Generation, optimisation, validation and implementation of bacterial whole-cell biosensors for the detection of novel antibiotics

Jennifer Katie Mitchell

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

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Abstract

Antibiotics revolutionised healthcare in the 20th century, reducing the prevalence of many infectious diseases that had been a burden for humankind throughout its history. Many of the antibiotics discovered during the last century were natural products isolated from bacteria found in soil. Eventually, not only did bacteria resistant to these antibiotics begin to emerge, but it became clear that natural product screening was offering diminishing returns as they were often rediscovering known compounds. The lack of success of target-based approaches and screening large small molecule libraries has cast the spotlight once again on screening natural products for novel antibacterial drugs. Unfortunately, this screening requires methods far more efficient than those currently available. One solution to this is to use whole-cell biosensors to indicate the presence of antibiotics. However, many of the biosensors in the literature are not specific enough for high-throughput screening.

The O'Neill lab produced three biosensors for detecting cell wall biosynthesis inhibitors (CBIs). In this thesis, one of these biosensors was used to screen a library of nearly 4,000 compounds. Only the 46 CBIs in this library induced the biosensor, demonstrating its high level of specificity. This biosensor was subsequently engineered to include resistance genes, generating a novel CBI biosensor that was simultaneously capable of dereplicating some classes of CBIs.

Most natural product antibiotics used in the clinic inhibit steps in bacterial cell wall or protein biosynthesis, and so biosensors capable of detecting protein biosynthesis inhibitors (PBIs), for example, would also be useful. With this aim, a transcriptional signature was obtained for *Staphylococcus aureus* exposed to a sub-inhibitory concentration of two PBIs to identify promoters suitable for inclusion in a PBI biosensor. Unfortunately, the three PBI biosensors generated were not specific for PBIs. However, the results obtained highlight the difficulties in generating PBI biosensors suitable for high-throughput screening.

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Abbreviations

4-MU	4-methylumbelliferone
AAC	aminoglycoside acetyltransferase
Amp	ampicillin
ANT	aminoglycoside nucleotidyl transferase
APH	aminoglycoside phosphotransferase
ARP	antibiotic resistance platform
alr	Alanine racemase
ATc	anhydrotetracycline
bp	base pair
CBI	cell wall biosynthesis inhibitor
Cm	chloramphenicol
DAP	diaminopimelate
Ddl	D-ala-D-ala ligase
DFC	drug-free control
DHPS	Dihydropteroate synthase
DMAPP	dimethylallyl diphosphate
DMSO	Dimethylsulfoxide
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EDGE	Empirical analysis of Digital Gene Expression
EDTA	ethylenediaminetetraacetic acid
EF-G	elongation factor G
EF-Tu	elongation factor Tu
EGCG	epigallocatechin gallate
Em	erythromycin
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa*, and *Enterobacter* species
FDA	US Food and Drug Administration
FPP	farnesyl diphosphate
g	grams
g	unit gravity sedimentation
GlcNAc	*N*-Acetylglucosamine

GTP	guanosine triphosphate
h	hours
HGT	horizontal gene transfer
HR-LCMS	High Resolution Liquid Chromatography Mass Spectrometry
HTS	high-throughput screen
IPP	isopentenyl diphosphate
IPTG	isopropyl β-D-1-thiogalactopyranoside
kb	kilo base pair
L	litre
LBA	Luria Bertani Agar
LBB	Luria Bertani Broth
m	metre
M	molar
MCS	multiple cloning site
MDR	multi-drug-resistant
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MIC	minimum inhibitory concentration
MOA	mode of action
MRSA	methicillin-resistant *Staphylococcus aureus*
MUG	4-methylumbelliferyl β-D-galactopyranoside
MurNAc	*N*-Acetylmuramic acid
NCI	National Cancer Institute
OD	Optical density
PBI	protein biosynthesis inhibitor
PBP	Penicillin binding protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RBS	ribosome binding site
RT	room temperature
S	Svedberg unit
SATMD	*Staphylococcus aureus* Transcriptome Meta-Database
S-gal	3,4-Cyclohexeneoesculetin-β-D-galactopyranoside
SNRI	serotonin–norepinephrine reuptake inhibitors

SSRI	serotonin-specific reuptake inhibitors
Tet	tetracycline
TSA	Tryptone Soya Agar
TSB	Tryptone Soya Broth
UDP	uridine diphosphate
Und-P	undecaprenyl phosphate
UppP	undecaprenyl diphosphate phosphatase
UppS	undecaprenyl diphosphate synthase
VRE	vancomycin-resistant enterococci
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
ZOI	zone of inhibition
β-gal	β-galactosidase

Chapter 1 Introduction

1.1 The rise of antibacterial resistance

Antibiotics have revolutionised healthcare in the 20th century, increasing the average life expectancy by around 30 years in developed countries (Finch *et al*., 2010). An example of just how important the introduction of antibiotics has been is the reduction in mortality associated with *Staphylococcus aureus* bacteremia (SAB); in the pre-antibiotic era, this was >80%, and antibiotics reduced this figure to approximately 20% by 2004 (van Hal *et al*., 2012). In addition to treating established infections, antibiotics are extensively used to prevent recurrent systemic infections and postoperative infections, which is particularly important when a patient is immunosuppressed in order to accept a new organ during transplantation or during cancer chemotherapy (Enzler *et al*., 2011; Nichols, 2001).

Unfortunately, there has been a dearth of new antibiotics since the 1970s, alongside rapidly increasing levels of infection caused by antibiotic resistant bacteria. Failure to develop novel drugs to treat infections caused by these resistant bacterial strains could ultimately return associated mortality rates to levels last seen a century ago and render simple medical procedures too risky to perform (Davies and Davies, 2010; Davis, 2013; Norrby *et al*., 2009). Current annual casualties associated with antibiotic resistance are 700,000 worldwide; without new antibiotics this figure is predicted to rise to 10 million by 2050, which would cost ~$100 trillion in lost GDP (O'Neill, 2016).

The origin of antibacterial resistance is the fact that bacteria evolved in an environment rich in small bioactive molecules, including peptides and antibiotics produced by other microbes competing for survival. Many bacteria found in the environment therefore have intrinsic resistance before they are exposed to antibiotics in hospital settings (Wright, 2010). Resistance can arise in one of two ways. The selection of bacteria in which a random mutation results in a gene that confers resistance to whichever compound is exerting the selection pressure is referred to as endogenous resistance (Silver, 2011). Alternatively, horizontal gene transfer (HGT) can transfer entire genes from

environmental organisms to human pathogens *via* transformation, transduction or conjugation, and this is termed exogenous resistance (Davies and Davies, 2010; Silver, 2011). A topical example of this is the recent widely reported emergence of exogenous resistance to colistin, a drug of last resort for the treatment of infections caused by Gram-negative bacteria (Liu *et al.*, 2015). Prior to this, resistance to colistin had occurred due to chromosomal mutation, which often incurred a fitness cost to the bacterial host and posed little threat of being transferred to other organisms. This was the first reported instance of resistance due to an easily-transferrable, plasmid-mediated gene (the *mcr*1 gene) in Enterobacteriaceae (Liu *et al.*, 2015).

Mechanisms of resistance to antibiotics include target modification, drug modification, target bypass, a decrease in membrane permeability, and efflux pumps (Wright, 2010). Modification of the antibiotic itself can be either the destruction of the drug or the addition of functional groups that reduce the affinity of the antibiotic for its target (Ramirez and Tolmasky, 2010; Zhang, 2001).

Below is outlined the history of antibacterial compound discovery, the issues surrounding modern drug discovery approaches, and how the work in this project could contribute to maintaining the lead in the battle against antimicrobial resistance.

1.2 The antibacterial mode of action of antibiotics

There are several essential biosynthetic pathways involved in bacterial metabolism that have been targeted by antibiotics so far. The major ones are nucleotide synthesis (which can be further categorised to DNA synthesis, RNA synthesis and folic acid synthesis), protein synthesis and cell wall synthesis (Brown and Wright, 2016; Coates *et al.*, 2002). In addition to these, triclosan can be used topically at low concentrations to avoid cytotoxicity and this drug inhibits fatty acid biosynthesis (Russell, 2004). Daptomycin was developed during the 1990s and has a unique mode of action: it binds specifically to the bacterial cell membrane and dissipates the membrane potential and distorts the membrane due to the drug aggregating, leading to cell death (Alborn *et al.*, 1991; Pogliano *et al.*, 2012). The two pathways on which this study focuses are

bacterial cell wall biosynthesis and bacterial protein biosynthesis, because the vast majority of antibiotics target these two pathways (Silver, 2011; Singh and Barrett, 2006).

The biosynthesis of the bacterial cell wall is a well-established target for antibiotics (Bugg *et al.*, 2011). Furthermore, the reactions involved in this pathway are essential to cell survival and the enzymes required to catalyse them are encoded for by genes that are highly conserved across bacterial species and that lack mammalian homologs (Silver, 2006). This means that antibiotics targeting this pathway are not only very unlikely to be cytotoxic due to having off-target interactions with mammalian cells, but also have the potential to be active against a wide range of bacteria. Details of CBIs and of peptidoglycan biosynthesis are provided in the introduction to Chapter 3.

Protein biosynthesis is the process by which the information encoded within mRNA molecules, which are synthesised from DNA by RNA polymerases, is translated into a sequence of amino acid residues by a molecular piece of machinery called the ribosome. This sequence of amino acid residues, connected by peptide bonds, then proceeds to fold in a specific manner as it emerges from the ribosome to form a protein (Berg *et al.*, 2002a). This is therefore an essential pathway for bacterial cell survival. Bacterial protein biosynthesis involves four major steps, including the formation of amino acyl tRNA-synthetases, chain initiation, chain elongation and chain termination (Walsh, 2003). There is, therefore, a plethora of targets in this pathway available to be exploited for antibacterial chemotherapy. In addition, targeting the proteins involved in these steps are unlikely to give rise to cytotoxicity, owing to the myriad differences between prokaryotic and eukaryotic translation (Berg *et al.*, 2002b; Russell and Chopra, 1996). Details of PBIs are provided in the introduction to Chapter 5.

1.3 Early 20th century antibiotics – the sulfa drugs and penicillin

Prior to the introduction of antibiotics, one of the most common causes of death globally was infectious diseases (Conly and Johnston, 2005). This began to change when the early sulphonamides and penicillins were discovered and

used clinically during the 1930's and 1940's (Drews, 2000; Fleming, 1929; Otten, 1986). The first antibacterial compound to be discovered was actually a synthetic compound. The Gram stain, used to differentiate between Gram-positive and Gram-negative bacteria, uses the stains crystal violet and safranin, both of which are examples of anilines. The fact that dyes derived from coal tar were capable of binding to bacteria led to the screening of hundreds of azo dyes in the hope of discovering novel antibacterial compounds (Wright et al., 2014). In 1932, Gerhard Domagk discovered a red dye that was able to prevent some bacterial infections in mice and this was released as the drug Prontosil, the first antibacterial drug to be used systemically (Otten, 1986). It was later discovered that Prontosil was actually a prodrug that was metabolised in the body to the active portion, sulphanilamide (Otten, 1986; Wright *et al.*, 2014). Its mode of action is the inhibition of dihydropteroate synthase (DHPS) (achieved due to the structural similarities between sulphanilamide and the middle section of dihydrofolate), thus preventing the synthesis of folic acid (Sköld, 2000). The sulpha drugs were not cytotoxic because not only do mammalian cells lack DHPS, but they also utilise a folate uptake system rather than synthesise folic acid endogenously (Sköld, 2000).

The discovery of penicillin was more serendipitous than that of the sulphonamide drugs. Alexander Fleming found that, on a petri dish inoculated with *Staphylococcus pyogenes*, there were no colonies growing in the vicinity of a sample of the mould *Penicillium notatum* (Fleming, 1929). He found that this mould was particularly effective at killing *Streptococcus pyogenes* and *Bacillus diphtheriae*. The drug was initially only extracted as crude material by Fleming, and it was the work carried out in Oxford by Edward Florey and Ernst Chain that was instrumental in obtaining pure penicillin (using alumina column chromatography to remove impurities) in quantities sufficient to administer to a patient. When production moved to the USA, a variety of strategies were implemented that successfully increased the yield of penicillin, including substituting lactose for sucrose in the growth medium, adding corn-steep liquor to the medium, adding penicillin precursors such as phenylacetic acid, and even using X-rays to obtain a mutant version of a strain of *Penicillium notatum* found on a mouldy cantaloupe (Royal Society of Chemistry, 1999).

1.4 Waksman platform and the 'Golden Age' of antibiotic discovery

At around the same time as the development of the sulphonamides, Selman Waksman was studying the occurrence and abundance of actinomycetes in soil (American Chemical Society, 2005). *Actinomycetes*, including the genus *Streptomyces*, are Gram-positive bacteria that play a crucial role in the decomposition of organic matter and nitrogen fixation (Waksman, 1950). They also produce secondary metabolites in abundance that display a combination of antibacterial, antifungal and antithelmitic properties. This impressive variety of functionality of their metabolites likely evolved due to the symbiotic relationship that developed between *Actinomycetes* and plants whereby the *Actinomycetes* would protect the plants from pathogens (de Lima Procópio *et al.*, 2012).

By the time penicillin had been discovered, Waksman and his students had embarked upon a more systematic way of screening cultures to discover novel antibiotics. They grew isolated soil microbes on agar plates and then looked for any colonies that were surrounded by a zone of inhibition. This strategy led to the discovery of around twenty antibiotics (American Chemical Society, 2005).

The first compound to be isolated from *Actinomycetes* was actinomycin (Waksman and Woodruff, 1940). Actinomycin was active against a range of Gram-positive bacteria but, unfortunately, it was also toxic to mammalian cells (Waksman, 1950; Waksman and Woodruff, 1941). The next compounds to be isolated, proactinomycin and micromonosporin, had a narrow spectrum of activity and were also deemed not suitable for therapeutic use (Gardner and Chain, 1942; Waksman *et al.*, 1942).

Streptothricin initially showed more promise than previously isolated compounds, since it was water-soluble and displayed *in vivo* activity against a wide range of bacteria and fungi (Waksman and Woodruff, 1942). Unfortunately, it became apparent that it had a delayed toxic effect in mammalian cells. In 1943, streptomycin was isolated from *Streptomyces griseus* (Schatz *et al.*, 1944). In contrast to streptothricin, streptomycin was not toxic to animals. It was also effective against tuberculosis and both Gram-positive and Gram-negative bacteria, including bacteria resistant to penicillin

(Schatz and Waksman, 1944). By 1946, the compound had entered clinical trials (Keefer *et al.*, 1946).

Many more antibacterial compounds were discovered between 1940 and 1970, such as the aminoglycosides, cephalosporins and tetracyclines (Conly and Johnston, 2005). This is often referred to in commentary on the history of antibiotic development as the 'Golden Age' of antibiotic discovery, owing to the fact that most of the classes of antibiotics in use today were discovered during this period (Conly and Johnston, 2005; Davies, 2006). However, the frequent rediscovery of known compounds ('replication') led to the abandonment of the Waksman platform and necessitated a change in strategy to the modification of existing antibiotics to overcome emerging resistance to the available antibiotics (Lewis, 2013).

1.5 Semi-synthetic antibiotics derived from natural product scaffolds

Many antibacterial compounds introduced in the 1960s and beyond were semi-synthetic, having been chemically modified to increase their potency or bioavailability (Lewis, 2013). The discovery and development of the cephalosporins provides an example of this process. Cephalosporin itself was discovered in 1945 by Giuseppe Brotzu, who found that the species *Cephalosporium acremonium* inhibited the growth of some Gram-positive and Gram-negative bacteria (Brotzu, 1948; Sader and Jones, 1992). The active antibacterial compound was cephalosporin C, which was not particularly potent but displayed acid stability and resistance to penicillinases (Abraham and Newton, 1961). It shared the beta-lactam ring with the structure of the penicillins and inhibited peptidoglycan synthesis via the same mechanism. This compound was hydrolysed to give 7-aminocephalosporinic acid (7-ACA), which was the basis for the 1st-generation cephalosporins (Hamilton-Miller, 2008) (Figure 1.1A).

The 1st-generation cephalosporins (such as cefradine, cefadroxil, cephalothin and cephalexin) typically had a methyl group at position 3' of the dihydrothiazine ring (García-Rodríguez *et al.*, 1995)(Figure 1.1B). This gave them a relatively low activity due to their low affinity for the common penicillin-

binding proteins. The α-amino group at position 7 made these compounds susceptible to β-lactamases (Sader and Jones, 1992). Despite this, the 1st-generation cephalosporins were most active against Gram-positive cocci, but only had moderate activity against some aerobic Gram-negative bacilli (Sader and Jones, 1992; Wright *et al.*, 2014).

By contrast, the 2nd-generation cephalosporins (such as cefuroxime) had increased potency for Gram-negative bacteria. They were less susceptible to β-lactamases owing to the addition of an α-methoxyimino group to position 7 (Sader and Jones, 1992)(Figure 1.1C). This group sterically hindered the β-lactamase from cleaving the β-lactam ring (Wright *et al.*, 2014).

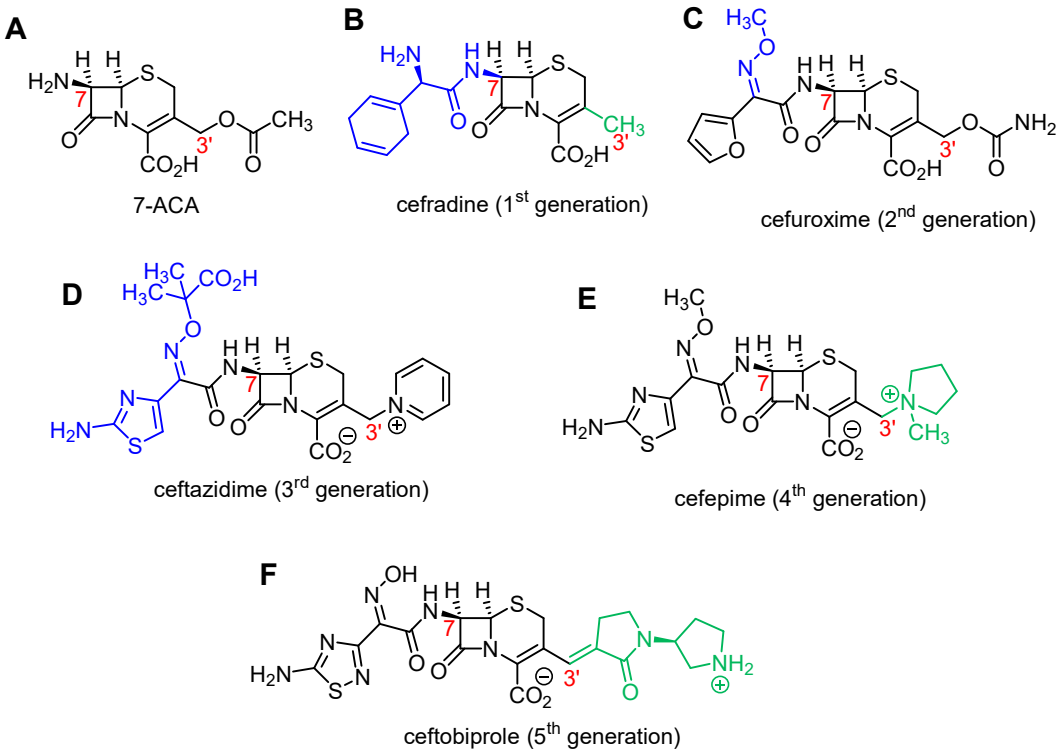


Figure 1.1 Examples of compounds in the cephalosporin class. A) 7-aminocephalosporinic acid (7-ACA), the basis for the 1st-generation cephalosporins with the C7 and C3' positions indicated in red; B) Cefradine (1st generation) with the α-amino group in blue and the methyl group in green; C) Cefuroxime (2nd generation) with the α-methoxyimino group in blue; D) Ceftazidime (3rd generation) with the aminothiazole oxime with a carboxylate side chain shown in blue; E) Cefepime (4th generation) with the positively charged quaternary amine group in green; F) Ceftobiprole (5th generation) with the improved side chain in green.

The 3rd-generation cephalosporins incorporated either an aminothiazolyl group or an iminomethoxy group at position 7, which further increased the stability of

the compounds to β-lactamases and gave them increased potency over the earlier generations, especially against Gram-negative bacteria (Sader and Jones, 1992). As an example, the MIC of cefuroxime (2nd generation) against *E. coli* was 8 μg.mL^{-1}, whereas that of cefotaxime (3rd-generation) was just 0.5 μg.mL^{-1} (Sader and Jones, 1992). Ceftazidime, another 3rd-generation cephalosporin, incorporated an aminothiazole oxime with a carboxylate side chain (Figure 1.1D). This improved the drug's capacity to penetrate the porins present in the outer membrane of Gram-negative bacteria (Wright *et al.*, 2014).

The 4th-generation cephalosporins offered a further increase in potency against Gram-negative bacteria over the earlier generations. For example, cefepime had an MIC against *E.coli* of 0.06 μg.mL^{-1} (Sader and Jones, 1992). They contain a positively charged quaternary nitrogen at the 3' position (Fung-Tomc, 1997) (Figure 1.1E). This positive charge allowed the compound to more readily diffuse through the membrane of Gram-negative bacteria as it is attracted to the porin channel (Fung-Tomc, 1997).

Ceftobiprole and ceftaroline are examples of fifth-generation cephalosporins (Chahine and Nornoo, 2011; Kaushik *et al.*, 2011; Wright *et al.*, 2014). Ceftobiprole was introduced in 2013 (Wright *et al.*, 2014)(Figure 1.1F). They are both administered as the prodrug form, and are then converted to the active drug by plasma esterases. Their side chain is specifically designed to have a strong affinity for the enzymes PBP2a and PBP2x, which are responsible for resistance in staphylococci and pneumococci, making them the only β-lactams to exert a bactericidal effect against MRSA (Bogdanovich *et al.*, 2005).

Another example of the evolution of antibacterial compounds is that of the fluoroquinolones, which are bacterial DNA synthesis inhibitors. The commercial preparation of chloroquine (an antimalarial agent) generated a by-product that was found to have antibacterial activity (Lesher *et al.*, 1962)(Figure 1.2A). This compound had moderate activity against some Gram-negative bacteria. The first clinically approved quinolone antibiotic, nalidixic acid, came about as a result of synthesising similar compounds following the initial discovery (Lesher *et al.*, 1962)(Figure 1.2B). Despite being unsuited for treating systemic infections due to its low absorption in the body, it was widely used to treat urinary tract infections (Appelbaum and Hunter, 2000; Wright *et al.*, 2014). The

incentive to generate derivatives of nalidixic acid was driven by its lack of activity against Gram-positive bacteria, its side effects, and the rapid development of resistance to the drug (Appelbaum and Hunter, 2000; Wright *et al.*, 2014).

Over the years, a few core functional groups were added to nalidixic acid to improve its utility in medicine. Initially, a piperazine ring was added at position 7 to give pipemidic acid, which displayed improved activity against Gram-negatives and some activity against Gram-positives (Appelbaum and Hunter, 2000)(Figure 1.2C). It also displayed improved bioavailability and metabolic stability over nalidixic acid (Shimizu *et al.*, 1975). The piperazine group increased the capacity of the quinolones to penetrate the bacterial cell wall, which explains why adding this group improved the activity against Gram-positive bacteria, since they have a thicker cell wall than Gram-negative bacteria (Appelbaum and Hunter, 2000). In flumequine, a fluoro group was added at position 6 and this also appeared to improve activity against Gram-positives (Appelbaum and Hunter, 2000)(Figure 1.2D).

These two moieties (a piperazine ring and a fluoro group) were combined in norfloxacin and this improved the activity against Gram-positive agents, although it could still not be used systemically owing to its pharmacokinetic profile (Appelbaum and Hunter, 2000; Ito *et al.*, 1980)(Figure 1.2E). Quinolones that were developed after this point included structural motifs that made them more amenable to being absorbed from the gastrointestinal tract and more active against anaerobic bacteria and Gram-positive bacteria (Wright *et al.*, 2014). The cyclopropyl group on the nitrogen at position 1, exemplified in ciprofloxacin (a 2nd generation quinolone), was a bulkier group than the ethyl group in the same position on nalidixic acid and the earlier quinolones (Appelbaum and Hunter, 2000)(Figure 1.2F). The 3rd-generation quinolone, levofloxacin, incorporated a tricyclic structure and a chiral centre (Hayakawa *et al.*, 1986)(Figure 1.2G). The limitations of appending functional groups to the nalidixic acid structure are that the carboxylic acid at position 3 and the ketone group at position 4 (Figure 1.2B) are essential for the activity of the quinolones because these are the sites that bind to DNA gyrase (Appelbaum and Hunter, 2000).

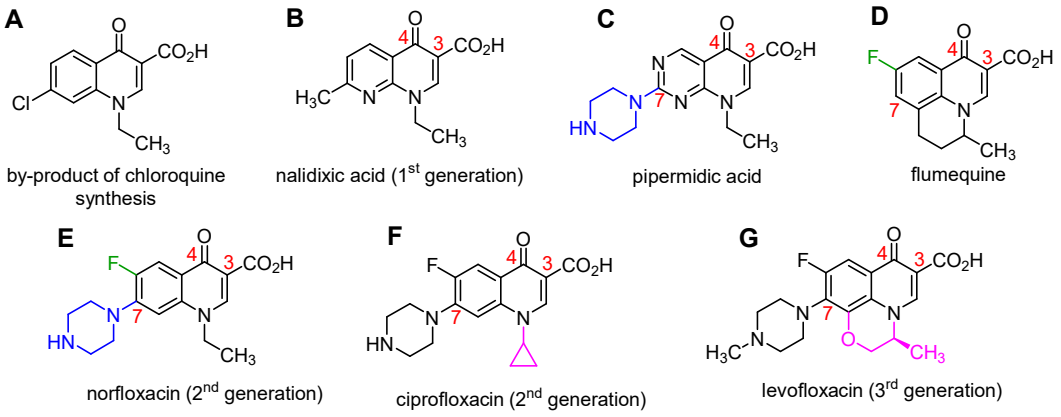


Figure 1.2 Examples of compounds in the quinolone class. A) The original antibacterial quinolone by-product from chloroquine synthesis; B) nalidixic acid, a 1st generation quinolone; C) pipermidic acid, with the piperazine ring in blue; D) flumequine, with the fluoro group in green; E) norfloxacin, a 2nd generation quinolone, with the piperazine ring in blue and the fluoro group in green; F) ciprofloxacin, a 2nd generation quinolone, with the cyclopropane group in pink; G) levofloxacin, a 3rd generation quinolone, with the tricyclic group and chiral centre in pink.

The strategy of modifying existing antibiotics worked well throughout the 1960s, but no new class of broad-spectrum antibiotic has been discovered since then (Fischbach and Walsh, 2009; Lewis, 2013; Silver, 2011). Daptomycin was discovered in 1986 and approved in 2003, but only for skin infections, since its mode of action is to disrupt the bacterial cell membrane potential (Alborn *et al.*, 1991; Pogliano *et al.*, 2012). It is a lipopeptide antibiotic that is active against most Gram-positive bacteria, including MRSA (Steenbergen *et al.*, 2005). Linezolid (an oxazolidinone) and retapamulin (a pleuromutilin) were introduced for clinical use in 2000 and 2007, although these had been discovered much earlier, in 1978 and 1952, respectively (Silver, 2011). As such, no completely novel antibacterial agent has been discovered since the 1980s (Silver, 2011).

1.6 Issues regarding target-based antibacterial drug discovery

Eventually, traditional innovations in antimicrobial drug design were not keeping up with the rise of resistance. For example, despite the success of the penicillins, we began to see strains of bacteria harbouring new β-lactamases (such as metallo-β-lactamases and an enzyme encoded by the NMD-1

plasmid) that were resistant to all β-lactams (Kumarasamy *et al.*, 2010; Yum *et al.*, 2002). This rise in resistance inspired renewed efforts to generate novel antibiotics at a time when new drug discovery approaches were becoming available across many therapy areas during the 1990s (Brown and Wright, 2016).

Advances in computing offered pharmaceutical companies the potential of handling much larger data sets and of employing computational rational drug-design based on protein crystal structures, which were also becoming easier to determine. Concurrently, there were also advances in the manipulation of recombinant DNA, in robotic liquid handling and in high-throughput synthesis. This introduced the possibilities of not only generating large chemical libraries that could be used in high-throughput biochemical assays, but also acquiring potential target proteins at high yields for structure determination (Brown and Wright, 2016). In 1995, the first genome was sequenced (of *Haemophilus influenzae*), followed by thousands more bacterial genomes becoming available (Brown and Wright, 2016; Fleischmann *et al.*, 1995). Surveys of these genomes aimed to find new, unexploited targets for rational drug design that would be unlikely to be susceptible to existing resistance mechanisms (Brown and Wright, 2005).

Altogether, these technologies heralded a target-based era of antibacterial drug discovery using synthetically-generated libraries of small molecules. This was more appealing to pharmaceutical companies than persevering with the more traditional empirical screening (see section 1.7), which by that point was offering diminishing returns due to the lack of novel scaffolds being discovered. Reorganisation within these companies encouraged antibacterial discovery groups to opt for screening libraries of combinatorial, synthetic small molecules, rather than performing natural product screens from crude microbial fermentation broths (Silver, 2011).

Unfortunately, this strategy has not resulted in a single antimicrobial drug being developed that is effective against important pathogens (Lewis, 2013; Payne *et al.*, 2007). The dawn of genomics brought about the possibility of dramatically increasing the range of targets that could be inhibited by future antibiotic compounds – most clinically used antibiotics inhibit a small number of

molecular targets. Researchers at GSK identified conserved genes across pathogenic strains of Gram-negative bacteria (*Haemophilus influenzae* and *Moraxella catarrhalis*) and Gram-positive bacteria (*Streptococcus pneumoniae*, *Staphylococcus aureus* and *Enterococcus faecalis*) (Payne *et al.*, 2007). These genes were narrowed down to potential broad-spectrum targets by determining which genes had highly conserved amino acid sequences across five pathogens and which were present as a single copy gene, making them less likely to develop resistance mechanisms through utilising backup copies of the gene (Brown and Warren, 1998). In total, 127 genes were identified as being essential *in vitro* in at least one of the strains surveyed.

Most of these were used in 67 high-throughput screens (HTSs) against the compound collections available in the company which comprised around 500,000 synthetic small molecules (Payne *et al.*, 2007). Only five of the HTSs provided the researchers with lead compounds, but there were various issues for all of these compounds. The MurB inhibitor lead was modified further but still was not potent enough. The inhibitor of ribonuclease P (RNaseP) turned out not to be specific to the enzyme, and was toxic to mammalian cells. The inhibitor leads of peptide deformylase (PDF), enoyl-acyl carrier protein reductase (FabI), 3-ketoacyl-acyl carrier protein III (FabH), methionyl tRNA synthetase (MetRS) and phenylalanyl-tRNA synthetase (PheRS) were all narrow spectrum compounds that did not even show activity against *S. pneunomiae*, which is surprising given that this was the species used as the basis for the gene selection in the first place. Overall, none of the compounds found through the HTS at GSK were potent enough against a sufficiently large pool of bacteria to deem this screening of small synthetic compounds a success.

The issue with screening large industrial collections of synthetic small molecules is that these compounds were designed to be used to find leads for many different research areas such as cancer treatment. As such, they obey Lipinski's 'rule of five' guidelines, which refer to the properties of drugs that typically interact with membrane proteins on human cells. The outcome of this is that it is possible to find compounds that inhibit bacterial targets, but it is often difficult to find compounds that are capable of penetrating the bacterial cell in order to reach those targets. This inefficient penetration into bacterial

cells also means that doses have to be between two and three orders of magnitude higher to be effective against the infection compared to an inhibitor of a eukaryotic target when the binding constants are comparable *in vitro* (Lewis, 2013). Aside from these scientific considerations, antibacterial drug discovery also offers a more modest monetary return on investment than research into treatments for long-lasting, chronic conditions. Furthermore, healthcare professionals were under pressure to reduce their prescriptions of antibiotics in order to avoid unnecessary increases in the spread of antibacterial resistance (Projan, 2003). These additional barriers to antibiotic discovery contributed to the lack of Big Pharma participation in this area and to the lack of success of target-based and structure-based drug design (Brown and Wright, 2016; Lewis, 2013; Silver, 2011).

1.7 Renewed interest in screening natural products to find novel antibiotics

There is a growing interest in returning to screening natural products for new antibiotics, given that natural products represent around 80% of all clinically used antibiotics (Brown and Wright, 2016; Butler and Buss, 2006; Gullo *et al.*, 2006; Lewis, 2013; Peláez, 2006; Silver, 2008; Watve *et al.*, 2001). The *Actinomycetes* class of bacteria has the potential to produce a huge range of natural products (Doroghazi and Metcalf, 2013; Fernandes, 2006; Silver, 2011). Many *Streptomyces* species have the capacity to produce far more secondary metabolites than they do in the laboratory due to the presence of silent operons that are not expressed under standard culture conditions (Lewis, 2013; Rutledge and Challis, 2015). For example, the *Streptomyces coelicolor* genome encodes 29 natural product biosynthetic pathways, but only four are routinely active (Bentley *et al.*, 2002; Craney *et al.*, 2013). Some solutions to this, such as cloning the silent operons into a heterologous host or disrupting negative regulatory genes, have been shown to dramatically increase the yield of known antibiotics from their natural product producers (Baltz, 2011).

However, the screening of natural products is reliant on traditional empirical methods, which are inefficient. They involve screening fermentation broths or microbial extracts blindly for the capacity to inhibit bacterial growth, initially

without knowing the mode of action (Chopra, 1997; Silver, 2011). This is a time- and resource-heavy method, given that all the compounds must be extracted first before the MOA can be determined. It costs around $50,000 and takes three months to isolate and identify an active compound from a natural product source (Dias *et al.*, 2012). This has resulted in pharmaceutical companies largely abandoning this area. Some slow-growing species may not be amenable to such screening, as the compounds may degrade by the time colonies form. In addition, known antibiotics are readily re-isolated (Baltz, 2007; Silver, 2011). This is extremely problematic since time will be wasted analysing cultures that may yield no new antibiotics. Such methods fail to identify compounds present at low, sub-inhibitory concentrations, which may be masked by these known antibiotics. There is not sufficient distinction between clinically viable, non-toxic inhibitors and those that would have toxic effects for humans.

For example, polymyxin B is a lipopeptide metabolite of *Bacillus polymyxa* and exerts its antibacterial effect by acting like a detergent and disrupting the outer membrane of Gram-negative bacteria by displacing the divalent cations that stabilise the lipopolysaccharides (Zavascki *et al.*, 2007). Whilst this drug is now returning to clinical use as a drug of last resort for the treatment of infections caused by MDR-Gram-negative bacteria, the fact that its mode of action of membrane perturbation can also affect mammalian cells means that it can cause side effects such as nephrotoxicity and neurotoxicity (Tam *et al.*, 2005; Zavascki *et al.*, 2007). Thus, compounds that initially appeared to be promising new leads when detected in a natural product screen may not offer as much clinical utility as desired.

Another challenge in relying on natural product producers is that 99% of the global microbiome remains uncultivable, *i.e.* they have not been grown under laboratory conditions (Lewis, 2013). Despite this, there has been a high profile case of antibiotic discovery recently involving the use of an ‘iChip’ to grow an uncultured microbial species (*Eleftheria terrae*) (Ling *et al*., 2015; Nichols *et al*., 2010). Briefly, soil was diluted such that only one bacterial cell in a single chamber was separated from its natural soil environment by two semi-permeable membranes that enabled the diffusion of nutrients and growth factors. By cultivating bacteria in this way, Nichols *et al*. discovered teixobactin,

an antibiotic effective against some drug-resistant Gram-positive strains, with peptidoglycan synthesis inhibition as its proposed mode of action.

The interest in returning to screening natural products, plus the case for the potential for activating silent gene clusters and the improving technology available to cultivate previously uncultivatable microbial species, suggests that a suitable high throughput natural product screening method for the detection of novel antibiotics will be in demand in the coming decades.

1.8 The potential of using whole-cell biosensors for screening natural products

Early screening for antibiotics relied on the observation of physical changes in the appearance of cells when exposed to natural product extracts (Gadebusch *et al.*, 1992). Fosfomycin was detected when *Proteus vulgaris* was exposed to *Streptomyces fradiae* (and also *Streptomyces viridochromogenes* and *Streptomyces wedmorensis*, separately). Direct light microscopy was used to observe that exposure to fosfomycin resulted in the formation of spheroplasts (cells that were previously rod-shaped, but have become spherical due to the loss of their cell wall), indicative that the mechanism of action of fosfomycin targeted the biosynthesis of the cell wall (Hendlin *et al.*, 1969). Assays of this type were useful during the first few decades of antibiotic discovery when screening only a moderate number of natural product producer extracts promised a reasonable chance of uncovering genuinely novel compounds. However, this format of assay is not viable for the high-throughput screening required in the present to detect scaffolds that are far less common than those detected during the 'Golden Age' of antibiotic discovery.

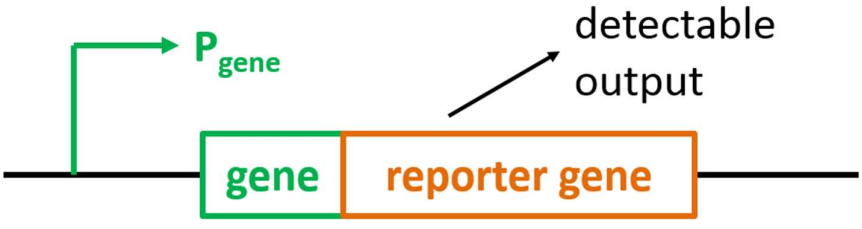


Figure 1.3 The general structure of the key genetic elements of a whole-cell biosensor/reporter.

A more appropriate solution presented here is to develop exquisitely-sensitive and specific 'biosensors': bacterial strains engineered to produce an easily quantifiable output when exposed to even sub-inhibitory concentrations of an antimicrobial agent inhibiting a particular cellular pathway. Whole-cell biosensors consist of a bacterial strain harbouring a reporter gene (such as one encoding green fluorescent protein) placed under the control of a promoter previously determined in transcriptional profiling experiments to be induced upon a specific stress condition (*e.g.* an antibiotic) being applied to the cell (Fischer *et al.*, 2003; Silver, 2011) (Figure 1.3). This allows for the detection of inhibitors of specific pathways based on the global regulatory genetic consequences of that inhibition, as opposed to the observation of its phenotypic effects. This makes assays using biosensors more amenable to high-throughput screens of either chemical libraries or natural product extracts.

Although biosensors have been developed for laboratory studies to investigate antibiotic mode of action (Alksne *et al.*, 2000; Bianchi and Baneyx, 1999; Blake *et al.*, 2009; Fischer *et al.*, 2003; Hutter *et al.*, 2004a; Mariner *et al.*, 2011; Silver, 2011, 2012; Urban *et al.*, 2007), they have not yet been properly developed as a means to discovering novel compounds. This is mostly due to them being neither sufficiently specific nor sensitive enough to be utilised for this purpose. Some fail to detect compounds with well-established modes of action, whilst others produce a signal in response to molecules that are off-target (*i.e.* do not inhibit the target pathway) (Czarny *et al.*, 2014; Urban *et al.*, 2007). These biosensors also did not distinguish between cell wall biosynthesis inhibitors (CBIs) and mammalian membrane damagers.

In the paper by Bianchi and Baneyx, reporters were generated with a translational fusion of the *lacZ* reporter gene with the promoters for cold shock response (*cspA*), cytoplasmic stress (*ibp*) and protein misfolding in the cell envelope due to heat shock (*P3rpoH*) (Bianchi and Baneyx, 1999). The idea behind using these promoters was that some antibiotics induced a heat shock response (*e.g.* streptomycin and neomycin) and other antibiotics induced a cold shock response (*e.g.* chloramphenicol and tetracycline) and so genes upregulated due to a temperature shift are also likely to be upregulated in the presence of antibiotics. The use of these genes was based on information from the literature and not on testing done by the group itself. The paper describes

how these reporters are induced by carbenicillin (cell wall inhibitor), polymyxin B (membrane perturber), nalidixic acid (DNA synthesis inhibitor) and chloramphenicol, tetracycline, streptomycin and neomycin (protein synthesis inhibitors). The fact that the reporters were induced by a membrane perturber should have been concerning as this would be toxic to mammalian cells. An agar-based assay was used in which X-gal was the substrate that reacted with the β-galactosidase enzyme produced by the *lacZ* gene. The *csp*::*lacZ* reporter was used to demonstrate that natural product antibiotics could be detected directly from its producing organism. Cultures of the chloramphenicol-producing *S. venezualae*, along with an isogenic mutant that was not able to synthesise chloramphenicol, were extracted and added to filter paper disks, which were then left on top of agar supplemented with X-gal and inoculated with the *csp*::*lacZ* reporter. After incubation, a blue ring was seen around the disk containing the wild type *S. venezualae*, but not around the mutant.

Alksne *et al.* (2000) used a reporter to explore the potential of exploiting bacterial protein secretion as a novel target to base finding novel antibacterial compounds on (Alksne *et al.*, 2000). The Sec-dependent secretion pathway comprises of five essential proteins, plus at least two other proteins, and these are distinct from their eukaryotic counterparts. These targets showed promise for leading to drugs with broad spectrum activity, as homologs for these proteins had been found in both Gram-positive and Gram-negative bacteria. The Sec-dependent secretion pathway is regulated by *secA*, which is expressed when secretion is inhibited. The group therefore constructed a *secA*::*lacZ* reporter and used this to find secretion pathway inhibitors in screens of synthetic chemical and natural product extract libraries. Two natural products and six synthetic compounds were identified as potential leads. One of the natural products was the previously isolated compound, pyrroindomycin, which displayed good activity *in vitro* but not in serum (Singh *et al.*, 1994). The other natural product that was identified was sulochrin, which had also been isolated before from a variety of fungal species and showed weak antifungal and antibacterial properties. The six synthetic compounds identified by the screen had MIC values against *S. aureus* ranging from 2 to 128 $\mu g.mL^{-1}$, and four of these compounds were still believed to inhibit protein secretion after further biochemical analysis. Unfortunately, these were all confirmed to be toxic to

more than one type of mammalian cell and it was concluded that the screen had a tendency to identify compounds that are membrane active, despite there being little resemblance between the prokaryotic and eukaryotic counterpart proteins.

The strategy of using promoter-inducible reporter assays for high-throughput screening was also used by Fischer *et al.* (2003). The group developed a 'Reference Compendium' to identify expression profiles of *B. subtilis* under stress conditions, with the aim of being able to identify inhibitors of specific bacterial biosynthetic pathways (Fischer *et al.*, 2003). A large set of microarray data from expression profiles following exposure to known antibiotics with specific modes of action was used to reconstruct the regulon for that pathway *in silico*. This paper focused on the implementation of a *yhfB*::*luc* reporter to identify inhibitors of the essential fatty acid biosynthesis pathway. The *luc* gene encodes the firefly luciferase gene, which generates a luminescence signal. This was tested with a selection of antibiotics inhibiting various pathways – the relative light units for the fatty acid biosynthesis inhibitors, triclosan and cerulenin, were much higher than the measurements for the drugs targeting other pathways. This reporter was then used in a high-throughput screening assay to screen a large compound library of around 900,000 compounds. From this, 60 confirmed hits were taken forward to a secondary hit confirmation screen and four novel chemically interesting compounds were analysed further. Three of these displayed inhibition of radiolabelled [^{3}H]acetate being incorporated into phospholipids, but not inhibition of any other pathway. They were active against *B. subtilis* and *S. aureus*, but only had MIC values beyond 100 μM. It appears that no further data has been published on these novel compounds.

