.3 down	Putative membrane protein	Amino acids, peptides and amines
SAOUHSC_00626	3.3 down	Putative monovalent cation/H+ antiporter subunit B	Cations and iron carrying compounds
Unknown function/unassigned function			
SAOUHSC_00028	2.3 down	Hypothetical protein of unknown function	
SAOUHSC_00029	3.0 down	Hypothetical protein of unknown function	
SAOUHSC_00280	2. down	Putative membrane protein	
SAOUHSC_00331	2.3 down	Hypothetical protein	

Table A1.2 (continued)

SAOUHSC_00465	2.1 down	Hypothetical protein of unknown function
SAOUHSC_00537	2.3 down	Hypothetical protein of unknown function
SAOUHSC_00589	2.4 down	Hypothetical protein of unknown function - no known homologues
SAOUHSC_00627	2.8 down	Putative monovalent cation/H+ antiporter subunit C
SAOUHSC_00663	3.2 down	Acetyltransferase, GNAT family
SAOUHSC_00678	2.7 down	Conserved hypothetical protein of unknown function
SAOUHSC_00680	3.6 down	LysR family transcriptional regulator
SAOUHSC_00697	2.2 down	Hypothetical protein
SAOUHSC_00702	2.2 down	Conserved hypothetical protein of unknown function
SAOUHSC_00704	2.8 down	Conserved hypothetical protein of unknown function
SAOUHSC_00734	2.4 down	5'(3')-deoxyribonucleotidase (DNA/RNA synth)
SAOUHSC_00736	3.1 down	Diacylglycerol kinase
SAOUHSC_00753	2.3 down	Conserved hypothetical protein of unknown function
SAOUHSC_00770	2.2 down	Hypothetical protein of unknown function
SAOUHSC_00819	2.5 down	Cold shock protein CspC
SAOUHSC_00824	2.0 down	Putative membrane protein
SAOUHSC_00825	3.9 down	Conserved hypothetical protein of unknown function
SAOUHSC_00826	4.3 down	Conserved hypothetical protein of unknown function
SAOUHSC_00837	2.3 down	Conserved hypothetical protein of unknown function
SAOUHSC_00862	2.2 down	Hypothetical protein
SAOUHSC_00919	2.0 down	Putative membrane protein
SAOUHSC_00938	2.0 down	Dithiol-disulfide isomerase/thioredoxin superfamily-protein, putative
SAOUHSC_00971	4.0 down	Conserved hypothetical protein of unknown function
SAOUHSC_00972	2.2 down	Hypothetical protein of unknown function
SAOUHSC_00975	2.7 down	DoxX family protein
SAOUHSC_00996	2.1 down	Conserved hypothetical protein of unknown function
SAOUHSC_01024	5.0 down	Hypothetical protein of unknown function
SAOUHSC_01036	2.1 down	Conserved hypothetical protein of unknown function
SAOUHSC_01037	3.3 down	Hypothetical protein of unknown function
SAOUHSC_01051	2.0 down	Conserved hypothetical protein of unknown function
SAOUHSC_01060	2.9 down	Conserved hypothetical protein of unknown function
SAOUHSC_01062	2.1 down	Hypothetical protein
SAOUHSC_01072	2.1 down	
SAOUHSC_01096	2.2 down	Cell division protein ZapA, putative
SAOUHSC_01097	2.0 down	Conserved hypothetical protein
SAOUHSC_01110	5.0 down	Fibrinogen-binding protein, putative
SAOUHSC_01115	2.8 down	Fibrinogen-binding protein, putative

Table A1.2 (continued)

SAOUHSC_01119	2.4 down	Conserved hypothetical protein of unknown function
SAOUHSC_01120	2.9 down	Conserved hypothetical protein of unknown function
SAOUHSC_01140	2.4 down	Hypothetical protein of unknown function
SAOUHSC_01155	2.0 down	Hypothetical protein of unknown function - no known homologues
SAOUHSC_01173	2.4 down	Conserved hypothetical protein of unknown function
SAOUHSC_01181	3.6 down	Conserved hypothetical protein
SAOUHSC_01230	2.7 down	Hypothetical protein of unknown function
SAOUHSC_01264	2.4 down	Conserved hypothetical protein of unknown function
SAOUHSC_01301	2.7 down	Conserved hypothetical protein of unknown function
SAOUHSC_01309	3.0 down	Hypothetical protein of unknown function
SAOUHSC_01315	3.4 down	Conserved hypothetical protein of unknown function
SAOUHSC_01324	2.0 down	Conserved hypothetical protein of unknown function
SAOUHSC_01340	2.0 down	Conserved hypothetical protein
SAOUHSC_01349	2.1 down	Conserved hypothetical protein
SAOUHSC_01353	2.0 down	Hypothetical protein of unknown function - no known homologues
SAOUHSC_01356	2.1 down	GlcA/GlcB -family transcription antiterminator
SAOUHSC_01357	2.0 down	Hypothetical protein of unknown function
SAOUHSC_01403	2.0 down	Cold shock protein, CspA, putative
SAOUHSC_01405	2.0 down	Hypothetical protein of unknown function
SAOUHSC_01421	2.5 down	Hypothetical protein of unknown function
SAOUHSC_01422	2.1 down	Conserved hypothetical protein of unknown function
SAOUHSC_01428	2.0 down	Hypothetical protein of unknown function
SAOUHSC_01464	2.2 down	Conserved hypothetical protein of unknown function
SAOUHSC_01468	2.7 down	Conserved hypothetical protein of unknown function
SAOUHSC_01489	2.0 down	Hypothetical protein of unknown function
SAOUHSC_01503	2.1 down	Conserved hypothetical protein of unknown function
SAOUHSC_01551	2.0 down	Conserved hypothetical phage protein
SAOUHSC_01560	2.1 down	Conserved hypothetical phage protein
SAOUHSC_01568	2.5 down	Hypothetical protein of unknown function
SAOUHSC_01607	2.3 down	Conserved hypothetical protein of unknown function
SAOUHSC_01608	2.6 down	Conserved hypothetical protein of unknown function
SAOUHSC_01636	2.4 down	Conserved hypothetical protein of unknown function
SAOUHSC_01665	2.0 down	CBS domain-containing protein
SAOUHSC_01719	2.5 down	Conserved hypothetical protein of unknown function

