THE ACTIVITY, MODE OF ACTION AND GENERATION OF RESISTANCE TO NOVEL ANTIBACTERIAL AGENTS

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This candidate confirms that the work submitted is her own and that the appropriate credit has been given where reference has been made to the work of others

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Abstract

The failure of antibiotics to treat infections caused by multi-drug resistant bacteria is a significant problem in the field of antimicrobial chemotherapy. The characterisation and development of antibacterial agents displaying novel modes of action (MOAs) or the modification of existing antibiotic scaffolds may address this problem. This study therefore sought to identify antibiotic candidates, establishing their antibacterial activity, bacterial specificity, MOA and propensity for resistance development. From nearly half a million compounds which were screened *in silico* against RNA polymerase (RNAP), D-alanine: D-alanine ligase and peptidoglycan transglycosylases, no inhibitors with specific activity against their target were identified, which highlights the difficulties of developing novel antibacterial agents. However, targeted inhibition of the cell envelope and RNAP were observed for the type B lantibiotic derivative NVB353 and corallopyronin A, respectively. The former may show greater promise as a chemotherapeutic candidate, due to lower propensity for resistance development. In addition, a number of compounds which appear to damage the bacterial cell membrane specifically were identified, and which may be suitable for treatment of persistent bacterial infections. Transcriptional profiling of *Staphylococcus aureus* treated with a panel of known membrane damagers was also used to identify upregulated genes which might be potential candidates for future development of biosensors solely responsive to membrane damage. These biosensors could be used to eliminate compounds which are likely to cause non-specific toxic side effects if administered to humans, but may also identify membrane damaging agents that could be developed for clinical use should they show bacterial specificity. The promoters of the genes encoding a single strand DNA-binding protein and the *kdp* (potassium transporting ATPase subunit) operon were

upregulated by known membrane damagers and therefore may be manipulated for future biosensor construction in an attempt to identify potential therapeutic agents to meet the challenge of the spread of resistant bacteria.

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Abbreviations

8-HQ	8-hydroxquinoline
ABC	ATP binding cassette
Abu	2-aminobutyric acid
Ac	Acetyl
ACP	Acyl Carrier Protein
Ala	Alanine
ArgJ	Arginine Biosynthesis Protein J
ASM	American Society of Microbiology
Asn	Asparagine
ATP	Adenosine triphosphate
BBSRC	Biotechnology and Biological Sciences Research Council
BGSC	*Bacillus* genetic stock center
BSAC	British Society for Antimicrobial Chemotherapy
Ca	Calcium
CA-MRSA	Community-acquired meticillin-resistant *Staphylococcus aureus*
CCCP	cyanide m-chlorophenyl hydrazone
cDNA	Complementary DNA
Clp	Caseinolytic protease
CLSI	Clinical Laboratory Standards Institute
CorA	Corallopyronin A
CTAB	Cetyltrimethylammonium bromide
DapE	N-succinyl-L-diaminopimelate-D-succinylase subunit E
Ddl	D-alanine:D-alanine ligase
dH_20	Distilled water
Dha	Dehydroalanine
Dhb	Dehydrobutyrine
DHF	Dihydrofolate
DHP	Dihydropteroate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
dTMP/dTTP	Deoxythymidine monophosphate/triphosphate
dUTP	Deoxyuridine triphosphate
EF-G	Elongation factor G
ESBL	Extended-spectrum β-lactamases
EtOH	Ethanol
FDA	Food and Drug Administration
FLIPr	Fluorescence imaging plate reader
fMET	Formyl-methionine
FMN	Flavin mononucleotide
GAF	Gamma interferon activation factor
GBS	Group B streptococci
GDP	Guanosine diphosphate
GlcNAc	N-acetyl glucosamine

Glu	Glutamate
Gly	Glycine
GMP	Guanosine monophosphate
GntR	Gluconate utilisation system repressor
GT	Glycosyltransferase
GTP	Guanosine triphosphate
Gyr	Gyrase
Hb	Haemoglobin
HCl	Hydrocholoric Acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HIV	Human Immuno Deficiency Virus
IC_{50}	Half maximal inhibitory concentration
IcaB	Intracellular adhesion protein B
Ile	Isoleucine
K^+	Potassium ion
KCl	Potassium chloride
Kdp	Potassium-transporting ATPase subunit
Lac	Lactate
LB	Luria Bertani
Leu	Leucine
LPS	Lipopolysaccharide
Lys	Lysine
LysM	Lysin motif
MarR	Multiple antibiotic resistance protein R
MBC	Minimum Bactericidal Concentration
MFS	Major Facilitator Superfamily
$MgCL_2$	Magnesium chloride
MHA	Müeller Hinton Agar
MHB	Müeller Hinton Broth
Met	Methionine
MIC	Minimum Inhibitory Concentration
MLS	Macrolide-Lincosamide-Streptogramin B
MOA	Mode of action
M_r	Relative molecular mass
MRSA	Meticillin-resistant *Staphylococcus aureus*
MSSA	Meticillin-susceptible *Staphylococcus aureus*
MurNAc	N-acetyl muramic acid
MutS	DNA mismatch repair protein
MyxA	Myxopyronin A
MyxB	Myxopyronin B
Na	Sodium
NAD(H)	Nicotinamide Adenine Dinucleotide (H)
NCI	National Cancer Institute
Ndh	NADH Dehydrogenase
NDM-1	New Delhi Metallo β-lactamase 1

NHS	National Health Service
NO	Nitric Oxide
OD	Optical Density
PABA	Para amino benzoic acid
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCR	Polymerase Chain reaction
Pdf	Peptide deformylase
PEP	Phosphoenolpyruvate
PGI	Phosphoglucose isomerase
Phe	Phenylalanine
PhoU	Negative regulator of phosphate transport system protein
PMBN	Polymyxin B nonapeptide
PO_4	Phosphate
Pro	Proline
PTS	Phosphate transport system
PVL	Panton-Valentine Leukocidin
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
R&D	Research and development
RibE	Riboflavin synthase β chain
RNA	Ribonucleic acid
RNAP	RNA polymerase
RND	Resistance nodulation division
RPMI	Roswell Park Memorial Institute
rpoA/B/C	RNA polymerase subunit α/β/β'
rRNA	Ribosomal RNA
SAICAR	2-[(5-amino-1-{3,4-dihydroxy-5-[(phosphonooxy) methyl] oxolan-2-yl}*1H*- imidazol-4-yl) formamido] butanedioic acid
SBDD	Structure-based drug design
SDS	Sodium dodecyl sulphate
Ser	Serine
ssrA RNA	Transfer-messenger RNA
TCA	Trichloroacetic acid
THF	Tetrahydrofolate
tmRNA	Transfer-messenger RNA
TP	Transpeptidase
tRNA	Transfer RNA
Trp	Tryptophan
TSB	Tryptone Soy Broth
UdP	Undecaprenyl phosphate
UDP	Uridine diphosphate
Val	Valine
VISA	Vancomycin intermediate *Staphylococcus aureus*
VRE	Vancomycin-resistant enterococci
WHO	World Health Organization

Chapter 1 – General Introduction

1.1 Introduction to antibiotics

'Antibiotics' are agents produced by bacteria or fungi that in low concentration inhibit the growth of, or kill, other microorganisms. However, the definition of the word antibiotic has now been extended to also include a variety of semi-synthetic and totally synthetic molecules (Fischbach & Walsh, 2009). At the beginning of the twentieth century, bacterial infections (especially tuberculosis, pneumonia and diarrhoeal disease) were among the top global causes of mortality (Wenzel & Edmond, 2000). The clinical introduction of antibiotics revolutionised the treatment and prevention of infection related illness, greatly reduced the burden of infectious diseases and contributed significantly to a thirty year average increase in life expectancy (Conly & Johnston, 2005). For example, Group B streptococci (GBS) were responsible for a fifty five per cent mortality rate in patients with neonatal sepsis in the United States at the beginning of the 1970s, which due to antibiotic prophylaxis and therapy regimes had dropped to less than five per cent by 2000 (Dermer *et al.*, 2004). Alexander Fleming's discovery of penicillin in 1928 and the development of the first commercially available sulphonamide antibiotic Prontosil (sulphanilamide) in 1932 led to antibiotic therapy becoming an integral part of medical practice (Davies, 2006). The rapid discovery and introduction of further antibiotic classes (e.g. aminoglycosides [1944], tetracyclines [1950], macrolides [1952] and glycopeptides [1956]) in the 'golden era' of antibiotic discovery accompanied by the refinement of manufacturing processes lead to further widespread production and use of antibiotics (Alanis, 2005; Conly & Johnston; 2005).

1.2 Mode of action (MOA) of antibacterial agents

Antibacterial agents are broadly classified into one of two groups according to whether they kill (bactericidal) or inhibit the growth (bacteriostatic) of bacteria, but may be further separated into more than fifteen distinct classes according to their chemical structure, antibacterial target and mechanism of action (Walsh, 2003). Currently classified antibacterial agents interfere with one of seven cellular targets, as outlined in Figure 1.1. Since the main theme of this thesis is characterisation of the mode of action of novel and underdeveloped antibacterial agents, the mechanism of antibacterial activity of established inhibitors (which will be used as comparator agents) is discussed and described in detail in the following pages.

1.2.1 Inhibitors of cell wall biosynthesis

The bacterial cell wall is composed of a layer of peptidoglycan (PG) which allows the cell to maintain internal osmotic pressure, rigidity and a defined shape (Vollmer *et al.*, 2008). Cell-wall biosynthesis is an attractive antibacterial target due to the unique nature of PG as a prokaryotic structure. Indeed many clinically available antibiotics target the synthesis of the cell wall of bacteria (Figure 1.2) (Bugg *et al.*, 2011). PG comprises β-linked chains of alternating sugars, namely N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) which are cross-linked via pentapeptides (L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala in Gram-positive bacteria, and L-Ala-γ-D-Glu-meso-diaminopimelic acid -D-Ala-D-Ala in Gram-negative bacteria) covalently attached to MurNAc (Schleifer & Kandler; 1972). Figure 1.3 shows the structure of Gram-positive PG and the mechanism of its biosynthesis in more detail.

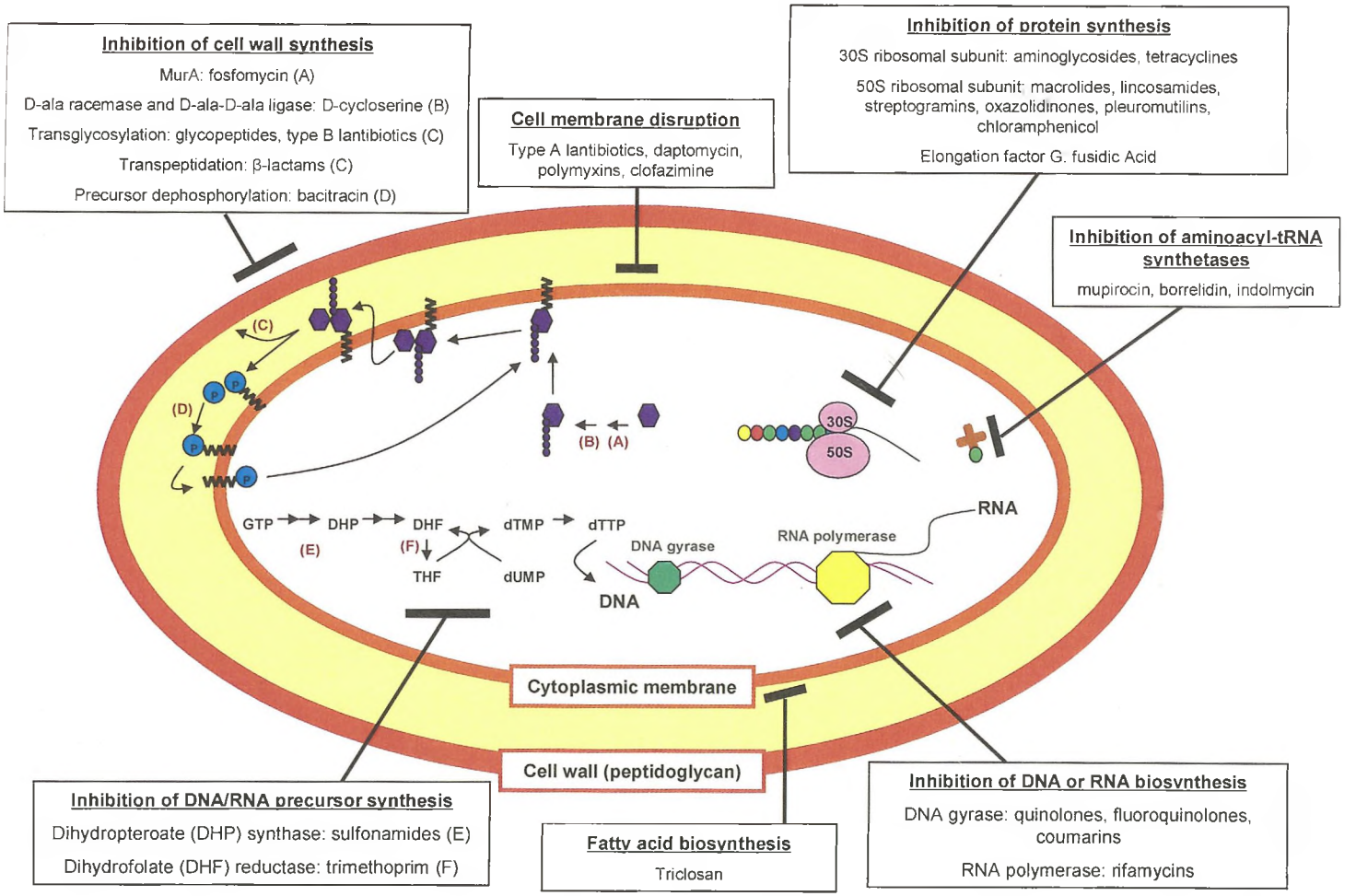


Figure 1.1 – Principal antibacterial agents with established modes of action and their target sites (Adapted from Walsh, 2003)

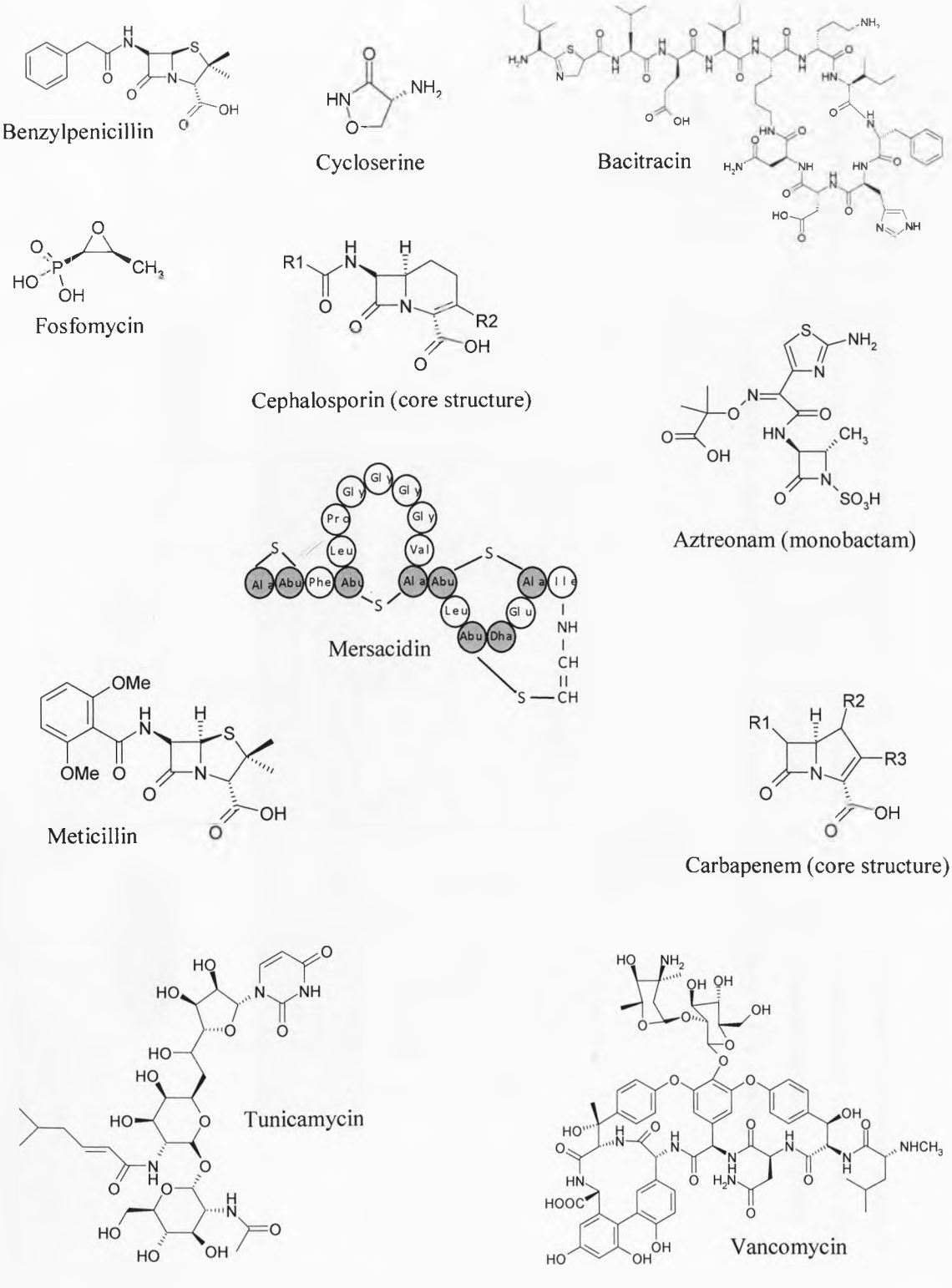


Figure 1.2 – Molecular structures of PG biosynthesis inhibitors.

Abu: 2-aminobutyric acid, Dha: dehydroalanine

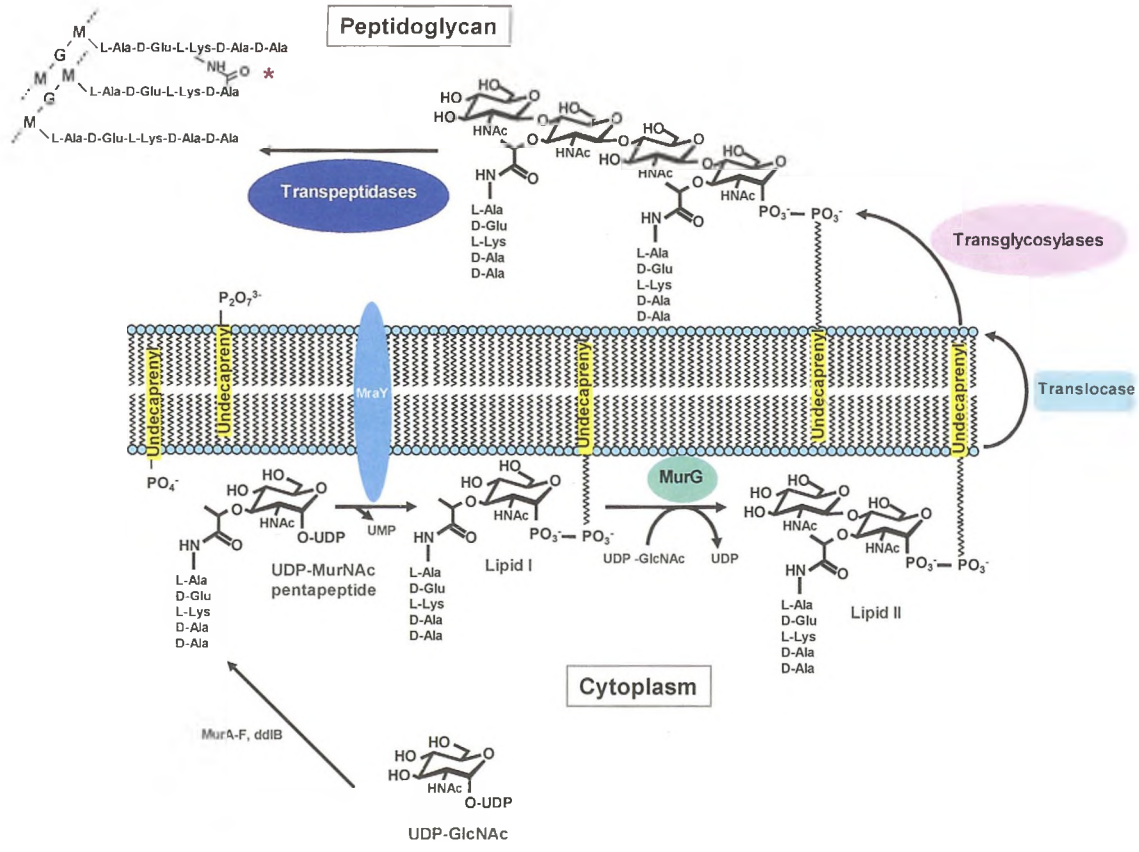


Figure 1.3 – Schematic of PG biosynthesis in Gram-positive bacteria

MurNAc: N-acetylmuramic acid, GlcNAc: N-acetylglucosamine (Adapted from Fang et al. 2006)

* *In Staphylococcus aureus there is no direct cross linkage between neighbouring pentapeptide chains, the cross link is created by the reaction of a nucleophilic terminal amine of a pentaglycine bridge (attached to Lys3 of the pentapeptide) with a neighbouring terminal D-ala.*

The first step of PG biosynthesis involves the reaction of UDP-GlcNAc with its co-substrate phosphoenolpyruvate (PEP) to form MurNAc, a process which is catalysed by phosphoenolpyruvate: UDP-GlCNAc-3-O-enolpyruvyltransferase (MurA) (Kahan *et al.*, 1974). Fosfomycin, a well-established PG biosynthesis inhibitor, targets MurA by acting as an inactivating analogue of PEP, binding covalently and irreversibly to the enzyme via the active site cysteine residue at position 115 in *Escherichia coli* (Eschenberg *et al.*, 2005; Walsh, 2003). The subsequent addition of three of the residues in the pentapeptide moiety to MurNAc is catalysed by MurC, D and E (Walsh, 2003). MurF adds on the terminal D-ala-D-ala as a dipeptide, which is formed from the conversion of L-alanine to D-alanine by alanine racemase and formation of an amide bond by D-alanyl-D-alanine ligase, both enzymes which are inhibited by D-cycloserine (Neuhaus & Hammes, 1981). After cytoplasmic synthesis of MurNAc-pentapeptide, the molecule is attached to the C55- undecaprenylphosphate carrier (catalysed by MraY, inhibited by tunicamycin) to form the Lipid I complex, which is further linked to GlcNAc to form Lipid II (Bugg *et al.*, 2011; Navarre & Schneewind, 1999). The addition of five glycines to L-Lysine of the pentapeptide is then followed by amidation of D-glutamate as the final stage of the PG precursor synthesis in *Staphylococcus aureus* (Linnett & Strominger, 1974). After the translocation of the precursor in the form of the Lipid-II complex across the cell membrane, cleavage of the C55 lipid carrier and linkage of the disaccharides into the cell wall polysaccharide (transglycosylation) occurs, followed by the cross-linkage of the pentapeptide with existing cell wall peptides (transpeptidation).

Transglycosylation is inhibited by both the glycopeptides and the globular type B lantibiotics. The glycopeptides (e.g. vancomycin, teicoplanin) bond via hydrogen bonds

to the D-Ala-D-Ala termini of the pentapeptides in the cell wall precursor (Perkins, 1969; Barna & Williams, 1984). As a result of the substrate binding action of glycopeptides to the D-Ala termini, the transpeptidases are unable to complete cross-linking and the steric hindrance of the bulky glycopeptide on the lipid II complex concurrently inhibits transglycosylation (Walsh, 2003; Anderson *et al.*; 1967). The type B lantibiotics (e.g. mersacidin and actagardine) inhibit transglycosylation by interaction with the pyrophosphate moiety of Lipid II (Brotz *et al.*, 1997; Brotz *et al.*, 1998). In the case of mersacidin, complexation of Ca^{2+} by Glu-17 bridges the anionic pyrophosphate of Lipid II and the lantibiotic, increasing the binding affinity (Bauer & Dicks, 2005), preventing precursor incorporation into PG and lipid II accumulation in the membrane.

The cellular transpeptidases are inhibited by β-lactam antibiotics (penicillins, cephalosporins, monobactams and carbapenems). The β-lactams act as suicide substrates for the transpeptidases, covalently bonding to the active site serine, rendering the enzymes inactive (Ghuysen, 1991). As for the glycopeptides, the reduction in cross-linkage leads to stimulation of cellular hydrolases and autolysins, disrupting the cellular osmotic pressure and causing cell lysis (Tomasz & Waks, 1975).

In the ultimate stages of PG biosynthesis, the C55 lipid pyrophosphate is hydrolysed by a membrane-bound phosphatase (inhibited by bacitracin) (Stone & Strominger, 1971) and recycled back to the intracellular face of the cytoplasmic membrane, completing the cycle (Bugg & Walsh, 1992).

1.2.2 Inhibitors of protein biosynthesis

Proteins have an essential role in the maintenance of bacterial structure and function. As such, there are a variety of both bactericidal and bacteriostatic antibiotic classes which target the protein biosynthetic pathway (Figure 1.4). The bacterial 70S ribosome, the ribozyme which catalyses protein synthesis, consists of two subunits (designated the 30S and 50S subunits) comprising ribosomal RNA (16S for the 30S subunit, 23S and 5S for the 50S subunit) and proteins (Clemons, 1999; Korostelev, 2006). The larger rRNA constituents are central to maintenance of ribosomal structure, recognition and catalysis (Dale & Park, 2010). The lack of structural homology between the 70S bacterial ribosome and the larger 80S eukaryotic ribosome permits selective targeting of bacterial protein synthesis at the level of the ribosome (Chopra, 1998).

Tetracyclines are broad-spectrum antibiotics which act on the 30S ribosomal subunit, preventing binding of an aminoacyl-tRNA to the aminoacyl (A) site of the peptidyltransferase centre (Chopra & Roberts, 2001). Electrostatic interactions mediate the binding of tetracyclines to the 16S rRNA via an Mg^{2+} ion bridge, and as such the presence of the antibiotic at this site inhibits rotation of the aminoacyl-tRNA into the A site (Pioletti *et al.*, 2001).

Aminoglycosides (e.g. gentamicin, streptomycin) are bactericidal protein synthesis inhibitors which also target the 30S ribosomal subunit. The generic structure of this class includes multiple carbohydrate rings which contain protonated amine groups (at physiological pH), available to electrostatically bind to 16S rRNA at the A site, preventing binding of an aminoacyl-tRNA (Carter *et al.*, 2000). The bactericidal activity

of this class of antibiotics is thought to arise from additional membrane damage, caused during entry of the agent to the cell and deposition of misread proteins in the lipid bilayer (Davis, 1987).

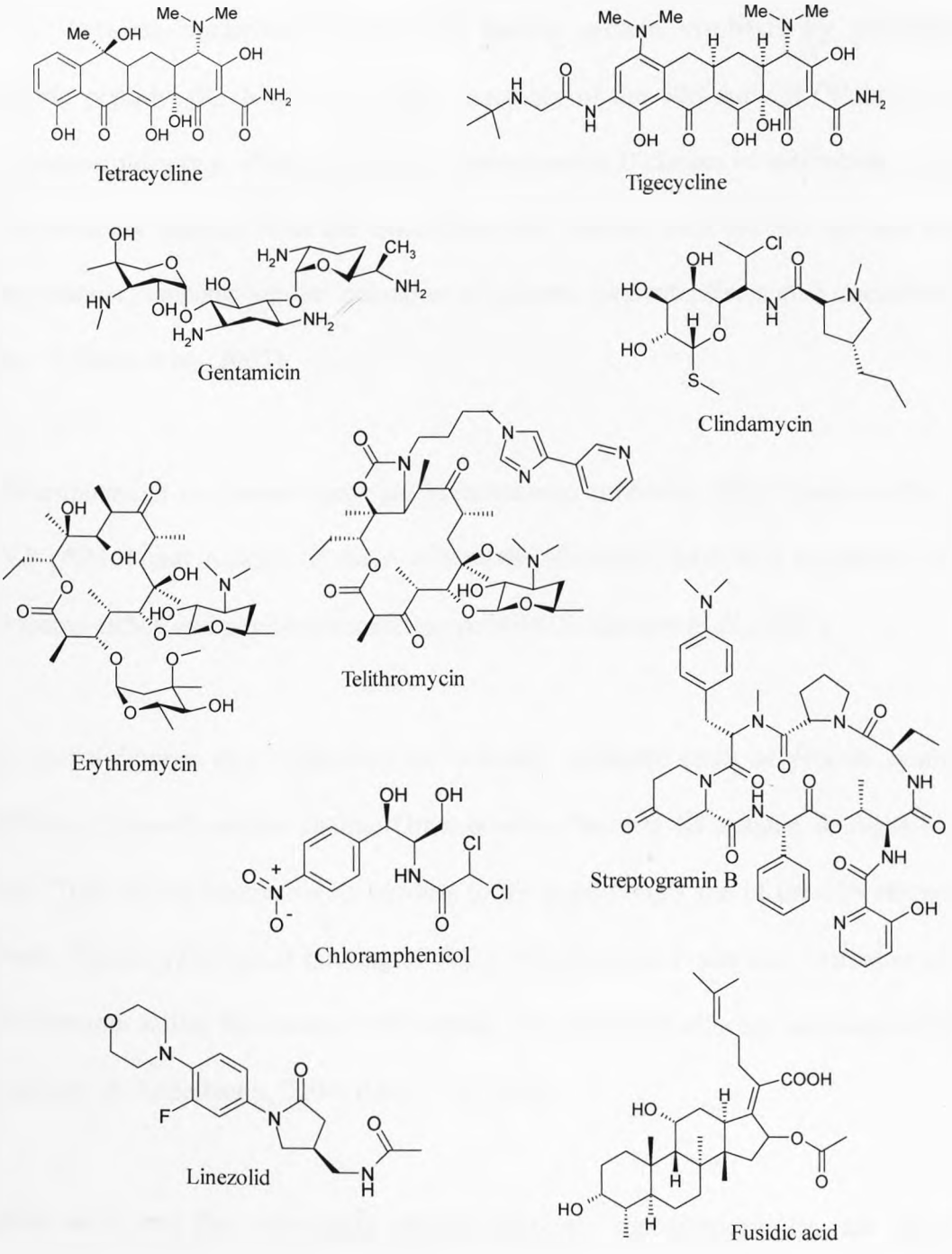


Figure 1.4 - Molecular structures of protein biosynthesis inhibitors

The larger 50S ribosomal subunit is the target of macrolides, lincosamides, chloramphenicol, streptogramins and oxazolidinones. The macrolide class (e.g. erythromycin, azithromycin) and structurally related ketolides (e.g. telithromycin) are natural product antibiotics that contain a 14-16 membered macrocyclic lactone fused to two carbohydrate rings. The sugar moieties interact specifically with the 23S ribosomal rRNA (between nucleotides 2058-2062) halting protein synthesis by prematurely releasing peptidyl-tRNA and preventing assembly of the 50S subunit (Walsh, 2003). The lincosamides (e.g. clindamycin) and streptogramin B classes of antibacterial agents are structurally distinct from the macrolides, but interact with the 50S subunit in the same manner, causing similar cessation of protein biosynthesis, and a bacteriostatic effect (Tenson *et al.*, 2003).

Chloramphenicol is a broad-spectrum bacteriostatic antibiotic, which binds to the 23S rRNA (A2451 and A2452) of the A site of the ribosome, inhibiting interaction of an aminoacyl-tRNA and peptidyl transferase activity (Schlunzen *et al.,* 2001).

The oxazolidinones (e.g. linezolid) are a totally synthetic class of protein synthesis inhibitors, primarily active against Gram-positive bacteria (Bozdogan & Appelbaum, 2004). They act by competitively binding to the peptidyl (P) site of the 50S ribosomal subunit, blocking the initial binding of fMET-tRNA to the P site and formation of the 70S ribosome and/or the creation of a peptide bond between adjacent aminoacyl-tRNAs (Bozdogan & Appelbaum, 2004; Patel *et al.*, 2001).

Fusidic acid, and the structurally related antibiotic cephalosporin P1, are steroidal antibacterial agents which interfere with the protein elongation step. By binding to

elongation factor G (EF-G), a protein responsible for translocation on the ribosome of the nascent polypeptide chain, they stabilise the protein in the inactive form of EF-G/GDP thus inhibiting the GTPase activity of EF-G and preventing elongation (Collignon & Turnidge, 1999; O'Neill *et al.*, 2002).

1.2.3 Inhibitors of bacterial aminoacyl-tRNA synthetases

The enzymatic activity of the aminoacyl-tRNA synthetases utilises ATP to catalyse the biosynthesis of the amino-acyl-tRNAs, molecules which are used by the ribosome to add the twenty amino acids into a growing polypeptide chain (Woese, 2000). Inhibition of the tRNA synthetases accumulates uncharged tRNA molecules which bind to the ribosome, leading to a bacteriostatic effect due to cessation of protein biosynthesis (Hurdle *et al.,* 2005). The isoleucyl tRNA synthetase inhibitor mupirocin (Figure 1.5) is an analogue of the isoleucyl-adenylate intermediate which prevents binding of both isoleucine and ATP to the enzyme (Nakama *et al.,* 2001). In addition, it has been shown that the activity of tryptophanyl-tRNA synthetase and threonyl-tRNA synthetase are inhibited by indolmycin and borrelidin respectively (Figure 1.5) (Kim *et al.*, 2003).

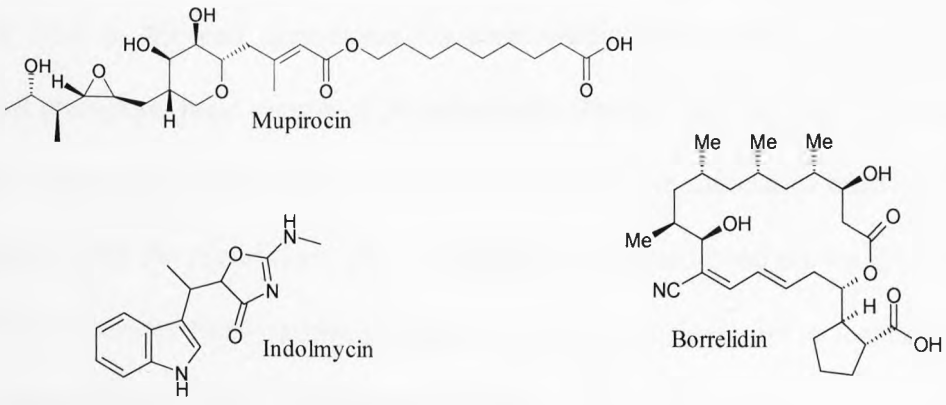


Figure 1.5 - Molecular structures of aminoacyl-tRNA synthetase inhibitors

1.2.4 Inhibitors of cell membrane structure and function

Of the wide variety of antimicrobial peptides which perturb bacterial cell membranes (Figure 1.6), only a few, including polymyxin and the cyclic lipopeptide daptomycin have been approved for clinical use. This is due to the fact that the majority of membrane-damaging compounds are cytotoxic in mammals (Kosmidis & Levine, 2010, Pacor *et al.*, 2002). Daptomycin, however, displays selectivity for bacterial membranes (Silver, 2007), utilising Ca^{2+} ions to bridge the anionic charges of the daptomycin molecule and the cell membrane, in order to bind to and insert itself into the bilayer (Jung *et al.*, 2004). The oligomerisation of daptomycin molecules forms a pore, leading to rapid loss of intracellular K^{+}, membrane depolarisation and ultimately cell death (Silverman *et al.*, 2003).

The type A lantibiotics (e.g. nisin) are amphipathic screw-shaped peptides, between twenty and thirty four amino acids in length, with an overall positive charge (McAuliffe *et al.*, 2001). The primary MOA of this class has been well characterised, and involves the ATP-dependent formation of transmembrane pores (McAuliffe *et al.*, 2001). Specifically, the cationic C-termini of a number of positively charged nisin molecules tightly bind to the cell membrane via electrostatic interactions with the negatively charged phosphate head groups of phospholipids (Breukink *et al.*, 1997). Following this initial interaction, the hydrophobic N-termini of the nisin molecules insert in a parallel orientation into the membrane, putting localised strain and bend on the bilayer, so that the nisin molecules finally adopt a membrane spanning orientation to form a short-lived pore approximately 1 nm in diameter (Sahl *et al.*, 1987). The presence of the pores leads

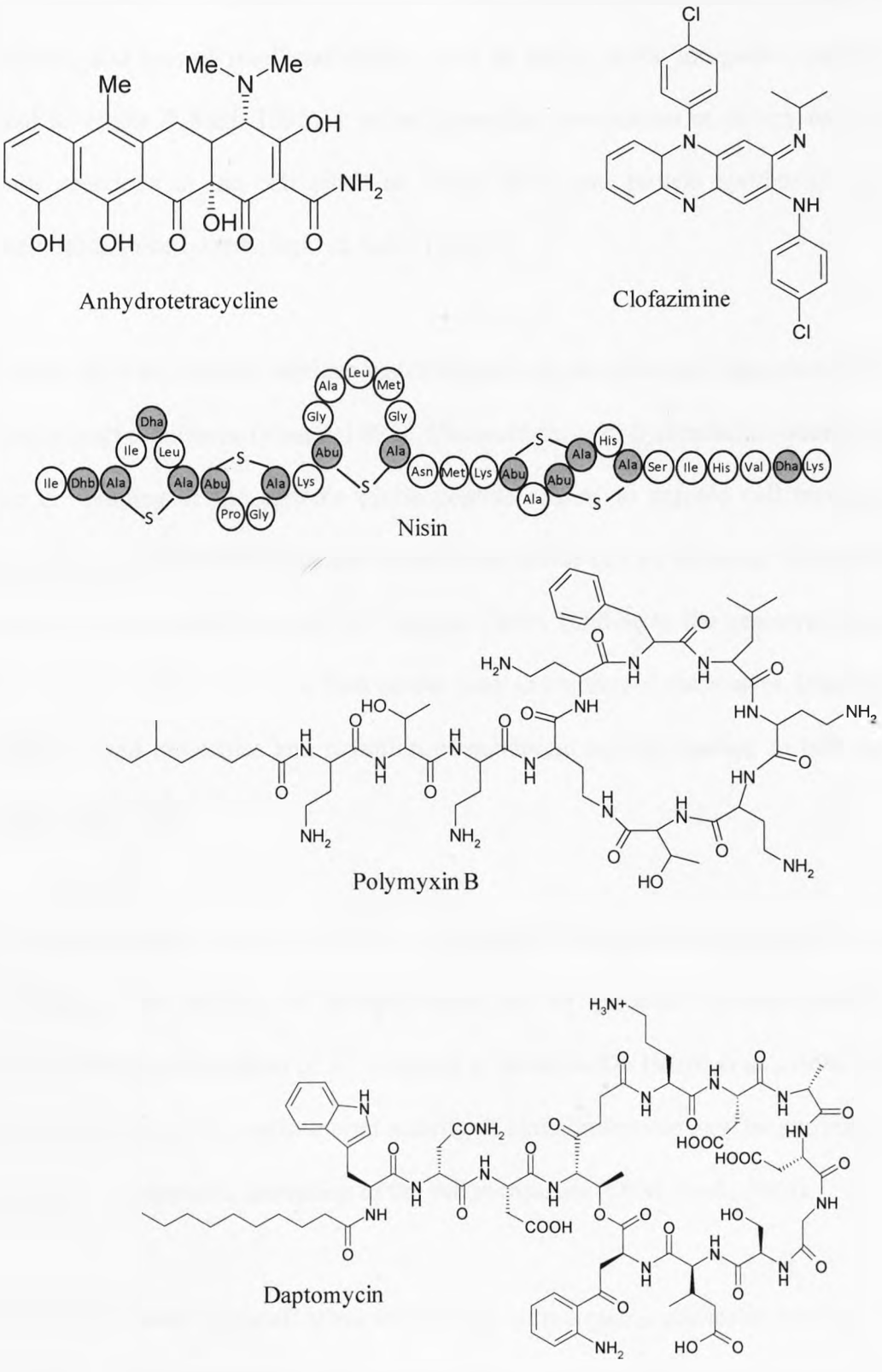


Figure 1.6 – Molecular structures of membrane-damaging agents

Abu: 2-aminobutyric acid, Dha: dehydroalanine, Dhb: dehydrobutyrine

to rapid dissipation of the proton motive force (i.e. loss of transmembrane potential and pH gradient) and loss of small metabolites such as amino acids, inorganic phosphate, ATP and K^+ (Ruhr & Sahl, 1985). It is the immediate termination of energy requiring metabolic reactions in the cell (such as DNA, RNA and protein synthesis) which ultimately leads to cell death (Ruhr & Sahl, 1985).

Polymyxins are also cationic peptides which target both the outer and inner membranes of Gram-negative bacteria (Vaara, 1992). Electrostatic and hydrophobic interactions mediate the binding of the cationic cyclic peptide moiety to anionic cell membrane components such as phospholipids and lipopolysaccharide (LPS), allowing the peptides to traverse the membrane (Hancock & Chapple, 1999). Binding to the negative charges on the inner side of the membrane then causes local disruption of the bilayer, leakage of intracellular small molecules and cessation of metabolic activity leading to cell death (Vaara & Vaara, 1983).

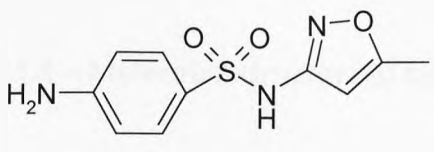
The anti-mycobacterial drug clofazimine was thought to disrupt cell membrane function by stimulating the activity of phospholipase A2 to generate lysophospholipids, ultimately leading to disruption of K^+ transport in bacteria (De Bruyn *et al.*, 1996, Steel *et al.*, 1999). However, the antibacterial activity of clofazimine can now be attributed to more general, non-specific disruption of the cell membrane (Oliva *et al.*, 2004).

A group of designated 'atypical' tetracyclines (e.g. anhydrotetracycline) do not have the same MOA as the classical tetracyclines (inhibition of protein biosynthesis at the level of the ribosome), and have therefore been shown to have activity against tetracycline-resistant species (Oliva & Chopra, 1992). In contrast to classical

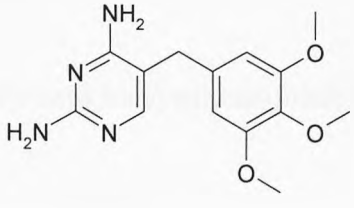
tetracyclines, the atypical tetracyclines are bactericidal due to perturbation of the cell membrane (Oliva *et al.*, 1992). However it is currently unclear whether they also maintain activity against the bacterial ribosome.

1.2.5 Inhibition of DNA/RNA precursor synthesis

The synthetic antibiotics that have been in longest clinical use are the sulphonamides (e.g. sulfamethoxazole) which are often administered in combination with trimethoprim (Figure 1.7) (Howe & Spencer, 1996). Both drugs target separate steps in folic acid synthesis (Figure 1.1), blocking the enzymatic activities of dihydropteroate (DHP) synthase and dihydrofolate (DHF) reductase respectively (Walsh, 2003). DHP is synthesised via a two step reaction from GTP and paraaminobenzoic acid (PABA) and as the sulphonamides are structural mimics of PABA, they bind competitively to and inhibit the DHP synthase active site (Dax, 1997). Trimethoprim blocks the reduction of 7, 8-Dihydrofolate to 5, 6, 7, 8-tetrahydrofolate (THF) by DHF reductase (Hitchings, 1973). THF is a co-substrate for the formation of thymidine monophosphate (dTMP); therefore inhibition of this pathway leads to a direct reduction in thymidine synthesis, which consequently disrupts DNA replication in the cell (Walsh, 2003).



Sulfamethoxazole

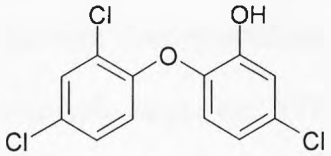


Trimethoprim

Figure 1.7 – Molecular structures of DNA/RNA precursor synthesis inhibitors

1.2.6 Inhibition of fatty acid biosynthesis

Fatty acid elongation in bacteria is a four step reaction, catalysed by fatty acid synthases, encoded by *fab* genes (Campbell & Cronan, 2001). The initial step involves the condensation of malonyl-ACP (Acyl Carrier Protein) and acetyl-ACP (mediated by FabH) to form 3-keto-acetyl-ACP, followed by a reduction to D-3-hydroxybutyryl hydroxylase-ACP (by FabG). In the third step, D-3-hydroxybutyryl hydroxylase-ACP is dehydrated to enoyl-ACP (by FabA and FabZ), which is reduced in the final step to butyryl-ACP, by enoyl-ACP reductase I (FabI). The newly formed butyryl adduct then reacts with further malonyl-ACPs (FabB/F) to elongate the fatty acid chain, until a 16C chain product is formed (palmitate) (Campbell & Cronan, 2001). Triclosan (Figure 1.8), a broad-spectrum antibiotic, inhibits fatty acid biosynthesis at the final step, by binding to both FabI and its NAD^+ cofactor, forming a stable complex which halts fatty acid synthesis by preventing the FabI substrate reaching the active site (Heath *et al.*, 1999).



Triclosan

Figure 1.8 – Molecular structure of triclosan, a fatty acid biosynthesis inhibitor

1.2.7 Inhibition of DNA or RNA biosynthesis

DNA replication and repair is essential to the growth and survival of any organism. In bacteria, topoisomerases are responsible for the supercoiling of DNA (Walsh, 2003). DNA gyrase (topoisomerase II) catalyses the separation of chromosomes by introducing negative supercoiling, allowing replication initiation proteins to bind to the DNA (Kampranis & Maxwell, 1998) and DNA topoisomerase IV mediates decatenation, a process by which the linkage between chromosomes is broken, allowing the DNA to separate into two daughter cells (Liu, 1994). Bacterial DNA replication is the antibacterial target of quinolones, fluoroquinolones and coumarins (Figure 1.9). The quinolones (e.g. nalidixic acid) and fluoroquinolones (e.g. ciprofloxacin) are totally synthetic broad-spectrum classes of antibacterial agent (Richard, 1992). They inhibit DNA gyrase and DNA topoisomerase IV by binding to the enzyme–DNA complex inducing conformational changes which stabilises the complex in an intermediate form where the DNA contains a double strand break, halting continuation of replication (Blondeau, 2004). With cessation of DNA replication, cell death rapidly results (Gorbach *et al.*, 2004). The coumarin class of antibacterial agents (e.g. novobiocin) also inhibit DNA gyrase, but alternatively target the ATPase activity of the GyrB subunit (Maxwell, 1993).

The rifamycins, and semisynthetic derivatives thereof (e.g. rifampicin) (Figure 1.9) are the only clinically used antibiotics which target RNA polymerase (RNAP), the enzymatic catalyst of bacterial transcription (Chopra, 2007). The core structure of RNAP is composed of an α, β, β' and γ subunit, which associates with transcriptional regulators (sigma factors) to form the holoenzyme (Vassylyev *et al.*, 2002). The β

subunit is the target for rifampicin, which binds via hydrophobic interactions and hydrogen bonding to the rifamycin binding region in the DNA/RNA tunnel, more than 12Å from the enzyme active site directly blocking elongation of RNA beyond 2-3 nucleotides (Campbell *et al.*, 2001).

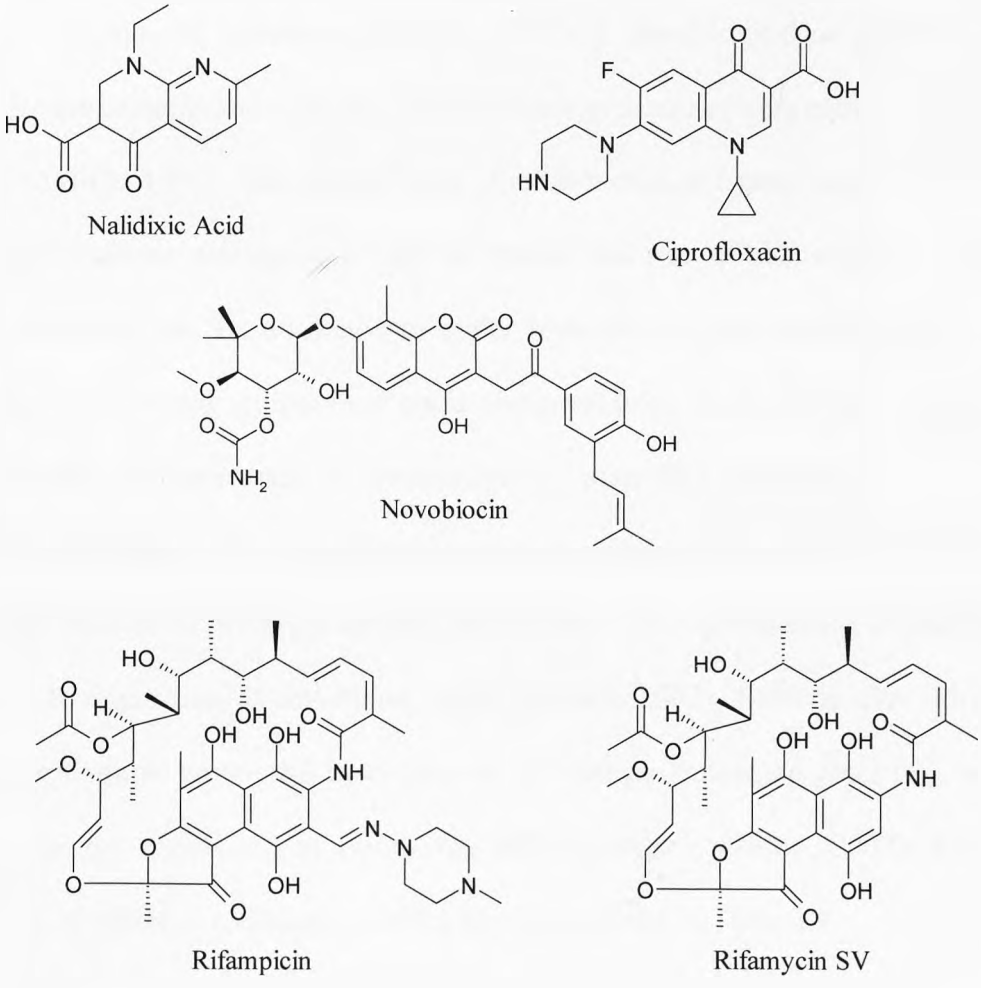


Figure 1.9 - Molecular structures of DNA and RNA synthesis inhibitors

1.3 Antibiotic resistance

1.3.1 Introduction to antibiotic resistance

Since the discovery of penicillin in 1928, the widespread use of antibacterial agents in medicine and the food and agricultural industries has lead to the selection and emergence of antibiotic resistance (Hawkey, 2008); a physiological or genetic-based capacity for the bacterium to withstand the long-term presence of high concentrations of the agent (Ayliffe, 1997). The genetic basis of antibacterial resistance may be intrinsic or acquired. Intrinsic resistance is due to natural features of the organism, while acquired resistance can be attributed to either mutation or horizontal acquisition of genetic material encoding antibacterial resistance genes from other resistant bacteria (on mobile genetic elements such as transposons or plasmids) (Ebrahim, 2010). The molecular mechanisms of resistance can be broadly classed into one of four groups a) antibiotic inactivation b) target absence, modification or overexpression c) antibiotic efflux and d) acquisition of alternative target (Russell, 2002). Bacteria may develop resistance to multiple antibiotics by acquisition of multiple resistance genes (e.g. on R plasmids), or the expression of multi-drug efflux pumps (Nikaido, 2009). Known mechanisms of bacterial resistance to antibiotics are outlined in Table 1.1.

1.3.2 History of Antibacterial resistance

Resistance to the first-discovered, natural product antibiotic penicillin (due to the production of β-lactamases) appeared shortly before its introduction into clinical use, and was closely followed by reports of resistance to other antibacterial agents such as

Table 1.1 – Major antibiotic resistance mechanisms in bacteria

Antibacterial Class	Example(s)	Resistance Mechanism	Reference
Glycopeptides	Vancomycin	VanHAX mediated modification of D-ala-D-ala termini in PG to D-ala-D-lactate (High level vancomycin resistance) RND (AcrF) mediated efflux. Vancomycin intermediate resistance associated with reduced cross linking and increased cell wall turnover leading to thickened PG.	(Bugg *et al.*, 1991). (Cui *et al.*, 2003)
Lantibiotics	(A) Nisin	1. Alterations in membrane composition (production of more phosphatidylgycerol). 2. Mutations in *nsaS* (putative histidine kinase sensor gene upstream of an ABC transporter operon).	(Verheul *et al.*, 1997) (Blake *et al.*, 2011)
	(B) Mersacidin	Autoimmunity in producing strains is mediated by expression of *lanI* (membrane bound protein acting as a physical barrier to lantibiotic binding) or the *lanFEG* operon (encoding ABC transporter).	(McAuliffe *et al.*, 2001)
β-lactams	Penicillins Cephalosporins Carbapenems	1. Production of β-lactamases (Class A, C and D – Serine enzymes, Class B – Zinc Enzyme). Enzymatic hydrolysis of β-lactam ring, inactivation of antibiotic. 2. RND (AcrAB, MexAB) mediated efflux	(Chain & Abraham, 1940) (Piddock, 2006)
	Meticillin	Production of penicillin binding protein PBP2a (encoded by *mecA* on the mobile genetic element SCC*mec*), enzyme with reduced affinity for antibiotic.	(Hartman & Tomasz, 1984)
Fosfomycin		1. Epoxide ring opening and inactivation by thiolate anion of plasmid encoded glutathione. 2. Mutations in *uhpT* and *glpT* transporters, responsible for fosfomycin import into the cell.	(Arca *et al.*, 1988) (Nilsson *et al.*, 2003)
D-cycloserine		1. Loss of L-alanine-glycine transport system responsible for D-cycloserine import into the cell. 2. Overexpression of alanine racemase (*alrA*)	(Wargel *et al.*, 1971) (Caceres *et al.*, 1997)
Mupirocin		1. Acquisition of *mupA*, plasmid encoded mupirocin resistant isoleucyl tRNA synthetase. 2. Point mutations in *ileS* (chromosomally encoded wild-type isoleucyl tRNA synthetase)	(Patel *et al.*, 2009) (Hurdle *et al.*, 2005)
Tetracyclines	Tetracycline	1. Efflux [*tetA-L, A (P), V, Y, Z, tet30, 31, 33, 35, 38-42, otrB/C, tcr3*]. 2. Ribosomal protection [*tetM, O, S, T, Q, B(P), W, otrA, tet32, 36*]. 3. Antibiotic oxidation (modification and inactivation) [*tetX, tet37*] 4. Other [*tetU, otrC*]	(Chopra & Roberts, 2001) Thaker *et al.*, 2010
	Tigicycline	5. Upregulation of RND (AcrAB, MexAB, MexCD, MexEF, MexXY) efflux pumps	(Hawkey & Finch, 2007).
Aminoglycosides	Gentamicin	1. Modification of antibiotic on hydrogen bonding groups (by ATP, Acetyl-CoA and phosphate) to interfere with binding to 16S rRNA. 2. Reduction in number of membrane porins for entry. 3. RND mediated efflux (AcrAD). 4. Altered ribosome binding site (rare).	(Poole, 2005) (Nikaido, 2009)

Table 1.1 continued...

Macrolides	Eythromycin	1. Mono or dimethylation of A_{2058} (23S rRNA) by Erm methyltransferase, interferes with binding of the antibiotic. 2. Antibiotic export by ATP binding cassette (ABC) type transport proteins. 3. Intracellular inactivation of antibiotic (phosphotransferases, esterases and glycosylases). 4. MFS or RND (AcrAB, MexAB, MexCD) mediated efflux.	(Leclercq, 2002) (Wright, 2005) (Nikaido, 2009)
Lincosamides	Clindamycin	1. Mono- or dimethylation of A_{2058} (23S rRNA) by Erm methyltransferase, interferes with binding of the antibiotic.2. ABC mediated efflux.	Leclercq (2002)
Streptogramins	(A) Dalfopristin (B) Quinupristin	(A) ABC mediated efflux (*vga* determinants), or O-acetylation of antibiotic –OH group. (B) 1. Mono or dimethylation of A_{2058} (23S rRNA) by Erm methyltransferase, interferes with binding of the antibiotic or antibiotic inactivation by ring opening lyase.	(Werner *et al.*, 2002)
Chloramphenicol		1. Reduced membrane permeability or 2. RND (AcrAB, MexAB, MexCD, MexEF) mediated efflux 3. Antibiotic inactivation by acetylation of –OH groups, reduced ribosomal binding affinity. 4. Mutations in 50S ribosomal subunit genes (rare).	(Moreira *et al.*, 2005) (Roberts & Schwarz, 2009)
Oxazolidinones	Linezolid	$G_{2576}U$ mutation in 23S rRNA and other mechanisms	(Pillai *et al.*, 2002)
Fusidic Acid		1. Mutations in *fusA* (encoding elongation factor G) lowers antibiotic binding affinity. 2. Acquisition of plasmid-borne FusB, which binds EF-G and inhibits interaction with antibiotic. 3. RND (AcrAD) mediated efflux	(Besier *et al.*, 2003) (G. Cox, pers. comm.) (Nikaido, 2009)
Lipopeptides	Daptomycin	1. Mutations in *mprF, yycG, rpoB* and *rpoC*. 2. Loss of an 81kDa membrane protein, resulting in reduced daptomycin binding to the cell membrane.	(Friedman *et al.*, 2006) (Kaatz *et al.*, 2006)
Polymyxins	Polymyxin B	Adaptations in cell membrane: reduction in LPS, divalent cation and membrane protein (porin) content and lipid alterations.	(Moore *et al.*, 1984)
Trimethoprim		1. RND (AcrAB, MexAB) mediated efflux. 2. Mutations in dihydrofolate reductase gene. 3. Acquisition of additional resistant dihydrofolate reductase	(Adrian & Klugman, 1997)
Sulphonamides	Sulfamethoxazole	1. RND (MexAB) mediated efflux. 2. Mutations in dihydropteroate synthase gene. 3. Acquisition of additional resistant dihydropteroate synthase	(Then, 1982)
Triclosan		1. Overexpression of *fabI* (containing $F_{204}C$ substitution -lowered triclosan binding affinity). 2. RND (AcrAB, MexAB, MexCD) mediated efflux.	(Fan *et al.*, 2002)
Fluoroquinolones	Ciprofloxacin	1. MFS (NorA) or RND (AcrAB, MexEF, MexXY) mediated efflux. 2. Point mutations in *gyrA* 3. *cfx-ofx* locus mediated low level antibiotic resistance (mechanism unknown)	(Kaatz *et al.*, 1993)
Coumarins	Novobiocin	1. Mutations in *gyrB* (ATP binding site), reduce affinity for antibiotic. 2. RND (AcrAB, MexAB, MexCD) mediated efflux	(Contreras & Maxwell, 1992)
Rifamycins	Rifampicin	1. Mutations in *rpoB* gene (β subunit of RNAP). 2. ADP ribosylation, glycosylation of antibiotic	(O'Neill *et al.*, 2000) (Wright, 2005)

Table 1.1 - RND (Resistance Nodulation Division), ABC (ATP binding cassette), MFS (Major Facilitator Superfamily)

the then newly discovered tetracyclines and macrolides (Finch, 2003). In less than ten years, the prevalence of β-lactamase producing strains of *S. aureus* in the UK had increased, with over fifty percent of isolates testing positive for production of the enzyme (Hawkey, 2008). It has been estimated that infections caused by β-lactamase producing *S. aureus* strains costs the US economy two to seven billion dollars per annum, highlighting it as a significant problem (Palumbi, 2001).

The discovery and development of aminoglycosides, fluoroquinolones, glycopeptides and β-lactamase stable, semi-synthetic penicillins (e.g. meticillin) initially solved the problem of treatment of β-lactamase producing strains. These compounds have different properties from penicillins, or in the case of meticillin, have large side chains which sterically hinder the activity of the β-lactamase (Bush, 2004; Chopra, 2003). However, the development of bacterial resistance to both the aminoglycosides and fluoroquinolones was documented within three years of their approval by the Food and Drug Administration (FDA) (Duckworth *et al.*, 1988, Shalit *et al.*, 1989; Bush, 2004) and the introduction of meticillin in 1960 was followed a year later by the emergence of meticillin-resistant *Staphylococcus aureus* (MRSA) strains (Aeschlimann *et al.*. 1999). The most recent analyses suggest approximately twenty percent of *S. aureus* isolates display meticillin resistance in the clinic (Dulon *et al*, 2011), with specific strains also having developed resistance to macrolides, aminoglycosides, tetracyclines, chloramphenicol and lincosamides (Nikaido, 2009). MRSA is also now the most prevalent pathogen isolated in the nosocomial setting (Wang & Barret, 2007), and has increasing prevalence in the community especially in areas where there is poor hygiene or close living conditions (Tristan *et al.*. 2007). As a pathogenic species, *S. aureus* is the causative agent of a wide range of infectious diseases ranging from superficial

infections of the skin and food poisoning, to debilitating diseases such as infective endocarditis, septicaemia, pneumonia and toxic shock syndrome (Noble 1998). In addition, upwards of sixty percent of community-acquired (CA) MRSA strains now carry the phage-borne Panton-Valentine Leukocidin (PVL) (David & Daum, 2010), a potent cytotoxin which is associated with higher virulence in producing strains. The acute onset of necrotizing pneumonia due to leukocyte destruction by PVL producing strains can lead to patient death within as little as 48 hours (Gillet *et al.*, 2002) making MRSA a significant and dangerous pathogen.

In recent years, the use of vancomycin (a last-line glycopeptides) was favoured for Gram-positive isolates resistant to the β-lactams, despite concerns about the adverse side effects of its usage (Barna & Williams; 1984, Hoffman-Terry *et al.*, 1999). However, vancomycin-intermediate *Staphylococcus aureus* (VISA) resistant clinical strains (Minimum inhibitory concentration [MIC] 8-16 μg/ml compared with 1-4 μg/ml for sensitive strains) emerged in 1996 (Hiramatsu *et al.*, 1997) and have since been associated with failure of vancomycin treatment to overcome serious infections (Moore *et al.*, 2003). In addition, a few cases of high-level vancomycin-resistant *S. aureus* have been reported (MICs >32 μg/ml) which express the enterococcal vancomycin resistance determinant, VanA (Centres for Disease Control and Prevention, 2002).

1.3.3 The growing requirement for novel antibacterial drugs

The failure of 'last resort' antibiotics to treat Gram-positive infections such as those caused by MRSA and vancomycin-resistant enterococci (VRE) has presented a significant problem in chemotherapeutics (Moore *et al.*, 2003; Schwarz *et al.*, 2008). An

additional predicament is the emergence and global spread of multi-drug resistant strains of *Mycobacterium tuberculosis* and Gram-negative bacteria displaying multiple antibiotic resistance phenotypes (Fischbach & Walsh, 2009). Some examples of the latter include virulent Enterobacteriaceae; particularly those producing extended-spectrum β-lactamases (ESBLs) (capable of hydrolysing third-generation cephalosporins and monobactams) (Paterson & Bonomo, 2005), plasmid-encoded AmpC type β-lactamases (which are not inhibited by clavulanic acid) (Bret *et al.*, 1998) or the newly identified carbapenemase NDM-1 (New Delhi Metallo-β-lactamase 1) which hydrolyses nearly all β-lactams in clinical use (Canton & Lumb; 2010). Also an issue are other 'pan resistant' pathogens such as *Pseudomonas aeruginosa* and *Acinetobacter* spp. (Bowden *et al.*, 2010; Bush, 2004). The increasing prevalence of multi-drug resistant bacteria causing life-threatening infections, combined with limited alternative therapies and the increased movement of multi-drug resistant pathogens from the nosocomial setting into the community (Alanis, 2005), has led to an increase in morbidity and mortality associated with bacterial infections, all of which is estimated to cost the National Health Service (NHS) over one billion pounds per year (Chopra, 2003).