Hutter *et al.* (2004) described using the firefly luciferase gene as part of a reporter in an attempt to identify novel antibiotics. In a previous study, the group had compiled the expression profiles of *B. subtilis* strain 168 following exposure to over 40 different antibiotics of various different modes of action (Hutter *et al.*, 2004b). This gene expression profile was analysed to find marker genes that could be used to construct reporters (Hutter *et al.*, 2004a). The researchers used relatively stringent criteria in that they only selected genes that were upregulated at least 5-fold with respect to the drug free control

sample and to other compounds that targeted other biosynthetic pathways. These genes were fused separately to the *luc* gene and the group generated twelve reporters in *B. subtilis* that covered a range of compound classes, with the genes shown in brackets: fatty acid biosynthesis (*fabHB* and *glpD*), protein biosynthesis (*yrzI*), cell wall biosynthesis (*ypbG*), quinolones (*dinB*, *yneA* and *yorB*), glycopeptides (*ytrA* and *ywoB*), cycloserine (*ydeK*), rifampicin (*yvgS*) and clindamycin (*expZ*). These reporters were tested against a panel of 37 antibiotics covering a range of modes of action and all of the reporters gave a response when exposed to sub-lethal concentrations of drug, so this demonstrated that they were relatively sensitive. However, the specificity was poor. The *fabHB*::*luc* reporter, which supposedly detected fatty acid synthesis inhibitors, responded only to clindamycin, which is a protein biosynthesis inhibitor. Furthermore, the *yrzI*::*luc* reporter detected six antibiotics corrently (chloramphenicol, clarithromycin, clindamycin, erythromycin, fusidic acid and neomycin) but it also gave false negative results for two compounds (actinonin and puromycin) and false positive results for three compounds (norfloxacin, 5-fluoruracil and nitrofurantoin).

The work described by Urban *et al.* (2007) expands on the work in (Fischer *et al.*, 2003) and (Hutter *et al.*, 2004a). In (Urban *et al.*, 2007), the researchers created five reporters, again using the firefly luciferase gene (*luc*) as the reporter gene. These reporters were designed to detect inhibitors of DNA synthesis (using the *yorB* gene promoter), RNA synthesis (*yvgS*), protein synthesis (*yheI*), cell wall synthesis and cell envelope stress (*ypuA*) and fatty acid synthesis (*fabHB*). The reporters therefore gave more comprehensive coverage of viable bacterial targets than the reporters described in the group's previous paper. A selection of 39 reference compounds were used to validate the specificity of the reporters. Unfortunately, the reporter designed to detect cell wall biosynthesis inhibitors (CBIs) also detected nisin and polymyxin B, which are cell membrane perturbers and would therefore be toxic to mammalian cells. Additionally, the reporter designed to detect protein biosynthesis inhibitors (PBIs) only detected four out of the ten PBIs tested (linezolid, doxycycline, fusidic acid and chloramphenicol). Erythromycin and azithromycin did not induce the *yheI*::*luc* reporter because they are both macrolides and the shuttle vector used for making the promoter-reporter

fusions expressed a macrolide-lincomycin-streptogramin B resistance marker in *B. subtilis*. There was no explanation given for the lack of response of this reporter to gentamicin, kanamycin, puromycin and actinonin. Two of the reporters were then tested against a library of 14,000 diverse, purified natural products. The induction threshold was set as five standard deviations above the mean induction level for the drug free control.

The DNA synthesis inhibitor reporter (*yorB*::*luc*) detected 12 compounds. Ten of these were known antibiotics, acting either by the inhibition of DNA gyrase and topoisomerase IV, or by strand breaking due to DNA binding. Two compounds were novel but were not described further. The PBI reporter (*yheI*::*luc*) detected 26 compounds. Most of the natural products that induced this reporter were inhibitors of the 50S portion of the ribosome. Unfortunately, some PBIs did not induce the *yheI*::*luc* reporter (actinonin, streptomycin, puromycin, fusidic acid and siomycin A). The paper then stated that only one compound (trinactin) out of the 26 hits induced the reporter and that this was known to be a membrane perturber. Four of the hits had not previously had their modes of action determined. One of these compounds was ferrimycin A1. To analyse the mode of action of this compound, precursors for DNA, RNA, protein, and cell wall biosynthesis were used in metabolite incorporation studies and these confirmed that ferrimycin A1 inhibited protein biosynthesis. This showed that these reporters demonstrated promise as tools to find novel antibiotics, although it was admitted that the reporters may still detect off-target activity of the identified compounds within the bacterial cell.

One of the more recent pieces of research in this area was described in (Czarny *et al.*, 2014). Wall teichoic acid (WTA) is the other component of the Gram-positive bacterial cell wall aside from peptidoglycan. The synthesis of WTA is an underexploited target for novel antibiotics. Previous work by the same group had identified that the *ywaC* gene was upregulated when WTA synthesis was inhibited by using a mutant of *B. subtilis* with the gene encoding the gene encoding for TagD deleted (Bhavsar *et al.*, 2001; D'Elia *et al.*, 2009). A reporter strain was generated with a P_{ywaC}-*lux* promoter-reporter system, with *lux* being an alternative firefly luciferase gene. This reporter was used to screen a library of around 26,000 diverse synthetic compounds, from which there were 54 initial hits, of which 35 were confirmed as active by determining their MIC

values against *B. subtilis* 168. These 35 confirmed hits were then tested with a membrane permeability assay in order to eliminate any compounds that were membrane damaging. This reduced the number of viable hits down to 25 compounds. The group then wanted to ascertain if the signal from the P_{ywaC}-*lux* reporter could be suppressed using osmoprotectants, such as MSM (20 mM $MgCl_2$, 0.5 M sucrose and 20 mM maleic acid, (Leaver *et al.*, 2009)), which assist bacterial survival when under extreme osmotic stress. They tested a panel of known CBIs and non-CBI antibiotics and confirmed that the induction was suppressed in the presence of MSM. When the growth media was supplemented with MSM for the assay with the reporter, 9 out of the 25 compounds also displayed suppressed inductions in the presence of MSM but not without it, suggesting that these were all novel CBIs. The only slight issue with this study is that the library consisted entirely of synthetic compounds, and as explained earlier, these stand a good chance of being unsuccessful as novel antibiotics *in vivo*.

Another recent use of whole-cell biosensors was described by de la Cruz *et al.*, (2017), which was work that followed on from the original generation of the biosensors in (Mascher *et al.*, 2004). A transcriptional signature of *Bacillus subtilis* exposed to sub-inhibitory concentrations of bacitracin revealed that the *liaRS* operon (lipid II cycle interfering antibiotic response Regulator and Sensor) was strongly upregulated in these conditions. The promoter for this operon was fused to the *lacZ* gene to form a biosensor that was able to accurately detect compounds that interfere with the recycling of lipid I in stage II of peptidoglycan synthesis inhibitors in an agar-based assay using X-gal as the β-galactosidase substrate (the “LiaRS assay”). This biosensor was specifically induced by bacitracin, nisin, ramoplanin and vancomycin, but did not respond to other cell wall biosynthesis inhibitors such as penicillin G and D-cycloserine. The biosensor was also weakly induced by fluoroquinolones and cephalosporins, but this result was also observed for another biosensor in which the *lacZ* gene had been disrupted. The researchers utilised the specificity of this biosensor for ramoplanin by using it to screen 37,000 natural product extracts (50% bacteria and 50% fungi) for other antibacterial compounds in this class. In total, there were 49 *Actinomycetes* strain extracts that induced the biosensor. Most of these extracts displayed activity against

Gram-positive organisms, such as *Staphylococcus aureus* and *Enterrococcus faecalis*, but unfortunately none of them were active against Gram-negative bacteria. The hits were analysed using HR-LCMS, which allowed dereplication of ramoplanin itself to be performed by comparing the results with the mass spectrometry signature known to correspond to ramoplanin. All of the extracts contained compounds related to the ramoplanin structural family and some of these represented novel analogs. These could potentially overcome the low tolerability of intravenously injected ramoplanin, which would be very useful given that ramoplanin exhibits activity against several MDR Gram-positive pathogens such as vancomycin-resistant *Enterrococcus* (VRE) and MRSA. However, this biosensor is only useful for detecting inhibitors of one particular step in the peptidoglycan biosynthesis pathway. It would be far more advantageous to have available a way of detecting inhibitors of cell wall biosynthesis that have a novel scaffold.

Overall, then, there has been some promising work in the area of using whole-cell biosensors with promoter-reporter systems, but there are still improvements that need to be made. A lot of the biosensors described above are not specific enough and still detect membrane damaging compounds even though they also appear to be specific for detecting CBIs. It seems there is also still the requirement that the compounds must be extracted and purified before they are used with the biosensor in a HTS.

1.9 Project aims

The O'Neill group have contributed to the field of biosensors by developing high-specificity whole-cell biosensors in the clinically-relevant bacterium, *Staphylococcus aureus* (Blake *et al*., 2009; O'Neill *et al*., 2009). Blake *et al*. reported the development of a staphylococcal biosensor that upregulated the expression of β-galactosidase when exposed specifically to CBIs (Blake, 2008; Blake *et al*., 2009). It was noted that exposure to fosfomycin left the expression from P_{murA} unchanged but increased the expression from P_{murZ} 3-fold. Strains with these promoters coupled to *lacZ* were tested using a selection of antibiotics with different modes of action and the β-galactosidase activity was increased significantly in the presence of early and late stage peptidoglycan

synthesis inhibitors. The P_{murZ}-*lacZ* (MurZ) system resides on the chromosome of *S. aureus* RN4220 with the native promoter and RBS, along with the first section of *murZ* fused to *lacZ* gene. It was formed by a cross-over event involving the promoter-reporter system on the plasmid pMUTIN4, resulting in chromosomal integration.

The group has since generated two further biosensors that have been subjected to extensive validation, and that show high sensitivity and 100% specificity for CBIs (C. Randall, unpublished data)(see Table 3.1). They are plasmid based biosensors with a backbone of pAJ129, itself derived from pAD123 (Dunn and Handelsman, 1999). The *gltA* and *oppB* genes were selected from a transcriptional profile following inhibition or depletion of enzymes involved in the first stage of cell wall biosynthesis in *S. aureus*. These genes were upregulated ≥2-fold above a drug-free control (O'Neill *et al*., 2009). In general, it was found that the genes that were upregulated were responsible for the synthesis or transport of precursors that are essential for cell wall biosynthesis. Indeed, the *gltA* gene is part of the *gltAB* operon that is responsible for glutamate synthesis and the *oppB* gene is part of the *oppABCDF* operon that encodes for enzymes involved in the oligopeptide transport system. The native promoter of the upregulated gene, along with the first section of the open reading frame were fused to *lacZ*. These plasmids were transformed into RN4220 to make the P_{gltA}-*lacZ* and P_{oppB}-*lacZ* biosensors.

The initial aim of this project was to validate the use of these three biosensors for detecting CBIs. Thus far, they had been used in a broth-based assay and early work in this study concentrated on demonstrating that they could also be used in an agar-based assay, in particular for detecting potential new antibiotics produced directly from an organism. This would mean that organisms could be screened for novel compounds without the need to generate chemical extracts first, thus streamlining the drug discovery process. It was also promising that the data gathered so far for these three biosensors suggested that they did not respond to membrane damaging compounds, but did still respond to CBIs.

The next aim was to modify the assay for use at a scale that would make it amenable to screening compound libraries available in the lab that were provided in a 96-well plate format. The libraries available were the NIH Clinical Collection (727 compounds with a history of use in human clinical trials), the Microsource Spectrum collection (2560 compounds comprising the US and International drug collections together with Microsource's natural product and discover libraries, of which 2000 were tested), and Tocris' discontinued Tocriscreen Total collection (1120 bioactive compounds that target a broad range of pharmacological targets). The plan was that the biosensor giving the most consistent results out of the three tested at the validation stage would be used for this screening.

There is clearly an issue in natural product screening with rediscovering known antibiotics, for which there are already widespread resistance mechanisms amongst pathogenic species of bacteria. Therefore, the second major aim of the project was to engineer a strain (based on whichever biosensor was used for the library screening) that would not be induced by many known CBIs. Whereas the work in (Czarny *et al.*, 2014) involved merely adding a supplement to the growth media used for the assay, the plan in this project was to also add genes that would confer resistance to selected major classes of CBIs to the biosensor itself. This would make it potentially more transferable to other assays in the future.

The third aim was to create a suite of biosensors for detecting protein biosynthesis inhibitors (PBIs). RNA would be extracted from bacterial cultures of *Staphylococcus aureus* that had been exposed to a sub-inhibitory concentration of a known PBI. This would give a snapshot of the transcriptional signature of *S. aureus* during the early stages of protein biosynthesis inhibition. The RNA would then be sequenced and analysed so that genes whose regulation was upregulated in the presence of PBIs could be determined. These genes would be compared to results held in the *Staphylococcus aureus* Transcriptome Meta-Database (SATMD), which comprises gene expression data for around 250 experiments, in order to disregard genes that are also upregulated in the presence of antibiotics that inhibit other pathways. Any suitable genes would then be cloned into a plasmid backbone suitable for translational fusion and the construct would then be transformed into

Staphylococcus aureus RN4220. The resulting biosensor would then be tested against a range of reference compounds to demonstrate the extent of their specificity and sensitivity.

Chapter 2 Materials and Methods

2.1 Bacterial strains, fungal strains and plasmids

Table 2.1 Plasmids used in this study.

Plasmid	Description/Application	Reference/Source
pRAB11	Vector with tight regulation of gene expression controlled by a $P_{xyl/tet}$ promotor containing two *tetO* operators, followed by a MCS (*Kpn*I, *Hpa*I, *Bgl*II, *Sac*I, *Eco*RI). Amp^R in *E. coli*, Cm^R in *S. aureus*.	(Helle *et al.*, 2011)
pZAB	Product of Gibson assembly with pRAB11 and two synthetic DNA fragments of the genes *fosB*, *blaZ*, and *bcrAB*.	This study
pPBIdr1	Product of Gibson assembly with pRAB11 and two synthetic DNA fragments of the genes *sat4*, *aadA*, and AAC(6')/APH(2").	This study
pPBIdr2	Product of Gibson assembly with pRAB11 and two synthetic DNA fragments of the genes *tetM*, *cfr*, and *ermC*.	This study
pAJ130	*E. coli – S. aureus* shuttle vector for creating *lacZ* biosensors *via* transcriptional or translational fusion. Lacks *lacZ* start and RBS. Amp^R in *E. coli*, Cm^R in *S. aureus*.	Alex O'Neill
pinfClac	Fusion of *infC* gene promoter to *lacZ* gene in pAJ130.	This study
p1910lac	Fusion of SAOUHSC_01910 gene promoter to *lacZ* gene in pAJ130.	This study
p2425lac	Fusion of SAOUHSC_02425 gene promoter to *lacZ* gene in pAJ130.	This study
pMUTIN4	Vector to perform insertional mutagenesis in the chromosome. Amp^R in *E. coli*, Em^R in *S. aureus*.	(Vagner *et al.*, 1998)
pMurZKO	Integration vector generated by ligating a 600 bp fragment of *murZ* into pMUTIN4.	(Blake, 2008)
pAJ129	*E. coli – S. aureus* shuttle vector for creating *lacZ* biosensors *via* transcriptional fusion. Includes	Alex O'Neill

Plasmid	Description/Application	Reference/Source
	lacZ start and RBS. Amp^R in *E. coli*, Cm^R in *S. aureus*.	
pgltAlac	The promoter, RBS and first few codons of the *gltA* gene was ligated upstream of *lacZ* in plasmid pAJ129.	Alex O'Neill
poppBlac	The promoter, RBS and first few codons of the *oppB* gene was ligated upstream of *lacZ* in plasmid pAJ129.	Alex O'Neill

Table 2.2 Bacterial strains used in this study.

Strain	Description/Application	Reference/Source
***Escherichia coli* strains:**		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*^{q} *Z*∆*M15* Tn*10* (Tet^r)]. Used for routine cloning.	Agilent Technologies
***Staphylococcus aureus* strains:**		
SH1000	Functional *rsbU* derivative of *S. aureus* 8325-4.	(Horsburgh *et al.*, 2002)
RN4220	Restriction deficient, modification proficient derivative of *S. aureus* 8325-4.	(Fairweather *et al*., 1983)
P_{murZ}-*lacZ* biosensor	Cross-over integration of pMurZKO with the native *murZ* gene on the chromosome of RN4220 produced strain KB02.	(Blake *et al.*, 2009) (Referred to as KB02 in this source)
P_{gltA}-*lacZ* biosensor	Plasmid pgltAlac was used to transform *S. aureus* RN4220.	Alex O'Neill
P_{oppB}-*lacZ* biosensor	Plasmid poppBlac was used to transform *S. aureus* RN4220.	Alex O'Neill
P_{murZ}-*lacZ*(derep) biosensor	Plasmid pZAB was used to transform the P_{murZ}-*lacZ* biosensor.	This study
P_{infC}-*lacZ* biosensor	Plasmid pinfClac was used to transform *S. aureus* RN4220.	This study
P_{01910}-*lacZ* biosensor	Plasmid p1910lac was used to transform *S. aureus* RN4220.	This study

Strain	Description/Application	Reference/Source
P_{02425}-*lacZ* biosensor	Plasmid p2425lac was used to transform *S. aureus* RN4220.	This study
RN4220(GC1)	Plasmid pPBIdr1 was used to transform RN4220.	This study
RN4220(GC2)	Plasmid pPBIdr2 was used to transform RN4220.	This study
***Streptomyces kanamyceticus*:**		
DSM 40500	Produces kanamycin A, B, C.	DSMZ
***Amycolatopsis rifamicinica*:**		
DSM 46095	Produces rifamycin SV.	DSMZ
***Streptomyces niveus*:**		
DSM 40088	Produces novobiocin.	DSMZ
***Streptomyces fradiae*:**		
NCIMB 8233	Produces neomycin.	Kenneth McDowall group
***Streptomyces coelicolor*:**		
M145	Produces actinorhodin.	Kenneth McDowall group
***Amycolatopsis orientalis*:**		
DSM 40040	Produces vancomycin.	DSMZ
***Bacillus licheniformis*:**		
DSM 603	Produces bacitracin.	DSMZ

Table 2.3 Fungal strains used in this study.

Strain	Description/Application	Reference/Source
Penicillium chrysogenum:		
DSM 848	Monospore isolate of NRRL 1249, derivative of Fleming strain, produces penicillin in surface culture.	DSMZ
Acremonium chyrysogenum		
DSM 2399	Produces cephaolosporin C.	DSMZ

2.2 Antibiotics and chemicals

All chemicals were purchased from Sigma-Aldrich (Poole, UK), with the exception of ammonium ferric citrate, cetyltrimethylammonium bromide, calclium carbonate, manganese sulphate, sodium carbonate (VWR [West Sussex, UK]); peptone, ISP-2 agar, YM broth (Becton Dickinson [Oxford, England]); flucloxacillin (CP Pharmaceuticals [Wrexham, UK]); bacteriological agar (Lab M Ltd [Lancashire, UK]); vancomycin, cefalexin (Cayman Chemical [Michigan, USA]); sodium chloride (Fisher Scientific [Loughborough, UK]) and X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) (Melford [Sulfolk, UK]); cephalosporin C zinc salt (Carbosynth [Berkshire, UK]); cefamandole, moxalactam (Cambridge Bioscience [Cambridge, UK]); aztreonam (MP Biochemicals [Illkirch, France]); Bax channel blocker, fenoldopam, EGCG (Bio-Techne [Abingdon, UK]); linezolid (ChemCruz [Heidelberg, Germany]); cefonicid, cefoperazone, cefprozil, cloxacillin, 5-nonyloxytryptamine, cetraxate, benserazide, topotecan, artemisinin, carfilzomib, mometasone furoate, genistein, actinonin (Insight Biotechnology [Wembley, UK]); daptomycin (Cubist Pharmaceuticals [Lexington, USA]). Nuclease-free (DEPC treated) water was purchased from Thermo-Fisher Scientific (Paisley, UK). Friulimicin was a gift from Tanja Schneider, University of Bonn, Germany.

The compound libraries that were screened were the NIH Clinical Collection, the Spectrum Microsource collection, and the now discontinued Tocriscreen Total collection. The 96 cyanobacterial extracts were kindly donated by Cyano-Biotech (Berlin, Germany) and the microbial extracts from the NCI Open Repository were kindly donated by the National Cancer Institute (Maryland, USA). These natural product extracts were dissolved in DMSO when required and stored at –20 °C.

Fresh stock solutions of the antibiotics used were prepared by dissolving the compound in water, with the following exceptions: chloramphenicol, fusidic acid and erythromycin were dissolved in 50% ethanol. D-cycloserine was dissolved in 1 mM disodium hydrogen phosphate. Cephalosporin C zinc salt was dissolved in warm 20 mM hydrochloric acid. X-Gal, S-Gal, benserazide, thymoquinone, topotecan, genistein, capsaicin, menadione, clofazimine, carfilzomib, mometasone furoate, artemisinin, actinonin, cefapirin, cefadroxil,

cefoxitin and ceftazidime were dissolved in DMSO. Anhydrotetracycline was dissolved in 100% ethanol. Friulimicin was dissolved in 12.5 mM aqueous calcium chloride. Daptomycin was dissolved in 1.25 mM aqueous calcium chloride. They were then stored at 4.0 °C and were used on the same day.

2.3 Culture conditions and bacteriological media

Bacteriological media were purchased from Oxoid Ltd (Basingstoke, UK). Bacteriological agar was from Lab M Ltd (Lancashire, UK). Media were prepared according to the instructions of either the manufacturer or DSMZ (Braunschweig, Germany) (in the case of the recommended media for the strains purchased from DSMZ). Cultures and colonies of bacteria were grown using either agar or broth as described in the table below. Appropriate antibiotics were used to maintain plasmids.

Table 2.4 Growth conditions for the bacterial and fungal strains used in this study.

Strain	Agar	Broth	Temperature	Duration
E. coli	Luria-Bertani agar (LBA)	Luria-Bertani broth (LBB)	37 °C	18 hours
S. aureus	Tryptone soya agar (TSA)	Tryptone soya broth (TSB) (or Müller Hinton Broth II (MHBII) for MICs)	37 °C	18 hours
P. chrysogenum	Sabourand dextrose agar (SAB)	LBB	37 °C	7 days
S. kanamyceticus	GYM agar (DSMZ medium 65)	GYM broth	28 °C	4–7 days
A. rifamicinica	ORG 79 agar (DSMZ medium 426)	ORG 79 broth	28 °C	4–7 days
S. niveus	GYM agar	GYM broth	28 °C	4–7 days
S. fradiae	International Streptomyces Project Yeast Malt Extract Agar medium 2 (ISP-2)	YM broth	26 °C	4–7 days
S. coelicolor	ISP-2	YM broth	26 °C	4–7 days
A. orientalis	GYM+S agar (DSMZ medium 214)	GYM+S broth	28 °C	4–7 days
A. chyrysogenum	YpSs agar (DSMZ medium 190)	YpSs broth	24 °C	4–7 days
B. licheniformis	Nutrient agar with 10 $mg.L^{-1}$ $MnSO_4.H_2O$ (DSMZ medium 1)	Nutrient broth with 10 $mg.L^{-1}$ $MnSO_4.H_2O$	37 °C	3 days

2.3.1 Antibiotic susceptibility determinations

Minimum inhibitory concentrations (MICs) of antibiotic compounds were determined by broth microdilution according to CSLI guidelines (Cockerill *et al*., 2012).

2.4 Molecular biology techniques

2.4.1 Extraction of plasmid DNA

A bacterial cell pellet was obtained using centrifugation (16000×*g* for 1 minute at RT) of a total of 3 mL of bacterial culture that had been incubated at 37 °C for 18 h overnight. Plasmid DNA was extracted from the pellet using either the QIAprep miniprep kit (Qiagen) or the Omega Bio-Tek Plasmid Mini Kit II (Omega Bio-Tek) according to the manufacturer's instructions, with the exception that the elution buffer was warmed to 65 °C before being used to elute the DNA from the column. For extractions from *S. aureus*, ~100 µg lysostaphin (8 µL of a stock solution of 13 $mg.mL^{-1}$ lysostaphin in TE buffer) was added following the addition of solution I and the cells were incubated at 37 °C with shaking for around 20 minutes until the contents of the microcentrifuge tube appeared clear following cell lysis. Plasmid DNA was stored at –20 °C.

2.4.2 Extraction of genomic DNA

A bacterial cell pellet was obtained using centrifugation (16000×*g* for 1 minute at RT) of a total of 3 mL of bacterial culture that had been incubated at 37 °C for 18 h overnight. The manufacturer's instructions for the QIAprep miniprep kit (Qiagen) or the Omega Bio-Tek Plasmid Mini Kit II (Omega Bio-Tek) were followed up until and including the centrifugal separation of the DNA from the RNA and protein following the addition of solution III. At this point, the supernatant containing the chromosomal DNA was transferred to a fresh microcentrifuge tube. A volume of 750 µL of isopropanol was added to each tube and the tube was inverted gently 10 times, before being centrifuged at 16000×*g* at 4 °C for 10 minutes. The supernatant was then removed carefully with a pipette and 700 µL of 70% ethanol was added to each tube. Each tube

was inverted gently 10 times and centrifuged at 16000×*g* at 4 °C for 3 minutes. The supernatant was then removed carefully with a pipette and then 100 µL of TE buffer (Life Technologies, Thermo Fisher Scientific) was added. The tubes were left in a heat block at 50 °C with the caps open before the DNA concentrations were measured and the extracted DNA was stored at –20 °C.

2.4.3 Determination of DNA concentration

The concentration of DNA was measured at 260 nm on a P300 nanophotometer (Implen, Munich, Germany). The ratios of wavelengths 260 nm/280 nm and 260 nm/230 nm were used to assess the purity of the DNA obtained (Sambrook *et al*., 2001). Ratios of around 2 and above indicated sufficient purity for use in further applications.

2.4.4 Ethanol concentration of DNA

Where necessary, plasmid DNA was concentrated using Pellet Paint (Novagen, Darmstadt, Germany), following the manufacturer's instructions.

2.4.5 PCR product purification (PCR clean up)

DNA amplified from a PCR reaction was purified before further manipulation using the E.Z.N.A. Cycle Pure kit (VWR Omega Biotek [Pennsylvania, USA]) according to the manufacturer's instructions, with the exception that the elution buffer was warmed to 65 °C before being used to elute the DNA from the column.

2.4.6 Preparation of chemically competent *E. coli*

Competent *E. coli* cells were prepared based on previously described methods (Sambrook *et al.*, 1989a). Fresh LBB (25 mL) was inoculated with 1 mL of bacterial overnight culture and this was incubated with aeration at 37 °C for 1 hour. The cells were then incubated on ice for 10 minutes before being collected by centrifugation (4000×*g* at 4 °C for 10 minutes). The cells were resuspended in 10 mL 0.1 M calcium chloride and incubated on ice for 20 minutes, before being collected by centrifugation again using the same conditions. The cells were then resuspended in 2.5 mL ice-cold 1:1 0.1 M calcium chloride and 15% glycerol. They were stored until use in 100 µL aliquots at –80 °C.

2.4.7 Chemical transformation into *E. coli*

Chemical transformation of DNA into *E. coli* was performed based on the instructions of the manufacturer of the XL1-Blue *E. coli* cells (Stratagene [Berkshire, UK]) and (Sambrook *et al.*, 1989a). Competent *E. coli* cells (XL1-Blue) were thawed on ice and 100 µL were aliquoted into a microcentrifuge tube. To this was added 1.7 µL of β-mercaptoethanol. The tube was then gently agitated and incubated on ice for 10 minutes, with further periodic gentle agitation approximately every 2 minutes. The DNA sample to be transferred was then added to the tube which was agitated again before being incubated on ice for 30 minutes. After this incubation, the mixture was heat shocked by being incubated in a 42 °C for 45 seconds, before being incubated on ice for 2 minutes. This mixture was then added to 900 µL LBB at 42 °C and incubated at 37 °C for one hour with shaking. A 100 µL volume of this mixture was used to inoculate a plate of LBA with the appropriate antibiotic selection (usually 100 $\mu g.mL^{-1}$ ampicillin), giving the 'neat' plate. A pellet was obtain from the remaining mixture by centrifugation (16000×*g* at RT for 1 minute). Sufficient supernatant was removed to leave approximately 100 µL of supernatant and this was used to resuspend the pelleted cells, which were then used to inoculate another LBA plate with selection to give the 'conc' plate. These plates were incubated at 37 °C overnight.

2.4.8 Preparation and electroporation of *S. aureus*

Electrocompetent *S. aureus* cells were prepared according to previously described methods (Monk *et al*., 2012). Briefly, 25 mL fresh TSB was inoculated with 1 mL of bacterial overnight culture and incubated with aeration at 37 °C for 40 minutes. The cells were harvested by centrifugation (4000×*g* at 4 °C for 10 minutes) and washed with 25 mL ice cold deionised water, before the cells were collected by centrifugation again using the same conditions. The cells were then washed and centrifuged in decreasing volumes of 10% glycerol (2.5 mL, then 1 mL, then 125 µL). They were stored in 50 µL aliquots at –80 °C until use. For the transformation, the cells were thawed on ice before being collected by centrifugation (5000×*g* for 1 minute) and resuspended in a 1:1 solution of 10% glycerol and 500mM sucrose. Up to 5 µg of plasmid DNA was added to the cells, which were transferred to an electroporation cuvette and

pulsed at 2.1 kV, 100 Ω, 25 µF. Fresh TSB (750 µL) and 500 mM sucrose (250 µL) were added to the cells and these were incubated with aeration at 37 °C for 2 hours before being plated onto TSA agar with the appropriate antibiotic selection and incubated overnight at 37 °C.

2.4.9 Polymerase chain reaction

Oligonucleotide primers were prepared for use by adding the appropriate volume of elution buffer (Qiagen or VWR) (to achieve a final concentration of 100 pmol.µL^{-1}) to the lyophilised primers. The primers were purchased from Eurofins MWG operon (Ebersberg, Germany), having been designed using Oligo software (Molecular Biology Insights inc., West Cascade, Colorado, USA). These were diluted to one tenth of the original concentration before being used in reactions. They were stored at –20 °C. PCR reactions (and also colony PCRs, restriction digests, ligation reactions and Gibson assembly reactions) were performed in a T100 Thermal Cycler (Bio-Rad [Hertfordshire, UK]). Phusion polymerase was purchased from New England Biolabs. The dNTPs were purchased as PCR Nucleotide Mix from Promega. The annealing temperature was optimised based on that suggested by the Tm calculator at http://tmcalculator.neb.com/#!/ (last accessed 23/09/17). The components, volumes and protocol were used according to the manufacturer's instructions. An alternative strategy was used to amplify the pRAB11 backbone in preparation for the Gibson assembly reactions, due to the relatively large size of the vector (6.5 kb). The LongRange PCR Kit was purchased from Qiagen and the manufacturer's instructions were followed.

2.4.10 Colony PCR

Primers were designed and prepared as described in 2.4.9. MyTaq Red Mix (incorporating MyTaq DNA polymerase, reaction buffer, dNTPs, $MgCl_2$, and a red dye for easy visualisation) was purchased from Bioline (London, UK) and the manufacturer's instructions were followed for the colony PCR reactions. Colony suspensions were prepared by suspending a single colony from the transformation plate concerned in 20 µL of nuclease-free water. In addition to being used for the colony PCR reactions, these colony suspensions were also subsequently used to inoculate fresh broth to produce an overnight bacterial

culture in the event of a colony PCR reaction indicating successful cloning for that colony.

2.4.11 Agarose gel electrophoresis

DNA agarose gels were used to either observe PCR products or to separate and purify DNA. Larger PCR products were measured against the HyperLadder 1 kb (Bioline) and shorter PCR products were measured against Quick-Load 100 bp Ladder (New England Biolabs). With the exception of colony PCR products (already dyed from the MyTaq Red Mix), the samples were mixed with 6× Purple Loading Dye (New England Biolabs) prior to loading into the gel. Diagnostic gels were performed using 0.8% agarose (w/v) and DNA gel extractions were generally performed using 0.5% agarose (w/v) dissolved in 1× TAE buffer. This agarose was warmed and added to a gel cast, where it was mixed with SYBR safe DNA stain (Life Technologies) (using 2 µL for a single row gel and 3 µL for a double row gel). The typical conditions used for running the gel were 90 V for 30–40 minutes.

2.4.12 Extraction of DNA from agarose gel

Agarose gels were visualised using a Dark Reader transilluminator (Clare Chemical Research [Colorado, USA]) and individual bands were separated and transferred to a microcentrifuge tube. The DNA was then extracted from the agarose using the E.Z.N.A. Gel Extraction kit (VWR Omega Biotek), according to the manufacturer's instructions, with the exception that the elution buffer was warmed to 65 °C before being used to elute the DNA from the column.

2.4.13 Restriction digests

Restriction enzymes were purchased from New England Biolabs. Plasmid DNA was digested according to the manufacturer's instructions.

2.4.14 Ligation reactions

Once both the vector and the insert had been digested using the appropriate restriction enzymes, the volumes of each component were calculated to achieve a 3:1 ratio of insert to vector based on using 50 ng of vector DNA using the "Ligation" tool on the NEBioCalculator website (https://nebiocalculator.neb.com/#!/ligation; last accessed 22/09/17). T4 DNA ligase was purchased from New England Biolabs and their instructions for

ligation reactions were followed. A control reaction, involving no insert, was also carried out in parallel with each set of ligation reactions. The reactions were incubated for longer than typical ligation reactions due to the *Sma*I restriction digest reaction producing a blunt end, as recommended in the manufacturer's instructions for this scenario. The completed reactions were concentrated using Pellet Paint, if necessary, and then used to transform XL1-Blue cells (see 2.4.7).

2.4.15 Gibson assembly reactions

Lyophilised DNA was rehydrated by adding the appropriate volume of nuclease free water to achieve a final concentration of 20 ng.μL^{-1} and incubating at room temperature for one hour before use. The volumes of each component were calculated to achieve a 2:1 ratio of insert to vector (recommended by the manufacturer) based on using 50 ng of vector DNA using the "Ligation" tool on the NEBioCalculator website (see 2.4.14). NEBuilder HiFi DNA Assembly Master Mix was purchased from New England Biolabs and their instructions for Gibson assembly reactions were followed. The completed reactions were concentrated using Pellet Paint, if necessary, and then transformed into XL1-Blue cells (see 2.4.7).

2.5 Generation of the biosensors

2.5.1 Creation of the P_{murZ}-*lacZ*(derep) biosensor

The pRAB11 vector was prepared by performing a PCR using oligonucleotide primers (pRAB11 LOb and pRAB11 ROb). The PCR product was then digested with DpnI enzyme to remove any remaining circular DNA, concentrated with ethanol using Pellet Paint and purified by DNA gel extraction. The genes *blaZ*, *fosB* and *bcrAB* were purchased as two fragments of synthetic DNA from Invitrogen (Waltham, MA, USA). The vector and the two inserts were ligated using Gibson assembly: the pRAB11 vector, insert 1 (*blaZ* and *fosB*), insert 2 (*bcrAB*), Gibson Assembly Master Mix and nuclease-free water were combined to a total of 20 µL as advised by the manufacturer. This mixture was incubated at 50 °C for 1 hour, after which it was concentrated with ethanol and used to transform *E. coli* (XL1-Blue) cells. Plasmid DNA was extracted (see section 2.4.1) from overnight bacterial cultures produced from the successfully

transformed colonies. These were again concentrated with ethanol and then used to transform electrocompetent P_{murZ}-*lacZ* biosensor cells (see section 2.4.8). MICs were determined for penicillin, bacitracin, fosfomycin and vancomycin to confirm that the correct phenotype had been obtained, indicated by a large increase in the MIC for the above compounds, with the exception of vancomycin. Anhydrotetracycline was added to the bacterial culture to a final concentration of between 0.01 μM and 0.4 μM, in order to induce the expression of the resistance genes.

2.5.2 Creation of the P_{infC}-*lacZ*, P_{01910}-*lacZ* and P_{02425}-*lacZ* biosensors

Fragment inserts (incorporating the promoter region, the ribosome binding site (RBS) and approximately the first five codons of each of the genes (*infC*, SAOUHSC_01910, SAOUHSC_02425)) were amplified from a genomic DNA preparation of SH1000. These PCR products were then purified (see section 2.4.5) and digested with the restriction enzyme *Sma*I (New England Biolabs [Ipswich, MA, USA])(see section 2.4.13). The digested products were both verified and purified further using agarose gel electrophoresis and extracted as described in 2.4.12. The vector, plasmid pAJ130 (a derivative of the shuttle vector pAD123, containing *lacZ* in place of *gfp*, permitting *lacZ* expression to be driven by the promoter of interest (Dunn and Handelsman, 1999)), was also digested with the restriction enzyme *Sma*I, purified and extracted in the same way. The vector and insert were ligated (see section 2.4.14) and the product of this reaction was used to transform XL1-Blue *E. coli* cells. A selection of colonies appearing on the plates the next day were used to inoculate fresh LBB, which was grown to saturation before plasmid DNA was extracted. Standard colony PCR reactions were used to confirm the presence of the correct insert. DNA preparations that gave the correct size of band on an agarose gel were used to transform electrocompetent RN4220 *S. aureus* cells. These three biosensors were tested in the β-glo assay with a selection of antibiotics listed in section 5.2.3 at 0.25×, 1× and 4×MIC.

2.5.3 Creation of RN4220(GC1) and RN4220(GC2)

The pRAB11 vector was prepared as described in 2.5.1. The genes *sat4*, *aadA* and APH(6')/AAC(2") were purchased as two fragments of synthetic DNA from

Invitrogen (Waltham, MA, USA) and the Gibson assembly of these two fragments and pRAB11 constitute PBI Gibson construct 1. The genes *tetM*, *cfr* and *ermC* were purchased as a further two fragments of synthetic DNA from Invitrogen and the Gibson assembly of these two fragments and pRAB11 constitute PBI Gibson construct 2. Three Gibson reactions for each construct were concentrated using Pellet Paint and were used to transform XL1-Blue *E. coli* cells. The primers pRMC seq U and pRMC seq L were used for colony PCRs to determine if the fragments had been inserted into the pRAB11 vector successfully. Correct colonies were used to inoculate LBB and these cultures were grown to saturation before plasmid DNA was extracted. This DNA was then used to transform electrocompetent RN4220 *S. aureus* cells.

MICs were determined for fusidic acid for both constructs, for streptothricin, spectinomycin and kanamycin for construct 1, and for tetracycline, linezolid and erythromycin for construct 2. A large increase in the MIC for the above compounds, with the exception of fusidic acid, indicated that the correct phenotype had been obtained. Anhydrotetracycline was added to the bacterial culture to a final concentration of 0.15 μM, in order to induce the expression of the resistance genes.

2.6 Creating transcriptional profiles

2.6.1 Growth of bacteria exposed to concentrations of antibiotics causing 25% growth inhibition

In order to produce results in triplicate, 585 μL (3 × 195 μL) of SH1000 bacterial overnight culture was added to 57.915 mL (58.5 mL – 3 × 195 μL) of MHBII in a 250 mL conical flask and this culture was incubated in a 37 °C water bath with shaking at 160 rpm until the OD_{600} had reached 0.2. This was then separated into three portions of 19.5 mL and 500 μL of antibiotic solution or drug free control was added to each culture portion. The cultures were incubated for 40 minutes at 37 °C as before, after which the OD_{600} was measured and recorded. Concentrations were found for the antibiotics tested for which the ratio of the OD_{600} of the treated culture to that of the drug free control was 0.75 ± 0.02, indicating growth inhibition of approximately 25%. When cultures containing such concentrations had been grown, 10 mL of this culture was transferred to a

50 mL Falcon tube. To this was added 20 mL of RNAprotect Bacteria Reagent (Qiagen [Manchester, UK]). The cells were then collected by centrifugation (4000×*g* at 4 °C for 10 minutes). The supernatant was removed and the tubes were drained upside down on a paper towel for 10 minutes before storing at –80 °C.

2.6.2 RNA extraction

Pure lysostaphin stock solution was prepared by dissolving 0.5 mg of lysostaphin in 100 µL to make a 5.0 $mg.mL^{-1}$. A volume of 20 µL of this was added to 5 mL TE buffer in an RNase-free 50 mL Falcon tube to produce a solution of 20 $\mu g.mL^{-1}$ lysostaphin in TE buffer. The RNA-protected pellet was thawed and washed with 1 mL of TE buffer pH 8.0. This solution was centrifuged at 5000×*g* at 4 °C for 10 minutes to obtain a pellet again. The supernatant was removed and 200 µL of TE buffer containing lysostaphin was added and the pellet was resuspended. The tube was vortexed for ten seconds and incubated for 90 minutes at 37 °C with occasional gentle mixing. Following this, 8 µg of proteinase K was added by adding 4 µL of a stock solution of 40 $\mu g.mL^{-1}$. The tube was vortexed for 10 seconds and then incubated at room temperature with shaking for 10 minutes. A volume of 40 µL of β-mercaptoethanol was added to 4.0 mL buffer RLT (provided with Midi Kit) and this was added to the tube along with 3.5 mL 80% ethanol, after which the tube was shaken vigorously.

At this point, the manufacturer's instructions were followed from point three onwards in the Qiagen RNeasy Midi Kit protocol. Point four was replaced by following the steps in Appendix E in the Qiagen RNeasy Midi/Maxi Handbook, on the subject of on-column DNA digestion with the RNase-free DNase set. The concentration, A_{260}/A_{280} and A_{260}/A_{230} ratios for the RNA was measured as in 2.4.3 and the presence of mRNA and rRNA was verified by running small volumes of the extracted RNA samples on an agarose gel as in 2.4.11. The RNA was stored at –80 °C until use.

2.6.3 RNA sequencing

For each RNA sample to be sequenced, 20 µL of RNA in RNase-free water was sent to the NGS Facility at St James' Hospital, University of Leeds. There, the samples were used to generate a ribosomal depleted library (the TruSeq

Stranded Total RNA Library (see http://dna.leeds.ac.uk/genomics/nationalprice.php; last accessed 22/09/17. The nine mRNA libraries (three each of two antibiotic treated cells and one drug free control) were run as paired reads on a single lane on the Illumina HiSeq 3000 machine.