Table A1.2 (continued)

SAOUHSC_01729	2.6 down	Conserved hypothetical protein of unknown function
SAOUHSC_01736	2.4 down	Conserved hypothetical protein of unknown function
SAOUHSC_01769	2.1 down	membrane protein AbrB, proposed regulator of AidB expression
SAOUHSC_01896	2.0 down	Hypothetical protein of unknown function
SAOUHSC_01899	2.3 down	Conserved hypothetical protein of unknown function
SAOUHSC_01930	2.8 down	Conserved hypothetical protein of unknown function
SAOUHSC_01957	2.9 down	Conserved hypothetical protein of unknown function
SAOUHSC_01963	2.8 down	Hypothetical protein of unknown function
SAOUHSC_01986	3.9 down	Conserved hypothetical protein of unknown function
SAOUHSC_02055	2.1 down	Conserved hypothetical phage protein
SAOUHSC_02065	2.2 down	Hypothetical protein phiETA_24 [Staphylococcus phage phiETA]
SAOUHSC_02068	2.8 down	Conserved hypothetical phage protein
SAOUHSC_02071	2.7 down	Single-strand DNA-binding protein, putative
SAOUHSC_02110	2.0 down	DNA polymerase III, alpha chain PolC-type/exonuclease
SAOUHSC_02135	2.0 down	Hypothetical protein
SAOUHSC_02141	2.1 down	Conserved hypothetical protein of unknown function
SAOUHSC_02145	2.9 down	Conserved hypothetical protein of unknown function
SAOUHSC_02214	2.2 down	Phi SLT orf 24-like protein [Staphylococcus phage phi13]
SAOUHSC_02248	3.4 down	GNAT family acetyltransferase
SAOUHSC_02249	3.6 down	Conserved hypothetical protein of unknown function
SAOUHSC_02256	2.6 down	Abortive infection protein
SAOUHSC_02294	2.6 down	Conserved hypothetical protein of unknown function
SAOUHSC_02320	2.6 down	Conserved hypothetical protein of unknown function
SAOUHSC_02378	2.0 down	Hypothetical protein of unknown function
SAOUHSC_02416	2.0 down	Hypothetical protein of unknown function
SAOUHSC_02471	3.0 down	Conserved hypothetical protein of unknown function
SAOUHSC_02473	2.0 down	Conserved hypothetical protein of unknown function
SAOUHSC_02515	2.2 down	Putative membrane protein
SAOUHSC_02521	2.4 down	Conserved hypothetical protein of unknown function
SAOUHSC_02523	2.2 down	Conserved hypothetical protein of unknown function
SAOUHSC_02568	2.2 down	Conserved hypothetical protein of unknown function
SAOUHSC_02666	2.3 down	Conserved hypothetical protein of unknown function
SAOUHSC_02668	2.1 down	Putative exported protein

Table A1.2 (continued)

SAOUHSC_02688	2.2 down	Conserved hypothetical protein of unknown function
SAOUHSC_02774	3.8 down	Conserved hypothetical protein
SAOUHSC_03001	2.8 down	Ica operon transcriptional regulator IcaR, putative
SAOUHSC_A00635	2.0 down	Hypothetical protein of unknown function - no known homologues
SAOUHSC_A01081	2.6 down	Conserved hypothetical protein of unknown function
SAOUHSC_A02013	3.7 down	Conserved hypothetical protein of unknown function
SAOUHSC_A02503	2.0 down	Conserved hypothetical protein of unknown function

Table A1.3. Putative integral membrane proteins up-regulated following exposure to Ag^{+}

Gene	Fold change in expression	Protein/Function	Additional comments	Number of predicted transmembrane domains	Presence of cysteine	JCVI Cellular Role Category
SAOUHSC_00010	2.4 up	Hypothetical protein	Putative azaleucine resistance protein AzlC	6	Yes	Transport and binding proteins: Amino acids, peptides and amines
SAOUHSC_00012	2.3 up	Hypothetical protein	Contains AzlD superfamily domain. AzlD is known to be involved in conferring resistance to 4-azaleucine although its exact role is uncertain.	3	Yes	Transport and binding proteins: Amino acids, peptides and amines
SAOUHSC_00051	3.2 up	1-phosphatidylinositol phosphodiesterase precursor, putative	Contains putatative metal binding domain - normally binds calcium	1	Yes	Fatty acid and phospholipid metabolism: Degradation
SAOUHSC_00053	2.0 up	Hypothetical protein	Tandem lipoprotein	1	Yes	Cell envelope: Other
SAOUHSC_00062	3.0 up	Hypothetical protein	DMT superfamily drug/metabolite transporter	10	Yes	Unknown function: General
SAOUHSC_00071	2.1 up	Lipoprotein, SirC, putative	Permease component of ABC-type Fe^{3+}-siderophore transport system	8	No	Transport and binding proteins: Cations and iron carrying compounds
SAOUHSC_00072	3.2 up	Lipoprotein, SirB, putative	Permease component of ABC-type Fe^{3+}-siderophore transport system	9	Yes	Transport and binding proteins: Cations and iron carrying compounds
SAOUHSC_00078	3.7 up	Hypothetical protein	Siderophore staphylobactin biosynthesis protein SbnD OR MFS family major facilitator transporter	10	Yes	Transport and binding proteins: Amino acids, peptides and amines
SAOUHSC_00084	2.0 up	Hypothetical protein	Hypothetical protein of unknown function	3	Yes	Hypothetical proteins/Unknown function

Table A1.3 (continued)