There is some evidence that reduction of antibacterial usage results in reduction in the incidence of resistance. For example, a reduction in pneumococcal resistance to penicillin in the UK followed a one third reduction in prescription of oral β-lactams (Livermore *et al.*, 2006). The EU has thus responded to the growing incidence of multi-drug resistant bacteria by increasing legislation to restrict the overuse of antibiotics. For example in 2000, fifteen percent of all antibiotics administered in the European Union were used as growth promoters or performance enhancers in animals (Van den Bogaard

& Stobberingh, 2000). However, this agricultural practice has now been banned within the European community (Dibner & Richards, 2005) due to evidence that animals act as reservoirs of bacterial pathogens, and are vectors for transfer of antibiotic-resistant bacteria (especially Salmonellae and Campylobacters) to humans (Hummel *et al.*, 1986). In addition, in delayed answer to the 1969 Swann report and 1997 World Health Organization (WHO) workshop, the American Society of Microbiology (ASM) alongside the FDA have responded to recommendations and called to end all non-therapeutic uses of antibacterial agents, including use in food production (Witte, 1998; Kolter *et al.*, 2010). However, federal bills to effect such changes were not passed (Hoffman & McGinnis, 2009) and due to the global movement of people and food as carriers of resistant bacteria, the spread of resistance genotypes has been allowed to continue unhindered (Warren *et al.*, 2008).

Additional issues that affect the treatment of infections caused by multi-drug resistant pathogens are the cost and time for the development of a new drug that are respectively upwards of one billion dollars and up to fifteen years (Alanis, 2005; DiMasi *et al.*, 2003). As such, many large pharmaceutical companies are no longer investing in antibacterial research and development (R&D) programs, and are focusing on other chemotherapeutic areas (e.g. chronic diseases) which offer better return on investment (Projan & Shales, 2004). This decrease in investment has resulted in the entrance of only a few new antibacterial drugs into clinical use in the last decade, e.g. linezolid (2000), daptomycin (2003), telithromycin (2004), tigecycline (2005), retapamulin and doripenem (2007), telavancin (2009) and ceftaroline (2010) (Baker, 2011; Brinker *et al.*, 2009; Cada *et al.*, 2010; Conley & Johnston, 2005; Davidovich *et al.*, 2007; Eliopoulos, 2009, Karlowsky *et al.*, 2009).

However, telithromycin, tigecycline, ceftaroline, doripenem, retapamulin and telavancin do not represent novel antibacterial structures, but rather variations on existing antibiotic scaffolds (macrolides, tetracyclines, cephalosporins, carbapenems, pleuromutilins and glycopeptides respectively), and as such resistance to all of these agents has already been reported (Brown & Traczewski, 2005; Clark *et al.*, 2011; Gentry *et al.*, 2007; Krause *et al.*, 2005). For example, high-level telithromycin resistant *Streptococcus pneumoniae* clinical isolates possess mutations in ribosomal proteins and *ermB*, which encodes the 23S rRNA A_{2508} methyltransferase associated with macrolide resistance (Wolter *et al.*, 2007). Tigecycline resistance has been observed in the Gram-negative pathogen *Klebsiella pneumoniae* (Dipersio & Dowzicky, 2007), and is attributed to upregulation of AcrAB efflux pumps (Hawkey & Finch, 2007).

Alarmingly, antibacterial resistance has also been reported for linezolid and daptomycin. Linezolid-resistant strains of *S. aureus* were reported just one year after its introduction into clinical use (Tsiodras *et al.*, 2001), with resistance due to a G_{2576} mutation to uridine in 23S rRNA (Pillai *et al.*, 2002). Reduced susceptibility to daptomycin in clinical MRSA strains isolated in septicaemia and osteomyelitis patients was reported just two years after FDA approval of daptomycin for treatment of complicated skin and soft tissue infections (Hayden *et al.*, 2005, Marty *et al.*, 2006). The mechanism of daptomycin resistance has been linked to mutations in *mprF* (lysylphosphatidylglycerol synthetase), *yycG* (sensory box histidine kinase), *rpoB* and *rpoC* (RNAP β and β' subunits) (Friedman *et al.*, 2006) and the loss of an 81kDa membrane protein, resulting in reduced binding of daptomycin to the cell membrane (Kaatz *et al.*, 2006).

The rapid development of resistance to all of these new antibiotics requires urgent attention. The failure of current hospital procedures to control the spread of resistant bacteria and the relative lack of success for development of non-antimicrobial therapies (e.g. bacterial vaccines) (Davies & Davies; 2010) alongside the threat of genetically modified (resistant) pathogens for use in bioterrorism (Gilligan, 2002) highlight the vital need for the discovery of new antibacterial agents.

1.4 Antibacterial drug discovery

1.4.1 Empirical Screening and the 'Golden Era' of antibacterial drug discovery

The earliest recorded antibacterial drug discovery programmes (which involved screening of chemicals and dyestuffs for inhibitory activity) were somewhat successful, and the first synthetic antibiotics, the sulphonamides, were discovered by such methods (Achilladelis, 1993). However, the majority of antibacterial drug discoveries have been due to accident or serendipity during empirical screening (Figure 1.10a) of chemical compounds and fermentation products in the so-called 'Golden era' (1944-1962) of antibacterial drug discovery (Chopra *et al.*, 1997; Silver, 2011). The subsequent emergence of resistant bacteria in the latter half of the twentieth century has largely been met with clinical introduction of newer members of existing antibacterial classes, analogues displaying improved properties such as greater spectrum of activity, lower toxicity and an ability to overcome existing resistance mechanisms such as efflux and antibiotic inactivation (Chopra *et al.*, 2002). This is exemplified by the development of a number of drugs including the semi-synthetic penicillins, cephalosporins, macrolides (e.g. azithromycin) and aminoglycosides (e.g. tobramycin, which lacks the antibiotic

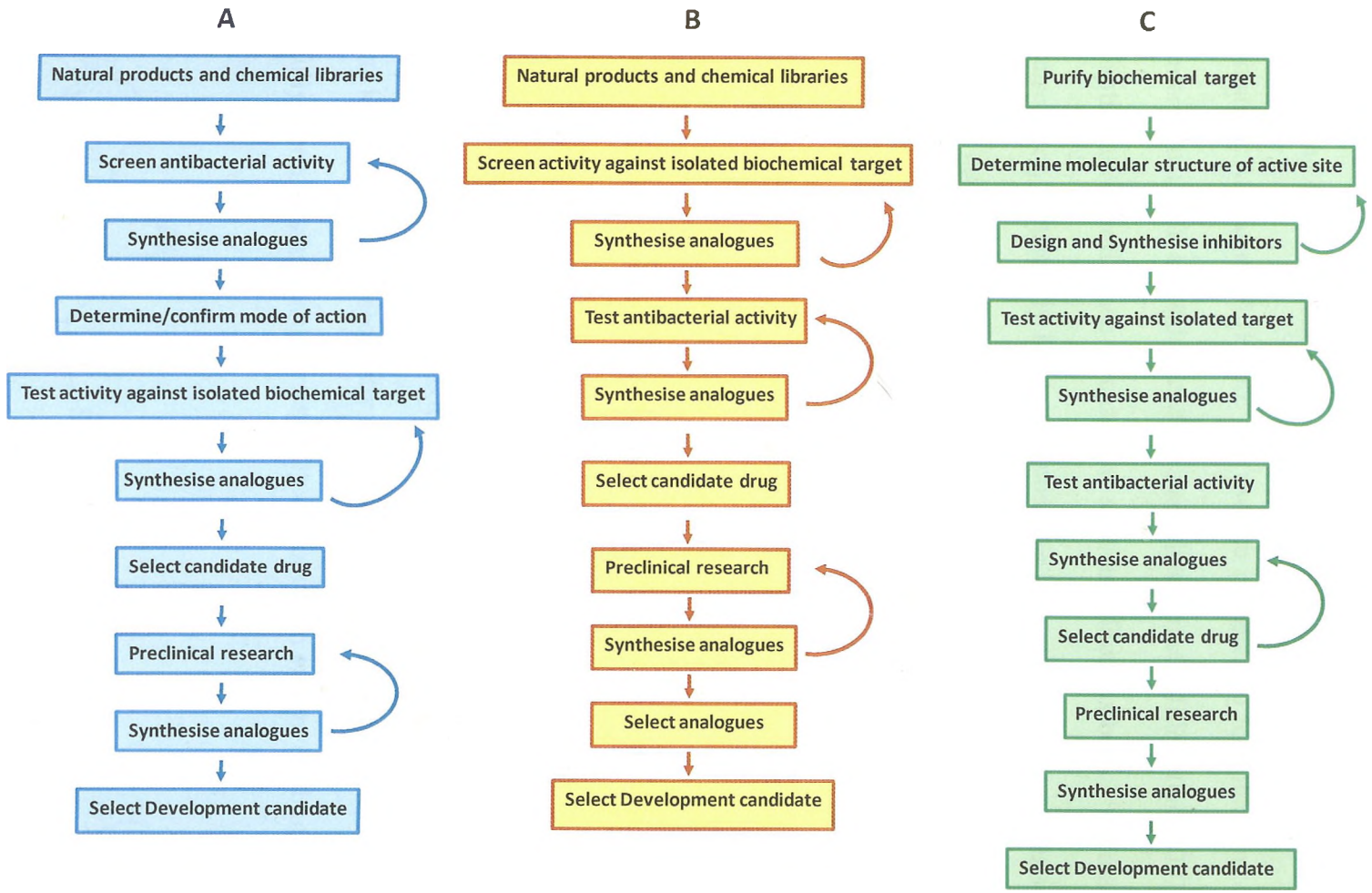


Figure 1.10 – Methodologies for antibacterial drug discovery (Adapted from Chopra, 1997).

A – Empirical screening using whole cells, B – Empirical screening using isolated biochemical targets, C – Structure-based drug design.

inactivation sites) (Wright, 2005). The development of resistance enzyme inhibitors to be used in combination therapy with an antibiotic has also previously been successful in overcoming resistance mechanisms and restoring the activity of the drug, a notable example being the amoxicillin (β-lactam) and clavulanic acid (β-lactamase inhibitor) combination, Augmentin® (Figure 1.11) which is indicated for infections of the lower respiratory tract, skin and skin structure and urinary tract (Miller *et al.*, 2001). Targeting other antibacterial resistance mechanisms such as efflux has been a popular concept in drug discovery, particularly for its potential use in combination therapies with existing antibacterial agents (Davies & Davies, 2010). Efflux pumps provide a particularly attractive target since several confer resistance to more than one antibiotic class (Table 1.1). However, this approach has been unsuccessful in producing any leads to development (Lomovskaya & Bostian, 2006). In 1977, Cohen suggested that the antibacterial drug discovery process be directed to screening for inhibitors of specific enzymes involved in bacterial metabolism, moving away from whole-cell screening (Figure 1.10b) (Cohen, 1977). The aim was to identify novel broad-spectrum biochemical targets which were not susceptible to rapid development of resistance, were essential to the survival of the bacteria, and were absent in humans to overcome issues such as host toxicity. Subsequently screening for inhibitors against those targets would hopefully identify compounds structurally distinct from current antibiotics which would

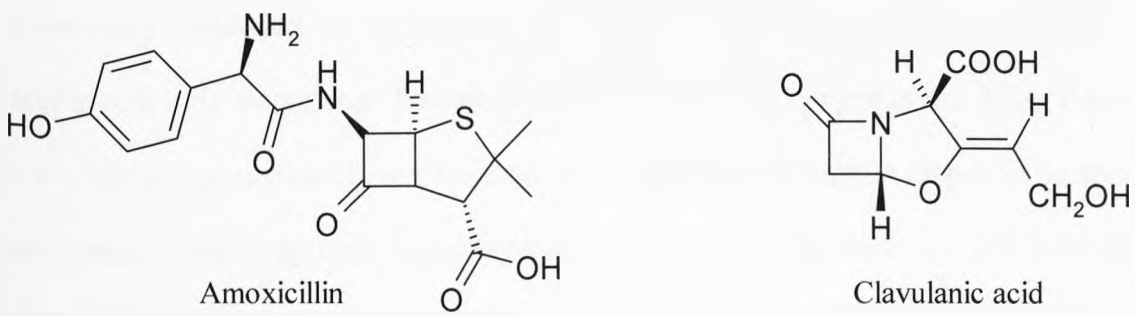


Figure 1.11 – Augmentin® (amoxicllin and clavulanic acid combination)

not be subject to existing resistance mechanisms (Silver, 2011). The screening of chemical compound libraries was particularly favoured due to the observed longer intervals between clinical introduction and reported transmissible resistance to synthetic molecules in comparison with natural products. For example, transmissible resistance to the sulphonamides was not seen for over twenty years after its clinical introduction, compared with almost concurrent introduction and resistance development of penicillins (Smith, 1966). However, *in vitro* screening of large compound libraries against novel protein targets was largely unsuccessful. Development of inhibitors against single enzyme targets can be hindered by issues including; potency against the enzyme *in vitro* but no accompanying antibacterial activity, or if MICs are detected, the inhibitory activity is not linked to inhibition of the enzyme and is attributable to non-specific activity (Silver, 2011). Also an issue may be the rapid development of resistance; therefore developed inhibitors might be limited to administration as a topical agent, or in combination therapy (Silver, 2011).

1.4.2 The genomic era of drug discovery and structure-based drug design

Since the sequencing of the entire *Haemophilus influenzae* genome in 1995, high-throughput genomic and bioinformatic approaches, involving the comparison of over one hundred genome sequences of significant bacterial pathogens have been increasingly employed in an attempt to identify highly conserved broad-spectrum targets with little mammalian homology (Chan *et al.*, 2003; Gwynn *et al.*, 2010; Payne *et al.*, 2007). Approximately one hundred and sixty essential enzyme targets in bacteria have been identified by such methods (Ascenzi *et al.*, 2002; Payne *et al.*; 2007; Perdih *et al.*, 2009) with only a small percentage exploited by currently used antibacterials

(Schmid, 1998). The evaluation of these sites has progressed to high-throughput screening of compound libraries *in silico*, by programs capable of virtual ligand screening and structure-based drug design (SBDD) (Figure 1.10c) (Vyas *et al.*, 2008). By utilising existing data on genome sequence and high-resolution crystallographic or nuclear magnetic resonance (NMR) structures of over six hundred bacterial proteins, it is possible to dock ligands (commercial compounds, fragments or *de novo* designed molecules) into specific sites on the protein via *in silico* modelling, make predictions and give scores on the binding affinities of the inhibitor, and then test potential inhibitors against the protein *in vitro* (Simmons *et al.*, 2010). Once a potential 'lead' compound has been identified, parallel *in silico* approaches may be utilised to screen compound libraries for molecules displaying similar structures and docking complementarities, creating a selection of analogues for *in vitro* biological testing (Barker, 2006). Recent advances in software for SBDD programs such as eHITS (SymBioSis Inc. Toronto, Canada), Autodock and Glide (Schrodinger) also mean that up to one hundred thousand compounds from a library may be virtually docked into a single target in one day, bringing another dimension to high throughput screening (Simmons *et al.*, 2010).

The choice of site for antibacterial drug design is complex, as not all targets display 'druggability' even if the proteins are conserved amongst bacterial genera and essential to cell survival. In fact, the majority of successful inhibitors with high binding affinities for their target may be found in deep clefts in the protein, and in the case of enzymes, usually at the active site (Blundell *et al.*, 2002). In addition, the reliance of SBDD methods on a single high-resolution crystal structure may be viewed as a limitation, as it only displays the protein in one position, whereas a biologically active enzyme may

adopt multiple conformations, although many of the newer SBDD programmes compensate by designing flexible ligands which can bind to the protein in a variety of poses (Simmons *et al.*, 2010).

Although SBDD has been successful for inhibitors of HIV protease (e.g. nelfinivir) (Kaldor *et al.*, 1997), it has lead to no antibacterial drugs. As an example, the pharmaceutical company GlaxoSmithKline undertook a seven year programme (1995-2001) in which sixty seven high-throughput screening programmes on antibacterial targets were run against over half a million compounds and from which only five leads were identified (including inhibitors of enzymes involved in the Mur pathway of PG biosynthesis, aminoacyl-tRNA synthetases, virulence factors such as the oedema factor and protective antigen toxins of *Bacillus anthracis*, peptide deformylase [Pdf] and enoyl reductases of fatty acid synthetase II [FAS II]), but none of which have successfully progressed through clinical trials (Hurdle *et al.*, 2005, Payne *et al.*, 2007). This low hit rate may be due to the fact that lead compounds often do not meet the requirements for development and/or clinical use, and suffer from issues such as weak antibacterial activity, poor compound penetration, lack of specificity, drug metabolism and efflux in the bacterium (Fischer *et al.*, 2004) or poor pharmacokinetic/ pharmacodynamic properties in the host (Payne *et al.*, 2007).

1.4.3 Development of underexploited antibacterial agents as a tool for drug discovery

An increasingly popular source of antibacterial leads includes earlier pharmacophores which have as yet been underexploited (Chopra, 2003). Of the four thousand reported

microbial metabolites displaying antibiotic activity, only one percent have been developed for clinical use and amongst the undeveloped inhibitors there may be a selection that could be taken forward for optimisation (Zahner & Fiedler, 1995). Examples of potential hits include proposed inhibitors of RNAP (myxopyronins, corallopyronins, ripostatins, thiolutin and holomycin), and the tRNA synthase inhibitor indolmycin. These compounds were all initially shown to have promising antibacterial activity, particularly against Gram-positive organisms, no cross resistance to existing antibiotic classes and low host toxicity (Irschik *et al.*, 1995; Irschik *et al.*, 1983; Irschik *et al.*, 1995; Oliva *et al.*, 2001; Rao, 1960) but were dropped from development due to the successful introduction of other currently used inhibitors (Zahner & Fiedler, 1995). Chopra remarked that 'many of these agents have structures and modes of action that are distinct from current antibiotics' and therefore optimisation of these leads may provide new chemotherapeutic candidates with minimal potential for cross resistance (Chopra, 2003).

1.4.4 Lead optimisation

The modification of existing scaffolds to improve antibacterial activity cannot overcome existing resistance mechanisms to the same degree as isolating a completely new target and inhibitor, although the former was favoured for many years due to the challenges of discovery and optimisation of completely novel chemotherapeutic candidates (Chan *et al.*, 2004). Several strategies have been employed to improve the properties of novel antibacterial agents identified in screening programmes. In 'lead optimisation', hits showing low antibacterial activity, poor spectrum of activity or inferior pharmacokinetic properties may be chemically modified to improve activity and potency against a wider

range of bacteria; although it is important to confirm that enhancements are still due to high affinity activity against the target (Chopra, 1997). In addition, it is beneficial to modify novel antibacterial candidates in order to have no structural features in common with clinically available antimicrobial agents, thereby surpassing existing resistance mechanisms (Chopra *et al.*, 1997).

The emergence of multi-drug resistance amongst Gram-negative bacteria is particularly problematic, due to the challenges of developing and optimising new agents which show activity against these species. The Gram-negative cell envelope is composed of an inner membrane, a thin layer of PG and an outer membrane structure composed of a bilayer of phospholipids and negatively charged LPS (Nikaido, 1988). It is the presence of the latter structure which impedes cell penetration by many of the larger, charged and more polar antibiotic classes (Denyer & Maillard, 2002). Reportedly successful examples of improving the accessibility of antibacterial agents to their target sites include i) production of liposomal preparations of hydrophobic antibiotics to ensure compound delivery (Khuller *et al.*, 2004), or ii) tethering an existing antibiotic (e.g. triclosan) to a β-lactam to utilise the activity of cellular β-lactamases to release an active compound (Stone *et al.*, 2004). However, these methods have not yet yielded any clinical candidates, possibly due to issues with compound metabolism, efflux or host toxicity. In addition to the outer membrane, many Gram-negative bacterial species remain insusceptible to antibiotic classes due to the activity of efflux pumps (e.g. AcrAB-TolC) (Nikaido, 2009). Many medicinal chemists have also suggested that modification of candidate antibacterial agents to avoid characteristics which make them prone to efflux, may overcome this significant resistant mechanism (Gwynn *et al.*,

2010). For example, membrane-permeable compounds that are less susceptible to efflux tend to be non-polar compounds with neutral or monovalent charge (Silver, 2011).

1.5 Establishing the MOA of novel antibacterial agents

1.5.1 Introduction to preclinical evaluation of novel antibacterial agents

Approval of a novel antibacterial agent is based upon a variety of factors, including pharmacokinetic/pharmacodynamic properties of the drug, efficacy/host response in clinical trials and comparative activity with existing antibiotics indicated for the same infection (Jacobs, 2004). However, prior to the clinical development of novel inhibitors, it is essential that drug candidates are tested to characterise their antibacterial target and bacterial specificity fully. Inhibition of growth as determined by MICs against a panel of organisms and the *in vitro* characterisation of the MOA of the agent are key to the prediction of clinical outcome of treatment and spectrum of activity (Spanu *et al.*, 2004). Antibacterial activity should be attributed to selective toxicity and not a non-specific mechanism, so as to rule out agents potentially displaying adverse effects in the host (Silver, 2011). In addition, characterisation of the MOA of a new agent may produce information that may be beneficial to pharmaceutical companies in the further development, appraisal and marketing of the compound (O'Neill & Chopra, 2004a).

1.5.2 MIC determinations

Prior to MOA analysis, MIC determinations should be conducted against a panel of organisms (representing Gram-positive and Gram-negative genera) and this approach

can classify pathogens as resistant or susceptible to the compound. This information is vital for predicting for which infections the drug might be indicated in clinical practice. In a laboratory setting, determination of the MIC may be achieved with doubling dilutions (up and down from 1 μg/ml) of the inhibitor in media containing the test organism. After an incubation period the MICs can be read visually as the lowest concentration of drug required to inhibit the growth of a microorganism (Andrews, 2001). Generally, antibacterial agents indicated for systemic use will usually need to have an MIC <1 μg/ml, but clinical progression may be possible for agents with an MIC up to 16-32 μg/ml (O'Neill & Chopra, 2004a). In addition, determination of the MIC gives further information on the most effective concentration at which the drug will need to be administered i.e. the serum concentration that will need to be maintained for optimal efficacy. In the case of fluoroquinolones and aminoglycosides for example, the antibacterial activity *in vivo* has been shown to be optimal when their peak concentration is 8-10X MIC, and with the majority of other agents, serum concentrations of at least 6X MIC give the highest probability of treatment success (Spanu *et al.*, 2004).

1.5.3 Characterisation of the bactericidal or bacteriostatic activity of the agent

Initially, it is useful to determine whether the antibacterial agent displays bactericidal or bacteriostatic activity (Jacobs, 2004). The bactericidal activity of a novel agent may be elucidated by i) the determination of the minimum bactericidal concentration (MBC) of the agent (concentration at which there is a 99.9% reduction in viability on subculture), where bacteriostatic agents are defined as having an MBC/MIC ratio of >4 or ii) the *in vitro* determination of the cell death kinetics upon treatment with suprainhibitory

concentrations of the compound (Pankey & Sabbath, 2004). The measurement of 'time kill' is particularly useful as it gives indications of the concentration or time dependency of the agent, and therefore vital information on likely dosage quantities and intervals (Gould *et al.*, 1990). For example time-dependent cell death or concentration-dependent killing will influence the clinical administration of drugs e.g. usually at time intervals for the former, and once daily for the latter (Vogelman & Craig, 1986).

1.5.4 Determination of MOA: inhibition of macromolecular synthesis

A useful method for monitoring the MOA of novel agents is measurement of the inhibition of DNA, RNA, protein and PG synthesis (Macromolecular synthesis or MMS). This is achieved by monitoring over a specific time period the incorporation of radiolabelled precursors into macromolecules in response to an inhibitor, relative to a drug free control (Cherrington *et al.*, 1990). This method may highlight inhibition of a specific biosynthetic pathway, or if all pathways are simultaneously inhibited, indicate a non-specific MOA such as membrane damage or cessation of energy producing metabolic pathways (Ooi *et al.*, 2009a).

1.5.5 Determination of MOA: expression profiling and use of antibiotic biosensors

Expression profiling and whole-cell biosensor approaches are becoming increasingly popular methods for elucidating the MOA of uncharacterised antibacterial agents (Freiberg *et al.*, 2004). Based upon transcriptional analysis of antibiotic induced stress in bacteria, promoters can be identified which are induced in response to exposure of bacteria to antibiotics with similar modes of action and these can be manipulated to

produce biosensor strains (Bianchi & Baneyx, 1999; Blake *et al.*; 2009; Fischer *et al.*, 2004; Urban *et al.*, 2007). Such strains contain promoter- luciferase/β-galactosidase reporter constructs which are induced by conditions of antibiotic-induced stress and can be used for whole-cell based screening and characterisation of inhibitors (Bianchi & Baneyx, 1999; Fischer *et al.*, 2004; Urban *et al.*, 2007). These biosensor strains signal the presence of inhibitors of various biosynthetic pathways and therefore represent a useful tool for high-throughput screening of novel antibacterial agents.

1.5.6 Determination of MOA: resistance development

Preclinical evaluation of the potential for resistance development to a novel antibacterial agent may give early indication to its clinical performance (Gwynn *et al.*, 2010). Such analyses involve the *in vitro* selection for resistant mutants against the target inhibitor, and subsequent characterisation of any mutants arising (e.g. cross-resistance analysis, genome sequence determination). Such analyses can give useful data on the mutation site and therefore the likely target of the drug, but also indicate whether the novel agent is able to overcome existing resistance mechanisms (O'Neill & Chopra, 2004a). For example, recent studies have shown that the introduction of point mutations into the β and β' subunits of RNAP confers high level resistance to the underdeveloped antibacterial agent, myxopyronin B (MyxB), confirming targeted inhibition of these RNAP subunits by this agent (Mukhopadhyay *et al.*, 2008). These mutations also resulted in cross-resistance to the structurally similar antibiotic, corallopyronin A but not to rifampicin and therefore MyxB may constitute a viable RNAP inhibitor for clinical development which is able to overcome existing resistance mechanisms.

1.5.7 Determination of MOA: underexpression of the target

Changes in the cellular levels of a target may lead to alterations in the susceptibility of bacteria to the inhibitor being tested. Therefore, confirmation of the target site for a novel antibacterial agent may be achieved by underexpression of the proposed target, which should cause the cells to become hypersusceptible to the agent, or conversely overexpression of the target should lead to reduction in susceptibility to the agent (O'Neill & Chopra, 2004a). The most widely reported example of the use of target titration is the employment of antisense RNA molecules with reverse complementarity to the mRNA transcript of the target (Forsyth *et al.*, 2002; Good, 2003). The binding of these antisense sequences to the target mRNA inhibits translation, resulting in a reduction in cellular concentration of the target. Antisense RNAs to isoleucine tRNA synthetase (IleS) and enoyl-[acyl-carrier-protein] reductase (FabI) have been shown in this manner to sensitise bacterial cells specifically to mupirocin and triclosan respectively (Yin *et al.,* 2004), highlighting this as a viable method for identification of antibacterial targets.

1.5.8 Characterisation of membrane damage and bacterial specificity of the agent

Novel antimicrobial agents that are identified via high-throughput screening programs and have antibacterial activity often suffer from lack of specificity against the target, promiscuous inhibition of other cellular proteins or non-specific membrane damage (Siedler *et al.*, 2003).

Membrane damage can be an undesirable characteristic in potential chemotherapeutic candidates as damage to bacterial membranes may indicate potential toxicity to humans. Consequently, novel antibacterial agents that display bacterial membrane damage are often disregarded. However, the recent introduction of daptomycin and telavancin (a lipoglycopeptide with additional membrane-damaging activity) into clinical use has regenerated interest in developing compounds with this MOA (Higgins *et al.*, 2005). Agents which perturb membrane structure, or damage the function of multiple membrane bound respiratory enzymes, have been suggested to be particularly efficacious against persistent infections (harbouring dormant or slow-growing bacterial cells) (Hurdle *et al.*, 2011). For example, the membrane-damaging agents XF-70 and XF-73 display potent activity against slow growing and biofilm cultures of *S. aureus* (Ooi *et al.*, 2009b). In addition, membrane-damaging agents have very low potential for resistance development, which makes them attractive chemotherapeutic candidates, provided that they also display acceptable bacterial specificity and low toxicity (Hurdle *et al.*, 2011). The ability of agents to perturb the membranes of microorganisms may be determined by a variety of methods, including fluorescence based assays (*Bac*Light™ and $DiSC_3[5]$) and measurement of leakage of small intracellular metabolites such as ATP and K^+ (Ooi *et al.*, 2009a).

Determination of the bacterial specificity of a novel agent may be determined by testing the inhibitory activity of the agent against eukaryotic enzymes or whole cells in cytotoxicity assays (Kafagi *et al.*, 2000; Miller *et al.*, 2010). The simplest of these assays involve testing the concentration of test agent effecting thirty five percent inhibition (IC_{35}) of mammalian enzymes (e.g. malate dehydrogenase and chymotrypsin), selecting for compounds which achieve this at a concentration of less

than 10 μM (Evans *et al.*, 2001). In addition, small inhibitors identified during screening programmes can be tested for their ability to inhibit the target enzyme specifically in the presence of a non-ionic surfactant (e.g. Triton X-100) to exclude aggregation. This is due to the fact that aggregates have previously been shown to cause non-specific (promiscuous) enzyme inhibition (Siedler *et al.*, 2003).

Whole cell MIC determinations of test agents against a mammalian cell line or lower eukaryote (e.g. yeast) can be equally as effective in highlighting compounds which may cause undesirable side-effects during therapy, and therefore are unsuitable for further development. As a general rule in this case, hit compounds should display greater than or equal to ten-fold higher activity against prokaryotes to be considered potential therapeutic candidates (Evans *et al.*, 2001).

1.6 Introduction to the research in this study: aims and objectives

There is growing governmental and public concern over the problem of antibiotic resistance (Gwynn *et al.*, 2010) which may put pressure for increased funding into antibacterial research. The characterisation and development of underexploited antibiotics or new antibacterial agents displaying novel modes of action offer the greatest potential to overcome existing issues of resistance. Prior to the clinical development of novel inhibitors, it is essential that any drug candidates identified be tested to fully characterise their antibacterial target and bacterial specificity. The research described in this thesis was primarily focused upon establishing the antibacterial activity, MOA and development of resistance to a selection of inhibitors

which may constitute future antibacterial drug candidates, in order to address the escalating problem of antibiotic resistance. These inhibitors represented:

- antibacterial agents with well-established modes of action, to validate and optimise the methods used (Chapter 3);
- chemically modified derivatives of existing antibiotic classes (i.e. the type B lantibiotic derivative: NVB353) (Chapter 4);
- older and underexploited compounds, comprising of putative inhibitors of RNAP (Chapter 5);
- hit compounds from virtual high throughput screening programmes designed to identify inhibitors of RNAP, D-ala-D-ala ligase (Ddl) and peptidoglycan transglycosylases (GTs) (Chapter 6).

As discussed above, current literature has demonstrated the value of expression profiling in the generation of antibiotic biosensors which can signal the presence of antibiotics inhibiting particular biosynthetic pathways. However as yet there remain no bacterial biosensors which can indicate bacterial membrane damage. Therefore transcriptional profiling of *S. aureus* treated with a panel of known membrane damagers was used to identify upregulated genes which might be potential candidates for future biosensor development (Chapter 7).

Sections of the work included in Chapters 3-6 have been published. A list of the publications is included as Appendix 3.

Chapter 2 - Materials and Methods

2.1 Microbial strains

All strains used (Table 2.1 and 2.2) were stored at -80°C in 8% (v/v) glycerol.

Table 2.1 – Gram-positive strains

Strain	Description	Reference/Source
***Staphylococcus aureus* strains**		
SH1000	*rsbU*⁻ derivative of 8325-4	Horsburgh *et al.*, 2002
MSSA 1-10	Clinical meticillin-susceptible isolates	Leeds General Infirmary
EMRSA-15	Clinical (Epidemic) MRSA isolate	Prof. M. Wilcox, University of Leeds
EMRSA-16	Clinical (Epidemic) MRSA isolate	Prof. M. Wilcox, University of Leeds
EMRSA- 17	Clinical (Epidemic) MRSA isolate	Prof. M. Wilcox, University of Leeds
MW2	Community-acquired (CA) MRSA isolate	Baba *et al.*, 2002
Mu3	Clinical heteroVISA	Hiramatsu *et al.*, 1997a
Mu50	Clinical VISA	Hiramatsu *et al.*, 1997b
	Reporter strains for inhibition of peptidoglycan biosynthesis	
RN4220 *gltA*	RN4220 - carriage of *gltA-lacZ* fusion on plasmid pAJ129.	Dr A. J. O'Neill, University of Leeds
RN4220 *oppB*	RN4220 - carriage of *oppB-lacZ* fusion on plasmid pAJ129.	Dr A. J. O'Neill, University of Leeds
RN4220 *murZ* KO	RN4220 - *murZ-lacZ* fusion by plasmid integration and *murZ* disruption.	Blake *et al.*, 2009
***Bacillus subtilis* strains**		
1S34	Parental strain of *B. subtilis* antibiotic biosensors	B. G. S. C (Ohio)
1S34 (pS63)	Reporter strain for inhibition of RNA biosynthesis. (Carries pHT304 with *yvgS* promoter and *luc* fusion).	Urban *et al.*, 2007
1S34 (pS72)	Reporter strain for inhibition of protein biosynthesis. (Carries pHT304 with *yheI* promoter and *luc* fusion).	Urban *et al.*, 2007
1S34 (pS77)	Reporter strain for inhibition of DNA biosynthesis. (Carries pHT304 with *yorB* promoter and *luc* fusion).	Urban *et al.*, 2007
1S34 (pS107)	Reporter strain for inhibition of cell envelope. (Carries pHT304 with *ypuA* promoter and *luc* fusion).	Urban *et al.*, 2007
1S34 (pNS14)	Reporter strain for inhibition of fatty acid biosynthesis. (Carries pHT304 with *fabHB* promoter and *luc* fusion).	Fischer *et al.*, 2004

Table 2.2 – Gram-negative and fungal strains

Strain	Description	Reference/ Source
***Escherichia coli* strains**		
1411	*lacI3, lacZ118, proB, trp, nalA, rpsL*	Miller *et al.*, 2002
SM1411	*lacI3, lacZ118, proB, trp, nalA, rpsL, ΔacrAB::Tn903kan*^{r}	O'Neill *et al.*, 2002
***Saccharomyces cerevisiae* strain**		
464	Clinical isolate	Leeds General Infirmary

2.2 Culture media and growth conditions

S. aureus strains were routinely cultured on Mueller-Hinton Agar (MHA) and in Mueller Hinton broth (MHB) [Oxoid Ltd, Cambridge, UK] with aeration. The *S. aureus* reporter strains were cultured in Tryptic Soy Broth (TSB) [Oxoid Ltd, Cambridge, UK], while the *Bacillus* reporter strains and strains of *E. coli* were grown in Luria Broth (LB) [Oxoid Ltd, Cambridge, UK] with aeration. Agar cultures were grown aerobically for sixteen hours at 37°C and liquid cultures were incubated with aeration under the same temperature and time conditions. *S. cerevisiae* strain 464 was grown aerobically for forty eight hours at 30°C on Saboraud dextrose agar [Oxoid Ltd, Cambridge, UK] for plate cultures, while liquid cultures were grown in RPMI (Roswell Park Memorial Institute) 1640 broth supplemented with L-arginine and 25mM HEPES (pH 7.0) [Invitrogen Ltd, Paisley, UK] for twenty four hours at 35°C with aeration.

2.3 Chemicals and antimicrobial agents

All chemicals used in this study were purchased from Sigma-Aldrich [Poole, UK] unless otherwise stated. The solvents used and sources for all antibacterial compounds

are displayed in Table 2.3. For assays undertaken using a buffered system in the place of bacteriological media, 5 mM HEPES and 5 mM glucose (pH 7.2) were used.

Table 2.3 – Solvents, supplements and sources of antibacterial agents

Antimicrobial Agent	Solvent	Source
Tetracycline, fosfomycin, anhydrotetracycline, polymyxin B, bacitracin, novobiocin, gentamicin, streptomycin, clindamycin, virginiamycin M1, 8-hydroxyquinoline, chromomycin, daunorubicin, Rose Bengal	Distilled water (dH_20)	Sigma Aldrich [Poole, UK]
Vancomycin	dH_20	LEK pharmaceuticals [Ljubljana, Slovenia]
Flucloxacillin	dH_20	CP Pharmaceuticals Ltd [Wrexham, UK]
Cetyltrimethylammonium bromide (CTAB)	dH_20	BDH Laboratory supplies [Poole, UK]
XF-70, XF-73	dH_20	Destiny Pharma [Brighton, UK]
Linezolid	dH_20	Pfizer [Kalamazoo, MI, USA]
Fosmidomycin	dH_20	Invitrogen Ltd [Paisley, UK]
Doxorubicin	dH_20	Merck Chemicals Ltd [Nottingham, UK]
Clofazimine, chlorhexidine, sulfamethoxazole, fusidic acid, actinonin, erythromycin, chloramphenicol, cephalosporin P1	50% Ethanol (EtOH) (v/v)	Sigma Aldrich [Poole, UK]
Triclosan	50% EtOH (v/v)	LG Life sciences [South Korea]
Baicalein	50% EtOH (v/v)	MP Biomedicals [Illkirch, France]
Rifampicin, trimethoprim, tunicamycin	50% Dimethylsulfoxide (DMSO) (v/v)	Sigma Aldrich [Poole, UK]
Sepracor 155342	50% DMSO (v/v)	University of Leeds
Indolmycin	50% DMSO (v/v)	Pfizer [Kalamazoo, MI, USA]
Borrelidin	50% DMSO (v/v)	Biotica Ltd [Cambridge, UK]
Holomycin, thiolutin	50% DMSO (v/v)	SmithKline Beecham Pharmaceuticals [Surrey, UK]
Ripostatin A, corallopyronin A	50% DMSO (v/v)	Dr. Gerhard Höfle, Helmholtz Zentrum fur Infektionsforschung, Braunschweig [Germany]
Myxopyronin B	50% DMSO (v/v)	Cubist Pharmaceuticals [Lexington, USA]

Table 2.3 continued...

NCI compound set	50% DMSO (v/v)	S. Gobec, University of Ljubljana [Slovenia]
Tocris compound set	50% DMSO (v/v)	Tocris Bioscience Ltd [Bristol, UK]
Rhodanine L339571	50% DMSO (v/v)	Chembridge Corporation [San Diego, USA]
Rhodanine VG2	50% DMSO (v/v)	Cheshire Sciences Ltd. [UK]
Moenomycin	50% DMSO (v/v)	E. Breukink, Utrecht University [The Netherlands]
Ciprofloxacin	20mM Hydrochloric acid (HCl)	Bayer-Leverkusen [Germany]
Nisin	20mM HCl	NBS Biologicals Ltd [Huntingdon, UK]
Mupirocin	20mM HCl	Sigma Aldrich [Poole, UK]
Valinomycin	100% DMSO (v/v) (supplemented with 0.1M KCl)	Sigma Aldrich [Poole, UK]
Mersacidin, deoxyactagardine B, NVB353	50% DMSO (v/v) (supplemented with 50 µg/ml $CaCl_2.2H_20$)	Novacta Biosystems Ltd [Hatfield, UK]
D-cycloserine	1mM Na_2HPO_4 (pH 8.0)	Sigma Aldrich [Poole, UK]
Daptomycin	dH_20 (supplemented with 50 µg/ml $CaCl_2.2H_20$)	Cubist Pharmaceuticals [Lexington, USA]
Telavancin	50% DMSO (v/v) (supplemented with 28.5mM HCl)	Theravance [San Francisco, USA]

2.4 Determination of the susceptibility of microorganisms to antibacterial agents

2.4.1 Bacteria

Minimum inhibitory concentration (MIC) determinations were performed using the British Society for Antimicrobial Chemotherapy (BSAC) broth microdilution method (BSAC, 1991). MICs were recorded as the modal value from nine replicates.

2.4.2 *S. cerevisiae*

MIC determinations were performed using the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method (CLSI, 2008) and were recorded as the modal value from nine replicates.

2.5 Analysis of cell death kinetics

Time-kill assays were carried out essentially according to Oliva *et al.* (2003), but with modifications. A 1:100 dilution of an overnight culture of *S. aureus* SH1000 in MHB was grown to an OD_{600nm} of 0.2. After washing and resuspension in 5 mM HEPES and 5 mM glucose buffer, 15 ml samples were exposed to agents at 4X MIC. Samples were taken at time points over a period of five hours, diluted in phosphate buffered saline (PBS) and spread onto triplicate MHA plates. Viable counts were then taken following overnight incubation at 37°C.

2.6 *S. aureus* reporters for inhibition of peptidoglycan biosynthesis

Dilutions (1 in 100) of overnight culture in fresh TSB were made for the three *S. aureus* strains (RN4220 *gltA*, RN4200 *oppB* and RN4220 *murZ* KO), which were grown to an OD_{600nm} of approximately 0.2. 900 µl samples of the strains were exposed to a range of eleven concentrations of antibiotics or experimental inhibitors. After one hour incubation at 37°C with aeration, the OD_{600nm} of the samples was recorded. The samples were then processed as described by Horsburgh *et al.* (2001) with modification. Briefly, resuspended cells were treated with 2 µl lysostaphin (5 mg/ml) for ten minutes at 37°C,

followed by the addition of 50 µl of 4-methyl-umbeliferyl-β-D-galactoside (10 mg/ml) in DMSO to each sample. Following ninety minutes' incubation at room temperature, the fluorescence of the samples was recorded in a BMG plate reader [BMG Labtech Ltd, Aylesbury, UK] (Excitation 365 nm, Emission 460 nm). The fold increase in β-galactosidase activity was determined relative to a drug-free control. The induction threshold was set at greater than or equal to twofold for a positive result.

2.7 *B. subtilis* antibiotic biosensors

The *B. subtilis* antibiotic biosensors were used in this study essentially as described by Fischer *et al.* (2004) and Urban *et al.* (2007) but with some modifications. Dilutions (1 in 100) of overnight culture in fresh LB were made of the biosensor strains, which were grown to an OD_{600nm} of approximately 0.2 units. In a white 96 well plate, 90 µl samples were exposed to antibiotics and experimental inhibitors at a range of eleven concentrations covering values below and above the MIC. After incubation for 1-3 hours (37°C) depending on the construct, 60 µl of 0.8 M luciferin in 0.1 M citrate buffer (0.1 M citric acid and 0.1 M sodium citrate [pH 5]) were added to each well. The luminescence of each sample was measured immediately in a BMG plate reader and determined as a value relative to the drug free control. Induction thresholds were as follows: ≥1.7 fold for the cell envelope biosensor, ≥2.5 fold for the DNA biosensor and ≥2 fold for the RNA, protein and fatty acid biosensors for a positive result (Urban *et al.*, 2007). Vancomycin, tetracycline, rifampicin, ciprofloxacin and triclosan were used as the positive reference agents for the cell-envelope, protein, RNA, DNA and fatty-acid biosensors, respectively.

2.8 Measurement of bacterial membrane damage

2.8.1 *Bac*Light™

The membrane damage inflicted by a panel of agents on *S. aureus* SH1000 was analysed using the LIVE/DEAD *Bac*Light™ bacterial viability kit (Invitrogen), essentially as described by Hilliard *et al.* (1999). *S. aureus* SH1000 was grown in MHB to an OD_{600nm} between 0.5 and 0.6. 0.5 ml samples were centrifuged twice at 10,000 X *g* (15 minutes) and washed twice with H_2O (1.5 ml). Cell pellets were resuspended in H_2O (50 µl), to which test and control antibacterial agents (950 µl) at a concentration of 4X MIC were added. Samples were then incubated at room temperature with gentle mixing for ten minutes. Following two washes with H_2O, the resuspended samples were adjusted to an OD_{600nm} of 0.15 units. Samples (0.5 ml) were then added to H_2O (1.5 ml) in an acrylic fluorescence cuvette. To each sample, 6 µl *Bac*light™ reagent (containing 1:1 red [propidium iodide] and green [SYTO® 9]) was added and incubated at room temperature in darkness for fifteen minutes. Fluorescence was measured in a Perkin-Elmer LS 45 luminescence spectrophotometer [Perkin-Elmer, Cambridge, UK] (Excitation – 485 nm, Red fluorescence emission – 645 nm and Green fluorescence emission – 530 nm). Percentage membrane damage of antibiotic- or inhibitor-treated SH1000 was expressed as the ratio of the green: red relative to a drug free control.

2.8.2 Atomic absorption spectroscopy

Leakage of intracellular potassium from *S. aureus* was examined over a time course as described previously (Hobbs *et al.*, 2008). Mid exponential phase cultures (OD_{600nm} of

0.2 units) were resuspended in 5 mM HEPES buffer (pH 7.2) supplemented with 5 mM glucose, and exposed to 4X MIC antibiotic or inhibitor for three hours. Samples were taken at time intervals and passed through a 0.2 μm syringe filter [Sartorius Stedim UK Ltd, Surrey, UK], following which the potassium (K^+) ion content was determined as previously described (Ioannou *et al.*, 2007) using a Perkin Elmer AAnalyst 100 atomic absorption spectrophotometer [Perkin-Elmer, Cambridge, UK] at the School of Chemistry, University of Leeds. The remaining intracellular concentration of K^+ at each timepoint was expressed as a percentage of total cellular K^+ (a value for which was obtained from a ten minute boiled sample of the untreated, resuspended culture at $OD_{600nm} = 0.2$).

2.9 Macromolecular synthesis assays

Inhibition of DNA, RNA, protein and peptidoglycan synthesis was examined in mid-exponential-phase (OD_{600nm} of 0.2) cultures of *S. aureus* SH1000. Cells were exposed to antibiotics and inhibitors and measurement of the incorporation of the radiolabelled precursors [methyl-^{3}H] thymidine (DNA), [5, 6-^{3}H] uridine (RNA) and L-[G-^{3}H] glutamine (protein) at 1 mCi/ml and [1-^{14}C] glycine (peptidoglycan) at 0.1 mCi/ml [Perkin-Elmer, Cambridge, UK], was performed as previously described (Cherrington *et al.*, 1990). Isotopes were added to growing cultures 10 minutes before addition of test antibiotics at 4X MIC. After ten minutes incubation at 37°C, culture samples were added to 5 ml volumes of ice cold trichloroacetic acid (TCA) and left on ice for twenty minutes to precipitate macromolecules. The samples were then processed as previously described (Wilson *et al.*, 1995), and the radioactivity measured using a Packard Tri Carb 2100TR liquid scintillation analyser [Packard Bioscience Ltd, Berkshire, UK].

2.10 Generation of antibiotic-resistant mutants

2.10.1 Generation of spontaneous antibiotic-resistant mutants and mutation frequencies

S. aureus SH1000 was used as the parental strain for the generation of antibiotic-resistant mutants. Spontaneous mutants were generated by plating out 20 µl of a 10X concentrated overnight culture (achieved by resuspension of the overnight culture in one tenth the original volume) onto 2 ml MHA with 4X MIC of each antibacterial agent in 8-well plates, which were subsequently incubated at 37°C for forty eight hours to select for resistance. The frequency of mutation was calculated as the number of mutants per total number of viable bacteria (O'Neill *et al.,* 2001).

2.10.2 Generation of antibiotic-resistant mutants: Continuous subculture

The generation of resistant mutants by continuous subculture in the presence of sub-inhibitory concentrations of agent was carried out essentially as previously described (Miller *et al.*, 2002). Briefly, 5 µl of an overnight culture of *S. aureus* SH1000 were used to inoculate MHB (9 ml) containing 0.25X MIC of the antibiotic or inhibitor and incubated overnight (eighteen hours) at 37°C. After incubation, 5 µl samples of the overnight culture were transferred into fresh broth containing 0.25X MIC of the agent to continue the selection. A 10X concentrated sample of the overnight culture was plated onto MHA containing 4X MIC of the agent and incubated at 37°C for a further forty eight hours to select for resistant mutants, and to ascertain at which passage resistance had emerged.

2.11 Growth rates determinations

Growth rate determinations were carried out by a method modified from Hurdle *et al.* (2004). Overnight cultures of selected *S. aureus* strains were diluted 1:100 in MHB and then grown to an OD_{600nm} of approximately 0.1 units. Optical density readings were then taken every three to four minutes until the OD_{600nm} reached approximately 0.2 units. The OD readings were transformed to log values, plotted on graphs and the doubling time for each mutant strain was calculated (doubling time = $\log_{10}$ (2)/gradient).

2.12 Molecular Biology Techniques

2.12.1 DNA manipulation and polymerase chain reaction (PCR)

S. aureus SH1000 genomic DNA was extracted using the Bacterial Genomic DNA purification kit [Edge Biosystems, Gaithersburg, USA] according to the manufacturer's instructions, from protoplasts generated by incubating whole cells with lysostaphin (40 μg/ml) and mutanolysin (10 KU/ml) for sixty minutes at 37°C.

PCR amplification and sequence determination of RNA polymerase subunit genes (*rpoA, rpoB* and *rpoC*) was carried out essentially as described (O'Neill *et al.*, 2006), in a final volume of 25 μl using 2X concentrated extensor Hi-fidelity PCR Master Mix [Thermo Scientific, Surrey, UK] consisting of a high fidelity DNA polymerase mix, dNTPs (350μM) and $MgCl_2$ (2.25mM), 2 μl genomic DNA, 8.5 μl H_20 and the primers described in Table 2.4. These primers were designed using Oligo 6.0 [MBI, Colorado, USA] and purchased from MWG Biotech [Milton Keynes, UK]. Amplification

Table 2.4 – Primers used for amplification and sequence determination of RNA polymerase subunit genes

RNA polymerase subunit gene	Primer	Type	Nucleotide Sequence (5' → 3')	Direction in gene
rpoA **(α subunit)**	rpoA1	Amplification	TAACTGCGATCAGAGACGTTACTCC	Forward
	rpoA2	Amplification	GCTGCATTACGACGAGAAGCTAAAT	Reverse
	rpoAseq	Sequencing	TACTATCTTCATTACCAGGTG	Forward
rpoB **(β subunit)**	rpoBFkpn	Amplification	TAGGGTACCGCGGATCACATAATTTTTGAG	Forward
	rpoBRsac	Amplification	TAGGAGCTCTTTGCCTGTTTTGTAAATTGC	Reverse
	F3	Sequencing	AGTCTATCACACCTCAACAA	Forward
	F4	Sequencing	TAATAGCCGCACCAGAATCA	Reverse
	Rif2	Sequencing	ACAGATGCTAAAGATGTTGTATAC	Forward
	Rif3	Sequencing	TCAATTAAAGTATATGTTCCTAAC	Forward
	Rif4	Sequencing	ACCAATATAAACGATACCACGATC	Reverse
	Rif5	Sequencing	ATCACGAGCCATACCAGCTTCTTC	Reverse
rpoC (β' subunit)	rpoC1	Amplification	GACGATGATGTTGTAGAACGCAAAG	Forward
	rpoC2	Amplification	TGTTGTTTGTTAAAGCGTGCAACT	Reverse
	rpoCseq1	Sequencing	AAAAATGGGTGCAGAAGGTA	Forward
	rpoCseq2	Sequencing	TACTTGGTAAACGTGTTGAC	Forward
	rpoCseq3	Sequencing	TAAACCTGTAGTTACACCATCACA	Forward
	rpoCseq4	Sequencing	GATAGAGGTTTATTAGTTTCTG	Forward
	rpoCseq5	Sequencing	TGCATACGGTAAACTTTTTGTA	Reverse

Table 2.5 – Conditions for PCR amplification of RNAP subunit genes

Stage	Temperature (°C)	Time	Cycles
1. Initial Denaturation	94	2 minutes	1
2. Denaturation	94	10 seconds	35
Annealing	52	30 seconds	
Extension	68	4 minutes	
3. Final Extension	68	7 minutes	1
4. Hold	4	-	-

conditions for the RNAP subunit genes are outlined in Table 2.5. The resultant amplicons were visualised using agarose gel electrophoresis (0.8% (w/v) agarose, 90V for 25 minutes) and purified using a MinElute gel extraction kit (Qiagen, Paisley, UK) according to the manufacturer's instructions. The concentration of DNA in the samples was quantified using an IMPLEN nanophotometer at an absorbance of 260nm [Geneflow Ltd. Staffordshire, UK]. The purified RNAP subunit PCR fragments were sequenced using the primers used for amplification and sequence determination (Table 2.4) by Beckman Coulter Genomics [Takely, UK].

2.12.2 Transcriptional profiling and isolation of total RNA from *S. aureus* SH1000

S. aureus SH1000 was grown at 37°C in MHB to an OD_{600nm} of 0.2 units, after which membrane-damaging agents were added at concentrations which inhibited growth of *S. aureus* by 25% relative to a drug free control over a forty minute time period (CTAB at 1.25 µg/ml, clofazimine at 3 µg/ml and sepracor 155342 at 0.01 µg/ml). After incubation with the inhibitor, 10 ml of the culture (4X 10^9 cells) was immediately dispensed into 20 ml RNA protect bacterial reagent. Cell pellets obtained by centrifugation (5000X *g*, 10 minutes) were washed in TE buffer (10 mM tris (hydroxymethyl) aminomethane, 1 mM ethylenediaminetetraacetic acid [pH 8]) to

remove excess RNAprotect reagent and resuspended in the same buffer containing RNAase-free lysostaphin (200 μg/ml). After incubation at 37°C for ninety minutes, proteinase K (40 μg/ml) was added, and the cell suspension was incubated at room temperature for ten minutes. Total RNA was harvested from the cells using a RNAeasy midi kit [Qiagen, Crawley, UK] according to the manufacturer's instructions, including an on-column DNA digestion step using DNAase 1 [Qiagen, Crawley, UK]. Purified RNA was quantified using an IMPLEN nanophotometer [Geneflow Ltd. Staffordshire, UK], and stored at -80°C for up to six months.

2.12.3 Microarray hybridisation and data analysis

Synthesis, labelling and hybridisation of cDNA and microarray analysis were performed by Roche Nimblegen [Madison, USA]. The data were analysed using ArrayStar® 4 [DNASTAR Inc, Madison, USA]. Differential expression was considered significant for genes displaying ≥2 fold up or downregulation.

Chapter 3 - Validation of assays for the characterisation of antimicrobial agents

3.1 Abstract

The successful development of novel antibacterial agents requires extensive preclinical evaluation to ensure that the observed antibacterial activity is correlated with inhibition of a specified cellular target and in addition there is no activity against eukaryotic homologues. To validate some of the methods described in this thesis for the characterisation of compounds with unknown modes of action (MOAs), and to determine the response and reliability of the assays, a panel of forty antibacterial agents with well defined MOAs were screened for antibacterial spectrum of activity, membrane-damaging activity (using the *Bac*Light™ assay), inhibition of cellular biosynthetic pathways (using antibiotic biosensors) and prokaryotic specificity (by determination of minimum inhibitory concentrations against *Saccharomyces cerevisiae*). Determination of the minimum inhibitory concentrations (MICs) against an AcrAB deficient *Escherichia coli* mutant and the wild-type *E. coli* treated with polymyxin B nonapeptide (PMBN), a Gram-negative outer membrane permeabiliser, also yielded information concerning the basis for intrinsic resistance in Gram-negatives to antibacterial agents of different structural classes. The *Bac*Light™ assay provided a system for preliminary determination of the membrane damaging effects of antibacterial agents since it was able to classify all of the agents tested into the expected categories of inactive against membranes or membrane damaging. The utility of the *Bacillus subtilis* antibiotic biosensors and *Staphylococcus aureus* peptidoglycan (PG) reporters for preliminary screening of the MOA of antibacterial agents was confirmed, as these systems were able to detect and identify the majority of inhibitors of peptidoglycan, DNA, RNA, fatty acid and protein biosynthesis correctly. In addition, susceptibility

determinations against *S. cerevisiae* confirmed that the majority of clinically relevant antibacterial inhibitors display ≥10 fold greater activity against bacteria, compared with yeast. Therefore this assay can be used as an indication of the prokaryotic specificity of experimental inhibitors.

3.2 Introduction

The increasing incidence of antibiotic resistance in bacteria is becoming a global health issue, and the associated need for novel antibacterial agents which overcome existing resistance mechanisms is not being met (Projan & Shales, 2004). The development of novel antibacterial agents is a complex process which requires extensive preclinical evaluation of inhibitors to ensure that any observed antibacterial activity (as determined by minimum inhibitory concentrations or MICs) is correlated with inhibition of a specific bacterial target in addition exhibiting low, or no activity against eukaryotic homologues (O'Neill & Chopra, 2004a). This is due to the fact that non-specific antibacterial activity is often linked to toxic side effects in humans (Silver, 2011). These stages of antibacterial development are key to the prediction of clinical outcome of treatment and spectrum of activity (Spanu *et al.*, 2004).

3.2.1 Antibacterial susceptibility profiling, determination of MICs

MIC determinations should be conducted against both Gram-positive and Gram-negative organisms to classify pathogens as resistant or susceptible to the compound, giving vital information in predicting which infections the drug might provide suitable chemotherapy. *Staphylococcus aureus* and *Escherichia coli* represent significant human pathogens in the categories of Gram-positive and Gram-negative bacteria respectively, and are routinely chosen as representative organisms since their genetics and physiology are well characterised (O'Neill & Chopra, 2004a). Gram-negative bacteria however, are innately resistant to many antibacterial classes due to i) the presence of the outer membrane, which acts as a barrier to hydrophobic antibiotics and hydrophilic

compounds greater than 600 Da in size (Sahalan & Dixon, 2008), and ii) the activity of multi-drug efflux systems, particularly the tripartite antibiotic efflux pump AcrAB-TolC (Sulavik *et al.*, 2001; Vaara, 1992). The addition of subinhibitory concentrations of polymyxin B nonapeptide (PMBN), which is an analogue of the membrane-damaging agent polymyxin B, to wild-type *E. coli* during susceptibility testing can permeabilise the outer membrane to such antibiotics (Kwon & Lu, 2006, Mamelli *et al.*, 2009). Similarly, deletion of the AcrAB component increases susceptibility of *E. coli* to antibiotics normally subject to efflux (O'Neill *et al.*, 2002; Stubbings *et al.*, 2004). By utilising deletion strains and outer membrane permeabilisers, the basis for intrinsic resistance in Gram-negatives to some antibacterial agents may be ascertained, in order that more active derivatives may be designed and synthesised.

3.2.2 Biosensor approaches for antibacterial mode of action (MOA) analyses

Whole cell-based biosensors are a useful tool for screening inhibitors during the process of anti-bacterial drug discovery. In specifically selecting for compounds with antibacterial activity, these methods overcome issues such as poor compound penetration or drug metabolism and efflux (Fischer *et al.*, 2004). Biosensors that contain promoter-reporter constructs which are induced by conditions of antibiotic-induced stress have previously been used for whole-cell based screening and characterisation of inhibitors (Bianchi & Baneyx, 1999; Shapiro & Baneyx, 2002; Urban *et al.*, 2007). Based upon a reference compendium of antibiotic-triggered microarray experiments, promoters which are induced in *Bacillus subtilis* by antibiotics have been identified and used to construct five promoter-luciferase reporter fusion strains (Fischer *et al.*, 2004; Urban *et al.*, 2007). These biosensors signal the presence of inhibitors of fatty-acid (*fabHB* promoter), DNA (*yorB*), cell-envelope (*ypuA*), RNA (*yvgS*) and protein (*yheI*)

biosynthesis and therefore represent an excellent tool for preliminary screening of novel antibacterial agents (Fischer *et al.*, 2004; Urban *et al.*, 2007). In addition, transcriptional analysis of *S. aureus* RN4220 demonstrated that inhibition of PG biosynthesis leads to induction of *gltA*, encoding a citrate synthase and *oppB,* an oligopeptide permease (O'Neill *et al.*, 2009). Further analysis identified that the UDP-GlcNAc enolpyruvyl transferase isoenzyme *murZ* was also upregulated in *S. aureus* in response to fosfomycin (Blake *et al.*, 2009). Following these studies, three *lacZ* reporter constructs were previously made which specifically show upregulation of these genes upon treatment with known or presumptive PG biosynthetic inhibitors (A. J. O'Neill and K. Blake, University of Leeds).

3.2.3 Determination of membrane damage caused by antibacterial agents

In addition to inhibitory effects on cellular biosynthetic pathways, it is important to determine whether novel antibacterial agent caused bacterial membrane damage, as this type of activity may suggest potential toxicity to mammals due to structural and functional homology between eukaryotic and prokaryotic membranes (O'Neill & Chopra, 2004a), indicating that such agents may not be suitable for systemic administration. The *Bac*light™ assay is a simple, quantitative measure of the maintenance of cell membrane integrity upon treatment with antibiotics and inhibitors (O'Neill *et al.*, 2004b). The fluorescent dyes used (SYTO-9 and propidium iodide) both bind to nucleic acid, however the latter is only able to penetrate cells with damaged membranes and in doing so reduces the amount of bound SYTO-9. The ratio of fluorescence intensity of the two dyes is therefore a direct measure of the membrane damage caused by an agent (O'Neill *et al.,* 2004b; Oliva *et al.*, 2004).

3.2.4 Antibacterial prokaryotic specificity analysis

Although membrane-damaging agents are often disregarded in antibacterial development programs, membrane damagers with specificity for bacteria versus eukaryotes may still be viable chemotherapeutic candidates (as evidenced by the recent introduction of daptomycin and telavancin into clinical use) (Hurdle *et al.*, 2011). The MICs of antibiotics against *Saccharomyces cerevisiae*, a budding yeast which is used extensively as a model organism in molecular and cellular biology (Botstein *et al.*, 1997; Fields & Johnston, 2005), is a simple screen to highlight compounds which could cause undesirable host toxicity during therapy against higher eukaryotes. Such compounds would therefore be unsuitable for further development as antibacterial agents.

3.2.5 Aims and objectives of the work described in this chapter

The methodology described above for antibacterial susceptibility testing is widely accepted as the most suitable method for the determination of the potency of novel agents. However, MOA analysis using the aforementioned biosensors has only been validated with a limited range of antibiotics (Blake *et al.*, 2009; Urban *et al.*, 2007). Therefore we sought to confirm their response to a wider panel of established antibacterial classes in order to confirm their validity for screening compounds with unknown MOAs. In addition, the *Bac*Light™ assay and *S. cerevisiae* susceptibility testing were performed for the same panel of compounds, to establish the utility of these assays for determining membrane damage and bacterial specificity for compounds with unknown MOAs to be described later in this thesis.

Therefore the aims and objectives of this chapter include:

- characterisation of the antibacterial spectrum of activity of antibacterial agents with established MOAs;
- confirmation of the MOA of these compounds using *Bac*Light™ and biosensor approaches;
- confirmation of the bacterial specificity of established antibacterial agents using *S. cerevisiae* as a model eukaryote.

3.3 Results and Discussion

3.3.1 MIC determinations

S. aureus SH1000 (a widely used standard laboratory strain) was chosen as the representative Gram-positive organism for susceptibility testing. *B. subtilis* 1S34 was tested as it is the parental organism for the biosensor strains (O'Neill, 2010; Urban *et al.*, 2007). *E. coli* was chosen as the representative Gram-negative organism. MICs of the agents against a wild-type *E. coli* strain (1411), an *acrAB* efflux pump deficient derivative strain (SM1411) and *E. coli* 1411 in the presence of 4 μg/ml PMBN were determined to elucidate the mechanism by which Gram-negative bacteria display intrinsic resistance to antibiotics (i.e. lack of penetration through the outer membrane). A standard concentration of 4 μg/ml PMBN was chosen as it is a subinhibitory concentration of the agent which has been utilised previously to permeabilise *E. coli* to fusidic acid, which is not normally able to penetrate the outer membrane (Dixon & Chopra, 1986b).