2.7 Biosensor assays

2.7.1 Agar assay validation

TSA was supplemented with 10 μg.mL^{-1} erythromycin and X-Gal to make a final concentration in agar of 100 μg.mL^{-1}. The agar was inoculated with the biosensor to achieve confluent growth. A volume of 2 μL of the reference antibiotic was pipetted onto the inoculated plate and the plate was incubated at 37 °C for 18 hours.

2.7.2 Natural product agar assays

For each plate, 18 mL (or 36 mL for the square plates) of the appropriate agar (see Table 2.4) was added to a round plate. A volume of 50 μL of resuspended producer organism spores in PBS was added to the centre of the plate and the natural product producer was left to grow according to the conditions listed in Table 2.4. Plugs of agar containing the producer organism were removed and transferred to TSA (supplemented with X-Gal) that had been inoculated with the P$_{murZ}$-*lacZ* biosensor with a hole in the middle of the agar. The plate was incubated for 2 days at 30 °C.

2.7.3 Full scale MUG assay

The extent of β-galactosidase acitivity was determined using the protocol according to Blake (Blake, 2008; Chan and Foster, 1998). Briefly, the cultures were incubated at 37 °C for 1 hour, centrifuged and resuspended in AB buffer (100 mM NaCl, 60 mM K_2HPO_4 and 40 mM KH_2PO_4 *i.e.* 5.85 g NaCl, 10.5 g K_2HPO_4 and 5.48 g KH_2PO_4 in 1 L of sterile distilled water) and lysostaphin. 4-methylumbelliferyl β-D-galactopyranoside (MUG, 10 mg.mL^{-1} in DMSO) was added and the reactions were incubated for 90 minutes before being quenched with 0.4 M Na_2CO_3. A volume of 100 μL from each tube was transferred to a separate well on a black 96-well plate. The relative fluorescence was

determined using a BMG Labtech FLUOstar Optima plate reader (excitation filter 355 nm, emission filter 460 nm).

A standard calibration curve was prepared by dissolving 4-methylumbelliferone (4-MU) in DMSO and diluting this with ABN buffer (1:1 AB buffer: 0.4 M Na_2CO_3) to obtain at least five solutions with increasing concentrations up to 20 $\mu g.mL^{-1}$. The gain on the fluorimeter was set to 80 % of the fluorescence of the 20 $\mu g.mL^{-1}$ solution.

2.7.4 96-well plate scale MUG assay

The protocol in the previous section was adapted to be carried out in 96- well plates. The antibiotic/test compound was added to the bacterial culture to a final volume of 200 µL in a flat-bottomed 96-well plate. Following measurement of the optical densities after 1 hour, 150 µL of the cultures was transferred to a conical-bottomed 96-well plate, which was centrifuged at 1500 × *g* for 15 minutes. The cells were resuspended in 67.5 µL of a 1:221 mixture of lysostaphin (3 $mg.mL^{-1}$):AB buffer. The volume of MUG used was 7.5 µL. The reaction was quenched with 75 µL 0.4 M Na_2CO_3 and the same procedure as in 2.7.3 was used to measure the fluorescence. For the library screening, 2 µL of a 1 mM solution of the compounds dissolved in DMSO was added to 198 µL bacterial culture to give a final concentration of compound of 10 µM. The relative fluorescence was determined using a BMG Labtech FLUOstar Optima plate reader (excitation filter 355 nm, emission filter 460 nm). The libraries of clinical compounds were obtained from Evotec (Hamburg, Germany), Microsource Discovery Systems (Connecticut, USA) and Tocris (Bristol, UK).

2.7.5 β-glo assay

Overnight bacterial culture of the biosensor being tested was used to inoculate fresh TSB in a 1:100 ratio, which was then incubated at 37 °C with shaking until the OD_{600} was approximately 0.2. The cultures were added to the antibiotic/test compound to a final volume of 200 µL (with the exception of the NCI collection, which was screened at a 50 µL scale due to the scarcity of the material and the requirement for a high concentration due to the unknown composition of each extract) in a flat-bottomed 96-well plate. The plate was incubated at 37 °C with agitation at 450 rpm for one hour, after which the OD_{600} was measured on a BMG Labtech FLUOstar Optima plate reader. A volume of 45 µL from each

well was transferred to the corresponding well on a white 96-well plate. In addition, 5 µL of Beta Glo Reagent (Promega [Southampton, UK]) was added to each well. The plate was agitated at 450 rpm for 30 seconds and was then incubated in the dark at room temperature with no agitation for 45 minutes. After this incubation time, the relative luminescence was determined using the same plate reader, with the gain set to 3600.

2.8 Data analysis

2.8.1 Standard DNA sequencing

Sufficient volumes of both the DNA sample and the relevant primers were sent to Beckman Coulter Genomics/Genewiz (High Wycombe, UK), where they were sequenced using standard Sanger sequencing. The sequencing data was trimmed and aligned using Sequencher Version 4.8 (Gene Codes Corporation, MI, USA).

2.8.2 MUG assay analysis

The OD_{600} and fluorescence values were scaled to the values given by blank TSB by subtracting the minimum value for the blank wells. The amount of 4-MU produced was calculated by dividing the fluorescence by the gradient of the line of best fit of the standard curve with a forced intercept at (0,0). This value was then divided by the corresponding OD_{600} reading to give a value for the 4-MU produced scaled to the number of cells present. The fold change in β-gal activity of the reference compounds was determined relative to the 4-MU produced by the drugless control. The increase in induction was considered to be significant if it was a 2-fold increase or greater. Averages were based on three biological replicates unless otherwise stated and error bars indicate the standard deviation across these replicates. Asterisks indicate results that were statistically significantly different from the drug-free control (2-tailed t-test, P = 0.05).

2.8.3 β-glo assay analysis

The OD_{600} and luminescence values were scaled to the values given by blank TSB by subtracting the minimum value for the blank wells. The luminescence values were divided by the corresponding OD_{600} reading to give a value for the

light produced scaled to the number of cells present. The fold change in β-gal activity of the reference compounds was determined relative to the light produced by the drugless control. The increase in induction was considered to be significant if it was a 3-fold increase or greater. Averages were based on three biological replicates and error bars indicate the standard deviation across these replicates. Asterisks indicate results that were statistically significantly different from the drug-free control (2-tailed t-test, $P = 0.05$).

2.8.4 RNA-seq

The RNA seq data was provided in fastq format and was analysed in CLC Genomics Workbench Version 8 (Qiagen Bioinformatics). The drug-free, fusidic acid and tetracycline sequences were aligned to the published sequence for *S. aureus* strain 8325 (GenBank number CP000253.1). The initial fold-changes in expression were calculated by performing an unpaired two-group comparison for Drug-free vs. Fusidic acid and for Drug free vs Tetracycline. This gave the raw mRNA reads and the fold-changes in expression, which were subjected to an EDGE test to determine whether or not the genes were significantly differentially expressed. The increase in expression was considered to be significant/upregulated if a 2-fold increase or greater EDGE-test fold-change in expression was observed for that gene (123 genes for fusidic acid and 411 genes for tetracycline). From these data, genes were identified that were upregulated both in the presence of fusidic acid and in the presence of tetracycline (50 genes). These were reduced to just 7 candidate genes following the process described in section 5.2.1.

2.8.5 SATMD

The 50 genes upregulated both in the presence of fusidic acid and in the presence of tetracycline were analysed in the *Staphylococcus aureus* Transcriptome Meta-Database (SATMD) (http://www.satmd.org/; Informatics LLC, Maryland, USA, last accessed 23/09/17), a database of gene expression data from 251 experiments involving *S. aureus* (Nagarajan and Elasri, 2007). This involved having to convert the *S. aureus* locus tags to those for strain N315, which was achieved using the BLAST search on the NCBI website (https://www.ncbi.nlm.nih.gov/; last accessed 23/09/17).

Chapter 3 Using whole-cell biosensors to detect antibiotics in high-throughput screening assays and from natural product producing organisms

Abstract

The validation of the three whole-cell biosensors generated by the O'Neill lab was completed and it was evident that all three biosensors were specific detectors of cell wall biosynthesis inhibitors. The P_{murZ}-*lacZ* biosensor was used to offer some insight into the possible mechanism of action of two antibacterial compounds from the literature – rhodomyrtone and clomiphene. These two compounds did not induce the biosensor, suggesting that they might not be cell wall biosynthesis inhibitors. The P_{murZ}-*lacZ* biosensor was also used in the high-throughput screening of nearly 4,000 library compounds consisting of clinically used drugs and other general bioactive compounds. The biosensor was able to detect the 46 cell wall biosynthesis inhibitors in this library, either within the library itself or from an external source, further validating the specificity of the P_{murZ}-*lacZ* biosensor. Furthermore, 19 pan-assay interference compounds did not induce the P_{murZ}-*lacZ* biosensor, reducing the likelihood that screening using this biosensor will result in false positive hits. This biosensor was then used to screen a total of 288 crude microbial extracts and 19 of these extracts induced the biosensor, demonstrating the biosensor could be used to detect antibacterial compounds prior to time-consuming purification.

The three whole-cell biosensors were also tested in an agar-based assay, with X-gal being used as the substrate for β-galactosidase detection, resulting in a blue colour being observed on the agar in the presence of cell wall biosynthesis inhibitors. Dual reporters were generated in an attempt to facilitate the detection of antibacterial compounds at even lower concentrations than the initial three biosensors had been capable of thus far. Finally, the P_{murZ}-*lacZ* biosensor was able to detect cell wall biosynthesis inhibitors specifically, directly from their producing organism, in a manner similar to the original discovery of penicillin.

3.1 Introduction

3.1.1 The structure and function of the bacterial cell wall

One common feature of both Gram-positive and Gram-negative bacterial cells is the presence of a layer of peptidoglycan beyond the cytoplasmic membrane. This peptidoglycan layer primarily gives the cell wall the mechanical strength required to defend against osmotic challenge (Typas *et al.*, 2011; Vollmer *et al.*, 2008). It also contributes to the maintenance of a uniform cell shape, as does the prokaryotic cytoskeleton which includes homologs to eukaryotic actin (MreB), tubulin (FtsZ) and intermediate filament proteins, along with the bacteria-specific MinD-ParA group (Shih and Rothfield, 2006). It has also been proposed that the presence of the cell wall reduced the frequency of horizontal gene transfer, thus improving the genome stability compared with more primitive cells and allowing for divergent bacterial evolution to occur (Errington, 2013).

Mammalian cells lack this peptidoglycan layer, which gives them the increased flexibility necessary for executing the more specialised functions they have evolved for, such as phagocytosis (Botelho and Grinstein, 2011). Inevitably, this means that enzymes involved in peptidoglycan synthesis lack mammalian homologs and this makes this pathway an attractive target to focus on for discovering and synthesising antibiotics because antibiotics that target bacterial cell wall synthesis are unlikely to be toxic to a mammalian host (Liu and Breukink, 2016; Silver, 2006).

Although a layer of peptidoglycan is common to both Gram-positive and Gram-negative bacteria, the amount of peptidoglycan present in these bacteria differs significantly – the cell wall of Gram-positive bacteria includes approximately 50% peptidoglycan by weight, whereas peptidoglycan only accounts for 10–20% of the weight of the cell wall in Gram-negative bacteria (Hammond *et al.*, 1984). Gram-positive bacteria are encased in a cell wall that comprises an inner cytoplasmic membrane (a phospholipid bilayer) covered by a relatively thick layer of peptidoglycan. Lipoproteins, lipoteichoic acids and teichoic acids lie in the peptidoglycan layers (Brown *et al.*, 2015). Small molecules can traverse the layers of peptidoglycan. The cell wall of Gram-negative bacteria consists of a cytoplasmic membrane and a thinner layer of peptidoglycan,

which is covered by an outer membrane containing porins and lipopolysaccharides (Brown *et al.*, 2015)(Figure 3.1). This outer membrane makes Gram-negative bacteria less susceptible to lysozyme, hydrolytic enzymes, surfactants, bile salts and hydrophobic antibiotics (Kim and Gadd, 2008). It is believed that the origin of the double membrane of Gram-negative bacteria was an endosymbiosis between a clostridium and an actinobacterium, which are both Gram-positive bacteria (Lake, 2009).

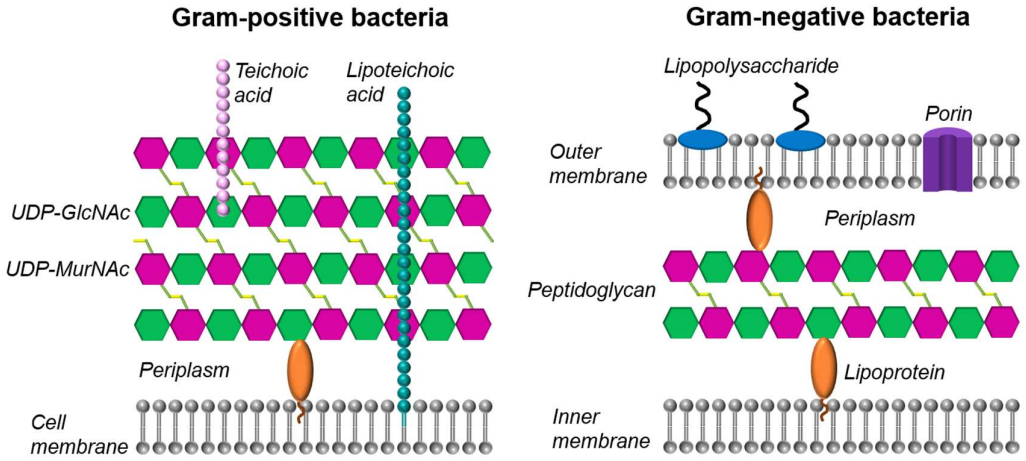


Figure 3.1 Cross sections of the structure of Gram-positive bacteria and Gram-negative bacteria. Inspired by Figure 1 in Brown *et al.*, 2015.

The peptidoglycan layers themselves consist of glycan stands held together by cross-linking peptide bonds. The glycan strands comprise alternating *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac) sugar residues linked by β-1→4 ether bonds (Vollmer *et al.*, 2008). Attached to the Mur*N*Ac residues are pentapeptide side chains with the sequence L-Ala/L-Gly–D-Glu–*m*-Dpm[1]/L-Lys–D-Ala–D-Ala. The exact pattern of cross-linking and the nature of any secondary modifications to the glycan strands (*N*-deacetylation, *O*-acetylation and *N*-glycolylation) are species-specific and these can translate into important differences in the defence mechanisms of particular bacterial species. For example, *Staphylococcus aureus* is highly resistant to lysozyme because its Mur*N*Ac residues are *O*-acetylated by *O*-acetyltransferase (*oatA*). Lysozyme is a muramidase that compromises the peptidoglycan layers by cleaving the β-1→4 bonds between Glc*N*Ac and Mur*N*Ac. The *O*-acetylation drastically decreases the stability of the conformation of the Mur*N*Ac residue

[1] *m*-Dpm stands for *meso*-diaminopimelic acid

during the transition state of its reaction with lysozyme, and so the reaction does not happen (Pushkaran *et al.*, 2015). This explains why it often colonises on the skin and mucosal areas where the lysozyme in tears and mucous would usually fend off most infections (Bera *et al.*, 2004).

The biosynthesis of peptidoglycan can be broken down into three major stages (Figure 3.5). First, the precursors (UDP-Glc*N*Ac and UDP-Mur*N*Ac with the pentapeptide motif attached) are synthesised in the cytoplasm. In the second stage, UDP-Mur*N*Ac is transferred to a lipid carrier molecule (undecaprenyl phosphate) and is also ligated to the Glc*N*Ac precursor. This unit is then transported across the cytoplasmic membrane by a flippase. Thirdly, the glycan units are polymerised by transglycosylases (formation of the β-1→4 bonds) and then the cross-linking peptide bonds are formed by transpeptidases, which inserts them into the cell wall (Bugg et al., 2011; Russell and Chopra, 1996; Silver, 2006). The secondary modifications mentioned above occur at this stage. This stage has provided the targets for most of the currently used cell wall biosynthesis inhibitors (CBIs).

3.1.2 The origin and mechanism of action of cell wall biosynthesis inhibitors

There are relatively few antibiotics currently available that target steps in either stage I or stage II, given the number of steps involved in these stages (Chopra *et al.*, 2002; El Zoeiby *et al.*, 2002; O'Neill *et al.*, 2009; Silver, 2006). D-cycloserine is an analogue of D-alanine and competitively inhibits the conversion of L-alanine to D-alanine by alanine racemase (alr) and the synthesis of the D-alanyl-D-alanine peptide by D-alanyl-D-alanine synthetase (Ddl) in the first stage (Russell and Chopra, 1996). It is a natural product of *Streptomyces garyphalus* and *Streptomyces lavendulae* and was discovered in 1955 (Kumagai *et al.*, 2015; Stromgaard *et al.*, 2016).

Fosfomycin is an analogue of phosphoenolpyruvate (PEP) that covalently binds to the enzyme MurA and thus inhibits the first committed step of peptidoglycan synthesis, namely the ligation of PEP to the UDP-Glc*N*Ac unit (Brown *et al.*, 1995; Halouska *et al.*, 2014; Russell and Chopra, 1996)(Figure 3.2). It was discovered in 1969 as a natural product from *Streptomyces fradiae,*

Streptomyces viridochromogenes and *Streptomyces wedmorensis* (Hendlin *et al.*, 1969).

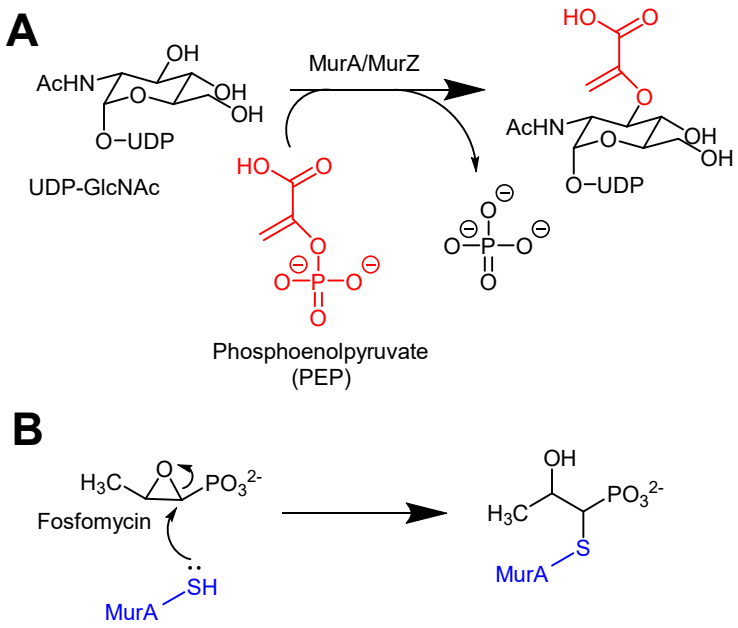


Figure 3.2 The mechanism of action of fosfomycin. A) Either MurA or MurZ catalyses the addition of PEP (shown in red) to UDP-GlcNAc in the first committed step of peptidoglycan synthesis; B) The MurA (and MurZ) enzyme (shown in blue) is inactivated by the ring-opening of fosfomycin.

The second stage of cell wall biosynthesis begins when the enzyme MraY (Phospho-*N*-acetylmuramoyl-pentapeptide translocase) transfers the MurNAc-pentapeptide unit from UDP to the undecaprenyl phosphate, anchored to the inside of the cytoplasmic membrane, forming lipid I (Stromgaard *et al.*, 2016; Walsh, 2003). The UDP-GlcNAc sugar unit is then added to lipid I to form lipid II, in a reaction catalysed by the enzyme MurG (Stromgaard *et al.*, 2016; Walsh, 2003). Lipid II is then translocated across the membrane to the extracytoplasmic side, ready for stage III of cell wall biosynthesis. The reaction involving MraY is inhibited by uridyl peptide antibiotics such as tunicamycin, liposidomycin B and mureidomycin A (Brandish *et al.*, 1996; Hakulinen *et al.*, 2017). Tunicamycin was discovered in 1982 as a product of *Streptomyces lysosuperificus* and is also produced by other *Streptomyces* species (Doroghazi *et al.*, 2011). The glycosyltransferase, MurG, is inhibited by murgocil, a small molecule that only exhibited activity against *Staphylococci* (Mann *et al.*, 2013).

Bacitracin prevents the dephosphorylation of the lipid carrier undecaprenyl phosphate, also known as C_{55}-isoprenyl pyrophosphate (IPP). This is achieved by forming a complex with the phosphate group of IPP and the thiazoline ring of the bacitracin compound, with the assistance of a divalent cation (Mg^{2+}) (Pollock *et al.*, 1994; Stone and Strominger, 1971; Walsh, 2003). This prevents the action of the phosphatase UppP on IPP, and so it cannot be reused to transfer another GlcNAc-MurNAc pentapeptide unit across the cytoplasmic membrane. Bacitracin is produced by *Bacillus licheniformis* and was discovered in 1945 (Craig *et al.*, 1952; Johnson *et al.*, 1945).

The majority of antibiotics that target bacterial cell wall biosynthesis inhibit steps in the third stage of peptidoglycan synthesis (Silver, 2006). Lantibiotics (lanthionine-containing antibiotics) such as mersacidin, along with lipodepsipeptides such as ramoplanin, form a complex with lipid II and prevent

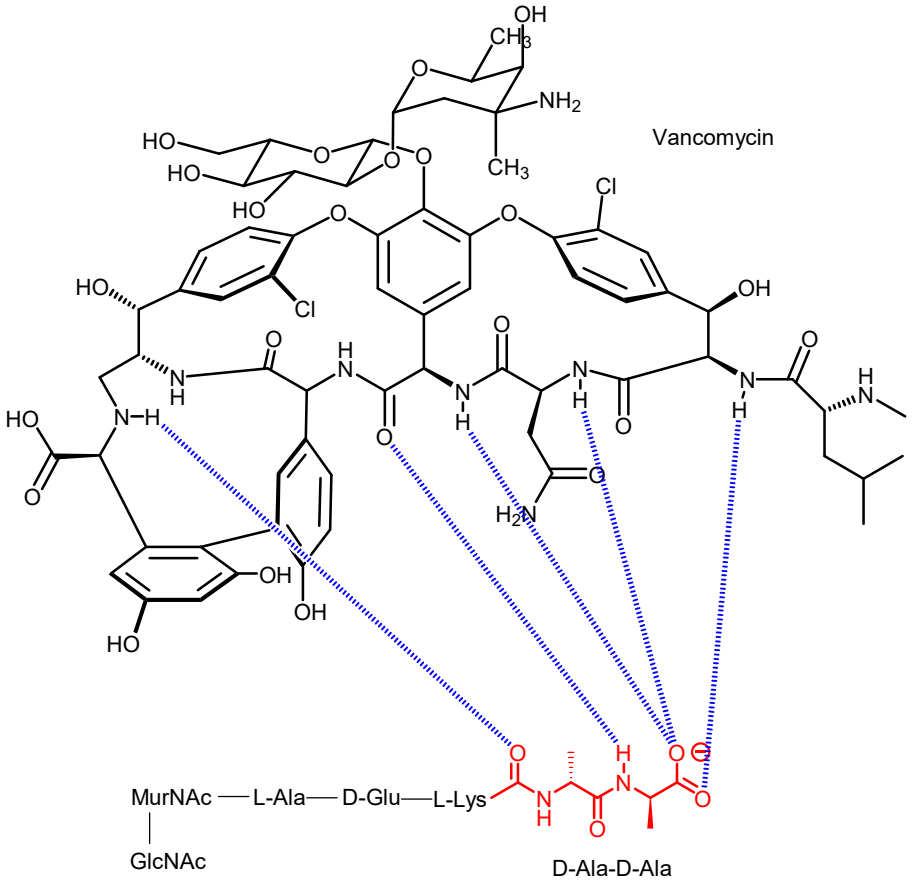


Figure 3.3 The interaction between vancomycin and the pentapeptide side chain. The five hydrogen bonding interactions are shown in blue. The D-Ala-D-Ala section of the pentapeptide chain is shown in red.

the GlcNAc-MurNAc-pentapeptide units being linked together by transglycosylases to form mature peptidoglycan polymer (Brötz *et al.*, 1998; de la Cruz *et al.*, 2017; Schmitz *et al.*, 2006; Walsh, 2003). Ramoplanin was discovered in 1984 as a natural product of *Actinoplanes* spp. ATCC 33076 (Cavalleri *et al.*, 1984). Mersacidin was isolated from a *Bacillus* strain found in a soil sample from India (Chatterjee *et al.*, 1992; Schmitz *et al.*, 2006).

The glycopeptides (*e.g.* vancomycin and teicoplanin) inhibit the final cross-linking reaction because they are able to form five strong hydrogen bond interactions with the D-Ala-D-Ala moiety at the end of the pentapeptide side chain (Figure 3.3). This sterically hinders the transpeptidase from catalysing the peptide bond formation (Walsh, 2003). Vancomycin was discovered in 1952 when it was isolated from a sample of *Streptomyces orientalis* found in Borneo, as a result of a program initiated by Eli Lilly to find new antibiotics that were active against staphlococcocal infections that had become penicillin-resistant by the 1950s. When such an antibacterial drug was found, it was named vancomycin after the word “vanquish” (Levine, 2006). Vancomycin-resistant Enterococci (VRE) emerged due to a plasmid borne operon consisting of five genes that encode for proteins that collectively alter the termini of the pentapeptide side chains formed during the early stages of peptidoglycan synthesis from *N*-acyl-D-Ala-D-Ala to *N*-acyl-D-Ala-D-lactate (Walsh, 2003). This means that there is one fewer hydrogen bonding interaction available and this decreases the affinity of vancomycin for its pentapeptide target by around 1000-fold, resulting in a reduction in MIC values by a similar magnitude (Bugg *et al.*, 1991).

The β-lactams (*e.g.* penicillins and cephalosporins) inhibit the cross-linking of glycan strands by undergoing an irreversible reaction with the transpeptidase that would, ordinarily, react with the penultimate D-Ala residue and catalyse the cross-linking reaction with the lysine residue in the adjacent pentapeptide side chain (Walsh, 2003)(Figure 3.4). Penicillin was discovered in 1929 as a metabolite produced by *Penicillium notatum* (Fleming, 1929). Cephalosporin was discovered a while later, in 1945, as a product of *Cephalosporium acremonium* (Brotzu, 1948; Sader and Jones, 1992). Strains of bacteria that are resistant to β-lactams contain β-lactamases that react irreversibly and even

more thermodynamically favourably with the β-lactam molecule than the lysine residue does (Beadle *et al.*, 2001; Tipper and Strominger, 1965).

A summary of antibiotics that target cell wall biosynthesis is shown in Figure 3.5.

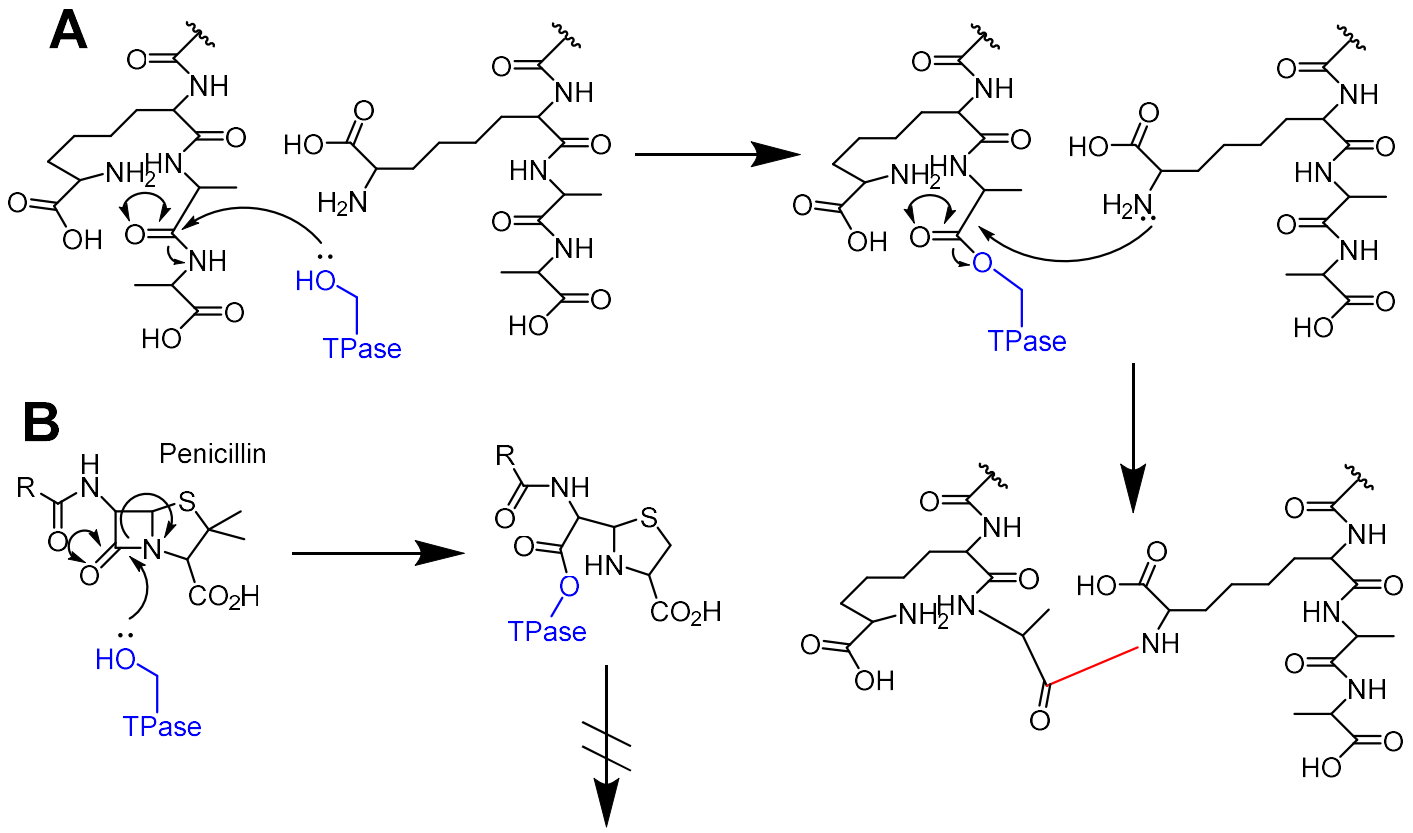


Figure 3.4 The mechanism of action of penicillin. A) The transpeptidase (TPase, shown in blue) catalyses the formation of the DAP-D-Ala isopeptide bond (shown in red); B) Penicillin irreversibly reacts with the TPase, preventing it from reacting with the D-Ala$_4$ residue and cross-linking the adjacent side chains.

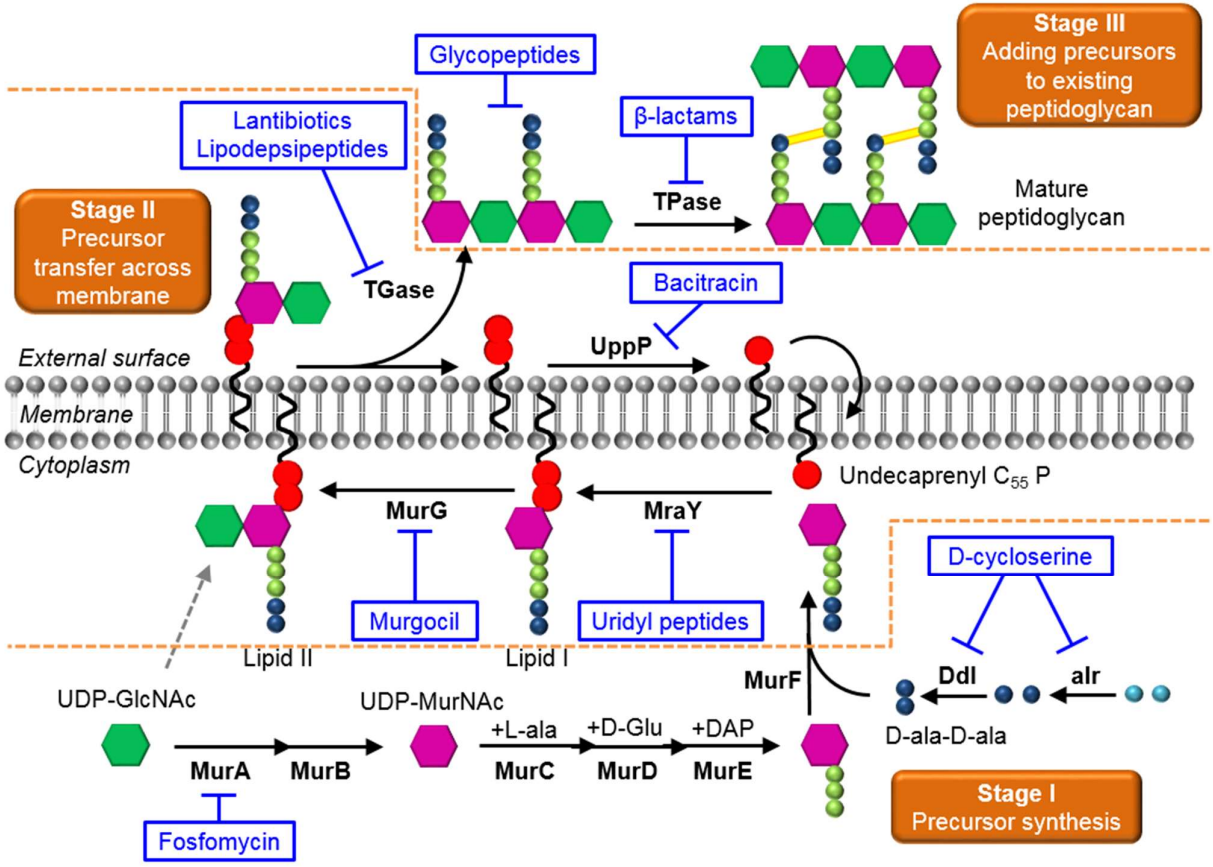


Figure 3.5 An overview of the peptidoglycan biosynthetic pathway and an indication of the targets of various CBIs. Inspired by Figure 23.3 in Stromgaard *et al.*, 2016.

3.1.3 Generation of the three CBI biosensors used in this study

The cell wall biosynthesis inhibitor (CBI) biosensors used in this study were the result of two different studies from the O'Neill lab, both focusing on the under-utilised first stage of peptidoglycan synthesis.

In a paper on the nature of the MurA and MurZ (UDP-*N*-acetylglucosamine enolpyruvyl transferase) enzymes in *Staphylococcus aureus* (Blake *et al.*, 2009), the initial aim was to determine how important these two enzymes were to the survival of the bacterium. MurZ is an isozyme of MurA which is able to catalyse the same step, albeit less efficiently, in species such as *Escherichia coli*. The MurA enzyme is the target of the antibiotic fosfomycin, which is one of the few drugs involved in inhibiting early stage peptidoglycan synthesis but has limited use as a way of treating infections caused by *Staphylococcus aureus*. The second aim of this study was to develop a way of discovering more potent novel MurA/MurZ inhibitors. Biosensors were produced using a gene inactivation strategy in the *S. aureus* strain RN4220 to generate a translational fusion of the native promotors of the *murA* and *murZ* genes to the reporter gene, *lacZ*, which encodes for the enzyme β-D-galactosidase. This generated the strains KB01 (P_{murA}-*lacZ*) and KB02 (P_{murZ}-*lacZ*), in which the level of β-galactosidase activity depended on the expression of the *murA* and *murZ* genes, respectively.

It was observed that inactivation of the *murA* or *murZ* gene increased the sensitivity of the strains to fosfomycin (the MICs of KB01 and KB02 were 8 $\mu g.mL^{-1}$ and 4 $\mu g.mL^{-1}$, respectively, whereas the MIC of the progenitor strain, RN4220, was 32 $\mu g.mL^{-1}$). This sensitivity was not observed for tetracycline (a protein synthesis inhibitor) or triclosan (a fatty acid synthesis inhibitor). These biosensors were used to monitor the level of β-galactosidase activity, as an indicator of their expression from the native promotor, following their exposure to fosfomycin. The substrate used was 4-methylumbelliferyl β-D-galactopyranoside (MUG), which is hydrolysed by β-galactosidase to give the fluorescent compound 4-methylumbelliferone (4-MU). The level of expression from P_{murZ} in KB02 increased approximately 3-fold, but the expression from P_{murA} in KB01 remained the same. Further compounds were tested and the conclusion was that exposure of the P_{murZ}-*lacZ* biosensor to antibiotics that

target early and late stage peptidoglycan synthesis resulted in an increase in β-galactosidase activity ≥2-fold compared to an untreated control (Blake *et al.*, 2009).

Another two biosensors were generated based on the work reported in a paper describing the transcriptional signature following treatment by early stage cell wall synthesis inhibitors (O'Neill *et al.*, 2009). The aim in this study was to ascertain the transcriptional signature of *S. aureus* when early stage cell wall biosynthesis is inhibited. Fosfomycin was used to inhibit the MurA and MurZ enzymes. The lack of antibiotics capable of inhibiting any of the other Mur enzymes led to genetic and posttranslational methods being employed to simulate inhibition. Inhibition of MurB was simulated by using a strain with a temperature-sensitive mutation in *murB* and harvesting these cells once the temperature was shifted above the permissive temperature. Inhibition of MurE was achieved by using a strain in which the expression of the *murE* gene was under the control of the P_{spac} promoter, and hence conditional on the presence of IPTG. RNA extracted from cells whose early stage cell wall biosynthesis had been inhibited was used to generate a labelled cDNA library to use for DNA microarray analysis to establish the gene expression levels. These results were considered alongside the transcriptional signature following the conditional depletion of MurF (Sobral *et al.*, 2007) and following the inhibition of later stages of cell wall biosynthesis (Nagarajan and Elasri, 2007). Two genes, that were upregulated across all of the inhibitory conditions considered, were used to make the other two biosensors. These genes were *gltA* (a citrate synthase) and *oppB* (an oligopeptide transport system permease). A translational fusion of the promotor region and the first few codons of these genes, together with the plasmid pAJ129, generated plasmids in which the expression of the reporter gene *lacZ* was regulated by either the P_{gltA} promoter or the P_{oppB} promoter. These plasmids were used to transform strain RN4220 to generate the P_{gltA}-*lacZ* and P_{oppB}-*lacZ* biosensors, respectively (unpublished data).

3.2 Results

3.2.1 Completing the validation of the P_{gltA}-*lacZ*, P_{oppB}-*lacZ* and P_{murZ}-*lacZ* biosensors

The O'Neill lab did some initial validation of the specificity and sensitivity of the P_{gltA}-*lacZ*, P_{oppB}-*lacZ* and P_{murZ}-*lacZ* biosensors by exposing them separately to 19 CBIs covering all three stages of cell wall biosynthesis. Each antibiotic tested was considered to have 'induced' the biosensor in the full scale MUG assay if the β-galactosidase activity ratio was ≥2-fold compared to a drug-free control (DFC). This threshold was an arbitrary cut-off point that is typically used in gene expression studies. The 19 CBIs all induced the reporters at concentrations of at least 4× the minimum inhibitory concentration (MIC) (Table 3.1).

More antibiotics were tested to add to the validation that had already been performed regarding the specificity and sensitivity of the biosensors. This was done using the full scale MUG assay (section 2.7.3) that was used to generate the rest of the data in Table 3.1 and that is based on a protocol described in (Chan and Foster, 1998) and (Blake, 2008). Briefly, the biosensors were incubated with the test antibiotics for one hour, after which point OD_{600} readings were taken to give an indication of growth inhibition. The cells were then resuspended in AB buffer and lysostaphin, then incubated with the MUG substrate for 90 minutes at room temperature. After this incubation time had elapsed, sodium carbonate was added to quench the reaction and the fluorescence values were recorded. The platereader was calibrated for each biosensor by setting the gain to 80% of the fluorescence emitted by a 20 $\mu g.mL^{-1}$ solution of 4-MU. The compound 4-MU was the fluorescent product of the reaction in which β-galactosidase cleaved the MUG substrate (Figure 3.6). The β-gal activity was calculated as the fluorescence reading divided by the corresponding OD_{600} reading. The β-gal activity ratio for each antibiotic and concentration was calculated as the β-gal activity divided by the β-gal activity of the drug-free control.

The additional compounds that were tested were the CBIs β-chloro-D-alanine, friulimicin, aztreonam and cloxacillin, the membrane damaging compounds EDTA and polymyxin B, and the protein biosynthesis inhibitor, actinonin. The

MIC values and the β-gal activity ratio obtained for all three biosensors with these compounds is shown in bold in Table 3.1 and graphically in Figure 3.7.

Overall, the biosensors were not induced by antibiotics that inhibited protein synthesis (*n*=13), DNA synthesis/maintenance (*n*=5), RNA synthesis (*n*=1), fatty acid synthesis (*n*=1), and compounds that damaged the cell membrane (*n*=9) (Table 3.1). They were induced by 23 CBIs in total (Table 3.1).

These results suggested that all three of the biosensors were specific for CBIs and were also sensitive enough to detect some CBIs at sub-inhibitory concentrations (0.25×MIC).

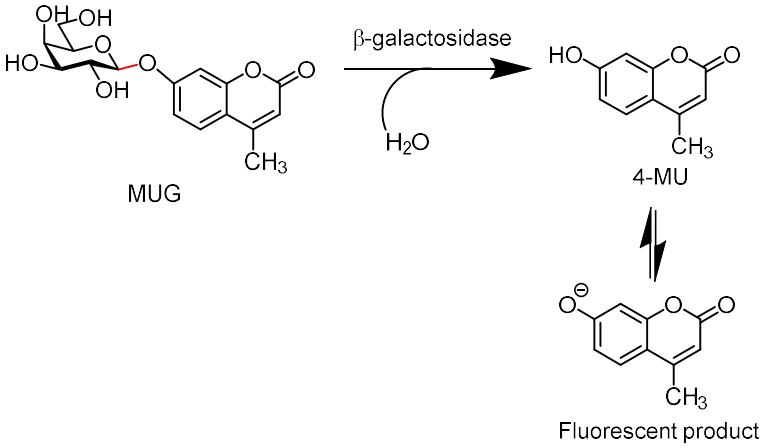


Figure 3.6 The reaction of β-galactosidase with MUG to produce the fluorescent product, 4-MU. The bond broken by β-galactosidase is shown in red.

Table 3.1 Validation of the P_{gltA}-*lacZ*, P_{oppB}-*lacZ* and P_{murZ}-*lacZ* biosensors. Red text indicates a fold-increase in fluorescence of at least 2-fold above that of the drug-free control (indicating a β-gal activity ratio ≥2-fold). Results obtained by C. Randall and K. Randall. Results in bold obtained by J. Mitchell (unpublished data). MICs may differ slightly from those assumed for most of this study.