SAOUHSC_00089	4.3 up	Hypothetical protein	Capsular polysaccharide biosynthesis glycosyltransferase (sugar transferase)	1	Yes	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
SAOUHSC_00091	2.4 up	Hypothetical protein	Probable capsular biosynthesis protein/O-antigen polymerase	12	Yes	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
SAOUHSC_00099	2.1 up	Hypothetical protein	MFS family transporter: tetracycline:cation symporter	11	No	Transport and binding proteins: Unknown substrate
SAOUHSC_00102	2.9 up	Phosphonates ABC transporter, permease protein CC0363, putative	Phosphonate ABC transporter, permease protein PhnE	6	Yes	Transport and binding proteins: Anions
SAOUHSC_00103	2.8 up	Phosphonates ABC transporter, permease protein CC0363, putative	Phosphonate ABC transporter, permease PhnE	6	Yes	Transport and binding proteins: Anions
SAOUHSC_00111	2.0 up	Hypothetical protein	Hypothetical protein of unknown function	3	Yes	Hypothetical proteins/Unknown function
SAOUHSC_00138	4.0 up	Hypothetical protein	Putative ABC-type nitrate sulfonate taurine bicarbonate transport system permease component	6	No	Transport and binding proteins: Anions
SAOUHSC_00146	2.1 up	Hypothetical protein	Integral membrane protein - homology to YagU in *Actinomyces* believed to be required for acid resistance	4	Yes	Cell envelope: Other
SAOUHSC_00158	2.9 up	PTS system component	Sucrose specific PTS	10	Yes	Transport and binding proteins: Carbohydrates, organic alcohols, and acids
SAOUHSC_00161	2.1 up	Hypothetical protein	Hypothetical protein of unknown function - no known homologues	3	No	Hypothetical proteins/Unknown function

Table A1.3 (continued)

SAOUHSC_00168	2.1 up	Hypothetical protein	Nickel ABC transporter, permease subunit, gene name NikB	9	Yes	Transport and binding proteins: Amino acids, peptides and amines
SAOUHSC_00169	3.7 up	Peptide ABC transporter, permease protein, putative	Peptide/nickel transport system permease	9	Yes	Transport and binding proteins: Amino acids, peptides and amines
SAOUHSC_00177	2.8 up	Maltose ABC transporter, permease protein, putative		8	No	Transport and binding proteins: Unknown substrate
SAOUHSC_00182	2.1 up	Hypothetical protein	Isoprenylcysteine carboxyl methyltransferase	4	Yes	Cell envelope: Other
SAOUHSC_00183	4.1 up	Sugar phosphate antiporter	UhpT - known in other species as hexose phosphate transport protein	12	Yes	Transport and binding proteins: Unknown substrate
SAOUHSC_00193	2.4 up	Hypothetical protein	Hypothetical protein of unknown function - no known homologues	1	Yes	Hypothetical proteins/Unknown function
SAOUHSC_00200	2.3 up	Hypothetical protein		8	No	Cell envelope: Other
SAOUHSC_00208	4.0 up	Hypothetical protein	Hypothetical protein of unknown function - no known homologues	1	No	Hypothetical proteins/Unknown function
SAOUHSC_00209	3.0 up	PTS system, glucose-specific IIBC component, putative		11	Yes	Transport and binding proteins: Carbohydrates, organic alcohols, and acids
SAOUHSC_00216	3.2 up	PTS system component	PTS enzyme IIC component galacticol or sorbitol specific	9	Yes	Transport and binding proteins: Carbohydrates, organic alcohols, and acids
SAOUHSC_00218	2.8 up	Hypothetical protein	Hypothetical protein of unknown function	1	No	Unknown function: General

Table A1.3 (continued)

SAOUHSC_00232	4.0 up	Murein hydrolase regulator LrgA	LrgAB inhibit extracellular murein hydrolase activity and promote penicillin tolerance	4	Yes	Unknown function: General
SAOUHSC_00233	5.2 up	Antiholin-like protein LrgB	LrgAB inhibit extracellular murein hydrolase activity and promote penicillin tolerance	7	Yes	Regulatory functions: Other
SAOUHSC_00241	2.0 up	Hypothetical protein	Ribose transporter RbsU	9	No	Transport and binding proteins: Amino acids, peptides and amines
SAOUHSC_00246	3.4 up	Drug transporter, putative	MFS family drug transporter 98% identity to quinolone resistance protein NorB - regulated by MgrA	14	Yes	Transport and binding proteins: Unknown substrate
SAOUHSC_00255	2.4 up	Hypothetical protein	Putative membrane protein	6	Yes	Cell envelope: Other
SAOUHSC_00259	2.2 up	Hypothetical protein	Virulence protein EssA (component of the ESAT-6 secretion system)	1	No	Unclassified: Role category not yet assigned
SAOUHSC_00281	2.5 up	Hypothetical protein	FNT family formate-nitrite transporter	6	Yes	Transport and binding proteins: Anions
SAOUHSC_00293	3.7 up	Hypothetical protein	Nucleoside permease NupC, putative. NupC has specificity towards the pyrimidine nucleosides, adenosine, and their deoxy derivatives.	8	No	Transport and binding proteins: Nucleosides, purines and pyrimidines
SAOUHSC_00294	4.3 up	Hypothetical protein	SSS family solute:sodium (Na^+) symporter	13	Yes	Transport and binding proteins: Unknown substrate
SAOUHSC_00299	3.0 up	Hypothetical protein	Putative membrane protein/gate domain protein/nucleoside recognition domain	12	Yes	Protein fate: Protein and peptide secretion and trafficking
SAOUHSC_00310	2.5 up	Ascorbate-specific PTS system enzyme IIC	Gene name: UlaA	10	Yes	Transport and binding proteins: Carbohydrates, organic alcohols, and acids

Table A1.3 (continued)