Inhibitors of peptidoglycan (PG) biosynthesis generally showed more activity against the Gram-positive organisms tested than *E. coli* (Table 3.1). This is a well described phenomenon due to the presence of the Gram-negative outer membrane which acts as a barrier to the penetration of the larger inhibitors (in this study, those >200 Da in size) to their periplasmic target (Mims *et al.*, 2004). This conclusion is supported by the results in Table 3.1, in which the addition of the outer membrane permeabiliser PMBN lead to 2-16 fold increases in susceptibility for five of the seven PG biosynthesis inhibitors tested.

Table 3.1 – Spectrum of activity of established antibacterial agents

Antibacterial Agent	M_r	MIC(μg/ml)				
		S. aureus SH1000	*E. coli* 1411	*E. coli* 1411 + PMBN (4 μg/ml)	*E. coli* SM1411	*B. subtilis* 1S34
PG biosynthesis inhibitors						
Vancomycin	1449.2	2	256	128	256	0.5
Flucloxacillin	453.9	0.25	>256	128	16	0.125
Fosfomycin	194.1	8	32	16	32	64
D-cycloserine	102.1	64	64	32	32	32
Bacitracin	1422.7	128	>256	>256	>256	>256
Tunicamycin	844.9	>256	>256	>256	>256	0.5
Fosmidomycin	183.1	>256	128	8	128	4
Cell membrane damagers						
Valinomycin	1111.4	2	>128	128	>128	4
Telavancin	1792.1	1	>256	>256	>256	0.031
Nisin	3354.1	4	>64	16	>64	4
CTAB	364.5	2	16	8	16	2
Clofazimine	472.1	2	>128	2	>128	2
Sepracor 155342	550.3	4	16	16	8	16
Chlorhexidine	897.8	1	1	1	0.5	1
Anhydrotetracycline	462.9	2	4	1	2	0.5
Polymyxin B	1385.0	>256	1	1	2	16
Daptomycin	1620.7	2	>256	>256	>256	2
XF70	694.9	0.5	8	4	8	0.5
XF73	694.9	0.5	64	64	64	0.25
Protein biosynthesis inhibitors						
Tetracycline	444.0	0.5	2	2	1	8
Fusidic Acid	516.7	0.125	>256	4	>256	0.25
Actinonin	385.5	32	>64	2	8	16
Erythromycin	733.9	0.5	128	2	32	0.25
Chloramphenicol	323.1	4	4	1	1	4
Gentamicin	575.0	0.25	2	1	2	1
Linezolid	337.4	4	>256	256	8	1
Streptomycin	1457.4	4	>256	>256	>256	256
Clindamycin	424.4	0.125	>256	>256	>256	>256
Cephalosporin P1	574.4	0.25	>256	64	>256	1
Virginiamycin M1	525.6	16	>64	32	>64	64

Table 3.1 continued...

RNA biosynthesis inhibitors						
Rifampicin	823.0	0.015	4	0.125	4	128
Rifamycin SV	697.8	0.125	64	0.5	64	16
DNA biosynthesis inhibitors						
Ciprofloxacin	331.3	1	0.5	0.5	0.125	0.125
Trimethoprim	290.3	8	1	1	0.5	0.5
Sulfamethoxazole	523.3	>256	>256	256	>256	>256
Novobiocin	634.6	0.125	256	8	8	1
Fatty acid biosynthesis inhibitor						
Triclosan	289.5	0.125	0.25	0.015	0.125	1
tRNA synthetase inhibitors						
Mupirocin	500.6	0.0625	32	0.25	1	0.015
Indolmycin	257.3	4	128	32	4	16
Borrelidin	489.7	32	>128	16	128	4

A similar increase in susceptibility of Gram-negative bacteria to vancomycin and β-lactams has been reported after PMBN supplementation (Kwon & Lu, 2006; Vaara, 1992). Amongst the β-lactams only flucloxacillin demonstrated a reduced MIC for the *acrAB* efflux pump deficient *E. coli* mutant, indicating that with the exception of penicillins, inhibitors of PG biosynthesis tend not to be subject to efflux. This is an observation which is supported in current literature and may aid in the characterisation of putative inhibitors of peptidoglycan synthesis (Nikaido *et al.*, 2009; Van Bembeke *et al.*, 2000).

A wider spectrum of activity was observed with the cell membrane-damaging agents, with the exception of valinomycin, telavancin, nisin, clofazimine and daptomycin, to which wild-type *E. coli* proved insusceptible, and polymyxin B, which showed preferential activity towards *E. coli.* The principal activity of polymyxin B on Gram-

negative bacteria is well described in current literature, and is due to rapid disruption of both the inner and outer membranes of Gram-negative bacteria, leading to loss of intracellular proteins and essential metabolites, resulting in cell death (Dixon & Chopra, 1986a; Storm *et al.*, 1977). As such, polymyxin B is currently only indicated for severe infections caused by the Gram-negative pathogen *Pseudomonas aeruginosa* (Greenwood *et al.*, 2007). The preferential activity of nisin, telavancin and daptomycin against Gram-positive organisms is also well documented (Eliopoulos *et al.*, 1986; Higgins *et al.*, 2005; Mattick & Hirsch, 1944), and in the case of the latter two antibiotics, provides an explanation for their sole clinical indication for treatment of serious Gram-positive infections, particularly in cases where *S. aureus* is the causative organism (Higgins *et al.*, 2005; Hobbs *et al.*, 2008). Similarly to inhibitors of cell wall biosynthesis, cell membrane damagers do not seem to be subject to efflux by AcrAB in Gram-negative organisms, with only three out of the twelve agents tested (i.e. sepracor 155342, chlorhexidine and anhydrotetracycline) displaying increased activity (two fold greater) against the *acrAB* knockout strain. Chlorhexidine is a known substrate for the QacA multi-drug efflux pump, a member of the Major Facilitator Superfamily of transporters (Paulsen *et al.*, 1996), and AcrAB is capable of effluxing other membrane damagers such as sodium dodecyl sulphate (SDS) and Triton X-100 (Nikaido 2009). In addition, six of the twelve membrane damaging agents showed increased activity (2->64 fold) against *E. coli* 1411 upon supplementation of the media with PMBN. Presumably, permeabilisation of the outer membrane with PMBN increases availability of the agents at the inner membrane, leading to increased damage at this site, and the observed decrease in MIC.

With the exception of tetracycline, erythromycin, chloramphenicol and gentamicin, the inhibitors of bacterial protein synthesis display a narrow spectrum of activity, primarily against the Gram-positive organisms tested. The broad-spectrum activity of the aforementioned compounds has been well documented in the literature (Greenwood *et al.*, 2007; Rasmussen *et al.*, 1991; Retsema *et al.*, 1987; Shakil *et al.*, 2008), and reflects the clinical administration of these agents against a wide range of bacterial infections (Greenwood *et al.*, 2007). The lack of activity of the remaining seven protein biosynthesis inhibitors against *E. coli* 1411 can be attributed to an inability to penetrate the Gram-negative outer membrane i.e. fusidic acid, cephalosporin P1 and virginiamycin M1 (a streptogramin A antibiotic), which is in some cases combined with antibiotic efflux by the AcrAB-TolC multi-drug efflux pump i.e. actinonin (a peptide deformylase inhibitor) and linezolid (Aller *et al.*, 2005). The activity of streptomycin and clindamycin against *E. coli* was not improved by the supplementation of the media by PMBN, or by deletion of AcrAB (Table 3.1). From this, it can be concluded that Gram-negative bacteria are innately resistant to these two antibiotics by an alternate mechanism. In *E. coli*, streptomycin is a known substrate of the AcrAD-TolC efflux pump, and the MexAB-OprM/MexXY-OprM efflux pump in *P. aeruginosa,* while clindamycin and related lincosamide antibiotics are reportedly extruded by a range of efflux pumps from the Major Facilitator superfamily and ATP-binding cassette transporter families (Nikaido, 2009; Van Bembeke *et al.*, 2000). In addition, fusidic acid and the structurally related antibiotic cephalosporin P1 which did not display increased activity in the *acrAB* knockout strain of *E. coli*, are still subject to efflux in Gram-negatives but are reportedly substrates for the structurally similar efflux pump, AcrAD-TolC (Nikaido, 2009).

Members of the rifamycin class of RNA polymerase inhibitors display a broad-spectrum activity, with particularly high potency against *Staphylococcus aureus* (Table 3.1). While mainly used in combination therapy for the treatment of tuberculosis, the observed broad-spectrum activity has allowed the indications for rifampicin to be expanded to include treatment of Legionnaires' disease, where it is used in combination with erythromycin (Greenwood *et al.*, 2007). While not subject to efflux by AcrAB, the supplementation of PMBN to the susceptibility assay increases the activity of these agents against *E. coli* 1411 by 32-128 fold, suggesting that in wild-type Gram-negative bacteria the activity of rifampicin is reduced due to the presence of the outer membrane. As previously mentioned, the outer membrane acts as a barrier to hydrophilic compounds greater than 600 Da in size (Sahalan & Dixon, 2008). Both the structures of rifamycin SV and rifampicin are over 600 Da in size, but are also composed of areas of both lipophilicity and hydrophilicity (Van Bambeke *et al.*, 2000). The lipophilicity of these compounds would account for the normal ability to penetrate the cell membranes of Gram-negative organisms, which is enhanced by the addition of an outer membrane permeabiliser due to their larger size.

The spectrum of activity of the four DNA synthesis inhibitors tested encompasses both the Gram-positive organisms tested and *E. coli*. The broad-spectrum nature of ciprofloxacin, trimethoprim and novobiocin has been well established in the literature, and is due to structural and functional homology of essential DNA synthetic machinery across bacterial genera (Beskid *et al.*, 1989; Brock, 1956; Coutourier *et al.*, 1998; Quinlivan *et al.*, 2000; Van de Casteele *et al.*, 1995). The poor antibacterial activity of suphamethoxazole and related sulphonamides is also well documented, and explains the administration of the former in combination with trimethoprim (co-trimoxazole) with

which it displays a synergistic effect (Hitchings, 1973). In agreement with the literature, ciprofloxacin, novobiocin and trimethoprim all appear to be substrates for the AcrAB efflux pump, as seen by the 2-32 fold increase in susceptibility of the AcrAB knockout *E. coli* mutant to these agents (Nikaido, 2009). In addition, however, the activity of both sulfamethoxazole and novobiocin is improved upon supplementation of the assay media with PMBN, suggesting that in wild-type *E. coli* these larger DNA synthesis inhibitors (with relative molecular masses of 523 and 635Da respectively) may have difficulty in penetrating the Gram-negative outer membrane.

Triclosan, an inhibitor of bacterial fatty acid biosynthesis, displays potent, broad-spectrum of activity across both the Gram-positive species and *E. coli* strains tested, which explains its widespread use as a biocide in soaps, detergents and toothpastes (Bhargava & Leonard, 1996). The broad-spectrum of activity of triclosan is due to the conserved nature of FabI, triclosan's intracellular target, across bacterial genera (Heath & Rock, 2000). From the results in Table 3.1 it is evident that the activity of triclosan is increased sixteen fold upon supplementation of the media with PMBN, suggesting that in wild-type *E. coli*, access of this agent to its intracellular target is hindered by the Gram-negative outer membrane.

The three aminoacyl-tRNA synthetase inhibitors tested also displayed broad-spectrum activity, but the Gram-positive organisms displayed higher susceptibility to these agents, in agreement with previous reports (Green *et al.*, 2009; Jarvest *et al.*, 2002; Sutherland *et al.*, 1985). The activity of all three inhibitors increased up to thirty-two fold in the AcrAB knockout mutant (in comparison to the wild-type *E. coli* strain) and up to one hundred and twenty-eight fold in wild-type *E. coli* with a permeabilised outer

membrane. Therefore, the tRNA synthetase inhibitors appear to be substrates for the AcrAB efflux pump, but are also obstructed from penetrating the Gram-negative outer membrane in wild-type *E. coli*.

From the results of Table 3.1, it can be concluded that, as expected, MICs determinations for inhibitors against *S. aureus*, *B. subtilis* and *E. coli* give a reliable indication of the spectrum of activity of the antibacterial agents tested. In addition, the activity of new agents against the AcrAB-deficient *E. coli* mutant and the wild-type treated with PMBN, may also yield valuable information concerning resistance mechanisms employed by Gram-negative bacteria to a new agent, in order that analogues with improved properties and a broader spectrum of activity may be designed and synthesised.

3.3.2 *Bac*Light™ measurement of membrane damage in *S. aureus* SH1000

The *Bac*Light™ assay is a quantitative measure of cell membrane damage effected upon treatment with test and control antibiotics at 4X MIC in comparison with a drug-free control. Untreated cells maintain 100% cell membrane integrity, while cells treated with the anionic surfactant SDS have 0% membrane integrity (100% membrane damage) after ten minutes exposure. *Bac*Light™ measurements of membrane damage caused by treatment of *S. aureus* SH1000 to the panel of agents with established MOAs is shown in Table 3.2. The data are discussed below in terms of the reliability of this assay for the characterisation of membrane damage caused by compounds with unknown MOAs. Agents with a *S. aureus* SH1000 MIC greater than 256 μg/ml (i.e. tunicamycin, fosmidomycin, polymyxin B and sulfamethoxazole) were unable to be tested in this

Table 3.2 - *Bac*Light™ measurement of membrane damage in *S. aureus* SH1000

Condition	*S. aureus* SH1000 MIC (μg/ml)	*Bac*light™ result (% membrane integrity)
Drug- free control	-	100
5% SDS (w/v)	-	0
PG biosynthesis inhibitors		
Vancomycin	2	83.5 ±4.4
Flucloxacillin	0.25	97.2 ±0.3
Fosfomycin	8	99.1 ±9.7
D-cycloserine	64	112.9 ±6.1
Bacitracin	128	93.3 ± 13.3
Cell membrane damagers		
Telavancin*	1	1.0 ±0.4
Daptomycin	2	94.7 ±14.1
Chlorhexidine	1	2.6 ±0.5
Anhydrotetracycline	2	91.1 ±10.1
Valinomycin	2	26.4 ±3.9
Nisin	4	14.9 ±1.0
CTAB	2	0
Clofazimine	2	1.3 ±0.3
Sepracor 155342	4	15.1 ±1.5
XF70*	0.5	0
XF73*	0.5	0
Protein biosynthesis inhibitors		
Tetracycline	0.5	99.9 ±4.6
Fusidic Acid	0.125	94.6 ± 12.8
Actinonin	32	94.2 ±7.4
Erythromycin	0.5	94.6 ±4.8
Chloramphenicol	4	105.2 ±4.4
Gentamicin	0.25	73.0 ±6.7
Linezolid	4	109.0 ±3.1
Streptomycin	4	105.8 ±13.3
Cephalosporin P1	0.25	87.6 ±7.2
Virginiamycin M1	16	123.0 ±6.0
Clindamycin	0.125	94.5 ±14.5
RNA biosynthesis inhibitors		
Rifampicin	0.015	80.8 ±6.1
Rifamycin SV	0.125	83.0 ±4.2
DNA biosynthesis inhibitors		
Ciprofloxacin	1	102.2 ±9.7
Trimethoprim	8	82.8 ±8.1
Novobiocin	0.125	105.0 ±4.9
Fatty acid biosynthesis inhibitor		
Triclosan	0.125	103.2 ±2.8
tRNA synthetase inhibitors		
Mupirocin	0.0625	107.7 ±12.2
Indolmycin	4	116.5 ±7.9
Borrelidin	32	66.7 ±2.2

* *Performed by Nicola Ooi, University of Leeds*

assay due to the possibility of the large concentrations required giving false readings. After ten minutes exposure to each antibacterial agent, all antibacterial agents not in the cell membrane-damaging class maintained between 66.7->100% membrane integrity, which is consistent with current literature describing the activities of these compounds (See Introduction). Within the group of membrane damagers, only *S. aureus* cells treated with daptomycin and anhydrotetracycline maintained close to 100% membrane integrity, while the remaining nine known membrane damagers caused a reduction in membrane integrity of *S. aureus* to <27% of that of the drug-free control, consistent with their reported MOAs (See Introduction; Higgins *et al.*, 2005; Oliva *et al.*, 2003; Ooi *et al.*, 2009a; Russel, 1986; Salton *et al.*, 1951 Tosteson *et al.*, 1967). Failure to observe membrane damage with daptomycin and anhydrotetracycline can be explained by the fact that in previous reports of the membrane damaging effects of these compounds, the effects were not observed until after ten minutes exposure to the agents. Exposure of *S. aureus* SH1000 to 4X MIC daptomycin resulted in a 90% reduction in intracellular potassium ions, although this effect was not apparent until 40 minutes after addition of the drug (Hobbs *et al.*, 2008). Similarly, a measurable release of the intracellular enzyme β-galactosidase from *E. coli* was not observed until after ten minutes exposure to anhydrotetracycline (Oliva *et al.*, 1992).

The *Bac*Light™ assay therefore provides a robust system for determination of membrane damage by antibacterial agents, as with the exception of two compounds, it was able to classify all of the agents tested into the expected categories of not active against membranes or membrane damaging. Since inhibitory effects on the cell membrane may indicate potential toxicity to mammals indicating that such agents *may* not be suitable for systemic administration, it may be prudent to perform the

*Bac*Light™ assay in the preliminary stages of antibacterial agent characterisation to discard compounds with undesirable characteristics at the earliest opportunity (O'Neill & Chopra, 2004a). From the results shown in Table 3.2 it can also be concluded that for MOA characterisation purposes in the following chapters, a *Bac*Light™ result of 40% membrane integrity represents the upper threshold for an agent to be classed as membrane damaging. However, this assay is only suitable for the preliminary detection of membrane damage, and therefore with agents of unknown MOAs which are suspected to cause membrane damage, it may be necessary to use further membrane damage assays such as determination of potassium ion (K^+) leakage over an extended period as previously described (Hobbs *et al.*, 2008).

3.3.3 *B. subtilis* antibiotic biosensors

The *B. subtilis* antibiotic biosensor strains have previously been shown to be capable of detecting the presence of inhibitors of fatty-acid, DNA, cell-envelope, RNA or protein biosynthesis when incubated with agents at three standard concentrations of 25 μg/ml, 6.25 μg/ml and 1.56 μg/ml (Fischer *et al.*, 2004; Urban *et al.*, 2007). The same set of biosensors was utilised to analyse their response to a selection of antibiotics with established MOAs, in order to confirm and expand confidence in their utility for analysis of agents with unknown or poorly characterised MOAs. It was expected that inhibitors of PG biosynthesis and cell membrane damagers might induce *ypuA*, protein biosynthesis inhibitors and tRNA synthetase inhibitors might induce *yheI* and RNA, DNA and fatty acid synthesis inhibitors should induce *yvgS, yorB* and *fabHB* respectively. The response of the *B. subtilis* antibiotic biosensors to the panel of well characterised antibacterial agents is shown in Table 3.3. The biosensors were used by

Table 3.3 - Induction of *B. subtilis* biosensors by established antibacterial agents

Antibacterial Agent	Upregulated biosensor promoter				
	Cell-envelope	*Protein*	*RNA*	*DNA*	*Fatty-acid*
PG biosynthesis inhibitors					
Vancomycin	+ **(2.7 ±0.2)**	- (0.9 ±0.1)	- (1.1 ±0.1)	- (1.1 ±0.2)	- (1.1 ±0.1)
Flucloxacillin	+ **(2.9 ±0.1)**	- (0.8 ±0.1)	- (1.1 ±0.3)	- (1.0 ±0.1)	- (1.1 ±0.1)
Fosfomycin	- (1.1 ±0.1)	- (0.9 ±0.1)	- (0.8 ±0.2)	- (0.9 ±0.1)	- (1.1 ±0.4)
D-cycloserine	- (1.1 ±0.3)	- (1.0 ±0.1)	- (1.3 ±0.2)	- (0.9 ±0.1)	- (1.1 ±0.2)
Tunicamycin	- (1.2 ±0.1)	- (1.5 ±0.3)	- (1.1 ±0.2)	- (1.7 ±0.2)	- (1.7 ±0.2)
Fosmidomycin	- (1.2 ±0.2)	- (0.8 ±0.2)	- (0.7 ±0.1)	- (0.8 ±0.2)	- (1.1 ±0.1)
Cell membrane damagers					
Telavancin[¥]	+ **(3.1 ±1.1)**	- (0.7 ± 0.2)	- (1.3 ± 0.3)	- (1.2 ± 0.4)	- (1.3 ± 0.2)
Daptomycin[¥]	+ **(2.3 ±0.5)**	- (1.2 ± 0.1)	- (1.2 ± 0.3)	- (1.0 ± 0.2)	- (1.2 ± 0.4)
Chlorhexidine	+ **(2.1 ± 0.1)**	- (0.7 ±0.1)	- (0.8 ±0.3)	- (0.9 ±0.1)	- (1.0 ±0.1)
Anhydrotetracycline	+ **(1.9 ± 0.1)**	+ **(10.7 ± 1.7)**	- (1.5 ± 0.2)	- (1.8 ± 0.1)	- (1.6 ± 0.2)
Valinomycin	- (1.0 ±0.1)	- (0.6 ±0.1)	- (1.6 ± 0.1)	- (1.6 ± 0.4)	- (1.2 ± 0.1)
Nisin	- (1.6 ±0.1)	- (0.8 ±0.1)	- (1.1 ±0.2)	- (1.2 ±0.4)	- (0.9 ±0.1)
CTAB	- (1.0 ±0.1)	- (1.0 ±0.4)	- (1.0 ±0.2)	- (0.9 ±0.1)	- (1.3 ±0.5)
Clofazimine	- (1.3 ±0.5)	- (0.8 ±0.3)	- (1.2 ±0.2)	- (0.9 ±0.1)	- (1.1 ±0.2)
Sepracor 155342	- (1.0 ±0.1)	- (1.3 ±0.2)	- (1.0 ±0.1)	- (1.1 ±0.1)	- (1.3 ±0.2)
Polymyxin B*	- (1.5 ±0.1)	- (1.0 ±0.1)	- (1.1 ±0.1)	- (1.1 ±0.1)	- (1.3 ±0.3)
XF70[¥]	- (1.5 ±0.1)	- (1.0 ±0.1)	- (1.1 ±0.1)	- (0.9 ±0.1)	- (0.9 ±0.1)
XF73[¥]	- (1.4 ±0.1)	- (1.0 ±0.1)	- (1.1 ±0.1)	- (1.0 ±0.1)	- (0.8 ±0.1)
Protein biosynthesis inhibitors					
Tetracycline	- (1.3 ±0.1)	+ **(2.1 ±0.1)**	- (1.5 ±0.3)	- (1.1 ±0.1)	- (1.2 ±0.2)
Fusidic Acid	- (0.7 ±0.1)	+ **(3.1 ±0.4)**	- (0.8 ±0.1)	- (0.9 ±0.1)	- (1.2 ±0.2)
Actinonin	- (1.3 ±0.1)	- (1.1 ±0.3)	- (1.3 ±0.1)	- (1.2 ±0.1)	- (1.2 ±0.1)
Erythromycin*	- (1.0 ±0.1)	- (1.0 ±0.2)	- (1.2 ±0.2)	- (1.2 ±0.1)	- (1.3 ±0.3)
Chloramphenicol*	- (1.0 ±0.1)	+ **(2.3 ±0.3)**	- (1.2 ±0.5)	- (1.1 ±0.2)	- (0.9 ±0.1)
Gentamicin*	- (1.1 ±0.1)	- (1.0 ±0.1)	- (1.2 ±0.1)	- (1.0 ±0.1)	- (1.0 ±0.1)
Linezolid*	- (1.0 ±0.1)	+ **(2.4 ±0.1)**	- (1.0 ±0.2)	- (1.0 ±0.1)	- (0.9 ±0.1)
Streptomycin*	- (1.0 ±0.1)	- (0.9 ±0.1)	- (1.3 ±0.1)	- (1.1 ±0.1)	- (1.2 ±0.1)
Cephalosporin P1	- (1.0 ±0.1)	+ **(5.2 ±0.6)**	- (0.6 ±0.1)	- (1.1 ±0.1)	- (1.1 ±0.1)
Virginiamycin M1	- (1.0 ±0.1)	+ **(5.3 ±0.7)**	- (1.0 ±0.4)	- (1.0 ±0.1)	- (1.1 ±0.1)
RNA biosynthesis inhibitors					
Rifampicin	- (1.0 ±0.1)	- (1.0 ±0.1)	+ **(2.7 ±0.2)**	- (1.0 ±0.1)	- (0.9 ±0.1)
Rifamycin SV	- (1.3 ±0.1)	- (0.9 ±0.1)	+ **(2.3 ±0.1)**	- (1.2 ±0.1)	- (1.2 ±0.2)
DNA biosynthesis inhibitors					
Ciprofloxacin	- **(1.8 ±0.4)**	- (1.0 ±0.1)	- (1.1 ±0.2)	+ **(74.9 ±5.6)**	- (1.7 ±0.2)
Trimethoprim	- (1.1 ±0.1)	- (0.8 ±0.1)	- (1.3 ±0.3)	+ **(2.7 ±0.1)**	- (1.2 ±0.4)
Novobiocin*	- (1.2 ±0.1)	- (0.9 ±0.1)	- (1.2 ±0.2)	+ **(6.7 ±0.7)**	- (0.8 ±0.4)

Table 3.3 continued...

Fatty acid biosynthesis inhibitors					
Triclosan	- (1.2 ±0.2)	- (0.6 ±0.1)	- (1.2 ±0.1)	- (1.4 ±0.4)	**+ (7.8 ±1.7)**
tRNA synthetase inhibitors					
Mupirocin	- (0.8 ±0.2)	- (0.9 ±0.1)	- (0.9 ±0.1)	- (0.9 ±0.1)	- (1.2 ±0.1)
Indolmycin	- (1.0 ±0.1)	- (1.5 ±0.3)	- (1.1 ±0.1)	- (1.2 ±0.2)	- (1.7 ±0.1)
Borrelidin	- (0.8 ±0.1)	- (0.9 ±0.1)	- (1.0 ±0.1)	- (1.1 ±0.1)	- (1.1 ±0.1)

*Table 3.3 - Reporter induction (+) or no induction (-). Threshold set at published values (Fischer et al., 2004; Urban et al., 2007), normalised against untreated samples. (Maximum reporter signal ± standard deviation). Figures in bold represent signals above the published threshold for induction of the respective biosensor. * Performed by Deborah Roebuck, ¥ Performed by Nicola Ooi, University of Leeds.*

measuring the luminescence emitted by the strains in response to a range of eleven concentrations of inhibitor (including values at, above and just below the MIC of the inhibitor). Induction was expressed as a value relative to the luminescence emitted by a drug-free control, and the thresholds for positive induction were defined as 2.5-fold for *yorB*, 2-fold for *yvgS*, *yhe1* and *fabHB* and 1.7-fold for *ypuA* as previously determined (Urban *et al.*, 2007). Agents exhibiting an MIC greater than 256 μg/ml against *B. subtilis* 1S34 MIC (i.e. bacitracin, clindamycin and sulfamethoxazole) could not be tested in this assay because of limited solubility of the antibiotics at concentrations above 256 μg/ml.

The biosensor responsive to cell envelope stress was induced by two of the established inhibitors of cell wall biosynthesis, namely vancomycin and flucloxacillin, and four of the twelve cell membrane-damaging agents (telavancin, daptomycin, chlorhexidine and anhydrotetracycline) (Table 3.3). The protein biosensor was induced over published thresholds in response to anhydrotetracycline and all protein biosynthesis inhibitors, with the exception of aminoglycosides, erythromycin and actinonin. The RNA, DNA and fatty acid biosensors were similarly capable of detecting the presence of all the control inhibitors in their respective MOA group. However, the biosensors did not

exhibit induction above the thresholds in response to tRNA synthetase inhibitors (mupirocin, indolmycin, and borrelidin), inhibitors of early stage PG synthesis (fosfomycin, D-cycloserine, tunicamycin and fosmidomycin [an inhibitor of the C55-lipid carrier synthesis]) (Shigi *et al.*, 1989) and the majority of membrane damaging agents (including the potassium ionophore valinomycin, nisin, the surfactant cetyltrimethylammonium bromide (CTAB), clofazimine, sepracor 155342, polymyxin B and the XF compounds) (Table 3.3). The inability of the protein biosensor to detect the presence of erythromycin was expected, since the biosensor strains possess a plasmid encoded macrolide-lincomycin- streptogramin B (MLS) resistance determinant (Urban *et al.*, 2007). However, the inability of the cell envelope stress biosensor to detect most of the cell membrane damagers is in direct contrast with the results of Urban *et al.* (2007) who found that the cell envelope biosensor was induced in response to agents with that MOA. Indeed, a cell envelope reporter should by definition be induced in response to inhibitors of both the cell membrane and cell wall biosynthesis. These results (Table 3.3) also do not support previous reports that clofazimine may target RNA polymerase (Morrison, 1972), as the biosensor responsive to RNA synthesis inhibition was not induced by this compound. However, the biosensor results are consistent with previous reports that clofazimine does not inhibit DNA or protein synthesis (Oliva *et al.*, 2004).

Anhydrotetracycline is considered to exert a bactericidal effect on the cell by damaging the cytoplasmic membrane (Oliva *et al.*, 1992), while having little effect on protein synthesis, in contrast to the classical tetracycline counterparts (Rasmussen *et al.*, 1991). Anyhydrotetracycline induced both *yheI* and *ypuA*, the biosensors responsive to inhibition of protein synthesis and cell envelope stress respectively, suggesting that the

mechanism of action of atypical tetracyclines may involve inhibition of both targets. The *B. subtilis* biosensors thus appear to be capable of detecting agents with more than one MOA. As well as anhydrotetracycline, the fluoroquinolone antibiotic ciprofloxacin induced both the *yorB* (DNA synthesis) and *ypuA* (cell envelope stress) promoters, suggesting that this established class of DNA biosynthesis inhibitors also affect the bacterial cell envelope, potentially causing membrane damage. The level of induction of *ypuA*, however, is just slightly above the published threshold and supports a weak secondary membrane damaging MOA for fluoroquinolones, an observation which has been noted in the literature (Linder *et al.*, 2002; Suerbaum *et al.*, 1987).

These results confirm and extend the utility of the biosensors for preliminary screening of antibacterial agents to determine MOA. However, the data also indicate that the biosensors may be limited to detecting only certain classes of bacterial inhibitors i.e. those against which they were originally validated (Urban *et al.*, 2007), and may be unable to detect other classes of antibacterial agents. Indeed, Urban *et al.* observed that a number of established inhibitors of protein biosynthesis were not detected by any of the biosensor strains (Urban *et al.*, 2007). These observations indicate that the use of these biosensors alone to characterise the MOA of antibiotics may not be sufficient.

Therefore it would appear prudent to use the *Bac*Light™ as described above in parallel to the biosensors to confirm any membrane damaging activity. Furthermore, if a compound is suspected of inhibiting protein biosynthesis or cell wall biosynthesis, additional assays may be needed to confirm this. In the case of putative protein biosynthesis inhibitors, macromolecular synthesis assays to measure the inhibition of incorporation of radiolabelled glutamine into protein over a time period in response to

the inhibitor is a well established method (Cherrington *et al.*, 1990, Ooi *et al.*, 2009a). For putative inhibitors of cell wall biosynthesis, *S. aureus* reporter systems which are specifically responsive to inhibition of PG biosynthesis may be used (Blake *et al.*, 2009).

3.3.4 *S. aureus* PG reporters

Microarray analysis has reportedly shown that treatment of *S. aureus* with inhibitors of PG biosynthesis leads to upregulation of a range of cell wall biosynthetic genes (O'Neill *et al.*, 2009). Following this initial work, three PG promoter: *lacZ* reporter constructs were previously made which upregulate expression of β-galactosidase (the protein product of *lacZ*) upon induction of the genes *gltA, oppB* and *murZ* (A. J. O'Neill and K. Blake, University of Leeds). The gene *gltA* encodes the enzyme citrate synthase which aids in the production of amino acids early-stage PG biosynthesis, OppB is an oligopeptide permease which promotes transport of oligopeptides and *murZ* encodes UDP-GlcNAc enolpyruvyl transferase (Blake *et al.*, 2009; Orchard & Blair, 2004; Park *et al.*, 1994). Since the *B. subtilis* antibiotic biosensors were only able to detect the presence of certain PG biosynthesis inhibitors, upregulation of *gltA*, *oppB* and *MurZ* in *S. aureus* was monitored in response to a range of known PG inhibitors, and control agents representing inhibitors of other biosynthetic pathways, at the concentrations tested in the *B. subtilis* biosensor assay (Table 3.4). For consistency in this assay, and in alignment with previous microarray studies with expression thresholds for upregulation, the threshold for a positive result was set as twofold or greater (Raju *et al.*, 2004; Wang *et al.*, 2004). PG biosynthesis inhibitors with an MIC greater than or equal to 128 μg/ml (i.e. bacitracin, tunicamycin and fosmidomycin) could not be tested in this assay due to

Table 3.4 – Response of *S. aureus* PG reporters to established PG biosynthesis inhibitors and comparator agents

Antibacterial Agent	Upregulated promoter		
	gltA	*oppB*	*murZ*
PG biosynthesis inhibitors			
Vancomycin	**+ (3.2 ±1.5)**	**+ (5.0 ±1.5)**	**+ (3.8 ±0.5)**
Flucloxacillin	**+ (5.1 ±0.6)**	**+ (6.0 ±1.1)**	**+ (8.6 ±1.6)**
Fosfomycin	**+ (2.9 ±0.3)**	**+ (5.9 ±0.8)**	**+ (4.6 ±0.9)**
D-cycloserine	**+ (2.9 ±0.5)**	**+ (3.4 ±0.7)**	**+ (2.8 ±0.2)**
Cell membrane damagers			
Valinomycin	- (0.9 ±0.1)	- (1.9 ±0.1)	- (1.3 ±0.1)
Nisin	- (1.2 ±0.2)	- (1.6 ±0.1)	- (1.6 ±0.1)
CTAB	- (1.9 ±0.1)	- (0.9 ±0.2)	- (0.9 ±0.1)
Clofazimine	- (1.5 ±0.1)	- (1.5 ±0.1)	- (1.4 ±0.1)
Protein biosynthesis inhibitors			
Tetracycline	- (1.1 ±0.2)	- (1.0 ±0.1)	- (1.0 ±0.2)
RNA biosynthesis inhibitor			
Rifampicin	- (1.4 ±0.3)	- (1.3 ±0.1)	- (1.0 ±0.4)
DNA biosynthesis inhibitor			
Ciprofloxacin	- (1.0 ±0.4)	- (1.7 ±0.1)	- (0.4 ±0.3)
Fatty acid biosynthesis inhibitor			
Triclosan	- (1.0 ±0.1)	- (1.4 ±0.2)	- (0.9 ±0.3)

Table 3.4 - Reporter induction (+) or no induction (-). Threshold set at greater than or equal to twofold for a positive result, normalised against untreated samples. (Maximum reporter signal ± standard deviation). Figures in bold represent signals above the published threshold for induction of the respective biosensor.

low susceptibility of *S. aureus* to these agents. Induction above the defined thresholds for all three PG reporter strains was seen in response to all of the PG biosynthesis inhibitors tested, consistent with their known inhibitory effects on PG biosynthesis (Table 3.4) (See introduction). No induction occurred with any of the inhibitors with other MOAs (Table 3.4), confirming that these agents do not inhibit early stage PG

biosynthesis. Inhibition of PG biosynthesis has been reported for nisin, however it occurs at a greatly reduced rate in comparison to the formation of membrane pores, which may explain its negative result in this assay (Brotz *et al.*, 1998b).

The positive induction observed for all the PG biosynthesis inhibitors tested in this assay suggests that the *S. aureus* PG reporters display enhanced sensitivity for the detection of cell-wall biosynthesis inhibitors compared with the *B. subtilis* biosensors. Therefore, the *S. aureus* reporters represent a more satisfactory system for the specific detection of inhibitors of cell wall biosynthesis and should be used to complement the *B. subtilis* biosensors in the characterisation of putative PG biosynthesis inhibitors.

3.3.5. *Saccharomyces cerevisiae* susceptibility testing as a model for prokaryotic specificity analysis of antibacterial agents

Antibacterial agents with the potential for chemotherapeutic use must display a high level of prokaryotic specificity, in order to avoid potential adverse side effects in humans (Payne *et al.*, 2007). One of the simplest and most rapid methods to ascertain the bacterial specificity of a new agent is to perform MIC determinations in a lower eukaryote, such as yeast (Khafagi *et al.*, 2000), and it has been suggested that inhibitors for clinical development should display at least ten-fold higher activity against prokaryotes to be considered as potential therapeutic candidates (Evans *et al.*, 2001).

To test this hypothesis, the antimicrobial activity of a selection of antibacterial agents (representatives from each MOA group) against the budding yeast *Saccharomyces cerevisiae* 464, a clinical strain (Table 3.5) was determined. In order to confirm the

validity of the *S. cerevisiae* susceptibility testing protocol, the MICs of the antibacterial agents were performed in parallel to the established antifungal agents amphotericin B and 5-fluorocytosine, which are mainly used in the clinical treatment of systemic fungal

Table 3.5 – Activity of antimicrobial inhibitors with established MOAs against *S. aureus* SH1000 and *S. cerevisiae* 464

Antimicrobial Agent	MIC(μg/ml)		Activity ratio[a]	Clinical use[b]
	S. cerevisiae 464	*S. aureus* SH1000		
Antifungal Agents				
Amphotericin B	0.5	>256	<0.001	Yes
5-fluorocytosine	0.125	>256	<0.001	Yes
PG biosynthesis inhibitors				
Vancomycin	>256	2	>128	Yes
Flucloxacillin	>256	0.25	>1024	Yes
Cell membrane damagers				
Valinomycin	2	2	1	No
Telavancin	>256	1	>256	Yes
Nisin	>256	4	>16	No
CTAB	1	2	0.5	No
Clofazimine	>256	2	>128	Yes
Sepracor 155342	8	4	2	No
Chlorhexidine	0.5	1	0.5	Yes
Anhydrotetracycline	>256	2	>128	No
Daptomycin	>256	2	>128	Yes
XF70	2	0.5	4	No
XF73	2	0.5	4	No
Protein biosynthesis inhibitors				
Tetracycline	256	0.5	512	Yes
RNA biosynthesis inhibitors				
Rifampicin	>256	0.015	>16,384	Yes
DNA biosynthesis inhibitors				
Ciprofloxacin	>256	1	>256	Yes
Fatty acid biosynthesis inhibitors				
Triclosan	2	0.125	16	Yes
tRNA synthetase inhibitors				
Mupirocin	>256	0.0625	>4,096	Yes

Table 3.5- (a) Ratio of S. aureus SH1000 MIC versus S. cerevisiae 464 MIC, (b) Clinical indications for treatment of human infections according to Greenwood et al. (2007).

infections and fungal endocarditis respectively (Mandell *et al.*, 2004). The MICs obtained for these compounds are within the published range (Barry *et al.*, 2000, Calhoun *et al.*, 1986).

As shown in Table 3.5, all antibacterial agents that are currently clinically available for systemic use display greater than tenfold higher activity against *S. aureus* SH1000 than *S. cerevisiae* 464. Nisin and anhydrotetracycline, while displaying high prokaryotic specificity, are not currently used in the treatment of systemic bacterial infections in humans. The therapeutic potential of nisin is limited by its instability in biological fluids and the fact that it is poorly soluble at blood pH (Maher & McClean, 2006). However, the lack of toxicity, colour and odour means that nisin meets the requirements of a food preservative, and consequently it obtained US FDA approval in 1988 (Abee *et al.*, 1995; Sobrino-Lopez & Martin-Belloso, 2008). Although anhydrotetracycline appears to display prokaryotic specificity (Table 3.5), the clinical development of anhydrotetracycline and other atypical tetracyclines has not been pursued due to preliminary observations of adverse side effects on the human nervous system (Chopra, 1994). Chlorhexidine, although displaying high inhibitory activity against both prokaryotic and eukaryotic cells (Table 3.5), is still used clinically in the specific treatment of bacterial infections. However, it can be qualified by the fact that chlorhexidine is solely used topically, due to systemic toxicity (Mandell *et al.*, 2005).

Despite some exceptions, these results show a high level of conformity to the observation that clinically relevant antibacterial agents display at least tenfold higher activity against bacteria, than the eukaryotic organism *S. cerevisiae*. Therefore, based on these results, performing an MIC against *S. cerevisiae* during the earliest stages of

antibacterial candidate evaluation, and comparing it with the prokaryotic activity, will be helpful in assessing the potential of a new inhibitor for further development as an antibacterial drug.

3.4 Conclusions

The successful development of a novel antibacterial agent is dependent on preclinical evaluation to ensure that the observed antibacterial activity is associated with inhibition of a specific bacterial target. Determination of the MICs of *S. aureus*, *B. subtilis* and *E. coli* gave a reliable indication of the spectrum of activity of the antibacterial agents tested. Furthermore, the activity of agents against the AcrAB-deficient *E. coli* mutant and the wild-type treated with PMBN, can also yield valuable information on resistance mechanisms employed by Gram-negative bacteria so that analogues with improved properties and a broader spectrum of activity may be designed and synthesised.

The *Bac*Light™ assay provides a good system for preliminary determination of the membrane-damaging effects of antibacterial agents, and a *Bac*Light™ result of 40% membrane integrity (60% membrane damage) has now been set as the upper threshold for an agent to be classed as membrane damaging for MOA characterisation purposes in the following chapters.

The utility of the *B. subtilis* antibiotic biosensors for preliminary screening of the MOA of antibacterial agents has also been confirmed, contributing to information on whether they might be suitable for development as new antibacterial agents. However, additional assays may be needed to fully characterise inhibitors such as macromolecular synthesis

and the *S. aureus* PG reporters. Indeed the latter displayed enhanced sensitivity for detection of cell-wall biosynthesis inhibitors compared with the *B. subtilis* biosensors.

The majority of clinically relevant antibacterial inhibitors were found to display at least tenfold higher activity against bacteria, versus the eukaryotic organism *S. cerevisiae*. Therefore, performing an MIC against *S. cerevisiae* during the earliest stages of antibacterial candidate evaluation (alongside bacterial MICs and *Bac*Light™), and comparing it to the prokaryotic activity, will give very useful information relevant to the potential development of a new inhibitor into a new antibiotic candidate.

Chapter 4 – Characterisation of NVB353, a type B lantibiotic derivative

4.1 Abstract

The class I bacteriocins or 'lantibiotics' are small post-translationally modified peptides which have bactericidal activity against Gram-positive species, including MRSA. The type B lantibiotics (e.g. mersacidin and deoxyactagardine B [DAB]) are thought to inhibit peptidoglycan (PG) biosynthesis specifically at the transglycosylation step, by a high affinity interaction with the MurNAc-pyrophosphate moiety of Lipid II. Results reported in this chapter address the *in vitro* antimicrobial activity of the DAB derivative NVB353. The susceptibility of a panel of *Staphylococcus aureus* strains (including MRSAs and VISAs) to inhibition and killing by NVB353 was assessed. NVB353 displayed bacterial-specific, potent activity against a range of clinically relevant Gram-positive pathogens, which was enhanced compared with DAB. Several lines of evidence suggest that the increased potency and bactericidal activity of NVB353 when compared to DAB was due to the fact that NVB353 exhibits a dual MOA against *S. aureus* SH1000, causing inhibition both of PG biosynthesis as well as perturbation of the cell membrane. The dual MOA of NVB353 could suggest that it would be indicated for infections caused by *S. aureus* strains which are resistant to existing Lipid II targeting antibiotics. In addition, *S. aureus* mutants displaying resistance to NVB353 were not generated; suggesting that resistance to this agent would be unlikely, or slow to develop clinically. As such, NVB353 is a viable candidate for chemotherapeutic development and should be taken forward for further preclinical evaluation.

4.2 Introduction

4.2.1 Class 1 bacteriocins: the 'lantibiotics'

The majority of bacteriocins are ribosomally synthesised peptide antibiotics that have bactericidal activity against Gram-positive species closely related to the producing strain (Bauer & Dicks, 2005). They are grouped into one of three classes according to their structure (Sahl *et al.,* 1995): the class I bacteriocins, renamed "lantibiotics" in 1988 (for ***l***anthionine containing ***antibiotics***) are small post-translationally modified peptides (Schnell *et al.,* 1988). The reaction of dehydrated serine/threonine with the thiol side chain of cysteine forms internal cyclic structures within the peptide, which are essential for maintenance of structure and antibacterial activity (Cotter *et al.,* 2005; McAuliffe *et al.,* 2001). As a family of around fifty peptide antibiotics, each with differing structures, sizes and modes of action (MOAs), this class is subdivided into the screw-shaped, amphipathic type A lantibiotics (e.g. nisin), and the globular type B lantibiotics (e.g. mersacidin and actagardine) (Chatterjee *et al.,* 2005) (Introduction: Figures 1.2 and 1.6).

Currently, telavancin, linezolid and daptomycin are at the forefront of the limited options for treatment of infections caused by meticillin-resistant *Staphylococcus aureus* (MRSAs) which display reduced susceptibility to vancomycin (Loffler & MacDougal, 2007). The type B lantibiotics show great therapeutic promise, as mersacidin is effective in clearing murine nasal staphylococcal infections, including those caused by MRSA (Chatterjee *et al.,* 1992; Kruszewska *et al.*, 2004). Mersacidin also displays good *in vitro* activity against the Gram-positive pathogen *Clostridium difficile* (Minimum

inhibitory concentration [MIC] 8 μg/ml), a significant nosocomial pathogen (Niu & Neu, 1991).

The MOA of type B lantibiotics is less well understood than that of the type A lantibiotics (Cotter *et al.,* 2005), but treatment with type B compounds leads to termination of cell growth and slow induction of lysis (Brotz *et al.,* 1995), as well as a time-dependent reduction in cell wall thickness (Brotz *et al.,* 1995; Molitor *et al.,* 1996). These observations, in addition to selective inhibition of incorporation of peptidoglycan (PG) components into the cell wall by mersacidin, have resulted in the current suggestion that the target of the type B lantibiotics is PG biosynthesis (Brotz *et al.,* 1995; Brotz *et al.,* 1998a). Specifically, mersacidin and actagardine are thought to inhibit PG biosynthesis at the transglycosylation step, by a high affinity interaction with the MurNAc-pyrophosphate moiety of Lipid II (Figure 4.1) (Breukink *et al.,* 2003). Indeed, binding of radiolabelled mersacidin to whole cells and isolated membranes is highly dependent upon the availability of the lipid precursor, and the presence of other Lipid II inhibitors hinders the binding and activity of mersacidin (Brotz *et al.,* 1998b). In the same study, the tight interaction of Lipid II and type B lantibiotics was demonstrated by the inability of Lipid II to dissociate from the peptides, even upon treatment of the membranes with 1% (w/v) sodium dodecyl sulphate (SDS). Therefore it was suggested that the mersacidin group do not inhibit the transglycosylase enzyme directly, but by binding to Lipid II prevent precursor incorporation into PG and Lipid II accumulation in the membrane (Brotz *et al.,* 1998a). In the case of mersacidin, the side chain of Glu-17 is responsible for interaction of the lantibiotic with Lipid II, with calcium cations required to form a bridge between anionic Lipid II and mersacidin, increasing both the binding affinity and antibacterial activity of the antibiotic (Barrett *et*

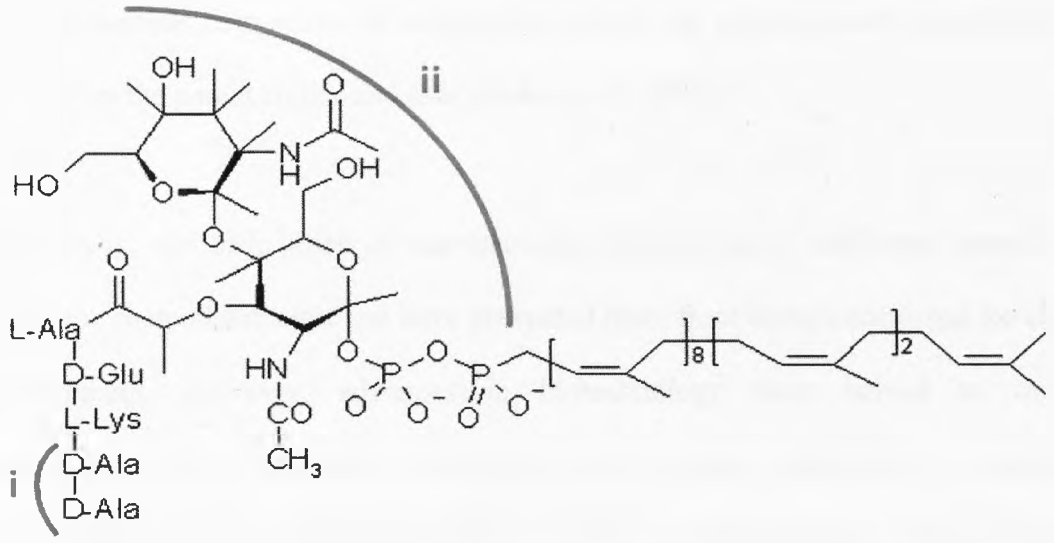


Figure 4.1 – Structure of the UDP MurNAc-GlcNAc-pentapeptide precursor (Lipid II) and the currently accepted binding sites of i) vancomycin, and ii) type B lantibiotics (Adapted from Breukink & de Kruijff, 2006).

al., 1992; Bauer & Dicks, 2005). Since the MurNAc-pyrophosphate moiety of Lipid II is not a target for clinically-available antibacterial agents (such as vancomycin) (Figure 4.1), cross resistance is unlikely to occur. This supports their potential for use against multi-drug resistant *S. aureus* strains (Hsu *et al.*, 2003).

4.2.2 Lantibiotic bioengineering and chemical modification

The type B lantibiotics have potential for derivatives to be engineered with greater potency, by cloning and modification of genes encoding lantibiotic biosynthetic enzymes (Dawson, 2006). Many studies have been undertaken in an attempt to synthesise more active peptide derivatives of the lantibiotics (Chen *et al.*, 2001; Kuipers *et al.*, 1992; Szekat *et al.*, 2003). The most promising study to date reports the synthesis

of carboxamide derivatives of actagardine which are approximately eightfold more active than the parent compound (Malabarba *et al.,* 1990).

Historically, the difficulties of manufacturing lantibiotics in sufficient quantity and purity for chemotherapeutic use have prevented them from being considered for clinical development. However, advances in biotechnology have helped to increase understanding of lantibiotic biosynthesis and refine protocols for lantibiotic manufacture (Boakes & Wadman, 2008). As such, the biotechnology company Novacta Biosystems Ltd has a focused drug discovery programme dedicated to development of natural product antibacterial agents, specifically generation of derivatives of mersacidin and deoxyactagardine B [DAB] (a non-oxidised actagardine variant with V15I and L16V substitutions) (Boakes *et al.*, 2010; Zimmerman *et al.*, 1995). One method used to derive lantibiotic analogues is based on cloning and manipulation of the lantibiotic biosynthetic genes, to generate gene variants, and thereby altering the amino-acid sequence of the mature lantibiotic. Genetic engineering approaches have been challenging to optimise, as successful *in vivo* expression depends on the production of expression systems for all of the genes in the lantibiotic cluster (including immunity and regulatory proteins), not just those involved with synthesising the mature peptide (Kuipers *et al.,* 1996). However, Novacta has also successfully developed methodologies to generate lantibiotic derivatives with improved properties via synthetic chemistry. NVB353 (undisclosed structure) is a derivative of DAB, which has been generated by the latter route, and in preliminary analyses has displayed improved antibacterial activity in comparison with the parental compound.

4.2.3 Aims and objectives of the work described in this chapter

The biosynthesis of peptidodoglycan is a well established target for antibacterials as evidenced by the large number of clinically relevant classes which target this pathway, including the β-lactams and glycopeptides (Bugg *et al.*, 2011). The biosynthesis of PG is well understood, unique to bacteria and conserved in both Gram-positive and Gram-negative genera, supporting the concept that novel broad-spectrum agents which target PG biosynthetic enzymes would be useful additions to the anti-bacterial repertoire (Barker, 2006).

Infections caused by antibiotic-resistant bacteria can be combated by the clinical development and introduction of novel agents derived from natural products. NVB353 is an example of a type B lantibiotic derivative, but is yet to be fully characterised in terms of its suitability for clinical development. Therefore the aims and objectives of this chapter include:

- characterisation of the antimicrobial spectrum of activity of NVB353 in comparison with mersacidin and DAB;
- characterisation of the bacterial specificity of NVB353, and its effect on staphylococcal membranes;
- analysis of the MOA of NVB353;
- determination of the potential for the development of resistance to NVB353.

4.3 Results and Discussion

4.3.1 Lantibiotic susceptibility testing – determination of the antibacterial spectrum of activity

Preclinical evaluation of any potential antibacterial chemotherapeutic candidate involves the determination of MICs against a panel of organisms representing Gram-positive and Gram-negative genera to determine possible clinical indications (O'Neill & Chopra, 2004a). MICs were therefore determined for mersacidin, DAB and NVB353 against the Gram-positive organism *S. aureus* SH1000 and the Gram-negative *Escherichia coli* wild-type strain (1411), an AcrAB (efflux pump)-deficient *E. coli* and *E. coli* 1411 strain treated with the outer membrane permeabiliser polymyxin B nonapeptide (PMBN) (Table 4.1). *Bacillus subtilis* 1S34 was also included as the parental strain for the biosensors (Urban *et al.*, 2007). As shown in Table 4.1, the antibacterial activity of the lantibiotics is limited to the Gram-positive organisms tested, in agreement with previous studies (Sahl & Bierbaum, 1998). *E. coli* do not become sensitised to the agents upon addition of an outer membrane permeabiliser or disruption of the AcrAB efflux pump. This would indicate that these lantibiotics are not candidates for broad-spectrum chemotherapy. The lack of activity of lantibiotics against *E. coli* is unsurprising, as these peptides are predominantly produced by Gram-positive species to target similar organisms (Bauer & Dicks, 2005). In addition, the comparatively high relative molecular masses of these compounds suggest that they are unlikely to penetrate the outer membrane of Gram-negative bacteria to exert an antibacterial effect at the cytoplasmic membrane. The lantibiotics may also be subject to extrusion by multi-drug resistant efflux pumps other than the AcrAB system.

Table 4.1 - Antibacterial spectrum of the type B lantibiotics

Antibacterial Agent	MIC (μg/ml)				
	***S. aureus* SH1000**	***E. coli* 1411**	***E. coli* 1411 + PMBN**	***E. coli* SM1411**	***B. subtilis* 1S34**
Mersacidin	32	>256	>256	>256	16
Deoxyactagardine B	32	>256	>256	>256	32
NVB353	4	>256	>256	>256	4

4.3.2 Lantibiotic susceptibility testing against *Staphylococcus aureus*

As shown in Table 4.1, the lantibiotics display antibacterial activity that is limited to Gram-positive organisms. The need for novel agents to treat multi-drug resistant strains of *S. aureus* (including MRSA and VISA) is a pressing issue in the field of chemotherapeutics (Hiramatsu *et al.,* 1997). Therefore, I sought to confirm the potential for NVB353 to be clinically developed, specifically as a treatment option for infections caused by staphylococci. Susceptibility determinations were performed against a wider selection of *S. aureus* strains, including *S. aureus* SH1000, a set of ten clinical meticillin-susceptible *S. aureus* (MSSA) strains, four MRSAs (EMRSA 15-17 and MW2) and two vancomycin-intermediate *S. aureus* (VISA) strains (Mu3 and Mu50). Mersacidin, DAB, vancomycin and daptomycin were used as comparator agents (Table 4.2). Previous studies have demonstrated the dependency of daptomycin on calcium ions for full antimicrobial activity (Eliopoulos *et al.,* 1986). A similar calcium dependent effect has also been observed with type B lantibiotics (Barrett *et al.*, 1992). Therefore MICs were determined for the lantibiotics, daptomycin and vancomycin in the presence and absence of physiological levels (50 μg/ml) of Ca^{2+}.

Table 4.2 - Antimicrobial activity of mersacidin, DAB, NVB353, vancomycin and daptomycin against a panel of *S. aureus* strains

S. aureus strain	Minimum Inhibitory Concentration (µg/ml)									
	Mersacidin (+ Ca^{2+})	Mersacidin (- Ca^{2+})	DAB (+ Ca^{2+})	DAB (- Ca^{2+})	NVB353 (+Ca^{2+})	NVB353 (-Ca^{2+})	Vancomycin (+Ca^{2+})	Vancomycin (-Ca^{2+})	Daptomycin (+Ca^{2+})	Daptomycin (-Ca^{2+})
SH1000	32	32	32	64	4	8	2	2	2	16
MSSA 1	8	8	8	32	4	4	2	2	4	2
MSSA 2	32	32	32	64	4	4	2	1	2	2
MSSA 3	16	32	64	128	4	4	1	1	2	2
MSSA 4	16	16	16	64	2	4	1	1	2	2
MSSA 5	32	32	16	256	4	4	2	1	2	4
MSSA 6	16	16	64	256	4	4	2	2	2	4
MSSA 7	4	8	8	16	2	2	2	1	2	4
MSSA 8	8	16	32	256	4	8	2	1	2	4
MSSA 9	16	32	32	256	2	4	2	1	2	4
MSSA 10	8	16	32	256	4	8	2	2	2	4
EMRSA-15	32	32	128	>256	4	8	4	4	2	4
EMRSA-16	32	32	64	128	8	8	4	4	2	4
EMRSA- 17	32	32	>256	>256	16	16	8	8	4	8
MW2	16	16	32	128	8	4	4	2	2	4
Mu3	64	64	64	128	8	8	8	8	8	16
Mu50	64	64	128	128	8	8	8	8	8	16

The lantibiotics displayed modest MICs against the *S. aureus* strains (Table 4.2). For mersacidin, the MICs varied between MSSA strains, with the lowest MIC against MSSA 7 (4 μg/ml) and highest for more than one strain at 32 μg/ml. The MICs for DAB were higher, but still varied between 8 μg/ml with calcium supplementation to 256 μg/ml without. With the exception of vancomycin, the activity of the agents appeared to increase with calcium supplementation to the growth medium. The activities of mersacidin, DAB, and to a lesser extent NVB353, are therefore dependent on the presence of calcium ions. This effect, however, is not as marked as that previously reported for daptomycin, as in the presence of the metal ion, the MICs were reduced on average twofold in comparison with cultures on non-supplemented media. These results are consistent with previous observations that the MIC for mersacidin approximately halves in the presence of physiological levels of calcium (Barrett *et al.*, 1992). In comparison with DAB, NVB353 displayed increased potency against the range of clinically relevant staphylococci. NVB353 MICs against MSSA varied between 2-8 μg/ml and with an MIC of 4 μg/ml for SH1000 is comparable with that of vancomycin and daptomycin (Table 4.2). Mersacidin and DAB were not as potent as vancomycin and daptomycin against MSSA, only displaying modest MICs (between 4-256 μg/ml) against these strains.

The antimicrobial activity of all three lantibiotics varies considerably between different meticillin-susceptible *S. aureus* strains. In contrast the MICs of daptomycin and vancomycin against the strains did not vary substantially (with MIC ranges of 2-4 and 2-8 μg/ml respectively). This is intriguing, and although further study is required, may be due to differences in the composition of the cell wall between strains, particularly in the relative abundances of Lipid II. Although this phenomenon has not been described

for the lantibiotics to date, differences in the susceptibility of *S. aureus* to glycopeptides have been described and accounted for by variation in PG composition (Koehl *et al.*, 2004). With respect to the results obtained for the MRSAs and VISAs, the MICs for mersacidin, DAB and NVB353 were generally higher against these strains than those observed for vancomycin and daptomycin. Therefore, the type B lantibiotics may be unsuitable alternative candidates for treatment of problematic infections caused by MRSAs and VISAs, but may still constitute a viable candidate for treatment of infections caused by other Gram-positive pathogens, although further study would be required to confirm this.

4.3.3 Determination of membrane damage and bacterial specificity of NVB353

In the previous chapter, analysis of the ability of a new antibacterial agent to cause membrane damage in *S. aureus* alongside determination of the MIC against *S. cerevisiae* were shown to be useful for prediction of potential eukaryotic toxicity. To examine the potential for mersacidin, DAB and NVB353 to cause membrane damage in *S. aureus*, the lantibiotics were tested in the *Bac*Light™ assay (Table 4.3). After ten minutes, cells treated with the test agents mersacidin, DAB and NVB353 maintained between 76 and ≥100% membrane integrity, compared with 0-15% for the positive control agents cetyltrimethylammonium bromide (CTAB) and nisin, and 82 and 100% for the negative control agents vancomycin and tetracycline, respectively (Table 4.3). These data suggest that the lantibiotics do not promote rapid membrane damage in *S. aureus*. The result for mersacidin is in agreement with published work, using electron microscopy and reporting that mersacidin-treated bacterial cells (ninety minutes at 10X

Table 4.3 - Determination of membrane damage and bacterial specificity of lantibiotics

Condition	*S. aureus* MIC (µg/ml)	*Bac*Light™ result (% membrane integrity)	*S. cerevisiae* 464 MIC (µg/ml)
Drug-free control	-	100	-
5% SDS (w/v)	-	0	-
CTAB	2	0	1
Nisin	2	14.9 ±1.0	>64
Tetracycline	0.5	99.9 ±4.6	256
Vancomycin	4	83.5 ±4.4	>256
Mersacidin	16	106.9 ±14.3	>256
DAB	32	99.0 ±5.3	>256
NVB353	4	75.8 ±14.6	>256

MIC) have intact cell membranes (Brotz *et al.*, 1995). In addition the maintenance of membrane integrity in *S. aureus* is promising with respect to the potential clinical use of NVB353, as it is unlikely to cause membrane damage and toxicity in humans. This conclusion is supported by the lack of activity of the lantibiotics against *Saccharomyces cerevisiae* (Table 4.3) and therefore NVB353 can be taken forward for further evaluation to attempt to link the observed antibacterial activity to cellular inhibition of PG biosynthesis in a manner similar to that of mersacidin and DAB.