Antibacterial Agent	MIC against *S. aureus* SH1000 (μg/ml)	Induction of promoter (± standard deviation)								
		P_{gltA}			P_{oppB}			P_{murZ}		
		0.25xMIC	1xMIC	4xMIC	0.25xMIC	1xMIC	4xMIC	0.25xMIC	1xMIC	4xMIC
Inhibitors of the first stage of peptidoglycan synthesis (precursor synthesis)										
β-chloro-D-alanine	**2048 (12800 μM)**	**2.0±0.3**	**2.2±0.7**	**1.0±0.3**	**2.0±0.5**	**2.0±0.3**	**0.5±0.02**	**1.9±0.3**	**2.4±0.3**	**1.1±0.4**
D-cycloserine	64 (627 μM)	2.8 ±0.2	2.1 ±0.4	2.0 ±0.7	1.7 ±0.7	2.6 ±0.4	3.7 ±0.4	1.0 ±0.5	2.8 ±0.2	3.7 ±0.7
Fosfomycin	8 (44.0 μM)	0.8 ±0.1	0.9 ±0.2	2.4 ±0.2	1.3 ±0.3	2.1 ±0.4	4.8 ±0.6	1.9 ±0.1	3.1 ±0.4	4.6 ±0.9
Inhibitors of the second stage of peptidoglycan synthesis (precursor transfer across membrane)										
Bacitracin	128 (90.0 μM)	3.0±0.3	2.5±0.3	2.6±0.2	5.4±0.7	4.7±0.6	3.8±0.8	5.5±0.4	6.7±0.7	5.3±0.3
Deoxyactagardine B	32 (18.4 μM)	2.5±0.3	3.0±0.1	1.7±0.1	2.4±0.2	3.4±0.5	2.6±0.8	1.6±0.1	3.2±0.5	2.1±0.2
Flavomycin	4 (2.53 μM)	7.0±0.5	7.2±0.9	7.4±0.6	1.6±0.2	2.3±0.1	2.5±0.05	1.2±0.1	4.2±0.4	3.9±0.3
Friulimicin	**4 (3.07 μM)**	**1.1±0.1**	**3.7±0.3**	**2.9±0.4**	**1.0±0.08**	**2.0±0.2**	**4.0±0.4**	**1.2±0.3**	**2.7±0.9**	**4.8±0.7**
Mersacidin	32 (17.5 μM)	2.7±0.2	2.8±0.5	2.0±0.1	2.0±0.4	2.5±0.1	2.8±0.5	2.4 ±0.3	3.7 ±0.8	2.9 ±0.3

Antibacterial Agent	MIC against *S. aureus* SH1000 (μg/ml)	Induction of promoter (± standard deviation)								
		P_{gltA}			P_{oppB}			P_{murZ}		
		0.25xMIC	1xMIC	4xMIC	0.25xMIC	1xMIC	4xMIC	0.25xMIC	1xMIC	4xMIC
Murgocil	8 (17.9 μM)	1.4±0.4	3.5±0.8	3.2±0.1	1.3±0.2	2.4±0.2	2.6±0.3	1.4±0.1	2.6±0.4	3.0±0.3
Ramoplanin	2 (0.783 μM)	1.2±0.4	3.1±0.7	2.4±0.7	1.3±0.3	2.7±0.2	2.1±0.2	1.6±0.3	2.3±0.6	2.3±0.2
Tunicamycin	8 (9.79 μM)	3.6±0.8	5.8±0.08	8.5±0.7	1.3±0.4	1.5±0.3	2.4±0.02	1.9±0.2	2.6±0.1	2.9±0.4
Inhibitors of the third stage of peptidoglycan synthesis (adding precursors to existing peptidoglycan)										
Aztreonam	**512 (1180 μM)**	**1.1±0.04**	**2.6±0.8**	**3.1±1.2**	**2.1±0.3**	**6.6±1.2**	**7.5±1.4**	**1.2±0.2**	**5.6±1.4**	**5.2±1.1**
Cefotaxime	2 (4.39 μM)	2.8±0.5	2.8±0.6	3.0±0.8	3.9±0.1	3.6±0.2	4.2±1.9	6.8±0.7	7.6±0.2	6.3±0.7
Cefoxitin	4 (9.36 μM)	0.9±0.08	1.6±0.5	5.0±0.4	1.1±0.1	1.8±0.6	3.0±0.4	1.4±0.1	5.5±0.6	7.3±0.4
Cefradine	8 (22.9 μM)	1.2±0.1	1.3±0.2	2.3±0.5	0.8±0.04	0.7±0.08	2.5±0.3	1.0±0.1	2.0±0.03	5.1±1.15
Cloxacillin	**0.125 (0.273 μM)**	**0.9±0.1**	**1.1±0.2**	**5.2±0.2**	**1.0±0.1**	**1.2±0.1**	**4.8±0.1**	**1.0±0.2**	**1.6±0.3**	**6.4±0.8**
Flucloxacillin	0.25 (0.525 μM)	1.0±0.5	2.3±0.2	3.9±1.2	1.1±0.1	3.7±1.0	4.1±0.7	1.6±0.1	7.2±0.9	7.8±1.9
Imipenem	0.0625 (0.197 μM)	1.0±0.09	2.8±0.7	3.1±0.5	1.5±0.6	2.6±0.4	2.5±0.6	1.3±0.04	2.9±0.4	4.6±0.3
Lysostaphin	16 (0.620 μM)	7.8±0.9	3.0±0.4	1.2±0.1	1.8±0.1	2.1±0.3	1.6±0.4	47±4.8	42±4.4	8.1±1.6

Antibacterial Agent	MIC against *S. aureus* SH1000 (μg/ml)	Induction of promoter (± standard deviation)								
		P_{gltA}			P_{oppB}			P_{murZ}		
		0.25xMIC	1xMIC	4xMIC	0.25xMIC	1xMIC	4xMIC	0.25xMIC	1xMIC	4xMIC
Methicillin	4 (9.94 μM)	1.0±0.01	1.5±0.02	4.9±0.4	1.0±0.02	2.6±0.2	2.4±0.1	0.8±0.02	1.4±0.5	3.3±0.7
Teicoplanin	4 (2.13 μM)	3.0±0.4	2.7±0.2	2.9±0.4	1.0±0.1	2.7±0.5	3.1±0.8	3.8±0.8	3.2±0.9	2.8±0.5
Telavancin	1 (0.570 μM)	2.6±0.3	2.0±0.3	1.6±0.03	3.4±0.9	3.7±0.4	2.8±0.2	4.6±0.8	4.5±0.4	4.3±0.5
Vancomycin	2 (1.35 μM)	1.9±0.5	3.2±1.5	2.0±0.1	1.1 ±0.2	4.0±0.3	5.0±1.5	0.7±0.1	3.7±0.4	3.8±0.5
Compounds that adversely affect cell membrane integrity										
Acriflavine	32 (123 μM)	0.8±0.3	1.2±0.3	0.5±0.08	0.8±0.2	0.9±0.1	0.7±0.3	1.1±0.1	1.0±0.1	0.6±0.02
CCCP	2 (9.77 μM)	1.1±0.2	1.5±0.2	1.6±0.08	1.4±0.2	1.5±0.07	1.6±0.4	1.1±0.02	1.2±0.2	1.6±0.3
Chlorhexidine	1 (1.98 μM)	0.8±0.2	1.3±0.3	1.1±0.3	1.3±0.2	1.4±0.3	1.2±0.4	1.4±0.1	1.3±0.09	1.1±0.03
CTAB	2 (5.49 μM)	1.4±0.3	1.6±0.2	1.8±0.1	0.8±0.1	0.4±0.2	0.9±0.1	0.5±0.1	0.9±0.3	0.2±0.1
EDTA	**16 (54.7 μM)**	**1.0±0.09**	**1.0±0.1**	**0.9±0.1**	**1.0±0.1**	**1.0±0.1**	**0.9±0.1**	**1.1±0.2**	**0.7±0.2**	**0.7±0.1**
Daptomycin	2 (1.23 μM)	1.1±0.06	1.2±0.2	1.4±0.1	1.0±0.3	1.3±0.2	1.3±0.1	1.0±0.08	1.4±0.05	1.1±0.4
Nisin	4 (1.19 μM)	0.6 ±0.3	1.0 ±0.5	1.0 ±0.2	1.4 ±0.1	1.1 ±0.4	1.2 ±0.1	1.3 ±0.3	1.1 ±0.2	0
Polymyxin B	**16 (11.5 μM)**	**0.8±0.1**	**1.1±0.5**	**0.9±0.1**	**1.0±0.1**	**1.1±0.09**	**1.5±0.6**	**1.2±0.2**	**1.2±0.1**	**1.3±0.02**

Antibacterial Agent	MIC against *S. aureus* SH1000 (μg/ml)	Induction of promoter (± standard deviation)								
		P_{gltA}			P_{oppB}			P_{murZ}		
		0.25xMIC	1xMIC	4xMIC	0.25xMIC	1xMIC	4xMIC	0.25xMIC	1xMIC	4xMIC
Valinomycin	2 (1.80 μM)	0.4±0.1	0.5±0.2	0.2±0.1	1.6±0.1	1.5±0.1	1.4±0.3	0.7±0.1	0.7±0.1	0.1±0.1
Inhibitors of DNA synthesis										
Ciprofloxacin	1 (3.02 μM)	1.2 ±0.1	1.0 ±0.4	0.9 ±0.1	1.7 ±0.1	1.1 ±0.2	0.9 ±0.2	0.4 ±0.1	0.4 ±0.1	0.3 ±0.1
Clofazimine	2 (4.22 μM)	1.4 ±0.1	1.3 ±0.2	1.6 ±0.2	1.0 ±0.1	1.1 ±0.1	1.8 ±0.2	1.1 ±0.1	0.8 ±0.2	1.0 ±0.1
Nalidixic acid	64 (276 μM)	1.0±0.02	1.2±0.07	1.0±0.1	0.9±0.1	1.1±0.3	1.2±0.3	0.9±0.07	1.1±0.2	1.1±0.2
Novobiocin	0.125 (0.204 μM)	1.1 ±0.4	1.0 ±0.3	1.1 ±0.3	1.0 ±0.2	1.0 ±0.2	1.0 ±0.1	1.3 ±0.4	1.2 ±0.4	1.8 ±0.1
Trimethoprim	8 (27.6 μM)	1.1 ±0.2	1.4 ±0.2	1.2 ±0.2	1.0 ±0.1	1.0 ±0.2	1.2 ±0.2	1.0 ±0.1	1.1 ±0.1	1.3 ±0.1
Inhibitors of RNA synthesis										
Rifampicin	0.015 (0.0182 μM)	0.9 ±0.2	1.2 ±0.2	0.8 ±0.3	0.4 ±0.1	1.1 ±0.5	1.1 ±0.2	0.7 ±0.3	0.5 ±0.1	0.4 ±0.1
Inhibitors of protein synthesis										
Actinonin	**4 (10.4 μM)**	**0.6±0.04**	**0.5±0.04**	**0.5±0.01**	**0.7±0.04**	**0.7±0.09**	**0.8±0.04**	**0.8±0.2**	**0.7±0.2**	**0.8±0.1**
Anhydrotetracycline	2 (4.32 μM)	0.7±0.04	0.7±0.1	1.0±0.3	1.2±0.4	1.0±0.3	0.8±0.3	1.3±0.2	1.1±0.03	1.1±0.1
Clindamycin	0.125 (0.271 μM)	0.7±0.03	0.7±0.1	0.7±0.1	1.3±0.1	1.4±0.3	1.3±0.3	0.8±0.1	1.1±0.02	1.1±0.02
Fusidic Acid	0.125 (0.242 μM)	0.7 ±0.2	0.8 ±0.2	0.7 ±0.1	0.9 ±0.2	0.8 ±0.1	0.6 ±0.1	1.2 ±0.1	1.1 ±0.1	1.1 ±0.1

Antibacterial Agent	MIC against *S. aureus* SH1000 (μg/ml)	Induction of promoter (± standard deviation)								
		P_{gltA}			P_{oppB}			P_{murZ}		
		0.25xMIC	1xMIC	4xMIC	0.25xMIC	1xMIC	4xMIC	0.25xMIC	1xMIC	4xMIC
Gentamicin	1 (2.09 μM)	1.0 ±0.2	0.9 ±0.1	0.9 ±0.1	0.7 ±0.1	0.6 ±0.1	1.4 ±0.4	1.1 ±0.2	1.1 ±0.1	1.0 ±0.1
Linezolid	1 (2.96 μM)	0.9 ±0.1	0.9 ±0.1	1.0 ±0.2	0.8 ±0.1	0.6 ±0.1	1.1 ±0.1	1.2 ±0.2	1.2 ±0.1	1.0 ±0.2
Mupirocin	0.0625 (0.125 μM)	1.3 ±0.2	1.1 ±0.1	1.6 ±0.1	1.0 ±0.3	1.0 ±0.2	1.3 ±0.4	1.2 ±0.1	1.1 ±0.2	1.1 ±0.1
Spectinomycin	64 (149 μM)	0.9±0.2	1.0±0.1	0.8±0.02	0.9±0.1	1.1±0.2	1.2±0.2	1.1±0.3	1.0±0.2	0.8±0.1
Streptomycin	4 (5.49 μM)	0.6±0.2	0.5±0.2	0.6±0.3	0.7±0.2	0.5±0.2	0.5±0.2	1.0±0.03	0.9±0.02	0.7±0.1
Tetracycline	0.5 (1.04 μM)	0.9 ±0.1	0.8 ±0.2	0.8 ±0.1	0.6 ±0.1	0.7 ±0.1	0.6 ±0.1	0.9 ±0.1	0.6 ±0.1	0.5 ±0.1
Tiamulin	1 (2.03 μM)	1.4±0.2	1.6±0.2	1.5±0.3	1.1±0.3	1.2±0.4	1.2±0.1	0.9±0.01	0.9±0.04	1.0±0.07
Tigecycline	0.5 (0.854 μM)	0.5±0.2	0.5±0.02	0.6±0.1	0.7±0.1	0.6±0.2	0.6±0.03	0.8±0.01	0.8±0.02	0.9±0.06
Virginiamycin	4 (2.96 μM)	0.6±0.02	0.5±0.02	0.6±0.01	0.9±0.3	0.8±0.3	0.7±0.3	0.9±0.2	0.9±0.06	0.9±0.2
Inhibitors of fatty acid synthesis										
Triclosan	0.125 (0.432 μM)	1.0 ±0.1	0.4 ±0.1	1.4 ±0.2	1.2 ±0.2	1.2 ±0.1	0.8 ±0.1	0.5 ±0.1	0.4 ±0.2	0.9 ±0.1

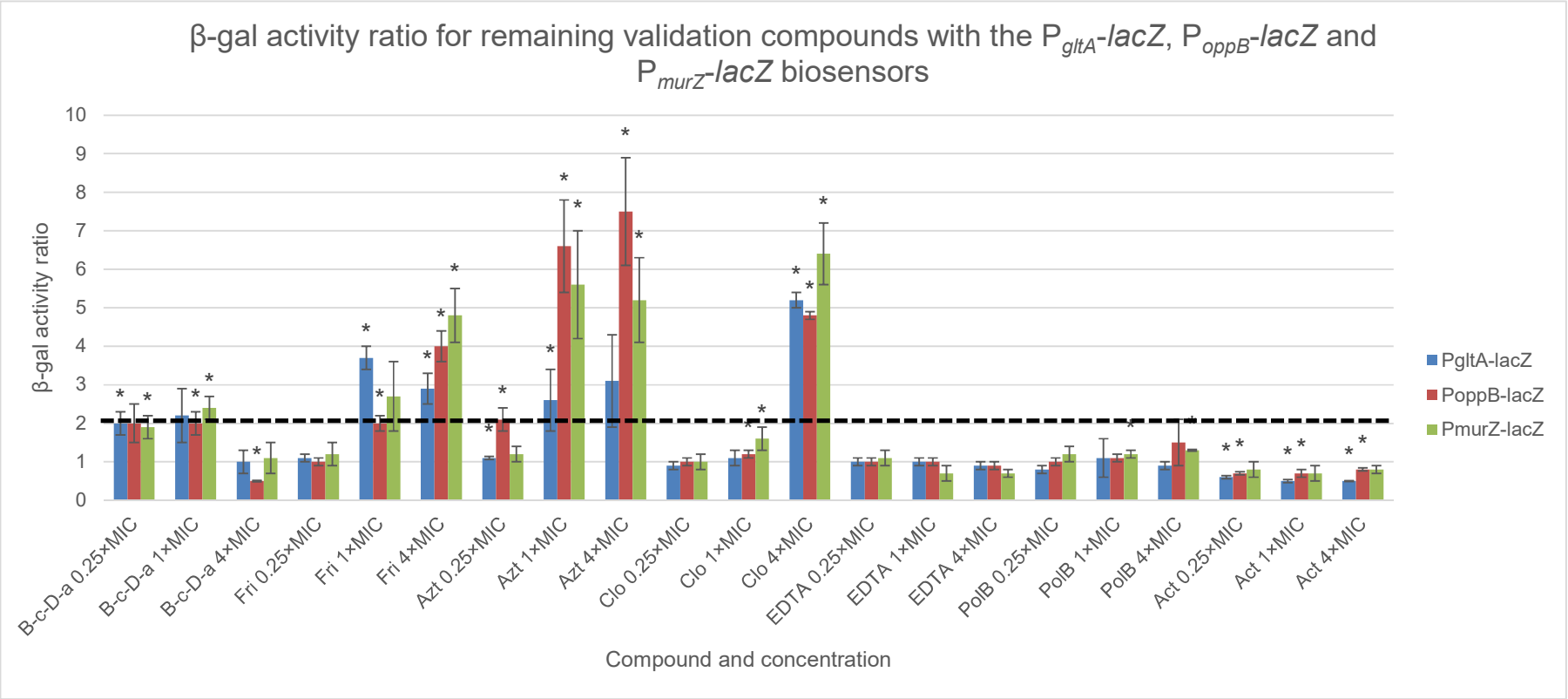


Figure 3.7 β-gal activity ratio for remaining validation compounds with the P_{gltA}-*lacZ*, P_{oppB}-*lacZ* and P_{murZ}-*lacZ* biosensors in the full scale MUG assay (induction threshold = 2-fold, represented by the black dotted line). B-c-D-a = β-chloro-D-alanine; Fri = friulimicin; Azt = aztreonam; Clo = cloxacillin; EDTA = ethylenediaminetetraacetic acid; PolB = polymyxin B; Act = actinonin.

3.2.2 Testing compounds from the literature using the P_{murZ}-*lacZ* biosensor

3.2.2.1 Testing rhodomyrtone

Rhodomyrtone is a natural product from the evergreen shrub *Rhodomyrtus tomentosa* (Hiranrat and Mahabusarakam, 2008). It had previously been reported that it displayed good inhibitory activity against a range of Gram-positive bacteria, including *Bacillus cereus, Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, methicillin-resistant *S. aureus* (MRSA), and *Streptococcus pyogenes* (Limsuwan *et al.*, 2009, 2011; Saising *et al.*, 2011). Electronmicrographs of MRSA cells after incubation with rhodomyrtone at a sub-inhibitory concentration over 18h revealed that the compound had altered the cell morphology – the cell walls were thicker in some locations but deformed overall, which had been observed when *S. aureus* had been exposed to penicillin and vancomycin (Sianglum *et al.*, 2011). Proteomic analysis using SDS-PAGE and Western immunoblotting revealed that there were several types of protein that were expressed in the presence of rhodomyrtone, including proteins involved in amino acid metabolism, carbohydrate metabolism, cell division, and cell wall biosynthesis (Sianglum *et al.*, 2011). Transcriptomic analysis of microarray data, obtained using qRT-PCR, identified 64 genes that were upregulated ≥2-fold in MRSA after exposure to rhodomyrtone for 1h (Sianglum *et al.*, 2012). Some of the most highly upregulated genes were part of the DAP operon, which coordinates the biosynthesis of diaminopimelate (DAP). DAP is not used by mammals but it is an essential component of peptidoglycan biosynthesis, making this pathway an attractive target for antibacterial drug development.

Given that the proposed mode of action of rhodomyrtone was related to cell wall biosynthesis, the P_{murZ}-*lacZ* biosensor was used in the full scale MUG assay to see if rhodomyrtone was able to induce this extensively validated biosensor. The positive control for this experiment was flucloxacillin and the negative control was tetracycline. Figure 3.8 shows that rhodomyrtone did not induce the P_{murZ}-*lacZ* biosensor, suggesting that it is not a CBI.

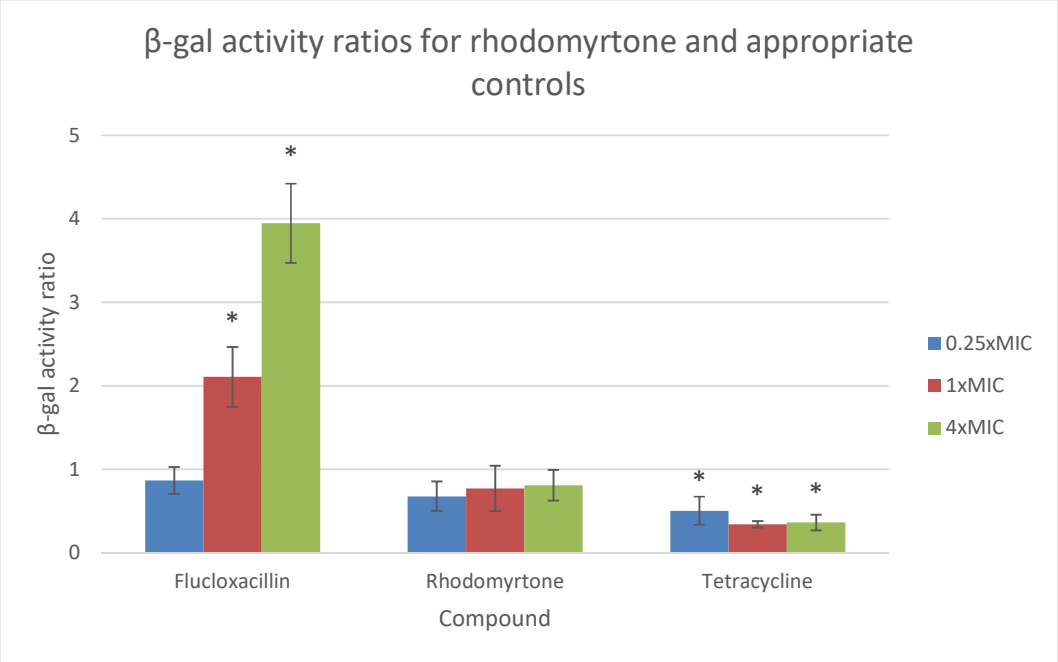


Figure 3.8 β-gal activity ratios for rhodomyrtone, flucloxacillin and tetracycline using the full scale MUG assay (induction threshold = 2-fold). Flucloxacillin MIC = 0.525 µM; rhodomyrtone MIC = 2.26 µM; tetracycline MIC = 1.04 µM.

3.2.2.2 Testing clomiphene

A paper by Farha *et al.* suggested that the drug clomiphene could exhibit an antibacterial effect as a consequence of inhibiting the cell wall biosynthesis enzyme, undecaprenyl diphosphate synthase (UppS) (Farha *et al.*, 2015). The researchers found that clomiphene displayed an MIC value of 8 µg.mL^{-1} against both *S. aureus* and *B. subtilis*. They also found that there was a synergistic relationship between clomiphene and bacitracin, which prevents the dephosphorylation of the lipid carrier undecaprenyl phosphate, usually catalysed by UppP (undecaprenyl diphosphate phosphatase). Their cumulative evidence for UppS being the target of clomiphene included depleting the *uppS* gene using antisense technology and observing that this enhanced the activity of clomiphene, along with overexpressing the UppS enzyme and observing that this increased the MIC of clomiphene 2-fold. They also noted that the addition of exogenous Und-P molecules suppressed the activity of clomiphene, but that the addition of exogenous IPP or FPP molecules had no such effect, implying that the target of clomiphene was downstream of FPP biosynthesis and upstream of Und-P biosynthesis. Figure 3.9 gives an overview of Und-P

biosynthesis. Finally, they investigated the morphological phenotype achieved upon exposure of *B. subtilis* cells to clomiphene. They observed that the cells grew wider and more swollen in the presence of clomiphene. This phenotype, they admitted, was actually more characteristic of cells lacking in WTA (wall teichoic acid) polymers, rather than in cells where peptidoglycan biosynthesis had been inhibited. Despite this, they proposed that clomiphene was an interesting lead compound in antibacterial drug discovery.

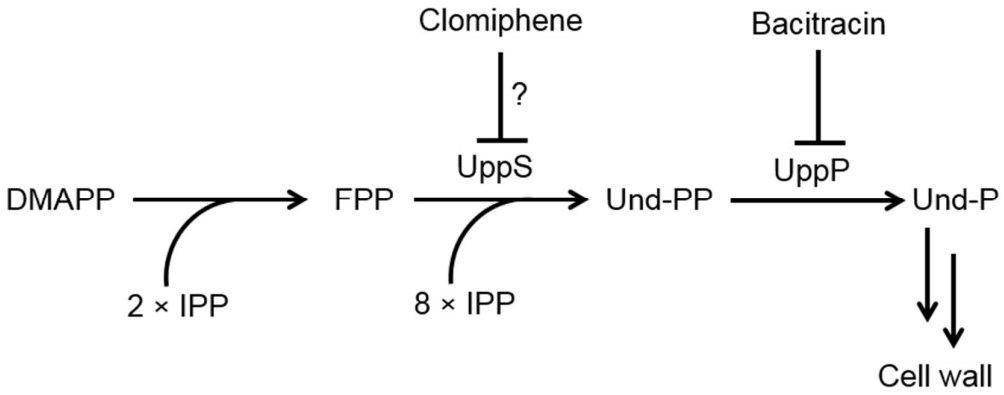


Figure 3.9 Overview of the Und-P biosynthesis pathway. DMAPP = dimethylallyl diphosphate. IPP = isopentenyl diphosphate. FPP = farnesyl diphosphate. UppS = undecaprenyl diphosphate synthase. UppP = undecaprenyl diphosphate phosphatase. Und-P = undecaprenyl phosphate.

A study by Feng *et al.*, (2015) investigated a range of antiinfective compounds for their capacity to act as uncoupling agents by targeting enzymes involved in the proton motive force. They found that clomiphene was an uncoupler, meaning that it could perturb cell membranes and could potentially be cytotoxic.

The P_{murZ}-*lacZ* biosensor was used to investigate the mechanism of action of clomiphene, since this biosensor appeared to be inducible by CBIs but not by membrane damaging compounds. Clomiphene was tested in the full scale MUG assay at 0.25×, 1× and 4×MIC, along with vancomycin as a positive control and tetracycline as a negative control. Figure 3.10 shows that clomiphene did not induce the P_{murZ}-*lacZ* biosensor.

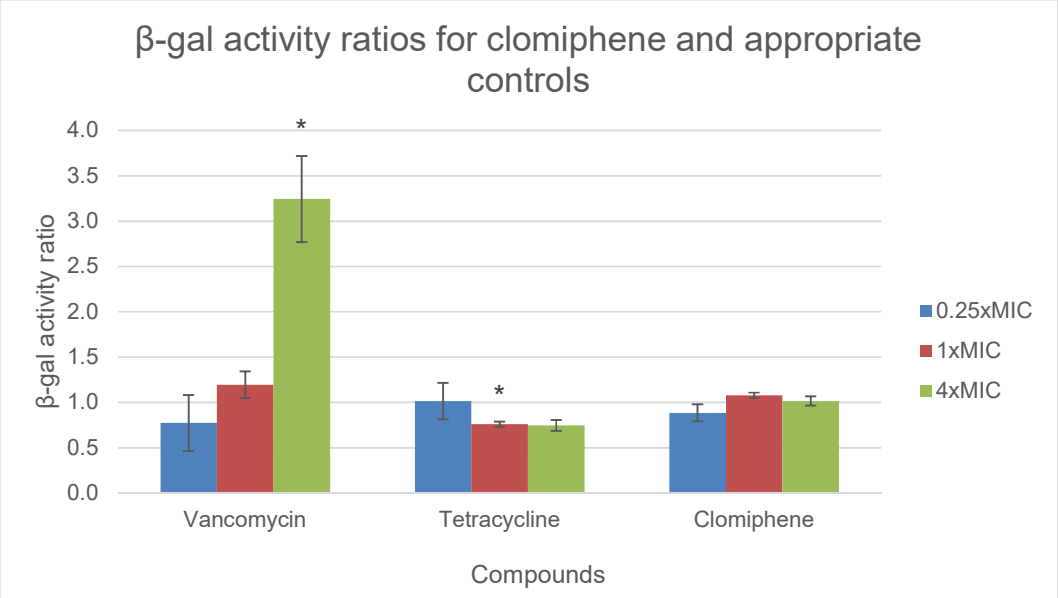


Figure 3.10 β-gal activity ratios for clomiphene, vancomycin and tetracycline using the full scale MUG assay (2 biological replicates, induction threshold = 2-fold). Vancomycin MIC = 0.673 µM; tetracycline MIC = 1.04 µM; clomiphene MIC = 13.4 µM.

3.2.3 Validating the 96-well plate scale MUG assay

The aim was to use these biosensors to screen collections of compounds and natural product extracts. The full scale broth assay was not amenable to the high-throughput screening of compound libraries due to the processing time and the volume of reagent used for each reaction. It was therefore necessary to scale the full scale MUG assay (1 mL cultures) down to a 96-well plate format (200 µL cultures). A small selection of antibacterial reference compounds were used to verify that the biosensor was still induced exclusively by CBIs at this scale (Table 3.2). Only the P*murZ*-*lacZ* biosensor was used from this point in the MUG assay as it had given the most consistent results both in the initial validation and in the agar based assay (see 3.2.8.2). The five positive controls (flucloxacillin, vancomycin, cefotaxime, D-cycloserine and bacitracin) successfully induced the P*murZ*-*lacZ* biosensor and the two negative controls (novobiocin and tetracycline) did not induce the biosensor at any of the concentrations used.

Table 3.2 Results of the MUG assay miniaturisation validation. Red text indicates a fold-increase in β-gal activity ratio of at least 2-fold above that of the drug-free control.

Compound (MIC)	β-gal activity ratio				
	0.25×MIC	0.5×MIC	1×MIC	2×MIC	4×MIC
Inhibitors of cell wall biosynthesis					
Bacitracin (45.0 μM)	1.8	2.0	2.5	2.3	1.7
Cefotaxime (4.39 μM)	4.8	4.0	4.2	4.5	4.6
D-cycloserine (313 μM)	1.4	1.7	1.6	1.7	2.4
Flucloxacillin (0.525 μM)	0.9	1.7	3.4	4.8	4.9
Vancomycin (1.35 μM)	1.0	1.0	2.1	3.5	3.4
Inhibitors of other pathways					
Novobiocin (0.204 μM)	0.9	0.9	0.9	1.2	1.5
Tetracycline (1.04 μM)	0.6	0.6	0.6	0.7	0.7

3.2.4 High throughput screen of the NIH Clinical Collection using the 96-well plate scale MUG assay

Following this validation of the new assay scale, the same method was used to screen a compound library. The library used at this point was the NIH Clinical Collection, which is a library of purified compounds that are either in current clinical use or have at least had a history of being used in human clinical trials (https://commonfund.nih.gov/molecularlibraries/tools). These drugs included established antibiotics with a range of MOAs, providing a broad range of internal control compounds, along with a variety of drugs used for other illnesses such as anticancer agents, antidepressants and anti-inflammatory drugs. External controls of vancomycin and tetracycline were also added to each plate at a concentration at which it was known that vancomycin would induce the biosensor (2×MIC). The compounds within the library were used in the assay at a concentration of 10 μM.

There were 727 compounds in the NIH library. All 44 antibacterial drugs within the collection that are known not to inhibit cell wall biosynthesis did not induce the P_{murZ}-*lacZ* reporter (three such antibiotics are duplicated in the collection). Of the 20 CBIs, 13 induced the biosensor and 6 did not (Table 7.2). The compounds that did not induce the biosensor were D-cycloserine, amoxicillin and all of the CBIs in plate 2302. The green data points in Figure 3.11 that lie below the black dotted line represent these compounds, including two instances of D-cycloserine. The non-CBI datapoints include cefpodoxime proxetil. This existed in the NIH Clinical Collection as the prodrug form of cefpodoxime, so this form would not have been expected to induce the biosensor. There were four compounds that induced (or nearly induced) the biosensor that were not antibiotics: two anti-inflammatory drugs and two serotonin receptor inhibitors.

In plate NIH 1875, the anti-inflammatory drug hydrocortisone gave a β-gal activity ratio of 1.7, which was reasonably close to the induction threshold of 2-fold. However, plate NIH 1974 contained hydrocortisone hemisuccinate, which gave a ratio of 1.1. Additionally, plate NIH 2073 contained cortisone acetate, which gave a ratio of 1.2. These results across the NIH plates suggest that hydrocortisone did not genuinely induce the P_{murZ}-*lacZ* biosensor. The SSRI 5-nonyloxytryptamine gave a result of 2.1 in plate NIH 2418. The SNRI milnacipran gave a value of 1.9 and the anti-inflammatory drug formoterol fumarate dehydrate also gave a result of 1.9.

The discovery of these results coincided with the intention to re-screen the NIH library using the BetaGlo reagent, described below (section 3.2.5). Consequently, it was decided that these anomalous results could be compared to the results from this next high throughput screen and that these non-antibiotic compounds could be investigated further if they induced (or nearly induced) the P_{murZ}-*lacZ* biosensor in both assays. The same rationale was applied to the lack of induction by the CBIs in plate NIH 2302 using the MUG assay.

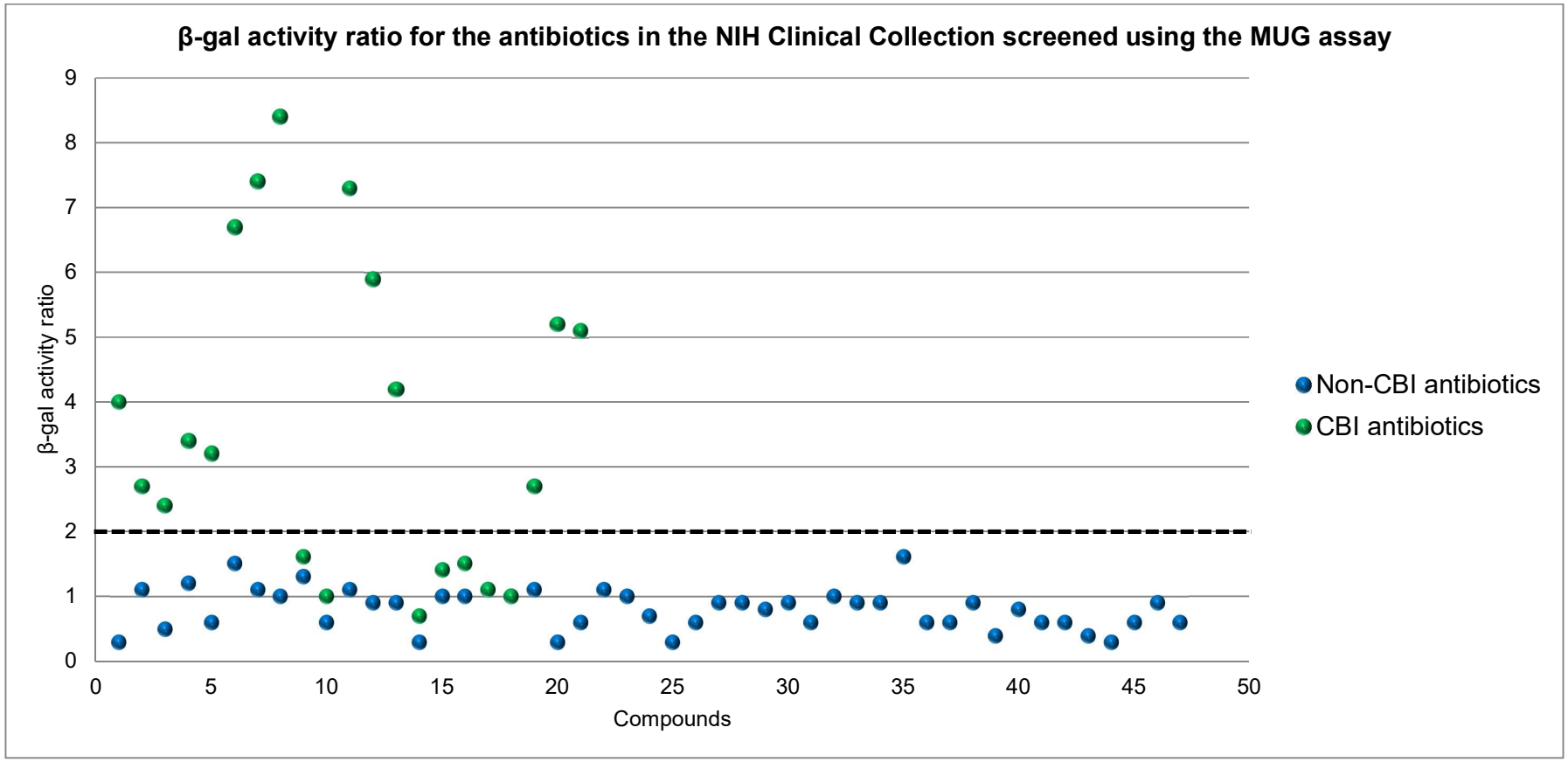


Figure 3.11 β-gal activity ratio for the antibiotics in the NIH Clinical Collection screened using the MUG assay. The black dotted line represents the 2-fold threshold.

3.2.5 High throughput screen of the NIH Clinical Collection, the Microsource Spectrum and the Tocris Totalscreen collections using the β-glo assay

At this point, an alternative reagent to MUG, called the BetaGlo reagent (Promega), was identified. This incorporated both 6-O-β-galactopyranosyl-luciferin and luciferase within the reagent. When this reagent is added to a solution containing β-galactosidase, 6-O-β-galactopyranosyl-luciferin is cleaved by β-galactosidase to achieve D-luciferin, which is then oxidised by luciferase to generate oxyluciferin (Figure 3.12). Luminescence light is emitted during this second step in the process, so measuring luminescence intensity gives an indication of β-galactosidase activity.

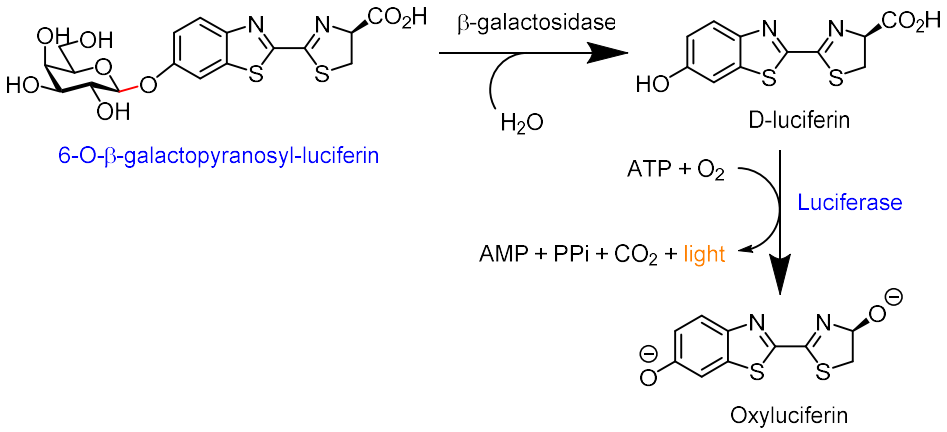


Figure 3.12 The reaction that takes place in the β-glo assay when the β-glo reagent is added. The bond broken by β-galactosidase is shown in red. The components provided in the β-glo reagent are shown in blue.

The protocol provided by the manufacturer involved fewer steps than the MUG assay, which made this assay more amenable to screening the other compound libraries available in the lab. The protocol also stated that the reagent should be added to the culture following exposure to the test compound in a 1:1 ratio. Since this reagent was relatively expensive, the β-gal activity ratio was determined for a selection of test compounds (positive controls flucloxacillin and vancomycin, and negative controls novobiocin and

tetracycline) using both a 1:1 ratio and a 1:9 ratio of reagent to challenged culture (Table 3.3). The induction values were higher when using a 1:9 ratio of reagent to culture, so this was implemented in the assay despite the manufacturer's instructions.

Table 3.3 Results of the β-glo assay for two positive controls (flucloxacillin and vancomycin) and two negative controls (novobiocin and tetracycline), comparing two possible ratios of reagent to challenged culture.

Compound	Concentration × MIC	β-gal activity ratio using 1:1 ratio reagent:culture	β-gal activity ratio using 1:9 ratio reagent:culture
Flucloxacillin	0.25	1.1	1.0
	1 (0.525 μM)	4.2	6.5
	4	5.8	9.1
Vancomycin	0.25	1.0	1.0
	1 (1.35 μM)	5.1	9.4
	4	3.8	6.8
Novobiocin	0.25	0.8	0.9
	1 (0.204 μM)	0.9	0.7
	4	1.3	1.2
Tetracycline	0.25	0.1	0.03
	1 (1.04 μM)	0.1	0.03
	4	0.2	0.04

The P_{murZ}-*lacZ* biosensor was used in the β-glo assay to screen three compound libraries that were available in the lab. The NIH Clinical Collection was screened along with the Microsource Spectrum collection and the Tocriscreen Total collection.

There were many compounds that were neither CBIs nor antibiotics that gave β-gal activity ratios of just over 2-fold, particularly in the Tocris collection. Having considered the β-gal activity ratios achieved by the genuine CBIs, it was decided that an activity threshold of a 3-fold increase of β-gal activity over the drug-free control was more suitable for assays using the β-glo reagent. This new threshold removed a lot of irrelevant compounds that could now be considered as 'noise'.

A total of 3,847 compounds were screened, of which 46 were known CBIs and 128 were antibacterial compounds that targeted other biosynthetic pathways (Figure 3.13). The CBIs induced the biosensor (≥3-fold level of β-gal expression), either in the library plates themselves or when tested from external sources (Table 7.3). The 128 library compounds that were antibiotics that inhibited other bacterial pathways can be categorised as follows: compounds that adversely affect cell membrane integrity (*n* = 17), inhibitors of DNA synthesis (*n* = 57), inhibitors of RNA synthesis (*n* = 6), inhibitors of protein synthesis (*n* = 44) and inhibitors of fatty acid synthesis (*n* = 4). There were no antibacterial drugs in the Tocriscreen Total collection. Figure 3.14 illustrates that the P_{murZ}-*lacZ* biosensor was induced specifically by CBIs and not by membrane damaging compounds or by antibacterial drugs that inhibit other biosynthetic pathways. The β-gal activity ratios for the CBIs are from the samples present in the library plates, the compound tested from an external source at 10 μM, or the compound tested from an external source at a higher concentration (Table 7.3). The one CBI datapoint below the black dotted line represents cefradine, which induced all three of the biosensors in the full scale MUG assay (Table 3.1). This means that this datapoint does not detract from the evidence suggesting that the P_{murZ}-*lacZ* biosensor is specific for CBIs.