SAOUHSC_00315	2.1 up	Hypothetical protein	Multidrug export protein MepA	12	Yes	Transport and binding proteins: Other
SAOUHSC_00317	3.0 up	Glycerol-3-phospha te transporter	MFS family protein	12	Yes	Transport and binding proteins: Other
SAOUHSC_00327	2.3 up	Hypothetical protein	Ferrous iron transport permease EfeU - functions as part of an iron uptake mechanism - membrane bound protein	6	Yes	Cell envelope: Other
SAOUHSC_00409	2.4 up	Hypothetical protein	Hypothetical protein of unknown function	2	Yes	Unknown function: General
SAOUHSC_00420	3.8 up	Hypothetical protein	NSS family amino acid:sodium (Na^+) symporter	11	No	Transport and binding proteins: Cations and iron carrying compounds
SAOUHSC_00583	3.6 up	Hypothetical protein	Hypothetical protein of unknown function	1	Yes	Unknown function: General
SAOUHSC_00932	2.3 up	Oligopeptide ABC transporter, permease protein, putative		6	No	Transport and binding proteins: Amino acids, peptides and amines
SAOUHSC_01387	2.7 up	Hypothetical protein	Phosphate ABC superfamily ATP binding cassette transporter, membrane protein	6	No	Transport and binding proteins: Anions
SAOUHSC_01450	2.7 up	Hypothetical protein	Amino acid permease/transporter protein	12	Yes	Transport and binding proteins: Amino acids, peptides and amines
SAOUHSC_01494	2.4 up	Hypothetical protein	Hypothetical protein of unknown function	1		Unknown function: General
SAOUHSC_01641	2.1 up	Hypothetical protein	Competence protein comgb -DNA transport	4	Yes	Protein fate: Protein and peptide secretion and trafficking
SAOUHSC_01764	2.0 up	Hypothetical protein	Peptidase, A24 type IV prepilin peptidase family protein	4	Yes	Cell envelope: Surface structures
SAOUHSC_01935	2.1 up	Serine protease SplF, putative	Spl serine proteases are secreted proteases and could be a virulence factor	2	No	Protein fate: Degradation of proteins, peptides, and glycopeptides

Table A1.3 (continued)

SAOUHSC_01941	3.6 up	Serine protease SplB	Spl serine proteases are secreted proteases and could be a virulence factor	1	No	Protein fate: Degradation of proteins, peptides, and glycopeptides
SAOUHSC_01984	2.0 up	Hypothetical protein	Hypothetical protein of unknown function	3	Yes	Unknown function: General
SAOUHSC_02270	2.7 up	Ammonium transporter	Ammonia or ammonium uptake transporter	11	Yes	Transport and binding proteins: Cations and iron carrying compounds
SAOUHSC_02597	2.3 up	PTS system component, putative	Maltose-specific EIICB component	10	Yes	Transport and binding proteins: Carbohydrates, organic alcohols, and acids
SAOUHSC_02658	2.2 up	Hypothetical protein	Contains multiple membrane-spanning domains	6	No	Transport and binding proteins: Unknown substrate
SAOUHSC_02671	2.4 up	Hypothetical protein	Nitrite extrusion protein	12	Yes	Transport and binding proteins: Anions
SAOUHSC_02711	2.8 up	Hypothetical protein	Biotin biosynthesis protein BioX in other organisms (80-95% identity)	4	No	Hypothetical proteins: Conserved
SAOUHSC_02719	2.1 up	Hypothetical protein	ABC transporter ATP-binding protein	5	Yes	Transport and binding proteins: Unknown substrate
SAOUHSC_02797	2.1 up	Hypothetical protein	Major facilitator superfamily transporter	8	Yes	Transport and binding proteins: Unknown substrate
SAOUHSC_02806	2.4 up	Gluconate permease, putative		12	No	Transport and binding proteins: Carbohydrates, organic alcohols, and acids
SAOUHSC_02815	2.1 up	Hypothetical protein	MFS family major facilitator transporter	11	No	Transport and binding proteins: Unknown substrate
SAOUHSC_02821	2.0 up	Membrane spanning protein, putative	Transmembrane protein SmpB/lantibiotic protection ABC transporter permease subunit	6	No	Energy metabolism: Electron transport
SAOUHSC_02863	2.2 up	Hypothetical protein	Conserved hypothetical protein of unknown function	1	Yes	Hypothetical proteins/Unknown function
SAOUHSC_02864	2.6 up	Ferrous iron transport protein B	Involved in iron uptake	10	Yes	Transport and binding proteins: Cations and iron carrying compounds

Table A1.3 (continued)

SAOUHSC_02872	2.6 up	Hypothetical protein		1	No	Hypothetical proteins: Conserved
SAOUHSC_02873	5.3 up	Cation transporter E1-E2 family ATPase CopA		8	Yes	Hypothetical proteins/Unknown function
SAOUHSC_02888	4.5 up	Hypothetical protein	Hypothetical phosphotransferase system IIC component	10	No	Cell envelope: Other
SAOUHSC_02902	2.7 up	Hypothetical protein	FeoB-family ferrous iron (Fe^{2+}) uptake protein	4	Yes	Protein fate: Protein and peptide secretion and trafficking
SAOUHSC_02948	2.9 up	Hypothetical protein	Hypothetical protein of unknown function	1	No	Unknown function: General
SAOUHSC_02967	2.2 up	Arginine/ornithine antiporter, putative		14	Yes	Transport and binding proteins: Amino acids, peptides and amines
SAOUHSC_02973	2.6 up	Hypothetical protein	Hypothetical protein of unknown function	1	No	Hypothetical proteins/Unknown function
SAOUHSC_03000	2.6 up	Capsular polysaccharide biosynthesis,		2	Yes	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
SAOUHSC_03002	3.3 up	N-glycosyltransferase, IcaA		4	Yes	Cell envelope: Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
SAOUHSC_03003	2.9 up	Hypothetical protein, IcaD	Gene name:	2	Yes	Unknown
SAOUHSC_03020	2.5 up	Hypothetical protein	Membrane protein of unknown function	5	Yes	Cell envelope: Other
SAOUHSC_03035	2.1 up	Hypothetical protein	Conserved hypothetical protein of unknown function	6	No	Cell envelope: Other
SAOUHSC_03037	2.0 up	Permease VraD, putative	Upregulated during cell membrane stress	10	Yes	Transport and binding proteins: Unknown substrate
SAOUHSC_03047	4.0 up	Hypothetical protein	Conserved membrane protein	4	Yes	Hypothetical proteins: Conserved