4.3.4 Analysis of the cell death kinetics following exposure of *S. aureus* to lantibiotics

As mentioned previously, it is useful to determine whether a novel antibacterial agent displays bactericidal or bacteriostatic activity to combine with pharmacokinetic/pharmacodynamic data to give an accurate prediction of the *in vivo*

efficacy of the drug (Jacobs, 2004). Therefore, time kill experiments were performed, using the antibiotics daptomycin, vancomycin, DAB, mersacidin and NVB353 at 4X MIC to determine the rate of kill of exponentially growing *S. aureus* SH1000 over a five hour time period (Figure 4.2). The observed bactericidal activity of nisin, daptomycin and vancomycin agrees with published data (Flandrois *et al.*, 1998; Hobbs *et al.*, 2008). The bactericidal response of *S. aureus* to treatment with mersacidin and DAB is not marked over five hours, but NVB353 effects a more rapid bactericidal response in *S. aureus* than DAB, with almost a two log reduction in cell viability over the five hour period. Bactericidal antibiotic activity is often a preferred characteristic for a novel antibacterial agent, particularly for use in the immunocompromised (Fuchs *et*

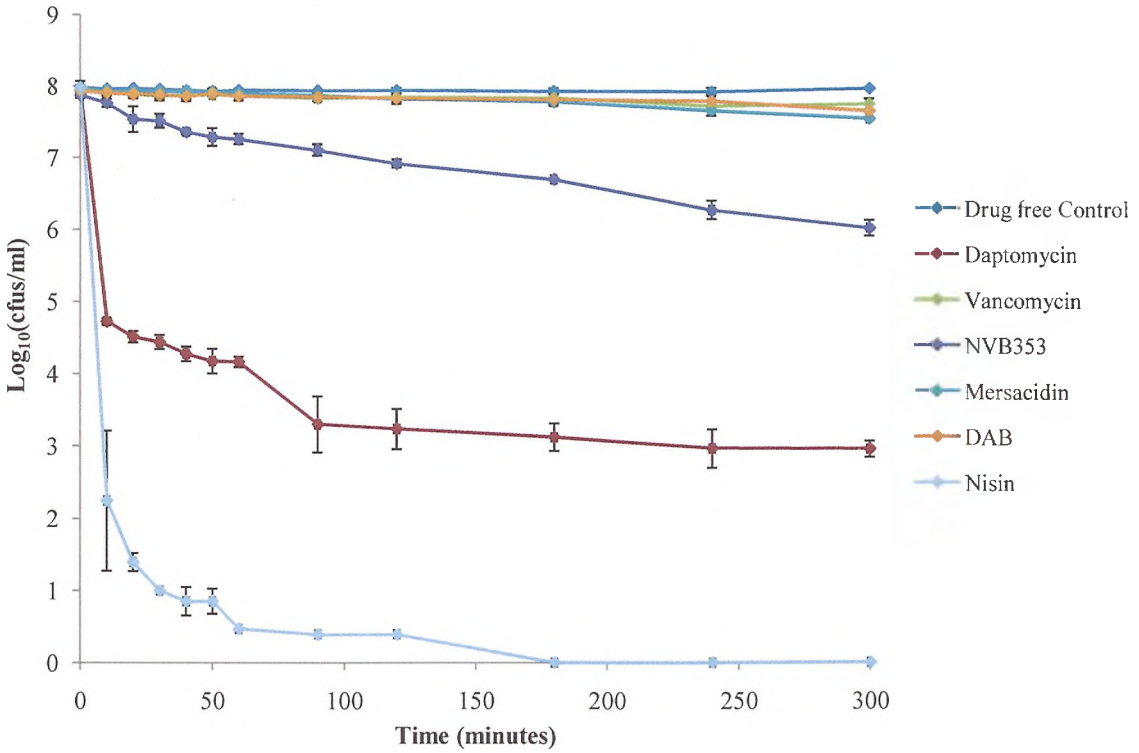


Figure 4.2 - Killing kinetics of *S. aureus* SH1000 upon treatment with a panel of antibiotics at 4X MIC *(Daptomycin, mersacidin, DAB and NVB353 time kills were performed in the presence of 50 µg/ml Ca^{2+}). Error bars represent the standard deviation from three replicates.*

al., 2002). However, the more rapid bactericidal activity of NVB353 in comparison with DAB may indicate a different MOA. Therefore, the *S. aureus* and *B. subtilis* biosensors and macromolecular synthesis assays were utilised to determine whether NVB353 had a similar MOA profile to that of DAB, or whether the increased potency could be attributed to differences in the antibacterial target of NVB353.

4.3.5 Analysis of antibacterial MOA using pathway specific biosensors

The MOA of NVB353 was examined by using the set of *B. subtilis* antibiotic biosensors. The 3-7 fold upregulation of the Bacillus biosensor responsive to cell envelope stress upon treatment with all three type B lantibiotics may suggest inhibition of PG biosynthesis (Table 4.4). However, none of the other biosensors were induced in

Table 4.4 – Induction of *B. subtilis* biosensors by type B lantibiotics and comparator agents

Antimicrobial Agent	Upregulated biosensor promoter				
	Cell envelope	***Protein***	***RNA***	***DNA***	***Fatty-acid***
Vancomycin	**+ (2.7 ±0.2)**	- (0.9 ±0.1)	- (1.1 ±0.1)	- (1.1 ±0.2)	- (1.1 ±0.1)
Nisin	- (1.6 ±0.1)	- (0.8 ±0.1)	- (1.1 ±0.2)	- (1.2 ±0.4)	- (0.9 ±0.1)
Tetracycline	- (1.3 ±0.1)	**+ (2.1 ±0.1)**	- (1.5 ±0.3)	- (1.1 ±0.1)	- (1.2 ±0.2)
Rifampicin	- (1.0 ±0.1)	- (1.0 ±0.1)	**+(2.7 ±0.2)**	- (1.0 ±0.1)	- (0.9 ±0.1)
Ciprofloxacin	**- (1.8 ±0.4)**	- (1.0 ±0.1)	- (1.1 ±0.2)	**+ (74.9 ±5.6)**	- (1.7 ±0.2)
Triclosan	- (1.2 ±0.2)	- (0.6 ±0.1)	- (1.2 ±0.1)	- (1.4 ±0.4)	**+ (7.8 ±1.7)**
Mersacidin	**+ (6.7 ±1.5)**	- (1.0 ±0.3)	- (1.3 ±0.5)	- (0.9 ±0.1)	- (1.0 ±0.2)
DAB	**+ (3.4 ±0.7)**	- (1.0 ±0.1)	- (1.3 ±0.4)	- (0.9 ± 0.1)	- (1.1 ± 0.1)
NVB353	**+ (3.6 ±0.6)**	- (1.1 ±0.2)	- (1.2 ±0.1)	- (1.2 ±0.1)	- (1.3 ±0.2)

Table 4.4 - Reporter induction (+) or no induction (-). Threshold set at published values (Fischer et al., 2004; Urban et al., 2007), normalised against untreated samples. (Maximum reporter signal ± standard deviation) Figures in bold represent signals above the published threshold for induction of the respective biosensor.

response to the lantibiotics, suggesting DNA, RNA, protein and fatty acid biosynthesis initially remain unaffected by type B lantibiotics, as supported by previous observations for mersacidin (Brotz *et al.*, 1995).

4.3.6 Analysis of inhibition of PG biosynthesis using *S. aureus* reporter constructs

As discussed in Chapter 3, the use of the *B. subtilis* biosensor strains alone to characterise the MOA of putative cell wall inhibitors is not sufficient. Therefore, the *S. aureus* cell-wall reporters were used to confirm the MOA of NVB3535 (Table 4.5). These are strains which are specifically responsive to PG biosynthesis inhibitors, which were found to be more robust for detection of inhibitors of cell wall biosynthesis

Table 4.5 – Response of *S. aureus* PG reporters to lantibiotics and comparator agents

Antimicrobial Agent	Upregulated gene		
	gltA	***oppB***	***murZ***
Vancomycin	**+ (3.2 ±1.5)**	**+ (5.0 ±1.5)**	**+ (3.8 ±0.5)**
Flucloxacillin	**+ (5.1 ±0.6)**	**+ (6.0 ±1.1)**	**+ (8.6 ±1.6)**
Fosfomycin	**+ (2.9 ±0.3)**	**+ (5.9 ±0.8)**	**+ (4.6 ±0.9)**
D-cycloserine	**+(2.9 ±0.5)**	**+ (3.4 ±0.7)**	**+ (2.8 ±0.2)**
Tetracycline	- (1.1 ±0.2)	- (1.0 ±0.1)	- (1.0 ±0.2)
Rifampicin	- (1.4 ±0.3)	- (1.3 ±0.1)	- (1.0 ±0.4)
Ciprofloxacin	- (1.0 ±0.4)	- (1.7 ±0.1)	- (0.4 ±0.3)
Triclosan	- (1.0 ±0.1)	- (1.4 ±0.2)	- (0.9 ±0.3)
Mersacidin	**+ (3.2 ±0.6)**	**+ (2.5 ±0.1)**	**+ (3.7 ±0.8)**
DAB	**+ (4.2 ±0.5)**	**+ (3.7 ±0.3)**	**+ (3.6 ±0.6)**
NVB353	**+ (2.8 ±0.3)**	**+ (2.2 ±0.4)**	**+ (2.7 ±0.1)**

Figure 4.5 - Reporter induction (+) or no induction (-). Threshold set at greater than or equal to twofold for a positive result, normalised against untreated samples. (Maximum reporter signal ± standard deviation) Figures in bold represent signals above the set threshold for induction.

(Chapter 3). In confirmation of the transcriptomic analysis from previous work (Blake *et al.*, 2009; O'Neill *et al.*, 2007), the genes *gltA, oppB* and *murZ* were upregulated in response to the PG biosynthetic inhibitors vancomycin, flucloxacillin, D-cycloserine and fosfomycin, (Table 4.5) in agreement with their known MOAs (See introduction, section 1.2). Treatment of *S. aureus* with mersacidin, DAB and NVB353 also induced the expression of *gltA, oppB* and *murZ* (Table 4.5). These results further support the hypothesis that NVB353 inhibits PG biosynthesis, and is consistent with previous data showing that mersacidin inhibits PG biosynthesis at the level of transglycosylation (Brotz *et al.*, 1997).

4.3.7 MOA analysis: Macromolecular synthesis

The results from the reporter assays (Table 4.4 and 4.5) strongly support the hypothesis that NVB353, like its parental compound DAB, inhibits PG biosynthesis. However, the time kill data (Figure 4.2) showing increased potency and more rapid bactericidal activity of NVB353 may indicate that NVB353 has an additional MOA. Examples exist in the literature that describe antibiotic analogues with altered inhibition mechanisms e.g. tetracyclines, whose MOA is disruption of the cytoplasmic membrane, as well as protein synthesis inhibition (Rassmussen *et al.*, 1991; Chopra, 1994). As mentioned in Chapter 3, the *B. subtilis* biosensors may be limited in their capacity to detect every class of antibacterial agent (particularly protein biosynthesis inhibitors), and therefore to examine whether more than one biosynthetic pathway was inhibited by the type B lantibiotics, macromolecular synthesis assays were performed to detect inhibition of incorporation of radiolabelled thymidine, uridine, glutamine and glycine into DNA, RNA, protein and PG respectively (Figure 4.3).

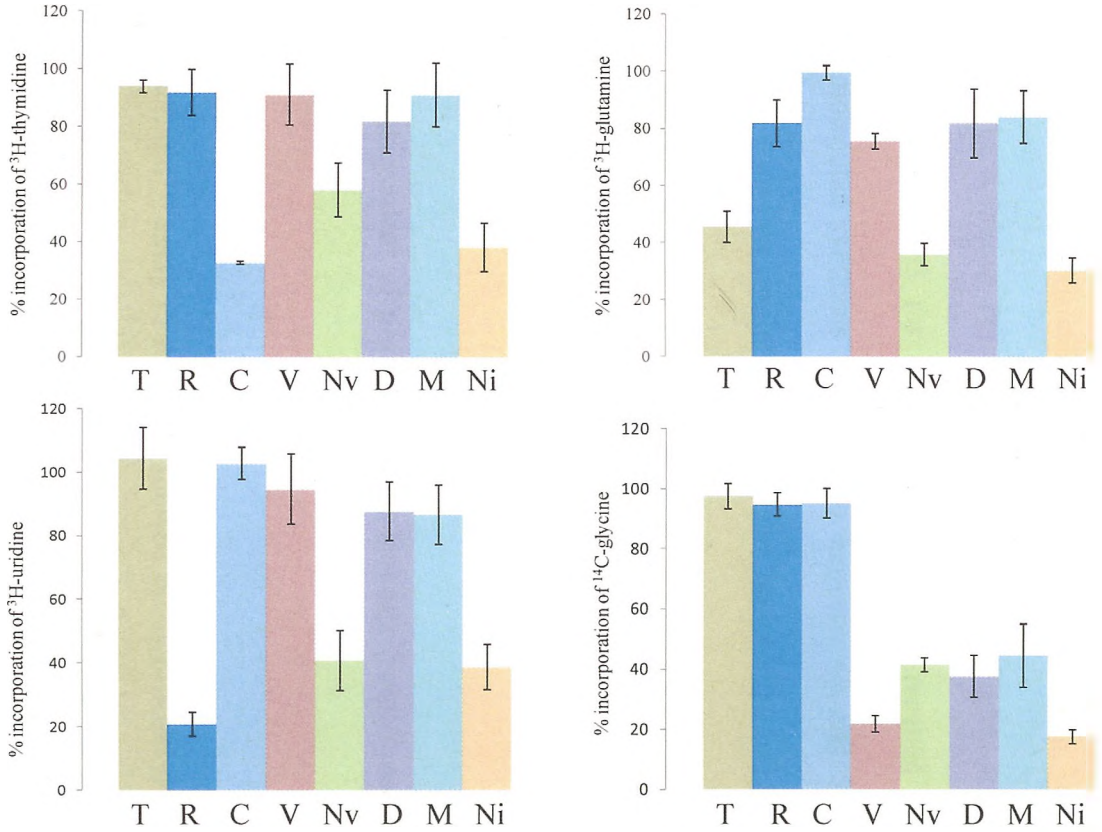


Figure 4.3 – Inhibition of macromolecular synthesis in *S. aureus* SH1000 in response to 10 minutes treatment with type B lantibiotics and comparator agents (T - tetracycline, R – rifampicin, C – ciprofloxacin, V- vancomycin, Nv – NVB353, D – DAB, M – mersacidin, N – nisin). *Data are expressed as percentage incorporation relative to a drug free control. Error bars represent the standard deviation from three replicates.*

Incorporation of DNA, RNA, protein and PG was preferentially inhibited (by >50% in ten minutes) by the control agents ciprofloxacin, rifampicin, tetracycline and vancomycin respectively, consistent with their activities as known inhibitors of these biosynthetic pathways (See Introduction). Mersacidin and DAB had no effect on the biosynthesis of DNA, RNA and protein after ten minutes in agreement with published studies (Brotz *et al.*, 1995), but preferentially inhibited the incorporation of ^{14}C labeled glycine into PG consistent with cell wall inhibition, comparable with that of vancomycin, a known PG biosynthesis inhibitor (Figure 4.3). Exposure of *S. aureus* SH1000 to NVB353 for ten minutes however, resulted in inhibition of all four macromolecular synthesis pathways to between 40 and 58% that of the drug-free control, in a manner similar to that of nisin. Inhibition of all four biosynthetic pathways indicates an additional non-specific MOA such as membrane damage (as in the case of nisin) or cessation of energy-producing metabolic pathways (Ooi *et al.*, 2009a; Silver, 2011).

4.3.8 Measurement of potassium (K^+) leakage from *S. aureus* cells in response to lantibiotics

The *Bac*Light™ assay (Table 4.3) showed no significant membrane damage after 10 minutes exposure to NVB353. However, an additional membrane-damaging MOA for NVB353 cannot be ruled out, as the *Bac*Light™ assay is only capable of detecting damage to the cell membrane which is great enough to permit permeation of the large relative molecular mass compound propidium iodide (668.4 Da) through the damaged bilayer. Therefore, the inhibition of macromolecular synthesis by NVB353 observed in Figure 4.3 may be a result of slight membrane perturbation. This additional MOA could

account for the increased bactericidal response of NVB353 in comparison with DAB as observed in Figure 4.2. To determine if the increased potency of NVB353 in comparison with DAB could be attributed to an additional membrane perturbation MOA, the effect of the type B lantibiotics on the cytoplasmic membrane of *S. aureus* SH1000 was determined, by measurement of K^+ leakage from the cell (Figure 4.4). It is necessary to perform this assay in buffer as opposed to growth media to avoid high background levels of K^+ which may interfere with the sensitivity of the spectrometer (Ooi *et al.*, 2009a).

Treatment of *S. aureus* with nisin and CTAB resulted in an immediate loss of greater than 70% of the intracellular potassium, consistent with their known MOAs (Ooi *et al.*, 2009). Exposure of the cells to 4X MIC of DAB and mersacidin caused only ~20% K^+

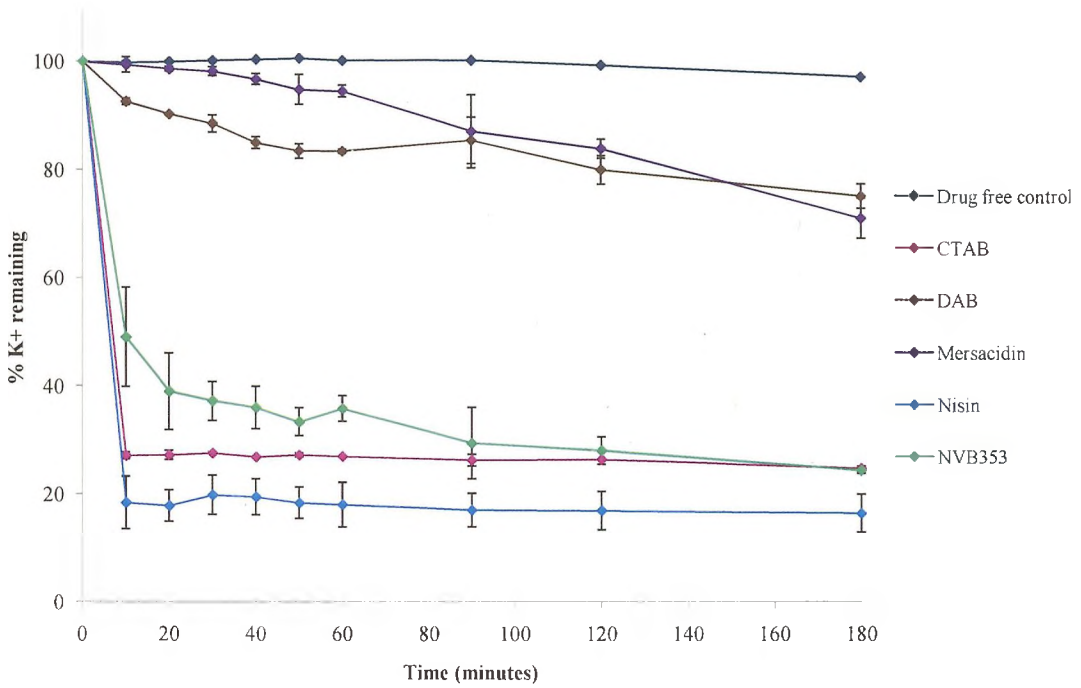


Figure 4.4 - K^+ leakage from *S. aureus* SH1000 cells treated with lantibiotics and control agents at 4X MIC. *Error bars represent the standard deviation from three replicates.*

leakage over the 3 hour period. However, treatment with NVB353 was associated with 70% K^+ leakage over the three hour time course, suggesting that the increased potency of NVB353 observed in the time kill experiments, compared to DAB, might be due to secondary disruption of the membrane, although this is not apparent in the first ten minutes of drug exposure as measured by the *Bac*Light™ assay.

Structural and functional similarities between the membranes of eukaryotes and prokaryotes have so far meant that antibacterial agents causing membrane damage are not suitable as chemotherapeutic candidates (Maher & McClean, 2006). There are however, notable exceptions to this (e.g. daptomycin and telavancin) which are sufficiently specific for bacteria to be used clinically (Kostrominova *et al.*, 2010; Stryjewski *et al.*, 2004). Therefore, if NVB353 exhibits low propensity for resistance development, it may still be a viable antibacterial candidate.

4.3.9 Lantibiotic resistance in *S. aureus*

NVB353 shows promise for clinical development, due to its good antibacterial activity against staphylococci, including MRSA and VISA (Table 4.2), and lack of activity against yeast, a representative eukaryotic cell (Table 4.3). However, it is necessary to determine the propensity of *S. aureus* to develop resistance to this novel agent, in order to estimate the chances of resistance developing during clinical use. In addition, generation of resistant mutants and subsequent identification of the genetic locus of resistance may provide further confirmation as to the likely drug target. Therefore, I attempted to generate *S. aureus* mutants displaying resistance to NVB353 and its parent DAB as currently there are no reports that describe acquired type B lantibiotic

resistance. By continuous subculture in 0.25X MIC of the agent, and plating onto 4X MIC, a DAB-resistant *S. aureus* mutant (DAB6) was selected within six passages, which displayed a fourfold increase in resistance (Table 4.6). Mutants displaying resistance to NVB353 were not generated, even after twenty passages, which further supports the hypothesis that NVB353 possesses an additional MOA against *S. aureus* in comparison with DAB. The inability to select for NVB353-resistant mutants suggests that resistance to this agent would be unlikely, or slow to develop clinically. In addition, the results suggest that type B lantibiotic resistance would require mutations at multiple sites (Miller *et al.*, 2002), such as those seen in staphylococcal resistance to vancomycin (Schaaf *et al.*, 2002).

The presence of an antibiotic-resistant phenotype may inflict a biological cost on the bacterium, limiting the fitness or virulence of resistant strains (Andersson, 2006) which may benefit the susceptible bacteria in the population, allowing them to outcompete their resistant counterparts in the absence of antibiotic selection pressure (Andersson & Hughes, 2010). However, compensatory genetic mutations may overcome such costs and lead to restoration of fitness and spread of antibiotic resistant strains (Bottger *et al.*, 1998). Determining the fitness of mutants arising *in vitro* gives an indication of the likelihood of mutants surviving *in vivo* (Hurdle *et al.*, 2004). To assess the likelihood of

Table 4.6 - Generation of a DAB-resistant *S. aureus* SH1000 mutant and comparative strain fitness

S. aureus strain	DAB MIC (µg/ml)	Generation time (min)
SH1000	32	37.2 ±3.1
DAB6	128	44.7 ±3.2

DAB mutants arising clinically, I determined the growth rate of the DAB-resistant mutant as an indicator of its fitness. The growth rate increased from 37.2 ±3.1 minutes in the wild-type, to 44.7 ±3.2 minutes in DAB6 (Table 4.6), suggesting that resistance to DAB is associated with a slight fitness cost in *S. aureus*. This may indicate that mutants are less likely to survive *in vivo* in the absence of selection and may revert, unless compensatory mutations are also introduced. The slight fitness cost associated with type B lantibiotic resistance in *S. aureus*, alongside the low propensity for resistance development to NVB353 highlights this compound as having potential for future clinical development. As previously mentioned, there are currently no reports which describe acquired type B lantibiotic resistance, and therefore the phenotypic characteristics of the DAB-resistant *S. aureus* mutant were analysed. DAB6 was

Table 4.7 – DAB6 cross resistance analysis

Antimicrobial agent	Minimum inhibitory concentration (µg/ml)	
	***S. aureus* SH1000**	***S. aureus* DAB6**
DAB	32	128
Mersacidin	32	64
NVB353	4	16
Rifampicin	0.015	0.015
Flucloxacillin	0.25	0.25
Tetracycline	0.5	0.5
Ciprofloxacin	1	1
Vancomycin	2	4
Daptomycin	2	2
Triclosan	0.125	0.125
Nisin	4	4

subjected to cross-resistance analyses to identify other agents to which it might display resistance (Table 4.7). DAB6 was cross-resistant to mersacidin, NVB353 and vancomycin. No cross-resistance was observed with any of the other agents with established MOAs, suggesting a distinct antibacterial target with respect to those agents as expected. The cross-resistance observed for mersacidin and NVB353 are most likely due to target similarities between the antibacterial compounds, and further confirms that although NVB353 possesses an additional membrane damaging MOA, the binding site of the derivative is likely to be the same as DAB and mersacidin.

4.4 Conclusions

The emergence of resistant bacteria has largely been met with clinical introduction of newer members of existing antibacterial classes displaying improved properties. NVB353 represents a DAB derivative with improved activity against *S. aureus* in comparison with the parental compound (improved activity against *S. aureus*, bacterial specificity and low propensity for resistance development), and therefore shows great promise as a potential chemotherapeutic candidate. Data from the MOA analysis suggest that the increased potency and rate of bactericidal activity of NVB353 when compared with DAB is due to the fact that NVB353 exhibits a dual MOA against *S. aureus* SH1000, causing inhibition of PG biosynthesis as well as perturbation of the cell membrane. NVB353 is a viable candidate for chemotherapeutic development and should be taken forward for further preclinical evaluation.

Chapter 5 - Characterisation of underexploited antibacterial agents: putative inhibitors of bacterial RNA polymerase (RNAP)

5.1 Abstract

The highly conserved but distinct structure of bacterial RNA polymerase (RNAP) in comparison with the eukaryotic counterpart and essential nature of the enzyme confirm the suitability of RNAP inhibitors as broad-spectrum antibacterial agents. Thus, RNAP remains an attractive target for novel antibiotics that bind to other regions on the enzyme distinct from the rifamycin binding site to overcome existing resistance mechanisms. Therefore, this experiment aimed to analyse a set of previously reported, but poorly characterised RNAP inhibitors, determining their antibacterial spectrum, bacterial specificity, mode of action (MOA) and propensity to develop resistance in *Staphylococcus aureus.* Rose Bengal, daunorubicin, doxorubicin, juglone and ripostatin A (hemiacetal isomer) caused damage to the staphylococcal membrane, while 8-hydroxyquinoline displayed an antifungal minimum inhibitory concentration (MIC) that was comparable with its antibacterial activity, and chromomycin induced the DNA biosensor, consistent with inhibition of DNA synthesis. As such these agents were deemed unsuitable for further development as clinical RNAP inhibitors. Thiolutin and holomycin displayed antibacterial potency and specificity. However, the observed antibacterial activity could not be linked with inhibition of RNAP, and therefore these agents were also eliminated from further consideration. Corallopyronin A (CorA) and myxopyronin B both displayed antistaphylococcal activity. They also induced the *Bacillus subtilis* biosensor responsive to RNA synthesis inhibition supporting the hypothesis that α-pyrone antibiotics inhibit bacterial RNAP. Eight spontaneous *S. aureus* mutants displaying high-level resistance to CorA were generated, and the

mutations conferring resistance mapped to the *rpoB* and *rpoC* subunits of RNAP, further confirming the proposed mode of action (MOA) of CorA. However, the limited anti-bacterial spectrum of CorA and its relatively high propensity for selection of resistance suggest that may not be a particularly promising antibacterial candidate, and may serve better as a scaffold for future drug development.

5.2 Introduction

5.2.1. RNA polymerase (RNAP) as an antibacterial target

DNA-dependent RNAP is an essential enzyme in bacterial transcription, catalysing the synthesis of RNA by the addition of ribonucleotides to the 3' end of a nascent transcript in four well characterised steps. The first includes the association of RNAP with a specific DNA promoter; second, the binding of ribonucleotides to the RNAP-promoter complex and formation of the first phosphodiester linkage (transcription initiation); third, the addition of more ribonucleotides to the 3' end of the transcript (elongation) and finally, cessation of elongation and release of the RNA transcript (termination) (Figure 5.1) (Puri, 2011). The activity of RNAP in bacteria is highly regulated, controlling the expression of genes responsible for basic metabolism and survival alongside responses to external stimuli and changes in the extracellular environment (Browning & Busby, 2004). The highly conserved core enzyme (~400 kDa) is composed of five subunits, two α chains (involved in promoter recognition and enzyme assembly), a β (polymerase) and β' (DNA binding) subunit and a γ subunit (promotes enzyme assembly) (Darst, 2001; Minakhin *et al.*, 2001). The core enzyme associates with promoter specific transcriptional regulators (σ factors) to form the holoenzyme and initiate transcription, but these factors are released when the RNA transcript is approximately ten nucleotides in length (Travers & Burgess, 1969). As yet RNAP remains an underexploited bacterial target as only the rifamycin group of antibiotics (e.g. rifampicin) are clinically approved, established inhibitors of RNAP. As rifampicin easily penetrates the bacterial cell and human tissues, its indication for treatment of

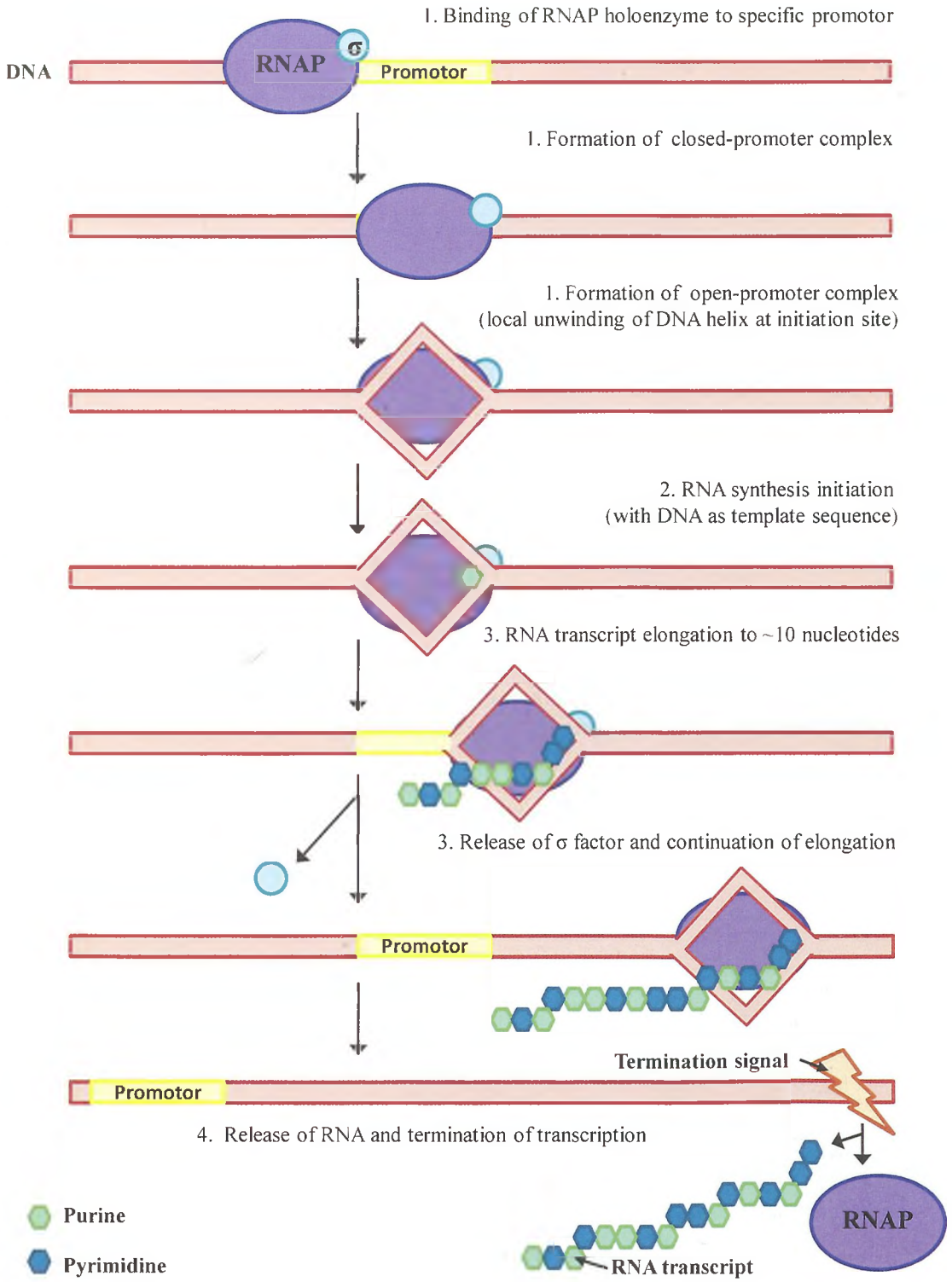


Figure 5.1 – Schematic representation of the four steps of prokaryotic transcription catalysed by RNAP

infections caused by intracellular pathogens (like *Mycobacterium tuberculosis*) is a logical choice (Campbell *et al.*, 2001). Inhibition at the level of transcription has the added benefit of a cidal, but non-lytic effect on the bacterial cell and the unique multi-subunit nature of RNAP provide a multitude of potential inhibitor binding sites (Villain-Guillot *et al.*, 2007a). In addition, the highly conserved but distinct structure of RNAP in comparison with the eukaryotic counterpart (which is a ≥12 subunit enzyme ~500kDa in size) and essential nature of the enzyme validate the potential for inhibitors of RNAP as broad-spectrum antibacterial agents (Chopra, 2007; Darst, 2001). However, the clinical efficacy of rifampicin is under threat due to the rapid emergence of resistance by point mutations in the β subunit of RNAP (O'Neill *et al.*, 2006). Thus, RNAP remains an attractive target for novel antibiotics if other regions on the enzyme distinct from the rifamycin binding site can be targeted to overcome existing resistance mechanisms.

5.2.2 Development of underexploited agents for antibacterial drug discovery

The increasing prevalence of antibiotic resistance in bacteria is creating an urgent need for antibacterial agents with novel modes of action and enhanced activity. This is particularly the case with infections caused by meticillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) for which antibiotic treatments are becoming increasingly ineffective (Hawkey, 2008). In the last fifteen years, however, strains of MRSA have been reported that are almost universally susceptible to the older sulphonamide-trimethoprim combination drug co-trimoxazole (Denis *et al.*, 2002), and infections caused by VRE have been reported to be successfully treated using chloramphenicol, which was also one of the earliest antibiotics to be introduced into clinical practice (Lautenbach *et al.*, 1998). As such,

there has been growing interest in the return to older and underexploited compounds to treat infections caused by 'new' resistant bacteria (Pitlik, 2003). Examples of older, reported RNAP inhibitors are shown in Figure 5.2 and discussed below.

5.2.3 α-pyrone antibiotics

Myxopyronin A and B (MyxA and MyxB) and corallopyronin A (CorA) are structurally related α-pyrone antibiotics, first isolated nearly thirty years ago as natural products from *Myxococcus* spp. (Irschik *et al.*, 1983; Irschik *et al.*, 1985). These antibiotics are active against Gram-positive bacteria and inhibit bacterial RNAP *in vitro* with IC_{50} values of ~8.4 μM and 3.8 μM for MyxA and CorA respectively (Irschik *et al.*, 1983; Irschik *et al.*, 1985). The activities of CorA and MyxB against *E. coli* RNAP *in vitro* have recently been confirmed by R. Trowbridge (Institute of Molecular and Cellular Biology, University of Leeds) using the Kool NC-45 universal RNAP template (Epicentre, Madison, WI). Although CorA and MyxB inhibited *E. coli* RNAP, rifampicin was a more potent inhibitor of the enzyme (IC_{50}s – RIF [11.5 ± 1.1 nM], CorA [0.73 ± 0.2 μM], MyxB [46.5 ± 5.9 μM]). The activities of CorA and rifampicin have also been compared with yeast RNA polymerase (Pol) II *in vitro* in a modified non-specific transcription assay (D. Bushnell, personal communication), using polyribose C template (Sigma, Poole, UK) and SYBR-Green I (Invitrogen, Paisley, UK) detection. The addition of CorA at 100 μM only inhibited yeast Pol II activity by 10.1% compared with 37.3% for rifampicin (R. Trowbridge, personal communication). The lack of activity of CorA against eukaryotic RNAP correlates with previous studies showing only 7% inhibition of wheat germ Pol II by 40 μg/ml (75.8 μM) of the antibiotic (Irschik *et al.*, 1985). In further confirmation of bacterial RNA synthesis

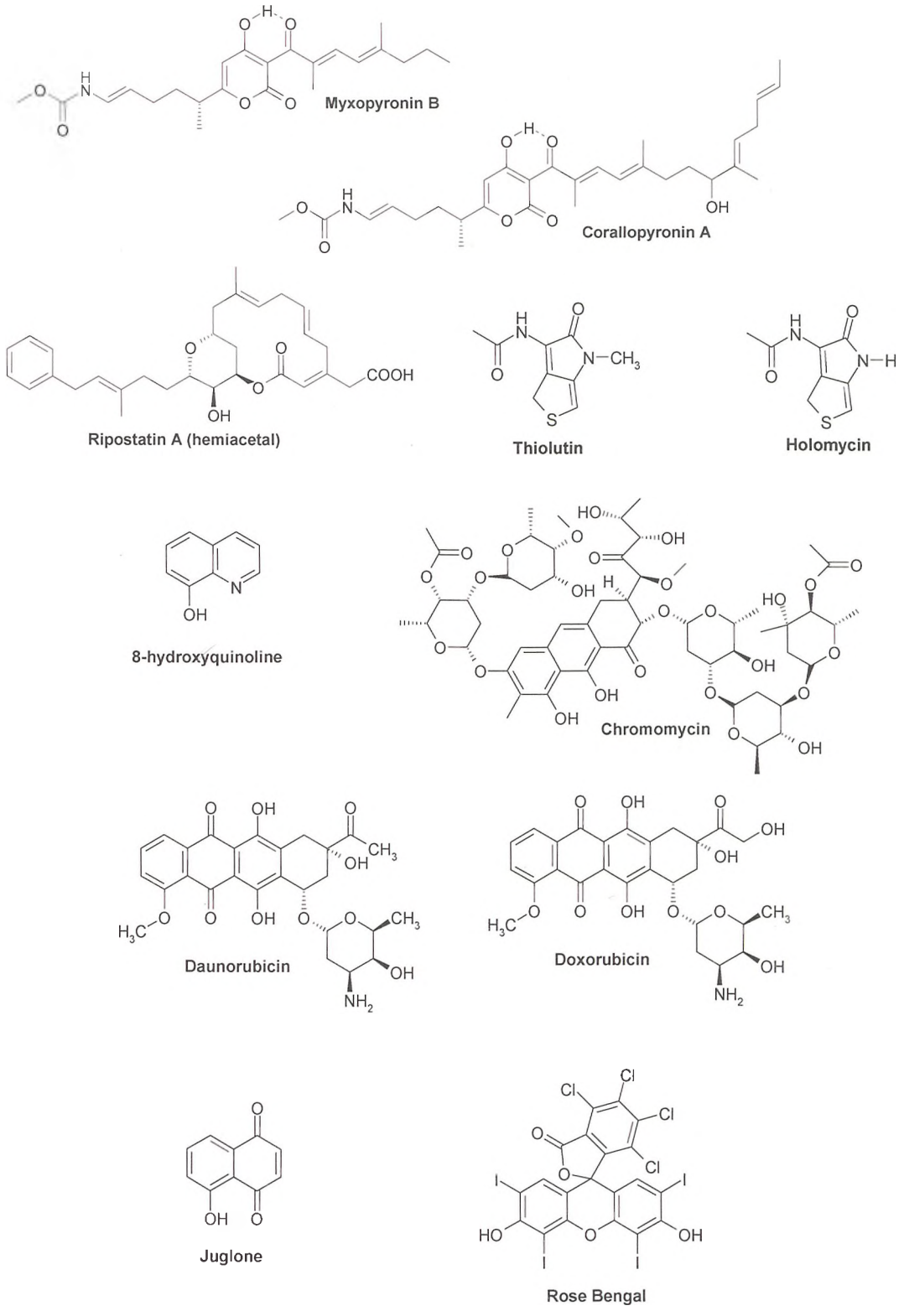


Figure 5.2 – Molecular structures of reported RNAP inhibitors

inhibition by α-pyrones, both CorA and MyxA significantly reduce incorporation of [^{14}C] uridine into *S. aureus* RNA after ten minutes exposure to the agent at concentrations approximating to 10X MIC (Irschik *et al.*, 1983, Irschik *et al.*, 1985). In addition, MyxB has more recently been co-crystallised with the β' subunit of RNAP (Belogurov *et al.*, 2009; Mukhopadhay *et al.,* 2008). The crystallographic analysis confirms that MyxB binds directly to the RNAP 'switch' region (which serves as the hinge between the β and β' subunits to control DNA binding), and prevents interaction of RNAP with the DNA template during transcription initiation (Mukhopadhay *et al.,* 2008).

5.2.4 The Ripostatins

The **ripo**statins (named for inhibitors of **ri**bonucleic acid **po**lymerase) were first described in 1995 as macrocyclic lactone carbonic acid antibiotics isolated as natural products from *Sorangium cellulosum* So ce377 in 1989 (Irschik *et al.*, 1995). Ripostatin A may be isolated as a mixture of two isoforms, the hemiacetal (45%) (Figure 5.2) and keto (55%) isomers (Augustiniak *et al.*, 1996). They display a narrow spectrum of activity (primarily against Gram-positive species due to lack of penetration into Gram-negative bacteria), but ripostatin A displays an IC_{50} of 0.2 μM against *E. coli* RNAP and completely inhibits incorporation of [2-^{14}C] uridine into *S. aureus* RNA after fifteen minutes exposure to the agent at concentrations approximating to 10X minimum inhibitory concentration (MIC) (Irschik *et al.*, 1995). Spontaneous mutants of *S. aureus* displaying resistance to ripostatin have been generated, but no cross resistance was seen to rifampicin, indicating that while ripostatin seems to inhibit RNAP, it acts at a unique site that is independent of the rifamycin binding site (Irschik *et al.*, 1995).

5.2.5 Thiolutin and holomycin

Holomycin and thiolutin are natural products containing a pyrrolinonodithiole nucleus and were first described in 1952 (Seneca *et al.*, 1952). These antibiotics display broad-spectrum antibacterial activity. Although their mode of action is poorly understood, it is currently thought to involve inhibition of RNA synthesis (Jimenez *et al.*, 1973; Khachatourians & Tipper, 1974). The bacteriostatic activity of holomycin is associated with rapid inhibition (in less than five minutes) of incorporation of [^{3}H] uridine into RNA, comparable with that seen for rifampicin (Oliva *et al.*, 2001). Holomycin also does not induce the stringent response in whole cells. Therefore inhibition of RNA synthesis is more likely to be due to targeted inhibition of RNAP than the secondary suppression of transcription that is observed during the stringent response (Oliva *et al.*, 2001). Despite this conclusion, holomycin only weakly inhibited *E. coli* RNAP *in vitro* at concentrations in excess of the MIC, and therefore it has been suggested that holomycin and thiolutin might act as prodrugs, which are converted intracellularly to the active antibiotic (Oliva *et al.*, 2001).

5.2.6 8- hydroxyquinoline (8-HQ)

8-HQ is a lipophilic metal ion chelator which is currently used a preservative, disinfectant and fungicidal agent (Leanderson & Tagesson, 1996). 8-HQ rapidly inhibits the activity of *Escherichia coli* RNAP by direct chelation of the enzyme's magnesium ion (Mg^{2+}) component, a dissociable bivalent cation which is required for polymerase activity (Fraser & Cranor, 1975; Ronald *et al.*, 1975; Collins *et al.*, 1979). Direct chelation can occur via the lone electron pair on the ring nitrogen and the negatively

charged oxygen on the hydroxyl group. However, an alternative mechanism of action has also been suggested by which the secondary inhibition of RNA synthesis is due to direct complexation and inhibition of the DNA template by 8-HQ (Cano *et al.*, 1973).

5.2.7 Chromomycin

Chromomycin is a glycosidic natural product antibacterial, antifungal and antitumour agent produced by a strain of *Streptomyces griseus* (Menendez *et al.*, 2006). Chromomycin has previously been shown to inhibit the DNA dependent synthesis of RNA (Behr *et al.*, 1969), but whether this inhibition is due to a direct interaction of the agent with RNAP or a secondary consequence of inhibition of DNA synthesis in whole bacterial cells is unknown.

5.2.8 Daunorubicin and doxorubicin

Daunorubicin and doxorubicin are members of the anthracycline class of antibiotics and are commonly used anti-cancer agents, being favoured for use against adult leukaemias and metastatic breast cancers, respectively (Sartiano *et al.*, 1979; Muggia *et al.*, 1981). Their mode of antibacterial action is poorly characterised, although they have previously been shown to bind with high affinity to DNA and are therefore thought to inhibit DNA replication and RNA transcription (Ward *et al.*, 1965). At the molecular level, high concentrations of daunorubicin directly interfere with the interaction of RNAP with DNA, but at lower concentrations there is evidence to suggest that the enzyme-DNA interaction is able to take place, but that RNAP-mediated RNA

elongation is inhibited (Kriebardis *et al.*, 1984). Consequently, more work is needed to elucidate the mechanism of action of these agents.

5.2.9 Juglone

Juglone is an established inhibitor of pyruvate decarboxylase, glutathione S transferase and peptidyl-prolyl isomerase (Pin1), an enzyme which is involved with the cis/trans isomerisation of amide bonds directly preceding prolyl residues (Gothel & Maraheil, 1999). The primary mechanism of inhibition of these enzymes by juglone lies in the modification of essential cysteines via the thiol moieties of the side chains (Hennig *et al.*, 1998). Limited studies have also shown that juglone directly interferes with the formation of the preinitiation complex of human RNAP II, however, the mechanism of this inhibition remains as yet uncharacterised (Chao *et al.*, 2001). Furthermore, whether juglone also inhibits bacterial RNAP is unknown.

5.2.10 Rose Bengal

Rose Bengal (a photosensitiser and bacterial dye) is a potent inhibitor of *E. coli* RNAP (Wu & Wu, 1973; Decraene *et al.*, 2006). This agent displays an IC_{50} against the enzyme of 1.4 μM, and inhibition is non-competitive with regards to the DNA template or ribonucleotides, and therefore Rose Bengal is thought to inhibit RNA elongation preferentially, rather than transcription initiation (Wu & Wu, 1973).

5.2.11 Aims and objectives of the work described in this chapter

Despite early reports of specific RNAP inhibition by the above inhibitors, the exact mechanism of action and biological activity of these compounds requires confirmation and further characterisation if they are to be re-considered for antibacterial chemotherapeutic application. Hence, this study explored the potential of the above agents to be developed for clinical use as inhibitors of bacterial RNAP by determination of:

- the antibacterial spectrum of activity of the putative RNAP inhibitors;
- the suitability of these compounds for clinical development using the *Bac*Light™ assay and *Saccharomyces cerevisiae* minimum inhibitory concentration determinations;
- the mode of action (MOA) of these compounds using *Bacillus subtilis* biosensors;
- the potential for resistance development to lead compounds, and characterisation of any mutants arising.

5.3 Results and discussion

5.3.1 Antibiotic susceptibility testing

In order to confirm the potential of underexploited, but reported inhibitors of RNAP for clinical development, MIC determinations of these agents were performed against a representative Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) organism (Table 5.1). In each case their activity was compared with that of the established RNAP inhibitors rifamycin SV and rifampicin. The putative bacterial RNAP inhibitors displayed good to modest activity (≤32 μg/ml) against the Gram-positive organisms tested, but were generally inactive against the Gram-negative strains, with the exception of 8-HQ, baicalein, juglone, holomycin and thiolutin (Table 5.1). The preferential activity of MyxB, CorA and ripostatin A observed for Gram-positive bacteria has previously been reported (Irschik *et al.*, 1983; Irschik *et al.*, 1985; Irschik *et al.*, 1995), and the inactivity of these compounds against *E. coli* SM1411 suggests that they are not a substrate for the AcrAB-TolC efflux pump. However, MyxB is more active against a TolC knockout mutant of *E. coli* in comparison with the wild-type (Doundoulakis *et al.*, 2004; Lira *et al.*, 2007), which may suggest that α-pyrone antibiotics are substrates for efflux pumps other than AcrAB which incorporate the TolC outer membrane component (Nikaido, 2009). However, the results from this study suggest that in wild-type *E. coli*, CorA, MyxB and ripostatin (like the rifamycins) also suffer from an inability to penetrate through the outer membrane, as supplementation of the media with polymyxin B nonapeptide (PMBN) leads to an increase in activity of these compounds against *E. coli* 1411, to a level that is comparable with that of the Gram- positive strains tested. The activity of Rose Bengal against wild-type *E. coli* is also enhanced in this

Table 5.1 – Antibacterial spectrum of activity of putative RNAP inhibitors

Antibacterial agent	M_r	MIC (μg/ml)				
		S. aureus SH1000	*E. coli* 1411	*E. coli* 1411 + PMBN	*E. coli* SM1411	*B. subtilis* 1S34
Rifampicin	823.0	0.015	4	0.125	4	128
Rifamycin SV	697.8	0.125	64	0.5	64	16
8-HQ	145.2	2	64	64	64	1
Chromomycin	1183.3	0.25	>128	>128	>128	1
Daunorubicin	527.5	16	128	16	32	4
Doxorubicin	543.5	16	128	128	32	4
Juglone	174.2	8	8	2	4	1
Rose Bengal	973.7	32	>256	32	>256	2
Holomycin	214.3	4	1	1	1	4
Thiolutin	228.3	2	4	4	4	4
Ripostatin A (Hemiacetal)	494.6	32	>128	32	>128	64
CorA	527.7	2	>128	32	>128	32
MyxB	431.5	2	>128	8	>128	32

manner, and therefore the same conclusion can be drawn. The relative molecular masses of these agents range between 430-973 Da (Table 5.1). For larger members of the RNAP inhibitor set (>600 Da), intact membranes would likely inhibit effective penetration to the proposed intracellular target.

The activity of 8-HQ, holomycin, thiolutin and chromomycin was not increased in the AcrAB knockout strain of *E. coli*, or in the wild-type treated by PMBN, suggesting that these compounds are not substrates for the AcrAB-TolC multi-drug efflux pump, nor is their ingress into the cell hindered by the presence of the Gram-negative outer membrane. For the former three, this may be due to the fact that these antibacterial agents constitute small hydrophobic molecules which could easily pass through the intact lipophilic cell membranes to reach their proposed intracellular target. This would

account for the observed broad spectrum of antibacterial activity, which is in accordance with current literature (Mirth *et al.*, 1978; Oliva *et al.*, 2001; Seneca *et al.*, 1952). Chromomycin constitutes the largest compound of the RNAP inhibitor set, and its lack of activity against *E. coli* 1411, *E. coli* 1411 with PMBN and *E. coli* SM1411 most likely is due to its size, as seen with other large antibiotics such as the lantibiotics (Chapter 4). The selective antibacterial activity of chromomycin against Gram-positive bacteria, has been well established in current literature (Menendez *et al.*, 2004).

Juglone (5–hydroxy–1,4–naphthoquinone) displayed a broad spectrum of antibacterial activity, in agreement with current literature (Didry *et al.*, 1994; Sharma *et al.*, 2009), that may be explained by its high structural similarity to 8-HQ. As explained above, 8-HQ can easily pass through the intact lipophilic cell membranes due to its small size to exert its antibacterial effect. Unlike 8-HQ however, the activity of juglone is increased against wild-type *E. coli* upon the addition of an outer membrane permeabiliser, and to a lesser extent upon the deletion of the AcrAB efflux pump. This would suggest that juglone is a substrate for the AcrAB-TolC tripartite multi-drug efflux system, as well as it being hindered to some degree by the presence of the outer membrane. In direct comparison with 8-HQ, the latter phenomenon may be explained by the addition of the two carbonyl moieties, which contribute to an overall increase in molecular polarity and poorer penetrative capability of juglone.

Daunorubicin and doxorubicin are anthracycline antibiotics with similar structures, and display an almost identical spectrum of antibacterial activity. In agreement with current literature, both antibiotics displayed preferential activity against the Gram-positive organisms tested (Gumpert *et al.*, 1982; Peiris & Oppenheim, 1993), and neither

antibiotic appeared to be a substrate for the AcrAB-TolC multi-drug efflux pump, as the susceptibility of *E. coli* SM1411 to both agents was not higher than that of the wild-type *E. coli*. Currently, daunorubicin and doxorubicin are known to be subject to efflux in Gram-negative organisms, but reportedly by ABC-type transporters (Srinivasan *et al.*, 2010). Interestingly, only the activity of daunorubicin was improved upon permeabilisation of the Gram-negative outer membrane with PMBN, suggesting this agent poorly penetrates wild-type Gram-negative organisms, a conclusion which has been alluded to in previous studies (Gumpert *et al.*, 1982). This result is surprising, due to the high structural similarity between daunorubicin and doxorubicin, but may yield important information concerning structural components of antibacterial agents which improve/impede cellular penetration. In the case of daunorubicin and doxorubicin, the addition of just one polar hydroxyl group to one of the molecular side chains results in lack of activity against PMBN-treated *E. coli*.

5.3.2 Determination of membrane damage and bacterial specificity of putative RNAP inhibitor compounds

To examine the suitability of the putative RNAP inhibitors to be developed for clinical use, the ability of the agents to cause membrane damage in *S. aureus* was determined using the *Bac*Light™ assay, and *S. cerevisiae* MICs were determined to evaluate toxicity against eukaryotic cells (Table 5.2). Exposure of *S. aureus* SH1000 to Rose Bengal, daunorubicin, doxorubicin, juglone and ripostatin A (hemiacetal) resulted in a reduction in cell membrane integrity to <40% of that of the drug-free control. These agents are therefore classed as membrane-damaging. As such, these compounds are likely to be unsuitable for further development as clinical RNAP inhibitors. In addition,

with the exception of ripostatin A, these agents also displayed activity against *S. cerevisiae* and therefore are likely to display cytotoxicity which supports the case for them being discarded from further evaluation. The lack of bacterial specificity of these agents is unsurprising, and correlates with reported activities as anti-cancer agents (Agarwala *et al.*, 2009; Aithal *et al.*, 2011; Sartiano *et al.*, 1979; Muggia *et al.*, 1981).

Table 5.2- Determination of membrane damage and bacterial specificity of putative RNAP inhibitors

Antimicrobial Agent	*S. aureus* SH1000 MIC (µg/ml)	*Bac*Light™ result (% membrane integrity)	*S. cerevisiae* 464 MIC (µg/ml)
Drug-free control	-	100	-
5% SDS (w/v)	-	0	-
CTAB	2	0	1
Nisin	2	14.9 ±1.0	>64
Tetracycline	0.5	99.9 ±4.6	256
Vancomycin	2	83.5 ±4.4	>256
Rifampicin	0.031	80.8 ±6.1	>256
Rifamycin SV	0.125	83.0 ±4.2	>256
Holomycin	4	116.3 ±10.2	64
Thiolutin	2	123.1 ±7.6	64
Rose Bengal	32	0.9 ±0.1	256
8-HQ	2	82.2 ±4.3	0.5
Chromomycin	0.25	111.6 ±5.1	>256
Daunorubicin	16	14.7 ±1.2	32
Doxorubicin	16	40.2 ±5.1	16
Juglone	8	15.1 ±1.2	1
Ripostatin A (Hemiacetal)	32	25.2 ±7.0	>128
Myxopyronin B	2	96.7 ±5.5	>128
CorA	2	97.9 ±10.4	>128

The cytoplasmic membrane of *S. aureus* SH1000 remained relatively intact (82% membrane integrity in comparison with the drug-free control) when exposed to 4X MIC of 8-HQ for 10 minutes. However the activity of the agent was not limited to *S. aureus*, and it was comparably active against *S. cerevisiae*. The lack of bacterial specificity is consistent with other studies (Leanderson & Tagesson, 1996; Shen *et al.*, 1999) and would indicate that 8-hydroquinoline is unsuitable for further development specifically as an antibacterial agent.

The five remaining agents (MyxB, CorA, chromomycin, holomycin and thiolutin) displayed MICs against *S. cerevisiae* that were at least tenfold greater that of the antibacterial activity, indicating a level of bacterial specificity. In addition, *S. aureus* SH1000 exposed to these agents maintained close to 100% membrane integrity. Therefore these agents are not classed as membrane-damaging compounds. As such, these compounds were taken forward for further evaluation to attempt to link the observed antibacterial activity to inhibition of RNAP at the cellular level.

5.3.3 *B. subtilis* antibiotic biosensors

The bactericidal activity of α-pyrone antibiotics and chromomycin, and the bacteriostatic effects of holomycin and thiolutin have already been established (Kamiyama & Kaziro, 1966; Li & Walsh, 2010; Mukhopadhay *et al.*, 2008). Therefore, time-kill analyses against *S. aureus* SH1000 were not performed for these compounds. Established bacterial RNAP inhibitors, such as rifampicin are bactericidal, resulting from irreversible inhibition of transcription (Boisivon *et al.*, 1990). The bactericidal activity of chromomycin and the α-pyrone antibiotics could support the hypothesis that

they inhibit RNAP. Previous reports utilising macromolecular synthesis assays suggest inhibition of RNA biosynthesis by MyxB, CorA, holomycin and thiolutin (Irschik *et al.*, 1983; Irschik *et al.*, 1985; Khachatourians & Tipper, 1974; Oliva *et al.*, 2001). Therefore, the *B. subtilis* antibiotic biosensors were used to further confirm the MOA of these compounds (Table 5.3).

The results in Table 5.3 suggest that the MOA of chromomycin in whole-cells is inhibition of DNA biosynthesis, a finding supported by previous work suggesting that inhibition of RNA synthesis by chromomycin is a secondary consequence of the complexation and hydrolysis of DNA by the agent (Behr *et al.*, 1969; Honikel & Hartman, 1969). The previous studies showing that these antibiotics inhibit RNA biosynthesis may be exploited by an effect on the DNA template used in the assays. As such, chromomycin is an unsuitable candidate for future development as an inhibitor of bacterial RNAP and was not evaluated further in this research.

The results also confirmed the initial reports that CorA and MyxB mediate their antibacterial effect through inhibition of RNA biosynthesis (Irschik *et al.*, 1983; Irschik *et al.*, 1985), since both caused induction of *yvgS*, the promoter responsive to inhibition of RNA synthesis. CorA also induced the fatty-acid biosynthesis biosensor. The reason for this is unclear, but suggests CorA might possess an additional mechanism of action involving inhibition of fatty acid synthesis. Although further studies will be required to confirm this suggestion, inhibition of fatty acid biosynthesis has been observed with other antimicrobial agents that contain an α-pyrone moiety (Giddens *et al.*, 2008). However, induction of *fabHB* by CorA is threefold lower than that exhibited by triclosan, a known inhibitor of fatty acid biosynthesis. In addition, if inhibition of fatty

acid synthesis was observed for CorA, it would be expected that a similar phenomenon would be observed for MyxB. For these reasons, induction of *fabHB* by CorA may be an artefact of the assay.

Table 5.3 - Induction of *B. subtilis* biosensors by putative RNAP inhibitors and comparator agents

Antimicrobial Agent	Upregulated biosynthetic pathway*				
	Cell-envelope	*Protein*	*RNA*	*DNA*	*Fatty-acid*
Vancomycin	**+(2.7 ±0.2)**	- (0.9 ±0.1)	- (1.1 ±0.1)	- (1.1 ±0.2)	- (1.1 ±0.1)
Flucloxacillin	**+(2.9 ±0.1)**	- (0.8 ±0.1)	- (1.1 ±0.3)	- (1.0 ±0.1)	- (1.1 ±0.1)
Nisin	- (1.6 ±0.1)	- (0.8 ±0.1)	- (1.1 ±0.2)	- (1.2 ±0.4)	- (0.9 ±0.1)
CTAB	- (1.0 ±0.1)	- (1.0 ±0.4)	- (1.0 ±0.2)	- (0.9 ±0.1)	- (1.3 ±0.5)
Tetracycline	- (1.3 ±0.1)	**+(2.1 ±0.1)**	- (1.5 ±0.3)	- (1.1 ±0.1)	- (1.2 ±0.2)
Fusidic Acid	- (0.7 ±0.1)	**+(3.1 ±0.4)**	- (0.8 ±0.1)	- (0.9 ±0.1)	- (1.2 ±0.2)
Rifampicin	- (1.0 ±0.1)	- (1.0 ±0.1)	**+(2.7 ±0.2)**	- (1.0 ±0.1)	- (0.9 ±0.1)
Rifamycin SV	- (1.3 ±0.1)	- (0.9 ±0.1)	**+(2.3 ±0.1)**	- (1.2 ±0.1)	- (1.2 ±0.2)
Ciprofloxacin	**+(1.8 ±0.4)**	- (1.0 ±0.1)	- (1.1 ±0.2)	**+(74.9 ±5.6)**	- (1.7 ±0.2)
Trimethoprim	- (1.1 ±0.1)	- (0.8 ±0.1)	- (1.3 ±0.3)	**+(2.7 ±0.1)**	- (1.2 ±0.4)
Triclosan	- (1.2 ±0.2)	- (0.6 ±0.1)	- (1.2 ±0.1)	- (1.4 ±0.4)	**+(7.8 ±1.7)**
Myxopyronin B	- (0.9 ±0.1)	- (1.5 ±0.1)	**+(6.0 ±0.4)**	- (1.4 ±0.1)	- (1.3 ±0.3)
Corralopyronin A	- (1.0 ±0.1)	- (1.3 ±0.3)	**+(8.4 ±1.0)**	- (1.7 ±0.2)	**+(2.9 ±0.4)**
Chromomycin	- (0.6 ±0.2)	- (1.2 ±0.1)	- (1.0 ±0.2)	**+(4.1 ±0.8)**	- (1.4 ±0.1)
Holomycin	- (1.0 ±0.1)	- (1.0 ±0.1)	- (1.1 ±0.1)	- (1.0 ±0.2)	- (1.0 ±0.1)
Thiolutin	- (1.1 ±0.1)	- (1.0 ±0.2)	- (1.3 ±0.3)	- (1.1 ±0.1)	- (1.0 ±0.1)

*Table 5.3 - * Reporter induction (+) or no induction (-). Threshold set at published values (Fischer et al., 2004; Urban et al., 2007), normalised against untreated samples. (Maximum reporter signal ± standard deviation). Figures in bold represent signals above the published threshold for induction of the respective biosensor.*

The biosensors were unable to confirm that any of the five cellular biosynthetic pathways were inhibited by holomycin or thiolutin. This appears to indicate that these agents are unlikely to be inhibitors of bacterial RNAP. However, as stated above previous macromolecular synthesis assays demonstrate that the bacteriostatic activity of holomycin is associated with rapid inhibition of incorporation of [^{3}H] uridine into RNA (Oliva *et al.*, 2001). Therefore, further analysis of the MOA of holomycin and thiolutin is needed to confirm or refute their potential for clinical development as inhibitors of bacterial RNAP. This might be achieved by generation of resistant *S. aureus* mutants and sequence determination of the RNAP subunit genes to identify possible mutations that are responsible for a thiolutin resistance phenotype (Section 5.3.4.2) (O'Neill & Chopra, 2004a).

5.3.4 Resistance analysis of putative RNAP inhibitors in *S. aureus* SH1000

5.3.4.1 Selection and characterisation of CorA resistant *S. aureus* mutants

Rifampicin resistance may arise through substitution of any but one of the twelve amino acids in the β' subunit of RNAP that are known to be involved with binding rifampicin (Villain-Guillot *et al.*, 2007a). Mutations which confer resistance to MyxB and CorA in *E. coli* have previously been selected by random mutagenesis of *rpoB* and *rpoC* (genes encoding the β and β' subunits of RNAP respectively) (Mukhopadhyay *et al.*, 2008). However, both the rate at which resistance arises to CorA and MyxB exposure, and the nature of any mutants emerging, have not been determined. Therefore, this study attempted to generate and characterise spontaneous mutants of *S. aureus* displaying CorA resistance in parallel with a similar study with MyxB, carried out by T. Moy,

Cubist Pharmaceuticals, U.S.A (Moy *et al*, 2011). Eight spontaneous mutants with high-level resistance to CorA were recovered following plating of *S. aureus* SH1000 on agar containing 4X MIC of CorA (Table 5.4). The frequency of mutation for resistance to CorA in *S. aureus* SH1000 at a selective concentration of 4X MIC was 7X 10^{-8} (number of mutants per total number of viable bacteria) which was comparable with that of rifampicin at 1X 10^{-7} and MyxB at 8X 10^{-8} (Moy *et al.*, 2011). Mutation frequencies between 10^{-6} and 10^{-9} usually indicate mutation of a single target (O'Neill & Chopra, 2004a; Silver, 2011), such as those point mutations in *rpoB* that confer rifampicin resistance (O'Neill *et al.*, 2006).