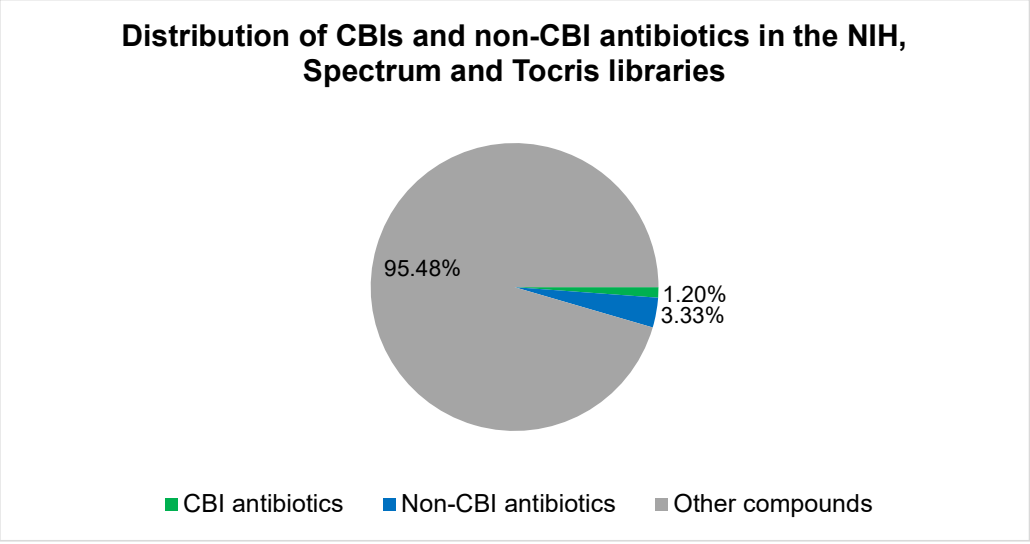


Figure 3.13 The distribution of CBIs and non-CBI antibiotics in the NIH, Spectrum and Tocris compound libraries.

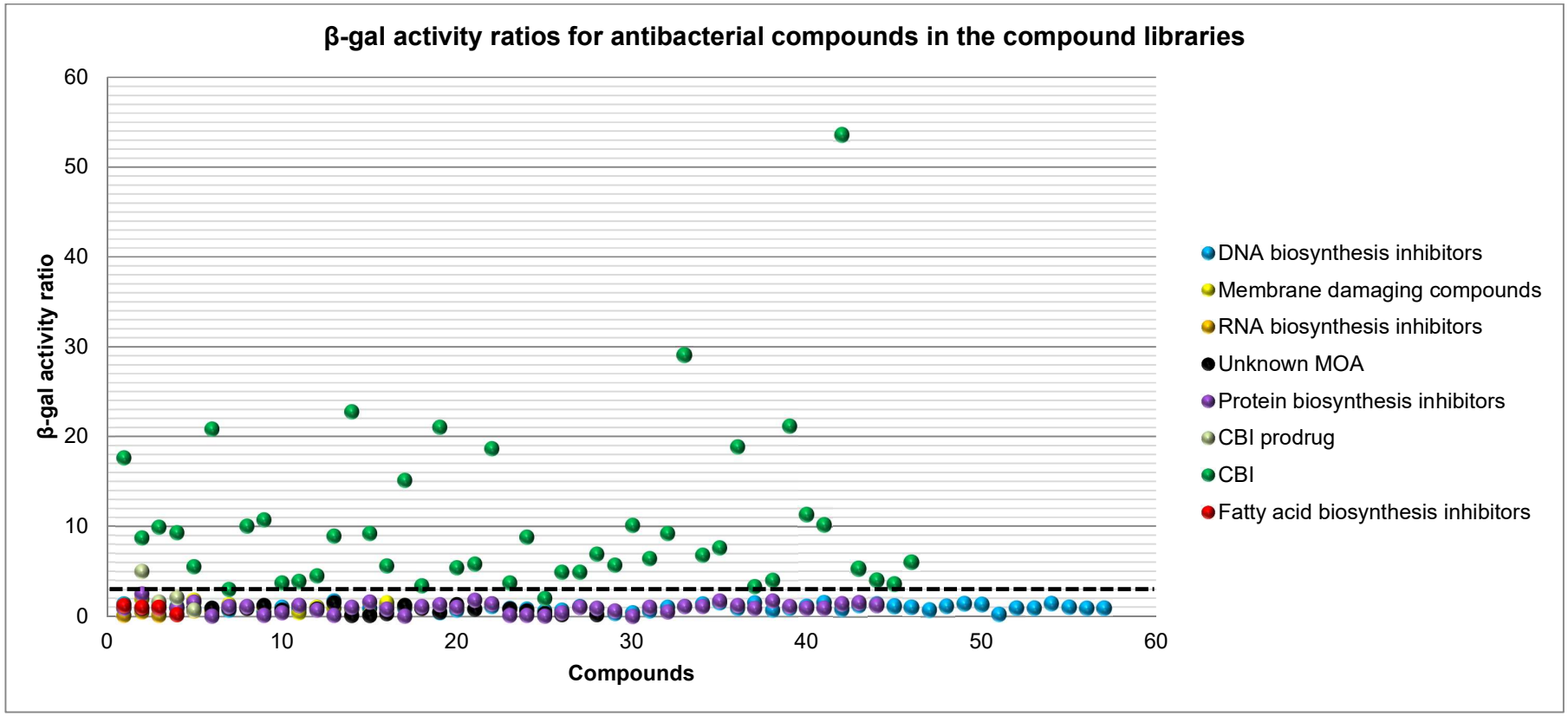


Figure 3.14 β-gal activity ratios for antibacterial compounds in the NIH Clinical Collection, the Microsource Spectrum collection, and the Tocris Totalscreen collection screened using the β-glo assay. The black dotted line represents the 3-fold induction threshold used in the β-glo assay.

There were several compounds in the libraries that induced the biosensor, but should not have done so. These compounds were either re-tested from external sources or re-tested from the plate concerned and were shown to not induce the biosensor again (Table 3.4). It was decided that, when conflicting results were obtained for two biological replicates, the accepted result would be that which verified the expected result for that compound.

Table 3.4 Compounds that were not CBIs or antibiotics and that induced the P_{murZ}-*lacZ* biosensor in the β-glo assay (threshold = 3-fold).

Compound	β-gal activity ratio in collection	MIC from external source	β-gal activity ratio from separate source
5-nonyloxytryptamine (NIH 2302, 2G)	5.3	2 μg.mL^{-1}	1.5 at 10 μM
Cetraxate (NIH 2302, 4H)	4.1	>256 μg.mL^{-1}	0.2 at 4×MIC, 0.5 at 1×MIC (749 μM), 1.0 at 0.25×MIC
Temozolomide (NIH 2302, 6H)	4.0	>256 μg.mL^{-1}	0.2 at 4×MIC, 0.7 at 1×MIC (1320 μM), 0.8 at 0.25×MIC
Idarubicin hydrochloride (NIH 2302, 8H)	6.5	4 μg.mL^{-1}	1.2 at 10 μM. Other similar compounds gave non-induction results throughout the NIH collection: daunorubicin (2172, 3E; 0.6), epirubicin (2333, 6H; 1.6), doxorubicin (2333, 10A; 0.9)
Stavudine (NIH 2302, 10H)	3.9	N/A	Gave a result of 1.0 on plate NIH 2073, well 11F
Podophyllin (Spectrum 519, 10H)	8.5	N/A	Gave a result of 1.2 on plate NIH 2073, well 10E
3,4',5,6,7-pentamethoxyflavone (Spectrum 525, 2E)	3.9	N/A	Gave a result of 1.0 when the plate was retested
Bax channel blocker (Tocris 461, 7E)	3.2	N/A	1.5 at 10 μM
Fenoldopam (Tocris 459, 6H)	3.3	N/A	1.0 at 10 μM

The anti-inflammatory drug and two of the serotonin receptor inhibitors that induced (or partially induced) the P_{murZ}-*lacZ* biosensor in the MUG assay screen of the NIH collection (hydrocortisone, milnacipran and formoterol), when tested in the β-glo assay, gave β-gal activity ratios of 1.3, 1.1 and 1.2,

respectively. These three values were satisfactorily below the 3-fold threshold. Conversely, 5-nonyloxytryptamine gave a result of 5.3 in the β-glo assay. This was tested from an external source and gave a result of 1.5.

3.2.6 Providing further validation of the P_{murZ}-*lacZ* biosensor in the β-glo assay by investigating its ability to detect PAINs

Pan-Assay interference compounds (PAINS) are promiscuous compounds that frequently account for around 10% of the hits from high-throughput screening programmes (Baell, 2016). They are false positives, despite occasionally displaying an early structure-activity relationship. They are a source of compounds for which optimisation efforts would be futile, in addition to previously discovered antibiotics. In many cases, they possess a structural motif that is prone to existing in a form that could interfere in assay readouts, such as metal chelation, redox cycling and protein reactivity. Many of these are natural products and it was therefore important to investigate the propensity of the P_{murZ}-*lacZ* biosensor to be induced by such compounds.

One such natural product is epigallocatechin gallate (EGCG), which is a plant phenolic catechol that is a component of green tea. It has been extensively reported as having many health benefits, but it is also a compound that appears as a hit in unrelated assays (Mereles and Hunstein, 2011). It contains a catechol motif, which is very likely to be redox active and readily chelates metals. This motif is also prone to reacting with nucleophiles in its oxidised form. This makes catechols a prime example of a class of compound that is likely to give a false positive result in a range of assays with no common mechanism.

In order to further demonstrate the specificity and utility of the P_{murZ}-*lacZ* biosensor, it was used to screen a representative selection of PAINS at a concentration much higher (100 μM) than the compound libraries had been at (10 μM). None of the PAIN compounds tested in these conditions induced the biosensor (Table 3.5). It should also be noted that the compounds daunorubicin, idarubicin, epirubicin and doxyrubicin can be classed as quinones and none of them induced the P_{murZ}-*lacZ* biosensor in the library screens (Table 3.4). In addition to this, rifampicin contains a hydroxyphenylhydrazone group (which can lead to PAIN compound behaviour

for reasons similar to those regarding the phenolic Mannich bases) and this did not induce the P*murZ*-*lacZ* biosensor in either the original MUG assay or in the library screen (Table 3.1 and Table 7.3). Curcumin, a membrane damaging compound, counts as a catechol and did not induce the biosensor either (Table 7.3). In addition to being tested here at 100 μM, clofazimine did not induce the biosensor in the NIH collection (NIH 2418, 3A) or in the original MUG assay (Table 3.1).

Table 3.5 β-gal activity ratios for pan-assay interference compounds (PAINs) using the P*murZ*-*lacZ* biosensor in the β-glo assay. Asterisks indicate statistically significant results (2-tailed t-test, *P* = 0.05).

PAIN compound	β-gal activity ratios at 100μM
Catechols	
Benserazide	1.0 ± 0.1
Dopamine	0.9 ± 0.1
Epigallocatechin gallate (EGCG)	1.0 ± 0.3
Quinones	
Menadione	0.02 ± 0.001*
Thymoquinone	0.3 ± 0.02*
Phenolic Mannich bases and hydroxyphenylhydrazones	
Clofazimine	0.1 ± 0.01*
Topotecan	0.9 ± 0.07
Natural products with other reactive groups (peroxide, epoxide, disulfide, enone, etc.)	
Artemisinin	0.8 ± 0.1
Carfilzomib	0.7 ± 0.07*
Mometasone furoate	0.6 ± 0.06*
Natural products with nonspecific global interference properties	
Capsaicin	0.8 ± 0.04*
Genistein	-0.07 ± 0.01*
Toxoflavin	0.4 ± 0.1*

3.2.7 Screening natural product extracts in a 96-well plate format

Up to this point in the study, the P*murZ*-*lacZ* biosensor had only been used in a high-throughput screen against panels of purified compounds. Some of these compounds had been natural products (in the Spectrum collection), but the vast

majority of the compounds across the three libraries screened were clinically used drugs or compounds that had a history of being used in clinical trials. Ultimately, the aim of using these biosensors was to screen mixtures of natural products and to be able to detect the presence of a single antibiotic of a specific pathway amongst the other, non-biologically active compounds.

In order to investigate the potential of the P_{murZ}-*lacZ* biosensor to be used in high-throughput screening of crude natural product mixtures, microbial extracts were obtained in a convenient 96-well plate format. Prior to screening the extracts themselves, the scale of the β-glo assay had to be reduced again. Had the standard 200 µL been used here and had the extracts been dissolved in 10 µL DMSO, the highest concentration of the extract in the assay would have been 50 $\mu g.mL^{-1}$ for the NCI microbial extracts. This would have been sufficient for a purified compound, as many antibacterial compounds would have had an MIC below this concentration. However, in a crude extract there would be many different compounds at much lower concentrations than 50 $\mu g.mL^{-1}$, so novel antibacterial compounds with moderate MIC values may easily have been missed. The test compounds flucloxacillin, vancomycin, novobiocin and tetracycline were tested in the β-glo assay in which the one hour incubation step was carried out in bacterial culture with volumes between 40 µL and 200 µL. A volume of 50 µL during this step seemed sufficient to maintain the specificity of the P_{murZ}-*lacZ* biosensor for CBIs and the general performance of the assay. This volume scale corresponded to an extract concentration of 200 $\mu g.mL^{-1}$, which should be more than high enough to detect weaker antibiotics or antibacterial compounds at sub-inhibitory concentrations.

In total, 96 extracts from cyanobacteria (Cyano-Biotech, Berlin, Germany) and 192 extracts from miscellaneous microbial extracts from the NCI Natural Products Open Repository (National Cancer Institute, Maryland, USA) were tested using the β-glo assay at a scale where the concentration of the natural product extracts was 200 $\mu g.mL^{-1}$ (50 µL scale for the NCI microbial extracts and 100 µL scale for the cyanobacterial extracts).

None of the extracts induced the P_{murZ}-*lacZ* biosensor in the Cyano-Biotech plate. However, three of the extracts caused an inhibition of growth across

three repeats of the same plate and one of these gave a β-gal activity ratio of 0.1.

Across the two plates screened from the NCI Open Repository, there were a total of 19 extracts that induced the P_{murZ}-*lacZ* biosensor in the β-glo assay (Figure 3.15). Also of interest was that 165 of the 192 extracts screened inhibited the growth of the biosensor by at least 25%.

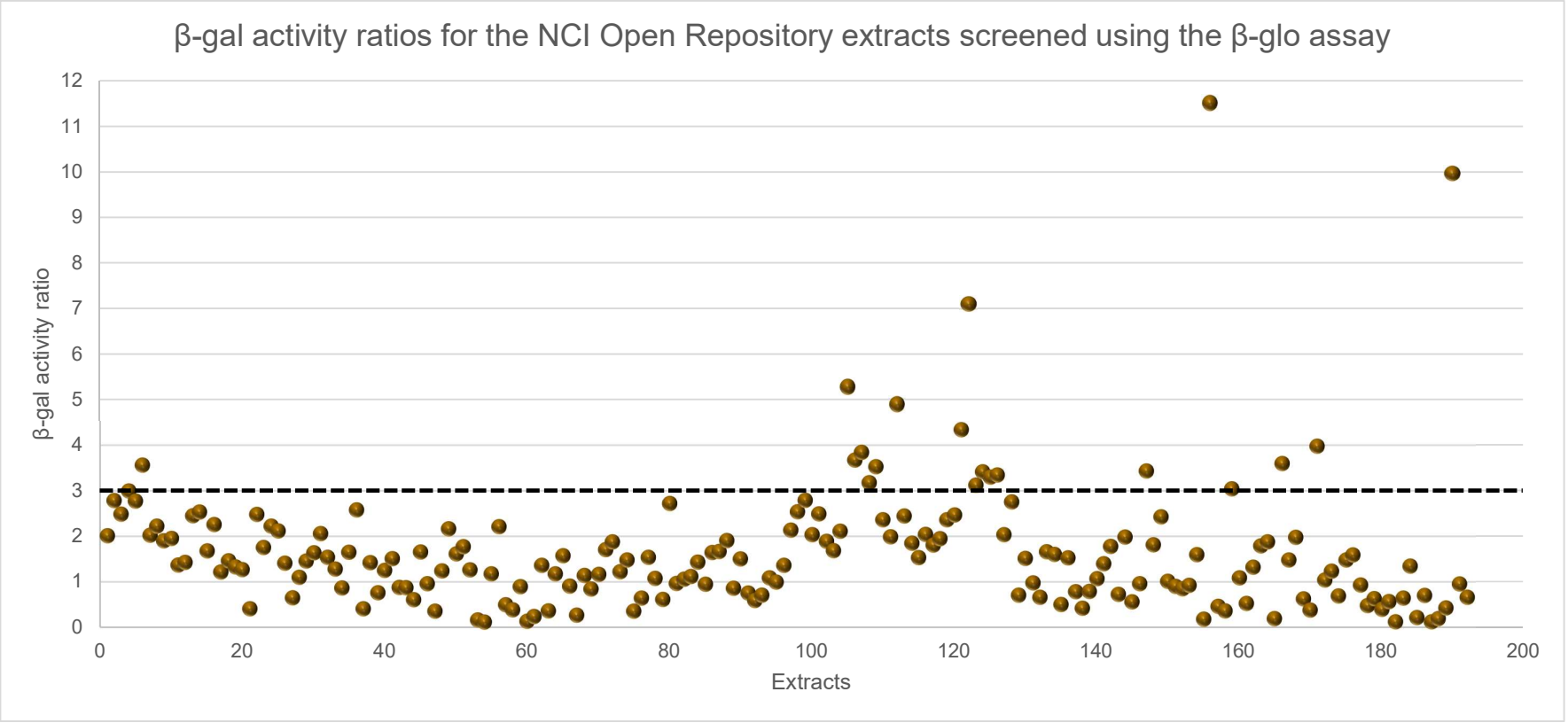


Figure 3.15 β-gal activity ratios for the NCI Open Repository extracts screened using the β-glo assay. There were 19 extracts that induced the P_{murZ}-*lacZ* biosensor above the 3-fold threshold.

3.2.8 Developing the agar-based assay

3.2.8.1 Rationale for the agar based assays

The original paper in which Alexander Fleming demonstrated the antibacterial properties of penicillium cultures reported that a growing mould of *Penicillium chrysogenum* (which, at the time, was categorised as *Penicillium notatum*) was surrounded by a zone of inhibition in a plate of staphylococcal colonies (Fleming, 1929). The compound responsible for creating this zone of inhibition was penicillin, a cell wall biosynthesis inhibitor. The aim of using an agar-based assay was to show that a CBI biosensor could be induced by natural products produced directly from their source. This would allow the biosensors to be used for the purpose of prioritising which natural product producers to take forward for resource-heavy compound purification. Any organism that induces the biosensor can have their natural products extracted and screened using a broth based assay to determine exactly which compound is the antibacterial agent of interest. In reference to the agar-based assay, 'induction' has a more qualitative threshold – here it simply means that sufficient β-galactosidase was produced to provide enough of the blue product of the cleavage of X-gal to be visible.

3.2.8.2 Validation using antibacterial test compounds

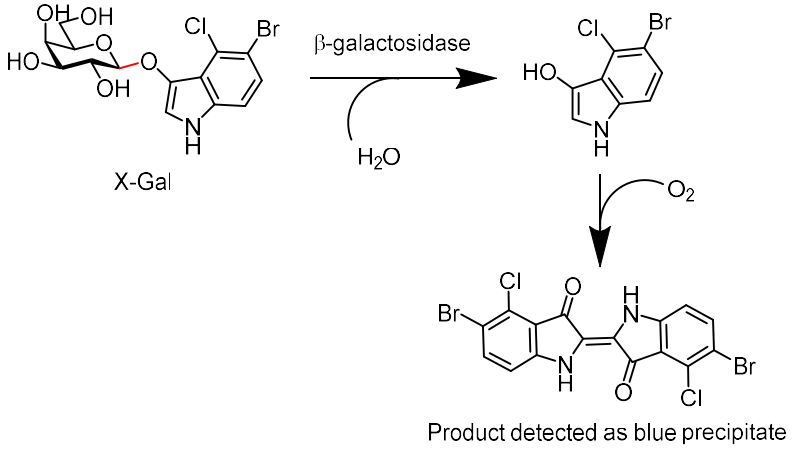


Figure 3.16 The reaction of β-galactosidase with X-gal to produce a product that forms a blue precipitate. The bond broken by β-galactosidase is shown in red.

In contrast to the broth based assay, the agar based assay required a visible signal, as opposed to a fluorescent or a luminescent signal necessitating the use of a plate reader. One of the more commonly used substrates used for detecting β-galactosidase is X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). This compound is cleaved by β-galactosidase to produce an indole product that dimerises to give a bright blue compound (Figure 3.16). In the presence of a cell-wall synthesis inhibitor a blue ring should be seen around the edge of the zone of inhibition owing to the reporter overexpressing β-galactosidase. In contrast, the zone of inhibition should be colourless when it is the result of antibiotics with a different mode of action.

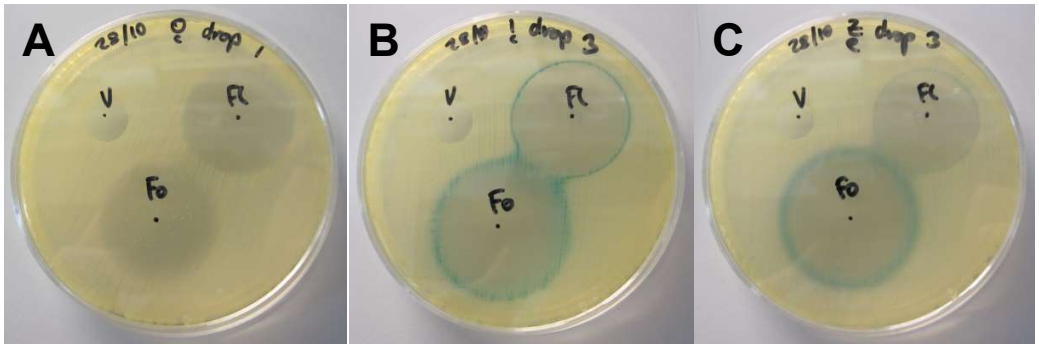


Figure 3.17 An agar based assay at the validation stage. A) P*gltA*-*lacZ* biosensor, showing almost no blue colour; B) P*oppB*-*lacZ* biosensor, showing a clear blue colour around fosfomycin and flucloxacillin; C) P*murZ*-*lacZ* biosensor, with a faint blue colour around flucloxacillin and a clear blue colour around fosfomycin.

Initially, two negative controls (tetracycline, cetyltrimethylammonium bromide) and three positive controls (flucloxacillin, fosfomycin, vancomycin) were used to test the P*gltA*-*lacZ*, P*oppB*-*lacZ* and P*murZ*-*lacZ* biosensors. Briefly, a plate of TSA containing the appropriate antibiotic selection (10 μg.mL^{-1} chloramphenicol for the P*gltA*-*lacZ* and P*oppB*-*lacZ* biosensors and 10 μg.mL^{-1} erythromycin for the P*murZ*-*lacZ* biosensor) and X-gal (80 μg.mL^{-1}) was inoculated on the surface with an overnight culture of the biosensor to be tested. Suitable zones of inhibition for the nature of the compounds tested in proportion to the size of the plate were obtained when a 2 μL drop was added of the antibiotic compound at a concentration of 1 mg.mL^{-1}. There was no blue colour visible around the negative controls or vancomycin, but a blue ring was seen around flucloxacillin

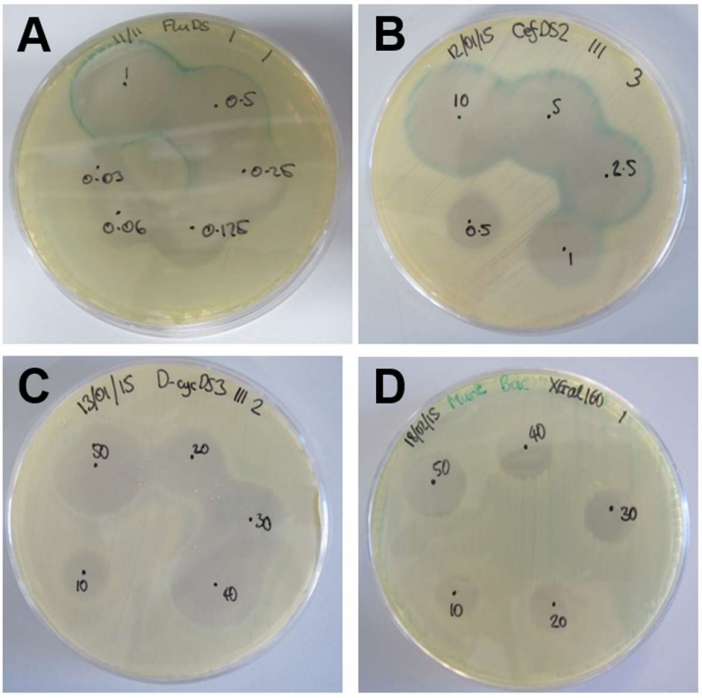


Figure 3.18 Limits of detection in the agar-based assay using the P_{oppB}-*lacZ* biosensor (A, B and C) and the P_{murZ}-*lacZ* biosensor (D). A) The limit of detection of flucloxacillin = 0.5 mg.mL^{-1}; B) The limit of detection of cefotaxime = 1 mg.mL^{-1}; C) The limit of detection of D-cycloserine = 40 mg.mL^{-1}; D) Bacitracin could not be detected at 50 mg.mL^{-1}, even when the X-gal concentration was increased to 160 µg.mL^{-1}.

and fosfomycin (Figure 3.17). Bacitracin and D-cycloserine were tested using the same conditions, but no blue colour was observed. The limit of detection was determined for flucloxacillin, cefotaxime and D-cycloserine in the agar-based assay using the P_{oppB}-*lacZ* biosensor and for bacitracin using the P_{murZ}-*lacZ* biosensor.

No blue colour was seen for bacitracin and D-cycloserine only seemed to induce faintly when the 2 µL drop was at a concentration of 40 mg.mL^{-1} (Figure 3.18). This was not surprising, given how high the MICs of those compounds were (32 µg.mL^{-1} and 64 µg.mL^{-1}, respectively). The limits of detection for flucloxacillin and cefotaxime were 0.5 mg.mL^{-1} and 1 mg.mL^{-1}, respectively (Figure 3.18).

One of the alternative substrates to X-gal is S-gal (3,4-Cyclohexeneoesculetin-β-D-galactopyranoside). When S-gal is cleaved by β-galactosidase in the presence of ferric ammomium citrate, a black by-product is formed. This substrate was tested with positive controls (vancomycin, bacitracin, D-cycloserine, methicillin, fosfomycin and flucloxacillin). This was done in the hope that the black rings would display a sharper contrast with the agar than the X-gal reaction product (*i.e.* the signal to noise ratio would be better), potentially reducing the limit of detection of CBIs (Figure 3.19). Although this prediction did turn out to be correct, S-gal was not used as a substrate in the agar-based assay again because the increase in both processing time and cost offered insufficient advantage over using X-gal.

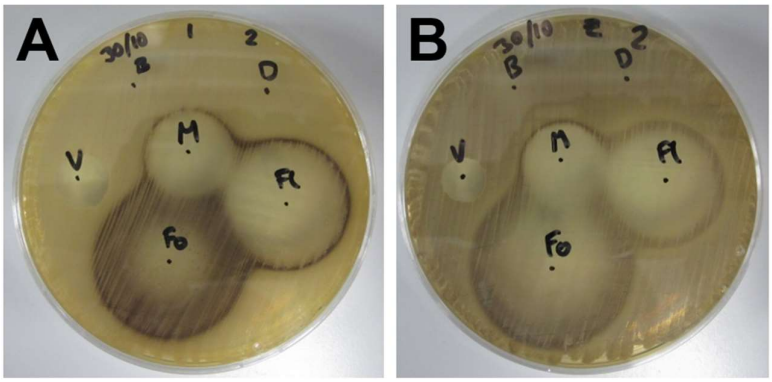


Figure 3.19 Positive controls with the S-Gal substrate in TSA (300 µg.mL^{-1}, plus 500 µg.mL^{-1} ferric ammonium citrate) with A) the P$_{oppB}$-*lacZ* reporter and B) the P$_{murZ}$-*lacZ* reporter.

3.2.8.3 Dual biosensors

Thus far in this study, the biosensors used had included only one instance of a promoter-reporter gene system. The P$_{murZ}$-*lacZ* biosensor just had the promoter for *murZ* controlling the expression of *lacZ* on the chromosome. The P$_{gltA}$-*lacZ* and P$_{oppB}$-*lacZ* biosensors just had the *gltA* or the *oppB* promoter, respectively, driving the expression of *lacZ* on a plasmid. The output signal from whole-cell biosensors, given a particular concentration of antibiotic, could be higher if there are multiple promoter-reporter systems within the same strain compared to strains with just one such system. Such strains would contain the promoters of two different genes that are upregulated in the presence of a CBI and both

would increase the expression of *lacZ*, resulting in even more β-galactosidase being produced and more of whichever substrate was used being cleaved to give a product emitting a signal.

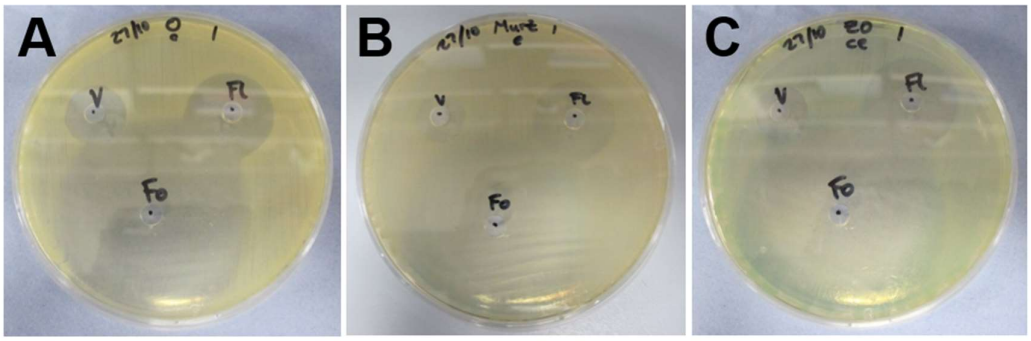


Figure 3.20 A comparison of the agar based assay with A) the P_{gltA}-*lacZ* single biosensor, B) the P_{murZ}-*lacZ* single biosensor and C) the Z0 dual biosensor. V = vancomycin, Fl = flucloxacillin, Fo = fosfomycin.

To this end, two dual biosensors were constructed by extracting the plasmid DNA from the P_{gltA}-*lacZ* and P_{oppB}-*lacZ* biosensors and then transforming the DNA into separate aliquots of the P_{murZ}-*lacZ* biosensor. This produced dual biosensors Z0 (containing the P_{murZ}-*lacZ* and P_{gltA}-*lacZ* systems) and Z1 (containing the P_{murZ}-*lacZ* and P_{oppB}-*lacZ* systems), both of which contained two *lacZ* genes capable of producing a measurable output. The dual biosensors were validated alongside the validation of the P_{murZ}-*lacZ*, P_{gltA}-*lacZ* and P_{oppB}-*lacZ* biosensors (referred to collectively in this section as the 'single biosensors'), using both the agar-based assay and the MUG assay.

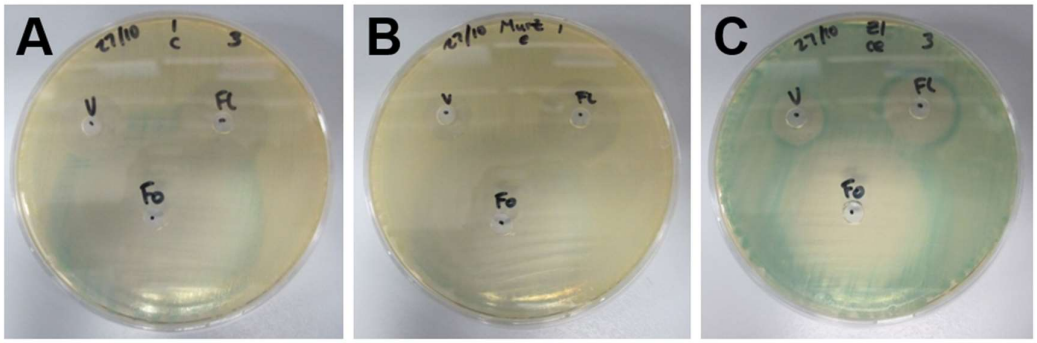


Figure 3.21 A comparison of the agar based assay with A) the P_{oppB}-*lacZ* single biosensor, B) the P_{murZ}-*lacZ* single biosensor and C) the Z1 dual biosensor. V = vancomycin, Fl = flucloxacillin, Fo = fosfomycin.

When tested in the agar-based assay the Z0 dual biosensor gave a slightly better response than either the P_{gltA}-*lacZ* or P_{murZ}-*lacZ* biosensor (Figure 3.20), but was not as sensitive as the P_{oppB}-*lacZ* biosensor. Therefore, the remainder of this section discusses the merits of the Z1 dual biosensor.

The initial comparison in Figure 3.21 illustrates that the Z1 dual biosensor showed a greater qualitative improvement in sensitivity compared with the Z0 dual biosensor. Limits of detection were determined for the single biosensors and the dual biosensors, in order to ascertain how effective this strategy was. The theory was that the dual biosensors should be induced by CBIs at a lower concentration that either of their corresponding single biosensors (*i.e.* the sensitivity of the dual biosensor should be higher).

The plates for the flucloxacillin dilution series are shown in Figure 3.22. Flucloxacillin could be detected by the Z1 dual biosensor from a stock solution of just 0.03125 mg.mL^{-1}. The minimum stock solution concentration to be detected by the corresponding single biosensors was 0.125 mg.mL^{-1} by the

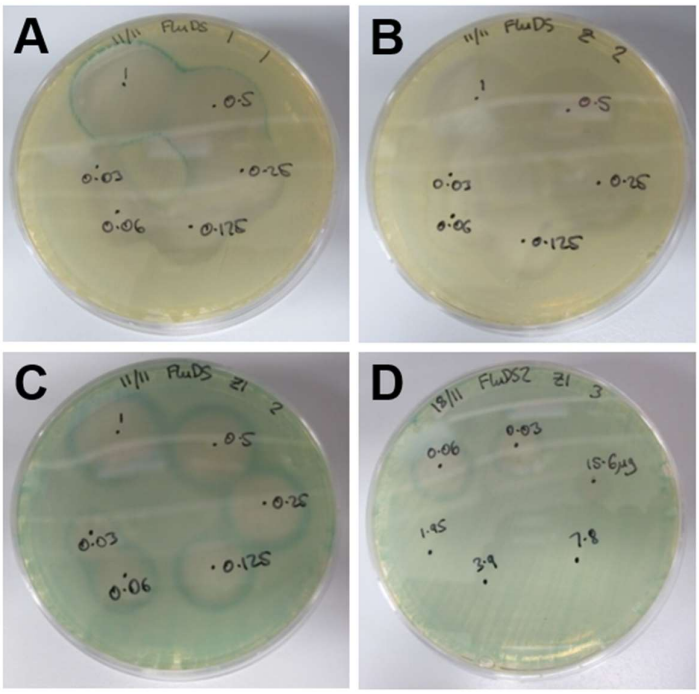


Figure 3.22 The flucloxacillin dilution series plates to determine the limit of detection for A) the P_{oppB}-*lacZ* biosensor, B) the P_{murZ}-*lacZ* biosensor and C/D) the Z1 dual biosensor. The numbers correspond to the concentration of the stock antibiotic solution used in mg.mL^{-1}.

P_{murZ}-*lacZ* single biosensor and 0.5 mg.mL^{-1} by the P_{oppB}-*lacZ* single biosensor. Although this suggests that the Z1 dual reporter is the best, when combined with the results from the other two antibiotics for which the limits of detection were determined for these three biosensors (cefotaxime and D-cycloserine – see Table 3.6), it seemed that the P_{murZ}-*lacZ* biosensor was the most sensitive overall.

The full scale MUG assay was used to determine the limits of detection of flucloxacillin, vancomycin, cefotaxime, D-cycloserine and bacitracin in the MUG assay. When combined with the results for the agar-based assay, these did not suggest that the Z1 dual biosensor was significantly more sensitive than the single biosensors (Table 3.6).

Table 3.6 A comparison of the limits of detection for agar-based assay and the full scale MUG assay for the P_{oppB}-*lacZ*, P_{murZ}-*lacZ* and Z1 biosensors. The coloured text for the Z1 biosensor indicates whether the limit was worse (red), better (green) or the same (blue) as at least one of the other reporters.

Antibiotic	MIC(μg.mL^{-1})	Limit in agar-based assay (mg.mL^{-1})	Limit in MUG assay (×MIC)
P_{oppB}-*lacZ* biosensor			
Flucloxacillin	0.25 (0.525 μM)	0.5 (2000 × MIC)	4
Vancomycin	2 (1.35 μM)	-	0.75
Cefotaxime	2 (4.39 μM)	1 (500 × MIC)	0.15
D-cycloserine	32 (313 μM)	40 (1200 × MIC)	0.5
Bacitracin	64 (45.0 μM)	-	0.75
P_{murZ}-*lacZ* biosensor			
Flucloxacillin	0.25	0.1 (400 × MIC)	1.25
Vancomycin	2	20 (100 × MIC)	1
Cefotaxime	2	0.1 (50 × MIC)	0.2
D-cycloserine	32	10 (300 × MIC)	2
Bacitracin	64	Did not induce	0.25
Z1 dual biosensor			
Flucloxacillin	0.25	0.03 (120 × MIC)	1
Vancomycin	2	-	0.75
Cefotaxime	2	0.1 (50 × MIC)	0.1
D-cycloserine	32	30 (1000 × MIC)	1
Bacitracin	64	-	1

3.2.8.4 Validation of agar assay to detect antibacterial compounds directly from their producing organisms

Initially, the penicillin-producing species *Penicillium chrysogenum* was used to develop an appropriate method for validating this use of the P_{murZ}-*lacZ* biosensor for this assay. The initial strategy was to add a loopful of *Penicillium chrysogenum* to the centre of a plate of the appropriate agar (Table 2.4) on which a bacterial lawn of the P_{murZ}-*lacZ* biosensor had been grown overnight. These plates were then incubated at three different temperatures (27 °C, 30 °C and 37 °C) because the difference between the optimum growth temperatures for the biosensor and the producer organisms that would potentially be used following the initial validation varied as much as 13 °C (37 °C for the P_{murZ}-*lacZ* biosensor and 24 °C for the cephalosporin C-producing strain, *A. chrysogenum*). It was therefore necessary to select a temperature that was a compromise between obtaining sufficient growth of the biosensor and sufficient production of the antibacterial natural product from the producer organism. It was found that incubating the plates at 30 °C offered the best visibility of the blue ring without sacrificing its definition with a high level of background blue colour. However, there was still untenable variability in the results due to the different growth rates of the natural product producing organism and the reporter. An alternative strategy was tested where *Penicillium chrysogenum* was grown separately on LBA before being transferred to the lawn of the P_{murZ}-*lacZ* biosensor on TSA (Figure 3.23). This consistently produced results such as those shown in Figure 3.24.

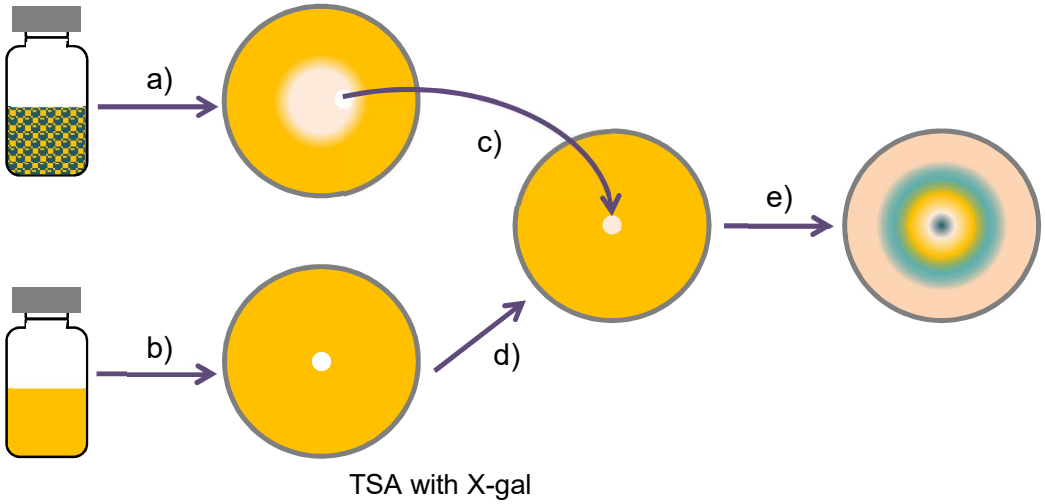


Figure 3.23 A schematic of the strategy for the natural product assays. a) Add 50 µL of a suspension of *Penicillium* spores in broth to LBA and incubate at 37 °C for 2 days; b) Plate out overnight of P_{murZ}-*lacZ* biosensor to make bacterial lawn; c) Use an agar hole punch to remove a plug of agar from the P_{murZ}-*lacZ* biosensor plate; d) Remove a plug of mould on LBA and place it in the hole on the TSA plate. Seal it with 20 µL of TSA; e) Incubate at 30 °C for ~2 days.

Figure 3.24 A *Penicillium* plate produced using the method illustrated in Figure 3.23, demonstrating the blue ring indicating the presence of penicillin at the edge of the ZOI.

Table 3.7 The bacterial or fungal strains used in the agar-based assay and the antibacterial compounds they produce.

Strain	Compound produced	Mode of action
Inhibitors of cell wall biosynthesis		
P. chrysogenum	Penicillin	Inhibits cell-wall biosynthesis (inhibits penicillin binding proteins, prevents peptide bridge formation between glycan molecules)
A. orientalis	Vancomycin	Inhibits cell-wall synthesis (prevents polymerisation of the phosphodisaccharide-pentapeptide lipid complex in stage II of cell wall synthesis)
A. chyrysogenum	Cephalosporin C	Inhibits cell-wall synthesis (see penicillin)
B. licheniformis	Bacitracin	Inhibits cell-wall synthesis (prevents the recycling of the undecaprenyl lipid carrier following the delivery of the peptidoglycan precursor across the membrane)
Inhibitors of other pathways		
S. kanamyceticus	Kanamycin	Inhibits protein synthesis (binds to 16S rRNA subunit)
A. rifamycinica	Rifamycin	Inhibits RNA synthesis (binds to bacterial RNA polymerase)
S. niveus	Novobiocin	Inhibits DNA synthesis (inhibits DNA gyrase, prevents DNA supercoiling)
S. fradiae	Neomycin	Inhibits protein synthesis (see kanamycin)
S. coelicolor	Actinorhodin	Unknown

Following the success of the strategy outlined above, several known producers of positive and negative control antibiotics were tested for their ability to induce β-galactosidase production in the P_{murZ}-*lacZ* biosensor (Table 3.7).

The strains *S. kanamyceticus*, *S. niveus*, *A. rifamycinica*, *S. fradiae* and *S. coelicolor* were tested along with *P. chrysogenum* (Figure 3.25). After two days of incubation at 30 °C, the *P. chrysogenum* gave a faint blue ring around the edge of the zone of inhibition. *S. kanamyceticus*, *S. fradiae* and *S. niveus* gave no blue ring, as expected. No zone of inhibition was observed for *A. rifamycinica*.