Table A1.3 (continued)

SAOUHSC_03048	4.3 up	Hypothetical protein	Conserved membrane protein	4	Yes	Hypothetical proteins: Conserved
SAOUHSC_A00084	4.9 up	Hypothetical protein	Hypothetical protein of unknown function	1	Yes	Hypothetical proteins/Unknown function
SAOUHSC_A00097	2.4 up	Hypothetical protein	Hypothetical protein of unknown function	2	Yes	Hypothetical proteins/Unknown function
SAOUHSC_A00379	6.1 up	Hypothetical protein	Conserved hypothetical protein of unknown function	2	Yes	Hypothetical proteins/Unknown function
SAOUHSC_A00526	2.8 up	Hypothetical protein	Conserved hypothetical protein of unknown function	1	Yes	Hypothetical proteins/Unknown function
SAOUHSC_A02445	2.3 up	Hypothetical protein	Conserved hypothetical protein of unknown function	1	No	Hypothetical proteins/Unknown function
SAOUHSC_A02680	2.1 up	Hypothetical protein	Conserved hypothetical protein of unknown function -no known homologues	2	Yes	Unknown function: General
SAOUHSC_A02795	2.3 up	Hypothetical protein		1	Yes	Unknown function: General
SAOUHSC_A02801	2.6 up	Hypothetical protein	Hypothetical protein of unknown function	1	Yes	Unknown function: General
SAOUHSC_A02856	2.6 up	Hypothetical protein	Hypothetical protein of unknown function	1	Yes	Unknown function: General

Appendix 2. Annotation of *E. coli* NCTC 50110

Table A2.1. Genes present in contiguous sequences corresponding to plasmid pMG101. Annotation and subsystem assignment carried out by the Rapid Annotation using Subsystem Technology (RAST) server. Start = nucleotide position corresponding to start of the gene. End= nucleotide position corresponding to end of the gene.

Contig	Start	Stop	Length (bp)	Protein/Function	Subsystem category
51	714	238	477	Lysin	- none -
51	1021	698	324	Phage holin	- none -
51	2276	1698	579	Phage antitermination protein Q	Phage nin genes - N-independent survival
51	2983	2318	666	Phage NinI serine/threonine phosphatase	- none -
51	3167	2961	207	Phage NinH	Phage nin genes - N-independent survival
51	3361	3164	198	Protein NinG	- none -
51	3938	3768	171	Phage NinF	Phage nin genes - N-independent survival
51	5144	4254	891	Phage NinC	Phage nin genes - N-independent survival
51	5563	5123	441	Phage NinB DNA recombination	Phage nin genes - N-independent survival
51	5927	5637	291	Ren protein	- none -
51	6625	5924	702	Hypothetical protein	- none -
51	7521	6622	900	Origin specific replication initiation factor	Phage replication
51	7691	7554	138	Phage repressor	- none -
51	8166	7966	201	Regulatory protein cro	- none -
51	8267	8980	714	Phage repressor	- none -
51	9093	9932	840	RexA protein	- none -
51	10847	11170	324	Phage antitermination protein N	Phage nin genes - N-independent survival
51	11998	12120	123	Phage restriction protein ral	- none -
60	1921	1589	333	Programmed cell death toxin PemK	MazEF toxin-antitoxing (programmed cell death) system
60	2180	1923	258	Programmed cell death antitoxin PemI	MazEF toxin-antitoxin (programmed cell death) system
60	2926	2273	654	Microcin M activity protein McmM	- none -
60	3164	3024	141	Hypothetical protein	- none -
60	3743	3910	168	Hypothetical protein	- none -
60	4723	3866	858	RepA1	- none -
60	5281	5027	255	Replication regulatory protein repA2 (Protein copB)	- none -
60	5654	7195	1542	Mobile element protein	- none -
60	7207	7956	750	Mobile element protein	- none -

Table A2.1 (continued)

60	8298	8164	135	Post-segregation killing protein	- none -
60	9296	9114	183	Hypothetical protein	- none -
60	10072	9512	561	Plasmid conjugative transfer fertility inhibition protein FinO	Conjugative transfer, Conjugal Transfer System
60	10873	10127	747	Plasmid conjugative transfer pilin acetylase TraX	Conjugative transfer, Conjugal Transfer System
60	16163	10893	5271	Plasmid conjugative transfer DNA-nicking and unwinding protein TraI	Conjugative transfer, Conjugal Transfer System
60	18316	16163	2154	Plasmid conjugative transfer protein TraD	Conjugative transfer, Conjugal Transfer System
60	19092	18313	780	YhfA Protein. In Tra region of some plasmids	Conjugative transfer, Conjugal Transfer System
60	20071	19295	777	Plasmid conjugative transfer surface exclusion protein TraT	Conjugative transfer, Conjugal Transfer System
60	23393	20574	2820	Plasmid conjugative transfer protein TraG	Conjugative transfer, Conjugal Transfer System
60	24763	23390	1374	Plasmid conjugative transfer pilus assembly protein TraH	Conjugative transfer, Conjugal Transfer System
60	25142	24750	393	Plasmid conjugative transfer protein TrbF	Conjugative transfer, Conjugal Transfer System
60	25410	25123	288	Plasmid conjugative transfer protein TrbJ	Conjugative transfer, Conjugal Transfer System
60	25945	25400	546	Plasmid conjugative transfer protein TrbB	Conjugative transfer, Conjugal Transfer System
60	26168	25932	237	Plasmid conjugative transfer protein TraQ	Conjugative transfer, Conjugal Transfer System
61	756	52	705	Mobile element protein	- none -
61	733	1089	357	Mobile element protein	- none -
61	1089	1547	459	Mobile element protein	- none -
61	1606	1977	372	Mobile element protein	- none -
61	2324	2617	294	Mobile element protein	- none -
61	3986	2676	1311	Glycerol-3-phosphate-binding protein	- none -
61	5052	3979	1074	Probable sugar ABC transporter, ATP-binding protein	- none -
61	5882	5058	825	Cyclic-nucleotide phosphodiesterase (EC 3.1.4.17)	CBSS-342610.3.peg.1794, cAMP signaling in bacteria