The *S. aureus* CorA mutants exhibited only a slight loss of fitness compared to strain SH1000 when mean generation times were measured (Table 5.4), suggesting that CorA

Table 5.4 - Generation of corallopyronin resistant *S. aureus* SH1000 mutants (COR1-8) and comparative strain fitness*

S. aureus strain	CorA MIC (µg/ml)	Generation time (min)
SH1000	2	37.2 ±3.1
COR1	>128	45.3 ±3.1
COR2	64	46.9 ±3.6
COR3	>128	41.1 ±4.9
COR4	>128	42.7 ±2.2
COR5	64	36.7 ±3.7
COR6	>128	38.1 ±4.4
COR7	128	44.5 ±8.0
COR8	64	47.8 ±1.1

** CorA resistant S. aureus mutant generation and CorA susceptibility analysis performed by C. Smith, University of Leeds*

mutants arising in the clinical setting might not suffer a competitive disadvantage compared with CorA sensitive strains. The rapid rate of generation of resistance in *S. aureus* to rifampicin makes it unsuitable for clinical monotherapy (Forrest & Tamura, 2010). CorA would also not be suitable for monotherapy, as it shows the same high propensity for selection of resistance as rifampicin, in addition to no fitness cost associated with the resistance phenotype. Rifampicin is highly effective in combination with third generation cephalosporins in the treatment of antibiotic-resistant *Streptococcus pneumoniae* (Loeffler, 1999) but since it shows broader spectrum of activity than CorA (i.e. activity against Gram-negative as well as Gram-positive bacteria), there is less likelihood of CorA being developed for similar use. Nonetheless, characterisation of the mutations responsible for the observed resistance to CorA may yield information on the MOA of the antibiotic, and also aid in the generation of CorA derivatives with improved features, which could be future antibiotic candidates.

In an attempt to further characterise the MOA of CorA, the CorA resistant *S. aureus* mutants were subjected to cross-resistance analyses to identify other agents to which these strains might display resistance. Identifying such agents may help to confirm the target site of the antibacterial agent and can indicate which antibiotic classes retain activity against the resistant mutants (O'Neill & Chopra, 2004a). A good example of well-characterised cross-resistance is the MLS genotype, which encodes resistance to macrolides, lincosamides and steptogramin B antibiotics (Leclercq, 2002), which all interact specifically with the 23S ribosomal rRNA between nucleotides 2058-2062 (Walsh, 2003). The cross-resistance analysis of the CorA mutants to antibacterial agents with well established MOAs was determined (Table 5.5).

Table 5.5 – Susceptibility of CorA resistant *S. aureus* mutants to antibacterial agents with well established modes of action

Antimicrobial agent	Minimum inhibitory concentration (µg/ml)								
	S. aureus SH1000	*S. aureus* COR1	*S. aureus* COR2	*S. aureus* COR3	*S. aureus* COR4	*S. aureus* COR5	*S. aureus* COR6	*S. aureus* COR7	*S. aureus* COR8
CorA	2	>128	64	>128	>128	64	>128	128	64
MyxB	2	>128	>128	>128	>128	>128	>128	128	>128
Rifampicin	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.015
Rifamycin SV	0.125	0.125	0.125	0.0625	0.125	0.125	0.125	0.125	0.125
Holomycin	4	4	4	2	2	4	2	4	4
Thiolutin	2	2	4	2	2	2	2	4	2
Flucloxacillin	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Tetracycline	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Ciprofloxacin	1	1	1	1	1	1	1	1	1
Gentamicin	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.125	0.25
Erythromycin	0.5	0.5	0.5	0.25	0.5	0.5	0.5	0.5	1
Vancomycin	2	2	2	2	2	2	2	2	2
Daptomycin	2	2	2	2	1	2	2	2	2
Mupirocin	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.0625	0.125	0.0625
Triclosan	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125

The CorA-resistant *S. aureus* mutants did not display cross-resistance to any of the agents with established MOAs, including rifampicin, suggesting a unique antibacterial target for CorA (Table 5.5). However, cross resistance was observed to MyxB, indicating in agreement with previous studies, that the CorA resistant mutants most likely possess mutations in the RNAP subunit gene to which CorA binds and that CorA inhibits RNAP in the same manner as MyxB (Mukhopadhyay *et al.*, 2008).

To confirm that the mutations responsible for the observed CorA resistance were located in RNAP, the genes encoding the RNAP α, β and β' subunits (*rpoA*, *rpoB* and *rpoC,* respectively) in the CorA mutants were subjected to polymerase chain reaction (PCR) amplification and DNA sequence determination as previously described (O'Neill *et al.*, 2006). In agreement with parallel studies with MyxB, mutations were identified in *rpoB* and *rpoC* (Table 5.6) (Moy *et al.*, 2011; Mukhopadhyay *et al.*, 2008). The mutations observed in COR2/COR8 and COR3/COR4 were identical, and in the case of COR1, COR3 and COR4 affected the same residue (Table 5.6). This may have resulted from clonal expansion from a common ancestor in the same overnight culture. COR5 and COR6 contained independent mutations. Mutations at the same location in *E. coli* RNAP as those observed in Table 5.6 in COR1, COR3, COR4, COR5 and COR7 (i.e. at S_{1322}, E_{1279} and L_{1326} of wild-type *E. coli rpoB*) and COR6 (i.e. K_{345} of wild-type *E. coli rpoC*) have also been shown to confer resistance to both Myx and CorA in *E. coli* (Mukhopadyay *et al.*, 2008). Similarly, spontaneous mutation of S1127 (β) to leucine and K334 (β') to asparagine were shown to confer high-level resistance to MyxB in *S. aureus* (Moy *et al.*, 2011).

Table 5.6 - Mutations responsible for CorA resistance in *S. aureus* SH1000

	CorA MIC (μg/ml)	Amino acid substitution (codon change)	
		β subunit	***β' subunit***
SH1000	2	-	-
COR1	>128	$S_{1127}P$ (TCA-CCA)	None
COR2	64	None	$L_{1165}R$ (CTT-CGT)
COR3	>128	$S_{1127}L$ (TCA-TTA)	None
COR4	>128	$S_{1127}L$ (TCA-TTA)	None
COR5	64	$E_{1084}K$ (GAA-AAA)	None
COR6	>128	None	$K_{334}N$ (AAA-AAC)
COR7	128	$L_{1131}F$ (TTG-TTT)	None
COR8	64	None	$L_{1165}R$ (CTT-CGT)

In order to rationalise how these amino acid substitutions in the β and β' subunits might give rise to CorA resistance, *In silico* docking of CorA into the previously characterised (Zsoldos *et al.*, 2006) MyxB binding site of *Thermus thermophilus* RNAP was performed using eHITS (M. McPhillie, School of Chemistry, University of Leeds). Subsequent ExPASy alignment (Gasteiger *et al.*, 2003) of the *S. aureus* and *T. thermophilus* RNAP subunit sequences by M. McPhillie permitted the location of the mutated residues in the *S. aureus* CorA resistant mutants. A *T. thermophilus* homology model was necessary for this analysis as a crystal structure of *S. aureus* RNAP is not available. The altered residues were all located directly within the characterised Myx binding site (Figure 5.3) (Belogurov *et al.*, 2009; Mukhopadhyay *et al.*, 2008). In addition, the substitutions responsible for CorA resistance in mutants

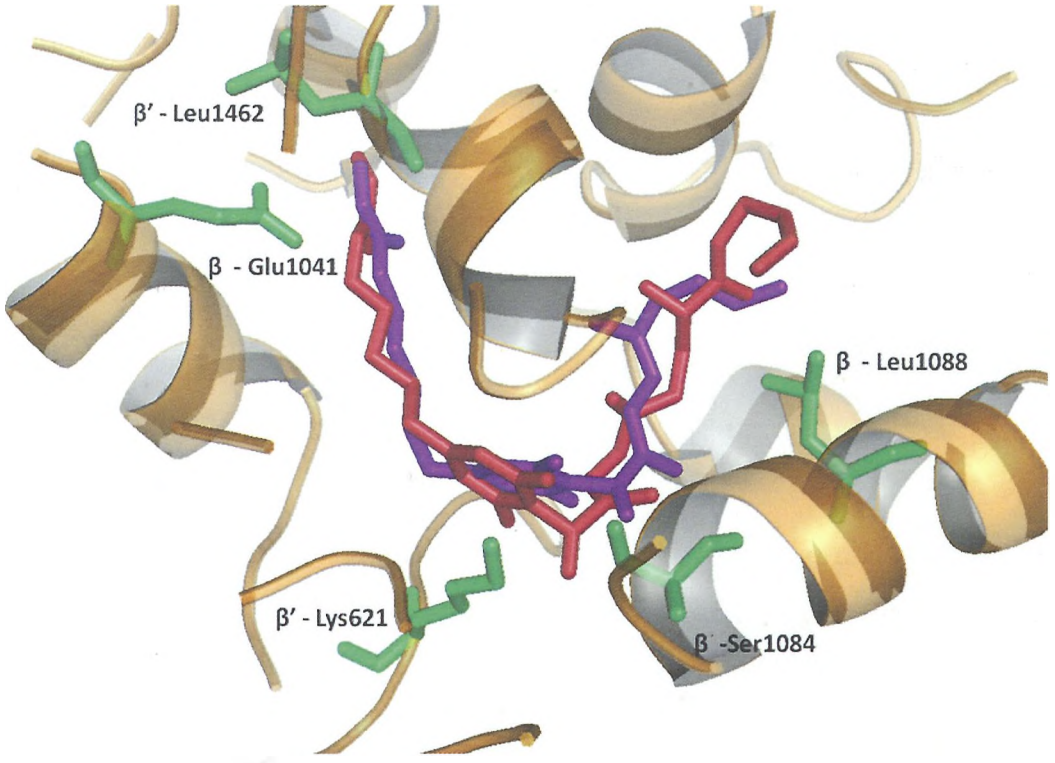


Figure 5.3 - Model of myxopyronin (purple) and CorA (red) bound to RNAP subunits, showing the proximity to the mutated residues in the RNAP of *S. aureus* mutants COR1-8. The residue numbers are those of *T. thermophilus* RNAP.

COR1-6 and COR8 involved residues which have previously been shown to interact directly with MyxB in the binding pocket via polar (COR1,3,4,5 and COR6) and van-der-waals (COR2 and COR8) forces (Moy *et al.*, 2011). Mutation of i) polar serine to non-polar and hydrophobic proline (COR1) or leucine (COR3 and COR4), ii) non-polar, hydrophobic leucine to positively charged arginine (COR2 and COR8) iii) negatively charged glutamate to positively charged lysine (COR5) and finally iv) positively charged lysine to non-charged asparagines would lead to loss of essential contacts between MyxB/CorA and the myxopyronin binding pocket, resulting in the observed resistance phenotype. With respect to the mutation of leucine 1131 to phenylalanine in COR7, it is currently unknown whether this residue interacts with CorA, however substitution of the alkyl side chain for a bulky aromatic constituent would potentially lead to steric hindrance to the binding of the antibiotic to RNAP.

The presence of amino acid substitutions in the β and β' subunits of RNAP in spontaneous CorA mutants strongly support the hypothesis that like MyxB, the MOA of CorA involves RNAP binding and inhibition of transcription. However, CorA displays inferior antibacterial activity in comparison with rifampicin (limited spectrum of activity) and exhibits a high propensity for selection of resistance. Similar observations have been made for MyxB (Moy *et al.*, 2011). In addition, previous studies have shown that although both MyxB and CorA show no acute toxicity in mice up to a concentration of 100 mg/kg, these concentrations were insufficient to clear experimental infections of *E. coli*, *S. aureus* and *Streptococcus pyogenes* (Irschik *et al.*, 1983; Irschik *et al.*, 1985). This is most likely due to high serum binding as observed for MyxB (Moy *et al.*, 2011). For the above reasons, it can be concluded that that CorA and MyxB are of limited interest for future clinical development, particularly for use in monotherapy. However, if derivatives of MyxB and CorA with improved characteristics can be developed, these may constitute viable future antibiotic candidates. For example, since the side chain at Carbon-3 of CorA is essential for antibacterial activity of CorA (Irschik *et al.*, 1985), modification of alternate sites of the structure may increase the binding affinity of the compound for RNAP (thus increasing overall susceptibility) and potentially overcome the ease with which resistance to CorA can be selected. Analogues of MyxB have already been synthesised (Doundoulakis *et al.*, 2004), but they did not exhibit improved antibacterial potency compared with the parent compound. Similarly, generation of inhibitors which also bind to the MyxB binding region of RNAP, but that exhibit improved potency compared to MyxB and CorA has been attempted. Using the *de novo* molecular design programme SPROUT, a series of novel inhibitors containing a substituted pyridyl-benzamide chemical scaffold were designed and synthesised (M. McPhillie, University of Leeds). Although a fraction of these compounds displayed

activity against the RNAP core enzyme *in vitro* (IC_{50} range 7.2 – 18.9 μM) (R. Trowbridge, personal communication) none displayed antibacterial activity up to 256 μg/ml.

5.3.4.2 Selection and characterisation of thiolutin resistant *S. aureus* mutants

A small number of previous studies have reported that dithiolopyrrolone antibiotics such as holomycin and thiolutin directly interfere with RNA synthesis (Jimenez *et al.*, 1973; Khachatourians & Tipper, 1974), although specific inhibition of RNAP *in vitro* has not been observed (Li & Walsh, 2011; Oliva *et al.*, 2001). The results from the *B. subtilis* biosensors (Table 5.3) also cannot substantiate inhibition of RNAP, and therefore thiolutin resistant *S. aureus* mutants were generated in an attempt to characterise the antibacterial target by identification of mutated loci. Thiolutin was chosen due to its slightly greater activity against *S. aureus* in comparison with holomycin (Table 5.1). In addition there are limited reports of resistance to thiolutin (Sivasubramanian & Jayaraman, 1980). Thiolutin resistance in *E. coli* maps to two loci, designated *tlnA* and *tlnB* (Sivasubramanian & Jayaraman, 1980). Mutation at these sites is thought to result in failure of the mutants to oxidise thiolutin from the pro-drug to the active form (Juhl & Clark, 1990). Thiolutin-resistant mutants of *Salmonella* Typhimurium have also been generated, although the mutations responsible for resistance were not characterised (Joshi *et al.*, 1982). Characterisation of mutations responsible for thiolutin resistance in *S. aureus* has not yet been achieved, and direct correlation of the thiolutin resistance phenotype to possible mutations in RNAP have also not yet been reported.

A spontaneous mutant (THIO1) with eightfold reduction in susceptibility to thiolutin (Table 5.7) arose following plating of a 10X concentrated culture of *S. aureus* SH1000 on agar containing thiolutin at 4X MIC. The frequency of mutation for resistance in *S. aureus* SH1000 was 2X 10^{-6}, which was higher than that of rifampicin at 1X 10^{-7} and that previously reported for thiolutin in *S.* Typhimurium at 3X 10^{-7} (Joshi *et al.,* 1982). The difference may suggest that mutations conferring thiolutin resistance arise more readily in *S. aureus* than in *S.* Typhimurium, and such a high mutation frequency would indicate mutation of a single target (Silver, 2011). The generation time of THIO1 was also only reduced by six minutes in comparison with *S. aureus* SH1000 suggesting that like the CorA resistant mutants, clinical thiolutin mutants if they arose would not suffer a competitive disadvantage compared with thiolutin sensitive strains. The rapid rate of generation of resistance in *S. aureus* to thiolutin, in addition to no observable biological cost associated with the resistance phenotype, could make thiolutin unsuitable for clinical monotherapy (Forrest & Tamura, 2010). However, characterisation of the mutation(s) responsible for the observed thiolutin resistance may yield vital information on the MOA of the antibiotic and whether it inhibits transcription.

To characterise the MOA of thiolutin further, THIO1 was subjected to cross-resistance analyses to identify other agents to which it might display resistance, giving further

Table 5.7 - Generation of a thiolutin resistant *S. aureus* SH1000 mutant and comparative strain fitness

S. aureus strain	Thiolutin MIC (µg/ml)	Generation time (min)
SH1000	2	37.2 ±3.1
THIO1	16	43.5 ±4.3

Table 5.8 - Cross resistance of CorA resistant *S. aureus* mutants to antibacterial agents with well established modes of action

Antimicrobial Agent	Minimum inhibitory concentration (μg/ml)	
	***S. aureus* SH1000**	***S. aureus* THIO1**
Thiolutin	2	16
Holomycin	4	8
MyxB	2	2
Rifampicin	0.015	0.015
Rifamycin SV	0.125	0.125
Flucloxacillin	0.25	0.25
Tetracycline	0.5	0.5
Ciprofloxacin	1	1
Gentamicin	0.25	0.25
Erythromycin	0.5	0.5
Vancomycin	2	2
Daptomycin	2	2
Mupirocin	0.0625	0.0625
Triclosan	0.125	0.125

evidence of a potential binding site of the inhibitor, and cellular mechanism of inhibition. Table 5.8 displays the cross resistance analysis of THIO1 to antibacterial agents with well established MOAs. As shown in Table 5.8, cross resistance of THIO1 was observed to the structurally similar antibiotic holomycin, but no cross-resistance was observed with any of the other agents with established MOAs, including rifampicin, rifamycin SV and MyxB, suggesting a unique antibacterial target for thiolutin/holomycin. Lack of cross-resistance of rifampicin resistant *S. aureus* mutants to both holomycin and thiolutin has previously been reported (O'Neill *et al.*, 2000), in

addition to absence of cross-resistance of my CorA resistant *S. aureus* mutants to these agents (Table 5.5). These results do not rule out holomycin or thiolutin as inhibitors of bacterial transcription, but suggest that if RNAP is the antibacterial target of dithiolopyrrolones, the target site is independent of the rifamycin and myxopyronin binding sites. As such, these agents may be indicated for infections caused by rifampicin resistant *S. aureus* strains.

To examine whether the mutations responsible for the observed reduction in thiolutin susceptibility might be located in RNAP, the genes encoding *rpoA*, *rpoB* and *rpoC* in THIO1 were amplified using PCR and the DNA sequence determined. No mutations were identified in any of the RNAP subunit genes, suggesting that RNAP is not the target of dithiolopyrrolones, and therefore these agents may be discounted for clinical development as inhibitors of bacterial RNAP.

5.4 Conclusions

RNAP remains an attractive target for novel antibiotics which might be developed to target other regions on the enzyme distinct from the rifamycin binding site to overcome existing resistance mechanisms. Therefore, we sought to analyse a set of previously reported, but poorly characterised RNAP inhibitors, determining their antibacterial spectrum of activity, bacterial specificity, MOA and propensity to develop resistance in *S. aureus.* It was not possible to link the observed antibacterial activity of Rose Bengal, daunorubicin, doxorubicin, juglone, ripostatin A (hemiacetal isomer), 8-HQ, holomycin, thiolutin and chromomycin to direct inhibition of RNA synthesis in whole bacterial cells, and therefore, these agents are considered to be unsuitable for further development

as clinical RNAP inhibitors. In addition, although CorA and MyxB induced the *B. subtilis* biosensor responsive to RNA synthesis inhibition, supporting the hypothesis that α-pyrone antibiotics inhibit bacterial RNAP, the narrow antibacterial spectrum of CorA and its relatively high propensity for selection of resistance suggest that it is not a promising antibacterial drug candidate, and may serve better as a starting scaffold for future drug development.

Chapter 6 – Characterisation of compounds with unknown mode of action (MOA)

6.1. Abstract

There is an increasing necessity for the discovery and development of novel antibacterial agents to combat the increasing incidence of antibiotic resistance in bacteria. However, any observed antibacterial activity in lead molecules must be associated with inhibition of a particular bacterial target (as non-specific antibacterial activity is often linked to toxic side-effects in humans) in addition to possessing low or no activity against mammalian homologues. Bacterial RNA polymerase (RNAP) and cell wall biosynthesis remain attractive antibacterial targets due to high conservation of the former across bacterial genera, and the unique nature of peptidoglycan (PG). In an attempt to identify putative inhibitors of bacterial RNAP, D-alanine: D-alanine ligase (Ddl) and PG glycosyltransferases (GTs), approximately half a million compounds were screened using virtual high-throughput methods to identify inhibitors. Twenty three *in vitro* inhibitors of bacterial RNAP were identified, but only two of these (Tocris 1610 and 2176) displayed good antibacterial activity, no membrane damage and no cross-activity against eukaryotes. However, it was not possible to link the antibacterial activity of these inhibitors to specific inhibition of RNAP, and therefore they were discarded. The lack of new antibacterial agents with specific bacterial targets for potential clinical development observed in this study, is representative of a wider problem, and highlights the many challenges which currently face large pharmaceutical companies in antibacterial drug discovery and development. Nevertheless, although inhibitors with specific activity against their target enzyme were not identified, a number of non-specific inhibitors were discovered (Tocris 1700, 1838, 2611 and furanyl-rhodanine L339571), that displayed membrane damage but greater than tenfold

higher activity against prokaryotes versus eukaryotes. These compounds might be developed as bacterial membrane damagers for persistent bacterial infections, such as those involving a biofilm.

6.2. Introduction

The increasing prevalence of multi-drug resistant strains of bacteria has highlighted the need for antibacterial agents with novel chemical scaffolds, inhibiting targets which are distinct from those of any drugs in current clinical use (Payne *et al.*, 2007). High-throughput screening of compound libraries against isolated biochemical targets and more recently using *in silico* methods remain popular techniques for the identification of potential antibacterial inhibitors (See Section 1.4).

6.2.1 Putative inhibitors of bacterial RNAP

Antibiotics of the rifamycin group are currently the only clinically available inhibitors of bacterial RNA polymerase (RNAP). Therefore there is a niche for the exploitation of this target in the discovery and development of novel antibacterial agents. Furanyl-rhodanines and a selection of compounds from the Tocris compound library have been identified as inhibitors of the RNAP core enzyme *in vitro*, and have therefore been chosen for further characterisation of their potential for chemotherapeutic development as inhibitors of RNAP at the cellular level.

6.2.1.1 The Tocris compound set

Since the first isolation of the RNAP core enzyme in 1962 (Chamberlin & Berg, 1962), further studies have gathered biochemical and structural data on this essential enzyme (Darst, 2001). Currently, the highest resolution crystal structure of the core enzyme of *Thermus aquaticus* is solved to 3.3Å, allowing detailed investigations on potential inhibitor binding sites (Campbell *et al.*, 2001). The α-pyrone antibiotic Myxopyronin B

(MyxB) has previously been co-crystalised with RNAP, and sits directly within the so-called 'switch region'; a hinge which mediates clamping of the β and β' subunits of RNAP onto a DNA template (Figure 6.1) (Mukhopadhyay *et al.*, 2008). MyxB and its structural analogue CorA have poor characteristics in terms of their potential for clinical development (Chapter 5). However, the switch region has been fully characterised in *Escherichia coli* (Mukhopadhyay *et al.*, 2008), and therefore represents an excellent binding pocket into which inhibitors may be designed by computational methods, or for screening of chemical libraries.

As part of an initial screening program, 491,200 compounds had been selected from a variety of chemical compound libraries were screened (A. Agarwal, University of Leeds), using the virtual high-throughput screening program eHiTS (Zsoldos *et al.*, 2006), against the myxopyronin binding region according to the myxopyronin/RNAP co-crystal structure. From this, 2071 compounds were selected for their high predicted

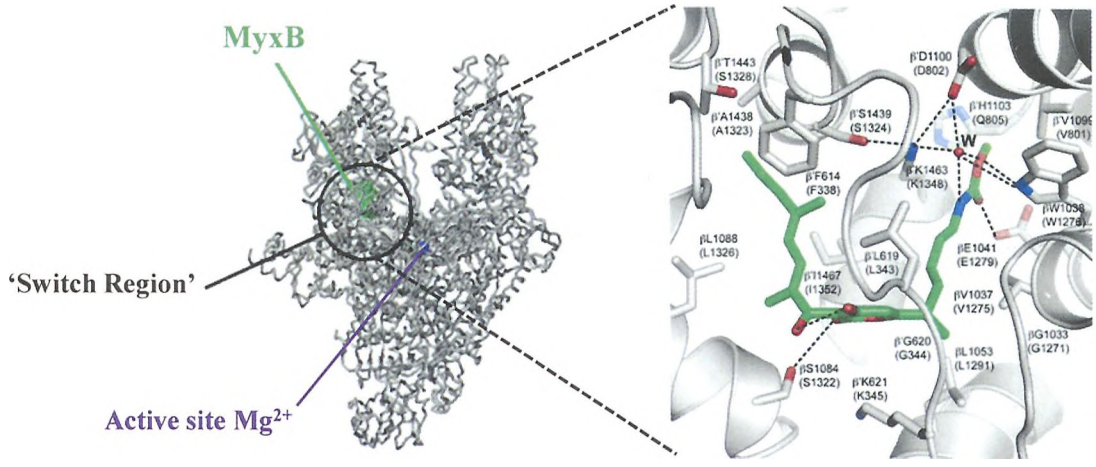


Figure 6.1 – Crystal Structures of the β and β' subunits of RNAP: *Shows the binding region of MyxB (left) and residues at the surface of the MyxB binding pocket (right). Residues are numbered both as in Thermus thermophilus RNAP and E. coli RNAP. Adapted from Mukhopadhyay et al., 2008*

binding affinities and tested against the *E. coli* RNAP core enzyme *in vitro* (R. Trowbridge, personal communication), using the Kool NC-45 RNAP activity and inhibitor screening kit (Epicentre, Madison, WI, USA). From the data obtained, 21 compounds from the Tocris Bioscience Ltd. compound database were chosen for microbiological analysis, on the basis that they displayed >50% inhibition of RNAP at 100 μM.

6.2.1.2 Furanyl-rhodanines

Synthetic chemical entities containing a furanyl-rhodanine core structure (Figure 6.2) were reported as specific inhibitors of bacterial RNAP based upon their ability to dissociate the core enzyme-σ^{70} complex, and to inhibit transcription at micromolar concentrations. They displayed antibacterial activity against *Staphylococcus epidermidis* (in planktonic cultures and biofilms) and a TolC-deficient mutant of *E. coli* without affecting growth of eukaryotic cells (e.g. Chinese hamster ovarian cells and *Candida albicans*) (Villain-Guillot *et al.*, 2007a). The most potent of this series, compound VG2 (Appendix 1), reportedly inhibited transcription by 98% at 10 μM, and displayed MICs against *S. epidermidis* CIP 105777 and *E. coli* TolC of 3.13 μg/ml and 12.5 μg/ml respectively (Villain-Guillot *et al.*, 2007b).

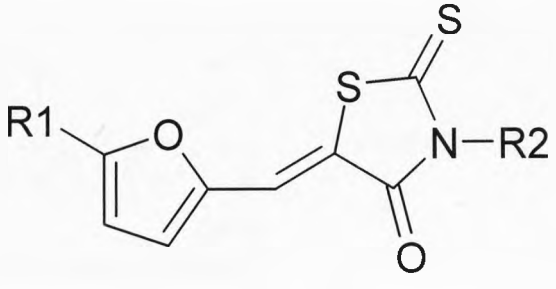


Figure 6.2 – Furanyl-rhodanine core structure

Compound VG2 was evaluated in more detail in terms of its microbiological activity and suitability for clinical development as an RNAP inhibitor. The activity of the structurally similar rhodanine compound L339571 (Appendix 1) was also assessed. Inhibition of *E. coli* RNAP *in vitro* by the rhodanines was confirmed as described for the Tocris compound set.

6.2.2 Putative inhibitors of bacterial D-alanine: D-alanine ligase (Ddl)

Peptidoglycan (PG) biosynthesis remains an attractive target for novel antimicrobial agents due to the prokaryotic-specific nature of PG and the enzymes which mediate its biosynthesis, and to the essential role played by the cell wall in the virulence and survival of bacteria (Bugg *et al.*, 2011). Ddl is an intracellular enzyme involved in the early stages of PG biosynthesis and is responsible for supplying the terminal D-alanine: D-alanine (D-ala: D-ala) dipeptide to the PG precursor (Figure 6.3) (Barreteau *et al.*,

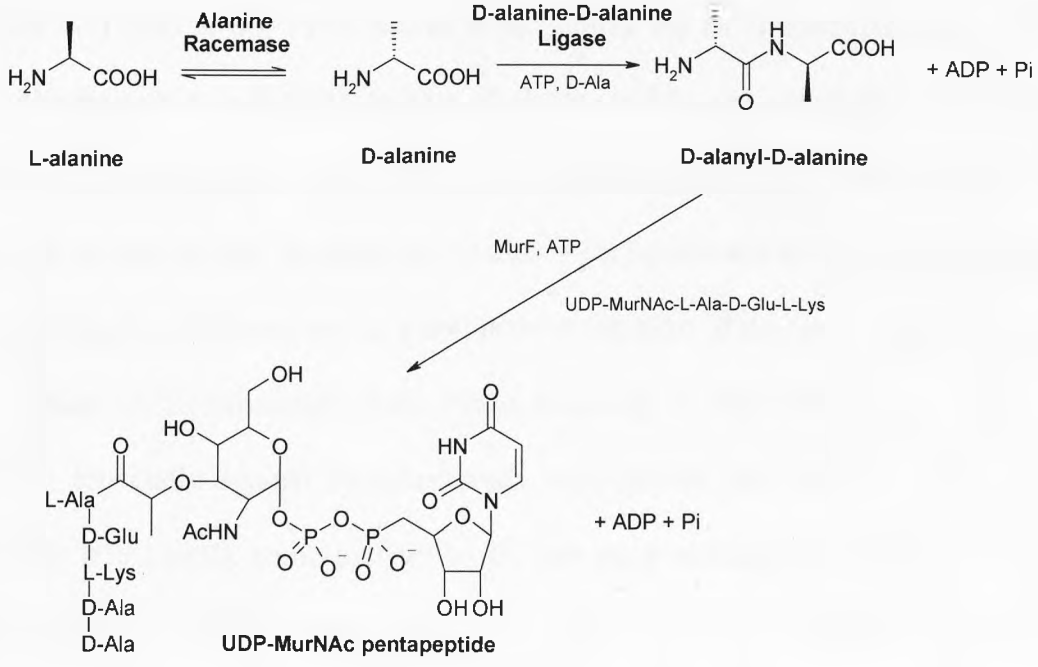


Figure 6.3 – Synthesis of the PG precursor, utilising D-alanine: D-alanine ligase

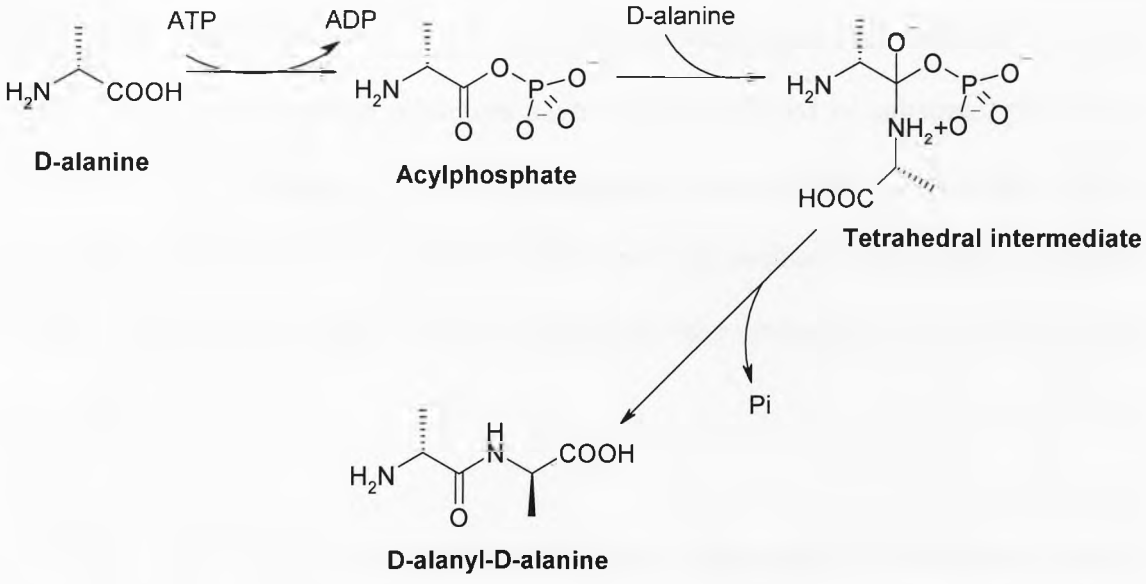


Figure 6.4 – Reaction scheme of the catalysis of D-ala: D-ala ligase. Adapted from Kovac *et al.* (2008)

2008). A member of the family of bacterial peptide synthases, Ddl utilises adenosine triphosphate (ATP) to form an amide bond between the carboxylate and amine moieties of two D-ala residues in a two-step reaction involving D-alanyl-phosphate intermediate (Figure 6.4) (Fan *et al.*, 1995; Kovac *et al.*, 2008). As an antibacterial target, Ddl is attractive as both its principal substrate, D-alanine and its primary product are unique to bacteria (Van Bambeke *et al.*, 1999). D-cycloserine represents the only clinically used inhibitor of both alanine racemase and D-ala: D-ala ligases and (as a structural analogue of the D-alanine substrate) acts as a competitive inhibitor of the latter enzyme (Lambert & Neuhaus, 1972; Strominger *et al.*, 1960). However, its use is limited to combination therapy, especially against *Mycobacterium tuberculosis* due to low activity (MICs generally ≥50 μg/ml), some toxicity issues and rapid resistance development in other species (David, 2001; Lu *et al.*, 2008). As such, its use has diminished in favour of antibiotics with improved properties (Rattan *et al.*, 1998). However, the elucidation of

seven x-ray crystal structures of Ddl enzymes, giving detailed information regarding the essential residues within the active site, has allowed the design of new inhibitors (Tytgat *et al.,* 2009). To date, these inhibitors have largely consisted of substrate, product or transition state analogues, or *in silico* designed novel scaffolds. However, a direct correlation between *in vitro* enzyme inhibition and antibacterial activity has been lacking, and therefore Ddl remains an underexploited antibacterial target, with great potential.

Previously, virtual high-throughput screening was employed using Autodock 4.0 for 1990 compounds from the National Cancer Institute (NCI) database, to dock a range of potential inhibitors into the active site of *Escherichia coli* DdlB (one of two Ddl enzymes in *E. coli*) (Kovac *et al.*, 2008). Of the 130 top-ranked compounds (selected by calculated binding affinities), two displayed *in vitro* IC_{50}s against the enzyme of <250 μM which was associated with MICs against *Staphylococcus aureus* 8325-4 of 32 μg/ml. Similarity searches of these two inhibitor scaffolds identified four additional potential DdlB inhibitors designated 155693, 155694, 352738 and 627505 (Appendix 1), which were selected for further microbiological characterisation (S. Gobec, University of Ljubljana, Slovenia). Preliminary *in vitro* enzyme inhibition data identified percentage enzyme inhibition values of 97%, 98%, 61% and 97% at 500 μM respectively (A. Kovac, University of Ljubljana, Slovenia). However, it is crucial that the observed *in vitro* enzyme activity is correlated with antibacterial activity and specific inhibition of DdlB *in vivo* for the future development of these compounds as potential chemotherapeutic candidates.

6.2.3 Putative inhibitors of bacterial PG-GTs

The ultimate stages of PG biosynthesis are mediated by the bifunctional penicillin binding proteins (PBPs), a family of essential enzymes involved in the PG biosynthesis pathway, catalysing the addition of the PG precursor (Lipid-II) into the nascent cell wall polymer via the glycosyltransferase (GT) domain, followed by cross-linkage of the pentapeptide side chains, catalysed by the transpeptidase (TP) domain (See Figure 1.3) (Sauvage *et al.*, 2008). These enzymes represent validated targets for the development of novel antibacterial agents, as the TP domain is the target of β -lactams (Tomasz & Waks, 1975). The GT domain is currently only the target of the natural product phosphoglycolipid moenomycin (El-Abadla *et al.*, 1999) (Figure 6.5). However this antibiotic is not used clinically due to poor pharmacokinetic properties (Welzel, 2005), and therefore the GT domain remains an attractive but underexploited antibacterial target.

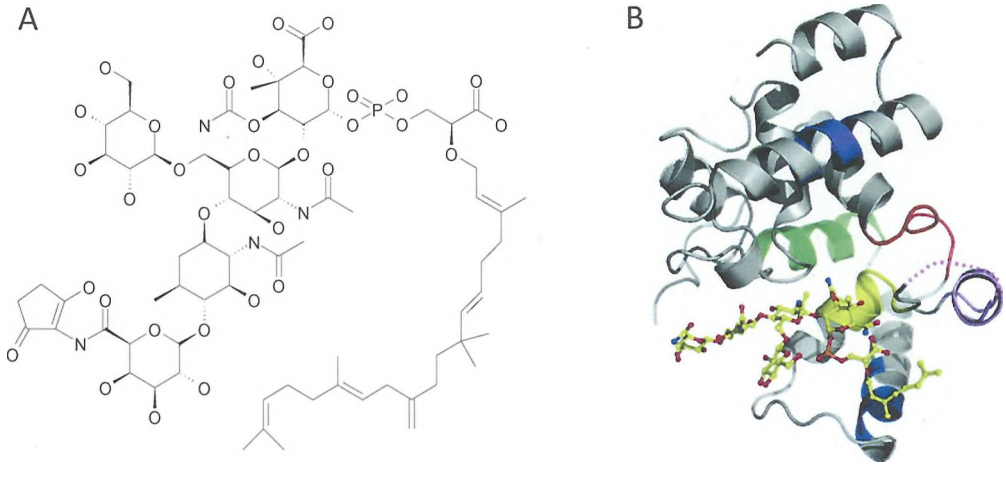


Figure 6.5 – Molecular structure of moenomycin A (A). Ribbon representation of moenomycin (ball and stick structure) co-crystalised with the GT domain of *Aquifex aeolicus* PBP1a (B) (coloured [non-grey] regions represent highly conserved residues). Adapted from Yuan *et al.*, 2008.

In recent years, moenomycin has been co-crystalised with various PBPs, allowing the extensive characterisation of the GT domain. This domain is composed of several conserved residues and two glutamate residues with essential catalytic activity (Heaslet *et al.*, 2009; Terrak *et al.*, 2008; Yuan *et al.*, 2008) (Figure 6.5). Virtual high-throughput screening using eHITS of the National Cancer Institute (NCI) database has been employed to dock potential inhibitors into the *S. aureus* PBP2 GT domain. From the initial screen, two inhibitors were identified, designated 5 and 5b (Appendix 1), which inhibited the activity of five GTs at micromolar concentrations (Derouaux *et al.*, 2011). Compounds 5 and 5b were chosen to be evaluated in an attempt to link the observed *in vitro* enzyme activity with cell wall biosynthesis inhibition at the cellular level and confirm their suitability for clinical development.

6.2.4 Aims and objectives of the work described in this chapter

The PG biosynthesis pathway and bacterial RNAP remain attractive targets for the design and synthesis of novel antibacterial agents. Separate virtual high-throughput screening programmes identified twenty-one compounds from the Tocris Bioscience Ltd database with inhibitory activity against *E. coli* RNAP, and six compounds from the NCI set, four with inhibitory activity against *E. coli* DdlB *in vitro* and two with inhibitory activity against various PBPs. However, there is currently no information regarding the antibacterial activity or cellular mechanism of action of these inhibitors. In addition, two putative inhibitors of bacterial transcription were identified from a series of furanyl-rhodanines, and require further microbiological characterisation to associate the observed antibacterial activity with inhibition of RNA synthesis at the

cellular level, and confirm their suitability for clinical development. Therefore, the aims of this chapter include:

- characterisation of the antimicrobial spectrum of activity of the RNAP, DdlB and PBP inhibitors;
- characterisation of the bacterial specificity of the agents, and effect on staphylococcal membranes;
- further analysis of the mode of action (MOA) of inhibitors which show desirable characteristics;
- determination of the potential for resistance development to potential lead molecules.

6.3. Results and Discussion

6.3.1 Putative inhibitors of bacterial RNAP – The Tocris compound set

6.3.1.1 Minimum inhibitory concentrations

The Tocris Bioscience Ltd. compound library consists of 1120 structurally unrelated biologically active compounds, but to date, no analysis of the antibacterial spectrum of activity of these compounds has been performed. To confirm the potential for clinical development as antibacterial agents, MIC determinations of twenty one compounds which displayed >50% inhibitory activity at 100 μM against the core RNAP enzyme *in vitro* were performed against the standard panel of bacterial strains (Table 6.1). Rifampicin and MyxB were analysed as comparator agents since these have established activity against RNAP.

The Tocris compounds generally displayed higher activity against the Gram-positive strains tested. However, Tocris compounds 723, 901, 985, 2002, 2160 and 2481 showed broad-spectrum antibacterial activity. The remaining fifteen Tocris compounds showed no activity against *E. coli* 1411, and therefore their narrow spectrum of activity would only make these compounds suitable for the treatment of infections caused by Gram-positive pathogens. For those compounds which were inactive against wild-type *E. coli*, only five of the fifteen (1547, 1610, 1867, 2176 and 2611) showed increased activity when the media was supplemented with polymyxin B nonapeptide (PMBN), indicating that these agents (like myxopyronin and rifampicin) are not substrates for the AcrAB-TolC efflux pump, but fail to penetrate the outer membranes of Gram-negative bacteria.

The Gram-negative outer membrane is an effective barrier to highly polar antibacterial molecules, and therefore the increased polarity of these compounds due to hydroxyl groups and halogenated side chains may account for this observation (Sahalan & Dixon, 2008). Two of the fifteen compounds showing inactivity against wild-type *E. coli* (1794 and 1979), only displayed increased activity (>2 fold) against *E. coli* SM1411 in

Table 6.1 –Spectrum of activity of the Tocris compounds in comparison with MyxB and rifampicin

Antibacterial compound	M_r	% Inhibition of RNAP (100μM)*	MIC (μg/ml)				
			S. aureus SH1000	*E. coli* 1411	*E. coli* 1411 + PMBN	*E. coli* SM1411	*B. subtilis* 1S34
Rifampicin	823.0	100.0 (at 3μM)	0.015	4	0.125	4	128
MyxB	431.2	78.8	2	>128	8	>128	32
378	433.4	66.1	16	>128	16	8	16
723	390.5	72.6	8	32	8	8	4
901	392.5	92.7	4	8	4	4	4
985	449.4	62.3	16	128	16	32	16
1547	310.4	83.2	16	>128	64	>128	8
1610	521.1	100.4	0.5	>128	4	>128	0.5
1700	987.1	101.4	32	>128	>128	>128	64
1794	565.1	64.8	64	>128	>128	64	64
1838	573.7	101.0	64	>128	>128	>128	32
1867	321.8	68.5	32	>128	128	>128	16
1979	599.6	72.3	32	>128	>128	64	64
2002	553.7	100.5	32	64	16	32	32
2160	558.1	98.3	16	16	16	16	16
2176	241.3	65.8	16	>128	128	>128	8
2199	512.1	100.1	16	>128	128	16	8
2252	580.0	74.2	32	>128	128	16	16
2481	448.0	102.5	8	16	16	16	8
2539	520.1	101.7	32	>128	32	32	32
2558	381.3	76.9	64	>128	32	32	64
2611	383.7	61.1	0.125	>128	0.5	>128	0.0625
2747	451.0	63.3	64	>128	32	32	32

* *Determined by R. Trowbridge, University of Leeds.*

comparison with *E. coli* 1411, indicating that these compounds are substrates for the AcrAB multi-drug efflux pump. Current studies have suggested that bacterial efflux pumps may recognise a variety of structural components in substrates for transport, however, molecules containing a hydrophilic head region attached to a hydrophobic moiety often constitute more likely candidates (Van Bambeke *et al.*, 2000). Tocris 1794 and to a lesser extent Tocris 1979, both have such structural elements, which may account for their apparent recognition by the AcrAB-TolC efflux pump. Of the remaining eight Tocris compounds, six displayed increased activity against both the AcrAB knockout *E. coli* mutant and the wild-type treated with PMBN (378, 2199, 2252, 2539, 2558 and 2747), indicating that in Gram-negative organisms, these compounds may be substrates for multi-drug efflux pumps, but may also be prevented from accessing their intracellular target by the presence of the outer membrane. Compounds 1700 and 1838 showed no increase in activity against PMBN-treated *E. coli* 1411, or *E. coli* SM1411, suggesting that they are not recognised by AcrAB-TolC or able to penetrate a permeabilised Gram-negative outer membrane. This is most likely due to the large size of the molecules and the relatively high number of bulky aromatic hydrocarbon moieties which constitute side chains in these molecules.

6.3.1.2 Determination of membrane damage and bacterial specificity

Determination of the ability of a novel antibacterial agent to cause membrane damage in *S. aureus* and also performing an MIC against *Saccharomyces cerevisiae* has been shown to be effective for excluding from further consideration a significant proportion of agents which may induce toxic side effects in mammals, and would therefore be unsuitable for clinical development (Chapter 3). These assays were performed on the

panel of Tocris compounds in an attempt to highlight compounds which should be disregarded from further preclinical evaluation (Table 6.2).

As shown in Table 6.2, with the exception of six inhibitors (985, 1547, 1610, 1867, 2002 and 2176), exposure of *S. aureus* SH1000 to all of the Tocris compounds caused a

Table 6.2 - Determination of membrane damage and bacterial specificity of the Tocris compounds

Condition	*S. aureus* MIC (µg/ml)	*Bac*Light™ result (% membrane integrity)	*S. cerevisiae* 464 MIC (µg/ml)
Drug-free control	-	100	-
5% SDS (w/v)	-	0	-
Rifampicin	0.015	80.8 ±6.1	>256
MyxB	2	96.8 ±5.5	>256
378	16	11.1 ±2.2	8
723	8	28.5 ±5.8	1
901	4	0	0.5
985	16	76.7 ±8.0	16
1547	16	121.4 ±2.6	32
1610	0.5	80.4 ±2.4	>128
1700	32	30.7 ±3.8	>128
1794	64	1.1 ±0.3	128
1838	64	11.0 ±3.3	>128
1867	32	108.4 ±3.2	64
1979	32	39.2 ±5.3	128
2002	32	55.8 ±4.9	64
2160	16	5.4 ±0.6	8
2176	16	124.3 ±9.1	>128
2199	16	47.3 ±3.5	128
2252	32	23.5 ±1.6	256
2481	8	38.6 ±3.1	16
2539	32	22.7 ±0.6	8
2558	64	12.4 ±2.7	4
2611	0.125	32.9 ±0.1	>128
2747	64	15.7 ±1.4	16

reduction in cell membrane integrity to <40% of the drug-free control. These compounds are therefore classed as membrane-damaging. Of these fifteen membrane-damaging compounds, twelve also displayed activity against *S. cerevisiae*, are likely to display cytotoxicity and were therefore not evaluated further. However, Tocris 1700, 1838 and 2611 did not display activity against *S. cerevisiae*, and therefore although they must be discarded as developmental inhibitors of RNAP, they may merit future development as membrane-damaging agents, possibly for persistent bacterial infections, such as those involving a bacterial biofilm (Hurdle *et al.*, 2011). It is worth noting that further work is required to evaluate the cytotoxicity of these compounds. Of the six Tocris compounds that do not damage membranes, only two (1610 and 2176) did not display any activity against *S. cerevisiae*. These two compounds were taken forward for further evaluation to attempt to link the observed antibacterial activity to inhibition of RNAP at the cellular level.

6.3.1.3 Time kill analysis of Tocris 2176 and Tocris 1610

As part of the mode of action analysis of the Tocris hit compounds, these compounds were analysed to determine whether they display bactericidal or bacteriostatic activity. Rifampicin and MyxB as established inhibitors of RNAP both display bactericidal activity (Martinez-Lacasa *et al.*, 2002; Mukhopadhyay *et al.*, 2008), and were therefore used as comparator agents (Figure 6.6).

Over a five hour incubation period, the viability of *S. aureus* SH1000 exposed to rifampicin and MyxB dropped by approximately 0.5 [Log_{10} (cfus/ml)] in each case, corresponding with a slow bactericidal activity, has been reported elsewhere (Martinez-

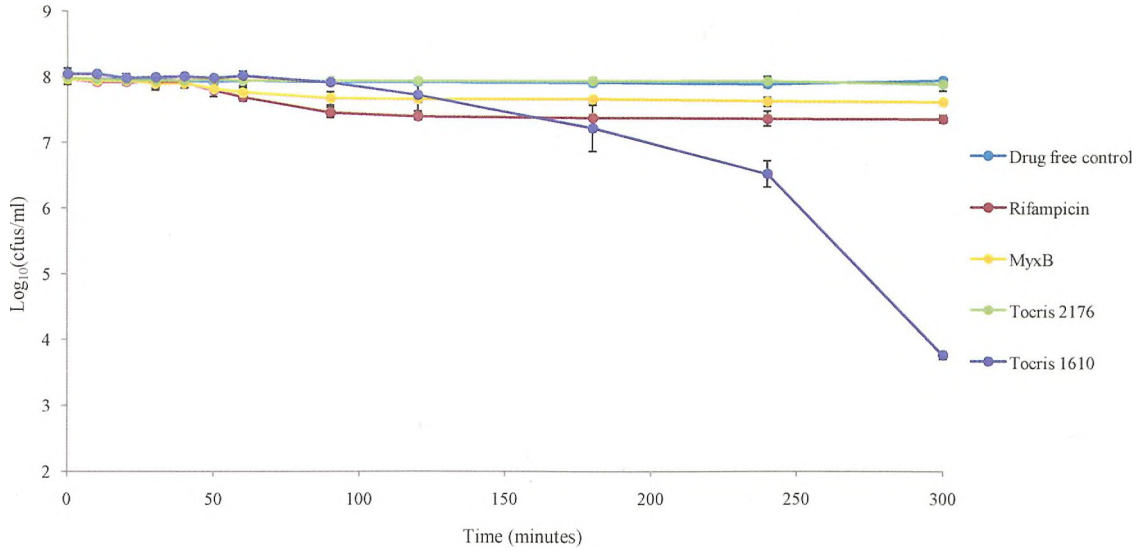


Figure 6.6 – Cell death kinetics of *S. aureus* SH1000 in response to the Tocris hit compounds and comparator agents. *Error bars represent the standard deviation from three replicates at each time point.*

Lacasa *et al.*, 2002; Mukhopadhyay *et al.*, 2008). Similarly, the log decrease in cfus/ml for Tocris 1610 was 4.3 over the five hour period, but an observable reduction in viability was not seen until after ninety minutes. This indicates that Tocris 1610 is a bactericidal antibiotic, but that this activity is preceded by a lag period. Tocris 2176 showed a bacteriostatic response against *S. aureus* SH1000, as dilution of the antibiotic in PBS and subsequent plating of the bacteria were sufficient to restore cell viability correlating with published data for other bacteriostatic inhibitors such as tetracycline (Hobbs *et al.*, 2008). This information may indicate that it is less likely that Tocris 2176 is an inhibitor of bacterial RNAP in whole cells, but additional assays are required to confirm this.

6.3.1.4 *Bacillus subtilis* antibiotic biosensors

In an attempt to evaluate the cellular mode of action of Tocris 1610 and 2176, the *B. subtilis* antibiotic biosensors (the utility of which in the preliminary screening of the MOA of antibacterial agents had been confirmed in Chapter 3), were utilized (Table 6.3). For inhibitors of RNAP, it was anticipated that these Tocris compounds might induce *vygS*, the biosensor responsive to inhibition of RNA synthesis. As shown in Table 6.3, the hit Tocris compounds did not induce any of the biosensors above.

Table 6.3 – *Bacillus subtilis* antibiotic biosensors for preliminary screening of the MOA of Tocris 1610 and 2176

Antimicrobial Agent	Upregulated biosynthetic pathway*				
	Cell-envelope	*Protein*	*RNA*	*DNA*	*Fatty-acid*
Vancomycin	**+(2.7 ±0.2)**	- (0.9 ±0.1)	- (1.1 ±0.1)	- (1.1 ±0.2)	- (1.1 ±0.1)
Flucloxacillin	**+(2.9 ±0.1)**	- (0.8 ±0.1)	- (1.1 ±0.3)	- (1.0 ±0.1)	- (1.1 ±0.1)
Nisin	- (1.6 ±0.1)	- (0.8 ±0.1)	- (1.1 ±0.2)	- (1.2 ±0.4)	- (0.9 ±0.1)
CTAB	- (1.0 ±0.1)	- (1.0 ±0.4)	- (1.0 ±0.2)	- (0.9 ±0.1)	- (1.3 ±0.5)
Tetracycline	- (1.3 ±0.1)	**+(2.1 ±0.1)**	- (1.5 ±0.3)	- (1.1 ±0.1)	- (1.2 ±0.2)
Fusidic Acid	- (0.7 ±0.1)	**+(3.1 ±0.4)**	- (0.8 ±0.1)	- (0.9 ±0.1)	- (1.2 ±0.2)
Rifampicin	- (1.0 ±0.1)	- (1.0 ±0.1)	**+(2.7 ±0.2)**	- (1.0 ±0.1)	- (0.9 ±0.1)
Rifamycin SV	- (1.3 ±0.1)	- (0.9 ±0.1)	**+(2.3 ±0.1)**	- (1.2 ±0.1)	- (1.2 ±0.2)
Myxopyronin B	- (0.9 ±0.1)	- (1.5 ±0.1)	**+(6.0 ±0.4)**	- (1.4 ±0.1)	- (1.3 ±0.3)
Ciprofloxacin	**+(1.8 ±0.4)**	- (1.0 ±0.1)	- (1.1 ±0.2)	**+(74.9 ±5.6)**	- (1.7 ±0.2)
Trimethoprim	- (1.1 ±0.1)	- (0.8 ±0.1)	- (1.3 ±0.3)	**+(2.7 ±0.1)**	- (1.2 ±0.4)
Triclosan	- (1.2 ±0.2)	- (0.6 ±0.1)	- (1.2 ±0.1)	- (1.4 ±0.4)	**+(7.8 ±1.7)**
Tocris 1610	- (0.9 ±0.1)	- (0.9 ±0.1)	- (1.0 ±0.1)	- (1.0 ±0.1)	- (1.0 ±0.5)
Tocris 2176	- (0.9 ±0.1)	- (1.0 ±0.1)	- (1.2 ±0.1)	- (0.9 ±0.2)	- (1.2 ±0.5)

*Table 6.3 - *Reporter induction (+) or no induction (-) (Maximum reporter signal ± standard deviation.) Figures in bold represent signals above the published threshold for induction of the respective biosensor.*

published thresholds (Urban *et al.*, 2007). This may suggest that these compounds do not specifically inhibit any of the cellular biosynthetic pathways tested. However, as previously described in this Chapter 3, the biosensors are limited to detecting only certain classes of bacterial inhibitors and therefore further analyses (i.e. macromolecular synthesis assays) were needed.

6.3.1.5 Macromolecular synthesis inhibition by Tocris 2176 and Tocris 1610

The inhibition of DNA, RNA and protein biosynthesis was monitored by following the incorporation of radiolabelled precursors into macromolecules following a brief exposure (ten minutes) to Tocris 1610 and Tocris 2176, relative to a drug-free control (Figure 6.7) (Cherrington *et al.*, 1990). Tetracycline, rifampicin, ciprofloxacin, vancomycin and nisin were included as positive and negative controls for the inhibition of protein, RNA, DNA and PG biosynthesis and membrane damage, respectively (Boaretti *et al.*, 1993; Henning *et al.*, 1986; Hobbs *et al.*, 2008).

Incubation of *S. aureus* SH1000 with 4X MIC of Tocris 1610 lead to 60-70% inhibition of all three synthetic pathways (Figure 6.7). The simultaneous inhibition of all three pathways is indicative of a non-specific MOA such as membrane damage or cessation of energy producing metabolic pathways (Ooi *et al.*, 2009a, Silver, 2011). Since it was not possible to detect membrane damage during ten minutes exposure by the BacLight™, it is more likely that the second inhibitory MOA applies in this case. There is evidence in the literature to suggest that Tocris 1610 (also known as rottlerin) is a potent inhibitor of protein kinases (enzymes involved in the regulation of a variety of cellular processes) which may account for the observed antibacterial activity of this

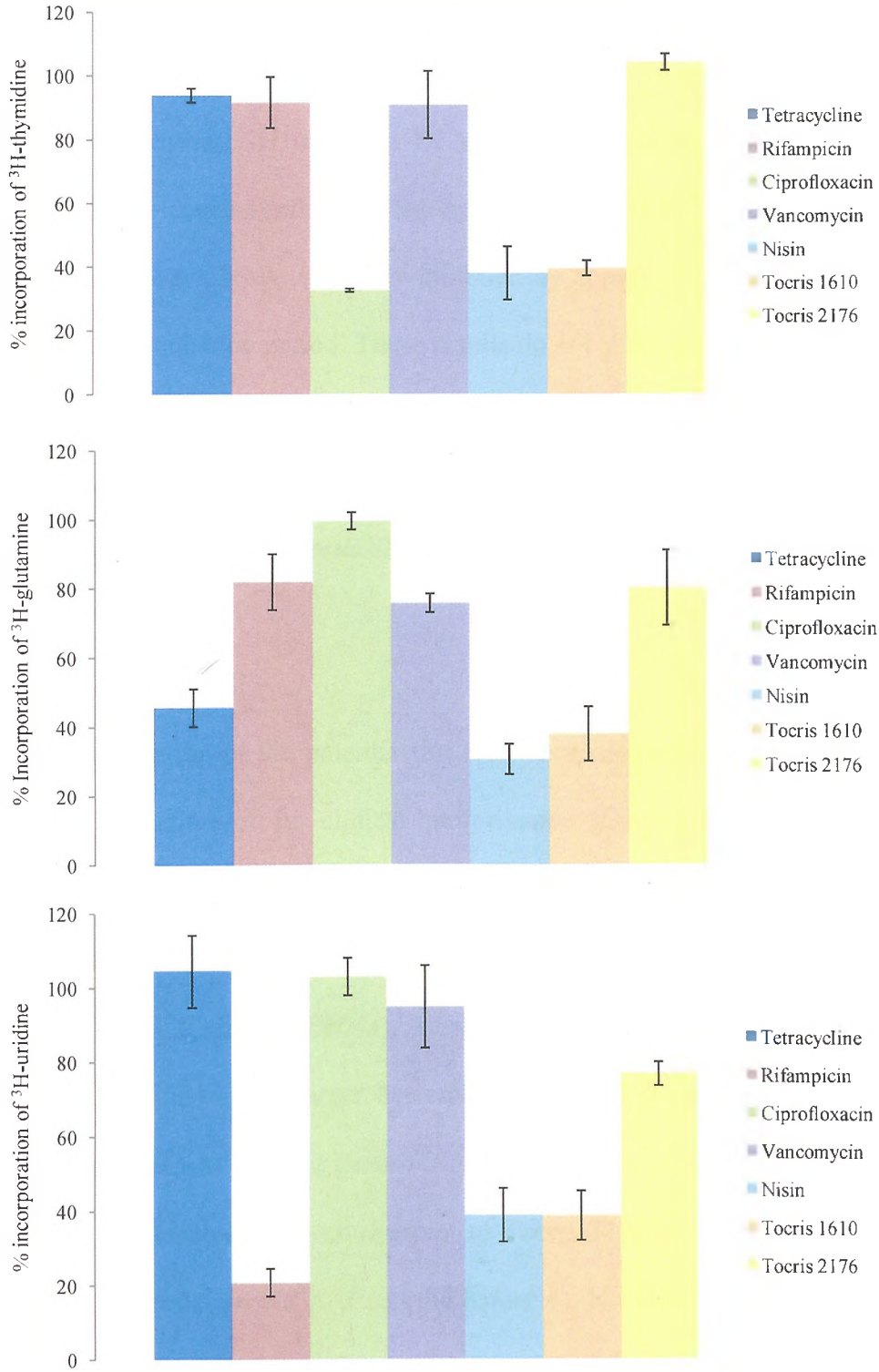


Figure 6.7 – Inhibition of macromolecular synthesis by 10 minutes exposure of *S. aureus* SH1000 to Tocris 1610 and Tocris 2176. *Error bars represent the standard deviation from three replicates.*

compound (Davies *et al.*, 2000; Gschwendt *et al.*, 1994). Nonetheless, Tocris 1610 may now be disregarded for development as an inhibitor of bacterial RNAP. Conversely, incubation of *S. aureus* SH1000 with 4X MIC of Tocris 2176 lead to minor effects on cellular macromolecular synthesis. DNA synthesis was not inhibited by this compound, and protein/RNA synthesis were only inhibited by approximately twenty percent over the ten minute incubation period. These results do not point to a single inhibitory target for Tocris 2176 and therefore more work is needed to characterise this compound fully.

6.3.1.6 Development of *S. aureus* SH1000 mutants displaying reduced susceptibility to Tocris 2176.

Preclinical evaluation of the potential for resistance development to Tocris 2176 may give early indication of its clinical performance (Gwynn *et al.*, 2010), but also characterisation of any mutants arising (e.g. cross-resistance analysis, genome sequence determination) can give useful data on the mutation site and therefore the likely target of the drug (O'Neill & Chopra, 2004a). As shown in Chapter 5, and in current literature, resistance to inhibitors that target bacterial RNAP tend to arise rapidly, due to point mutations in the RNAP subunit genes (O'Neill *et al.*, 2006). Therefore, I first attempted to generate spontaneous mutants resistant to Tocris 2176 by the same method as that employed for corallopyronin A (CorA) (Chapter 5). No mutants arose by this method, and therefore, the generation of resistant mutants by continuous subculture in the presence of sub-inhibitory concentrations of Tocris 2176 was attempted as described for the generation of mutants to deoxyactagardine B (DAB) (Chapter 4). After twenty passages, only a twofold reduction in susceptibility was observed. These results indicate that the potential for clinical resistance development to Tocris 2176 is low. However it

was not possible to ascertain the antibacterial target by this method, and as yet the MOA of action of Tocris 2176 remains uncharacterised. In addition, the results from all of the MOA analyses suggest that RNAP is unlikely to be the target of Tocris 2176. Therefore, since there is no link between *in vitro* enzyme activity and cellular MOA, this compound must be disregarded for clinical development as a bacterial RNAP inhibitor. Although there remains the potential for Tocris 2176 to be taken forward for development as a antibacterial agent with unknown MOA, a previous study has reported that this compound may also inhibit human protein tyrosine phosphatases (Liljebris *et al.*, 2004), and therefore evaluation of the cytoxicity of Tocris 2176 would be the most logical progression for its assessment.

6.3.2 Putative inhibitors of bacterial RNAP: The rhodanines

6.3.2.1 Minimum inhibitory concentration determinations

Furanyl-rhodanines have previously been reported to inhibit bacterial transcription, and possess antibacterial activity (Villain-Guillot *et al.*, 2007b). We sought to confirm the mode of action of two representative rhodanine compounds (VG2 and L339571) as inhibitors of RNAP in whole cells. The antibacterial spectrum of activity of the furanyl-rhodanines is shown in Table 6.4.

Compound VG2 lacked antibacterial activity against any of the bacterial strains tested. The inability of VG2 to inhibit the growth of wild-type *S. aureus* and *E. coli* is in accordance with previous studies, where the activity of VG2 was limited to a small number of Gram-positive species, including *S. epidermidis* and *Bacillus anthracis* (Villain-Guillot *et al.*, 2007b). The lack of activity of VG2 against *E. coli* 1411 treated

Table 6.4 – Antibacterial spectrum of activity of rhodanines

Antibacterial compound	M_r	IC_{50} for *E. coli* RNAP (μM)*	MIC (μg/ml)				
			S. aureus SH1000	*E. coli* 1411	*E. coli* 1411 + PMBN	*E. coli* SM1411	*B. subtilis* 1S34
Rifampicin	823.0	0.01 ±0.001	0.015	4	0.125	4	128
MyxB	431.2	46.5 ±5.9	2	>128	8	>128	32
VG2	407.9	37.3 ±3.9	>256	>256	>256	>256	>256
L339571	371.4	16.3 ±2.5	4	>128	16	>128	1

* *Performed by Rachel Trowbridge, University of Leeds*

with PMBN and the AcrAB deficient *E. coli* mutant suggest that the inactivity of this compound against Gram-negative species is not due to hindrance by the outer membrane or efflux by the AcrAB-TolC multi-drug transporter pump. Since previous work has identified that this compound is more active against a TolC knockout mutant than its corresponding wild-type strain (Villain-Guillot *et al.*, 2007b), this would suggest that VG2 is a substrate for efflux by another multi-drug efflux pump incorporating the TolC outer membrane component. The apparent narrow spectrum of activity of VG2, but particularly its lack of activity against *S. aureus* SH1000 prevented further study of this compound, and signifies that it would not constitute a viable candidate for clinical development.