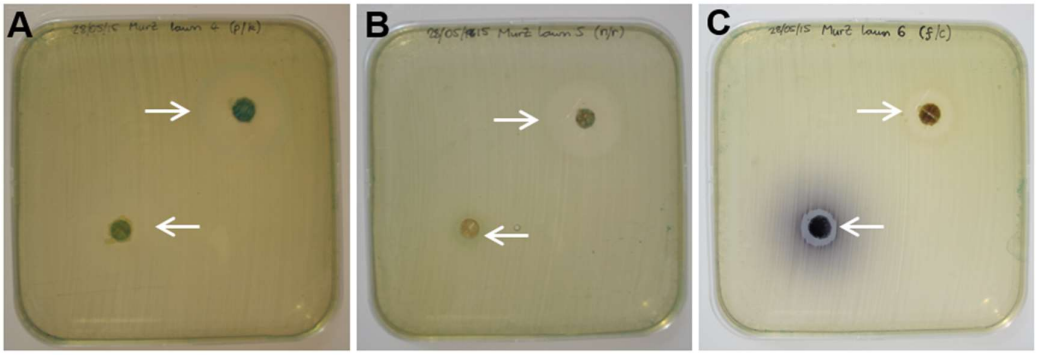


Figure 3.25 Plugs of natural product producers inserted into P_{murZ}-*lacZ* biosensor lawns. The plates were incubated at 30 °C for 2 days. A) *P. chrysogenum* top right, *S. kanamyceticus* bottom left; B) *S. niveus* top right, *A. rifamicinica* bottom left; C) *S. fradiae* top right, *S. coelicolor* bottom left. The arrows indicate the edge of the ZOI.

Three further strains were tested in the same way (Figure 3.26). *A. orientalis* gave a zone of inhibition and a blue ring, as expected. *B. licheniformis* gave a zone of inhibition, with a faint blue ring around the edge and a bright blue ring just 1 mm out from the plug. In contrast to vancomycin, the relatively large size of bacitracin still impeded its diffusion across the agar, although it did show a bold blue ring, suggesting that *B. licheniformis* was producing bacitracin at much higher concentrations than in Figure 3.18. No blue ring was observed with *A. chrysogenum*, the cephalosporin C producer.

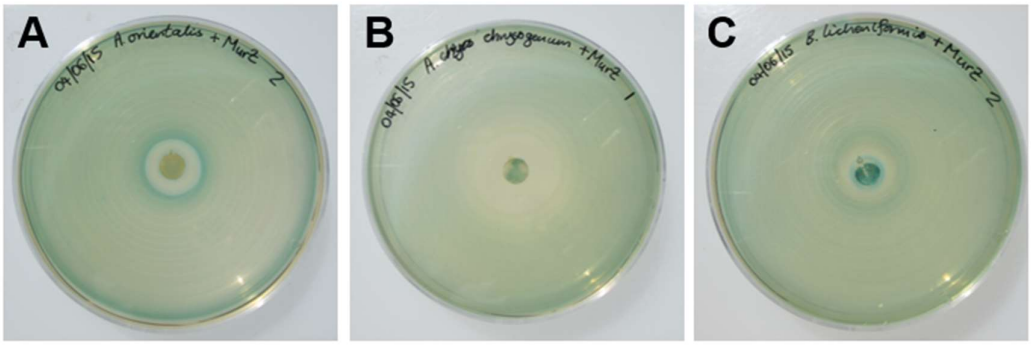


Figure 3.26 Further plugs of natural product producers inserted into P*murZ*-*lacZ* biosensor lawns. A) *A. orientalis*, a vancomycin producer; B) *A. chrysogenum*, a cephalosporin C producer; C) *B. licheniformis*, a bacitracin producer.

It was noted that the characteristic blue colour of the X-gal cleavage product appeared within the mould on plates that did not display a blue ring (such as the *S. kanamyceticus* in Figure 3.25A and the *S. niveus* in Figure 3.25B). Other replicates of the plate in Figure 3.24 were prepared, but with the bacterial lawn being RN4220. Under the same incubation conditions, a blue colour was seen under the mould plug, but not around the edge of the zone of inhibition, suggesting that the natural product producers generate their own β-galactosidase locally. The cell density on the plug was far greater than the inoculated reporter on the agar surface, so it is not surprising that the background activity seemed to be very high on the plug.

The lack of false positive results using this strategy implies that this could be a useful assay to use with more sensitive biosensors.

3.3 Discussion

The additional CBIs tested with the P*gltA*-*lacZ*, P*oppB*-*lacZ* and P*murZ*-*lacZ* biosensors in the full scale MUG assay (β-chloro-D-alanine, friulimicin, aztreonam and cloxacillin) all induced all three biosensors when used at at least one of the concentrations used (0.25×, 1× or 4×MIC). This was not the case for the membrane damaging compounds EDTA and polymyxin B and the protein biosynthesis inhibitor, actinonin. When considered along with the previously obtained validation data (Table 3.1), these results completed the extensive validation of the specificity of the three CBI biosensors generated in the O'Neill lab.

It was surprising that rhodomyrtone did not induce the P_{murZ}-*lacZ* biosensor, given that it was reported to upregulate the expression of genes involved in DAP biosynthesis and this pathway is related to cell wall biosynthesis (Sianglum *et al.*, 2012). However, it has also been reported that other Gram-positive organisms can synthesise DAP *via* either a succinylase pathway or a dehydrogenase pathway (Wehrmann *et al.*, 1998), and the gene involved in the dehydrogenase pathway (*ddh*) was not affected by the exposure of MRSA to rhodomyrtone (Sianglum *et al.*, 2012). Furthermore, the amino acid at the position in the pentapeptide side chain occupied by DAP can also be occupied by lysine and the residue at this position is not involved in the reaction catalysed by the transpeptidase that cross-links the MurNAc and GlcNAc chains together (Chopra *et al.*, 2002; Walsh, 2003)(Figure 3.4). DAP is a precursor to lysine which, as an amino acid residue, can proceed to be utilised in a wide variety of pathways. It is therefore potentially not specific to the peptidoglycan biosynthesis pathway, and so the fact that rhodomyrtone did not induce the highly CBI-specific P_{murZ}-*lacZ* biosensor provides more evidence of the specificity that this biosensor has for detecting CBIs.

Although clomiphene is approved by the FDA as a fertility drug (Farha *et al.*, 2015), despite also being a membrane damaging compound (Feng *et al.*, 2015), it may not be suitable for use as an antibiotic. The dose required to eradicate a bacterial infection may be much higher than that required to increase fertility because the latter merely requires interaction with receptors on eukaryotic cells, whereas treating a bacterial infection would involve clomiphene being able to penetrate into a bacterial cell (Lewis, 2013). The fact that clomiphene did not induce the P_{murZ}-*lacZ* biosensor provides further evidence to suggest that the P_{murZ}-*lacZ* biosensor is highly specific for CBIs and does not respond to potentially cytotoxic membrane damaging compounds.

The results from the validation of the 96-well plate scale MUG assay did not completely match those from the full scale MUG assay. For example, bacitracin induced the P_{murZ}-*lacZ* biosensor at 1×MIC at the 96-well plate scale but at 0.25×MIC in the full scale assay. D-cycloserine induced the P_{murZ}-*lacZ* biosensor at 4×MIC at the 96-well plate scale but at 1×MIC in the full scale assay. However, since the MIC of the compounds would be unknown in any subsequent high throughput screening, the criterion for a compound

successfully inducing the biosensor was that it gave ≥2-fold β-gal activity ratio at any concentration, not necessarily the minimum inducing concentration identified in the full scale MUG assay.

Of the six CBIs that did not induce in the MUG assay screen of the NIH collection, cefatrizine induced the biosensor sufficiently in the β-glo assay (β-gal activity ratio = 9.2) and the other five compounds (amoxicillin, D-cycloserine, cephalexin, meropenam and mecillinam) induced the biosensor at various concentrations, as detailed in Table 7.2. Five of the drugs across all three libraries (bacampicillin, hetacillin, metampicillin, cefditorin pivoxil and cefpodoxime proxetil) were present in the collection as prodrugs that should only become active compounds in physiological conditions. For example, cefpodoxime proxetil is hydrolysed in the intestinal lumen to its active form, cefpodoxime. Administering the drug as the prodrug improves its oral bioavailability (Crauste-Manciet *et al.*, 1997). Given that the β-glo assay does not replicate these conditions, any prodrugs of CBIs would not have been expected to induce the P_{murZ}-*lacZ* biosensor.

The compound 5-nonyloxytryptamine gave a false positive result when the NIH Clinical Collection was screened with both the MUG assay and the β-glo assay. It also had an MIC of 2 μg.mL^{-1} with *S. aureus* RN4220. It has recently been reported in the literature that 5-alkyloxytryptamines are broad-spectrum antibiotics that target the cell membrane (Faulkner *et al.*, 2016). In that study, the MIC of a range of 5-alkyloxytryptamines with alkyl chain lengths from 4 to 15 were determined, along with their corresponding percentage of membrane permeability. It was found that the maximum membrane permeability and minimum MIC was attained with an alkyl chain length of 9, as it is in 5-nonyloxytryptamine. However, the most potent 5-alkyloxytryptamines tested also showed activity against the membranes of human embryonic kidney cells and it was admitted that the compounds would have to be used below their MIC to achieve acceptably low levels of mammalian cell toxicity. This also indicates that this is yet another cell membrane damaging compound that does not induce the P_{murZ}-*lacZ* biosensor, bringing the total number of non-inducing negative controls across the compound libraries to 129.

The results from the screening of these three libraries of purified compounds demonstrates the high specificity that the P_{murZ}-*lacZ* biosensor has for CBIs over antibiotics that inhibit other pathways, including potentially toxic membrane damaging compounds. There were several CBIs that did not induce the P_{murZ}-*lacZ* biosensor when only the library plates were screened. The library plates had been stored at –20 °C for around four years, and so it is highly likely that some of the compounds had degraded in that time. Indeed, this may account for the fact that cefditorin pivoxil, supposedly present in the collection in its prodrug form, actually induced the biosensor with a β-gal activity ratio of 5.0 – it is likely that this compound had degraded to cefditorin itself. In total, 19 PAIN compounds have been shown to not induce the P_{murZ}-*lacZ* biosensor, suggesting that using this biosensor to screen for novel natural product antibacterial compounds is unlikely to produce false positive hits of this variety.

Using the P_{murZ}-*lacZ* biosensor to screen nearly 4,000 purified compounds and observing that it was only induced in the presence of CBIs that were not membrane damaging compounds provides substantial evidence to suggest that this is a highly specific biosensor that could be a very useful tool in screening for novel antibiotics that inhibit steps in the cell wall biosynthesis pathway.

Based on the results from screening the purified compound libraries, a β-gal activity ratio as low as 0.1 for a compound that also exhibited a noticeable antibacterial effect based on the optical density readings seemed to be characteristic of protein biosynthesis inhibitors. It may be that the extract that gave this low ratio when the Cyano-Biotech plate was screened using the β-glo assay contains an antibacterial compound that inhibits protein biosynthesis. Cyanobacteria produce a plethora of secondary metabolites that have displayed a range of bioactivity, including having antibacterial, anticancer and antiviral properties (Singh *et al.*, 2011).

Screening the NCI plates using the β-glo assay and the P_{murZ}-*lacZ* biosensor gave a hit rate of 9.9% (19 out of 192 extracts). Given that most of the antibiotics that are natural products or derivatives thereof inhibit the biosynthesis of either proteins or the bacterial cell wall (Silver, 2011), it is not surprising that this hit rate is relatively high. Neither is it surprising that 85.9% of

the extracts screened displayed antibacterial activity, insofar as they inhibited the growth of the biosensor by at least 25% after the one hour incubation period. These extracts were all microbial extracts, and bacteria produce antibacterial compounds as a survival strategy in competition with other species of bacteria (van der Meij *et al.*, 2017). Given the high specificity of the P_{murZ}-*lacZ* biosensor, as implied by the screening of the compound libraries in section 3.2.5, it is likely that these 19 extracts all contain a CBI. However, many, if not all, of these CBIs will be antibacterial compounds that have already been discovered, and this issue of dereplication will be addressed in the next chapter. Nevertheless, even this limited screening of natural product extracts suggests that the P_{murZ}-*lacZ* biosensor can be used to detect unpurified CBIs from a crude mixture.

The P_{gltA}-*lacZ* biosensor did not perform well in the validation of the agar-based assay with vancomycin, fosfomycin and flucloxacillin. Referring to Table 3.1, it is apparent that this biosensor was the least responsive of the three biosensors to these three antibiotics in the MUG assay, so it is not surprising that it is not a suitable biosensor to use in the agar-based assay.

The hypothesis for the lack of an observable blue ring for vancomycin and bacitracin in the initial validation of the agar-based assay was that their molecular size could impede their diffusion through the agar (they both have molecular weights of approximately 1,400 $g.mol^{-1}$). This would explain why they perform so much better in the MUG assay and the β-glo assay because, in those cases, the whole solution is sampled. In contrast, antibiotics such as flucloxacillin and cefotaxime have molecular weights of around 450 $g.mol^{-1}$ and gave a much more noticeable blue ring in the agar-based assay.

The aim of making the dual biosensors was to generate a tool that could detect CBIs at lower concentrations than the single biosensors. This would have been useful in detecting antibacterial compounds present in a mixture at sub-inhibitory concentrations. The limits of detection for flucloxacillin and cefotaxime appeared to be at lower concentrations with the Z1 biosensor compared to the corresponding single biosensors (the P_{oppB}-*lacZ* biosensor and the P_{murZ}-*lacZ* biosensor)(Table 3.6). However, it is clear from the appearance of the plates in the agar-based assay (Figure 3.22C and Figure 3.22D) that the Z1 dual

biosensor also gave a higher background level of β-galactosidase activity. The concept of making a dual biosensor in this way is therefore an unsuitable strategy for increasing the sensitivity of the CBI biosensors.

There were a few unexpected results from using the agar-based assay to detect antibacterial compounds directly from their producing organisms. *S. fradiae* gave no blue ring, despite initially being selected as a fosfomycin producer, and fosfomycin should have induced the biosensor because it is a CBI. There was no ZOI visible around the plug of *A. rifamycinia*, despite the fact that this should have been producing the antibiotic rifamycin. There was no visible blue ring around the plug of *A. chrysogenum*, even though this was producing some cephalosporin C, which was evident from the presence of a ZOI.

The particular strain of *S. fradiae* used (NCIMB 8233) produces neomycin (Fan *et al.*, 2008). However, further literature searching revealed that *S. fradiae* can produce neomycin, tylosin and fosfomycin, depending on the exact strain (Butler *et al.*, 1999; Majumdar and Majumdar, 1965; Rogers and Birnbaum, 1974). Neomycin is a DNA biosynthesis inhibitor, tylosin inhibits bacterial protein biosynthesis and fosfomycin is a CBI. It could be that the strain used is capable of producing these other antibiotics, but at a much lower concentration than neomycin. This illustrates one of the drawbacks of screening natural product producing organisms directly as it is unknown whether or not they will produce the desired natural product at a high enough concentration to be detected by the current biosensors. This may explain why the strain of *S. fradiae* used in the laboratory produced a zone of inhibition but did not induce the P_{murZ}-*lacZ* biosensor.

Banerjee *et al.* noted that the form of rifamycin produced by the *A. rifamicinica* strain used in this study is rifamycin B (Banerjee *et al.*, 1992). (The strain in the paper is called *Nocardia mediterranei* after the first of the three name changes to the strain *A. rifamicinica* since its discovery (Bala, 2004; Lechevalier *et al.*, 1986; Thiemann *et al.*, 1969).) Rifamycin B is a relatively inactive precursor of the highly active rifamycin S. The conversion between the two requires acidic conditions for the oxidation step, catalysed by rifamycin oxidase, to form the spiro intermediate, rifamycin O. This is then hydrolysed to give rifamycin S.

This suggests that reducing the pH of the media used to grow *A. rifamicinica* could induce the production of rifamycin S, which should result in a zone of inhibition and a correct negative result. *S. coelicolor* showed no blue ring, although any faint blue colour may easily have been masked by the striking purple colour of the actinorhodin produced by this organism.

The fact that *A. chrysogenum* gave a zone of inhibition but no blue ring was surprising given that the P_{murZ}-*lacZ* biosensor had been able to detect many different types of cephalosporin derivatives in the high-throughput library screens. It was therefore assumed that there was simply an insufficient concentration of cephalosporin C produced by *A. chrysogenum* in the agar based assay. Furthermore, cephalosporin C was detected in the β-glo assay with a β-gal activity ratio of 3.9 at 1×MIC (Table 7.3).

In contrast to these unexpected results, using the agar-based assay to screen the producing organisms of kanamycin, neomycin and novobiocin all gave a ZOI and no blue colour, as expected. The assay also showed a ZOI with a blue ring for the producers of penicillin, vancomycin and bacitracin. This demonstrates that the P_{murZ}-*lacZ* biosensor can be used to detect the production of a CBI without even having to extract the contents of the bacterial cells first. In addition, this proves that the P_{murZ}-*lacZ* biosensor is capable of detecting vancomycin, and so the lack of a response to vancomycin in the validation of the agar-based assay (Figure 3.17) must have been due to the vancomycin being present at an insufficient concentration.

Chapter 4 Tackling the issue of dereplication – using growth media supplementation and resistance genes in the P_{murZ}-*lacZ* biosensor to eliminate known antibiotics

Abstract

One of the main barriers to screening extracts of organisms that produce bioactive natural products for novel antibacterial compounds is the re-isolation of known compounds. This makes screening for natural products relatively unproductive, which is a problem given that tackling the rise in antimicrobial resistance by finding new antibiotics is likely to depend on screening for natural products synthesised less frequently than 10^{-7} (Baltz, 2007). Many techniques have been developed over the last half a century in an attempt to focus on sources that produce antibacterial natural products based on genuinely novel scaffolds (Gaudêncio and Pereira, 2015). One of the methods that can be used for dereplication is to use indicator strains of bacteria that confer resistance to specific classes of antibiotics (Cox *et al.*, 2017; Gullo *et al.*, 2006).

In this chapter, the β-glo assay was developed further – to not detect CBIs that are β-lactams, fosfomycins, bacitracins or D-cycloserines. This represents the dereplication of four classes of CBI. The detection of D-cycloserine was suppressed by adding D-alanine to the growth media for the duration of the β-glo assay. The detection of β-lactams, fosfomycins and bacitracins was suppressed by cloning the resistance genes *blaZ*, *fosB* and *bcrAB* into the pRAB11 vector and transforming this construct into the P_{murZ}-*lacZ* biosensor to generate the P_{murZ}-*lacZ*(derep) biosensor. When used in conjunction with the D-alanine supplementation, this dereplication biosensor was still capable of detecting CBIs apart from those members of the four classes mentioned above. This is a powerful tool, as it can potentially be used to detect novel CBIs in complex crude natural product extracts.

4.1 Introduction

One of the reasons that antibacterial drug discovery began to be less productive was the increasing frequency at which known compounds were being rediscovered (Baltz, 2006, 2007; Lewis, 2013; Moloney, 2016). In general, the older the biosynthetic pathway of the compound, the more abundant it is amongst natural product producers (Baltz, 2007). For example, the pathways for the biosynthesis of erythromycin and vancomycin are hundreds of millions of years old, and these are typically found in soil samples of *Actinomycetes* bacteria at a frequency of between 5×10^{-6} and 1.5×10^{-5}. It has been estimated that there may be many more antibacterial compounds to be discovered at a frequency of the magnitude 10^{-7}, but these are unlikely to be found soon given that the past 50 years have only seen around 10^{7} strains of *Actinomycetes* bacteria screened for metabolites with antibacterial activity (Baltz, 2007). It has also been estimated that there could be in the region of 10^{25} to 10^{26} species of bacteria in this class present in just the top 10 cm of soil globally (Baltz, 2007). When the likely low frequency of discovering genuinely novel antibacterial compounds from cultures from soil (and other natural product producers) is considered, it is clear that there will need to be some way of discriminating against the far more common compounds. If such a tool for achieving this is available, then screening bacteria from soil will be far more productive because less time and fewer resources are likely to be wasted investigating the activity of what turn out to be previously discovered compounds.

A variety of analytical methods of dereplication have been described in the literature. For example, Lang *et al.*, (2008) describes a workflow that involves assessing the antibacterial properties of a crude extract, before analysing active extracts by HPLC and comparing the results to an in-house library of known fungal and bacterial metabolites. Any compounds that do not have a match at this point were then compared to external databases that also includes basic NMR data. To demonstrate this method, they found a compound with antibacterial activity that was produced by an unidentified nonsporulating endophyte. Using this method, they were able to identify it as phomosine A. In a study conducted by Genilloud *et al.*, around 28,000 bioactive extracts were

analysed by LC-MS over the course of 2 years, revealing 190 compounds that were already present in the lab's own antibiotic library (Genilloud *et al.*, 2011). Many other hyphenated techniques, that combine two or more separation and detection methods, have been employed to aid in dereplication (Gaudêncio and Pereira, 2015).

The major drawback of the above solutions is that they are low throughput and so are unlikely to be useful for screening extracts for compounds found at the low frequencies mentioned above. They also focus on the physicochemical properties of the compounds screened and not on their antibacterial activity. Anti***bio***tic ***m***ode of ***a***ction ***p***rofile (BioMAP) screening is a higher-throughput method which works on the basis that antibacterial compounds can be categorised by structure based on a biological 'fingerprint' of their MIC values against a panel of six Gram-positive strains and nine Gram-negative strains (Wong *et al.*, 2012). The accuracy of this method was determined by using a 'training set' of 72 antibiotics with a range of target pathways. This method was then used to screen a library of 3,120 prefractionated natural product extracts and a novel naphthoquinone-based antibiotic with a unique carbon skeleton (arromycin) was discovered. Unfortunately, this was mildly toxic to HeLa cells (LD_{50} = 60 μM).

Dereplication through considering antibiotic resistance is a long established strategy, although the earliest attempts at applying this involved looking at cross-resistance in poorly characterised mutant strains of bacteria (Stapley, 1958). Some of the most recent work on using antibiotic resistance to aid dereplication was presented by Cox *et al.*, (2017). They have recently developed an antibiotic resistance platform (ARP) which consists of a library of *E. coli* cells expressing individual resistance genes. Over 40 resistance genes were incorporated, mediating resistance to over 15 classes of antibiotics (mainly aminoglycosides, β-lactams and macrolides). One of the ways in which this ARP was used was to place a plug of the natural product producing organism onto a lawn of an indicator ARP strain, much like the technique used when the P_{murZ}-*lacZ* biosensor was used to observed CBIs produced directly from the producing organism in the agar-based assay described in section 3.2.8. A smaller ZOI would give some indication as to the identity of the compound responsible for the antibacterial effect. This technique was used to

screen an in-house library of around 60 strains of *Actinobacteria* that displayed activity against some ESKAPE pathogens. The ARP rapidly detected that 42% of these strains exerted their antibacterial effect by producing streptothricin, one of the most common antibacterial compounds produced by *Actinobacteria* (Baltz, 2006). Another 7% produced streptomycin, one strain produced oxytetracycline and another strain produced the macrolide, pikromycin. Eliminating these strains from consideration in one step allowed attention to be focussed on finding producers of more unusual molecules – two of the strains isolated produced the poorly characterised antibiotic, mayamycin (Cox *et al.*, 2017).

In a similar manner, Cubist Pharmaceuticals generated a Gram-negative strain that incorporated multiple resistance genes, along with a modified version of this strain with increased permeability to increase the sensitivity of the strain to the antibacterial compounds tested (Gullo *et al.*, 2006). They developed an assay in which macrodroplets encapsulated an entire individual environment of *Actinomycetes* bacteria, allowing an impressive 8 million *Actinomycetes* species to be screened for antibacterial activity in under a year. Using their resistance-gene rich strains alongside the antibiotic-susceptible parent strain streamlined this process, resulting in a discovery rate of 17:1 known to novel compounds and an overall chemical follow-up rate of 0.001%.

Whilst both the ARP and Cubist's dereplication strains represent powerful tools to rapidly eliminate strains that produce known antibiotics from a screening process, it would be even more advantageous if there was a method for dereplicating in a high-throughput screen that also detects antibacterial compounds that target specific bacterial biosynthetic pathways. Given that it had already been demonstrated that the P_{murZ}-*lacZ* biosensor was able to identify CBIs specifically that are not membrane damaging compounds, it seemed like a sensible strategy to try to engineer this strain to tackle the dereplication problem using antibiotic resistance. The idea was to introduce genes that confer resistance to a selection of CBIs, which would hopefully reduce the β-gal activity ratios due to the biosensor cells being able to grow in the presence of these selected classes of antibiotic. In addition to this, D-alanine was used to supplement the TSB in order to suppress the antibacterial effect and, subsequently, the β-gal activity ratio, of D-cycloserine.

4.2 Results

4.2.1 Using D-alanine to suppress the induction of the P_{murZ}-*lacZ* biosensor by D-cycloserine

4.2.1.1 Rationale for supplementation of the growth media

D-cycloserine is an analogue of D-alanine and is thus capable of inhibiting the action of two enzymes involved in the formation of the pentapeptide for attachment to the UDP-MurNAc unit. These two enzymes are alanine racemase, responsible for transforming L-alanine into D-alanine, and D-Ala-D-Ala ligase, which catalyses the formation of a peptide bond between two D-alanine molecules (Halouska *et al.*, 2014; Prosser and de Carvalho, 2013)(Figure 4.1). It was therefore proposed that supplementing the growth media used in the β-glo assay (TSB) with D-alanine would enable the P_{murZ}-*lacZ* biosensor by D-cycloserine to not be induced by D-cycloserine whilst continuing to be induced by all of the other CBIs.

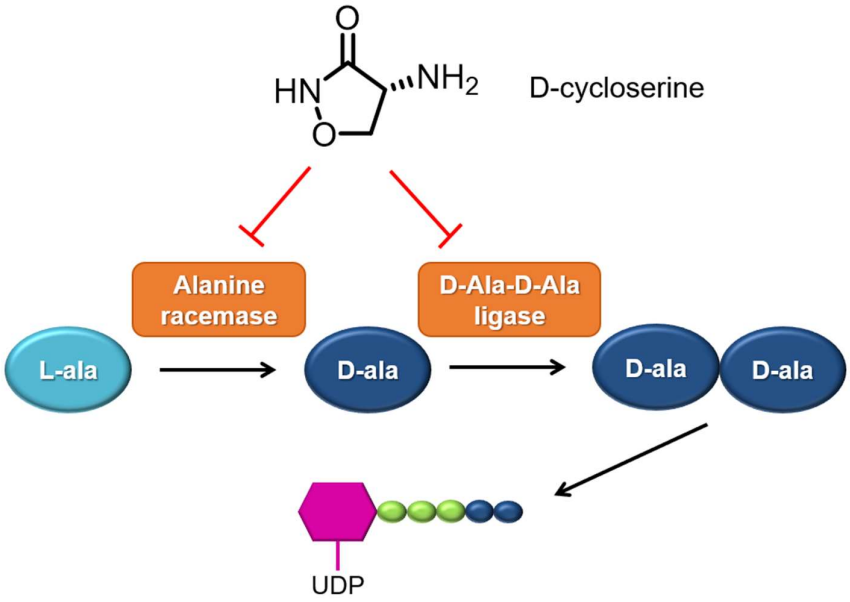


Figure 4.1 An overview of the steps in peptidoglycan biosynthesis that are inhibited by the antibiotic D-cycloserine.

4.2.1.2 Identifying the best D-alanine concentration to use

MIC values were determined for D-cycloserine in the presence of increasing concentrations of D-alanine using the P_{murZ}-*lacZ* biosensor (Table 4.1). This

demonstrated that this strategy did at least make the reporter less susceptible to D-cycloserine as the concentration of D-alanine increased.

The β-glo assay was then used to determine the β-gal activity ratios for D-cycloserine at various multiples of its MIC of 32 μg.mL^{-1} for a range of D-alanine concentrations (Figure 4.2).

Table 4.1 MIC values of D-cycloserine with the P*murZ*-*lacZ* biosensor in the presence of increasing concentrations of D-alanine.

D-alanine concentration (μg.mL^{-1})	MIC D-cycloserine (μg.mL^{-1})
0	32 (313 μM)
16	32
32	32
64	64
128	64
256	128
512	128
1024	256
2048	512
4096	512 (5020 μM)

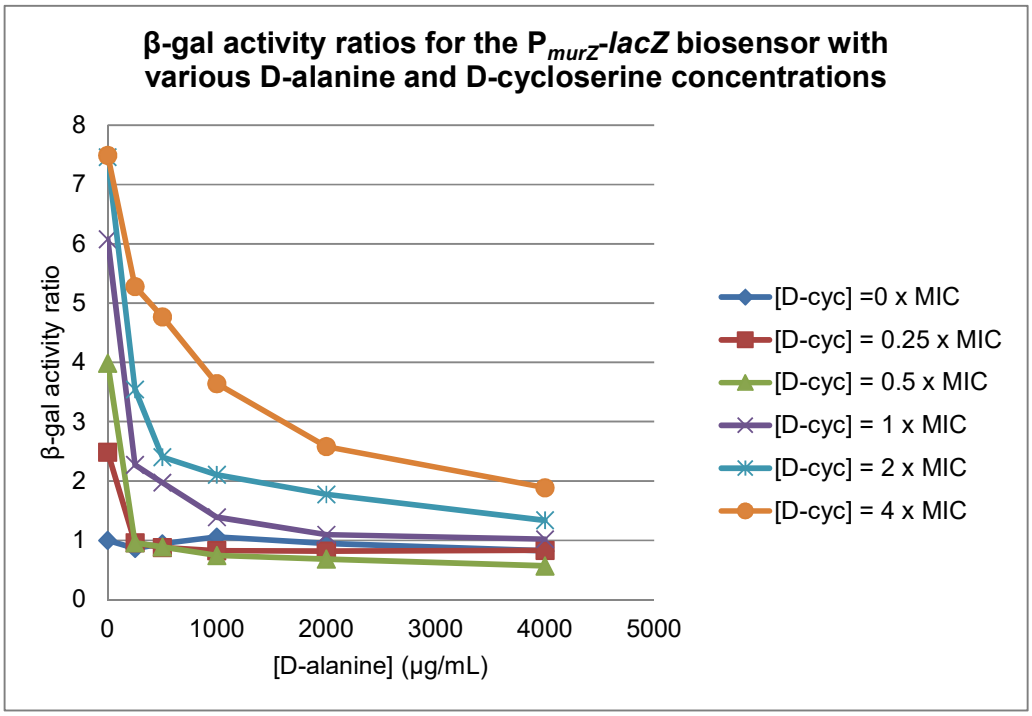


Figure 4.2 β-gal activity ratios of the P_{murZ}-*lacZ* biosensor with various D-alanine and D-cycloserine concentrations.

This showed that a concentration of 2 mg.mL^{-1} was sufficient to suppress the β-gal activity ratio of D-cycloserine to below the 3-fold induction threshold. However, it was also important to ensure that the addition of such high concentrations of D-alanine to the TSB media would not impact the function of the P_{murZ}-*lacZ* biosensor when it was used with other CBIs that should still induce the biosensor. Therefore, a D-alanine concentration of 8 mg.mL^{-1} was used to determine the β-gal activity ratio of a selection of antibacterial compounds at a concentration of 2×MIC to show that the ratios for the other CBIs were largely unaffected by the addition of D-alanine (Figure 4.3). Ultimately, a D-alanine concentration of 5 mg.mL^{-1} was chosen to suppress the β-gal activity ratio of D-cycloserine when the P_{murZ}-*lacZ* biosensor was used in the β-glo assay.

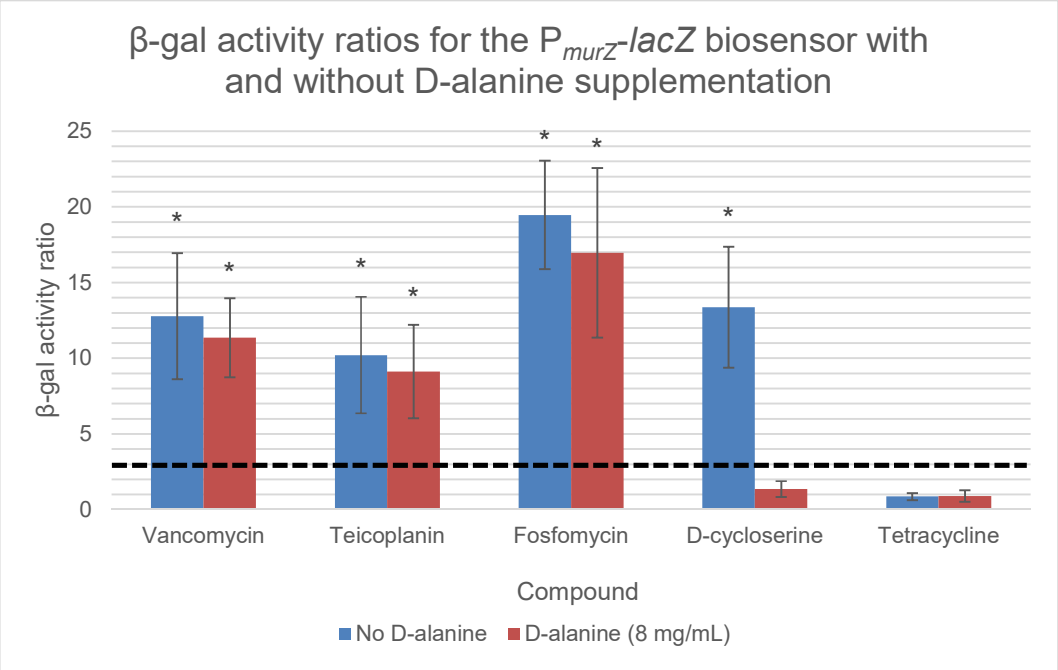


Figure 4.3 β-gal activity ratios for the P_{murZ}-*lacZ* biosensor with and without D-alanine supplementation. Antibiotic compounds were used at 2×MIC. The black line indicates the 3-fold threshold for induction in the β-glo assay. 2×MIC vancomycin = 2.69 μM; 2×MIC teicoplanin = 2.13 μM; 2×MIC fosfomycin = 176 μM; 2×MIC D-cycloserine = 627 μM; 2×MIC tetracycline = 2.08 μM.

4.2.2 Design and generation of the P_{murZ}-*lacZ*(derep) biosensor

Following the extensive testing of the specificity of the P_{murZ}-*lacZ* biosensor detailed in the previous chapter, the next aim was to engineer a further strain that would not be induced by some of the more common known CBIs. The idea of this strategy was that this would increase the likelihood that any hits in a natural product screen using the new biosensor would be genuinely novel antibiotics.

A Gibson assembly strategy was used to insert the genes *blaZ*, *fosB* and *bcrAB* (conferring resistance to β-lactams, fosfomycin and bacitracin, respectively) into the multiple cloning site of the vector plasmid pRAB11, where their expression was tightly controlled by an anhydrotetracycline-inducible promoter (Helle *et al.*, 2011). These genes were selected primarily because they would

make the dereplication biosensor blind to many of the known CBI classes (β-lactams, cyclic peptides and the phosphonic antibiotics).

4.2.2.1 Overview of the Gibson assembly strategy

The plan was to amplify the pRAB11 vector from a point just downstream of the MCS and ligate the three required genes in two synthetic DNA fragments. Synthetic fragments were used in order to replace the native ribosome binding sites (RBS) with the optimised sequence for *S. aureus* (AGGAGG). The length of the pRAB11 vector (~6.5kbp) necessitated the use of Qiagen's LongRange PCR kit for the amplification of the vector prior to the Gibson assembly. This kit included an enzyme mix of thermostable DNA polymerases and a buffer system specifically formulated for the amplification of DNA sequences up to 40 kbp in length.

Figure 4.4 shows the overall construction strategy for the pZAB plasmid. Figure 4.5 shows more details of where the homology sites for the Gibson assembly strategy were. The plan was to then use the pZAB plasmid DNA to transform the P_{murZ}-*lacZ* biosensor, which had its promoter-reporter gene system on the chromosome.

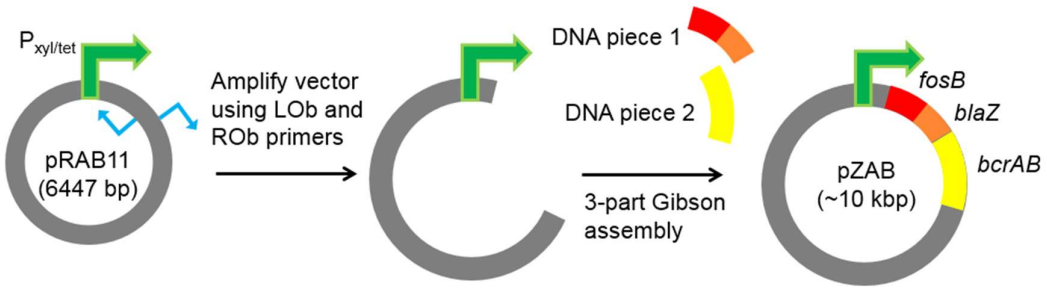
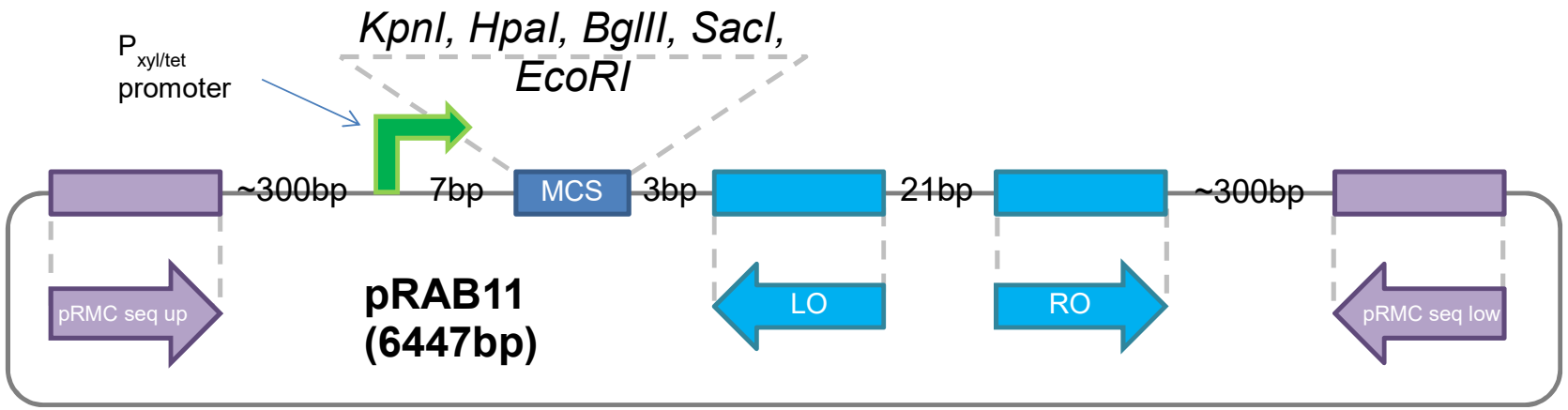


Figure 4.4 The construction strategy for the pZAB plasmid.



DNA piece 1:

DNA piece 2:

Figure 4.5 Diagrams of the relevant sites on the pRAB11 vector.

4.2.2.2 Generation of the P_{murZ}-*lacZ*(derep) biosensor

The product of the Gibson assembly reaction of the amplified pRAB11 vector and the two synthetic DNA pieces was used to transform ultra-competent *E. coli* (XL1-Blue) cells. A colony PCR reaction was performed for ten of the colonies from this transformation and this indicated that eight of these colonies contained an assembly product. These colonies were used to prepare overnight bacterial cultures, from which plasmid DNA was extracted and then used to transform electrocompetent cells of the P_{murZ}-*lacZ* biosensor. Seven of these eight transformations resulted in colonies and two colonies from each of these seven transformation plates were used to prepare overnight bacterial cultures. MIC values were determined for these fourteen transformation strains, initially for just penicillin G and bacitracin to test for the phenotype conferred by the *blaZ* and *bcrAB* genes, respectively (Table 4.2). Four of the fourteen strains were just as susceptible to penicillin G as the control strain, SH1000, and another two of the actually appeared to be more susceptible to bacitracin than SH1000. This indicated that either one or both of the *bcrAB* or *blaZ* genes had not been ligated into the pZAB construct properly. MIC values for fosfomycin and a control of vancomycin were subsequently determined for the remaining eight strains that had displayed the correct phenotype with penicillin G and bacitracin. This eliminated another strain that was susceptible to fosfomycin, implying that the *fosB* gene had not been ligated correctly into the assembly product in this strain. This left seven candidate P_{murZ}-*lacZ*(derep) biosensor strains that displayed the expected phenotype – increased resistance to penicillin G, fosfomycin and bacitracin compared to SH1000, but no change in the susceptibility to vancomycin.

The nomenclature “MZAB6” was used because these strains were the result of the sixth attempt overall at the Gibson assembly. In Table 4.2, the second number in the strain name refers to the number of the transformation into the P_{murZ}-*lacZ* biosensor cells and the third number denotes whether that strain comes from the first or second colony picked from that transformation plate.

Table 4.2 MIC values for the MZAB6 transformation colonies with penicillin, bacitracin, fosfomycin and vancomycin, compared with the MIC values for SH1000. Green text indicates the correct resistance profile (higher MIC) for that compound and red text indicates the incorrect phenotype.

Strain	MIC (μg.mL^{-1})				Correct phenotype?
	Penicillin G	Bacitracin	Fosfomycin	Vancomycin	
SH1000	0.03125	128	4	1	
MZAB611	2	32			No
MZAB612	0.5	32			No
MZAB631	2	1024	256	0.5	Yes
MZAB632	4	1024	256	0.5	Yes
MZAB641	0.5	512	128	0.5	Yes
MZAB642	16	1024	128	0.5	Yes
MZAB661	2	512	64	0.5	Yes
MZAB662	1	512	32	0.5	Yes
MZAB671	2	512	0.5	0.5	No
MZAB672	8	512	128	0.5	Yes
MZAB691	0.03125	1024			No
MZAB692	0.03125	>1024			No
MZAB6(10)1	0.03125	1024			No
MZAB6(10)2	0.03125	1024			No

The seven candidate P$_{murZ}$-*lacZ*(derep) biosensor strains were tested in the β-glo assay using the same four antibiotics for which MICs had been determined. The three candidate strains that performed the best in the assay, in terms of their suppression of the β-gal activity ratios for penicillin G, fosfomycin and bacitracin, compared with the ratio for vancomycin, were used for further repeats and these results are shown in Figure 4.6.