Table A2.1 (continued)

61	6780	5893	888	Putative SN-glycerol-3-phosphate transport system permease	- none -
61	7636	6770	867	N-Acetyl-D-glucosamine ABC transport system, permease protein	- none -
61	8006	7779	228	Alcohol dehydrogenase	5-FCL-like protein, Butanol Biosynthesis, Fermentations: Mixed acid, Glycerolipid and Glycerophospholipid Metabolism in Bacteria
61	8219	8037	183	Alcohol dehydrogenase	5-FCL-like protein, Butanol Biosynthesis, Fermentations: Mixed acid, Glycerolipid and Glycerophospholipid Metabolism in Bacteria
61	8193	8387	195	Putative cytoplasmic protein	- none -
61	8620	9510	891	Dihydrodipicolinate synthase	Lysine Biosynthesis DAP Pathway, Lysine Biosynthesis DAP Pathway, GJO scratch
61	9535	9915	381	Probable translational inhibitor protein	- none -
61	9948	10913	966	2-keto-3-deoxygluconate permease (KDG permease)	D-Galacturonate and D-Glucuronate Utilization
61	11711	10959	753	Transcriptional regulator, IclR family	- none -
61	12316	12600	285	Hypothetical protein	- none -
61	12600	12875	276	FIG01069112: hypothetical protein	- none -
61	12981	13274	294	Stability (stb) locus of IncFII plasmid NR1; similar to SwissProt accession number P11907	- none -
61	14639	13470	1170	FIG00642200: hypothetical protein	- none -
61	16085	16825	741	Resolvase	- none -
61	18063	17110	954	RepFIB replication protein A	- none -
61	18857	19189	333	Mobile element protein	- none -
61	19489	19836	348	Error-prone, lesion bypass DNA polymerase V (UmuC)	DNA repair, bacterial UmuCD system
67	215	84	132	FIG00242751: hypothetical protein	- none -
67	431	718	288	FIG00638017: hypothetical protein	- none -

Table A2.1 (continued)

67	837	1658	822	Unnamed protein product	- none -
67	2463	1954	510	X polypeptide	- none -
67	3404	4081	678	Plasmid conjugative transfer regulator TraJ	Conjugative transfer, Conjugal Transfer System
67	4214	4396	183	Plasmid conjugative transfer regulator TraY	Conjugative transfer, Conjugal Transfer System
67	4430	4792	363	Plasmid conjugative transfer pilin protein TraA	Conjugative transfer, Conjugal Transfer System
67	4797	5108	312	Plasmid conjugative transfer pilus assembly protein TraL	Conjugative transfer, Conjugal Transfer System
67	5130	5696	567	Plasmid conjugative transfer pilus assembly protein TraE	Conjugative transfer, Conjugal Transfer System
67	5683	6411	729	Plasmid conjugative transfer pilus assembly protein TraK	Conjugative transfer, Conjugal Transfer System
67	6411	7862	1452	Plasmid conjugative transfer pilus assembly protein TraB	Conjugative transfer, Conjugal Transfer System
67	7852	8418	567	Plasmid conjugative transfer protein TraP	Conjugative transfer, Conjugal Transfer System
67	8405	8716	312	Plasmid conjugative transfer protein TrbD	Conjugative transfer, Conjugal Transfer System
67	8716	9231	516	Plasmid conjugative transfer pilus assembly protein TraV	Conjugative transfer, Conjugal Transfer System
67	9366	9587	222	Plasmid conjugative transfer protein TraR	Conjugative transfer, Conjugal Transfer System
67	9541	9684	144	FIG00641102: hypothetical protein	- none -
67	9747	12374	2628	Plasmid conjugative transfer pilus assembly protein TraC	Conjugative transfer, Conjugal Transfer System
67	12371	12757	387	Plasmid conjugative transfer protein TrbI	Conjugative transfer, Conjugal Transfer System
67	12856	13386	531	Plasmid conjugative transfer pilus assembly protein TraW	Conjugative transfer, Conjugal Transfer System
67	13383	14384	1002	Plasmid conjugative transfer pilus assembly protein TraU	Conjugative transfer, Conjugal Transfer System
67	14793	15098	306	Mobile element protein	- none -
67	15098	15445	348	Mobile element protein	- none -

Table A2.1 (continued)