Rhodanine L339571 displayed low MICs against the Gram-positive strains tested, but no activity against the wild-type *E. coli* strain. Activity was not improved in the AcrAB-deficient mutant strain, suggesting that L339571 is not a substrate for AcrAB-mediated efflux in Gram-negative organisms. However, the activity of this rhodanine against *E. coli* with a permeabilised outer membrane was improved, suggesting that in wild-type *E. coli*, penetration of this compound into the cell is poor. The selectivity of antibacterial activity displayed by this compound against Gram-positive species, agrees

with previous studies in which MICs of 25 μg/ml and >200 μg/ml against *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 respectively have been reported (Zervosen *et al.*, 2004).

6.3.2.2 Determination of membrane damage and bacterial specificity of rhodanines

Prior to further MOA analysis and determination of the effect of rhodanine L339571 on RNA synthesis in whole cells, the *Bac*Light™ assay was utilised to determine membrane damage in *S. aureus* SH1000 caused by L339571, and *S. cerevisiae* MIC determinations performed to ascertain whether this compounds is generally suitable for clinical development (Table 6.5). The membrane integrity of *S. aureus* SH1000 was reduced by over 70% when the cells were exposed to 4X MIC of L339571 for ten minutes (Table 6.5). Rhodanine L339571 has also been reported to inhibit PBP2a from meticillin-resistant *S. aureus* MRSA (Zervosen *et al.*, 2004). Similarly, the inhibitory activities of L339571 at 100 μM have also been determined for bovine chymotrypsin and porcine malate dehydrogenase, using previously described methodologies (Siedler *et al.*, 2003). This compound inhibited the activity of both enzymes at this concentration by 88.1% and 96.7% respectively (R. Trowbridge, personal communication). These data suggest that compound L339571 is not only a non-specific enzyme inhibitor, but also causes membrane damage. Therefore, this rhodanine is unsuitable for clinical development as an inhibitor of RNAP.

Since L339571 displayed activity against *S. cerevisiae* 464 that was greater than tenfold higher than its antibacterial activity, it may merit future studies, alongside Tocris 1700, 1838 and 2611, but further work would be required to evaluate the cytotoxicity of this

Table 6.5 - Determination of membrane damage and bacterial specificity of L339571

Condition	*S. aureus* MIC (µg/ml)	*Bac*Light™ result (% membrane integrity)	*S. cerevisiae* 464 MIC (µg/ml)
Drug-free control	-	100	-
5% SDS (w/v)	-	0	-
Rifampicin	0.015	80.8 ±6.1	>256
MyxB	2	96.8 ±5.5	>256
L339571	4	27.3 ±4.2	128

compound. In addition, preliminary assays to determine the extent of VG2 binding to human serum proteins have indicated that the physiochemical properties of this compound class (i.e. high hydrophobicity) lead to high levels of serum binding (Villain-Guillot *et al.*, 2007b), which would reduce plasma concentrations of agent and reduce the efficacy of these compounds. In conclusion, rhodanines do not have the required characteristics which would make them suitable for chemotherapeutic development.

6.3.3 Putative inhibitors of bacterial Ddl

6.3.3.1 MIC determinations

To confirm the potential for clinical development, MIC determinations of the DdlB inhibitors were performed against the standard panel of bacterial strains (Table 6.6). D-cycloserine was analysed as a comparator agent since it has established activity against DdlB.

The DdlB inhibitors generally displayed a broad spectrum of antibacterial activity, with at most only sixteen-fold difference between the activity of the compounds against *S. aureus* SH1000 and *E. coli* 1411. A broad spectrum of activity for D-cycloserine was also observed, and consistent with the conserved nature of the active site of Ddl enzymes across bacterial genera (Tytgat *et al.*, 2009). The differences in susceptibility of *S. aureus* SH1000 to the Ddl inhibitors may be accounted for by subtle structural differences between the compounds. For example, the similarity in activities of 155693 and 627505 could result from the fact that these compounds represent the iodide and acetate salt respectively of the same compound (Appendix 1). This may also explain why both compounds appear to have similar IC_{50}s against *E. coli* DdlB and are both substrates for the for the AcrAB-TolC multi-drug efflux pump (both displaying four-fold increase in activity against the AcrAB deletion *E. coli* mutant in comparison with the wild-type). Ddl 155694, while a substrate for the AcrAB-TolC efflux pump due to its similar structure to 155693, was also more active then the latter against *S. aureus* and

Table 6.6 - Antibacterial spectrum of activity of DdlB inhibitors

Antibacterial compound	M_r	IC_{50} against *E. coli* DdlB (μM)	MIC (μg/ml)				
			S. aureus SH1000	*E. coli* 1411	*E. coli* 1411 + PMBN	*E. coli* SM1411	*B. subtilis* 1S34
D-cycloserine	102.1	1083*	64	64	32	32	32
Ddl 155693	418	65¥	32	128	128	32	256
Ddl 155694	432	43¥	4	64	16	4	32
Ddl 352738	413	40%#	256	128	128	128	128
Ddl 627505	350	89¥	64	128	128	32	256

**IC_{50} determination performed by J. Bostock, University of Leeds, in the presence of 250mM D-alanine substrate. ¥ IC50 determination performed by A. Kovac, University of Ljubljana, Slovenia in the presence of 700μM D-alanine substrate. # Residual activity at 500 μM; IC50 not measured*

is hindered by the presence of the outer membrane in Gram-negatives, as the permeabilisation of the outer membrane with PMBN increased the susceptibility of the wild-type strain to this agent by fourfold. The improved antibacterial activity against *S. aureus* appears to result from the simple addition of a methyl group onto the secondary amine. The loss of a polar N-H group would contribute to 155694 being overall less basic/more hydrophobic than 155693, meaning that it could penetrate the cells more readily, especially in Gram-negative bacteria where the outer membrane has been permeabilised by PMBN. The activity of 352738 was poor against all of the bacterial strains tested, and is not enhanced in *E. coli* upon permeabilisation of the outer membrane, or in the AcrAB deletion mutant. While comprising the same molecular scaffold as the other three DdlB inhibitors, this compound contains more polar constituents (halogenated side chain, two tertiary and one secondary amine), which would impede its penetration into the bacterial cell across the lipophilic membrane bilayer. An activity which is also not improved upon addition of an outer membrane permeabiliser.

6.3.3.2 Determination of membrane damage and bacterial specificity of the DdlB inhibitors

The *Bac*Light™ assay was utilised to examine membrane damage in *S. aureus* SH1000 caused by the DdlB inhibitors, and *S. cerevisiae* MIC determinations performed to ascertain whether the compounds might be suitable for clinical development and therefore warrant further evaluation (Table 6.7). Exposure of *S. aureus* SH1000 to all of the DdlB inhibitors caused a reduction in cell membrane integrity to <40% that of the

Table 6.7 - Determination of membrane damage and bacterial specificity of the DdlB inhibitors

Condition	*S. aureus* MIC (µg/ml)	*Bac*Light™ result (% membrane integrity)	*S. cerevisiae* 464 MIC (µg/ml)
Drug-free control	-	100	-
5% SDS (w/v)	-	0	-
D-cycloserine	64	112.9 ±6.1	>256
Ddl 155693	32	16.0 ±2.2	8
Ddl 155694	4	17.8 ±1.2	4
Ddl 352738	256	37.5 ±5.5	128
Ddl 627505	64	7.8 ±1.1	8

drug-free control (Table 6.7), and therefore these inhibitors were classed as membrane-damaging. The compounds also displayed activity against *S. cerevisiae* and are likely to display cytotoxicity. They are therefore unsuitable for development as clinical inhibitors of bacterial DdlB.

6.3.4 Putative inhibitors of PG-GTs: compounds 5 and 5b

6.3.4.1 Minimum inhibitory concentration determinations

Virtual high-throughput screening of small molecule inhibitors against the *S. aureus* PBP2 GT domain has identified 2 compounds, 5 and 5b which inhibit enzymatic activity of various PBPs at micromolar concentrations (Derouaux *et al.*, 2011). We sought to examine the suitability of these compounds for clinical development by confirming the mode of action of these compounds as inhibitors of cell wall

biosynthesis in whole cells. The antibacterial spectrum of activity of the GT inhibitors is shown in Table 6.8.

Vancomycin and moenomycin (an established GT inhibitor) displayed low MICs against *S. aureus* SH1000 and little activity against *E. coli* 1411 (Table 6.8). The preferential activity of cell wall inhibitors such as these against Gram-positive organisms has been well established (Chapter 3; Cheng *et al.*, 2008). Compounds 5 and 5b displayed modest MICs against all of the strains tested. As broad-spectrum agents, these agents may be indicated for use against a wide range of bacterial infections. It is apparent however, that the marginally lower activity of both 5 and 5b against *E. coli* in comparison with the Gram-positive organisms tested may be due to hindrance by the outer membrane and efflux by the AcrAB-TolC multi-drug efflux pump, as the activity of these agents was improved upon addition of an outer membrane permeabiliser in the wild-type and in the AcrAB deletion mutant. As discussed in Chapter 3, with the exception of antibiotics from the β -lactam class, the activity of cell wall biosynthesis inhibitors in Gram-negative bacteria tends to be improved by the addition of PMBN, but they are not substrates for efflux by AcrAB. This is exemplified by the spectrum of activity observed for vancomycin and moenomycin (Table 6.8). The difference in activities between moenomycin and 5/5b may indicate variance in antibacterial target. However, it is more likely to be due to structural differences between the three antibiotics which allows substrate recognition of the latter two, but not moenomycin by AcrAB. Further analysis of the MOA of 5 and 5b should resolve this discrepancy.

Table 6.8 – Antibacterial spectrum of activity of compounds 5 and 5b

Antibacterial compound	M_r	MIC (μg/ml)				
		S. aureus SH1000	*E. coli* 1411	*E. coli* 1411 + PMBN	*E. coli* SM1411	*B. subtilis* 1S34
Vancomycin	1449.2	2	256	128	256	0.5
Moenomycin	1583.6	0.5	>128	4	>128	>128
Compound 5	381.0	16	64	16	16	16
Compound 5b	415.5	8	16	8	4	4

6.3.4.2 Determination of membrane damage and bacterial specificity of compounds 5 and 5b

Prior to further MOA analysis and determination of the effect of compounds 5 and 5b on PG biosynthesis in whole cells, the *Bac*Light™ assay and *S. cerevisiae* MIC determinations were performed to ascertain whether these compounds might be generally suitable for clinical development (Table 6.9).

The membrane integrity of *S. aureus* SH1000 exposed to the established GT inhibitor moenomycin did not vary significantly from that of the vancomycin treated samples or the drug free control, indicating that this compound does not cause membrane damage. In addition, neither vancomycin nor moenomycin displayed activity against *S. cerevisiae* 464. Vancomycin has been used clinically for many years in the treatment of severe Gram-positive infections including those caused by staphylococci, streptococci and clostridia (Greenwood *et al.*, 2007), but as mentioned previously, clinical use of moenomycin has not been possible because of the poor pharmacokinetic properties of this compound (Welzel, 2005). Exposure of *S. aureus* SH1000 to both GT inhibitors resulted in a reduction in cell membrane integrity to <40% that of the drug-

Table 6.9 - Determination of membrane damage and bacterial specificity of compounds 5 and 5b

Condition	*S. aureus* MIC (µg/ml)	*Bac*Light™ result (% membrane integrity)	*S. cerevisiae* 464 MIC (µg/ml)
Drug-free control	-	100	-
5% SDS (w/v)	-	0	-
Vancomycin	2	83.5 ±4.4	>256
Moenomycin	0.5	85.9 ±8.0	>128
Compound 5	16	3.5 ±1.1	4
Compound 5b	8	32.9 ±1.1	4

free control (Table 6.9), and therefore these compounds were classed as membrane-damaging. Compounds 5 and 5b also displayed activity against *S. cerevisiae*, which was two to fourfold lower than the observed antistaphylococcal activity. Therefore it is likely that these compounds will display cytotoxicity and be unsuitable for chemotherapeutic development and therefore they have not been evaluated further.

6.4. Conclusions

The increasing prevalence of multi-drug resistant strains of bacteria has highlighted the need to develop antibacterial agents with novel scaffolds, possessing targets that are distinct from those of drugs in current clinical use. In this study, amongst nearly half a million compounds screened *in silico* against RNAP, DdlB and bacterial GTs, only Tocris 1610 and Tocris 2176 displayed the promising characteristics of good antibacterial activity, no membrane damage and no cross-activity against eukaryotes. For these two potential hit compounds with suspected inhibition of RNAP, I attempted to analyse their MOA to link the *in vitro* enzyme activity with RNA synthesis inhibition

in whole bacterial cells. However, it was not possible to detect inhibition of RNA synthesis by either compound utilising the *B. subtilis* antibiotic biosensors or macromolecular synthesis assays. Consequently, these compounds were discarded for future development as specific inhibitors of bacterial RNAP. The lack of new antibacterial agents to put forward for clinical development as observed in this chapter is representative of the wider problem, and may explain the deficit of new antibacterial drugs in the antibiotic pipeline. This study also highlights the many challenges which currently face large pharmaceutical companies in antibacterial drug discovery and development, particularly in being able to demonstrate a direct correlation between prokaryotic specific antibiotic activity and selective inhibition of a bacterial target. However, although it was not possible to identify any inhibitors with specific activity against their proposed target enzyme, a number of non-specific inhibitors were identified displaying membrane-damaging activity and sufficient preferential inhibitory activity for prokaryotes over eukaryotes. These compounds might be developed as bacterial membrane damagers for persistent bacterial infections e.g. those involving a biofilm.

Chapter 7 - Transcriptional analysis of antibacterial induced membrane damage in *Staphylococcus aureus*

7.1 Abstract

Evidence from current literature and work described in this thesis suggests that it is prudent to establish during the earliest stages of antibacterial drug development whether candidate agents cause bacterial membrane damage, since membrane damagers are likely to cause non-specific toxic side effects if administered to humans. Current assays to determine membrane damage caused by novel antimicrobial agents suffer from slow processing times, high expense or the requirement for large amounts of the test antibacterial agent. As such, new methods are required for the more rapid screening of membrane-damaging agents, to process potential chemotherapeutic candidates faster, and eliminate compounds with unattractive characteristics. The construction of biosensors responsive to membrane damage would enable high-throughput detection of membrane-damaging antibacterial agents. However, no such reporter strains have been described to date. To facilitate the generation of such reporters, the transcriptional response of *Staphylococcus aureus* SH1000 to exposure with the established membrane-damaging agents clofazimine, cetyltrimethylammonium bromide (CTAB) and sepracor 155342 was determined by DNA microarray analysis (literature data are also available for transcriptional profiles in the presence of daptomycin, carbonylcyanide m-chlorophenylhydrazone [CCCP] and nisin). The objective was to identify upregulated promoters which could be employed for the future construction of biosensors responsive to the presence of membrane damagers. These could then be used in high-throughput screening programmes of novel antibacterial agents. The transcriptional profiling of membrane damage revealed that genes involved in

maintenance of membrane integrity and energy production were significantly upregulated. In addition, expression of a gene encoding a single-strand DNA-binding protein was induced in response to all three membrane-damaging agents tested, but not by other antibacterial agents inhibiting different metabolic pathways in *S. aureus*. Therefore, the promoter of this gene potentially constitutes a viable candidate for the construction of a biosensor uniquely responsive to membrane damage in *S. aureus*. In addition, the promoters of genes in the *kdp* (potassium-transporting ATPase subunit) operon may also constitute viable options for the construction of membrane damaging biosensors, as *kdpA*, *kdpB* and *kdpF* are all induced in response to sepracor 155342, clofazimine, daptomycin and CCCP. However, further quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis is needed to verify that these genes are not upregulated in response to antibacterial agents with other modes of action (MOAs) which would negate their use in the construction of biosensors specifically induced in response to membrane damagers.

7.2 Introduction

7.2.1 Membrane-damaging antibacterial agents

As previously mentioned, novel antimicrobial agents that are identified via high-throughput screening programs often exert non-specific membrane-damaging effects (Siedler *et al.*, 2003). This characteristic frequently leads to such agents being disregarded, due to their potential harmful toxicity to humans. Conversely, bacterial-specific membrane damagers (such as daptomycin and telavancin) can be particularly efficacious against persistent infections, and have a low potential for resistance development (Hurdle *et al.*, 2011).

It is prudent, therefore, to establish during the earliest stages of antibacterial drug development whether a new agent causes prokaryotic-specific membrane damage. Currently, high thoughput systems exist for testing bacterial specificity and cytotoxicity of novel drugs (Khafagi *et al.*, 2000; Niles *et al.*, 2009). However no such assays exist for the rapid identification and quantification of membrane damage caused by an agent. Current assays such as *Bac*Light™ and quantification of potassium ion (K^+) leakage may take up to a day to perform, are expensive, and are limited in the number of samples it is possible to screen in one session. As a result, new methods are required for rapid and high-throughput screening of potential membrane damaging agents to select chemotherapeutic candidates faster, and disregard compounds with unattractive characteristics (Payne *et al.*, 2007).

7.2.2 Construction of antibiotic biosensors as a tool for antibacterial drug development

Cellular biosensors represent a rapid tool for high-throughput detection of novel antibacterial agents. Reporter strains such as the *Bacillus subtilis* and *Staphylococcus aureus* constructs described in the previous chapters are becoming widely used for the screening of existing antibiotics and developmental compounds (Bianchi & Baneyx, 1999; Shapiro & Baneyx, 2002; Fischer *et al.*, 2004; Hutter *et al.*, 2004; Urban *et al.*, 2007).

As yet, no bacterial biosensors have been described in the literature to detect membrane-damaging agents specifically. The *ypuA* promoter of *B. subtilis* 1S34 has previously been reported to detect both cell-wall active and membrane-damaging agents (Urban *et al.*, 2007), although induction of *ypuA* was only observed in response to some membrane damagers and most peptidoglycan biosynthesis inhibitors (Chapter 3). Similarly, the *P3rpoH* promoter of *E. coli* is upregulated in response to the cell membrane damager polymyxin B, but this promoter is also upregulated in response to inhibitors of peptidoglycan synthesis such as carbenicillin (Bianchi & Baneyx, 1999).

The construction of biosensor solely responsive to membrane damage would be particularly useful due to the fact that only small concentrations of agent are required for screening in comparison with other mode of action (MOA) techniques (e.g. macromolecular synthesis) (Shapiro & Baneyx, 2002). The development and application of such biosensors might also eliminate the need for extensive

characterisation of membrane-damaging agents by such methods as the *Bac*Light™ assay and K^+ leakage and provide a cheaper, rapid and high-throughput alternative.

DNA microarray analyses can provide vital information on the cellular response to bacterial inhibitors at the level of gene expression (O'Neill *et al.*, 2009). Bacteria will regulate the expression of genes to compensate for the presence of an inhibitor, generally upregulating genes that will remove the inhibitor or increase production of the antibacterial target (Odenholt, 2001; Utaida *et al.*, 2003). Transcriptional analysis of membrane damage in *S. aureus* induced by growth inhibiting concentrations of the agent could enable the identification of genes which are upregulated in response to membrane damage. Those that are significantly induced in response to membrane damagers, and not other antibiotic classes, might be candidates for the construction of promoter reporter constructs, for future use as biosensors which can identify potential membrane damagers.

Previous work has identified the cellular responses of *S. aureus* N315 to exposure of the prokaryotic-specific membrane damagers, daptomycin and nisin, and the membrane uncoupler, carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (Muthaiyan *et al.*, 2008). This study revealed that treatment of *S. aureus* with daptomycin leads to upregulation of four hundred and seventy four genes, including those involved with the staphylococcal cell wall stress stimulon (e.g. *pbpB, murAB, tcaA*), signal transduction (*vraSR*) and amino acid biosynthesis (e.g. *dapA, thrC, cysK*). Treatment of *S. aureus* with CCCP and nisin lead to upregulation of four hundred and one hundred genes respectively, including those involved with macromolecular biosynthesis and metabolism. Significant induction of the *kdpA* and *kdpB* potassium transporter subunit

genes was also reported, and thought to be a response to disruption of the membrane potential. Although this study highlights genes that are responsive to specific membrane damage in *S. aureus*, more analysis is required of the effect of exposure of the organism to non-specific membrane damagers, in an attempt to identify genes that could be manipulated in the construction of membrane damage responsive biosensor strains.

7.2.3 Aims and objectives of the work described in this chapter

To facilitate screening of membrane damage by new inhibitors, the transcriptional response of *S. aureus* SH1000 following exposure to the established membrane-damaging agents clofazimine, cetyltrimethylammoium bromide (CTAB) and sepracor 155342 was attempted by DNA microarray analysis. The objective was to identify upregulated promoters in common with those identified by Muthaiyan *et al.*, which could be manipulated in the future construction of biosensors responsive to the presence of membrane damagers. These could then be used in high-throughput screening programmes of novel antibacterial agents. The overall aims and objectives of the research described in this chapter were therefore to:

- perform transcriptional profiling analysis of *S. aureus* exposed to growth inhibiting concentrations of membrane damagers (clofazimine, CTAB and sepracor 155342);
- compare the analysed microarray data in cross reference to published data to identify upregulated promoters with the potential for use in the construction of biosensors responsive to membrane damage.

7.3 Results and Discussion

For comparison of gene expression in *S. aureus* SH1000, total RNA was extracted in exponentially growing cells in the absence and presence of membrane damaging agents representing the concentration which inhibited growth of *S. aureus* by 25% relative to a drug-free control (Sepracor 155342 - 0.01 μg/ml, CTAB - 1.25 μg/ml, clofazimine - 3 μg/ml) for transcriptional profiling experiments (DNA microarray). Differentially expressed genes were identified using ArrayStar® 4 software as displaying at least two-fold up or downregulation (Raju *et al.*, 2004).

7.3.1 Transcriptional analysis of clofazimine induced membrane damage in *S. aureus* SH1000

In the cells treated with clofazimine (3 μg/ml), 58.7% of the *S. aureus* SH1000 genome (which comprises 2892 genes in total) (Baba *et al.*, 2008) showed no differential expression in comparison with the untreated control (Figure 7.1). Of the remainder, only 0.6% (17 genes) of the genome was upregulated in comparison with untreated cells (Table 7.1) while 40.7% of the genome (1208 genes) was downregulated (Appendix 2). This contrasts with four hundred and seventy four genes that were upregulated in response to daptomycin exposure in *S. aureus* 29213 and three hundred and ninety five genes which were downregulated. The difference observed is most likely due to the suprainhibitory concentrations of daptomycin that were used in the previous transcriptional profiling, in addition to the fact that daptomycin also appears to upregulate genes involved with the cell wall stress stimulon (Muthaiyan *et al.*, 2008).

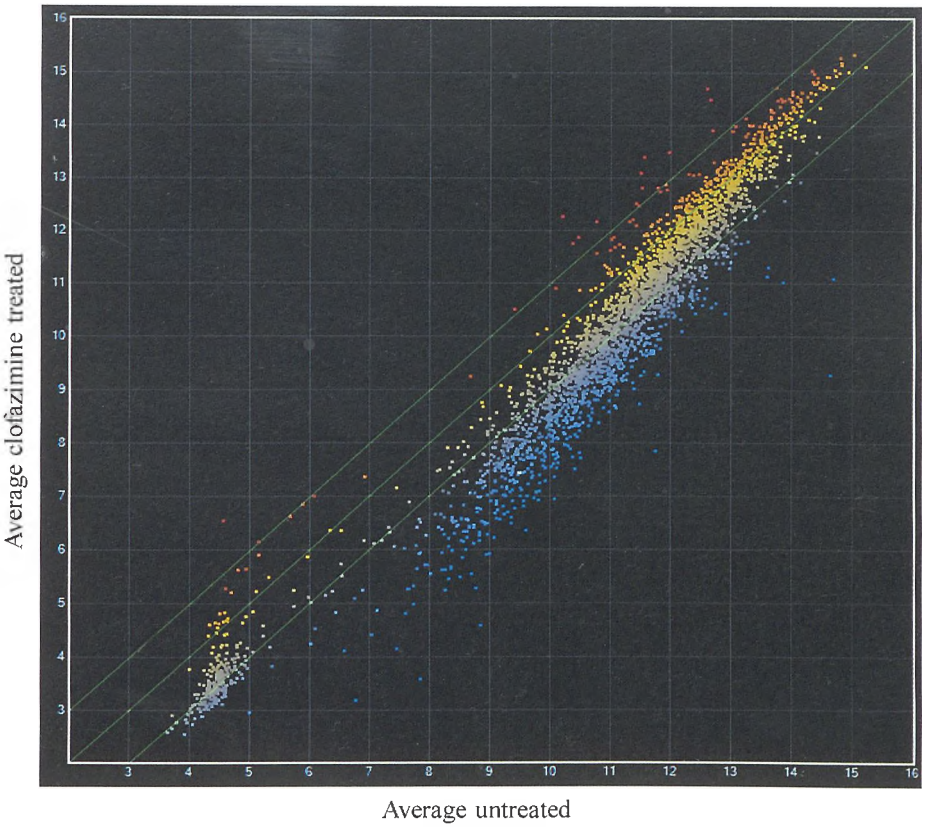


Figure 7.1 – Scatter-plot of differentially expressed genes in *S. aureus* SH1000 in response to clofazimine (3 µg/ml) *The lines represent the threshold (twofold) for differential gene expression in clofazimine treated S. aureus SH1000 versus an untreated control. Upregulated genes are shown in red, down-regulated in blue.*

Of the seventeen genes that were upregulated in response to clofazimine, seven represented encoded hypothetical proteins of unknown function. Four of the remaining ten upregulated genes were involved with cellular energy metabolism. NADH dehydrogenase (Ndh) mediates entry of electrons into the electron transport chain at the cell membrane (Friedrich & Scheide, 2000), while L-/D-lactate dehydrogenase, precorrin-2 dehydrogenase and glutamate synthase all behave as oxidoreductases which catalyse the metabolism of lactate, porphyrins and L-glutamate/α-ketoglutarate respectively, with NAD^+ or $NADP^+$ acting as the electron acceptor (Garvie, 1980; Goss *et al.*, 2001; Raux *et al.*, 2003). The potassium/sodium-transporting ATPase subunits

Table 7.1- Upregulated genes in *S. aureus* SH1000 in response to clofazimine challenge

SEQ_ID	Fold change	DESCRIPTION	Gene	Also upregulated by	Reference
SAOUHSC_00412	4.339	NADH dehydrogenase subunit 5	*ndhF*	Oxacillin	Muthaiyan *et al.*, 2008
SAOUHSC_00206	4.222	L- lactate dehydrogenase	*ldh2*	Nisin	Muthaiyan *et al.*, 2008
SAOUHSC_02071	4.033	Single-strand DNA-binding protein	-	CTAB, sepracor 155342	Table 7.2 and 7.3
SAOUHSC_00413	3.633	Conserved hypothetical protein	-		
SAOUHSC_02830	3.579	D- lactate dehydrogenase	*ddh*		
SAOUHSC_02312	3.044	K/Na transporting ATPase subunit A	*kdpA*	Daptomycin, CCCP, D-cycloserine Mild Acid, pH 5.5 (6 hours), Sepracor 155342	Muthaiyan *et al.*, 2008 Nagarajan & Elasri, 2007 Weinrick *et al.*, 2004 Table 7.2
SAOUHSC_02311	2.874	K/Na transporting ATPase subunit B	*kdpB*	Daptomycin, CCCP, D-cycloserine Mild Acid, pH 5.5 (6 hours), Sepracor 155342	Muthaiyan *et al.*, 2008 Nagarajan & Elasri, 2007 Weinrick *et al.*, 2004 Table 7.2
SAOUHSC_02243	2.671	Conserved hypothetical protein	-		
SAOUHSC_00435	2.659	Glutamate synthase large subunit	*gltB*	Oxacillin, Stringent Response, Silver Fosfomycin, Sepracor 155342	Muthaiyan *et al.*, 2008 Nagarajan & Elasri, 2007 C. Randall (unpublished data) Table 7.2
SAOUHSC_02866	2.638	Conserved hypothetical protein	-		
SAOUHSC_02388	2.471	Conserved hypothetical protein	-		
SAOUHSC_02945	2.340	Precorrin-2 dehydrogenase	*cysG*	Silver	C. Randall (unpublished data)
SAOUHSC_02313	2.160	K/Na transporting ATPase subunit F	*kdpF*	Sepracor 155342	Table 7.2
SAOUHSC_00414	2.128	Conserved hypothetical protein	-		
SAOUHSC_02389	2.062	Cation efflux family protein	-		
SAOUHSC_00721	2.039	conserved hypothetical protein	-	Silver	C. Randall (unpublished data)
SAOUHSC_01551	2.019	conserved hypothetical phage protein	-		

and which form part of this essential membrane protein, were also upregulated by exposure to clofazimine (Table 7.1). The Na^+/K^+ ATPase is responsible for maintaining the electrochemical gradient across the cytoplasmic membrane (Moller *et al.*, 1996; Walderhaug *et al.*, 1992), a role which may also be shared by the cation efflux family protein, the function of which is poorly characterised. Single-strand DNA-binding protein was also upregulated by clofazimine-treated *S. aureus* (Table 7.1). This protein binds to single strands of DNA during replication, to prevent premature re-annealing (Mijakovic *et al.*, 2006). The identities of these various genes suggest that exposure of *S. aureus* SH1000 to growth inhibitory concentrations of clofazmine result in upregulation of genes that restabilise the cell membrane and cellular genetic material, but also re-establish and increase oxidative phosphorylation at the cell membrane to supply energy in the form of ATP to the dying cell.

7.3.2. Transcriptional analysis of sepracor 155342 induced membrane damage in *S. aureus* SH1000

In cells treated with sepracor 155342 (0.01 μg/ml), 60.1% of the *S. aureus* SH1000 genome showed no differential expression in comparison with the untreated control (Figure 7.2). Of the remainder, only 0.3% (8 genes) of the genome was upregulated in comparison with untreated cells (Table 7.2) while 39.6% of the genome (1176 genes) was downregulated (Appendix 2). Of the eight genes upregulated in *S. aureus* SH1000 exposed to sepracor 155342, five were in common with the genes upregulated in response to clofazmine, involving stabilisation of DNA (single-strand DNA-binding protein), increased energy metabolism (*gltB*) and maintenance of membrane integrity

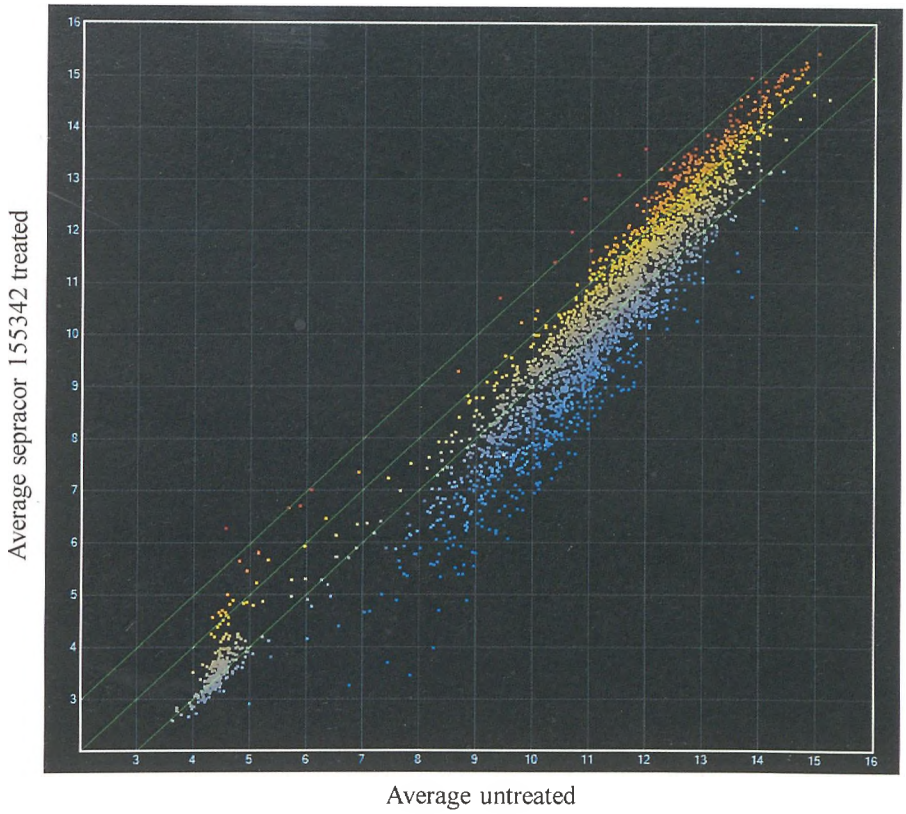


Figure 7.2 – Scatter-plot of differentially expressed genes in *S. aureus* SH1000 in response to sepracor 155342 (0.01 µg/ml) *The lines represent the threshold (twofold) for differential gene expression for sepracor 155342 treated S. aureus SH1000 versus an untreated control. Upregulated genes are shown in red, down-regulated in blue.*

(*kdp* operon genes). In addition, two subunits of cytochrome d ubiquinol oxidase were upregulated in response to sepracor 155342. Cytochrome d ubiquinol oxidase represents one of two terminal oxidases in the prokaryotic aerobic respiratory chain, which is responsible for the final reduction of molecular oxygen into water (Dueweke & Gennis, 1990). Upregulation of these subunit genes would lead to an increase in activity of energy production pathways in order to compensate for cell membrane stress.

Table 7.2 - Upregulated genes in *S. aureus* SH1000 in response to sepracor 155342 challenge

SEQ_ID	Fold change	DESCRIPTION	Gene	Also upregulated by	Reference
SAOUHSC_01032	3.359	Cytochrome d ubiquinol oxidase subunit II	*cydB*	Daptomycin,Oxacillin Silver	Muthaiyan *et al.*, 2008 C. Randall (unpublished data)
SAOUHSC_02071	3.290	Single-strand DNA-binding protein	-	Clofazimine, CTAB	Table 7.1 and 7.3
SAOUHSC_02311	3.098	K/Na transporting ATPase subunit B	*kdpB*	Daptomycin, CCCP D-cycloserine, Mild Acid, pH 5.5 (6 hours) Clofazimine	Muthaiyan *et al.*, 2008 Nagarajan & Elasri, 2007 Weinrick *et al.*, 2004 Table 7.1
SAOUHSC_02312	3.071	K/Na transporting ATPase subunit A	*kdpA*	Daptomycin, CCCP D-cycloserine, Mild Acid, pH 5.5 (6 hours) Clofazimine	Muthaiyan *et al.*, 2008 Nagarajan & Elasri, 2007 Weinrick *et al.*, 2004 Table 7.1
SAOUHSC_01031	2.499	Cytochrome d ubiquinol oxidase subunit I	*cydA*	Daptomycin, Oxacillin, Bacitracin, Silver	Muthaiyan *et al.*, 2008 Nagarajan & Elasri, 2007 C. Randall (unpublished data)
SAOUHSC_02313	2.465	K/Na transporting ATPase subunit F	*kdpF*	Clofazimine	Table 7.1
SAOUHSC_A01912	2.230	conserved hypothetical protein	-		
SAOUHSC_00435	2.078	glutamate synthase large subunit	*gltB*	Oxacillin, Stringent Response Fosfomycin, Silver, Clofazimine	Muthaiyan *et al.*, 2008 Nagarajan & Elasri, 2007 C. Randall (unpublished data) Table 7.1

7.3.3 Transcriptional analysis of CTAB induced membrane damage in *S. aureus* SH1000

In the cells treated with CTAB (1.25 μg/ml), 59.7% of the *S. aureus* SH1000 genome showed no differential expression in comparison with the untreated control (Figure 7.3). Of the remainder, only 0.4% (12 genes) of the genome was upregulated in comparison with untreated cells (Table 7.3) while 39.9% of the genome (1184 genes) was downregulated (Appendix 2).

Of the twelve upregulated genes, six represented genes encoding conserved hypothetical proteins and a UvrB/UvrC motif domain protein, while a further two were found to be the single-strand DNA-binding protein and NADH dehydrogenase genes which were upregulated in response to clofazimine. The UvrB and UvrC proteins interact during the nucleotide excision repair process and are involved with the recognition of damaged DNA and guidance to the bacterial repair systems (Theis *et al.*, 2000). Similar to the single-strand DNA-binding protein, the upregulation of this protein in response to membrane damage is most likely to be a compensatory mechanism for the dying cell. ATP: guanidophosphotransferase was also upregulated in response to CTAB (Table 7.3). This enzyme is involved with the reversible transfer of the terminal phosphate of ATP onto other phosphogens, and is involved with ATP generation within the bacterial cell (Stein *et al.*, 1990). The upregulation of the phosphate ATP binding cassette (ABC) transporter was most likely a consequence of the increased requirement for inorganic phosphate in ATP production within the cell. As a phosphate binding protein this ABC transporter can mediate rapid uptake of phosphate from the extracellular environment (Gebhard *et al.*, 2006).

A subunit of the ATP-dependent Clp protease was also upregulated in response to CTAB (Table 7.3). Clp (**C**aseino**l**ytic **p**rotease) enzymes are capable of degrading misfolded or accumulated polypeptides up to six residues in length, and are mainly involved with protein turnover and maintenance of cellular homeostasis (Clarke, 1999). The heat shock chaperone gene *grpE* was similarly upregulated (Table 7.3), and is known to be induced in cellular stress response (Gamer *et al.*, 1992). The upregulation

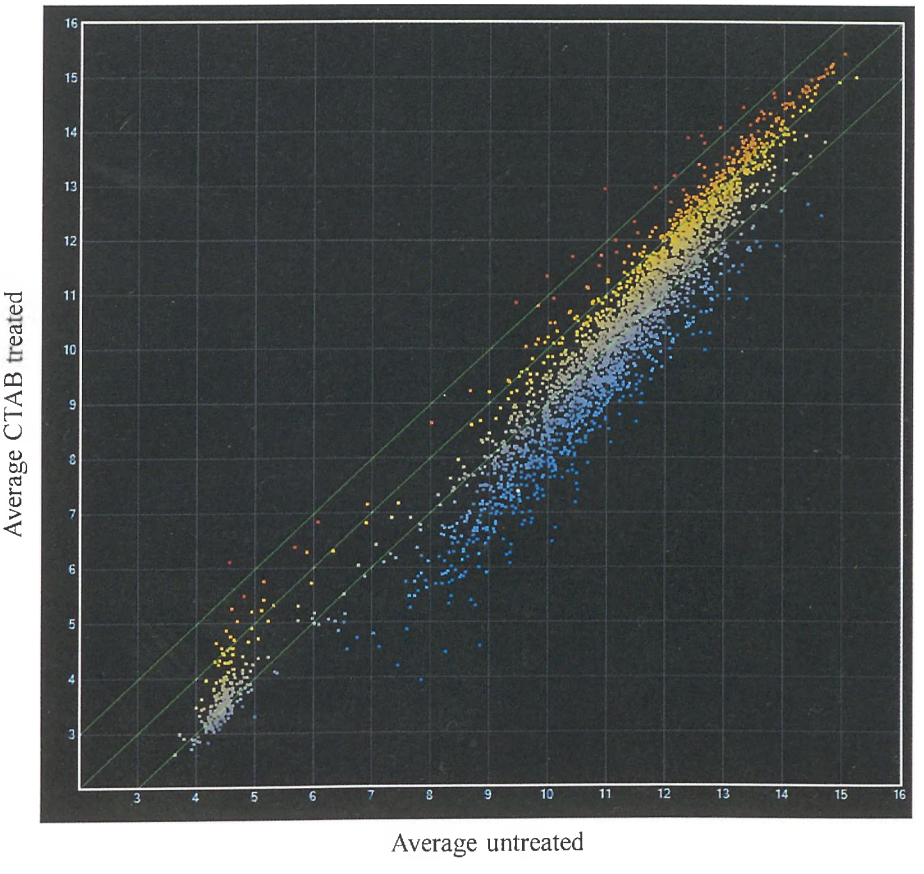


Figure 7.3 – Scatter-plot of differentially expressed genes in *S. aureus* SH1000 in response to CTAB (1.25 µg/ml). *The lines represent the threshold (twofold) for differential gene expression in CTAB treated S. aureus SH1000 versus an untreated control. Upregulated genes are shown in red, down-regulated in blue.*

Table 7.3- Upregulated genes in *S. aureus* SH1000 in response to CTAB challenge

SEQ_ID	Fold change	DESCRIPTION	Gene	Also upregulated by	Reference
SAOUHSC_02645	4.023	Conserved hypothetical protein	-		
SAOUHSC_02071	2.979	Single-strand DNA-binding protein	-	Clofazimine, sepracor 155342	Table 7.1 and 7.2
SAOUHSC_00912	2.909	ATP-dependent Clp protease, ATP binding subunit ClpB	*clpB*	Daptomycin, Oxacillin CCCP, Cefoxitin Fosfomycin	Muthaiyan *et al.*, 2008 Nagarajan & Elasri, 2007
SAOUHSC_01387	2.593	conserved hypothetical protein	-	Silver	C. Randall (unpublished data)
SAOUHSC_00412	2.538	NADH dehydrogenase subunit 5	*nuoF*	Oxacillin	Muthaiyan *et al.*, 2008
SAOUHSC_02646	2.462	conserved hypothetical protein	-		
SAOUHSC_00504	2.267	ATP: guanidophosphotransferase	-	Daptomycin, CCCP	Muthaiyan *et al.*, 2008
SAOUHSC_00503	2.235	UvrB/UvrC motif domain protein	-		
SAOUHSC_00413	2.229	Conserved hypothetical protein	-		
SAOUHSC_00502	2.205	Conserved hypothetical protein	-		
SAOUHSC_01684	2.104	Heat shock protein	*grpE*	Daptomycin, CCCP, Mild Acid, pH 5.5 (6 hours)	Muthaiyan *et al.*, 2008 Weinrick *et al.*, 2004
SAOUHSC_01389	2.660	Phosphate ABC transporter periplasmic phosphate-binding protein	*pstS*		

of these genes in response to CTAB most likely occurs to aid the downregulation of dispensable cellular metabolic processes by degrading non-essential enzymes and proteins (Michel *et al.*, 2006).

7.3.4 Selection of candidate genes for membrane damage biosensor strains

The purpose of this study was to identify promoters that are upregulated in response to membrane damage. These could then be employed in the future construction of biosensors responsive to membrane damage. The transcriptional profiling outlined above identified one gene (encoding a single-strand DNA protein) that appeared to be upregulated in response to all three membrane-damaging agents, which did not appear in current literature to be induced in response to cellular inhibition of any other metabolic pathway. Therefore, the promoter of SAOUHSC_02071 would be a good candidate for the construction of a biosensor which might be solely responsive to membrane damage. In addition, upregulation of potassium-transporting ATPase subunit genes *kdpA*, *kdpB* and *kdpF* was observed in response to both sepracor 155342 and clofazimine, and also to daptomycin and CCCP according to current literature (Muthaiyan *et al.*, 2008). Therefore the genes of this operon may also constitute viable options for candidate promoters for membrane damaging biosensors. However, these promoters are also upregulated by the peptidoglycan synthesis inhibitor D-cycloserine (Muthaiyan *et al.*, 2008). Therefore, to confirm the suitability of selected promoters for use in membrane damage biosensors, it would be necessary to perform quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on mRNA extracted from antibiotic treated cells as described previously (Cardoso *et al.*, 2010) in order to confirm

that these genes are not upregulated in response to antibacterial agents with other MOAs. In addition, it would be necessary to confirm the upregulation of the *kdp* promoters in response to daptomycin and CCCP, as in published work induction of these genes was observed in response to suprainhibitory concentrations of the agent and differing assay conditions in comparison with those used above (Muthaiyan *et al.*, 2008).

7.4 Conclusions

Transcriptional profiling of membrane damage in *S. aureus* SH1000 reveals that genes involved in maintenance of membrane integrity and energy production are significantly upregulated. Expression of the gene encoding a single-strand DNA-binding protein was induced in response to all three membrane-damaging agents tested, but not by other antibacterial agents inhibiting different metabolic pathways in *S. aureus*. Therefore, the promoter of this gene may constitute a viable candidate for the construction of a biosensor uniquely responsive to membrane damage in *S. aureus*. In addition, the promoters of genes in the Kdp (potassium-transporting ATPase subunit) operon may also constitute options for the construction of membrane-damaging biosensors, as *kdpA*, *kdpB* and *kdpF* are induced in response to sepracor 155342, clofazimine, daptomycin and CCCP. However, further qRT-PCR analysis is needed to verify that these genes are not also upregulated in response to antibacterial agents with other MOAs.

Chapter 8 – General discussion, conclusions and future work

8.1 General discussion and conclusions

The failure of 'last resort' antibiotics to treat Gram-positive infections such as those caused by meticillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE), and Gram-negative infections such as those caused by extended-spectrum β-lactamase (ESBL) producing Enterobacteriaceae, pan-resistant strains of *Pseudomonas aeruginosa* and *Acinetobacter* spp. is presenting a significant problem to antibacterial chemotherapy (Bowden *et al.*, 2010; Bush, 2004; Moore *et al.*, 2003; Paterson & Bonomo, 2005; Schwarz *et al.*, 2008). The characterisation and development of underexploited antibiotics or new antibacterial agents displaying novel modes of action offer the potential to address this problem. However, there has been a reduction in the investment by large pharmaceutical companies into antibacterial research and development (R&D) (Projan & Shales, 2004). This decrease has resulted in the entrance of only a few new antibacterial drugs into clinical use in the last decade, a supply that is currently not meeting the increasing demand.

Prior to the clinical development of novel antibacterial inhibitors, it is essential that drug candidates are tested to characterise their antibacterial target and bacterial specificity thoroughly. In addition, characterisation of the mode of action (MOA) of a new agent may produce information that can be beneficial to pharmaceutical companies in the further development, appraisal and marketing of the compound (O'Neill & Chopra, 2004a). The research described in this thesis was primarily focused on identification of novel antibacterial inhibitors, utilising various drug discovery

methodologies. These included establishing the antibacterial activity, MOA and development of resistance to a selection of inhibitors. Compounds included antibacterial agents with well-established modes of action (to validate and optimise the methods used), chemically modified derivatives of existing antibiotic classes (i.e. the type B lantibiotic derivative: NVB353), older and underexploited compounds, comprising proposed inhibitors of RNA polymerase (RNAP) and hit compounds from virtual high-throughput screening of RNAP, D-ala-D-ala ligase (Ddl) and peptidoglycan (PG) transglycosylases.

It has been established that analysis of the minimum inhibitory concentrations (MICs) of Gram-positive (*S. aureus* and *Bacillus subtilis*) and Gram-negative (*Escherichia coli)* bacteria will give an indication of the spectrum of activity of the antibacterial agents tested (O'Neill & Chopra, 2004a). The activity of agents against the AcrAB-deficient *E. coli* mutant and the wild-type treated with polymyxin B nonapeptide (PMBN), also yielded information about resistance mechanisms of *E. coli* to an antibacterial agent. The *Bac*Light™ assay and *B. subtilis/S. aureus* reporter systems in combination also provided reliable methodologies for preliminary determination of the membrane damaging effects of antibacterial agents for MOA analysis. In addition, the majority of clinically established antibacterial inhibitors displayed greater than or equal to tenfold higher activity against bacteria versus *Saccharomyces cerevisiae*. Therefore, performing an MIC determination against *S. cerevisiae* and comparing it with the prokaryotic activity, should provide an indication of the likelihood of a developmental compound having sufficient prokaryotic specificity for potential clinical use.

With respect to the test agents described in this thesis, the type B lantibiotic derivative NVB353 shows promise as a potential chemotherapeutic candidate with much improved MICs and bactericidal activity against SH1000 in comparison with DAB, greater than tenfold higher activity against prokaryotes versus eukaryotes and low propensity for resistance development in *S. aureus*. Data from the MOA analysis also suggested that the increased potency and rate of bactericidal activity of NVB353 when compared with DAB is due to the fact that NVB353 exhibits a dual MOA against *S. aureus* SH1000, causing inhibition of PG biosynthesis as well as perturbation of the cell membrane.

Concerning bacterial RNAP inhibitors, it was possible to link the observed antibacterial activity of corallopyronin A (CorA) and myxopyronin B (MyxB) to direct inhibition of RNAP. However, the limited spectrum of antibacterial activity and high propensity for selection of resistance lead to the conclusion that CorA is of little interest for development as a future drug candidate, except possibly in combination therapy. However, if CorA derivatives could be designed and synthesised with improved characteristics, these might be future antibiotic candidates for monotherapy.

From the total of nearly half a million compounds which were screened against RNAP, DdlB and bacterial glycosyltransferases (GTs), only Tocris 1610 and Tocris 2176 displayed the promising characteristics of antibacterial activity, absence of membrane damage and no cross-activity against eukaryotes. However, although these compounds inhibited RNAP *in vitro*, it was not possible to detect inhibition of RNA synthesis in whole cells by either compound.

The results presented in this thesis are testament to the difficulties which currently face large pharmaceutical companies, i.e. the inability to make a direct correlation between

in vitro enzyme inhibition and whole cell, targeted antibacterial activity for compounds identified by high-throughput screening (Payne *et al.*, 2007). The lack of new antibacterial agents with specific bacterial targets for clinical development is representative of the wider problem and highlights the low probability of success, which may explain the reduction in numbers of larger antibacterial R&D programs, and concurrent shift to other chemotherapeutic areas which offer better return on investment (Projan & Shales, 2004).

Although it was not possible to identify any inhibitors of specific molecular targets that would be suitable for chemotherapeutic development, a number of non-specific inhibitors were identified (Tocris 1700, 1838, 2611 and furanyl-rhodanine L339571), that displayed membrane-damaging activity and yet exhibited preferential inhibition of prokaryotes versus eukaryotes. There may still be potential for these compounds to be developed as bacterial membrane damagers, perhaps with an indication for persistent bacterial infections (Hurdle *et al.*, 2011). To aid in the identification of such compounds, transcriptional profiling of *S. aureus* treated with a panel of known membrane damagers was used to identify upregulated genes that might be potential candidates for future biosensor development. The microarray analysis revealed that genes involved in maintenance of membrane integrity and energy production are significantly upregulated. However, the promoter of the gene encoding a single strand DNA-binding protein, and the promoters of genes in the *kdp* (potassium transporting ATPase subunit) operon were found to be viable candidates for the construction of biosensors uniquely responsive to membrane damage in *S. aureus*.

In conclusion, the results from this thesis show that current and developing methods for determining the MOA of novel antibacterial agents represent useful tools for the identification of compounds with suitable characteristics for chemotherapeutic development. Therefore, increased investment in antibacterial drug discovery programmes and compound library screening may help to identify therapeutic agents to meet the challenge of the rapidly and continually increasing spread of resistant bacteria.

8.2 Future work

A number of additional experimental procedures could be undertaken to extend the analysis of the compounds described in this thesis.

Firstly, many of the compounds described in this thesis displayed a lack of activity against *E. coli* 1411, which was not improved upon addition of PMBN or with the AcrAB knockout strain (SM1411). The inactivity of such agents against the latter only rules out efflux of these compounds by the multi-drug transporters comprising the AcrAB component. Therefore, it would be necessary to test the activity of these compounds against other mutants containing knockouts of other efflux pump components, such as the TolC outer membrane protein (Shapiro & Baneyx, 2002). Ascertaining the basis for intrinsic resistance in Gram-negative bacteria may enable more active derivatives to be designed and synthesised which are not substrates for efflux. In addition, determining the exact multi-drug efflux system that is utilised in response to the new agent may provide information as to which bacterial species may be treated by the agent i.e. those in which the particular efflux pump is not present.

The variable susceptibility of different *S. aureus* strains to type B lantibiotics could also be evaluated further. The variability as described in Chapter 4 may be due to differences in the composition of the cell wall between strains, particularly in the relative abundances of lipid II. In *S. aureus,* the number of precursor molecules in the cell wall has been estimated to be approximately fifty thousand (Van Heijenoort, 2007). Quantification of lipid II abundance in different strains could be achieved as previously described (Somner & Reynolds, 1990). This involves incubation of a standard number of *S. aureus* cells with a lipid II binding antibiotic (such as ramoplanin or vancomycin) for a short period, after which the cells are harvested by centrifugation (27,000X *g* for twenty minutes). The amount of antibiotic in the supernatant is then quantified by liquid chromatography and mass spectrometry (Pozo *et al.*, 2006), from which the amount of bound antibiotic can be established, which is directly proportional to the quantity of lipid II.

As discussed in Chapters 4 and 5, the mutations that give rise to type B lantibiotic and thiolutin resistance in *S. aureus* remain uncharacterised. As such, sequencing of the entire genome of DAB6 and THIO1 as previously described (Chen *et al.*, 2007) would identify the mutated loci, and give better understanding as to the antibacterial target and MOA of, in particular, thiolutin. This information would also aid in the generation of derivatives which may overcome the resistance mechanisms and indicate how resistance might arise if these compounds were employed clinically.

As described in Chapter 5, it was discovered that CorA might possess an additional mechanism of action involving inhibition of fatty acid synthesis using *B. subtilis* biosensor assay. However, to complete the analysis of this compound, further

investigations are needed particularly in comparison with triclosan (an established fatty acid synthesis inhibitor). Triclosan-resistant MRSAs have been isolated with point mutations in FabI (Brenwald & Fraise, 2003). Therefore, sequence determination for *fabI* in the CorA resistant mutants may highlight mutations which confer CorA resistance thereby possibly confirming that α-pyrone antibiotics also inhibit fatty acid biosynthesis. An alternative option might be to assay the ability of CorA to inhibit the incorporation of [^{14}C] acetic acid into fatty acid macromolecules utilising standard methodology as described in Chapter 2 for DNA, RNA, PG and protein (King & Wu, 2001).

Confirmation of the ability of the promoters of a single-strand DNA-binding protein and *kdp* operon genes to act as biosensors for detection of membrane damaging compounds will be required. As described in Chapter 7, this could be achieved by performing quantitative reverse transcription polymerase chain reaction (qRT-PCR) on mRNA extracted from antibiotic-treated cells as described previously (Cardoso *et al.*, 2010) in order to confirm that these genes are not upregulated in response to antibacterial agents of other MOA classes. In addition, construction of the biosensors (by fusion of these promoters to a luciferase or β-galactosidase reporter gene as previously described) would enable validation of these promoters to solely detect inhibition at the level of the cell membrane (Blake *et al.*, 2009, Fischer *et al.*, 2004; Urban *et al.*, 2007).

Finally, compounds highlighted by this study as having potential for chemotherapeutic development either as targeted inhibitors (i.e. NVB353) or non-specific membrane damagers (Tocris 1700, 1838, 2611 and furanyl-rhodanine L339571) with preferential activity for prokaryotes versus eukaryotes will require further analysis. For example, although these compounds showed little activity against *S. cerevisiae*, it would be

necessary for the cytotoxicity of these compounds to be assessed against higher eukaryotes. This could be achieved by a variety of methods as previously described (Niles *et al.*, 2009). Arguably the most simple of these is to determine the ability of the agent to effect mammalian membrane damage by haemolysis of red blood cells extracted from mice or humans (Oliva *et al.*, 2003). Briefly this involves extraction of erythrocytes, which are subsequently exposed to antibiotics at a fixed concentration (e.g. 4X MIC), after which the OD_{540} is read to quantify the extent of haemolysis.

In addition, while the work described in this thesis represents the earliest stages of chemotherapeutic research and development, there is a multitude of additional assays which need to be performed with potential lead compounds before they can enter clinical trials, as shown in Figure 8.1. The compound must be assessed for its

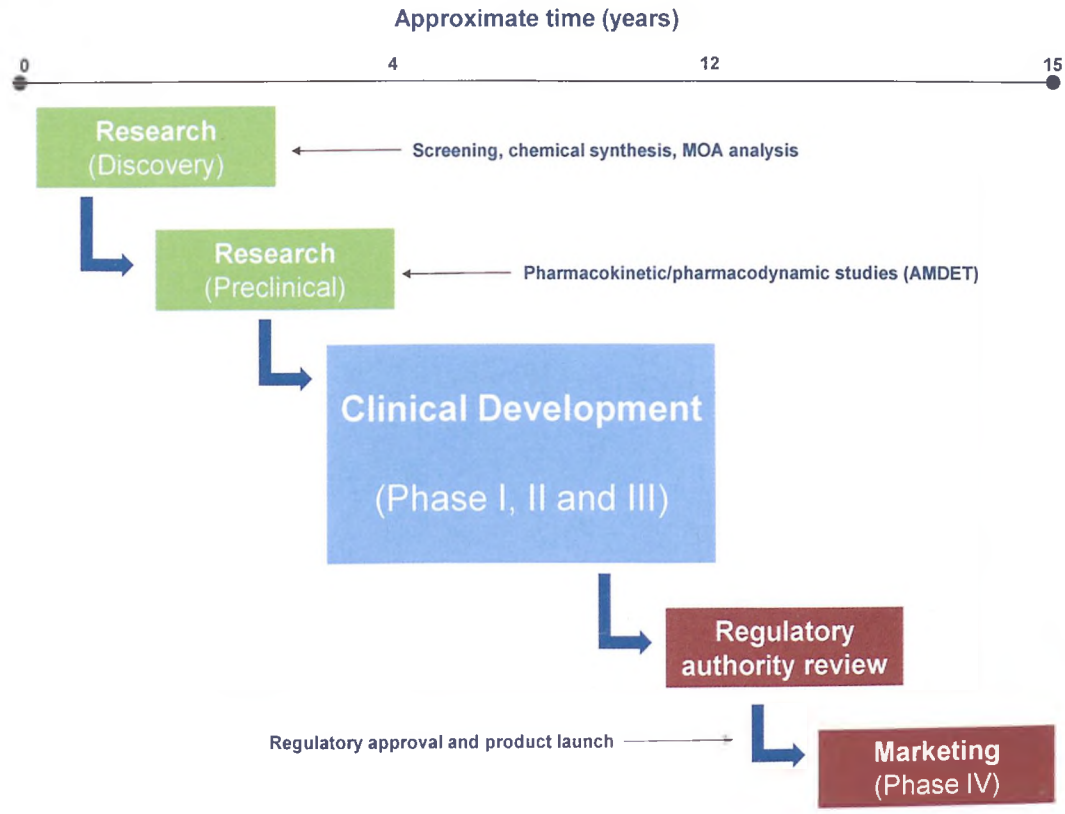


Figure 8.1 Research and development process of a novel antibacterial agent

pharmacokinetic and pharmacodynamic properties in animal studies (particularly the absorption, distribution, metabolism, excretion and toxicity [ADMET] profile of the agent) as *in vitro* activity does not provide a guarantee for *in vivo* efficacy (Gootz, 1990). The stages of ADMET testing in animals yield important information on the chemical stability, solubility, quantity in biological fluids, rate of metabolism and toleration/safety levels, which can give reliable indicators of the likely response in humans.