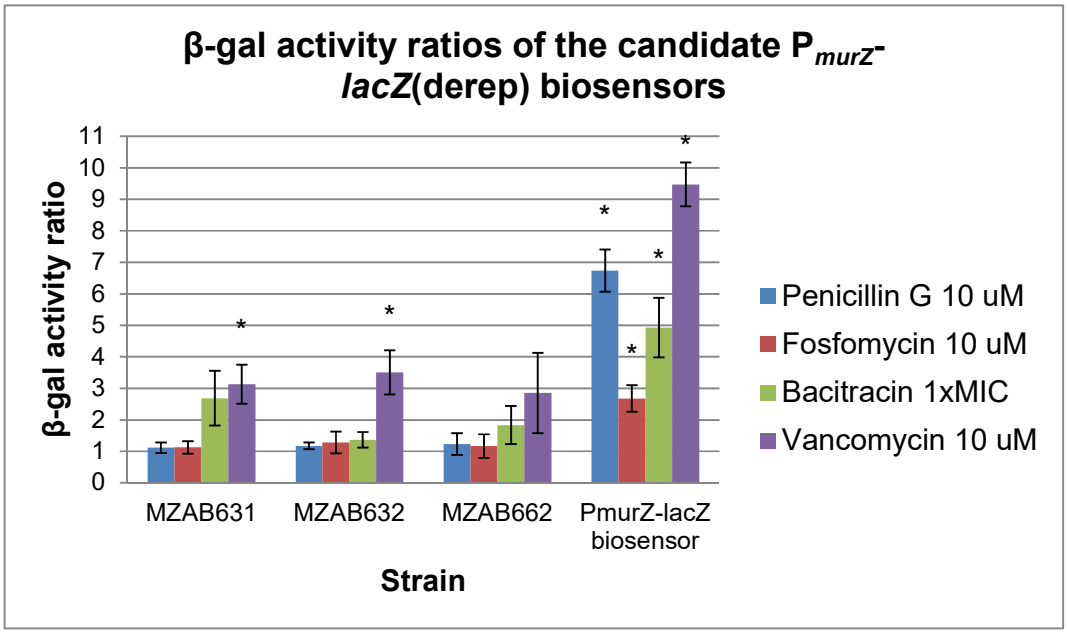


Figure 4.6 β-gal activity ratios of the best three candidate P*murZ-lacZ*(derep) biosensors, tested using the β-glo assay with 5 mg/mL D-alanine. 1×MIC bacitracin = 45.0 µM.

Out of these three better MZAB6 strains, MZAB632 displayed the most noticeable difference between the β-gal activity ratio for vancomycin and that of the other compounds that the candidate P$_{murZ}$-*lacZ*(derep) biosensor strains should have conferred resistance to. Therefore, MZAB632 was used as the P$_{murZ}$-*lacZ*(derep) biosensor.

4.2.2.3 Optimising the conditions for the β-glo assay with the P$_{murZ}$-*lacZ*(derep) biosensor

Inducing the expression of the resistance genes in the P$_{murZ}$-*lacZ*(derep) biosensor using 0.4 µM ATc (as was recommended in the literature, (Helle *et al.*, 2011)) clearly resulted in too much of a fitness cost. The cells did not grow at a rate sufficient for a significant output from the β-glo assay, which resulted in the β-gal activity ratio being significantly suppressed for vancomycin, which was not the intention (Figure 4.6). The fact that the promoter on the pRAB11 vector was under tight regulational control from the concentration of anhydrotetracycline meant that this parameter could be altered in order to find an optimum level of expression of the resistance genes that would be high enough to suppress the β-gal activity ratios of penicillin G, fosfomycin and

bacitracin, but not so high that the P_{murZ}-*lacZ*(derep) biosensor could not be used in the β-glo assay.

The MIC values were determined for penicillin G with anhydrotetracycline (ATc) concentrations of 0.4, 0.2 and 0.1 μM. The P_{murZ}-*lacZ*(derep) biosensor was slightly less susceptible to penicillin G in the presence of 0.1 μM compared to 0.4 μM (Table 4.3), suggesting that using an ATc concentration of 0.1 μM would result in an improved performance of the P_{murZ}-*lacZ*(derep) biosensor in the β-glo assay.

Table 4.3 MIC values for penicillin with the P_{murZ}-*lacZ*(derep) biosensor induced by different ATc concentrations.

ATc concentration	P_{murZ}-*lacZ*(derep) MICs (μg.mL^{-1})
0.4 μM	32
0.2 μM	64
0.1 μM	64

Even lower concentrations of ATc were used for further MIC determinations to see if the induction could be improved even further than when 0.1 μM was used. Table 4.4 shows the MIC values for penicillin, fosfomycin, bacitracin and vancomycin for these lower concentrations. These suggested that it might be worth determining the β-gal activity ratios using these lower concentrations of ATc, given that the MIC values were still higher compared with those for no ATc (which should, in theory, be equivalent to the P_{murZ}-*lacZ* biosensor given that the expression of the resistance genes should not be induced under these conditions). The results of this, shown in Figure 4.7, show that using 0.01 μM ATc means that the resistance genes are not sufficiently expressed, and so the β-gal activity ratios for penicillin, fosfomycin and bacitracin ended up being greater than the 3-fold threshold. Using 0.05 μM ATc did not offer much advantage over using 0.1 μM ATc, so it was decided at this stage that 0.1 μM was the most appropriate concentration of ATc to use with the P_{murZ}-*lacZ*(derep) biosensor.

Table 4.4 MIC values for penicillin, fosfomycin, bacitracin and vancomycin with the P*murZ*-*lacZ*(derep) biosensor using 0.01, 0.05 and 0.1 µM ATc.

Antibiotic MICs in µg/mL	Concentration of anhydrotetracycline (ATc)			
	0 µM	0.01 µM	0.05 µM	0.1 µM
Penicillin	0.125	16	128	128
Fosfomycin	32	128	512	512
Bacitracin	256	512	1024	>2048
Vancomycin	1	1	1	1

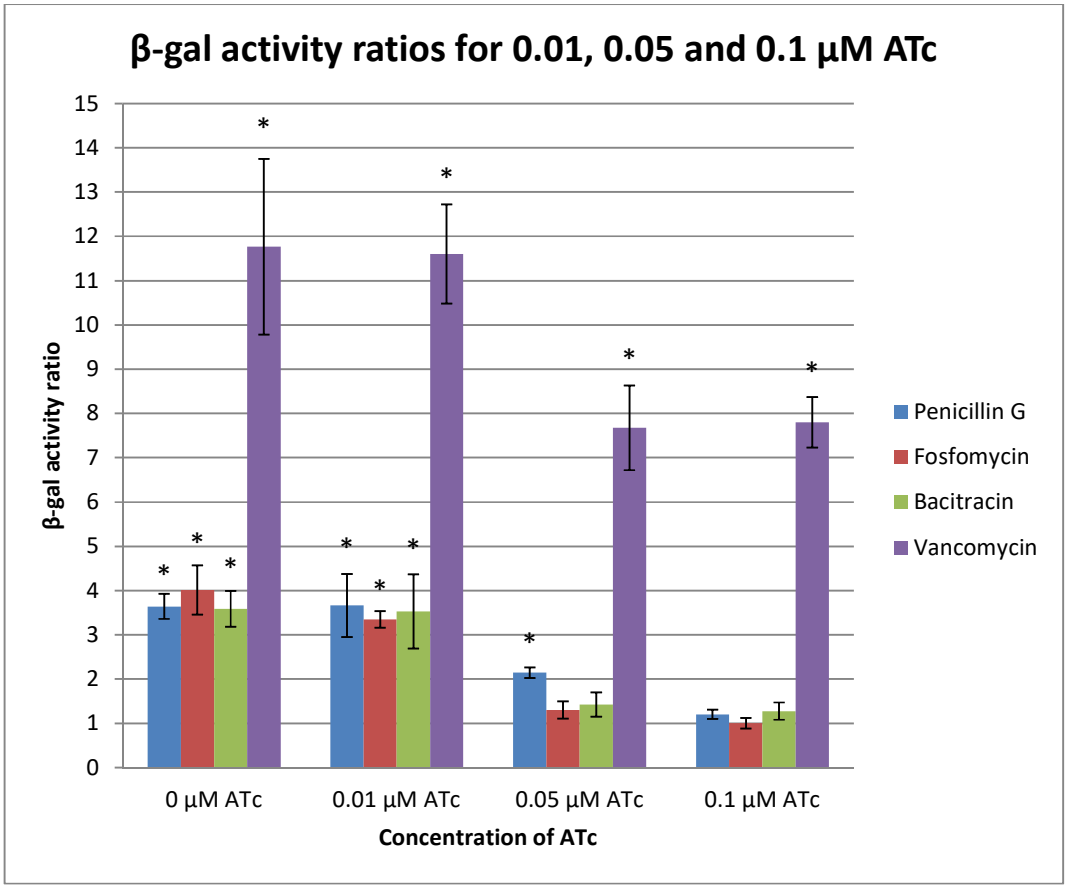


Figure 4.7 The β-gal activity ratios for the P*murZ*-*lacZ*(derep) biosensor with penicillin (10 µM), fosfomycin (10 µM), bacitracin (1 × MIC) and vancomycin (10 µM) with 0.01, 0.05 and 0.1 µM ATc. 1×MIC bacitracin = 45.0 µM.

4.2.3 Using the P*murZ*-*lacZ*(derep) biosensor with one of the NIH library plates

The utility of the P*murZ*-*lacZ*(derep) biosensor was demonstrated further by screening one of the plates from the library screens and comparing the results

with those obtained with the P_{murZ}-*lacZ* biosensor for the same plate. Plate 1974 from the NIH Clinical Collection was screened with both biosensors in the presence of 0.1 µM ATc (Figure 4.8). The only two compounds in this plate for which the β-gal activity ratios were expected to be suppressed by the P_{murZ}-*lacZ*(derep) biosensor were cefazolin in well 5C and penicillin G in well 9A. This expected result was obtained.

A

	1	2	3	4	5	6	7	8	9	10	11	12
A		1.05	0.81	0.73	0.61	0.54	0.60	0.59	**8.58**	0.52	0.59	
B	**1.00**	0.59	**1.04**	0.76	0.74	0.75	0.84	0.55	0.54	0.54	0.59	**9.21**
C	**1.00**	0.74	**1.11**	0.79	**5.63**	0.58	0.82	0.71	0.92	0.06	0.68	**6.91**
D	**1.00**	0.88	**0.99**	0.82	0.61	0.61	0.49	0.51	0.61	0.72	0.57	**9.94**
E		0.96	0.67	0.67	0.57	0.11	0.62	0.37	0.61	0.56	**0.02**	
F	**1.00**	0.47	0.66	0.59	**3.68**	0.68	0.59	0.80	**0.51**	0.58	0.38	**0.02**
G	**1.00**	0.75	**0.91**	0.69	0.84	0.65	0.82	0.51	0.53	0.81	0.53	**0.03**
H	**1.00**	**0.61**	0.87	0.91	1.05	0.52	0.71	0.55	0.99	0.89	**6.75**	**0.01**

B

	1	2	3	4	5	6	7	8	9	10	11	12
A		0.70	0.79	0.68	0.87	0.98	0.91	0.69	**1.07**	1.01	0.81	
B	**1.00**	0.86	**0.87**	0.79	1.06	1.38	0.92	1.27	1.22	1.84	1.12	**6.97**
C	**1.00**	0.79	**0.90**	0.95	**2.76**	1.11	0.95	0.91	1.15	0.13	1.04	**8.41**
D	**1.00**	0.89	**0.97**	0.81	1.12	1.05	1.61	0.85	1.28	0.99	1.31	**5.91**
E		0.98	0.87	0.84	0.82	0.17	0.97	0.77	1.12	1.08	**0.19**	
F	**1.00**	0.93	1.02	0.88	**5.10**	1.11	1.14	0.91	**1.35**	1.21	1.01	**0.22**
G	**1.00**	0.90	**0.87**	0.81	0.95	0.81	1.08	1.13	1.46	1.12	0.88	**0.18**
H	**1.00**	**0.94**	1.02	1.08	1.62	1.04	1.28	1.33	2.70	1.22	**7.18**	**0.22**

CBIs

5C=cefazolin (1st gen cephalosporin)
5F=cefoxitin (2nd gen cephalosporin)
9A=penicillin G, pfizerpen
11H=nafcillin (beta-lactamase resistant penicillin)

Non-CBI antibiotics

2H=pyrazinamide (antibiotic for TB)
3B=sulfisoxazole (DNA biosynthesis)
3C=sulfamethoxazole (DNA biosynthesis)
3D=sulfacetamide (DNA biosynthesis)
3G=tetracycline (protein biosynthesis)
9F=spectinamycin (protein biosynthesis)
11E=demeclocycline (protein biosynthesis)

Figure 4.8 β-gal activity ratios for the NIH Clinical Collection 1974 plate with the P_{murZ}-*lacZ* biosensor and the P_{murZ}-*lacZ*(derep) biosensor. A) P_{murZ}-*lacZ* biosensor; B) P_{murZ}-*lacZ*(derep) biosensor with 0.1 µM ATc. Dark blue boxes = vancomycin positive control, yellow boxes = tetracycline negative control, green boxes = drug-free control

4.2.4 Finalising the conditions and testing the P_{murZ}-*lacZ*(derep) biosensor with the D-alanine supplementation

To verify that the 0.1 μM ATc was definitely the optimum concentration of ATc to use for the P_{murZ}-*lacZ*(derep) biosensor in the β-glo assay with higher concentrations of antibacterial compound, several CBIs were tested at 4×MIC with other concentrations of ATc ranging from 0.05 μM to 1 μM (Figure 4.9). It was decided that the final optimum ATc concentration was 0.15 μM, as these conditions suppressed the β-gal activity ratio for penicillin G, fosfomycin and bacitracin sufficiently, but kept the ratios high enough for D-cycloserine and vancomycin for the P_{murZ}-*lacZ*(derep) biosensor to be able to distinguish between those CBIs it should have been capable of detecting and those CBIs it should be blind to (β-lactams, fosfomycins and bacitracins). Using 0.05 μM ATc did not suppress the β-gal activity ratio for bacitracin sufficiently and using 0.2 μM ATc suppressed the β-gal activity ratio for D-cycloserine too much.

The final stage of optimising the conditions was to incorporate the strategy of supplementing the TSB growth media with D-alanine. Figure 4.10 shows that adding 5 $mg.mL^{-1}$ D-alanine to the growth media suppressed the β-gal activity ratio for D-cycloserine without significantly reducing the β-gal activity ratio for vancomycin. Unfortunately, the P_{murZ}-*lacZ*(derep) biosensor was still induced by some β-lactamase-resistant β-lactams, such as cefotaxime.

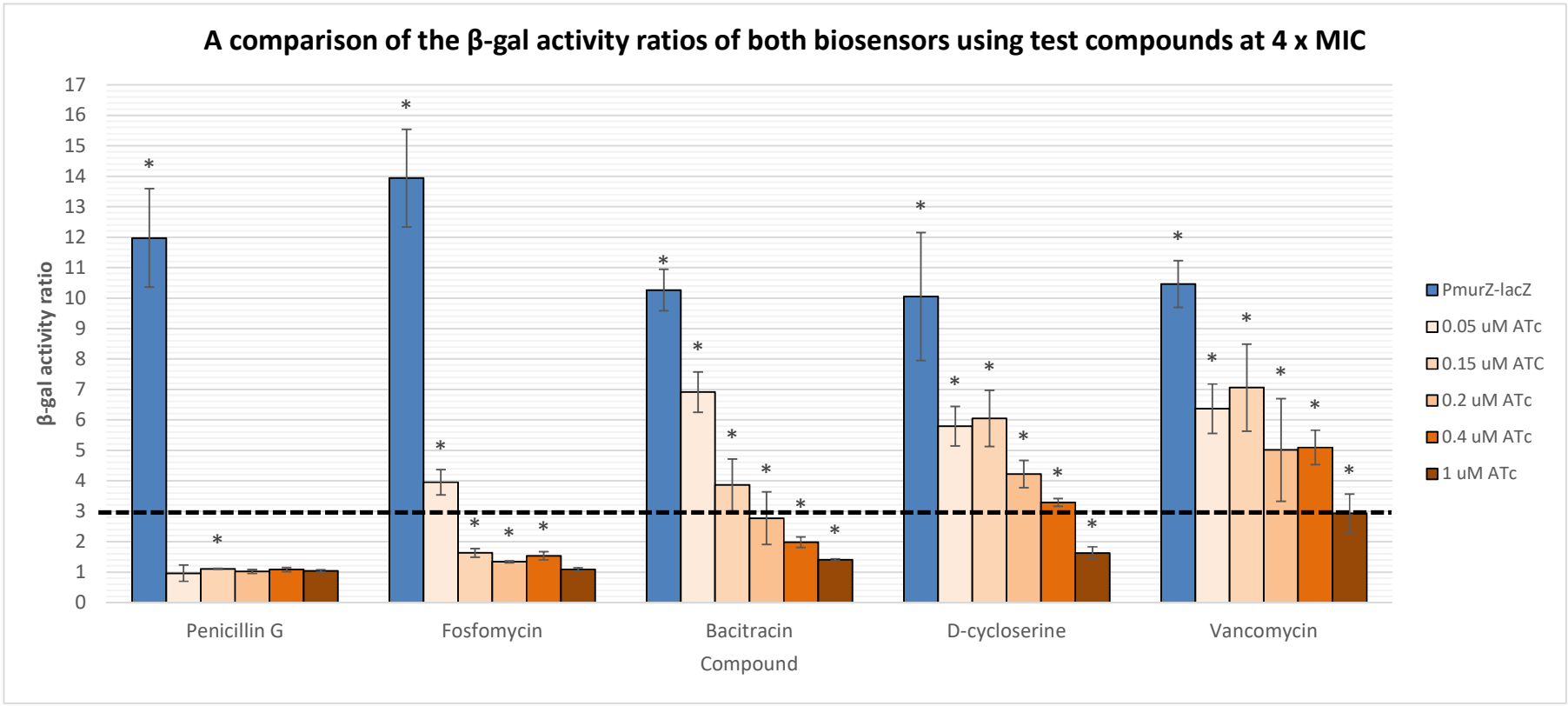


Figure 4.9 A comparison of the β-gal activity ratios of both biosensors with D-alanine supplementation combined with various concentrations of ATc. The black line indicates the 3-fold threshold for induction for the β-glo assay. 4×MIC penicillin G = 0.702 µM; 4×MIC fosfomycin = 352 µM; 4×MIC bacitracin = 45.0 µM; 4×MIC D-cycloserine = 313 µM; 4×MIC vancomycin = 1.35 µM.

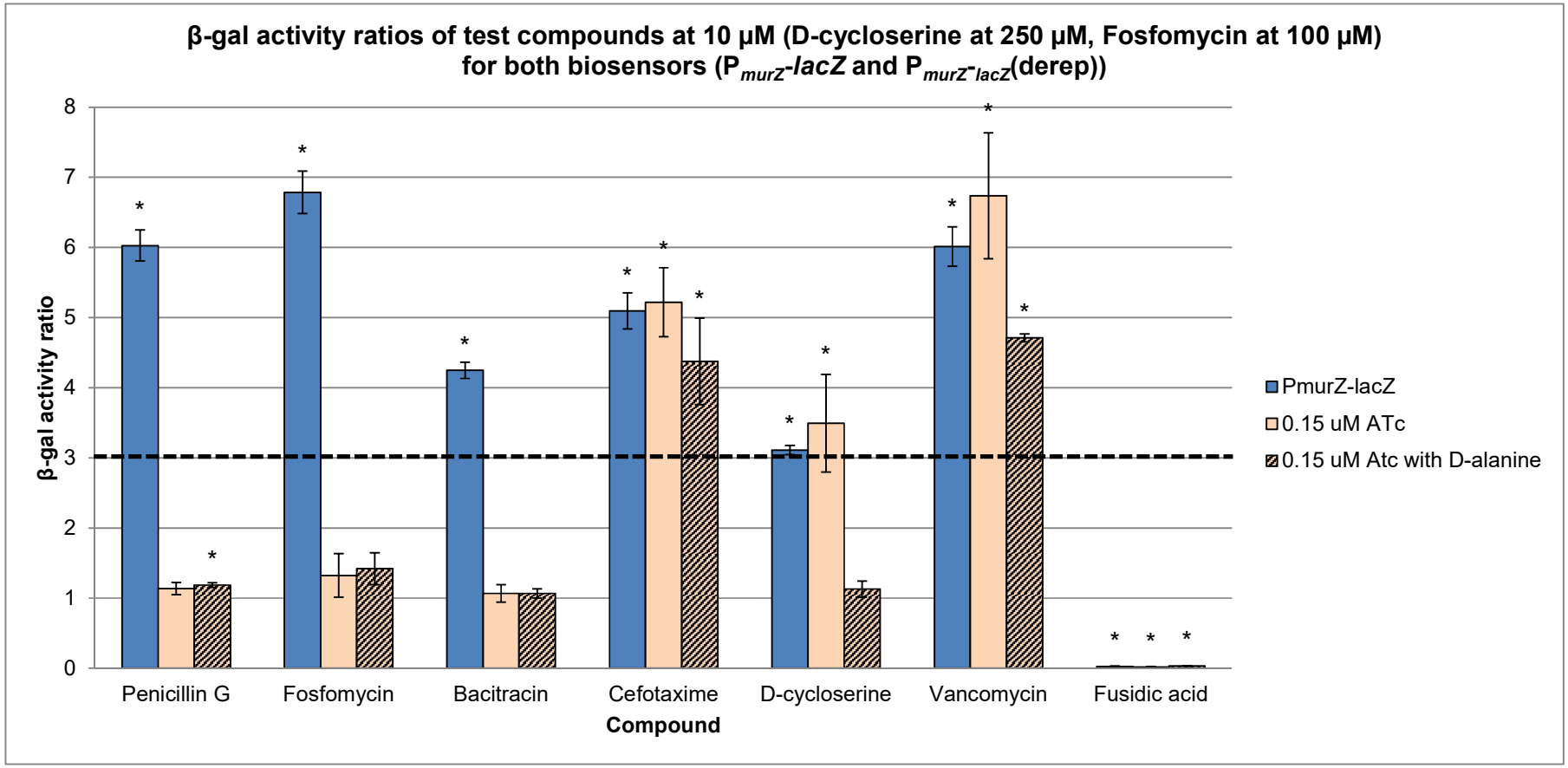


Figure 4.10 β-gal activity ratios of test compounds for both biosensors using 0.15 μM ATc both with and without D-alanine supplementation. The black line indicates the 3-fold threshold for induction for the β-glo assay.

4.3 Discussion

The suppression of β-gal activity ratios for D-cycloserine by supplementing the growth media with D-alanine demonstrated a simple way of eliminating one class of CBI from further consideration as a hit when screening compounds. A D-alanine concentration of 5 $mg.mL^{-1}$ ensured that the β-gal activity ratio for high concentrations (several times the MIC) of D-cycloserine would be well below the 3-fold induction threshold for the β-glo assay.

For the purpose of engineering the P_{murZ}-*lacZ* biosensor, it was decided that a Gibson assembly strategy would be used because this would be more reliable than traditional restriction-digest based cloning for cloning large pieces of DNA (the sizes of the *blaZ*, *fosB* and *bcrAB* genes were approximately 850bp, 420bp and 1,700bp, respectively). The pRAB11 vector was chosen because the $P_{xyl/tet}$ promoter was inducible by tetracycline derivatives. This meant that the level of expression of the resistance genes could be tightly regulated by altering the concentration of anhydrotetracycline which, unlike tetracycline, does not exert an antibacterial effect. Using a constituitive promoter to regulate the expression of these genes would not have allowed for any control if high levels of expression of the resistance genes incurred a fitness cost (Andersson and Levin, 1999; Silver, 2011), rendering the dereplication biosensor dysfunctional as far as using it in the β-glo assay was concerned.

The gene *blaZ* encodes for β-lactamase, which irreversibly inhibits the action of penicillins by hydrolysing the β-lactam ring (Zhang, 2001). The gene *fosB* encodes for the FosB protein – one of three distinct classes of fosfomycin resistance enzymes, the other two being FosA and FosX proteins (Thompson *et al.*, 2015). They are all metalloenzymes that inactivate fosfomycin by opening its epoxide ring, thus preventing the drug from inactivating the MurA enzyme by alkylating a cysteine residue that is crucial for its role in catalysing the first committed step in peptidoglycan biosynthesis. FosB is more typically found in Gram-positive bacteria and is responsible for the Mn^{2+}-dependent nucleophilic addition of either an L-cysteine group or a bacillithiol (BSH) group to carbon C1 of fosfomycin (Thompson *et al.*, 2014). The gene *bcrAB* encodes for the two-component ABC transporter system, BcrAB (Nawrocki *et al.*, 2014).

This system alone is able to confer resistance to bacitracin (Han *et al.*, 2015). This confers resistance to bacitracin by removing it from the cell membrane undecaprenol peptidoglycan carrier (Podlesek *et al.*, 2006). The component BcrA is an ATP-binding cassette (nucleotide-binding domain) and the component BcrB is a permease (transmembrane domain).

Dereplication of the glycopeptides, such as vancomycin, could have extended the utility of the P*murZ*-*lacZ*(derep) biosensor even further. However, vancomycin is considered to be an antibiotic of last resort to treat infections caused by MDR Gram-positive bacteria (Boneca and Chiosis, 2003). It was therefore decided that it would not be wise to generate a strain that was simultaneously resistant to glycopeptides, β-lactams, fosfomycin and bacitracin, in case this strain was inadvertently released into the environment.

In the β-glo assay with NIH plate 1974, the non-CBI antibiotics all had β-gal activity ratios below the 3-fold threshold with both of the biosensors (Figure 4.8). Cefoxitin and nafcillin are both β-lactamase resistant and induced both biosensors, as expected. Cefazolin induced the P*murZ*-*lacZ* biosensor, but narrowly missed the 3-fold threshold with the P*murZ*-*lacZ*(derep) biosensor. This is a first generation cephalosporin and was therefore unlikely to be as β-lactamase resistant as the second generation cephalosporin, cefoxitin, so this result was not unexpected. Nafcillin is a β-lactamase resistant member of the penicillin class, so unfortunately this was not dereplicated by the *blaZ* gene in the P*murZ*-*lacZ*(derep) biosensor (Tan and Fink, 1992). Importantly, penicillin G induced the P*murZ*-*lacZ* biosensor considerably higher than the threshold and did not induce the P*murZ*-*lacZ*(derep) biosensor. Even though this compound had already been tested outside the plate and had had its induction successfully suppressed by the P*murZ*-*lacZ*(derep) biosensor, it was encouraging to see that the same result could be obtained in a plate from a compound library.

A concentration of 100 μM for fosfomycin and 250 μM for bacitracin had to be used due to their relatively high MIC values, but other than that, the rest of the antibiotics were all tested at the same concentration of 10 μM (Figure 4.10). Cefotaxime and vancomycin induced the P*murZ*-*lacZ* biosensor and the P*murZ*-*lacZ*(derep) biosensor in all conditions, as expected. Penicillin, fosfomycin and

bacitracin all induced the P*murZ*-*lacZ* biosensor but not the P*murZ*-*lacZ*(derep) biosensor. D-cycloserine induced the P*murZ*-*lacZ* biosensor and the P*murZ*-*lacZ*(derep) biosensor in the absence of D-alanine, but it did not induce the P*murZ*-*lacZ*(derep) biosensor in the presence of 5 mg/mL D-alanine. This implies that, overall, resistance genes and growth media supplementation can be combined to give a tool with which to eliminate known antibiotics with specific mechanisms of action in a high-throughput screen.

Chapter 5 Towards the generation of whole-cell biosensors for the detection and dereplication of protein biosynthesis inhibitors

Abstract

Of all the antibiotics derived from natural products, the vast majority are either cell wall biosynthesis inhibitors or protein biosynthesis inhibitors. Having demonstrated the utility of the P_{murZ}-*lacZ* biosensor for specifically detecting CBIs and of the P_{murZ}-*lacZ*(derep) biosensor for suppressing the response to several known CBIs, the aim of the work reported in this chapter was to use the same process, but for generating biosensors to detect protein biosynthesis inhibitors (PBIs). RNA-seq analysis was used to generate a transcriptional profile for *S. aureus* exposed separately to sub-inhibitory concentrations of two PBIs targeting different steps in the bacterial protein biosynthesis pathway – fusidic acid and tetracycline. Genes that were upregulated ≥2-fold above a drug-free control upon exposure to both fusidic acid and tetracycline were narrowed down by eliminating any genes that had been upregulated in the presence of antibiotics that target other pathways, according to experimental data held in the *Staphylococcus aureus* Transcriptome Meta-Database. Once this process was complete, seven candidate genes remained. The plan was to clone the region containing the promoter and the RBS for each of these seven genes into a shuttle vector designed to allow for a translational fusion into the *lacZ* gene. Unfortunately, it was only possible to generate biosensors corresponding to three of these genes: the P_{infC}-*lacZ*, P_{01910}-*lacZ* and P_{02425}-*lacZ* biosensors. These three PBI biosensors were tested in the β-glo assay with a total of 13 antibiotics targeting a range of biosynthetic pathways, and the results suggested that the PBI biosensors were not specific for PBIs.

Should functional PBI biosensors have been generated, the issue of dereplication would still have needed to be addressed. The plan was to prepare two separate constructs by Gibson assembly similar to that included in the P_{murZ}-*lacZ*(derep) biosensor. The first construct included genes conferring resistance to all aminoglycosides and the second construct included genes

conferring resistance to many members of the tetracycline class, the $PhLOPS_A$ compounds (Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins and Streptogramin As) and the MLS_B compounds (Macrolides, Lincosamides and Streptogramin Bs). The first construct was made successfully and its phenotype was confirmed by determining MIC values for relevant compounds. The second construct was not made successfully.

5.1 Introduction

5.1.1 Bacterial protein biosynthesis and antibiotics that inhibit this process

The majority of antibiotics are microbial natural products or derivatives thereof (Brown and Wright, 2016). The fluoroquinolines (inhibitors of DNA biosynthesis), and the folate biosynthesis inhibitors, trimethoprim and the sulphonamides, are the only antibiotics that do not fit into the natural product category (Cox *et al.*, 2017). Table 5.1 and Figure 5.1 illustrate that most of the natural product antibiotics inhibit steps in either the cell wall biosynthetic pathway or the protein biosynthetic pathway. This is not to be unexpected given the large number of steps involved in these two pathways, in addition to the central role that protein formation has in bacterial cell viability (Walsh, 2003). Furthermore, the significant differences between the prokaryotic and eukaryotic ribosomes mean that many inhibitors of bacterial protein biosynthesis are not cytotoxic and are therefore suitable for clinical use (Osterman *et al.*, 2016).

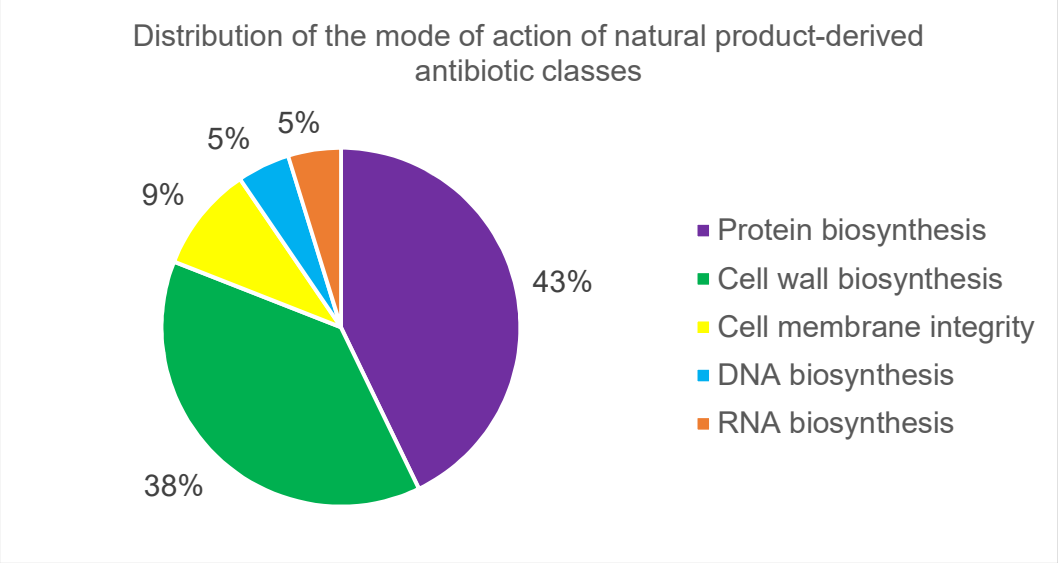


Figure 5.1 The distribution of the mode of action of natural product-derived antibiotic classes. Data from (Silver, 2011), see Table 5.1.

Table 5.1 The class, biosynthetic process affected and the year of discovery for representative natural product antibiotics for each class. Data from (Silver, 2011).

Antibiotic	Class	Biosynthetic process affected	Year of discovery
Penicillin	β-lactams	Cell wall biosynthesis	1928
Streptomycin	Aminoglycosides	Protein biosynthesis	1943
Bacitracin	Cyclic peptide	Cell wall biosynthesis	1945
Chloramphenicol	Phenicol	Protein biosynthesis	1947
Polymyxin	Polymyxins	Cell membrane integrity	1947
Chlortetracycline	Tetracyclines	Protein biosynthesis	1948
Cephalosporin	β-lactams	Cell wall biosynthesis	1948
Pleuromutilin	Pleuromutilins	Protein biosynthesis	1950
Erythromycin	Macrolides	Protein biosynthesis	1952
Vancomycin	Glycopeptides	Cell wall biosynthesis	1954
Pristinamycin	Streptogramins	Protein biosynthesis	1952
Cycloserine	Cycloserines	Cell wall biosynthesis	1955

Antibiotic	Class	Biosynthetic process affected	Year of discovery
Novobiocin	Coumarin	DNA biosynthesis	1956
Rifamycin	Ansamycins	RNA biosynthesis	1957
Lincomycin	Lincosamides	Protein biosynthesis	1961
Fusidic acid	Steroids	Protein biosynthesis	1961
Fosfomycin	Phosphonic acids	Cell wall biosynthesis	1969
Mupirocin	Pseudomonic acids	Protein biosynthesis	1971
Carbapenem	β-lactams	Cell wall biosynthesis	1976
Monobactam	β-lactams	Cell wall biosynthesis	1981
Daptomycin	Lipopeptides	Cell membrane integrity	1987

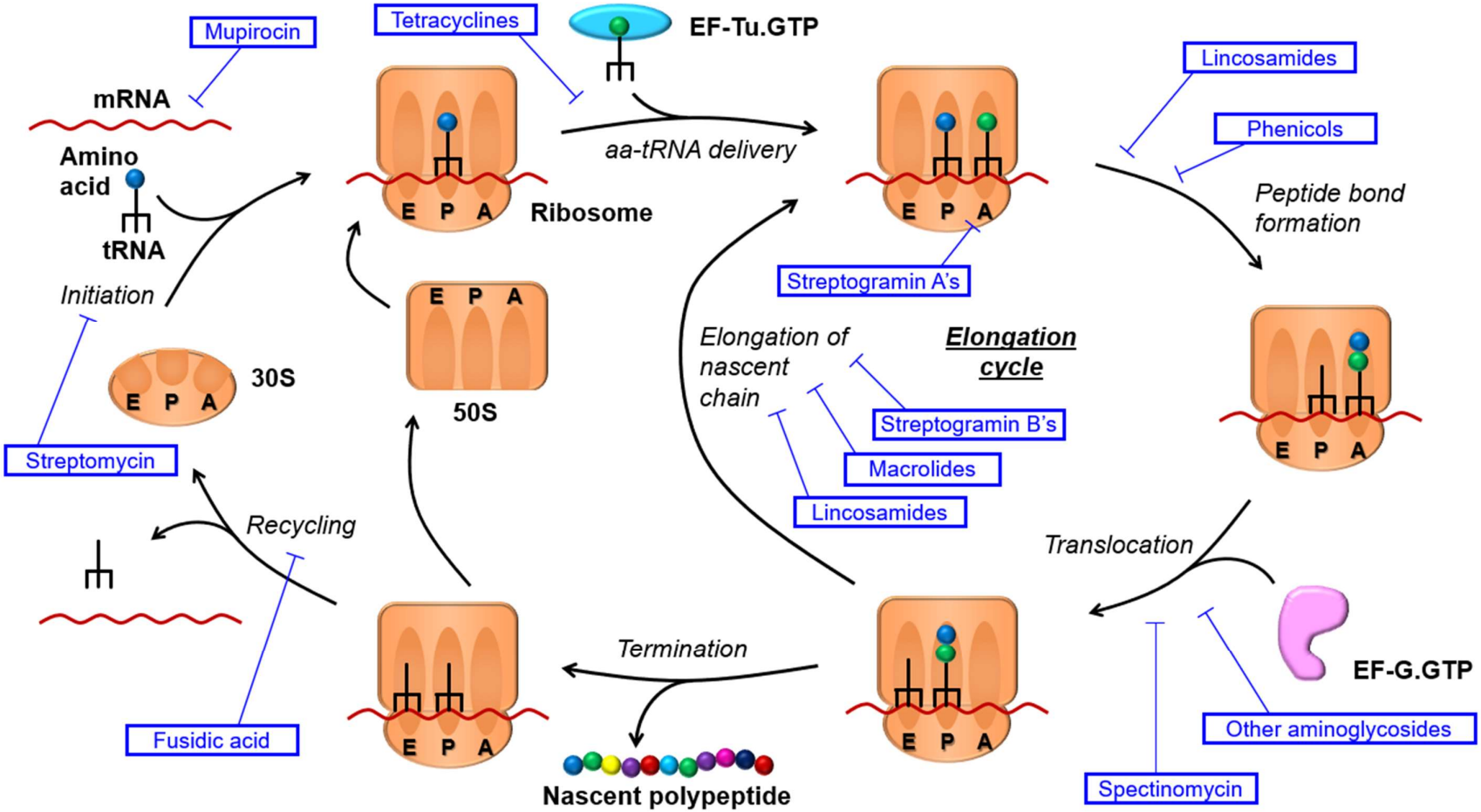


Figure 5.2 The bacterial translation process. Blue boxes show various protein biosynthesis inhibitors (PBIs) and which part of the translation process they inhibit. Inspired by Figure 1 in Wilson, 2013.

There are antibiotics that inhibit protein biosynthesis at most stages in the process, with the majority of the compounds interfering with ribosome functionality (Figure 5.2). The bacterial ribosome comprises of a 50S subunit and a 30S subunit, which come together for the purpose of RNA translation to form a 70S ribosome (Russell and Chopra, 1996). The 30S subunit consists of 16S rRNA and 21 ribosomal S proteins, and the 50S subunit consists of 23S rRNA, 5S rRNA, and 33 ribosomal L proteins (Wilson and Nierhaus, 2005).

The register for the mRNA translation is set when the Shine-Dalgarno sequence on the mRNA molecule base pairs with the anti-Shine-Dalgarno sequence at the 3' end of the 16S rRNA molecule (Walsh, 2003). Upstream of this, in the tunnel formed by the 30S subunit, is the exit (E) site, the peptidyl (P) site, and the aminoacyl (A) site. The first tRNA, charged with a methionine amino acid residue, is transferred to the P-site, where one end presents an anti-codon sequence of three bases that match with the start codon (AUG) on the mRNA molecule and the aminoacylated end protrudes into the 50S subunit. Subsequent cognate tRNA molecules are chaperoned to the A-site by EF-Tu.GTP (Wilson, 2013). The ribosome catalyses a peptide bond formation between the amino acid residues on the tRNA molecules occupying the P-site and the A-site. The tRNA molecules are then translocated to the E-site and P-site, assisted by EF-G.GTP, and the nascent peptide leaves the ribosome through the exit tunnel in the 50S subunit. Protein synthesis terminates when the ribosome encounters a stop codon on the mRNA molecule, the nascent peptide chain is released, and the ribosome dissociates into the separate 30S and 50S subunits. This is the recycling step.

Mupirocin is a metabolite of *Pseudomonas fluorescens* and was first isolated in 1971 (Fuller *et al.*, 1971). The addition of an amino acid to tRNA is performed by enzymes called amino acid tRNA synthetases (Berg *et al.*, 2002a). Mupirocin inhibits the action of isoleucyl-tRNA synthetase because it is able to structurally mimic isoleucine, the ribose and the adenine of the intermediates required to produce $tRNA^{Ile}$ (Thomas *et al.*, 2010). This means that peptide synthesis will cease when an isoleucine codon appears because the corresponding tRNA will not be attached to an amino acid.

The first tetracyclines were discovered from *Actinomycetes* soil bacteria in the 1940s (Nelson and Levy, 2011). Aureomycin (chlortetracycline) was extracted from *Streptomyces aureofaciens* and terramycin (oxytetracycline) was isolated from *Streptomyces rimosus* (Duggar, 1948; Finlay *et al.*, 1950). The removal of the chloro- group on chlortetracycline generated the semi-synthetic derivative, tetracycline, which had greater potency and solubility. Second generation tetracyclines include doxycycline, sancycline and minocycline. The mechanism of action of the tetracyclines is to bind to a site on the 16S rRNA of the 30S subunit (Ross *et al.*, 1998). This prevents aa-tRNA molecules binding to the A-site and also prevents the binding of release factors RF-1 and RF-2 to the stop codon on the mRNA, thus impeding the hydrolysis of the peptidyl-tRNA bond and subsequent release of the nascent polypeptide chain (Brodersen *et al.*, 2000).

The peptidyltransferase centre incorporates the P-site and the A-site. This is the binding site occupied by the antibiotics belonging to the $PhLOPS_A$ class (Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins and Streptogramin A's). Occupation of this site prevents the correct positioning of the aminoacylated ends of the tRNA molecules for peptide bond formation (Wilson, 2013). Antibiotics in the MLS_B class (Macrolides, Lincosamides and Streptogramin B's), inhibit protein biosynthesis by occupying the peptide exit tunnel (Tenson *et al.*, 2003).

Phenicols and lincosamides (exemplified by chloramphenicol and clindamycin, respectively) act at the peptidyltransferase centre (PTC) and occupy the binding site of A-site tRNA (Wilson *et al.*, 2008). The first lincosamide to be discovered was lincomycin, which was extracted from *Streptomyces lincolnensis* from a soil sample (MacLeod *et al.*, 1964). Another species of bacteria from soil, *Streptomyces venezuelae*, produced chloramphenicol (Ehrlich *et al.*, 1947). Tiamulin and valnemulin are semi-synthetic derivatives of the original pleuromutilin produced by *Pleurotus* fungi and which displayed activity against *Staphylococcus aureus* (Kavanagh *et al.*, 1951). Pleuromutilins occupy both the A-site and the P-site of the PTC (Eyal *et al.*, 2016).

In contrast, the oxazolidinones are entirely synthetic and were originally designed to eliminate plant pathogens (Brickner, 1996). They do not inhibit

peptidyl transferase or translation termination, despite using the same binding site as the phenicols and the lincosamides (Lin *et al.*, 1997). Instead, they inhibit the initiation step by preventing fMet-tRNA from binding to the 50S subunit and by preventing EF-G mediated elongation (Wilson *et al.*, 2008).