67	15465	17036	1572	Mobile element protein	- none -
67	17112	17417	306	Conjugative transfer protein PSLT093	Conjugative transfer, Conjugal Transfer System
67	17426	18064	639	Plasmid conjugative transfer protein TrbC	Conjugative transfer, Conjugal Transfer System
67	18061	19911	1851	Plasmid conjugative transfer protein TraN	Conjugative transfer, Conjugal Transfer System
67	19938	20180	243	Plasmid conjugative transfer protein TrbE	Conjugative transfer, Conjugal Transfer System
72	39	203	165	Hypothetical protein	- none -
72	1281	259	1023	Hypothetical protein	CBSS-1496.1.peg.2937
72	2831	1266	1566	Predicted ATP-dependent endonuclease of the OLD family	CBSS-1496.1.peg.2937
72	3322	2906	417	VagD	- none -
72	3549	3319	231	Virulence-associated protein vagC	- none -
72	3930	7724	3795	Hypothetical protein	- none -
72	8185	7769	417	VagD	- none -
72	8297	8485	189	VagD	- none -
72	9190	9963	774	K88 minor fimbrial subunit faeH precursor	- none -
72	10130	11263	1134	Hypothetical protein	- none -
72	11809	11297	513	Hypothetical protein	- none -
72	12086	12199	114	Hypothetical protein	- none -
72	12376	12594	219	CcdA protein (antitoxin to CcdB)	Toxin-antitoxin replicon stabilization systems
72	12596	12901	306	CcdB toxin protein	Toxin-antitoxin replicon stabilization systems
72	12950	13708	759	Resolvase	- none -
72	13902	14021	120	Hypothetical protein	- none -
72	14482	15237	756	Replication initiation protein RepE	- none -
72	15869	15717	153	Hypothetical protein	- none -
72	15825	16991	1167	Chromosome (plasmid) partitioning protein ParA	Bacterial Cell Division, Bacterial Cytoskeleton, Cell Division Subsystem including YidCD, RNA modification and chromosome partitioning cluster
72	16991	17962	972	Chromosome (plasmid) partitioning protein ParB	Bacterial Cytoskeleton, Cell Division Subsystem including YidCD, RNA modification and chromosome partitioning cluster
72	18590	19516	927	Hypothetical protein	- none -
72	19793	19668	126	Hypothetical protein	- none -

Table A2.1 (continued)

72	19901	20584	684	Adenine-specific methyltransferase	CBSS-257314.1.peg.752
72	20585	20806	222	Putative cytoplasmic protein	- none -
72	20820	21254	435	YcgB	- none -
72	21305	22081	777	Hypothetical protein	- none -
72	22498	22923	426	Putative antirestriction protein	- none -
72	22970	23392	423	Adenine-specific methyltransferase	CBSS-257314.1.peg.752
72	23389	23580	192	Hypothetical protein	- none -
85	544	110	435	Mercuric resistance operon regulatory protein, MerR	Mercury resistance operon
85	616	966	351	Mercuric transport protein, MerT	Mercury resistance operon
85	980	1255	276	Periplasmic mercury(+2) binding protein, MerP	Mercury resistance operon
85	1300	1713	414	Mercuric transport protein, MerC	Mercury resistance operon
85	1837	3459	1623	Mercuric ion reductase, MerA	Mercuric reductase, Mercury resistance operon
85	3477	3839	363	Mercuric resistance operon coregulator	Mercury resistance operon
85	3836	4072	237	Mercuric transport protein, MerE	Mercury resistance operon
85	4108	4776	669	Tn21 protein of unknown function	- none -
85	5367	6530	1164	TniA putative transposase	CBSS-203122.12.peg.188
85	6533	7393	861	TniB NTP-binding protein	CBSS-203122.12.peg.188
85	8988	7444	1545	Mobile element protein	- none -
85	9120	10634	1515	Mobile element protein	- none -
85	10624	11406	783	Mobile element protein	- none -
85	11690	11908	219	Hypothetical protein	- none -
85	13049	12210	840	Dihydropteroate synthase	Folate Biosynthesis
85	14345	13554	792	Streptomycin 3-O-adenylyltransferase /Spectinomycin 9-O-adenylyltransferase	Aminoglycoside adenylyltransferases, aminoglycoside adenylyltransferases
85	15333	14458	876	β-lactamase	Beta-lactamase
85	15498	16511	1014	Integron integrase IntI1	Integrons
85	16864	17064	201	Mobile element protein	- none -
85	17190	17750	561	Resolvase	- none -

Table A2.1 (continued)

85	17753	20719	2967	Mobile element protein	- none -
85	20786	21163	378	YbjA protein	- none -
85	22023	21364	660	Chloramphenicol acetyltransferase	- none -
94	2541	343	2199	Aerobactin siderophore receptor IutA/TonB-dependent siderophore receptor	Siderophore Aerobactin, Siderophore assembly kit, Ton and Tol transport systems, Ton and Tol transport systems
94	3903	2626	1278	L-lysine 6-monooxygenase [NADPH], aerobactin biosynthesis protein IucD/Siderophore biosynthesis protein, monooxygenase	Siderophore Aerobactin, Siderophore assembly kit
94	5642	3900	1743	Citrate:6-N-acetyl-6-N-hydroxy-L-lysine ligase, alpha subunit, aerobactin biosynthesis protein IucA/Siderophore synthetase superfamily, group C/Siderophore synthetase component, ligase	Siderophore Aerobactin, Siderophore assembly kit
94	6589	5642	948	N6-hydroxylysine O-acetyltransferase, aerobactin biosynthesis protein IucB/Siderophore synthetase small component, acetyltransferase	Siderophore Aerobactin, Siderophore assembly kit
94	8314	6590	1725	Citrate:6-N-acetyl-6-N-hydroxy-L-lysine ligase, alpha subunit, aerobactin biosynthesis protein IucA/Siderophore synthetase superfamily, group A/Siderophore synthetase large component, acetyltransferase	Siderophore Aerobactin, Siderophore assembly kit
94	8450	9643	1194	Hypothetical protein	- none -
94	11094	10744	351	Enolase	Entner-Doudoroff Pathway, Glycolysis and Gluconeogenesis
94	12503	11646	858	Manganese ABC transporter, inner membrane permease protein SitD	- none -
94	13357	12500	858	Manganese ABC transporter, inner membrane permease protein SitC	- none -
94	14181	13354	828	Manganese ABC transporter, ATP-binding protein SitB	- none -
94	14972	14181	792	Manganese ABC transporter, periplasmic-binding protein SitA	- none -
122	118	276	159	Secreted protein, suppressor for copper-sensitivity ScsC	Copper homeostasis: copper tolerance
122	266	772	507	Membrane protein, suppressor for copper-sensitivity ScsD	Copper homeostasis: copper tolerance