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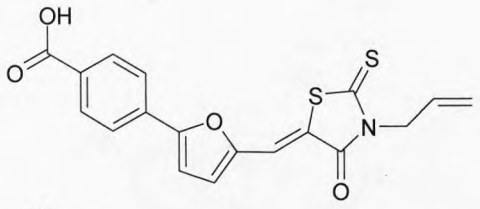
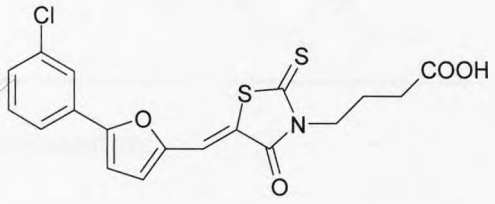
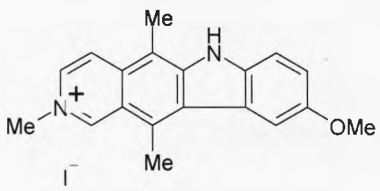
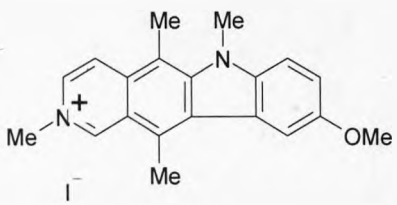
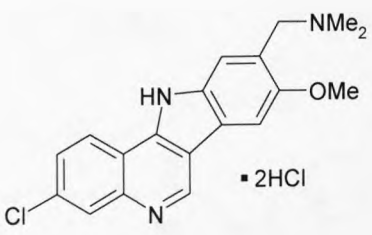
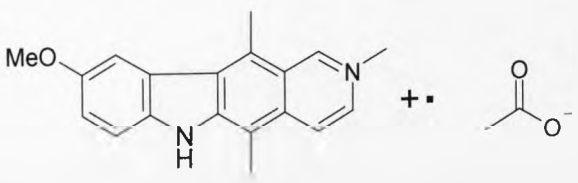
Appendix 1

Table A1 - Molecular structures of test agents

COMPOUND DESIGNATION	STRUCTURE
Furanyl-rhodanines	
VG2	
L339571	
National Cancer Institute (NCI) compound set	
155693	
155694	
352738	
627505	

NCI compound set continued…

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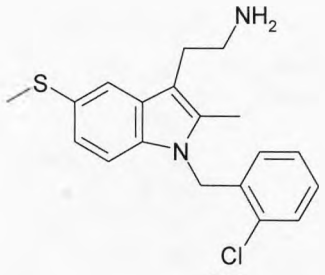

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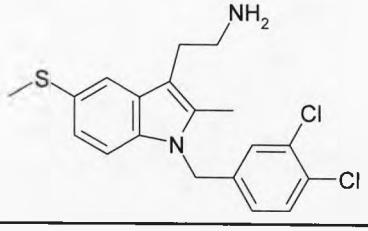

Tocris compound set

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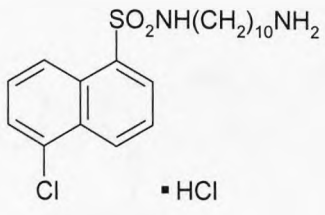

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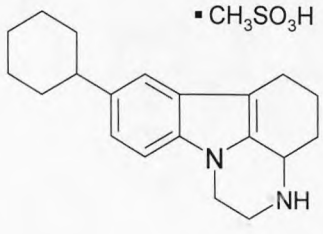

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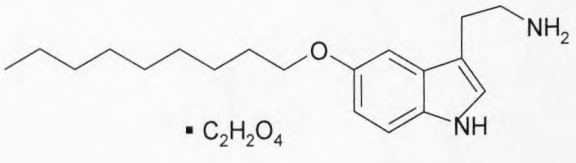

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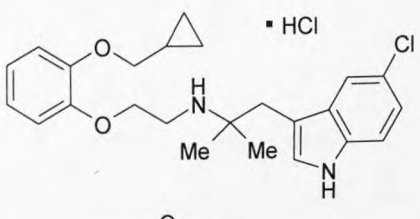

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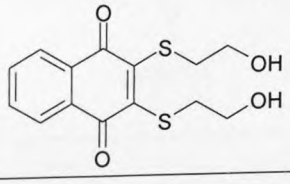

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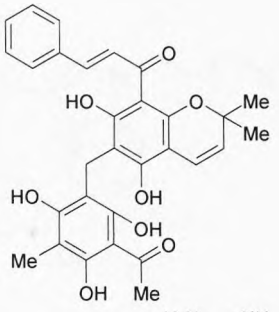

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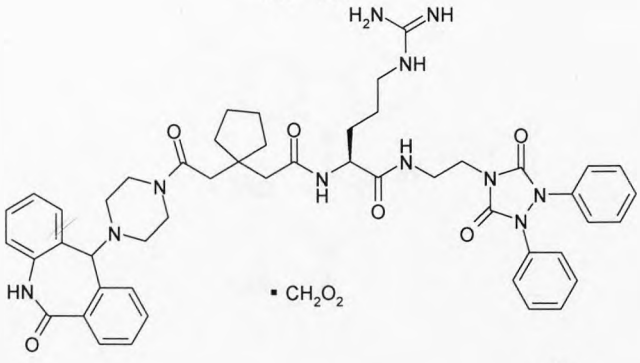

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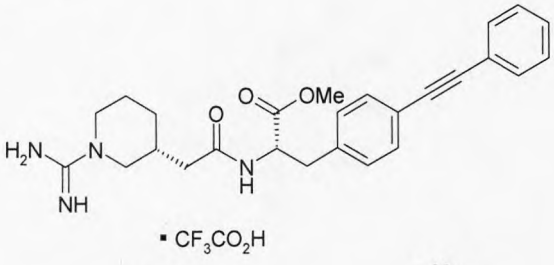

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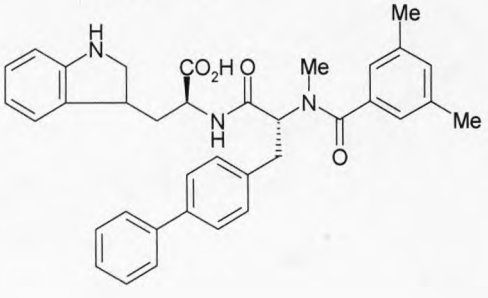

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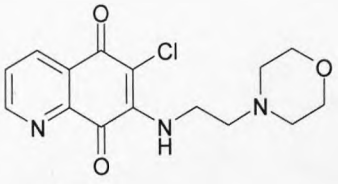

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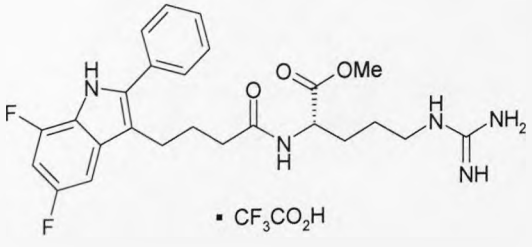

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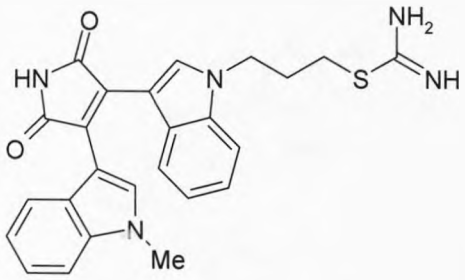

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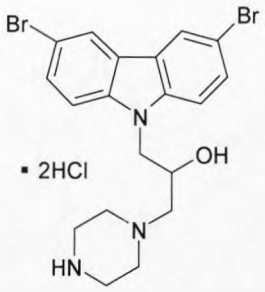

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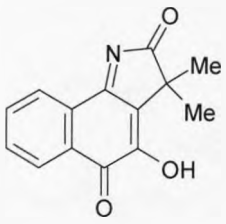

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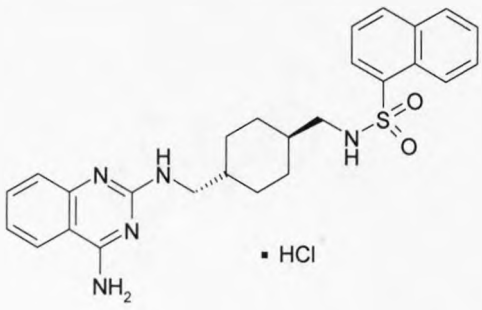

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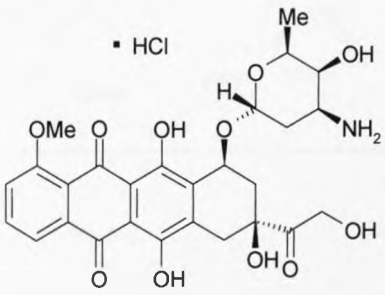

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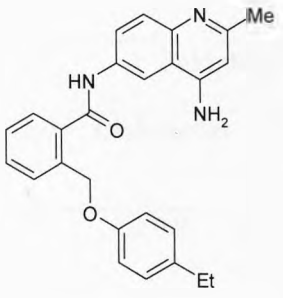

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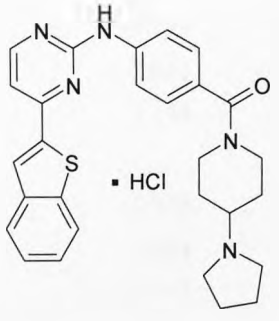

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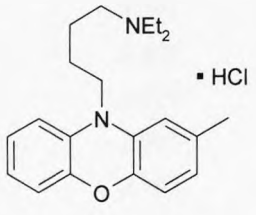

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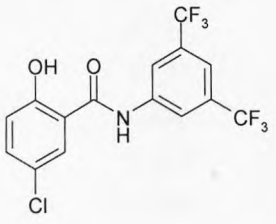

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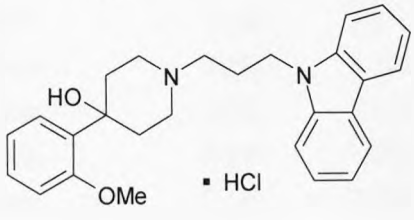

Appendix 2

Table A2 - Down-regulated genes in *S. aureus* SH1000 in response to membrane damagers

Gene	Description	Fold down-regulation		
		Clofazimine	CTAB	Sepracor 155342
SAOUHSC_00002	DNA binding protein	2.025		3.157
SAOUHSC_00006	Unknown function			2.328
SAOUHSC_00007	Conserved hypothetical protein	2.883	2.868	2.419
SAOUHSC_00009	tRNA synthesis-linked protein	2.295	2.474	2.520
SAOUHSC_00012	Conserved hypothetical protein	2.041		
SAOUHSC_00013	Conserved hypothetical protein	2.134		
SAOUHSC_00023	Conserved hypothetical protein			2.864
SAOUHSC_00024	Conserved hypothetical protein	2.073		2.413
SAOUHSC_00025	Conserved hypothetical protein	2.052		
SAOUHSC_00028	Unknown function	2.942	3.752	3.672
SAOUHSC_00029	Conserved hypothetical protein	3.214	4.908	4.573
SAOUHSC_00030	Conserved hypothetical protein	2.291	2.702	2.935
SAOUHSC_00031	Conserved hypothetical protein	2.805	2.192	2.716
SAOUHSC_00032	Conserved hypothetical protein	3.497	2.372	2.875
SAOUHSC_00034	Conserved hypothetical protein	2.092		
SAOUHSC_00037	Conserved hypothetical protein	2.792	4.123	3.01
SAOUHSC_00038	Conserved hypothetical protein	4.325	3.528	4.016
SAOUHSC_00040	Conserved hypothetical protein	2.182	2.181	2.192
SAOUHSC_00042	Conserved hypothetical protein	3.693	3.029	3.197
SAOUHSC_00043	Conserved hypothetical protein	4.260	3.461	3.507
SAOUHSC_00044	Conserved hypothetical protein	2.169	2.378	2.498
SAOUHSC_00045	Conserved hypothetical protein	4.924	3.818	2.799
SAOUHSC_00046	Conserved hypothetical protein	2.956	2.637	2.673
SAOUHSC_00047	Conserved hypothetical protein	2.245	2.225	2.158
SAOUHSC_00050	Conserved hypothetical protein	2.523	2.377	2.343
SAOUHSC_00052	Conserved hypothetical protein	2.009	2.053	
SAOUHSC_00053	Conserved hypothetical protein	2.239	2.110	
SAOUHSC_00054	Conserved hypothetical protein	2.056		
SAOUHSC_00057	Conserved hypothetical protein	2.498	2.086	
SAOUHSC_00058	Conserved hypothetical protein	2.403		
SAOUHSC_00060	Conserved hypothetical protein	3.066		2.323
SAOUHSC_00061	Unknown function	2.995	3.063	2.455
SAOUHSC_00062	Conserved hypothetical protein	2.030		
SAOUHSC_00064	Conserved hypothetical protein	2.117	2.152	
SAOUHSC_00065	Conserved hypothetical protein	2.559	3.062	

SAOUHSC_00069	Unknown function		2.112	2.188
SAOUHSC_00070	Unknown function	4.445	4.673	3.578
SAOUHSC_00072	Unknown function	2.069		
SAOUHSC_00075	Conserved hypothetical protein	2.991	2.135	
SAOUHSC_00076	Unknown function	2.254		
SAOUHSC_00077	Conserved hypothetical protein	2.073		
SAOUHSC_00078	Conserved hypothetical protein	2.246		
SAOUHSC_00079	Conserved hypothetical protein	2.574		
SAOUHSC_00080	Conserved hypothetical protein	2.660		
SAOUHSC_00081	Conserved hypothetical protein	2.243		
SAOUHSC_00083	Conserved hypothetical protein	2.120		
SAOUHSC_00086	3-ketoacyl-acyl carrier protein reductase, putative			2.148
SAOUHSC_00090	Conserved hypothetical protein	2.428	2.179	2.062
SAOUHSC_00092	Conserved hypothetical protein	2.193		
SAOUHSC_00093	Unknown function	2.154	3.724	3.766
SAOUHSC_00094	Conserved hypothetical protein	3.991	4.017	3.575
SAOUHSC_00096	Transcriptional regulator, GntR family, putative	2.727	2.541	2.700
SAOUHSC_00102	Unknown function	2.773	2.071	2.228
SAOUHSC_00103	Unknown function	2.520	2.007	2.177
SAOUHSC_00105	Unknown function	2.320	2.163	2.194
SAOUHSC_00109	Unknown function	2.122	2.578	2.059
SAOUHSC_00110	Unknown function	2.182	3.079	2.286
SAOUHSC_00111	Unknown function	3.000	2.961	2.745
SAOUHSC_00112	Transposase, IS200 family, putative	2.759	3.231	3.389
SAOUHSC_00113	Alcohol dehydrogenase, iron-containing, putative	2.408	2.188	2.010
SAOUHSC_00114	Unknown function		3.561	
SAOUHSC_00115	Unknown function		3.029	
SAOUHSC_00116	Unknown function	2.070	4.015	
SAOUHSC_00117	Unknown function		2.332	
SAOUHSC_00118	Unknown function	2.047	3.521	
SAOUHSC_00119	Unknown function		2.307	
SAOUHSC_00120	Unknown function		3.050	
SAOUHSC_00121	Unknown function		4.750	
SAOUHSC_00122	Unknown function		3.141	
SAOUHSC_00123	Unknown function	2.379	4.795	
SAOUHSC_00124	Unknown function	2.252	4.521	
SAOUHSC_00125	Unknown function		2.908	
SAOUHSC_00126	Unknown function	2.170	2.880	
SAOUHSC_00127	Unknown function		2.527	
SAOUHSC_00129	Unknown function	2.156	3.165	
SAOUHSC_00131	Conserved hypothetical protein	2.119		2.254
SAOUHSC_00132	Unknown function			2.957

SAOUHSC_00133	Conserved hypothetical protein	2.499	2.289	
SAOUHSC_00134	Conserved hypothetical protein	4.884	4.019	3.535
SAOUHSC_00135	Conserved hypothetical protein	6.657	3.425	3.307
SAOUHSC_00136	Conserved hypothetical protein	2.937		
SAOUHSC_00137	Conserved hypothetical protein	4.952	2.680	2.417
SAOUHSC_00138	Conserved hypothetical protein	2.763		
SAOUHSC_00139	Conserved hypothetical protein	3.485	2.609	2.015
SAOUHSC_00141	Conserved hypothetical protein			3.054
SAOUHSC_00142	Carbon dioxide oxidising protein	2.498	2.404	2.353
SAOUHSC_00145	Conserved hypothetical protein	3.116	4.842	2.380
SAOUHSC_00146	Unknown function		2.197	2.630
SAOUHSC_00147	Unknown function	2.520		2.500
SAOUHSC_00148	Bifunctional arginine biosynthesis protein, ArgJ	2.439		2.000
SAOUHSC_00149	Arginine biosynthesis linked protein	2.716		
SAOUHSC_00151	Unknown function	2.210	2.132	
SAOUHSC_00152	Conserved hypothetical protein	3.733	4.075	5.655
SAOUHSC_00153	Unknown function			2.160
SAOUHSC_00156	Conserved hypothetical protein	4.459	2.673	2.787
SAOUHSC_00157	Involved in MurNAc dissimilation pathway	2.350		
SAOUHSC_00160	Conserved hypothetical protein	2.158		
SAOUHSC_00161	Conserved hypothetical protein	2.927	2.186	2.46
SAOUHSC_00163	Unknown function		2.467	2.695
SAOUHSC_00164	Unknown function		2.209	2.010
SAOUHSC_00166	Conserved hypothetical protein	2.812	3.256	3.153
SAOUHSC_00167	Unknown function	2.540		
SAOUHSC_00168	Conserved hypothetical protein	2.755	2.068	2.039
SAOUHSC_00169	Unknown function	2.002		
SAOUHSC_00170	Conserved hypothetical protein	2.110		
SAOUHSC_00171	Unknown function	2.022		
SAOUHSC_00172	Conserved hypothetical protein	3.220	2.837	2.812
SAOUHSC_00173	FMN-dependent protein	2.923	3.083	3.523
SAOUHSC_00174	M23/M37 peptidase domain protein			2.070
SAOUHSC_00178	Unknown function	2.397		
SAOUHSC_00182	Conserved hypothetical protein	2.472	2.303	2.495
SAOUHSC_00184	Response regulator receiver domain protein	3.391	3.028	3.204
SAOUHSC_00189	Conserved hypothetical protein	2.094		
SAOUHSC_00190	Conserved hypothetical protein	2.965	2.335	2.232
SAOUHSC_00191	Conserved hypothetical protein	4.040	3.999	5.445
SAOUHSC_00193	Unknown function	2.175		2.463
SAOUHSC_00195	Unknown function	2.003		2.112
SAOUHSC_00196	Conserved hypothetical protein	2.099		2.222
SAOUHSC_00199	Conserved hypothetical protein	2.574		2.306

SAOUHSC_00201	Conserved hypothetical protein	3.649	4.282	5.258
SAOUHSC_00202	Conserved hypothetical protein	2.668	2.877	2.226
SAOUHSC_00203	Conserved hypothetical protein	3.354	4.021	3.125
SAOUHSC_00204	Unknown function		2.191	
SAOUHSC_00206	Unknown function		2.504	
SAOUHSC_00211	Conserved hypothetical protein	3.406	3.336	3.728
SAOUHSC_00213	Conserved hypothetical protein	2.223		
SAOUHSC_00214	Unknown function	3.042	2.357	2.699
SAOUHSC_00215	Unknown function	2.213		2.101
SAOUHSC_00231	Two-component response regulator, putative	2.284	2.095	2.180
SAOUHSC_00232	Negative regulator of murein hydrolase activity	2.179		
SAOUHSC_00234	Conserved hypothetical protein	3.213	3.153	4.086
SAOUHSC_00237	Conserved hypothetical protein	2.963		2.413
SAOUHSC_00238	Unknown function	6.737	4.749	5.022
SAOUHSC_00242	Conserved hypothetical protein	3.217	2.908	2.736
SAOUHSC_00244	Conserved hypothetical protein	4.016	3.998	3.972
SAOUHSC_00245	Truncated transposase	6.021	4.041	5.377
SAOUHSC_00248	Peptidoglycan hydrolase, putative	2.655	2.390	2.912
SAOUHSC_00249	Conserved hypothetical protein	4.068	3.344	3.632
SAOUHSC_00250	Conserved hypothetical protein	3.143	2.772	2.560
SAOUHSC_00251	Conserved hypothetical protein	2.382		
SAOUHSC_00254	Conserved hypothetical protein	6.082	4.799	6.941
SAOUHSC_00255	Conserved hypothetical protein	3.210	4.046	3.016
SAOUHSC_00257	Conserved hypothetical protein	2.279	2.338	
SAOUHSC_00258	Conserved hypothetical protein	2.540		
SAOUHSC_00259	Conserved hypothetical protein	2.371		
SAOUHSC_00260	Conserved hypothetical protein	4.835	2.997	2.833
SAOUHSC_00261	Conserved hypothetical protein	3.179	2.224	
SAOUHSC_00262	Conserved hypothetical protein	2.815		
SAOUHSC_00264	Conserved hypothetical protein	2.466		
SAOUHSC_00265	Conserved hypothetical protein	2.249		
SAOUHSC_00266	Conserved hypothetical protein	2.610		
SAOUHSC_00267	Conserved hypothetical protein	2.189		
SAOUHSC_00270	Conserved hypothetical protein	2.680	2.648	2.494
SAOUHSC_00271	Conserved hypothetical protein	2.636	2.582	2.353
SAOUHSC_00272	Conserved hypothetical protein	3.871	3.044	2.616
SAOUHSC_00278	Conserved hypothetical protein	2.301	2.705	
SAOUHSC_00279	Conserved hypothetical protein	2.102	3.107	
SAOUHSC_00280	Conserved hypothetical protein	2.839	3.209	
SAOUHSC_00282	Unknown function			2.161
SAOUHSC_00284	5'-nucleotidase, lipoprotein e(P4) family	2.069	3.155	
SAOUHSC_00285	Conserved hypothetical protein		2.271	

SAOUHSC_00287	Unknown function		2.178	
SAOUHSC_00289	Unknown function	3.018	2.678	2.545
SAOUHSC_00293	Conserved hypothetical protein	2.110	2.221	
SAOUHSC_00300	Unknown function		2.589	
SAOUHSC_00301	Conserved hypothetical protein	3.437	2.864	3.017
SAOUHSC_00302	Conserved hypothetical protein	3.082		
SAOUHSC_00303	Unknown function	2.261	2.202	
SAOUHSC_00309	Conserved hypothetical protein	3.011	4.575	2.409
SAOUHSC_00310	Membrane associated protein	2.282		
SAOUHSC_00313	Conserved hypothetical protein	2.290		
SAOUHSC_00314	Conserved hypothetical protein	2.120		2.660
SAOUHSC_00317	Unknown function			2.418
SAOUHSC_00318	Conserved hypothetical protein	2.400	2.259	
SAOUHSC_00320	Unknown function	6.215	5.825	5.972
SAOUHSC_00322	Conserved hypothetical protein	2.116	2.213	2.490
SAOUHSC_00323	Conserved hypothetical protein	3.092	2.850	2.891
SAOUHSC_00325	Conserved hypothetical protein	2.364	2.088	
SAOUHSC_00327	Conserved hypothetical protein	2.132	2.092	2.212
SAOUHSC_00328	Conserved hypothetical protein	2.013		
SAOUHSC_00329	Unknown function	2.417		
SAOUHSC_00330	Conserved hypothetical protein	2.450		
SAOUHSC_00331	Conserved hypothetical protein	2.595	2.945	2.340
SAOUHSC_00335	Conserved hypothetical protein	2.924	2.159	2.198
SAOUHSC_00337	Unknown function			2.267
SAOUHSC_00339	Unknown function	2.275		
SAOUHSC_00340	Unknown function	2.237		
SAOUHSC_00347	Conserved hypothetical protein	2.575		
SAOUHSC_00350	16S rRNA stabilising protein	6.656	8.447	8.636
SAOUHSC_00351	Conserved hypothetical protein	4.216	4.189	4.016
SAOUHSC_00352	Unknown function	3.019	2.238	
SAOUHSC_00353	Conserved hypothetical protein	2.756	2.089	
SAOUHSC_00354	Conserved hypothetical protein	3.869	4.037	3.120
SAOUHSC_00355	Conserved hypothetical protein	2.472	3.446	2.598
SAOUHSC_00356	Conserved hypothetical protein		5.416	
SAOUHSC_00357	Conserved hypothetical protein	4.817	5.986	4.892
SAOUHSC_00358	Conserved hypothetical protein	4.337	5.482	3.153
SAOUHSC_00359	Unknown function	3.413	2.340	2.292
SAOUHSC_00360	Conserved hypothetical protein	3.148		2.110
SAOUHSC_00362	Conserved hypothetical protein			2.063
SAOUHSC_00363	Conserved hypothetical protein		2.350	2.177
SAOUHSC_00366	Unknown function			2.192
SAOUHSC_00367	Conserved hypothetical protein	3.568	2.759	3.403
SAOUHSC_00368	Conserved hypothetical protein	6.152	4.655	5.993

SAOUHSC_00369	Conserved hypothetical protein	2.328	2.350	3.826
SAOUHSC_00371	Unknown function			2.524
SAOUHSC_00372	Phosphoribosyltransferase	2.106		
SAOUHSC_00376	Unknown function	4.053	3.905	4.437
SAOUHSC_00377	Conserved hypothetical protein	4.370	4.496	3.950
SAOUHSC_00378	Conserved hypothetical protein	3.811	3.873	4.653
SAOUHSC_00380	Unknown function	3.220	3.254	3.256
SAOUHSC_00381	Conserved hypothetical protein		2.284	2.545
SAOUHSC_00384	Structural homologue of superantigen protein	2.007	2.308	2.011
SAOUHSC_00392	SSL7, SET1	2.531	3.143	2.927
SAOUHSC_00393	Complement associated protein	2.589	3.874	2.352
SAOUHSC_00394	SSL7, SET1	2.028	2.029	2.001
SAOUHSC_00395	SSL7, SET1	2.001	2.174	
SAOUHSC_00398	Unknown function		2.009	
SAOUHSC_00400	Conserved hypothetical protein	2.512	3.207	3.004
SAOUHSC_00401	Conserved hypothetical protein			2.987
SAOUHSC_00409	Conserved hypothetical protein	2.428	2.337	2.745
SAOUHSC_00410	Conserved hypothetical protein	2.917	2.571	2.456
SAOUHSC_00411	Conserved hypothetical protein	14.835	2.874	3.494
SAOUHSC_00420	Conserved hypothetical protein	2.650		2.232
SAOUHSC_00421	Conserved hypothetical protein	4.183		2.263
SAOUHSC_00422	Unknown function	3.661		2.565
SAOUHSC_00423	Conserved hypothetical protein	2.716	2.101	
SAOUHSC_00424	Unknown function	3.530	2.604	
SAOUHSC_00426	Unknown function	3.377	2.548	2.119
SAOUHSC_00427	Unknown function	2.489	2.136	2.307
SAOUHSC_00428	Conserved hypothetical protein	3.178	2.394	4.011
SAOUHSC_00429	Unknown function	2.428	2.181	2.886
SAOUHSC_00430	Conserved hypothetical protein	2.280	2.078	2.635
SAOUHSC_00431	Conserved hypothetical protein	2.048		2.358
SAOUHSC_00439	Conserved hypothetical protein			2.188
SAOUHSC_00440	Conserved hypothetical protein	3.375	2.058	4.337
SAOUHSC_00445	DNA recombination associated protein	2.619	2.364	3.184
SAOUHSC_00446	Unknown function	2.2		2.479
SAOUHSC_00453	Unknown function		2.123	
SAOUHSC_00456	Unknown function	2.534		2.003
SAOUHSC_00460	Conserved hypothetical protein	2.586	2.632	2.037
SAOUHSC_00464	Methyltransferase	2.132		
SAOUHSC_00465	Conserved hypothetical protein		2.072	2.217
SAOUHSC_00469	Stage V sporulation protein G	4.372	6.454	4.561
SAOUHSC_00470	Conserved hypothetical protein	4.175	3.898	2.865
SAOUHSC_00473	Unknown function	2.383	2.015	3.163
SAOUHSC_00474	Unknown function	2.645	2.217	3.37

SAOUHSC_00482	Conserved hypothetical protein			2.213
SAOUHSC_00483	S1 RNA binding domain protein	2.156		
SAOUHSC_00488	Conserved hypothetical protein	2.852	3.289	3.308
SAOUHSC_00490	Unknown function		2.023	
SAOUHSC_00497	Conserved hypothetical protein	2.761	2.714	2.955
SAOUHSC_00501	Conserved hypothetical protein	3.091	3.269	3.989
SAOUHSC_00506	Unknown function			4.948
SAOUHSC_00521	Multicopy ribosomal protein			2.040
SAOUHSC_00532	2-amino-3-oxobutanoate synthase		2.096	2.944
SAOUHSC_00537	Conserved hypothetical protein		2.660	
SAOUHSC_00538	Unknown function		2.091	2.154
SAOUHSC_00539	Conserved hypothetical protein			2.177
SAOUHSC_00540	Conserved hypothetical protein	2.059	3.187	2.160
SAOUHSC_00541	Conserved hypothetical protein	2.079		
SAOUHSC_00543	Conserved hypothetical protein		3.233	3.720
SAOUHSC_00544	Unknown function	2.027	2.259	
SAOUHSC_00545	Unknown function		2.112	
SAOUHSC_00547	Conserved hypothetical protein	3.422	3.410	2.963
SAOUHSC_00548	Conserved hypothetical protein			2.257
SAOUHSC_00555	Unknown function	5.214	5.362	4.547
SAOUHSC_00556	Unknown function	2.266	3.263	2.517
SAOUHSC_00557	Conserved hypothetical protein	2.022	2.741	
SAOUHSC_00561	Conserved hypothetical protein			2.470
SAOUHSC_00562	Unknown function			2.028
SAOUHSC_00567	Conserved hypothetical protein	2.852	2.604	2.925
SAOUHSC_00569	Conserved hypothetical protein	2.022	2.716	
SAOUHSC_00571	Conserved hypothetical protein	3.201		2.209
SAOUHSC_00573	Conserved hypothetical protein		2.156	3.076
SAOUHSC_00574	Unknown function		2.371	2.427
SAOUHSC_00575	Conserved hypothetical protein		2.691	2.710
SAOUHSC_00580	Conserved hypothetical protein	3.016	2.381	3.049
SAOUHSC_00581	Conserved hypothetical protein	2.808	2.556	2.598
SAOUHSC_00585	Conserved hypothetical protein	2.472	2.986	2.111
SAOUHSC_00586	Conserved hypothetical protein		2.006	2.693
SAOUHSC_00587	Conserved hypothetical protein	2.883	2.734	2.964
SAOUHSC_00588	Conserved hypothetical protein	3.200	2.958	2.925
SAOUHSC_00589	Unknown function	3.517	3.711	7.337
SAOUHSC_00591	Conserved hypothetical protein	6.588	5.184	4.892
SAOUHSC_00592	Conserved hypothetical protein	3.410	3.232	5.353
SAOUHSC_00593	Conserved hypothetical protein	3.792	3.426	3.793
SAOUHSC_00595	Conserved hypothetical protein	2.591	2.588	4.598
SAOUHSC_00596	Conserved hypothetical protein	3.424	3.178	4.080
SAOUHSC_00598	Conserved hypothetical protein	3.825	3.471	

SAOUHSC_00602	Conserved hypothetical protein	2.711	2.449	2.015
SAOUHSC_00607	Conserved hypothetical protein	3.881	4.413	4.510
SAOUHSC_00609	Conserved hypothetical protein	2.939	3.602	3.275
SAOUHSC_00611	Aminoacyl-tRNA synthesis linked protein			2.126
SAOUHSC_00612	Unknown function	4.569	4.389	5.162
SAOUHSC_00613	Unknown function		2.170	
SAOUHSC_00615	Unknown function	2.409	2.319	2.699
SAOUHSC_00617	Conserved hypothetical protein	2.787	2.647	3.757
SAOUHSC_00618	Conserved hypothetical protein	2.983	2.775	2.808
SAOUHSC_00619	Conserved hypothetical protein		4.259	2.101
SAOUHSC_00620	Staphylococcal accessory regulator T, putative	2.831	4.227	6.097
SAOUHSC_00621	Conserved hypothetical protein	3.652	3.682	2.959
SAOUHSC_00622	Conserved hypothetical protein	3.714	3.378	2.930
SAOUHSC_00624	Unknown function	3.125	3.217	
SAOUHSC_00625	Subunit A of alkali metal ion antiporter	2.508	2.024	
SAOUHSC_00626	Subunit B of alkali metal ion antiporter	3.590	2.995	2.028
SAOUHSC_00627	Subunit C of alkali metal ion antiporter	3.015	2.651	
SAOUHSC_00628	Subunit D of alkali metal ion antiporter	2.739		
SAOUHSC_00629	Subunit E of alkali metal ion antiporter	2.422	2.154	
SAOUHSC_00630	Unknown function	2.568		
SAOUHSC_00632	Subunit G of alkali metal ion antiporter	4.158		2.727
SAOUHSC_00638	Conserved hypothetical protein	3.405		4.229
SAOUHSC_00639	Conserved hypothetical protein	2.370		2.182
SAOUHSC_00640	Conserved hypothetical protein	2.703	2.283	2.413
SAOUHSC_00641	Teichoic acid export protein			2.241
SAOUHSC_00642	Unknown function	3.688	3.677	5.027
SAOUHSC_00645	Unknown function	2.573	2.558	2.759
SAOUHSC_00648	Conserved hypothetical protein	2.070	2.250	2.559
SAOUHSC_00650	Conserved hypothetical protein	2.205		2.036
SAOUHSC_00660	Conserved hypothetical protein		2.072	2.332
SAOUHSC_00661	Conserved hypothetical protein	3.312	2.941	3.994
SAOUHSC_00662	Conserved hypothetical protein	4.635	4.810	8.643
SAOUHSC_00663	Conserved hypothetical protein	2.593	3.536	3.043
SAOUHSC_00664	Conserved hypothetical protein			2.049
SAOUHSC_00665	Conserved hypothetical protein			2.085
SAOUHSC_00666	Conserved hypothetical protein			2.106
SAOUHSC_00670	Conserved hypothetical protein	4.376	3.237	4.894
SAOUHSC_00671	Unknown function	2.116	3.061	3.462
SAOUHSC_00672	Conserved hypothetical protein		2.169	2.297
SAOUHSC_00673	Conserved hypothetical protein	2.463	2.129	
SAOUHSC_00674	Conserved hypothetical protein	5.069	4.763	2.505
SAOUHSC_00676	Conserved hypothetical protein	2.177	2.635	2.657

SAOUHSC_00677	Conserved hypothetical protein	4.618	3.068	2.960
SAOUHSC_00680	Unknown function	2.801	3.748	3.469
SAOUHSC_00681	Unknown function			2.248
SAOUHSC_00682	Conserved hypothetical protein	2.194	2.037	3.567
SAOUHSC_00683	Conserved hypothetical protein	2.716	2.413	2.202
SAOUHSC_00684	Conserved hypothetical protein	3.170	3.600	
SAOUHSC_00686	Conserved hypothetical protein	2.55	3.218	2.682
SAOUHSC_00687	Conserved hypothetical protein	2.332	3.144	2.588
SAOUHSC_00688	Conserved hypothetical protein	2.679	3.150	2.672
SAOUHSC_00689	Conserved hypothetical protein	2.174	3.074	
SAOUHSC_00690	Conserved hypothetical protein	2.196	2.909	
SAOUHSC_00691	Unknown function	2.107	2.152	2.887
SAOUHSC_00694	Conserved hypothetical protein	3.872	5.307	6.280
SAOUHSC_00695	Conserved hypothetical protein	2.380	2.792	4.029
SAOUHSC_00697	Conserved hypothetical protein	2.226	2.701	2.855
SAOUHSC_00700	Conserved hypothetical protein	4.628	3.549	3.320
SAOUHSC_00701	Conserved hypothetical protein	3.650	3.282	3.974
SAOUHSC_00702	Conserved hypothetical protein	2.806	3.075	3.881
SAOUHSC_00704	Conserved hypothetical protein	2.013	2.891	3.031
SAOUHSC_00705	Conserved hypothetical protein	2.094	2.293	
SAOUHSC_00709	Unknown function	4.014	4.827	9.286
SAOUHSC_00718	Conserved hypothetical protein	2.602	3.321	5.779
SAOUHSC_00728	Conserved hypothetical protein			2.558
SAOUHSC_00729	Unknown function	2.284		
SAOUHSC_00730	Unknown function	2.960		2.613
SAOUHSC_00733	Unknown function	2.378		
SAOUHSC_00734	Conserved hypothetical protein	3.534	3.083	3.440
SAOUHSC_00735	Conserved hypothetical protein	4.654	4.946	3.308
SAOUHSC_00736	Conserved hypothetical protein	3.250	5.951	2.972
SAOUHSC_00737	Unknown function	4.568	4.902	6.967
SAOUHSC_00738	Conserved hypothetical protein			2.056
SAOUHSC_00739	Queuosine biosynthetic enzyme	2.941	3.082	3.688
SAOUHSC_00744	Conserved hypothetical protein	2.836	2.548	2.460
SAOUHSC_00746	Conserved hypothetical protein	4.276	2.931	3.243
SAOUHSC_00747	Conserved hypothetical protein	2.939		2.084
SAOUHSC_00748	Conserved hypothetical protein	3.721	2.370	2.326
SAOUHSC_00749	Conserved hypothetical protein	3.480	4.098	3.836
SAOUHSC_00751	Conserved hypothetical protein		2.008	2.255
SAOUHSC_00753	Conserved hypothetical protein	2.357	3.549	3.734
SAOUHSC_00754	Conserved hypothetical protein		2.309	2.529
SAOUHSC_00756	Conserved hypothetical protein			2.073
SAOUHSC_00760	Conserved hypothetical protein	2.316	2.767	3.706
SAOUHSC_00763	Conserved hypothetical protein	2.821	2.795	3.728

SAOUHSC_00764	Conserved hypothetical protein	3.050	2.757	2.934
SAOUHSC_00765	Conserved hypothetical protein	2.305		
SAOUHSC_00766	Conserved hypothetical protein	2.241	2.300	2.248
SAOUHSC_00770	Conserved hypothetical protein	4.369	2.896	3.275
SAOUHSC_00772	Conserved hypothetical protein	5.724	3.912	5.045
SAOUHSC_00773	LysM domain protein	2.899	2.870	3.216
SAOUHSC_00774	Conserved hypothetical protein		2.378	2.366
SAOUHSC_00775	Conserved hypothetical protein	2.448	2.499	2.713
SAOUHSC_00785	Unknown function	2.432	2.286	3.366
SAOUHSC_00786	Conserved hypothetical protein	2.453	2.812	2.085
SAOUHSC_00790	Unknown function			3.192
SAOUHSC_00792	Conserved hypothetical protein	3.926	3.084	3.092
SAOUHSC_00799	Enolase			2.353
SAOUHSC_00804	ssrA RNA (tmRNA) binding protein	2.169		2.259
SAOUHSC_00805	Conserved hypothetical protein	2.809	2.749	2.698
SAOUHSC_00806	Conserved hypothetical protein	6.020	8.412	5.277
SAOUHSC_00807	Conserved hypothetical protein	3.269	4.512	2.410
SAOUHSC_00808	Conserved hypothetical protein	2.888	3.874	
SAOUHSC_00809	Conserved hypothetical protein	5.311	6.587	2.177
SAOUHSC_00810	Conserved hypothetical protein	2.528	3.727	
SAOUHSC_00811	Conserved hypothetical protein	2.287	2.272	
SAOUHSC_00812	Unknown function		2.118	
SAOUHSC_00813	Unverified frameshift	2.282	2.799	2.537
SAOUHSC_00814	Unverified frameshift	2.122	3.106	2.874
SAOUHSC_00816	Unknown function		3.015	
SAOUHSC_00817	Conserved hypothetical protein		2.773	2.551
SAOUHSC_00818	Unknown function	2.874	3.450	2.505
SAOUHSC_00819	Conserved hypothetical protein	3.744	3.420	5.006
SAOUHSC_00820	Conserved hypothetical protein	2.168	3.183	
SAOUHSC_00821	Conserved hypothetical protein	2.046	2.643	
SAOUHSC_00823	Conserved hypothetical protein	7.203	5.197	5.490
SAOUHSC_00824	Conserved hypothetical protein	5.031	5.293	5.167
SAOUHSC_00825	Conserved hypothetical protein		4.454	
SAOUHSC_00826	Conserved hypothetical protein	2.492	5.381	2.021
SAOUHSC_00827	Conserved hypothetical protein	2.952	2.610	2.912
SAOUHSC_00828	Conserved hypothetical protein	4.157	4.424	4.788
SAOUHSC_00830	Conserved hypothetical protein		2.878	
SAOUHSC_00831	Conserved hypothetical protein		3.408	
SAOUHSC_00833	Conserved hypothetical protein		2.252	2.191
SAOUHSC_00834	Unknown function	2.222	2.704	3.990
SAOUHSC_00835	Conserved hypothetical protein		2.235	2.116
SAOUHSC_00836	Unknown function		2.386	2.577
SAOUHSC_00837	Conserved hypothetical protein	2.485	3.745	2.922

SAOUHSC_00839	Conserved hypothetical protein	2.829	4.444	5.376
SAOUHSC_00840	Conserved hypothetical protein	3.385	5.137	3.858
SAOUHSC_00841	Conserved hypothetical protein	4.705	5.566	4.235
SAOUHSC_00842	Unknown function	2.558		2.389
SAOUHSC_00843	Conserved hypothetical protein	2.132		2.297
SAOUHSC_00844	Conserved hypothetical protein	2.638		2.836
SAOUHSC_00845	Conserved hypothetical protein		3.693	
SAOUHSC_00846	Conserved hypothetical protein	2.808	2.235	
SAOUHSC_00852	Conserved hypothetical protein	3.378	2.716	4.137
SAOUHSC_00853	Conserved hypothetical protein	4.769	3.548	5.095
SAOUHSC_00862	Conserved hypothetical protein	3.105	3.579	4.283
SAOUHSC_00863	Conserved hypothetical protein	3.059	2.979	3.726
SAOUHSC_00867	Conserved hypothetical protein		3.088	
SAOUHSC_00872	Unknown function		2.413	2.433
SAOUHSC_00873	Conserved hypothetical protein		2.879	3.586
SAOUHSC_00874	Conserved hypothetical protein	2.626	2.395	2.946
SAOUHSC_00876	Conserved hypothetical protein			2.507
SAOUHSC_00877	Conserved hypothetical protein	2.246	2.899	4.302
SAOUHSC_00878	Conserved hypothetical protein			2.030
SAOUHSC_00879	Probable cytosol aminopeptidase		2.261	2.258
SAOUHSC_00880	Conserved hypothetical protein	2.011		3.318
SAOUHSC_00881	Conserved hypothetical protein	2.720	2.035	4.147
SAOUHSC_00882	Conserved hypothetical protein	2.922	2.380	2.623
SAOUHSC_00883	Subunit G of alkali metal ion antiporter	3.677	2.267	5.32
SAOUHSC_00884	Subunit F of alkali metal ion antiporter	3.234		4.739
SAOUHSC_00890	Conserved hypothetical protein		2.014	
SAOUHSC_00891	Unknown function	2.217	2.333	2.761
SAOUHSC_00892	Conserved hypothetical protein		2.389	
SAOUHSC_00896	Conserved hypothetical protein	3.503	2.948	3.637
SAOUHSC_00897	Conserved hypothetical protein	3.639	3.343	3.906
SAOUHSC_00898	Arginine synthase			2.410
SAOUHSC_00900	Member of PGI family			2.615
SAOUHSC_00901	Conserved hypothetical protein		2.325	2.984
SAOUHSC_00902	Unknown function		2.197	3.087
SAOUHSC_00903	Unknown function	2.532	2.934	4.389
SAOUHSC_00907	Conserved hypothetical protein	3.860	2.959	3.113
SAOUHSC_00911	Conserved hypothetical protein	3.318	3.282	3.618
SAOUHSC_00913	Conserved hypothetical protein	2.574	2.404	2.898
SAOUHSC_00915	Conserved hypothetical protein	4.283	3.683	2.967
SAOUHSC_00916	Conserved hypothetical protein	3.206	3.104	2.272
SAOUHSC_00917	Conserved hypothetical protein	2.386	2.118	
SAOUHSC_00918	Unknown function		2.220	
SAOUHSC_00919	Conserved hypothetical protein	2.919	3.232	3.847

SAOUHSC_00922	Conserved hypothetical protein	8.083	5.570	8.609
SAOUHSC_00928	Unknown function		2.039	
SAOUHSC_00929	Unknown function	2.466	2.541	2.055
SAOUHSC_00930	Unknown function	2.760	2.719	2.372
SAOUHSC_00931	Unknown function	3.161	2.968	2.634
SAOUHSC_00932	Unknown function	2.959	2.621	2.783
SAOUHSC_00935	Involved in negative regulation of competence			2.491
SAOUHSC_00936	Conserved hypothetical protein	2.164		
SAOUHSC_00938	Conserved hypothetical protein		2.151	2.477
SAOUHSC_00939	Conserved hypothetical protein			2.064
SAOUHSC_00946	Conserved hypothetical protein			2.135
SAOUHSC_00947	Fatty acid biosynthetic enzyme			2.581
SAOUHSC_00950	Conserved hypothetical protein	2.153	2.691	2.519
SAOUHSC_00951	Similar to 2'-5' RNA ligase		2.036	2.137
SAOUHSC_00952	Conserved hypothetical protein	2.986	2.596	4.288
SAOUHSC_00953	Membrane glycolipid synthase			2.778
SAOUHSC_00957	Conserved hypothetical protein	2.646		
SAOUHSC_00959	Conserved hypothetical protein	3.281	3.436	4.213
SAOUHSC_00961	Conserved hypothetical protein	4.532	3.211	2.425
SAOUHSC_00962	Conserved hypothetical protein			2.135
SAOUHSC_00963	Unknown function			2.073
SAOUHSC_00964	Conserved hypothetical protein		2.133	2.032
SAOUHSC_00965	Conserved hypothetical protein	5.621	4.516	6.931
SAOUHSC_00966	Conserved hypothetical protein	3.628	2.688	3.936
SAOUHSC_00968	Conserved hypothetical protein	2.530	2.642	2.326
SAOUHSC_00969	Conserved hypothetical protein	4.041	4.361	3.869
SAOUHSC_00970	Unknown function	3.290	3.889	2.922
SAOUHSC_00971	Conserved hypothetical protein	3.726	4.167	6.704
SAOUHSC_00972	Conserved hypothetical protein	2.193	3.509	2.334
SAOUHSC_00973	Conserved hypothetical protein		2.940	
SAOUHSC_00974	Unknown function	2.142	3.155	2.501
SAOUHSC_00975	Conserved hypothetical protein	3.665	3.989	4.923
SAOUHSC_00976	Conserved hypothetical protein	2.902	2.793	2.495
SAOUHSC_00977	Conserved hypothetical protein	3.783	4.205	3.522
SAOUHSC_00978	Conserved hypothetical protein	2.462	3.459	4.970
SAOUHSC_00980	Dimethylmenaquinone synthase			2.028
SAOUHSC_00985	1,4-dihydroxy-2-naphthoate synthase			2.818
SAOUHSC_00986	Unknown function	3.936	2.931	3.604
SAOUHSC_00987	Unknown function	2.975	3.344	
SAOUHSC_00988	Unknown function	3.431	3.345	
SAOUHSC_00991	Conserved hypothetical protein	3.272	2.338	3.575
SAOUHSC_00992	Transcriptional regulator, MarR family, putative	3.228	2.935	3.311

SAOUHSC_00995	Conserved hypothetical protein	2.635	2.214	
SAOUHSC_00996	Conserved hypothetical protein	3.880	3.794	6.044
SAOUHSC_00998	Unknown function			2.222
SAOUHSC_00999	Unknown function	2.414	3.344	2.682
SAOUHSC_01003	Unknown function	2.171	2.516	2.245
SAOUHSC_01005	Conserved hypothetical protein		2.237	
SAOUHSC_01007	Unknown function			3.898
SAOUHSC_01008	Unknown function	2.950		
SAOUHSC_01009	*De novo* purine nucleotide biosynthetic enzyme	2.479		
SAOUHSC_01010	SAICAR synthase	3.537		
SAOUHSC_01011	Unknown function	2.738		
SAOUHSC_01012	Acetamidine synthase	3.175		
SAOUHSC_01013	Acetamidine synthase	3.020		
SAOUHSC_01014	De novo purine nucleotide biosynthetic enzyme	2.366		
SAOUHSC_01015	1-(5-phosphoribosyl)-5-aminoimidazole synthase	2.415		
SAOUHSC_01016	Unknown function	4.091		
SAOUHSC_01017	Involved in de novo purine biosynthesis	2.927		
SAOUHSC_01018	5-phospho-D-ribosyl glycinamide synthase	2.485		
SAOUHSC_01019	Conserved hypothetical protein	3.880	4.488	5.612
SAOUHSC_01023	Unknown function	3.721	5.543	4.316
SAOUHSC_01024	Unknown function	4.401	9.734	4.094
SAOUHSC_01029	Unknown function			2.224
SAOUHSC_01030	Conserved hypothetical protein	6.219	5.375	6.992
SAOUHSC_01037	Unknown function	2.014	2.285	
SAOUHSC_01038	Peptide deformylase			2.452
SAOUHSC_01039	Conserved hypothetical protein			2.272
SAOUHSC_01044	Conserved hypothetical protein	4.398	3.502	3.970
SAOUHSC_01048	Unknown function	2.011		
SAOUHSC_01051	Conserved hypothetical protein	4.480	3.773	6.242
SAOUHSC_01053	Conserved hypothetical protein	3.194	2.155	3.613
SAOUHSC_01057	Unknown function	5.968	6.604	8.005
SAOUHSC_01060	Conserved hypothetical protein	3.722	5.208	8.461
SAOUHSC_01061	Conserved hypothetical protein	2.078	2.901	2.540
SAOUHSC_01062	Unknown function	2.570	3.834	4.095
SAOUHSC_01063	Conserved hypothetical protein			3.272
SAOUHSC_01065	Unknown function	2.387	2.864	3.198
SAOUHSC_01067	Conserved hypothetical protein	3.167	3.494	2.688
SAOUHSC_01068	Conserved hypothetical protein		2.340	
SAOUHSC_01070	Conserved hypothetical protein	2.453	2.632	2.359
SAOUHSC_01071	Unknown function		2.082	2.413
SAOUHSC_01074	Conserved hypothetical protein			2.165

SAOUHSC_01075	Diphosphate and 3'-dephospho-coA synthase	3.187	2.405	4.548
SAOUHSC_01078	Unknown function	2.558	2.159	3.489
SAOUHSC_01079	Unknown function	2.621	2.642	2.330
SAOUHSC_01081	Conserved hypothetical protein		2.460	
SAOUHSC_01082	Conserved hypothetical protein	3.878	3.334	2.510
SAOUHSC_01084	Conserved hypothetical protein	4.894	4.196	2.875
SAOUHSC_01085	Conserved hypothetical protein	2.479	2.156	
SAOUHSC_01086	Unknown function	2.144		
SAOUHSC_01087	Unknown function	2.036		
SAOUHSC_01088	Conserved hypothetical protein	3.071	2.875	2.211
SAOUHSC_01089	Involved with haem release from Hb	2.924	2.818	2.496
SAOUHSC_01095	RNA endonuclease	2.532	2.588	3.002
SAOUHSC_01096	Conserved hypothetical protein	2.569	2.764	2.598
SAOUHSC_01097	Conserved hypothetical protein	2.095	2.325	2.160
SAOUHSC_01100	Unknown function	2.202	2.010	2.285
SAOUHSC_01105	Component of succinate dehydrogenase			2.478
SAOUHSC_01109	Conserved hypothetical protein	6.333	7.351	12.552
SAOUHSC_01110	Unknown function		3.612	5.423
SAOUHSC_01111	Unknown function	2.925	4.429	4.151
SAOUHSC_01112	FLIPr	3.462	3.550	5.319
SAOUHSC_01113	Conserved hypothetical protein	3.027	3.248	4.682
SAOUHSC_01115	Conserved hypothetical protein		2.241	2.638
SAOUHSC_01118	Conserved hypothetical protein	2.640	2.332	
SAOUHSC_01119	Conserved hypothetical protein	2.785	3.418	4.200
SAOUHSC_01120	Conserved hypothetical protein	2.996	4.772	3.511
SAOUHSC_01121	Unknown function	3.664	4.312	5.879
SAOUHSC_01122	Conserved hypothetical protein	4.244	5.415	8.749
SAOUHSC_01123	Conserved hypothetical protein	5.182	6.073	8.167
SAOUHSC_01124	Structural homologue of superantigen protein	4.740	3.881	3.957
SAOUHSC_01125	Structural homologue of superantigen protein	3.803	3.466	3.593
SAOUHSC_01127	Structural homologue of superantigen protein	4.733	4.097	3.703
SAOUHSC_01128	L-citrulline synthase		2.634	2.284
SAOUHSC_01130	Conserved hypothetical protein	2.294	2.657	
SAOUHSC_01131	Conserved hypothetical protein	2.253	2.855	3.138
SAOUHSC_01134	Conserved hypothetical protein	3.022	2.770	3.193
SAOUHSC_01135	Conserved hypothetical protein	11.940	4.453	
SAOUHSC_01136	Conserved hypothetical protein	40.867	3.064	5.883
SAOUHSC_01137	Conserved hypothetical protein	4.545		3.136
SAOUHSC_01150	GTPase			2.011
SAOUHSC_01154	Conserved hypothetical protein	2.460		2.384
SAOUHSC_01155	Conserved hypothetical protein	3.204	2.138	2.359

SAOUHSC_01156	Conserved hypothetical protein	2.008		
SAOUHSC_01160	Conserved hypothetical protein			3.001
SAOUHSC_01161	Truncated transposase	3.999	3.882	4.803
SAOUHSC_01162	Lipoprotein signal peptidase			2.206
SAOUHSC_01163	Conserved hypothetical protein			2.514
SAOUHSC_01164	Pyrimidine biosynthetic regulator	3.556	3.022	3.862
SAOUHSC_01165	Uracil permease, putative	2.682		
SAOUHSC_01166	Carbamoyl transferase	3.358		
SAOUHSC_01168	N-carbamoyl-L-aspartate synthase	2.039		
SAOUHSC_01169	Carbamoyl phosphate synthase	2.693		
SAOUHSC_01172	Pyrimidine biosynthetic enzyme	2.589		
SAOUHSC_01173	Conserved hypothetical protein	2.497	2.957	
SAOUHSC_01174	Conserved hypothetical protein		2.570	2.470
SAOUHSC_01175	Unknown function			2.110
SAOUHSC_01176	Essential for recycling GMP			2.102
SAOUHSC_01177	Promotes RNA polymerase assembly	2.842	2.661	3.175
SAOUHSC_01180	Conserved hypothetical protein	2.864	3.266	3.379
SAOUHSC_01181	Conserved hypothetical protein		2.534	
SAOUHSC_01183	Unknown function	2.280		
SAOUHSC_01190	Conserved hypothetical protein	4.137	4.677	6.284
SAOUHSC_01191	Required for 70S ribosome assembly	2.009	2.074	2.385
SAOUHSC_01200	Conserved hypothetical protein	18.888	14.904	20.896
SAOUHSC_01201	Unknown function	3.744	2.738	2.326
SAOUHSC_01203	rRNA processing	2.396		
SAOUHSC_01208	30S assembly and stabilisation protein	2.422		2.649
SAOUHSC_01211	Unknown function	4.770	5.769	8.000
SAOUHSC_01212	Unknown function	5.185	5.188	4.368
SAOUHSC_01218	Protein kinase			2.445
SAOUHSC_01219	Unknown function	2.631	2.082	2.838
SAOUHSC_01220	Conserved hypothetical protein	3.341	2.778	3.614
SAOUHSC_01221	Conserved hypothetical protein	2.354		
SAOUHSC_01230	Conserved hypothetical protein	3.483	2.448	3.303
SAOUHSC_01246	Formylmethionyl-tRNA protection protein			2.126
SAOUHSC_01247	16S rRNA processing protein	2.710	2.278	3.643
SAOUHSC_01248	Conserved hypothetical protein			2.247
SAOUHSC_01249	Unknown function			2.253
SAOUHSC_01259	Conserved hypothetical protein			2.112
SAOUHSC_01260	Unknown function	5.260	5.082	7.857
SAOUHSC_01261	Unknown function		2.009	2.093
SAOUHSC_01264	Conserved hypothetical protein	4.755	6.738	8.107
SAOUHSC_01268	Conserved hypothetical protein	2.109	2.768	
SAOUHSC_01274	Glycerol uptake operon antiterminator regulator			2.240

SAOUHSC_01279	Hydrolase, alpha/beta fold family domain protein		2.323	2.068
SAOUHSC_01280	IPP transferase		2.195	
SAOUHSC_01281	Unknown function	5.881	5.910	9.059
SAOUHSC_01287	Unknown function		2.036	
SAOUHSC_01288	Conserved hypothetical protein	4.379	4.605	5.346
SAOUHSC_01289	Conserved hypothetical protein	6.148	4.736	5.156
SAOUHSC_01290	Conserved hypothetical protein	4.747	5.051	3.791
SAOUHSC_01291	Conserved hypothetical protein	3.137	4.081	2.061
SAOUHSC_01292	Conserved hypothetical protein	3.524	3.792	3.796
SAOUHSC_01293	Conserved hypothetical protein	4.703	5.868	5.374
SAOUHSC_01294	Conserved hypothetical protein	2.551	2.363	2.850
SAOUHSC_01295	Conserved hypothetical protein	5.156	4.457	3.932
SAOUHSC_01296	Conserved hypothetical protein	2.718	3.060	2.690
SAOUHSC_01297	Conserved hypothetical protein	2.499	3.507	3.019
SAOUHSC_01298	Unknown function	2.027	2.579	
SAOUHSC_01301	Conserved hypothetical protein	4.615	4.676	2.334
SAOUHSC_01302	Conserved hypothetical protein	2.325	2.303	
SAOUHSC_01303	Conserved hypothetical protein	3.659	4.051	3.715
SAOUHSC_01304	Conserved hypothetical protein	4.506	5.170	4.474
SAOUHSC_01305	Conserved hypothetical protein	5.887	7.101	5.644
SAOUHSC_01306	Conserved hypothetical protein	7.225	7.880	6.460
SAOUHSC_01307	Conserved hypothetical protein	2.048	2.418	
SAOUHSC_01309	Unknown function	2.987	4.005	3.292
SAOUHSC_01311	Unknown function	2.533	3.191	2.761
SAOUHSC_01312	Conserved hypothetical protein	5.027	6.324	4.985
SAOUHSC_01313	ATPase domain protein		2.413	
SAOUHSC_01314	Conserved hypothetical protein		2.360	2.091
SAOUHSC_01315	Conserved hypothetical protein	3.007	4.585	2.740
SAOUHSC_01316	Conserved hypothetical protein	3.513	2.549	2.546
SAOUHSC_01317	Conserved hypothetical protein		2.054	2.772
SAOUHSC_01318	Conserved hypothetical protein		2.413	3.990
SAOUHSC_01319	4-phospho-L-aspartate synthase	2.643	2.384	3.065
SAOUHSC_01323	Conserved hypothetical protein		2.376	2.884
SAOUHSC_01324	Conserved hypothetical protein	2.726	3.483	3.789
SAOUHSC_01325	Unknown function	6.164	5.154	6.462
SAOUHSC_01328	Unknown function		2.940	4.449
SAOUHSC_01329	Involved in assembly of 30S ribosome subunit	3.442	3.394	4.027
SAOUHSC_01331	Conserved hypothetical protein	2.357	2.112	2.034
SAOUHSC_01332	Conserved hypothetical protein	2.953	3.821	4.86
SAOUHSC_01333	DNA damage gene repressor	2.67	3.324	4.008
SAOUHSC_01334	Conserved hypothetical protein	2.513	3.010	3.100
SAOUHSC_01336	Conserved hypothetical protein		2.053	

SAOUHSC_01339	Conserved hypothetical protein	2.008		2.084
SAOUHSC_01340	Conserved hypothetical protein	2.061		2.026
SAOUHSC_01342	Conserved hypothetical protein	2.566	2.362	2.939
SAOUHSC_01343	Conserved hypothetical protein	6.263	4.005	5.979
SAOUHSC_01344	Conserved hypothetical protein	2.810	2.777	2.280
SAOUHSC_01346	Unknown function			2.902
SAOUHSC_01348	Conserved hypothetical protein			2.080
SAOUHSC_01349	Conserved hypothetical protein	5.350	3.887	5.284
SAOUHSC_01350	Conserved hypothetical protein	3.049	2.079	2.512
SAOUHSC_01353	Conserved hypothetical protein	2.551	2.411	
SAOUHSC_01354	Unknown function	2.768	2.662	4.025
SAOUHSC_01356	Transcriptional antiterminator		2.056	
SAOUHSC_01357	Conserved hypothetical protein		3.149	3.971
SAOUHSC_01358	Conserved hypothetical protein	2.079	2.238	3.351
SAOUHSC_01359	Conserved hypothetical protein		2.537	2.597
SAOUHSC_01360	Provides protection against oxidative stress		2.450	2.283
SAOUHSC_01361	Unknown function	3.935	4.705	6.859
SAOUHSC_01362	Unknown function	2.034	2.016	
SAOUHSC_01363	Conserved hypothetical protein		2.128	
SAOUHSC_01364	4-hydroxyphenylpyruvate synthase		2.181	2.450
SAOUHSC_01365	Conserved hypothetical protein		2.265	2.453
SAOUHSC_01366	Glutamine amidotransferase	2.267		
SAOUHSC_01368	Anthranilate synthase	3.320	2.607	2.843
SAOUHSC_01369	Tryptophan biosynthetic enzyme	2.129		
SAOUHSC_01370	1-deoxy-D-ribulose 5-phosphate synthase	2.504		2.075
SAOUHSC_01371	L-tryptophan synthase	2.203		
SAOUHSC_01372	Indole and glyceraldehyde 3-phosphate synthase	2.490	2.409	2.538
SAOUHSC_01374	Unknown function			2.318
SAOUHSC_01376	Conserved hypothetical protein	2.162	2.312	2.179
SAOUHSC_01378	Unknown function	2.025	2.003	2.345
SAOUHSC_01381	Unknown function	4.267	3.311	3.552
SAOUHSC_01382	Conserved hypothetical protein	3.642	4.539	5.421
SAOUHSC_01384	PhoU family, putative	4.851		4.783
SAOUHSC_01385	Unknown function	3.236		2.710
SAOUHSC_01386	Unknown function	3.117		2.446
SAOUHSC_01387	Conserved hypothetical protein	2.278		
SAOUHSC_01388	Unknown function	5.550		4.386
SAOUHSC_01389	Unknown function	2.553		
SAOUHSC_01390	Truncated transposase	3.055	2.985	2.900
SAOUHSC_01391	Conserved hypothetical protein		2.068	2.406
SAOUHSC_01397	Lysine and diaminopimelate biosynthetic enzyme			2.004

SAOUHSC_01401	Unknown function		2.254	3.049
SAOUHSC_01402	Conserved hypothetical protein	2.867	4.051	5.477
SAOUHSC_01403	Unknown function	3.495	3.865	4.725
SAOUHSC_01404	Unknown function	8.005	13.945	19.225
SAOUHSC_01405	Conserved hypothetical protein	3.301	4.699	5.982
SAOUHSC_01409	Truncated transposase	2.228	2.205	2.288
SAOUHSC_01410	Truncated transposase	3.001	2.910	2.692
SAOUHSC_01411	Unknown function	3.173	2.295	2.321
SAOUHSC_01414	Conserved hypothetical protein	2.457		
SAOUHSC_01419	Conserved hypothetical protein	3.304	4.981	2.774
SAOUHSC_01420	Unknown function	2.051	2.832	
SAOUHSC_01421	Unknown function	4.472	4.398	2.959
SAOUHSC_01422	Conserved hypothetical protein	5.235	4.267	3.611
SAOUHSC_01423	Conserved hypothetical protein	2.141	2.360	
SAOUHSC_01425	Conserved hypothetical protein	2.362	2.483	
SAOUHSC_01427	Unknown function		2.229	2.062
SAOUHSC_01428	Unknown function	2.473	3.520	4.628
SAOUHSC_01429	Conserved hypothetical protein	2.408	3.532	5.368
SAOUHSC_01430	Unknown function		2.782	3.635
SAOUHSC_01431	Provides protection against oxidative stress			2.043
SAOUHSC_01434	Unknown function	2.076	2.199	
SAOUHSC_01440	Conserved hypothetical protein			3.214
SAOUHSC_01441	Conserved hypothetical protein	2.612	2.614	2.250
SAOUHSC_01442	Conserved hypothetical protein	2.376	2.052	2.825
SAOUHSC_01447	Conserved hypothetical protein	2.416	2.248	2.463
SAOUHSC_01448	Conserved hypothetical protein	3.895	3.126	2.900
SAOUHSC_01456	Conserved hypothetical protein		2.366	
SAOUHSC_01459	Conserved hypothetical protein	3.633	4.238	5.404
SAOUHSC_01465	Conserved hypothetical protein	2.157		
SAOUHSC_01468	Conserved hypothetical protein	2.593	2.881	3.596
SAOUHSC_01469	Unknown function			2.430
SAOUHSC_01477	Conserved hypothetical protein		2.240	3.562
SAOUHSC_01478	Conserved hypothetical protein			2.397
SAOUHSC_01484	Conserved hypothetical protein			2.247
SAOUHSC_01485	Nucleoside triphosphate synthase	3.536	3.844	4.505
SAOUHSC_01489	Unknown function		2.047	
SAOUHSC_01493	Unknown function			2.137
SAOUHSC_01494	Unknown function	2.066		
SAOUHSC_01495	Conserved hypothetical protein	5.269	3.932	3.461
SAOUHSC_01499	Conserved hypothetical protein			2.114
SAOUHSC_01500	Conserved hypothetical protein	3.573	2.620	3.252
SAOUHSC_01502	Unknown function	2.309	2.331	
SAOUHSC_01503	Conserved hypothetical protein	2.067	2.163	

SAOUHSC_01504	Ferredoxin, putative		2.439	2.002
SAOUHSC_01505	Conserved hypothetical protein	2.824	3.830	5.503
SAOUHSC_01507	Conserved hypothetical protein	2.568	2.591	3.388
SAOUHSC_01508	Conserved hypothetical protein	2.160	2.474	2.644
SAOUHSC_01510	Conserved hypothetical protein		2.091	
SAOUHSC_01513	Conserved hypothetical protein	2.163	2.002	
SAOUHSC_01516	Unknown function	2.438	2.163	2.194
SAOUHSC_01517	Conserved hypothetical phage protein	2.159	2.164	2.104
SAOUHSC_01518	Conserved hypothetical phage protein	2.363	2.146	2.252
SAOUHSC_01520	Unknown function	2.068		
SAOUHSC_01521	Unknown function	2.061		
SAOUHSC_01522	Conserved hypothetical phage protein			2.018
SAOUHSC_01523	Unknown function		2.081	
SAOUHSC_01524	Unknown function	2.077	2.126	
SAOUHSC_01526	Conserved hypothetical phage protein	2.140	2.050	2.115
SAOUHSC_01528	Bacterial Ig-like domain group 2 family protein	2.194		2.051
SAOUHSC_01529	Unknown function	2.275	2.369	2.264
SAOUHSC_01530	Conserved hypothetical phage protein	2.135		
SAOUHSC_01531	Unknown function	2.208	2.068	
SAOUHSC_01532	Unknown function	2.183	2.026	2.130
SAOUHSC_01535	Conserved hypothetical phage protein	2.026		
SAOUHSC_01536	Unknown function	2.444	2.375	2.269
SAOUHSC_01537	Phage portal protein, HK97 family	2.093		2.046
SAOUHSC_01538	Unknown function	2.034		
SAOUHSC_01541	Conserved hypothetical phage protein	2.143		2.065
SAOUHSC_01544	Hypothetical phage protein			2.020
SAOUHSC_01545	Conserved hypothetical phage protein	2.495	2.320	2.251
SAOUHSC_01546	Conserved hypothetical phage protein	2.041	2.167	2.074
SAOUHSC_01548	Conserved hypothetical phage protein	2.111	2.202	2.055
SAOUHSC_01550	Conserved hypothetical phage protein	2.318	2.336	2.212
SAOUHSC_01555	Conserved hypothetical phage protein		2.188	
SAOUHSC_01556	Unknown function	2.020		2.025
SAOUHSC_01558	Unknown function	2.472	2.142	2.259
SAOUHSC_01559	Conserved hypothetical phage protein	2.147	2.019	2.110
SAOUHSC_01562	Unknown function	2.375	2.476	2.273
SAOUHSC_01565	Phage-related protein	2.033	2.097	
SAOUHSC_01567	Conserved hypothetical phage protein	2.187	2.161	
SAOUHSC_01569	Conserved hypothetical phage protein		2.181	2.211
SAOUHSC_01572	Conserved hypothetical phage protein	2.090	2.092	2.082
SAOUHSC_01576	Exonuclease family	2.174	2.120	2.214
SAOUHSC_01577	Conserved hypothetical phage protein	2.096	2.141	2.070
SAOUHSC_01578	Conserved hypothetical phage protein	2.292	2.110	2.162
SAOUHSC_01580	Unknown function	2.346	2.288	