Table A2.1 (continued)

122	1009	1770	762	Na(+)-translocating NADH-quinone reductase subunit C	Na(+)-translocating NADH-quinone oxidoreductase and rnf-like group of electron transport complexes
122	1767	1886	120	Hypothetical protein	- none -
122	1984	1844	141	Hypothetical protein	- none -
122	2063	4003	1941	Putative high-affinity iron permease	- none -
122	4044	4571	528	Periplasmic protein p19 involved in high-affinity Fe^{2+} transport	- none -
122	4675	6054	1380	Putative membrane protein	- none -
122	6057	7340	1284	Hypothetical ABC transporter	- none -
122	7330	8460	1131	Putative membrane protein	- none -
122	8465	9160	696	ABC transporter, ATP-binding subunit precursor	- none -
122	9147	9632	486	Putative thioredoxin precursor	- none -
122	9657	10142	486	Hypothetical protein	- none -
122	10264	10818	555	Glucose-1-phosphatase	- none -

Table A2.2. Genes present in contig 10: containing a Tn*7*-like transposable element harbouring the *sil* operon. Annotation and subsystem assignment carried out by the Rapid Annotation using Subsystem Technology (RAST) server

Start	Stop	Length (bp)	Function	Subsystems
1283	1167	117	FIG00640193: hypothetical protein	- none -
1811	1386	426	Arsenate reductase	Anaerobic respiratory reductases, Arsenic resistance, Transcription repair cluster
3113	1824	1290	Arsenic efflux pump protein	Arsenic resistance
3520	3167	354	Arsenical resistance operon repressor	Arsenic resistance
4232	4059	174	Hypothetical protein	- none -
4227	4343	117	hypothetical protein	- none -
5749	4397	1353	Glutathione reductase	Glutathione: Redox cycle
6663	5821	843	Protein involved in catabolism of external DNA	DNA processing cluster
6974	8908	1935	Oligopeptidase A	Protein degradation
8916	9668	753	Hypothetical protein	- none -
11186	9717	1470	Di/tripeptide permease DtpB	Proton-dependent Peptide Transporters
11937	11503	435	Universal stress protein A	Universal stress protein family
12382	12663	282	Universal stress protein B	Universal stress protein family
12854	12726	129	Hypothetical protein	- none -
14406	12907	1500	Low-affinity inorganic phosphate transporter	Phosphate metabolism
14638	15840	1203	NAD(FAD)-utilizing dehydrogenases	- none -
16288	17130	843	Tn7-like transposase TnsA	- none -
17162	19240	2079	Tn7-like transposase TnsB	- none -
19240	20688	1449	Tn7-like transposition protein TnsC	- none -
20729	22285	1557	Tn7-like transposition protein TnsD	- none -
22297	23223	927	Conserved hypothetical protein	- none -
24439	26265	1827	Hypothetical protein	- none -
26784	26434	351	Putative exported protein	- none -
27363	26932	432	Periplasmic silver-binding protein SilE	- none -
29089	27608	1482	Putative membrane histidine-kinase silver-sensor SilS	- none -
29762	29082	681	Putative transcriptional regulator/responder SilR	- none -
29952	31337	1386	Putative silver-efflux system, outer membrane protein SilC	- none -
31365	31718	354	Periplasmic silver-binding protein SilF	- none -
31832	33124	1293	Putative silver-efflux system, membrane-fusion protein SilB	- none -

Table A2.2 (continued)

33135	36281	3147	Putative silver-efflux system, proton/cation antiporter SilA	- none -
36368	36808	441	CopG protein	- none -
36936	39377	2442	Putative cation-tranporting P-type ATPase SilP	- none -
39418	39615	198	FIG00643033: hypothetical protein	- none -
40386	39649	738	Cell wall endopeptidase, family M23/M37	Glutaredoxins
41110	40661	450	Hypothetical protein	- none -
41345	43162	1818	Multicopper oxidase	Copper homeostasis
43168	44058	891	Copper resistance protein, PcoB	Copper homeostasis
44140	44478	339	Copper resistance protein PcoC	Copper homeostasis
44590	44465	126	Hypothetical protein	- none -
44669	45412	744	Copper resistance protein PcoD	Copper homeostasis
45467	46147	681	Transcriptional regulatory protein PcoR	- none -
46144	47544	1401	Copper sensor histidine kinase, PcoS	Cobalt-zinc-cadmium resistance
47760	48194	435	Periplasmic copper-binding protein PcoE	- none -
49559	48552	1008	FIG00638244: hypothetical protein	- none -
49797	50000	204	FIG01068562: hypothetical protein	- none -
50163	50044	120	FIG00638049: hypothetical protein	- none -
50218	51225	1008	FIG00638558: hypothetical protein	- none -
51855	53477	1623	FIG00637996: hypothetical protein	- none -
53667	53780	114	Hypothetical protein	- none -
53843	54910	1068	Putative membrane protein	- none -
54907	57642	2736	ABC-type multidrug transport system, permease component	- none -
57642	58766	1125	Putative transporter	- none -

Appendix 3. List of publications

The following publications have arisen as a result of work carried out during the course of my PhD studies:

Randall CP; Mariner KR; Chopra I; O'Neill AJ. The target of daptomycin is absent from Escherichia coli and other Gram-negative pathogens. *Antibacterial Agents and Chemotherapy* **57;** 637-639, 2013

Randall CP; Oyama LB; Bostock JM; Chopra I; O'Neill AJ The silver cation (Ag+): antistaphylococcal activity, mode of action and resistance studies. *Journal of Antibacterial Chemotherapy* **68** 131-138, 2013.

Randall C; Oyama L; Bostock J; Chopra I; O'Neill AJ. Antibacterial mode of action and resistance studies with the silver cation (Ag^{+}) in *Staphylococcus aureus*. Presented at the 21st European congress of clinical microbiology and infectious diseases 7-10th May 2011. Poster presentation, abstract number P1454