SAOUHSC_01582	Unknown function	2.400	2.166	2.250
SAOUHSC_01584	Conserved hypothetical protein		2.109	
SAOUHSC_01589	Conserved hypothetical protein		2.087	
SAOUHSC_01590	Conserved hypothetical protein	2.635	2.429	3.136
SAOUHSC_01591	Unknown function		2.085	2.341
SAOUHSC_01594	Conserved hypothetical protein	2.473	2.428	2.018
SAOUHSC_01597	Unknown function	2.049	2.882	2.591
SAOUHSC_01598	Unknown function	2.780	3.943	3.805
SAOUHSC_01600	Conserved hypothetical protein		2.390	2.662
SAOUHSC_01601	Alpha-glucosidase, putative		2.077	3.016
SAOUHSC_01602	Transcriptional regulator, putative		2.021	2.578
SAOUHSC_01603	Unknown function	2.765	2.746	2.963
SAOUHSC_01604	Conserved hypothetical protein	2.622	2.925	3.157
SAOUHSC_01607	Conserved hypothetical protein	2.443	2.737	2.295
SAOUHSC_01608	Unknown function	3.784	3.886	4.183
SAOUHSC_01610	Conserved hypothetical protein		2.139	2.111
SAOUHSC_01625	Involved in peptide bond synthesis	3.473	3.875	3.412
SAOUHSC_01627	Conserved hypothetical protein	2.357	3.272	3.119
SAOUHSC_01628	Conserved hypothetical protein	3.448	3.537	4.101
SAOUHSC_01629	Conserved hypothetical protein			2.455
SAOUHSC_01630	Conserved hypothetical protein	3.174	2.491	4.605
SAOUHSC_01635	Unknown function	2.276	3.205	2.652
SAOUHSC_01636	Unknown function	3.563	4.837	4.496
SAOUHSC_01637	Conserved hypothetical protein	4.568	4.474	4.378
SAOUHSC_01638	Conserved hypothetical protein	4.112	3.287	3.381
SAOUHSC_01639	Conserved hypothetical protein	2.892	2.643	2.313
SAOUHSC_01640	Conserved hypothetical protein	3.081	2.639	2.42
SAOUHSC_01641	Conserved hypothetical protein	8.766	9.762	9.663
SAOUHSC_01643	Conserved hypothetical protein	3.095	3.128	3.107
SAOUHSC_01651	Unknown function	2.371	2.315	2.443
SAOUHSC_01655	Conserved hypothetical protein	5.224	4.402	7.938
SAOUHSC_01657	Unknown function	2.239		2.042
SAOUHSC_01661	Conserved hypothetical protein	2.030		2.514
SAOUHSC_01665	CBS domain protein		2.100	
SAOUHSC_01666	tRNA synthesis-linked protein			2.292
SAOUHSC_01675	Conserved hypothetical protein		2.177	2.823
SAOUHSC_01678	Binds central domain of 16S rRNA		2.387	2.137
SAOUHSC_01681	Unknown function			3.174
SAOUHSC_01686	Protoporphyrinogen-IX synthase	2.140		2.217
SAOUHSC_01687	Conserved hypothetical protein	2.598	2.962	2.019
SAOUHSC_01689	Inhibitor of arginine and ornithine decarboxylase	2.071	2.051	2.297
SAOUHSC_01690	DNAX complex assembly protein	3.321	3.636	5.148
SAOUHSC_01691	Unknown function	3.706	3.160	3.226

SAOUHSC_01692	Conserved hypothetical protein	2.555	2.798	3.336
SAOUHSC_01693	Conserved hypothetical protein	2.883	2.975	3.876
SAOUHSC_01694	Conserved hypothetical protein		2.192	
SAOUHSC_01703	Unknown function	3.202	2.895	3.306
SAOUHSC_01704	Conserved hypothetical protein	4.050	3.335	4.591
SAOUHSC_01705	Unknown function	2.497		2.555
SAOUHSC_01706	Conserved hypothetical protein	2.160	2.152	3.464
SAOUHSC_01707	Conserved hypothetical protein			3.086
SAOUHSC_01710	Unknown function		2.093	
SAOUHSC_01711	Conserved hypothetical protein		2.017	
SAOUHSC_01714	Transcription elongation protein	5.372	4.033	5.560
SAOUHSC_01718	Conserved hypothetical protein	2.214		
SAOUHSC_01719	Conserved hypothetical protein	2.441	2.274	2.964
SAOUHSC_01720	Conserved hypothetical protein			2.043
SAOUHSC_01725	Unknown function	2.029		
SAOUHSC_01728	Conserved hypothetical protein	2.459	2.246	2.771
SAOUHSC_01729	Conserved hypothetical protein	8.664	11.992	5.822
SAOUHSC_01730	Conserved hypothetical protein		2.541	
SAOUHSC_01733	Unknown function	2.861	2.276	3.099
SAOUHSC_01736	Conserved hypothetical protein		2.725	2.754
SAOUHSC_01737	Aminoacyl adenylate tranferase			2.325
SAOUHSC_01743	Involved in the formation of AMP			2.198
SAOUHSC_01744	Conserved hypothetical protein			2.094
SAOUHSC_01746	Part of the preprotein secretory system		2.164	2.733
SAOUHSC_01747	Unknown function	2.544	2.305	4.241
SAOUHSC_01755	Peptidyltransferase			2.857
SAOUHSC_01756	Conserved hypothetical protein			2.224
SAOUHSC_01760	Conserved hypothetical protein	2.359	2.963	4.829
SAOUHSC_01761	Conserved hypothetical protein			2.801
SAOUHSC_01762	Conserved hypothetical protein	2.907	2.500	2.862
SAOUHSC_01763	DNA recombination protein	2.390	2.236	2.286
SAOUHSC_01764	Conserved hypothetical protein	2.883	3.219	2.961
SAOUHSC_01765	Unknown function	2.021	2.132	2.012
SAOUHSC_01768	Conserved hypothetical protein	2.535	2.836	2.364
SAOUHSC_01769	Conserved hypothetical protein		2.073	
SAOUHSC_01770	Conserved hypothetical protein		2.399	2.452
SAOUHSC_01780	Unknown function		2.285	
SAOUHSC_01783	Unknown function	4.019	3.190	4.228
SAOUHSC_01784	23S ribosomal RNA binding protein	5.627	4.455	6.806
SAOUHSC_01787	Conserved hypothetical protein			2.430
SAOUHSC_01791	Primosomal protein			2.057
SAOUHSC_01794	Glyceraldehyde-3-P dehydrogenase, type I	3.999	3.421	5.080
SAOUHSC_01795	Coenzyme A synthase			2.578

SAOUHSC_01798	Conserved hypothetical protein	2.765	2.829	3.219
SAOUHSC_01801	Converts isocitrate to alpha ketoglutarate			2.260
SAOUHSC_01804	Unknown function	2.628	2.243	2.433
SAOUHSC_01805	Truncated transposase, putative	2.617	2.161	2.263
SAOUHSC_01806	Unknown function	2.815	3.097	3.124
SAOUHSC_01814	Conserved hypothetical protein		2.009	
SAOUHSC_01815	Conserved hypothetical protein	2.062	4.164	
SAOUHSC_01816	Conserved hypothetical protein		2.063	
SAOUHSC_01817	Conserved hypothetical protein	8.282	5.440	9.113
SAOUHSC_01818	Unknown function			3.413
SAOUHSC_01819	Conserved hypothetical protein		2.870	4.059
SAOUHSC_01820	Acetyl-CoA synthase	2.301	2.418	
SAOUHSC_01823	Conserved hypothetical protein	2.309		
SAOUHSC_01825	Conserved hypothetical protein	2.586		
SAOUHSC_01826	Conserved hypothetical protein	5.182	2.177	2.732
SAOUHSC_01827	Negative regulator of ftsZ ring formation	2.146		2.896
SAOUHSC_01828	GAF domain protein		2.097	2.471
SAOUHSC_01829	Primary rRNA binding protein	3.006	2.990	4.318
SAOUHSC_01830	Conserved hypothetical protein	2.147	2.528	2.395
SAOUHSC_01834	Unknown function	3.278	2.626	2.946
SAOUHSC_01835	Conserved hypothetical protein		2.089	2.822
SAOUHSC_01837	Acyltransferase domain protein	2.104	2.691	
SAOUHSC_01839	Unknown function		2.243	
SAOUHSC_01840	Transglycosylase domain protein	2.435	2.666	3.390
SAOUHSC_01841	Unknown function	4.040	3.382	4.287
SAOUHSC_01844	Conserved hypothetical protein	3.155	4.931	4.453
SAOUHSC_01847	Unknown function			2.002
SAOUHSC_01851	Unknown function	2.669	5.232	2.426
SAOUHSC_01852	Ulosonate 7-phosphate synthase	2.694	3.204	3.881
SAOUHSC_01853	Unknown function	6.402	5.422	3.172
SAOUHSC_01860	Conserved hypothetical protein			2.302
SAOUHSC_01863	Conserved hypothetical protein	6.724	6.841	11.170
SAOUHSC_01864	Conserved hypothetical protein	3.311	3.261	4.726
SAOUHSC_01869	Conserved hypothetical protein		2.628	
SAOUHSC_01873	Conserved hypothetical protein	2.122		
SAOUHSC_01876	Conserved hypothetical protein			2.058
SAOUHSC_01878	Conserved hypothetical protein	2.836	2.833	3.930
SAOUHSC_01879	Unknown function	2.792	3.599	4.001
SAOUHSC_01880	Conserved hypothetical protein	2.007		2.093
SAOUHSC_01881	Conserved hypothetical protein	3.074	2.486	3.085
SAOUHSC_01882	Conserved hypothetical protein		2.560	3.308
SAOUHSC_01883	Conserved hypothetical protein	8.06	6.419	6.126

SAOUHSC_01884	Conserved hypothetical protein	3.739	3.211	2.823
SAOUHSC_01886	RibE	3.259	3.259	2.436
SAOUHSC_01887	Riboflavin biosynthesis protein, putative		2.061	
SAOUHSC_01890	Conserved hypothetical protein	2.054	2.957	
SAOUHSC_01891	Unknown function	2.197		
SAOUHSC_01894	Arsenite synthase	4.063	3.085	3.916
SAOUHSC_01895	Conserved hypothetical protein		2.193	2.439
SAOUHSC_01896	Conserved hypothetical protein		2.167	2.128
SAOUHSC_01897	Conserved hypothetical protein	2.456		2.135
SAOUHSC_01898	Conserved hypothetical protein	3.014	2.195	2.515
SAOUHSC_01899	Conserved hypothetical protein	4.121	3.611	3.570
SAOUHSC_01900	Conserved hypothetical protein	4.073	3.870	4.150
SAOUHSC_01902	Conserved hypothetical protein	3.834	3.327	5.305
SAOUHSC_01903	Conserved hypothetical protein	2.521	2.036	3.210
SAOUHSC_01904	Conserved hypothetical protein	4.018	3.443	4.672
SAOUHSC_01905	Truncated transposase, putative	2.678	2.214	2.482
SAOUHSC_01906	Truncated transposase, putative	2.587	2.669	2.459
SAOUHSC_01907	Conserved hypothetical protein		2.347	2.008
SAOUHSC_01908	Conserved hypothetical protein	5.163	4.797	6.427
SAOUHSC_01909	Methionine adenosyltransferase	2.928		3.493
SAOUHSC_01910	PEP carboxykinase			3.14
SAOUHSC_01911	Transposase, IS200 family	2.595	2.607	3.298
SAOUHSC_01917	Conserved hypothetical protein	2.388	3.032	2.762
SAOUHSC_01918	Conserved hypothetical protein			2.692
SAOUHSC_01919	Conserved hypothetical protein			2.394
SAOUHSC_01920	Conserved hypothetical protein			2.555
SAOUHSC_01921	Conserved hypothetical protein	2.253	2.110	4.373
SAOUHSC_01922	Conserved hypothetical protein	3.135	2.410	3.085
SAOUHSC_01923	Conserved hypothetical protein	5.438	3.590	5.015
SAOUHSC_01924	Conserved hypothetical protein	3.310	3.221	2.615
SAOUHSC_01925	Conserved hypothetical protein	4.674	4.425	3.274
SAOUHSC_01927	Transposase, IS3 family	2.713	3.099	2.412
SAOUHSC_01928	Unknown function	2.236	2.828	2.306
SAOUHSC_01929	Conserved hypothetical protein	3.262	4.813	4.115
SAOUHSC_01930	Conserved hypothetical protein	6.339	8.274	10.544
SAOUHSC_01931	Conserved hypothetical protein	2.470	2.393	
SAOUHSC_01932	S subunit, EcoA family, putative	3.154	2.841	3.186
SAOUHSC_01935	Unknown function	2.384	2.330	2.152
SAOUHSC_01936	Unknown function	2.876	2.823	
SAOUHSC_01937	Unknown function	4.267	6.008	2.029
SAOUHSC_01938	Unknown function	2.994	3.212	
SAOUHSC_01939	Unknown function	2.155		
SAOUHSC_01942	Unknown function	2.532	3.114	

SAOUHSC_01943	Conserved hypothetical protein	3.519	2.717	2.918
SAOUHSC_01944	Conserved hypothetical protein	5.487	4.696	3.87
SAOUHSC_01945	Membrane protein, putative		2.304	
SAOUHSC_01947	Membrane protein, putative	2.305	2.955	
SAOUHSC_01948	ABC transporter domain protein	3.918	4.478	2.698
SAOUHSC_01950	Unknown function	2.668	2.588	
SAOUHSC_01951	Unknown function	4.614	4.227	2.639
SAOUHSC_01952	Unknown function	3.206	3.452	2.249
SAOUHSC_01953	Gallidermin superfamily epiA, putative	5.233	4.897	3.072
SAOUHSC_01954	Pathogenicity island SaPIn3	2.587	2.532	2.040
SAOUHSC_01955	Pathogenicity island SaPIn3	2.114	2.147	
SAOUHSC_01956	Conserved hypothetical protein	2.694	3.130	2.348
SAOUHSC_01957	Conserved hypothetical protein		2.591	
SAOUHSC_01958	Conserved hypothetical protein	2.942	2.841	3.434
SAOUHSC_01966	Conserved hypothetical protein			2.521
SAOUHSC_01968	Conserved hypothetical protein		2.028	
SAOUHSC_01969	Conserved hypothetical protein		2.750	2.300
SAOUHSC_01971	Conserved hypothetical protein	6.746	7.133	8.399
SAOUHSC_01972	Unknown function		2.522	3.241
SAOUHSC_01973	Nucleioside 5'-phosphates synthase	3.606	4.002	6.578
SAOUHSC_01976	Conserved hypothetical protein	4.446	2.508	3.364
SAOUHSC_01977	Conserved hypothetical protein	2.270	2.637	3.187
SAOUHSC_01978	Conserved hypothetical protein	2.900	3.829	3.400
SAOUHSC_01979	Conserved hypothetical protein	3.055	2.889	4.110
SAOUHSC_01980	Unknown function		2.084	
SAOUHSC_01982	RluD subfamily, putative	3.706	3.510	3.499
SAOUHSC_01983	Reversibly converts (S)-malate to fumarate			2.459
SAOUHSC_01984	Conserved hypothetical protein	5.981	6.360	6.263
SAOUHSC_01985	Conserved hypothetical protein	7.343	5.422	5.168
SAOUHSC_01986	Conserved hypothetical protein	6.793	8.450	5.432
SAOUHSC_01987	Conserved hypothetical protein		3.199	2.286
SAOUHSC_01988	Conserved hypothetical protein		2.006	
SAOUHSC_01989	Conserved hypothetical protein		2.108	
SAOUHSC_01990	Unknown function	2.040		
SAOUHSC_01992	Phosphotransferase system, EIIC domain protein	2.715	2.851	2.815
SAOUHSC_01993	Unknown function		2.285	2.303
SAOUHSC_01997	Unknown function		2.651	3.669
SAOUHSC_02001	Conserved hypothetical protein		2.314	2.254
SAOUHSC_02002	Unknown function	3.212	2.635	3.233
SAOUHSC_02003	Unknown function			2.605
SAOUHSC_02004	Conserved hypothetical protein	2.100	2.104	
SAOUHSC_02007	Unknown function	5.383	3.854	4.808

SAOUHSC_02012	Monofunctional PBP		2.475	2.380
SAOUHSC_02013	Conserved hypothetical protein		2.291	
SAOUHSC_02014	Conserved hypothetical protein		2.141	
SAOUHSC_02015	Hypothetical phage protein	2.140	2.040	2.000
SAOUHSC_02016	Conserved hypothetical phage protein	2.059		
SAOUHSC_02017	Conserved hypothetical phage protein	2.040	2.008	2.051
SAOUHSC_02021	Unknown function	2.050	2.095	2.078
SAOUHSC_02025	Unknown function	2.243	2.215	2.189
SAOUHSC_02031	Conserved hypothetical phage protein	2.135	2.176	
SAOUHSC_02034	Conserved hypothetical phage protein		2.086	
SAOUHSC_02037	Conserved hypothetical phage protein		2.176	
SAOUHSC_02040	Conserved hypothetical phage protein		2.105	2.097
SAOUHSC_02042	Unknown function	2.431	2.114	2.193
SAOUHSC_02043	Unknown function	2.210	2.218	2.041
SAOUHSC_02046	Conserved hypothetical phage protein			2.068
SAOUHSC_02049	Phage terminase, large subunit, PBSX family	2.122	2.028	
SAOUHSC_02050	Unknown function	2.327	2.155	2.216
SAOUHSC_02052	Hypothetical phage protein	2.179	2.117	
SAOUHSC_02053	Unknown function	2.897	2.411	2.929
SAOUHSC_02054	Conserved hypothetical phage protein	2.261	2.016	
SAOUHSC_02056	Conserved hypothetical phage protein	2.669	2.367	2.605
SAOUHSC_02057	Unknown function	2.119		
SAOUHSC_02059	Unknown function		2.042	
SAOUHSC_02062	Unknown function	2.003		
SAOUHSC_02064	Unknown function	2.213	2.004	2.027
SAOUHSC_02069	Unknown function	2.133		
SAOUHSC_02075	Conserved hypothetical phage protein	2.253	2.453	2.228
SAOUHSC_02076	Unknown function	2.452	2.264	2.500
SAOUHSC_02079	Conserved hypothetical phage protein	2.207	2.330	2.317
SAOUHSC_02080	Unknown function	2.231	2.136	2.216
SAOUHSC_02083	Unknown function	2.131		
SAOUHSC_02086	Unknown function	2.425	2.167	2.239
SAOUHSC_02087	Conserved hypothetical phage protein	2.279		2.193
SAOUHSC_02088	Unknown function	2.425	2.144	2.342
SAOUHSC_02090	Conserved hypothetical phage protein		2.191	2.126
SAOUHSC_02095	Conserved hypothetical protein	2.495	4.419	
SAOUHSC_02096	Conserved hypothetical protein	2.543	3.688	2.491
SAOUHSC_02097	Conserved hypothetical protein	2.286	4.094	
SAOUHSC_02098	Unknown function			2.074
SAOUHSC_02100	Conserved hypothetical protein			2.011
SAOUHSC_02103	Conserved hypothetical protein		2.360	
SAOUHSC_02104	Conserved hypothetical protein	4.232	3.708	5.834
SAOUHSC_02109	Conserved hypothetical protein	2.367	4.091	6.337

SAOUHSC_02110	Conserved hypothetical protein	2.216		3.205
SAOUHSC_02112	Conserved hypothetical protein	2.230	2.063	3.224
SAOUHSC_02114	Similar to YegS from *E. coli*	2.120	2.123	2.040
SAOUHSC_02115	Conserved hypothetical protein	2.331		
SAOUHSC_02119	Unknown function			2.433
SAOUHSC_02125	Conserved hypothetical protein	2.607	2.926	4.147
SAOUHSC_02127	Unknown function	4.200	3.247	2.837
SAOUHSC_02129	Conserved hypothetical protein	5.188	4.590	5.097
SAOUHSC_02130	Conserved hypothetical protein	3.655	4.031	4.820
SAOUHSC_02131	Conserved hypothetical protein	2.896	3.428	3.708
SAOUHSC_02134	NO synthase, oxygenase domain, putative		2.259	
SAOUHSC_02135	Conserved hypothetical protein	3.899	4.480	4.273
SAOUHSC_02136	Unknown function	3.767	3.735	3.176
SAOUHSC_02137	Unknown function		2.829	
SAOUHSC_02138	Conserved hypothetical protein		2.080	
SAOUHSC_02140	Hydrolyses pyrophosphate to phosphate		2.152	2.666
SAOUHSC_02141	Conserved hypothetical protein		2.180	
SAOUHSC_02143	Conserved hypothetical protein			2.135
SAOUHSC_02144	Conserved hypothetical protein	2.817		2.171
SAOUHSC_02145	Conserved hypothetical protein	5.068	4.427	4.466
SAOUHSC_02146	Conserved hypothetical protein	2.141		
SAOUHSC_02149	Conserved hypothetical protein	2.136	2.130	
SAOUHSC_02151	Conserved hypothetical protein	3.805	2.883	2.491
SAOUHSC_02152	Unknown function	3.236	2.047	
SAOUHSC_02153	Conserved hypothetical protein	3.551	2.347	
SAOUHSC_02154	Unknown function	2.683		
SAOUHSC_02155	Conserved hypothetical protein	4.125	3.051	
SAOUHSC_02156	Conserved hypothetical protein	4.488	3.952	3.457
SAOUHSC_02157	Conserved hypothetical protein	5.989	5.630	6.115
SAOUHSC_02160	Conserved hypothetical protein	3.630	4.162	3.927
SAOUHSC_02161	Unknown function		3.076	2.067
SAOUHSC_02163	Conserved hypothetical phage protein		2.864	
SAOUHSC_02164	Conserved hypothetical phage protein	2.060	2.125	2.224
SAOUHSC_02166	Hypothetical phage protein	2.133		2.218
SAOUHSC_02169	Formylated peptide receptor binding protein	2.142	2.059	
SAOUHSC_02171	Unknown function	2.197		2.008
SAOUHSC_02173	Unknown function			2.036
SAOUHSC_02175	Hypothetical phage protein	4.104	4.878	4.912
SAOUHSC_02176	Conserved hypothetical phage protein	4.753	4.853	6.411
SAOUHSC_02178	Unknown function	2.200	2.308	
SAOUHSC_02179	Conserved hypothetical phage protein	2.427	2.260	2.083
SAOUHSC_02181	Unknown function	2.088	2.025	2.148

SAOUHSC_02183	Conserved hypothetical phage protein	2.019		
SAOUHSC_02184	Unknown function		2.012	2.126
SAOUHSC_02186	Unknown function	2.148	2.072	
SAOUHSC_02191	Phage major capsid protein, HK97 family	2.149	2.161	2.204
SAOUHSC_02193	Unknown function		2.121	
SAOUHSC_02195	Unknown function	2.329	2.268	2.356
SAOUHSC_02198	Conserved hypothetical phage protein		2.012	2.087
SAOUHSC_02199	Unknown function	2.097	2.167	
SAOUHSC_02202	Conserved hypothetical phage protein			2.035
SAOUHSC_02203	Conserved hypothetical phage protein	2.269		2.373
SAOUHSC_02206	Hypothetical phage protein	2.021		
SAOUHSC_02207	Unknown function	2.258	2.297	2.023
SAOUHSC_02210	Unknown function	2.313	2.122	
SAOUHSC_02212	Conserved hypothetical phage protein	2.063		
SAOUHSC_02215	Conserved hypothetical phage protein		2.200	2.020
SAOUHSC_02218	Conserved hypothetical phage protein	2.252	2.076	2.186
SAOUHSC_02224	Unknown function	2.477	2.561	2.389
SAOUHSC_02225	Conserved hypothetical phage protein	2.365	2.144	2.126
SAOUHSC_02226	Conserved hypothetical phage protein	2.164	2.136	2.285
SAOUHSC_02227	Conserved hypothetical phage protein			2.038
SAOUHSC_02231	Unknown function	2.173		
SAOUHSC_02234	Unknown function	2.109	2.298	2.320
SAOUHSC_02236	Conserved hypothetical phage protein	2.527	2.165	2.318
SAOUHSC_02240	Unknown function		2.047	
SAOUHSC_02241	Conserved hypothetical protein		5.214	3.888
SAOUHSC_02244	DapE	2.453	3.937	
SAOUHSC_02245	Conserved hypothetical protein	2.803	3.167	2.221
SAOUHSC_02246	Conserved hypothetical protein	2.764	2.734	2.117
SAOUHSC_02247	Unknown function	3.395	3.746	3.495
SAOUHSC_02248	Conserved hypothetical protein	2.305	3.951	2.217
SAOUHSC_02249	Conserved hypothetical protein	2.464	4.041	2.162
SAOUHSC_02250	Unknown function	2.038		2.168
SAOUHSC_02251	Conserved hypothetical protein	2.411		2.589
SAOUHSC_02256	Abortive infection protein	3.802	4.796	6.235
SAOUHSC_02257	Conserved hypothetical protein	2.120	2.090	2.875
SAOUHSC_02258	Conserved hypothetical protein	2.449	2.345	2.306
SAOUHSC_02260	Unknown function	2.325		
SAOUHSC_02265	Unknown function	5.492	3.256	4.033
SAOUHSC_02266	Conserved hypothetical protein	6.207	4.280	3.843
SAOUHSC_02271	Conserved hypothetical protein	4.741	3.657	3.688
SAOUHSC_02272	Conserved hypothetical protein	4.069	2.175	2.191
SAOUHSC_02273	Transcriptional modulator			2.408
SAOUHSC_02275	Unknown function	6.471	5.324	5.504

SAOUHSC_02276	MutS domain V protein	2.855	2.887	2.571
SAOUHSC_02286	3-carboxy-4-methyl-2-oxopentanoate synthase	2.138		
SAOUHSC_02288	3-isopropylmalate isomerase	2.173		2.230
SAOUHSC_02289	2-oxobutanoate synthase	4.262	4.003	3.77
SAOUHSC_02294	Unknown function	4.176	4.676	3.916
SAOUHSC_02309	Conserved hypothetical protein		2.934	
SAOUHSC_02310	KDP component protein		2.587	
SAOUHSC_02311	KDP component protein		2.592	
SAOUHSC_02312	ATP hydrolytic enzyme		4.716	
SAOUHSC_02313	Unknown function		5.284	
SAOUHSC_02314	Unknown function		3.709	
SAOUHSC_02315	Unknown function		3.698	
SAOUHSC_02316	ATP-dependent RNA helicase	2.133		3.040
SAOUHSC_02319	Conserved hypothetical protein	2.720	2.924	3.784
SAOUHSC_02320	Conserved hypothetical protein	5.387	5.900	5.446
SAOUHSC_02321	Conserved hypothetical protein	3.218	3.293	2.936
SAOUHSC_02322	Conserved hypothetical protein	2.199	2.132	
SAOUHSC_02323	Unknown function	2.158		2.557
SAOUHSC_02324	Conserved hypothetical protein	2.213	2.021	3.341
SAOUHSC_02325	Unknown function	2.577		3.006
SAOUHSC_02326	Conserved hypothetical protein	2.157		2.674
SAOUHSC_02328	Thiamine monophosphate synthase			2.192
SAOUHSC_02332	Conserved hypothetical protein		2.528	2.254
SAOUHSC_02333	Unknown function	3.168	3.922	2.138
SAOUHSC_02334	Unknown function	6.331	7.637	7.694
SAOUHSC_02335	Conserved hypothetical protein	3.292	3.346	4.054
SAOUHSC_02336	Unknown function	6.523	4.947	7.577
SAOUHSC_02338	Conserved hypothetical protein		2.637	
SAOUHSC_02339	Unknown function	2.904	2.143	2.459
SAOUHSC_02340	Part of catalytic core of ATP synthase	2.008	2.156	3.167
SAOUHSC_02360	Thymidine 5'-phosphate synthase	2.269		
SAOUHSC_02361	RpmE2	5.933	4.728	8.430
SAOUHSC_02367	Conserved hypothetical protein	3.330	4.096	4.201
SAOUHSC_02371	(R)-4'-phosphopantothenate synthase	2.349	2.013	2.014
SAOUHSC_02372	Conserved hypothetical protein	3.350	3.200	4.676
SAOUHSC_02373	Conserved hypothetical protein			2.289
SAOUHSC_02375	Homocysteine and autoinducer-2 synthase	2.373	2.658	3.252
SAOUHSC_02376	Conserved hypothetical protein	3.423	4.711	8.293
SAOUHSC_02377	Pyrimidine phosphorolytic enzyme			2.231
SAOUHSC_02380	(2'-deoxy)ribose-1- phosphate synthase		2.101	2.808
SAOUHSC_02381	Conserved hypothetical protein		3.372	2.211
SAOUHSC_02382	Conserved hypothetical protein	2.118	2.809	

SAOUHSC_02384	Conserved hypothetical protein	2.546	2.525	2.197
SAOUHSC_02385	Unknown function	2.126	2.660	
SAOUHSC_02386	Conserved hypothetical protein	2.613	3.407	
SAOUHSC_02387	Conserved hypothetical protein	2.215	2.923	
SAOUHSC_02389	Unknown function			2.387
SAOUHSC_02390	Unknown function	2.685	3.037	3.272
SAOUHSC_02391	Conserved hypothetical protein	3.427	3.371	4.030
SAOUHSC_02392	Unknown function	2.096		
SAOUHSC_02393	Conserved hypothetical protein	2.199	2.419	
SAOUHSC_02394	Unknown function	3.858	4.379	3.761
SAOUHSC_02396	Conserved hypothetical protein			2.017
SAOUHSC_02400	Unknown function		2.125	
SAOUHSC_02401	Conserved hypothetical protein	2.340	3.924	
SAOUHSC_02402	PTS system, mannitol-specific IIa component		3.334	
SAOUHSC_02403	Mannitol-1-phosphate 5-dehydrogenase, putative		3.732	
SAOUHSC_02409	Unknown function		2.634	3.529
SAOUHSC_02411	Unknown function	2.144	2.286	
SAOUHSC_02412	Unknown function	3.591	2.955	3.565
SAOUHSC_02416	Unknown function		2.319	
SAOUHSC_02418	Conserved hypothetical protein			2.067
SAOUHSC_02419	Conserved hypothetical protein			2.762
SAOUHSC_02420	Conserved hypothetical protein			2.053
SAOUHSC_02422	Conserved hypothetical protein		2.114	3.090
SAOUHSC_02425	Conserved hypothetical protein			3.031
SAOUHSC_02427	Conserved hypothetical protein	2.992	2.401	3.364
SAOUHSC_02428	Conserved hypothetical protein			2.178
SAOUHSC_02430	Unknown function	2.617	2.664	3.167
SAOUHSC_02432	Unknown function	4.257	3.651	4.095
SAOUHSC_02433	Conserved hypothetical protein	3.188	3.550	2.803
SAOUHSC_02434	Conserved hypothetical protein	2.639	2.909	
SAOUHSC_02435	Conserved hypothetical protein	2.265		
SAOUHSC_02436	Conserved hypothetical protein		2.127	
SAOUHSC_02437	Conserved hypothetical protein	2.637	2.124	2.269
SAOUHSC_02438	Conserved hypothetical protein	3.127	2.383	2.611
SAOUHSC_02440	Conserved hypothetical protein	2.896	2.889	2.836
SAOUHSC_02441	Conserved hypothetical protein		3.298	2.355
SAOUHSC_02442	Conserved hypothetical protein		2.089	
SAOUHSC_02447	Conserved hypothetical protein			2.040
SAOUHSC_02449	6-phospho-galactosesynthase	2.667	2.466	2.622
SAOUHSC_02450	Unknown function	2.311		
SAOUHSC_02451	PTS system lactose-specific IIA component	2.677		2.077
SAOUHSC_02452	Tagatose 1,6-bisphosphate synthase	2.282		

SAOUHSC_02453	Tagatose 1,6-bisphosphate synthase	3.020	2.048	2.228
SAOUHSC_02454	Tagatose 1,6-bisphosphate synthase	2.249		
SAOUHSC_02455	Tagatose 6-bisphosphate synthase	2.657		
SAOUHSC_02456	Lactose phosphotransferase repressor, putative	2.107	2.641	2.431
SAOUHSC_02458	Conserved hypothetical protein	3.441	4.364	4.128
SAOUHSC_02459	Conserved hypothetical protein	3.278	3.796	4.074
SAOUHSC_02460	Conserved hypothetical protein		2.627	3.413
SAOUHSC_02461	Transcriptional regulator, merR family, putative		2.768	2.878
SAOUHSC_02462	Unknown function	5.001	3.497	2.627
SAOUHSC_02463	Unknown function	2.705	2.259	2.096
SAOUHSC_02464	Conserved hypothetical protein	3.498	3.234	3.916
SAOUHSC_02466	Unknown function	2.775	3.853	2.092
SAOUHSC_02467	Unknown function	3.185	2.573	3.60
SAOUHSC_02469	Conserved hypothetical protein	2.930	2.560	2.289
SAOUHSC_02470	Conserved hypothetical protein	4.931	4.421	3.602
SAOUHSC_02471	Conserved hypothetical protein	2.416	3.687	2.326
SAOUHSC_02472	Conserved hypothetical protein	2.145	2.636	2.075
SAOUHSC_02473	Unknown function	2.103	2.864	
SAOUHSC_02475	Conserved hypothetical protein	2.170	2.550	
SAOUHSC_02476	Conserved hypothetical protein	2.207	2.565	
SAOUHSC_02477	tRNA interacting protein	3.005	2.172	3.718
SAOUHSC_02515	Conserved hypothetical protein		2.942	3.558
SAOUHSC_02516	Conserved hypothetical protein	5.115	4.052	5.829
SAOUHSC_02518	Conserved hypothetical protein	3.774	3.562	3.032
SAOUHSC_02521	Conserved hypothetical protein	4.289	3.264	4.069
SAOUHSC_02522	Conserved hypothetical protein	2.572	2.116	2.78
SAOUHSC_02523	Conserved hypothetical protein	3.415	3.468	3.849
SAOUHSC_02524	Conserved hypothetical protein	4.594	4.474	6.187
SAOUHSC_02529	Conserved hypothetical protein	2.140	2.431	
SAOUHSC_02530	Conserved hypothetical protein	2.841	2.000	
SAOUHSC_02532	Conserved hypothetical protein	3.317	2.849	4.193
SAOUHSC_02533	Conserved hypothetical protein	2.952	2.292	
SAOUHSC_02534	Conserved hypothetical protein	3.496	3.496	2.599
SAOUHSC_02535	Conserved hypothetical protein	3.161	3.133	2.569
SAOUHSC_02549	Unknown function		2.044	
SAOUHSC_02550	Formate dehydrogenase-H activator		2.449	4.113
SAOUHSC_02551	Conserved hypothetical protein	2.426		3.045
SAOUHSC_02554	Conserved hypothetical protein	2.883	2.294	2.206
SAOUHSC_02555	Conserved hypothetical protein	2.220		2.282
SAOUHSC_02557	Unknown function	3.673	2.721	4.959
SAOUHSC_02562	Urease metallocenter assembly protein			2.062
SAOUHSC_02563	Urease accessory protein UreF, putative			2.238

SAOUHSC_02564	Unknown function			2.382
SAOUHSC_02565	Urease accessory protein UreD, putative	2.917	3.083	6.703
SAOUHSC_02566	Conserved hypothetical protein	2.468	4.146	2.267
SAOUHSC_02567	Unknown function	9.774	9.389	13.251
SAOUHSC_02568	Conserved hypothetical protein	2.106	3.217	3.531
SAOUHSC_02569	Conserved hypothetical protein	3.450	3.032	2.645
SAOUHSC_02570	Conserved hypothetical protein	4.682	3.460	2.661
SAOUHSC_02572	Conserved hypothetical protein	9.326	6.689	8.431
SAOUHSC_02573	Unknown function	2.437	2.689	
SAOUHSC_02575	Conserved hypothetical protein	3.698	3.181	2.947
SAOUHSC_02576	Unknown function		2.425	2.131
SAOUHSC_02577	NAD binding domain protein		2.399	3.083
SAOUHSC_02580	Unknown function	2.339	2.194	2.042
SAOUHSC_02581	Conserved hypothetical protein	2.431	3.613	4.012
SAOUHSC_02585	Conserved hypothetical protein			2.573
SAOUHSC_02588	Conserved hypothetical protein	2.621	2.964	2.444
SAOUHSC_02590	Conserved hypothetical protein	2.020		2.904
SAOUHSC_02591	Conserved hypothetical protein			2.216
SAOUHSC_02592	Conserved hypothetical protein	2.459		2.898
SAOUHSC_02593	Conserved hypothetical protein	2.478		2.832
SAOUHSC_02594	Conserved hypothetical protein	2.037	2.040	2.040
SAOUHSC_02596	Conserved hypothetical protein	2.860	2.984	3.066
SAOUHSC_02599	Hex regulon repressor, putative	3.726	3.936	5.004
SAOUHSC_02602	Unknown function	3.463	2.485	2.095
SAOUHSC_02603	Conserved hypothetical protein	2.961	2.649	2.541
SAOUHSC_02604	Conserved hypothetical protein	2.183	3.460	
SAOUHSC_02606	N-formimidoyl-L-glutamate synthase	2.141		2.145
SAOUHSC_02608	Conserved hypothetical protein	3.513	2.824	2.999
SAOUHSC_02609	Fosfomycin resistance determinant	5.239	4.254	4.253
SAOUHSC_02610	Glutamate and formamide synthase	2.635	3.927	2.025
SAOUHSC_02611	Conserved hypothetical protein			2.226
SAOUHSC_02612	Pentose phosphate pathway protein	2.838	2.939	2.715
SAOUHSC_02614	Unknown function	2.503	2.907	3.313
SAOUHSC_02616	Unknown function		2.070	
SAOUHSC_02618	Conserved hypothetical protein	2.378		2.245
SAOUHSC_02620	Conserved hypothetical protein	2.594	2.333	
SAOUHSC_02621	Unknown function	2.766	2.120	2.356
SAOUHSC_02622	Unknown function	3.200	3.028	4.281
SAOUHSC_02623	Isopentenyl pyrophosphate isomerase			2.360
SAOUHSC_02625	Conserved hypothetical protein			2.089
SAOUHSC_02626	Conserved hypothetical protein	2.863	2.599	3.501
SAOUHSC_02627	Conserved hypothetical protein	2.233	2.112	2.484
SAOUHSC_02628	Conserved hypothetical protein	3.218	2.948	2.961

SAOUHSC_02631	Conserved hypothetical protein	2.710	2.982	3.498
SAOUHSC_02632	Conserved hypothetical protein	2.485	2.341	2.570
SAOUHSC_02633	Conserved hypothetical protein	2.151		
SAOUHSC_02635	Conserved hypothetical protein			2.073
SAOUHSC_02636	Conserved hypothetical protein	2.760	3.318	2.115
SAOUHSC_02637	Conserved hypothetical protein	2.545	3.171	
SAOUHSC_02639	Truncated transposase	3.052	2.510	2.710
SAOUHSC_02640	Conserved hypothetical protein	2.509		
SAOUHSC_02641	Permease, putative domain protein	2.423		
SAOUHSC_02644	Unknown function	2.824	2.642	2.778
SAOUHSC_02645	Conserved hypothetical protein	2.220		3.236
SAOUHSC_02646	Conserved hypothetical protein	3.073		3.978
SAOUHSC_02647	Unknown function			2.342
SAOUHSC_02648	Malate dehydrogenase		2.617	2.257
SAOUHSC_02650	Lipoprotein, putative		2.627	
SAOUHSC_02653	Conserved hypothetical protein	2.726		2.764
SAOUHSC_02655	Conserved hypothetical protein	2.292		
SAOUHSC_02656	Conserved hypothetical protein	3.051	3.873	3.609
SAOUHSC_02657	Conserved hypothetical protein	2.023		2.050
SAOUHSC_02658	Membrane protein, putative		2.010	2.141
SAOUHSC_02660	Conserved hypothetical protein			2.358
SAOUHSC_02661	Unknown function		2.003	3.137
SAOUHSC_02662	Unknown function		2.203	2.883
SAOUHSC_02663	Conserved hypothetical protein			2.850
SAOUHSC_02664	Unknown function	3.154		2.419
SAOUHSC_02665	Conserved hypothetical protein	4.706	7.117	5.658
SAOUHSC_02666	Conserved hypothetical protein	3.587	5.063	3.391
SAOUHSC_02667	Conserved hypothetical protein			2.316
SAOUHSC_02669	Conserved hypothetical protein	4.299	4.538	4.332
SAOUHSC_02670	Conserved hypothetical protein		2.524	2.881
SAOUHSC_02671	Conserved hypothetical protein	2.675		
SAOUHSC_02672	Conserved hypothetical protein	3.694	3.069	2.881
SAOUHSC_02673	Conserved hypothetical protein	3.235	2.292	2.385
SAOUHSC_02674	Conserved hypothetical protein	3.088	2.849	2.699
SAOUHSC_02675	Conserved hypothetical protein	2.349	2.104	
SAOUHSC_02678	Unknown function	2.394	2.743	2.018
SAOUHSC_02679	Unknown function	2.715	2.561	2.330
SAOUHSC_02680	Unknown function	2.180	2.062	
SAOUHSC_02687	Unknown function	2.306	2.083	2.679
SAOUHSC_02688	Conserved hypothetical protein	5.634	5.920	4.381
SAOUHSC_02689	Conserved hypothetical protein	2.484	2.682	2.413
SAOUHSC_02690	Conserved hypothetical protein	2.594	3.932	2.013
SAOUHSC_02691	Conserved hypothetical protein	2.992	2.913	4.132

SAOUHSC_02692	Conserved hypothetical protein	2.333	2.415	3.070
SAOUHSC_02693	Conserved hypothetical protein	3.687	3.854	3.236
SAOUHSC_02694	Conserved hypothetical protein		2.648	
SAOUHSC_02695	Conserved hypothetical protein		2.792	
SAOUHSC_02696	Unknown function	3.014	2.348	2.671
SAOUHSC_02697	Unknown function	3.815	2.773	3.410
SAOUHSC_02698	Unknown function	2.656		2.531
SAOUHSC_02699	Conserved hypothetical protein	2.280		
SAOUHSC_02700	Conserved hypothetical protein	2.159		
SAOUHSC_02701	Unknown function	3.807	3.122	2.632
SAOUHSC_02702	Conserved hypothetical protein	3.771	2.642	2.835
SAOUHSC_02703	Unknown function	2.251	2.457	2.583
SAOUHSC_02705	Conserved hypothetical protein	4.011	3.617	3.415
SAOUHSC_02708	Gamma-hemolysin h-gamma-ii subunit, putative		2.216	2.528
SAOUHSC_02709	Unknown function		2.127	2.036
SAOUHSC_02711	Conserved hypothetical protein	2.821	2.370	2.572
SAOUHSC_02712	Pimeloyl-CoA synthase	2.921		2.806
SAOUHSC_02718	Conserved hypothetical protein	2.182	2.063	
SAOUHSC_02719	Conserved hypothetical protein	2.157		
SAOUHSC_02721	Conserved hypothetical protein	5.039	6.297	5.981
SAOUHSC_02722	Conserved hypothetical protein	5.166	6.073	5.971
SAOUHSC_02725	Conserved hypothetical protein	2.002		
SAOUHSC_02726	Positive transcriptional activator, putative	2.373	2.499	
SAOUHSC_02727	Conserved hypothetical protein	2.065		2.786
SAOUHSC_02728	Conserved hypothetical protein	2.173		2.198
SAOUHSC_02729	Unknown function		2.076	2.037
SAOUHSC_02731	Conserved hypothetical protein	3.133	2.297	2.018
SAOUHSC_02732	Unknown function	2.556	2.273	2.383
SAOUHSC_02733	Membrane protein, putative	2.588	3.000	2.242
SAOUHSC_02734	Conserved hypothetical protein	6.937	6.630	6.560
SAOUHSC_02736	Conserved hypothetical protein	3.605	4.113	4.928
SAOUHSC_02737	Unknown function	3.956	3.592	4.933
SAOUHSC_02738	Conserved hypothetical protein	2.885		
SAOUHSC_02740	Unknown function	2.325		
SAOUHSC_02746	Conserved hypothetical protein		2.071	2.575
SAOUHSC_02751	Conserved hypothetical protein	2.758	3.094	
SAOUHSC_02752	Conserved hypothetical protein	3.551	2.697	2.436
SAOUHSC_02753	Membrane protein, putative	2.001	2.769	
SAOUHSC_02754	Unknown function		2.218	
SAOUHSC_02756	Conserved hypothetical protein	2.783		3.058
SAOUHSC_02757	Conserved hypothetical protein	2.198		2.561
SAOUHSC_02758	Conserved hypothetical protein	2.790	2.110	2.919

SAOUHSC_02760	Unknown function			2.444
SAOUHSC_02761	Conserved hypothetical protein	3.357	3.319	3.153
SAOUHSC_02763	Unknown function	2.195		
SAOUHSC_02764	Unknown function	2.446	2.067	
SAOUHSC_02766	Unknown function	2.595	2.520	
SAOUHSC_02767	Unknown function	2.311		
SAOUHSC_02768	Conserved hypothetical protein	3.768	2.781	2.128
SAOUHSC_02769	Conserved hypothetical protein	2.652		
SAOUHSC_02770	Conserved hypothetical protein	2.761	2.117	
SAOUHSC_02771	Conserved hypothetical protein	5.639	10.517	6.309
SAOUHSC_02772	Conserved hypothetical protein	2.270	3.944	
SAOUHSC_02773	Unknown function	2.281		
SAOUHSC_02774	Conserved hypothetical protein		3.935	
SAOUHSC_02775	Conserved hypothetical protein	2.076	2.228	2.977
SAOUHSC_02779	Conserved hypothetical protein	2.170	2.455	2.717
SAOUHSC_02780	Conserved hypothetical protein	3.492	3.993	4.223
SAOUHSC_02781	Conserved hypothetical protein		2.463	
SAOUHSC_02782	Conserved hypothetical protein	2.158	2.030	2.37
SAOUHSC_02783	Conserved hypothetical protein	2.623	2.414	2.757
SAOUHSC_02784	Conserved hypothetical protein	2.168		
SAOUHSC_02791	Unknown function	2.214	2.562	
SAOUHSC_02793	Conserved hypothetical protein		2.377	2.634
SAOUHSC_02794	Conserved hypothetical protein	2.716	2.435	2.723
SAOUHSC_02795	Conserved hypothetical protein	3.526	3.452	3.442
SAOUHSC_02796	Conserved hypothetical protein	11.152	10.964	10.322
SAOUHSC_02798	Conserved hypothetical protein	2.016		
SAOUHSC_02799	Unknown function	4.651	3.849	2.436
SAOUHSC_02800	Conserved hypothetical protein	3.383	3.111	3.041
SAOUHSC_02804	Conserved hypothetical protein	2.174	2.565	5.195
SAOUHSC_02812	Conserved hypothetical protein	5.124	6.820	4.154
SAOUHSC_02813	Conserved hypothetical protein	2.726	2.107	3.027
SAOUHSC_02814	Conserved hypothetical protein	2.383		2.694
SAOUHSC_02815	Conserved hypothetical protein		2.588	2.874
SAOUHSC_02816	Conserved hypothetical protein	4.898	5.448	6.035
SAOUHSC_02817	Unknown function			2.083
SAOUHSC_02818	Conserved hypothetical protein	2.602	2.999	3.182
SAOUHSC_02819	Conserved hypothetical protein	3.577	4.037	4.390
SAOUHSC_02820	Conserved hypothetical protein	3.524	3.500	2.450
SAOUHSC_02821	Unknown function	3.104	3.871	2.930
SAOUHSC_02822	Conserved hypothetical protein			2.613
SAOUHSC_02823	Conserved hypothetical protein	3.269	2.761	2.533
SAOUHSC_02827	Conserved hypothetical protein	4.108	4.207	3.475
SAOUHSC_02828	Unknown function			2.551

SAOUHSC_02830	Pyruvate synthase		2.354	2.344
SAOUHSC_02831	Conserved hypothetical protein	3.471	3.108	3.031
SAOUHSC_02832	Conserved hypothetical protein	4.802	4.546	5.694
SAOUHSC_02833	Conserved hypothetical protein	2.910	2.216	2.926
SAOUHSC_02834	Sortase, putative			2.449
SAOUHSC_02836	Conserved hypothetical protein	2.402		
SAOUHSC_02837	Conserved hypothetical protein	2.614	2.153	
SAOUHSC_02838	Conserved hypothetical protein			2.098
SAOUHSC_02839	Unknown function	2.298		2.140
SAOUHSC_02842	Conserved hypothetical protein	7.457		5.339
SAOUHSC_02843	Conserved hypothetical protein	2.816		2.308
SAOUHSC_02852	Conserved hypothetical protein		2.184	2.494
SAOUHSC_02855	LysM domain protein	2.218	2.443	2.359
SAOUHSC_02856	Unknown function	7.226	9.658	9.105
SAOUHSC_02857	Conserved hypothetical protein	5.024	7.464	7.284
SAOUHSC_02858	Conserved hypothetical protein	3.753	4.747	5.020
SAOUHSC_02860	Unknown function			2.474
SAOUHSC_02862	Unknown function	2.053	4.884	
SAOUHSC_02863	Conserved hypothetical protein	4.138	2.776	2.303
SAOUHSC_02864	Unknown function	2.662		2.096
SAOUHSC_02865	Conserved hypothetical protein	2.416		
SAOUHSC_02866	Conserved hypothetical protein		2.178	
SAOUHSC_02867	Conserved hypothetical protein		2.081	2.464
SAOUHSC_02868	Conserved hypothetical protein	2.981	2.423	3.103
SAOUHSC_02872	Conserved hypothetical protein	2.320	3.279	4.516
SAOUHSC_02873	Cation-transporting ATPase, putative			2.518
SAOUHSC_02874	Cation-transporting ATPase, putative	3.078	2.372	5.745
SAOUHSC_02877	Squalene synthase, putative	2.034	2.676	
SAOUHSC_02879	Unknown function	2.747	3.984	
SAOUHSC_02880	Conserved hypothetical protein		2.575	
SAOUHSC_02881	Conserved hypothetical protein	2.012	3.100	
SAOUHSC_02882	Conserved hypothetical protein	3.363	5.625	
SAOUHSC_02883	LysM domain protein	2.589	2.196	2.536
SAOUHSC_02886	Conserved hypothetical protein	3.489	2.548	
SAOUHSC_02887	Immunodominant antigen A, putative			2.011
SAOUHSC_02890	Conserved hypothetical protein	3.807	3.850	3.570
SAOUHSC_02891	Conserved hypothetical protein	2.891	2.389	2.936
SAOUHSC_02892	Conserved hypothetical protein	2.587		3.081
SAOUHSC_02893	Conserved hypothetical protein	2.052		2.754
SAOUHSC_02896	Conserved hypothetical protein	2.461		
SAOUHSC_02897	Conserved hypothetical protein	2.654	2.005	
SAOUHSC_02898	Conserved hypothetical protein	2.291		
SAOUHSC_02899	Conserved hypothetical protein		2.425	

SAOUHSC_02900	Conserved hypothetical protein	2.465	3.900	2.007
SAOUHSC_02901	Conserved hypothetical protein	2.151		
SAOUHSC_02903	Conserved hypothetical protein	2.389		2.003
SAOUHSC_02904	Conserved hypothetical protein	2.884	2.053	2.209
SAOUHSC_02905	Conserved hypothetical protein	3.142	3.488	3.179
SAOUHSC_02906	Conserved hypothetical protein	2.262	2.404	
SAOUHSC_02907	Conserved hypothetical protein	2.174	2.416	
SAOUHSC_02908	Conserved hypothetical protein	3.453	3.834	2.391
SAOUHSC_02910	Conserved hypothetical protein	3.171	3.440	2.628
SAOUHSC_02911	Conserved hypothetical protein		2.128	
SAOUHSC_02912	Conserved hypothetical protein	2.973	2.826	3.587
SAOUHSC_02914	Conserved hypothetical protein	2.804	2.320	2.454
SAOUHSC_02916	Beta alanine synthase	2.766	2.358	2.657
SAOUHSC_02922	Unknown function		2.055	
SAOUHSC_02923	Conserved hypothetical protein			2.016
SAOUHSC_02925	Conserved hypothetical protein	4.263	6.171	3.668
SAOUHSC_02928	Conserved hypothetical protein	2.650	2.918	3.204
SAOUHSC_02930	Conserved hypothetical protein	2.252	2.797	
SAOUHSC_02931	Conserved hypothetical protein	4.293	3.494	3.808
SAOUHSC_02932	Betaine aldehyde synthase	7.143		8.718
SAOUHSC_02933	Unknown function	4.915		5.180
SAOUHSC_02934	Conserved hypothetical protein	11.947		11.267
SAOUHSC_02935	Conserved hypothetical protein	2.006		
SAOUHSC_02936	Conserved hypothetical protein	3.244		
SAOUHSC_02937	Choline transporter, putative	5.483		6.163
SAOUHSC_02941	Conserved hypothetical protein			2.061
SAOUHSC_02942	2'-deoxynucleoside 5'-triphosphate synthase	2.234		2.506
SAOUHSC_02943	Unknown function	2.419		
SAOUHSC_02944	Conserved hypothetical protein		2.867	3.321
SAOUHSC_02948	Unknown function		2.846	
SAOUHSC_02949	Conserved hypothetical protein	3.663	4.323	3.511
SAOUHSC_02950	Unknown function	3.736	2.204	3.202
SAOUHSC_02954	Unknown function			2.146
SAOUHSC_02958	Unknown function	2.538		
SAOUHSC_02964	Conserved hypothetical protein	5.509	4.167	4.505
SAOUHSC_02972	Conserved hypothetical protein		2.074	2.427
SAOUHSC_02974	Conserved hypothetical protein	3.086	2.860	3.437
SAOUHSC_02976	Mannose-6-phosphate isomerase, class I	2.112		2.119
SAOUHSC_02979	Conserved hypothetical protein			2.501
SAOUHSC_02983	Conserved hypothetical protein	2.677		
SAOUHSC_02985	Peptide export protein	2.285		
SAOUHSC_02986	Conserved hypothetical protein	3.307		

SAOUHSC_02987	Conserved hypothetical protein	2.818		
SAOUHSC_02988	Conserved hypothetical protein	3.260		2.008
SAOUHSC_02989	Membrane protein, putative	4.513	2.199	2.977
SAOUHSC_02990	Conserved hypothetical protein	3.793		2.582
SAOUHSC_02991	Conserved hypothetical protein	5.333	3.580	2.877
SAOUHSC_02992	Conserved hypothetical protein	4.659	4.137	2.775
SAOUHSC_02993	Conserved hypothetical protein	3.921	3.641	2.296
SAOUHSC_02994	Conserved hypothetical protein	3.924	3.862	
SAOUHSC_02995	Conserved hypothetical protein	2.426	2.267	
SAOUHSC_03000	Unknown function	3.090	2.075	
SAOUHSC_03001	Unknown function	3.017	3.547	5.322
SAOUHSC_03004	IcaB protein, putative	2.201	2.209	2.378
SAOUHSC_03005	Unknown function	3.064	3.247	3.802
SAOUHSC_03006	Unknown function	2.971		
SAOUHSC_03016	Conserved hypothetical protein	2.215		
SAOUHSC_03017	Conserved hypothetical protein	2.510	2.052	
SAOUHSC_03022	Conserved hypothetical protein	3.174	3.041	3.425
SAOUHSC_03023	Unknown function		2.016	2.365
SAOUHSC_03024	Conserved hypothetical protein			2.056
SAOUHSC_03025	5-oxoproline removal protein	2.356		
SAOUHSC_03026	Conserved hypothetical protein	2.285	2.366	2.955
SAOUHSC_03027	Conserved hypothetical protein	2.099	2.026	
SAOUHSC_03028	Conserved hypothetical protein	4.731	6.710	5.116
SAOUHSC_03030	Unknown function	3.353	2.907	2.631
SAOUHSC_03032	Conserved hypothetical protein	3.435	3.952	2.529
SAOUHSC_03033	Unknown function		2.600	2.447
SAOUHSC_03034	Conserved hypothetical protein		2.603	2.686
SAOUHSC_03035	Conserved hypothetical protein	3.585	4.374	2.515
SAOUHSC_03036	Unknown function		2.387	
SAOUHSC_03037	Unknown function		2.104	
SAOUHSC_03038	Unknown function		5.099	4.651
SAOUHSC_03040	Unknown function		2.867	3.131
SAOUHSC_03041	Conserved hypothetical protein	4.095	4.929	5.573
SAOUHSC_03042	Integrase/recombinase, core domain family	2.518	2.914	2.729
SAOUHSC_03043	Unknown function	3.079	4.347	3.921
SAOUHSC_03045	Unknown function	5.460	4.539	4.386
SAOUHSC_03046	Helix-turn-helix domain protein	2.234	2.463	
SAOUHSC_03049	Conserved hypothetical protein			2.499
SAOUHSC_03055	Unknown function	3.092	2.626	4.256
SAOUHSC_A00097	Unknown function			2.656
SAOUHSC_A00219	Unknown function		2.160	
SAOUHSC_A00283	Unknown function	3.218	3.797	
SAOUHSC_A00354	Conserved hypothetical protein	18.893	19.246	15.312

SAOUHSC_A00635	Unknown function	4.363	4.698	7.52
SAOUHSC_A00703	Unknown function	3.651	2.671	2.651
SAOUHSC_A00747	Conserved hypothetical protein		2.111	2.126
SAOUHSC_A00992	Conserved hypothetical protein	2.263	2.914	2.216
SAOUHSC_A01079	Unknown function	5.513	7.672	2.788
SAOUHSC_A01081	Conserved hypothetical protein	2.365	3.966	3.134
SAOUHSC_A01332	Conserved hypothetical protein	6.157	3.356	4.427
SAOUHSC_A01436	Unknown function	4.717	3.590	3.364
SAOUHSC_A01455	Hypothetical phage protein	2.255	2.197	2.109
SAOUHSC_A01514	Hypothetical phage protein	2.059		2.190
SAOUHSC_A01723	Unknown function	2.408		4.032
SAOUHSC_A01754	Unknown function	4.052	4.286	5.070
SAOUHSC_A01909	Unknown function	2.163		
SAOUHSC_A01910	Unknown function	3.441	3.168	2.746
SAOUHSC_A02013	Conserved hypothetical protein	2.798	4.774	3.872
SAOUHSC_A02094	Hypothetical phage protein	2.074	2.000	2.075
SAOUHSC_A02254	Unknown function	6.525	5.019	5.959
SAOUHSC_A02273	Unknown function	3.570	3.743	3.910
SAOUHSC_A02331	Conserved hypothetical protein	3.085	2.910	3.17
SAOUHSC_A02350	Unknown function	4.007	3.123	3.056
SAOUHSC_A02445	Conserved hypothetical protein	2.886	2.148	
SAOUHSC_A02450	Conserved hypothetical protein	2.642	3.242	3.106
SAOUHSC_A02483	Unknown function	5.289	4.402	6.717
SAOUHSC_A02503	Unknown function		2.939	
SAOUHSC_A02635	Unknown function	2.177		2.160
SAOUHSC_A02680	Conserved hypothetical protein	2.414	2.467	5.016
SAOUHSC_A02771	Unknown function	2.174	2.763	3.286
SAOUHSC_A02794	Unknown function	2.054	3.025	2.552
SAOUHSC_A02795	Unknown function	3.396		3.633
SAOUHSC_A02811	Unknown function	2.346		2.183
SAOUHSC_A02856	Unknown function	2.482	3.347	2.866

Appendix 3

List of publications

The following publications have arisen as result of the work carried out over the course of my PhD studies.

Derouaux, A., Turk, S., Olrichs, N. K., Gobec, S., Breukink, E., Amoroso, A., Offant, J., Bostock, J., Mariner, K., Chopra, I., Vernet, T., Zervosen, A., Joris, B., Frère, J-M., Nguyen-Distèche, M. and M. al Terrak. (2011) Small molecule inhibitors of peptidoglycan synthesis targeting the lipid II precursor. *Biochemical Pharmacology* **81**: 1098-1105.

Mariner, K., McPhillie, M., Trowbridge, R., Agarwal, A. K., Smith, C., O'Neill, A. J., Fishwick, C. W. G. and I. Chopra. (2010) Antibacterial Activity and Development of Resistance to Corallopyronin A (CorA), an Inhibitor of RNA Polymerase (RNAP). 50th Interscience Conference of Antimicrobial Agents and Chemotherapy (ICAAC), **Presentation No. F1-1616**

Mariner, K., McPhillie, M., Trowbridge, R., Smith, C., O'Neill, A. J., Fishwick, C. W. G. and I. Chopra (2011) Activity of and Development of Resistance to Corallopyronin A, an Inhibitor of RNA Polymerase. *Antimicrobial Agents and Chemotherapy* **55**: 2413-2416.

Mariner, K. R., O'Neill, A. J. and I. Chopra (2009) Biological activity and mode of action of type B lantibiotics against Gram-positive bacteria. UKCanBACWAN Conference, Warwick, United Kingdom.

Mariner, K. R., Ooi, N., Roebuck, D., O'Neill, A. J. and I. Chopra (2011) Further Characterization of *Bacillus subtilis* Antibiotic Biosensors and Their Use for Antibacterial Mode-of-Action Studies. *Antimicrobial Agents and Chemotherapy* **55**: 1784-1786.

Mariner, K. R., Trowbridge, R., Agarwal, A. K., Miller, K., O'Neill, A. J., Fishwick, C. W. G. and I. Chopra. (2010) Furanyl-Rhodanines Are Unattractive Drug Candidates for Development as Inhibitors of Bacterial RNA Polymerase. *Antimicrobial Agents and Chemotherapy* **54**: 4506-4509.

McPhillie, M., Trowbridge, R., Mariner, K., O'Neill, A. J., Johnson, A. P., Chopra, I. and C. W. G. Fishwick. (2011) Structure-based Ligand Design of Novel Bacterial RNA Polymerase Inhibitors. *ACS Medicinal Chemistry Letters*. **2:** 729-734

Vehar, B., Hrast, M., Kovac, A., Konc, J., Mariner, K., Chopra, I., Janezic, D. and S. Gobec. (2011) Ellipticines and 9-acridinylamines as inhibitors of D-alanine:D-alanine ligase. *Bioorganic & Medicinal Chemistry*. **19:** 5137-5146