Streptogramins are combinations of a depsipeptide that binds to the P-site (streptogramin A's) and a macrolide that binds to the peptide exit tunnel between the PTC and the small r-proteins L4 and L22, thus blocking the exit of the growing peptide from the tunnel of the ribosome and also facilitating the release of the peptidyl tRNA from the ribosome (Hansen *et al.*, 2002). The first combination to be discovered was pristinamycin I and pristinamycin II, which are both produced by *Streptomyces pristinaespiralis* (Mast *et al.*, 2011). They act synergistically to exert a bactericidal effect, whereas they are merely bacteriostatic when used in isolation. This is partially because the binding of streptogramin A molecules results in a conformation change in the ribosome that increases its affinity for streptogramin B molecules (Beyer and Pepper, 1998). A range of other streptogramins have been extracted from natural sources, and the semi-synthetic combination of quinupristin/dalfopristin has also been produced (Beyer and Pepper, 1998).

Lincosamides and macrolides (such as erythromycin) also bind to the same site, and use the same mechanism of action, as streptogramin Bs (Tenson *et al.*, 2003). Erythromycin was the first macrolide to be discovered, in 1949, as a polyketide metabolite of *Streptomyces erythryus* (Mcguire *et al.*, 1952). A range of further natural macrolides have been discovered (such as oleandomycin and spiramycin) and semi-synthetic derivatives have been produced (Mazzei *et al.*, 1993).

The aminoglycosides are another major class of PBI, and they generally bind to the A-site, stabilising the ribosome in a conformation ordinarily achieved upon a cognate tRNA molecule base pairing with the codon currently presented in the A-site (Becker and Cooper, 2013). This leads to non-cognate tRNA molecules (tRNA with an anti-codon that does not match the mRNA codon in the A-site at that point) being accommodated instead, which results in the wrong amino acid residue being incorporated into the nascent peptide chain (nonsense translations) (Carter *et al.*, 2000). Aminoglycosides can be categorised as 4,5-

disubstituted deoxystreptamines (such as neomycin), 4,6-disubstituted deoxystreptamines (such as kanamycin) and other aminoglycosides (such as streptomycin, streptothricin and spectinomycin) (Russell and Chopra, 1996). The first two aminoglycosides to be discovered were streptothricin and streptomycin, extracted from *Actinomyces lavendulae* and *Actinomyces griseus*, respectively (Schatz *et al.*, 1944).

Fusidic acid is unusual for a protein biosynthesis inhibitor, in that it does not bind to the ribosome directly. Instead, it prevents the recycling of EF-G for use in subsequent rounds of translocation by forming a stable complex between the enzyme and the ribosome (Bodley *et al.*, 1969; Cundliffe, 1972). It also forms a stable complex with the eukaryotic EF-2 enzyme, but it is not toxic to mammalian cells because its accumulation in such cells is poor (Russell and Chopra, 1996). Fusidic acid is a steroidal compound, originally isolated from a fungal species called *Fusarium coccineum* that was obtained from faecal matter (Godtfredsen *et al.*, 1962; White *et al.*, 2012). It is effective against *Staphylococcus aureus* and there was low prevalence of resistance to it for several decades following its clinical introduction (McLaws *et al.*, 2011).

As is hopefully evident from the above summary of protein biosynthesis inhibitors (PBIs), many antibiotics from this class were produced from natural sources, as opposed to being designed synthetically. It was therefore deemed an effective strategy to apply the method of creating whole-cell biosensors for detecting novel CBIs to also detecting novel PBIs. As was mentioned in Chapter 1, generating such reporters has been attempted previously (Osterman *et al.*, 2016; Urban *et al.*, 2007). However, the PBI reporters described in (Urban *et al.*, 2007) only detected four out of the ten PBIs tested, partially because there was a gene on the shuttle vector that conferred resistance to several PBIs even though this had not been consciously engineered into the reporter, in contrast to the P_{murZ}-*lacZ*(derep) biosensor described in Chapter 4.

The P_{gltA}-*lacZ* and P_{oppB}-*lacZ* biosensors, described in section 3.1.3, were generated using data describing the transcriptional signature following treatment by early stage cell wall synthesis inhibitors (O'Neill *et al.*, 2009). These data were obtained using the technique of DNA microarray – a

technique commonly used for the generation of earlier promoter-reporter systems (Fischer, 2001; Fischer *et al.*, 2003; Moir *et al.*, 2007; Sass *et al.*, 2008; Wecke and Mascher, 2011). Since this paper from the O'Neill lab was published, the technique of RNA-seq has become a more popular choice for investigating transcriptomic profiles, as it offers several advantages over conventional microarray techniques (Wang *et al.*, 2009). The hope here was that using RNA-seq to identify the transcriptional profile upon exposure to protein biosynthesis inhibitors (PBIs) might give a more accurate genetic profile from which promoters could be chosen for the design of PBI biosensors.

5.1.2 Dereplication of protein biosynthesis inhibitors

In Chapter 4, it was demonstrated that it is possible to incorporate resistance genes into a pathway-specific biosensor, in order to supress the induction of the biosensor by antibiotic compound to which the genes confer resistance. This offers a method of screening for compounds with specific modes of action, whilst minimising the chance of re-isolating known compounds. This is an issue that has been encountered in other studies, described below, that aimed to screen natural product extracts for PBI antibiotics.

Pratt *et al*. used a luciferase reporter in a strain of *S. pneunomiae* to screen a library of around 220,000 small compounds in which 10 compounds were present in each well at a concentration of 10 μM (Pratt *et al.*, 2004). Several issues were encountered with this screen. Around 80% of the compounds tested positive for luciferase inhibition, and so they had to use a β-galacosidase based reporter in *E. coli* as a secondary screen. This had the added advantage of eliminating narrow-spectrum inhibitors that only affected *S. pneunomiae* but not *E. coli*. Macrolides constituted around 1% of the library and these had to be manually discounted. Once accounting for these factors, only 50 compounds remained in consideration. However, most of these compounds proved to be non-specific for bacteria, also affecting eukaryotic translation targets. There was only one compound remaining from these screening efforts once cytotoxic compounds and compounds in chemical classes with "known liabilities" were eliminated. This compound was structurally similar to the DNA biosynthesis inhibiting quinolones, although the mechanism of action was not similar. This compound exhibited moderate activity compared with levofloxacin against a

small number of Gram-negative organisms. The main issue to highlight with this case is that the discovery of this compound required many different screens and manual elimination of compounds and that it would be useful to have a screening system that was more streamlined.

In a study reported by Dandliker *et al.*, (2003), a luciferase reporter was used in a screening method adapted to be run at a 2 µL scale. This method was tested in a preliminary screen of a collection of 320 compounds, which returned 11 hits. Of these, seven were known ribosome inhibitors (which consisted of a mixture of macrolides, aminoglycosides, tetracyclines and chloramphenicols) and the other four were either library plate contaminants or genuinely had unreported activity against *E. coli*. Following on from these results, around 30,000 natural product extracts were screened in 1,536-well plates over a timespan of only three days. This natural product extract collection (which is around the same size as the total NCI Open Repository mentioned in Chapter 3) comprised of 99% *Actinomycetes* species and 1% fungi, cyanobacteria, lichens, and myxobacteria. This screen returned 1,327 extracts, representing a 4.6% hit rate. These were retested once they had been diluted to a quarter of their original volume and were also subjected to a counterscreen to ascertain whether or not the extracts contained components that inhibited luciferase itself, rather than the bacterial translation pathway. The final selection of 110 extracts were all obtained from *Actinomycetes* species. As was alluded to in section 4.1, known PBIs such as erythromycin and vancomycin are typically isolated from soil samples of *Actinomycetes* bacteria at a frequency of between 5×10^{-6} and 1.5×10^{-5} (Baltz, 2007). It is highly likely that these 110 extracts contained many previously isolated PBIs and it would clearly be useful to have tools for screening such extracts for these, negating the requirement of purifying individual compounds.

One of the most common antibacterial compounds produced by *Actinomycetes* species is streptothricin, at a frequency of 1 in 10 (Baltz, 2006). Streptomycin and tetracycline are also relatively abundant metabolites in this class of bacteria, being produced at frequencies between 1 in 100 and 1 in 1000. Macrolides such as erythromycin have been produced at frequencies of around 1×10^{-6}. The discovery of natural products that inhibit bacterial protein biosynthesis and that have not been discovered previously would be far more

efficient if tools were available to identify the more common compounds in natural product extracts that display antibacterial activity. In addition, streptothricins are cytotoxic and would not be suitable for clinical use, offering another reason for it to be desirable to eliminate them when screening for potential novel antibacterial compounds (Ji *et al.*, 2008).

In this chapter, a similar approach for developing a PBI dereplication strain is described as was used to develop the P_{murZ}-*lacZ*(derep) biosensor. This work occurred concurrently with the development of the PBI biosensors, and it was envisaged that, should constructs with appropriate PBI resistance genes be made successfully, they could easily be used to transform the best PBI biosensor.

5.1.3 Rationale for the resistance genes used in the dereplication constructs

Resistance to aminoglycosides can occur *via* a variety of mechanisms, including the modification of the binding site on the ribosomal 16S subunit, reducing the permeability of the outer membrane or the inner membrane transport, and exporting the compound using efflux pumps (Ramirez and Tolmasky, 2010). However, the most common mechanism responsible for resistance within clinical settings is the modification of the antibiotic itself. Acetyltransferases (AACs), nucleotidyl transferases (ANTs) and phosphotransferases (APHs) all add a modifying group to either the –OH or the $–NH_2$ groups of either the 2-deoxystreptamine nucleus or the sugar moieties of specific aminoglycosides (Ramirez and Tolmasky, 2010). These modifications all render the antibiotic incapable of binding to the ribosomal 30S subunit and inhibiting translocation.

The *sat4* gene is one of several streptothricin acetyltransferases that confer resistance to streptothricin by adding an acetyl group to the compound (Jacob *et al.*, 1994). It was imperative to use a gene conferring resistance to streptothricin, given that this antibiotic is cytotoxic and is the most abundant natural product found in extracts of *Actinomycetes* species (Baltz, 2006; Ji *et al.*, 2008).

Several aminoglycoside modifying enzymes exist as bifunctional enzymes that are coordinately encoded for by a single gene (Zhang *et al.*, 2009). Such genes

were desirable for incorporation into the PBI dereplication strain because they would be able to confer resistance to many different aminoglycosides. A selection of bifunctional modifying enzyme genes were considered and the genes chosen were the *aadA* gene and the gene encoding for the AAC(6')/APH(2") enzyme.

The *aadA* gene encodes for the ANT(3")(9) enzyme, which adds a nucleotidyl group to either the (3") –OH group of streptomycin or to the (9) –OH group of spectinomycin (Clark *et al.*, 1999; Ramirez and Tolmasky, 2010). The AAC(6')/APH(2") enzyme adds an acetyl group to the (6') $–NH_2$ group and also adds a phosphate group to the (2") –OH group of all aminoglycosides, with the exception of streptomycin, spectinomycin and streptothricin (Daigle *et al.*, 1999; Ferretti *et al.*, 1986; Zhang *et al.*, 2009) (Figure 5.3).

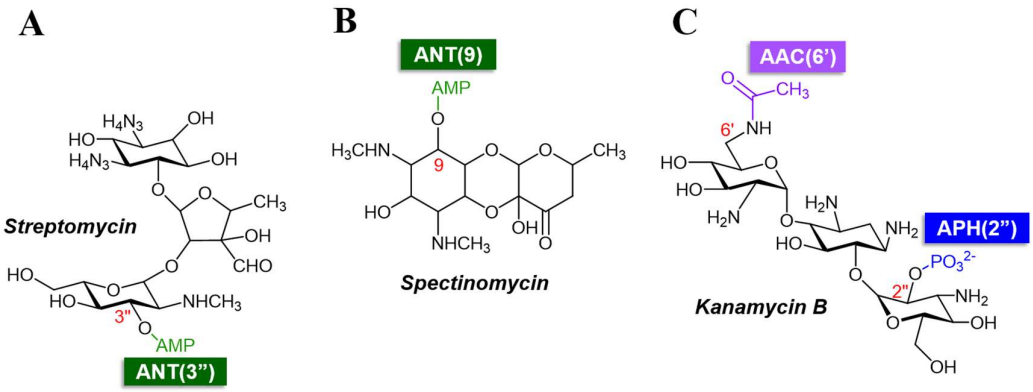


Figure 5.3 Positions of aminoglycoside modification. (A) The ANT(3")(9) enzyme adds an adenosine monophosphate (AMP) group to streptomycin at the position shown in green; (B) The ANT(3")(9) enzyme adds an adenosine monophosphate (AMP) group to spectinomycin at the position shown in green; (C) The AAC(6')/APH(2") enzyme adds an acetyl group and a phosphate group to aminoglycosides such as kanamycin at the positions shown in purple and blue, respectively. Inspired by Figure 1 in Ramirez and Tolmasky, 2010.

Tetracyclines are also one of the most abundant classes of antibacterial compounds produced by *Actinomycetes* species (Baltz, 2006). Resistance to compounds in the tetracycline class is due to either efflux proteins or ribosomal protection (Roberts, 1996). Both of these mechanisms have been discovered in *S. aureus* (Trzcinski *et al.*, 2000). Efflux proteins are encoded by genes such as *tetK* and *tetL*, but these only confer resistance to tetracycline itself and not to its derivatives. The *tetM* and *tetO* genes encode for ribosomal protection proteins

(RPPs), which confer resistance to all first and second generation tetracycline compounds, including derivatives such as doxycycline and minocycline (Trzcinski *et al.*, 2000). The TetM and TetO RPPs have high sequence similarity with the elongation factors EF-G and EF-Tu, and so it was initially proposed that they acted as tetracycline-resistant elongation factors (Sanchez-Pescador *et al.*, 1988). However, it was later discovered that their mechanism of action was the GTP-dependent displacement of tetracycline compounds from the ribosome, thus allowing aa-tRNA molecules to bind to the A-site of the ribosome (Burdett, 1996; Trieber *et al.*, 1998). RPPs do not confer resistance to the glycylcyclines (such as tigecycline) due to the steric hindrance between the 9-t-butylglycylamido moiety on the D-ring of the glycylcycline and the RPP (Bauer *et al.*, 2004; Jenner *et al.*, 2013).

Unfortunately, a third mechanism of resistance to compounds in the tetracycline class, including tigecycline, emerged. The *tetX* gene encodes for a flavin-dependent monooxygenase, which adds a hydroxyl group to tigecycline at a position that compromises its propensity for the magnesium coordination crucial for binding to the ribosome (Moore *et al.*, 2005). However, a recent review of the relevant epidemiological data implied that insusceptibility to tigecycline is still relatively low at <10%, so antibacterial compounds as potent as tigecycline need not be discounted by a PBI dereplication strain (Pournaras *et al.*, 2016). Taking this into consideration, the *tetM* gene was chosen for the second construct for a potential PBI dereplication strain.

As was alluded to in section 5.1.1, there are several classes of antibacterial compound that bind to the peptidyltransferase centre (PTC). Phenicols, lincosamides and oxazolidinones bind to the A-site (Lin *et al.*, 1997; Wilson *et al.*, 2008). Pleuromutilins occupy both the A-site and the P-site (Eyal *et al.*, 2016). Streptogramin As bind to the P-site (Hansen *et al.*, 2002). Common to all of these binding sites is that they are spatially close to 23S rRNA nucleotide A2503 (Long *et al.*, 2006).

The *cfr* gene (chloramphenicol-florfenicol resistance gene) exploits this commonality and transfers a methyl group to nucleotide A2503 (in addition to suppressing the methylation of C2498, although this is not involved in the PTC). This modification causes the antibiotic compound to occupy the binding

site in a less optimal position, thus reducing its affinity and, therefore, its ability to inhibit bacterial protein biosynthesis (Kehrenberg *et al.*, 2005). The *cfr* gene confers moderate to high level resistance to the $PhLOPS_A$ compounds (Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins and Streptogramin As) (Long *et al.*, 2006). The fact that this one gene can confer resistance to a relatively wide variety of antibiotic classes, most of which are natural products or derivatives thereof, made it an important gene to include in the PBI dereplication strain.

The peptide exit tunnel, adjacent to the PTC, is the binding site of the MLS_B compounds (Macrolides, Lincosamides and Streptogramin Bs) (Tenson *et al.*, 2003). Individual compounds classes in this group can be modified to by genes such as the erythromycin esterases (*ere* genes) and the macrolide phosphotransferases (*mph* genes) (Roberts *et al.*, 1999). They can also be subject to active efflux transporters, encoded by genes such as the *msr* genes and the *lsa* genes (Roberts, 2008).

Alternatively, in a manner similar to the mechanism of the *cfr* gene, a 23S subunit rRNA nucleotide in close proximity to the common binding site (A2058) to all of the MLS_B compounds can be modified so as to reduce the efficiency of the compounds binding to the site (Pernodet *et al.*, 1996). This is achieved by the erythromycin ribosome methylation (*erm*) genes, of which around 30 have been identified (Roberts, 2008).

The common *erm* genes found in *S. aureus* isolates are *erm(A)* and *erm(C)* (Leclercq, 2002). The *ermC* gene encodes for a 23S rRNA methyltransferase that converts a single adenine residue into N^6, N^6-dimethyladenine (Denoya and Dubnau, 1988). Its resistance profile based on the available data for the macrolides (Piątkowska *et al.*, 2012), suggested that *ermC* would be a good example of an *erm* gene to use in the PBI dereplication strain to confer resistance to multiple common PBI antibiotics, for which resistance is widespread and re-isolation during screening would be undesirable (Pernodet *et al.*, 1996).

These genes were chosen to confer resistance across a wide range of classes of protein biosynthesis inhibitors derived from natural products. Figure 5.4 expands on Figure 5.1 and illustrates the approximate proportion of natural

product-derived classes of PBIs that would be dereplicated by the two PBI biosensors that would be generated should each of the Gibson constructs be transformed into a functional PBI biosensor. In total, these PBI(derep) biosensors would be blind to around 33% of the natural product-derived antibiotics, or around 78% of the natural product-derived PBIs.

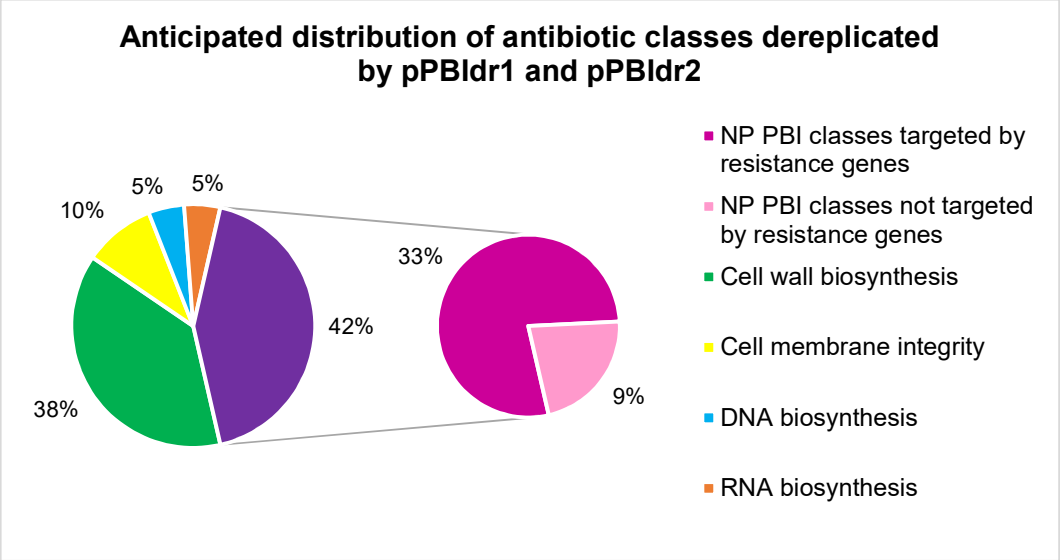


Figure 5.4 Distribution of the natural product-derived antibiotic classes theoretically dereplicated by the resistance genes in the Gibson constructs.

5.2 Results

5.2.1 RNA-seq data

The first step in obtaining a transcriptional signature of protein biosynthesis inhibition was to identify sub-inhibitory concentrations of relevant antibiotics that caused a 25% inhibition in SH1000 cell growth after 40 minutes of incubation at 37 °C. The reason that the transcriptional signature was obtained at only 25% growth inhibition was that it was necessary to identify levels of gene expression at the early onset of protein biosynthesis inhibition for the purposes of designing PBI biosensors.

A range of concentrations (as a fraction of their MIC) were trialled for tetracycline and fusidic acid. RNAprotect was added to cultures that had been

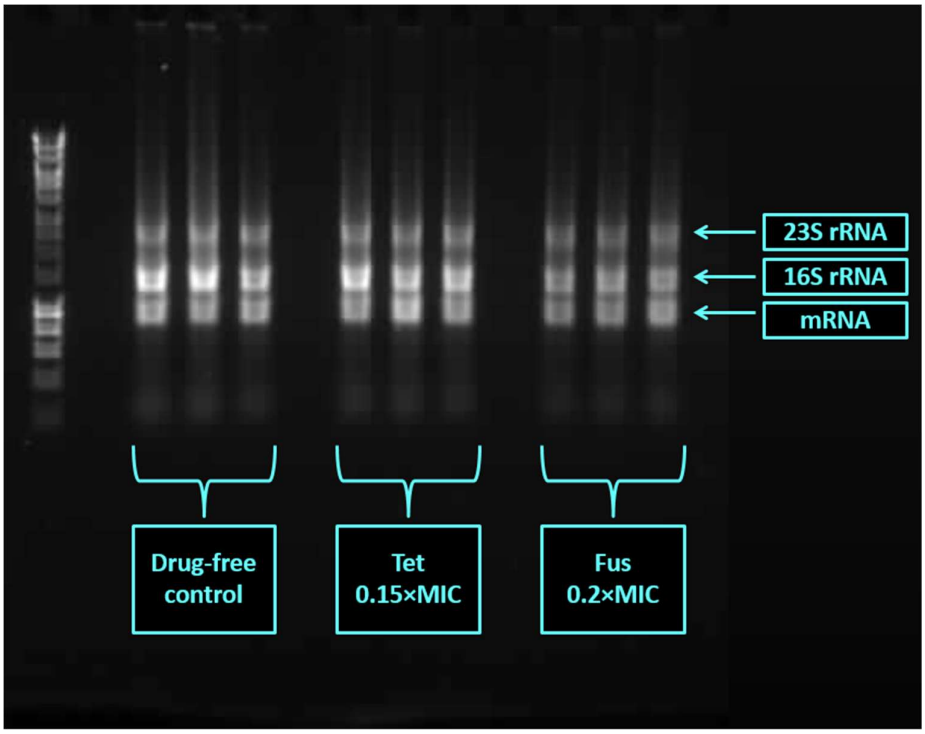


Figure 5.5 Agarose gel of RNA samples. The ladder used was Bioline's HyperLadder 1kb.

inhibited close to 25% when exposed to 0.15×MIC of tetracycline and 0.2×MIC of fusidic acid, separately. Cell pellets were obtained from this mixture and stored at –80 °C. Only two antibiotics were used to obtain RNA samples because it was decided that this would still give a sufficiently broad coverage of gene expression. It would therefore have been a waste of money to get data sequenced for more antibiotics because those selected inhibited different stages in protein biosynthesis and far more compounds could be tested later, once the PBI reporters had been generated.

RNA was extracted from the samples using the RNeasy Midi Kit from Qiagen. In addition, Qiagen's RNase-free DNase set was incorporated into the protocol in order to eliminate as much DNA as possible. The RNA samples were analysed using agarose gel electrophoresis, as shown in Figure 5.5. For all nine samples, ribosomal RNA appeared as bands at 1.5 kb and 2.9 kb (corresponding to 16S rRNA and 23S rRNA, respectively). Messenger RNA appeared as a band at 0.8 kb, as expected. tRNA was not present in quantities sufficient to make it visible on a standard agarose gel. Values were also recorded for the A_{260nm}/A_{280nm} ratio, for which a value of 1.8–2.2 indicated an acceptable lack of protein or amino acid impurities. The A_{260nm}/A_{230nm} values

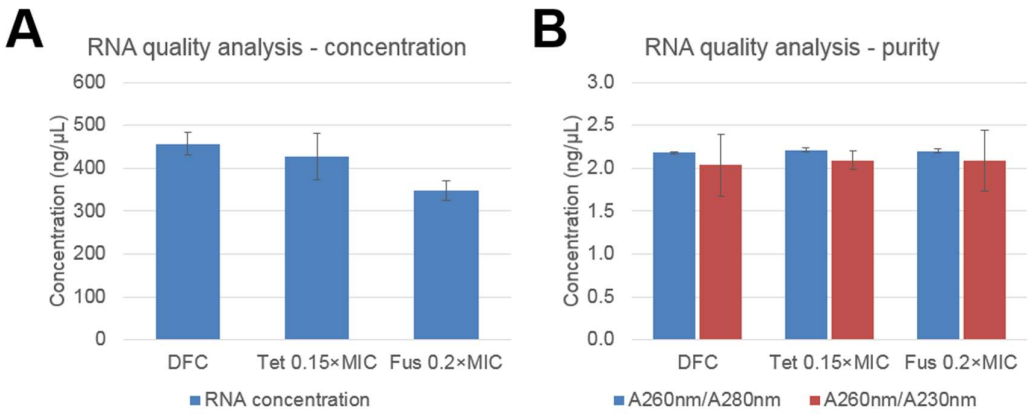


Figure 5.6 Quality analysis of the extracted RNA. A) Average concentration for each set of conditions; B) Average A_{260nm}/A_{280nm} ratio and A_{260nm}/A_{230nm} ratio for each set of conditions.

indicated a lack of other residual impurities from the RNA extraction procedure if the value was >1.7. The averages for these values across the three biological replicates of the same conditions, along with the average concentrations of RNA extracted, are shown in Figure 5.6.

Three biological replicates for each of SH1000 cells treated with 0.15×MIC tetracycline, SH1000 cells treated with 0.2×MIC fusidic acid, and SH1000 cells treated with just water, were subjected to RNA-seq. A ribosomal RNA depleted library was generated from the samples – this contained all long RNA molecules and therefore not tRNA or rRNA. The libraries generated from all nine RNA extractions were run on a single lane of an Illumina HiSeq flow cell. A single lane will usually produce around 150 million paired end reads. Paired reads were used to resolve any ambiguous alignments. Previous studies suggest that a sequencing depth of only 5–10 million non-rRNA fragments is sufficient to cover a bacterial genome (Haas *et al.*, 2012). Multiplexing is a method that allows short DNA pieces to be added to the ends of every sequencing read and act as molecular barcodes. Each sample will have unique barcodes, which means that several samples can be run on the same lane and still generate separate sets of data (Lennon *et al.*, 2010).

The RNA-seq data was mapped onto the annotated genome of *S. aureus* 8325 in CLC Genomics Workbench. Two transcriptomics analysis experiments were then set up – one that compared the data for the three biological replicates of SH1000 exposed to tetracycline to SH1000 exposed to water (DFC), and

another experiment to compare the data for the SH1000 cells exposed to fusidic acid to the three biological replicates of the DFC. An unpaired two-group comparison was performed for each of these scenarios, using the existing expression values from the samples. This experiment calculated the fold change in expression as the mean unique gene reads of a gene in one group divided by the mean reads of the same gene across the DFC replicates.

It was then necessary to determine if the genes were significantly differentially expressed beyond what would be expected given the biological variability across the replicates in each group. An EDGE test (Empirical Analyses of Digital Gene Expression) was performed for this purpose. This was an 'exact test' for two-group comparisons designed specifically to analyse data where there are many features being studied simultaneously (*i.e.* the ~2800 genes in the 8325 genome) but only a few biological replicates are available in each

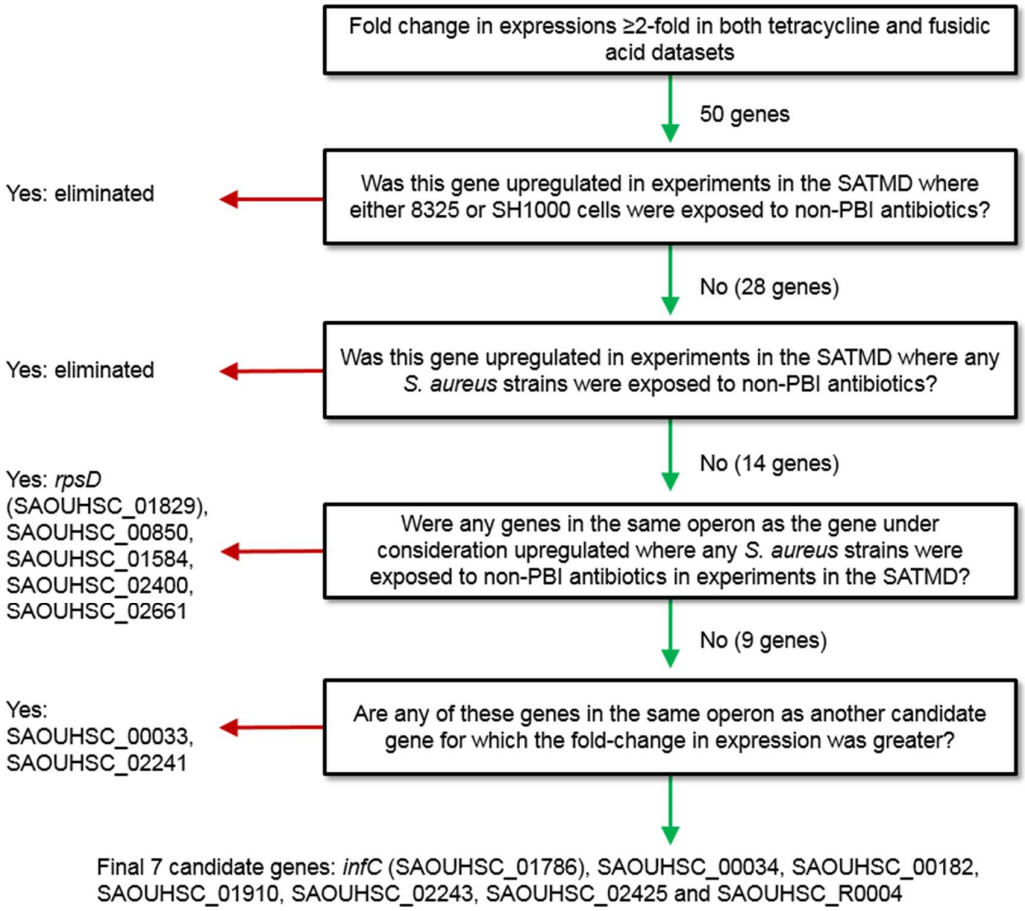


Figure 5.7 Summary of the stages in choosing the candidate genes for the PBI biosensors from the processed RNA-seq data.

group, as is usually the case with RNA-seq data (Robinson and Smyth, 2007; Robinson *et al.*, 2010). This generated *P*-values for the exact test, along with new fold change values comparing the average counts per million for the two groups in each experiment.

The data for any genes for which the mean average fold change in expression after the EDGE test was ≥2 (which here is the definition of 'upregulated', since this is a standard threshold used to define upregulated genes in transcriptional profiling experiments) were transferred into Microsoft Excel. Out of a total of 2,872 genes in the genome of *S. aureus* 8325, there were 123 genes upregulated in the presence of fusidic acid and 411 genes upregulated in the presence of tetracycline. There were 50 of these genes that were common to both sets of data, once the six genes with P-values exceeding 0.05 had been eliminated.

A flow chart of the process of narrowing these genes down to the candidate genes to be cloned into the pAJ130 vector is shown in Figure 5.7. These 50 genes were compared with the results for previous transcriptomics experiments that had been submitted to the *Staphylococcus aureus* Transcriptome Meta-Database (SATMD). The SATMD is a database of gene expression data from 251 transcriptional profiling experiments involving *S. aureus*. Initially, experiments involving either strain 8325 or SH1000 specifically were selected and any genes that were upregulated in the presence of antibiotics that did not inhibit protein biosynthesis were discounted. For example, SAOUHSC_00438 and SAOUHSC_00851 were both upregulated when challenged with 1.2 $\mu g.mL^{-1}$ oxacillin (a CBI) for 15 minutes (Muthaiyan *et al.*, 2008). SAOUHSC_00706 and SAOUHSC_00707 were both upregulated after incubation with 0.05 μM triclosan (a fatty acid biosynthesis inhibitor) for 60 minutes (Jang *et al.*, 2008).

The second round of exclusion involved considering the data on upregulated genes for all of the *S. aureus* experiments in the database, not just 8325 or SH1000. Some genes were eliminated if they were upregulated by a non-PBI antibiotic, even if they were also upregulated when SH1000 cells were treated with 2 $\mu g.mL^{-1}$ fusidic acid for 15 minutes (Delgado *et al.*, 2008). By this point,

the potential genes that could be used to make the PBI biosensors were reduced to just 14 genes.

At this point, it was necessary to establish where the promoters were for these genes. No literature was available on operon prediction in strain 8325, but there was information available on operon prediction in *S. aureus* strain Mu50 (Wang *et al.*, 2004). The final round of exclusion eliminated any genes that were part of an operon that included at least one gene that was upregulated when exposed to non-PBI antibiotics, according to the experimental data held in the SATMD. This left only seven genes from which the promoter and RBS were to be used in the PBI biosensors, listed in Table 5.2: *infC* (SAOUHSC_01786), SAOUHSC_00034, SAOUHSC_00182, SAOUHSC_01910, SAOUHSC_02243, SAOUHSC_02425 and SAOUHSC_R0004. SAOUHSC_00033 was also suitable, but it was in a two gene operon along with SAOUHSC_00034 and the fold change in expression was higher for SAOUHSC_00034, so only one biosensor using the promoter region for both of these genes was designed.

Table 5.2 The average genes reads, fold changes in expression and the general functions of the protein encoded by those genes (if known) of the 7 candidate genes.

Gene locus tag	Average gene reads (DFC)	Fusidic acid		Tetracycline		Average fold change in expression	Function in SH1000/8325	Function in N315 (for comparison)
		Average gene reads	Fold change in expression	Average gene reads	Fold change in expression			
infC (SAOUHSC_01786)	33,404.00	71,262.67	2.02	171,762.33	5.54	**3.78**	translation initiation factor IF-3	
SAOUHSC_00034	69.67	284.33	3.9	411.67	6.35	**5.125**	hypothetical protein	hypothetical protein
SAOUHSC_00182	165.33	530	3.04	531.67	3.24	**3.14**	hypothetical protein	hypothetical protein
SAOUHSC_01910	105,021.67	430,354.00	3.9	225,036.67	2.26	**3.08**	phosphoenolpyruvate carboxykinase	
SAOUHSC_02243	70	189	2.55	219.67	3.34	**2.945**	hypothetical protein	succinyl-diaminopimelate desuccinylase
SAOUHSC_02425	20,259.33	107,596.00	5.04	100,538.33	5.24	**5.14**	hypothetical protein	hypothetical protein
SAOUHSC_R0004	2,411.67	5,645.67	2.23	5,041.00	2.31	**2.27**	16S Ribosomal RNA	

5.2.2 Design and generation of the PBI biosensors

Oligonucleotide primers were designed to amplify the promoter and RBS for the candidate genes from the SH1000 chromosomal DNA, creating amplicons harbouring a site for a restriction enzyme at either end (*Kpn*I on the upper primer and *Sma*I on the lower/reverse primer).

The promoter regions for the candidate genes were amplified using PCR amplification and these amplicons were digested with *Kpn*I and *Sma*I, as was the vector pAJ130. Unfortunately, these restriction sites partially overlapped on the pAJ130 vector, and so the digest reaction was too inefficient to successfully digest the plasmid. Consequently, the strategy was altered to a *Sma*I-only digest procedure. The products of the PCR reactions to amplify the promoter regions from the SH1000 chromosome could be digested with *Sma*I only and still produce blunt-ended products because the Phusion DNA Polymerase generated blunt ends. Ligation reactions were carried out using these *Sma*I-digested vectors and inserts. These were then used to transform XL1-Blue (*E. coli*) cells and plated out onto TSA containing the ampicillin selection antibiotic for the vector, along with 40 $\mu g.mL^{-1}$ X-gal. Blue colonies indicated that the insert had been ligated into the vector in the correct orientation. Colony PCR reactions were performed to verify that only one insert of the correct length had been cloned into the pAJ130 vector. Any correct constructs were transformed into RN4220, which is restriction deficient and is therefore amenable to hosting DNA native to *E. coli* (Herbert *et al.*, 2010). Unfortunately, it was only possible to generate constructs including the promoter and RBS regions for the genes *infC* (SAOUHSC_01786), SAOUHSC_01910 and SAOUHSC_02425, despite repeated attempts to clone the inserts in both *E. coli* and *S. aureus*.

5.2.3 Testing the PBI biosensors using the β-glo assay

The P_{infC}-*lacZ* biosensor, the P_{01910}-*lacZ* biosensor and the P_{02425}-*lacZ* biosensor were tested for their specificity using the β-glo assay. The compounds used initially were fusidic acid and tetracycline as positive controls, along with vancomycin as a negative control. These were tested at concentrations corresponding to 0.25×, 1× and 4× their MIC. Disappointingly, none of the PBI biosensors that had been made responded to the initial test compounds in the manner expected (*i.e.* inducing β-galactosidase production

at least 2-fold over the drug free control for both fusidic acid and tetracycline, but not inducing expression of the *lacZ* gene when exposed to vancomycin, a CBI).

The three PBI biosensors were subsequently tested with antibiotics with various different modes of action, again at 0.25×, 1× and 4× their MIC, to see if the biosensors had specificity for any particular type of antibiotic. These results are shown, along with the earlier results for fusidic acid, tetracycline and vancomycin, in Figure 5.8, Figure 5.9 and Figure 5.10.

In order to check that the *lacZ* gene being regulated by the promoters on the biosensors was capable of producing functional β-galactosidase, the β-glo assay was performed again. This time, only the luminescence from the blank wells (just TSB) and the DFC for the three biosensors and the parental strain, RN4220, was recorded. The luminescence for the DFC for each strain was divided by the luminescence for the blank wells containing just the TSB growth media and no cells to give the luminescence ratio. The ratio for the RN4220 DFC was 1.2±0.2 which, within the standard deviation, demonstrated that the parent strain gave no background β-galactosidase activity. The ratios for the three PBI biosensors were all significantly greater than 1, indicating that the background β-galactosidase activity for the drug-free controls was all due to the expression of the *lacZ* gene on the plasmid (Figure 5.11). This confirmed that there was nothing about the pAJ130 vector that was causing the lack of specificity of the PBI biosensors for PBI antibiotics in the β-glo assay. In Figure 5.8, Figure 5.9 and Figure 5.10, green bars represent CBIs, blue bars represent DNA biosynthesis inhibitors, orange bars represent RNA biosynthesis inhibitors, red bars represent fatty acid biosynthesis inhibitors, yellow bars represent compounds that affect cell membrane integrity, and finally pink/purple bars represent PBIs. None of the antibiotics tested induced any of the three biosensors, based on an induction threshold of a β-gal activity ratio of 3-fold in the β-glo assay.

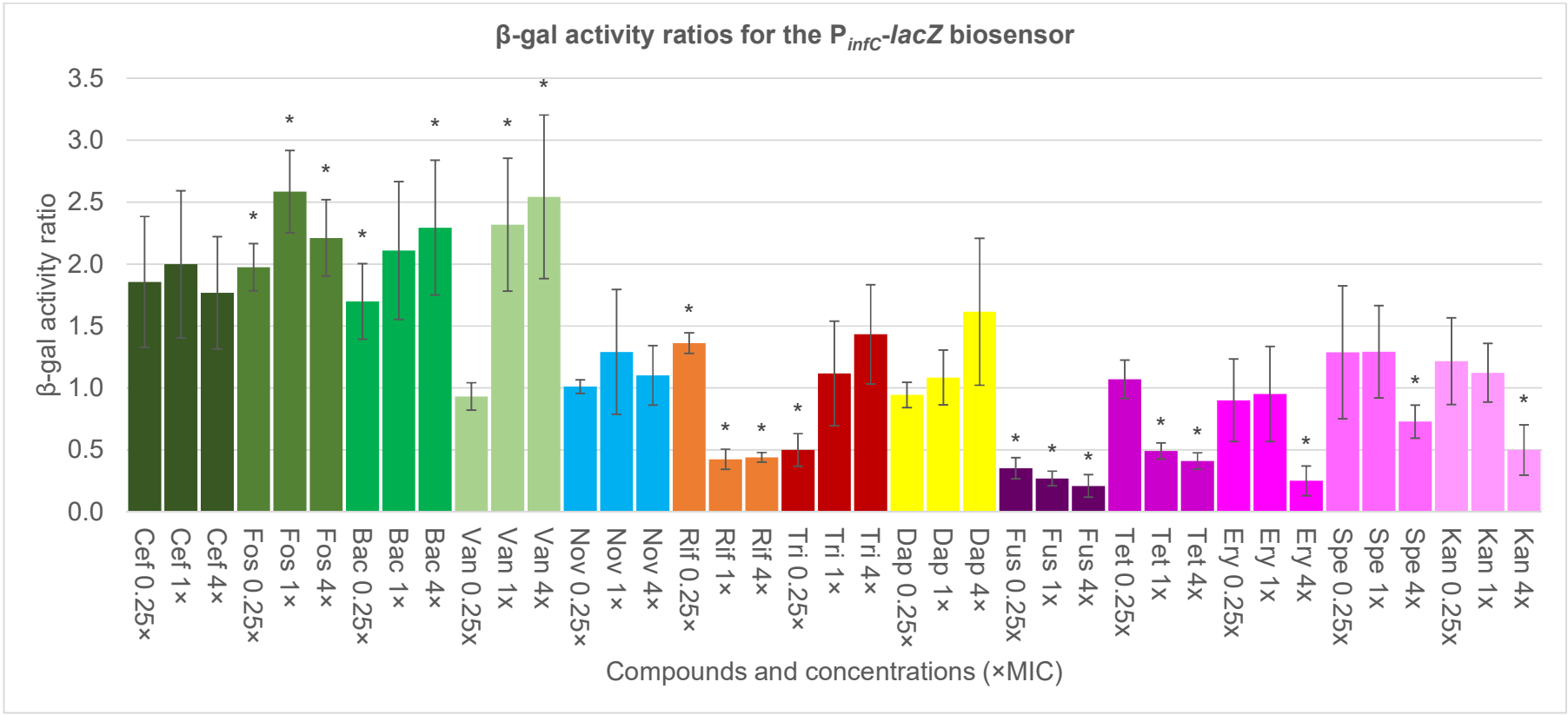


Figure 5.8 β-gal activity ratios for the P*infC*-*lacZ* biosensor. Cef = cefotaxime, MIC = 1.10 μM; Fos = fosfomycin, MIC = 87.9 μM; Bac = bacitracin, MIC = 45.0 μM; Van = vancomycin, MIC = 1.35 μM; Nov = novobiocin, MIC = 0.204 μM; Rif = Rifampicin, MIC = 0.00972 μM; Tri = triclosan, MIC = 6.91 μM; Dap = daptomycin, MIC = 0.617 μM; Fus = fusidic acid, MIC = 0.484 μM; Tet = tetracycline, MIC = 1.04 μM; Ery = erythromycin, MIC = 0.341 μM; Spe = spectinomycin, MIC = 74.3 μM; Kan = kanamycin, MIC = 1.72 μM.

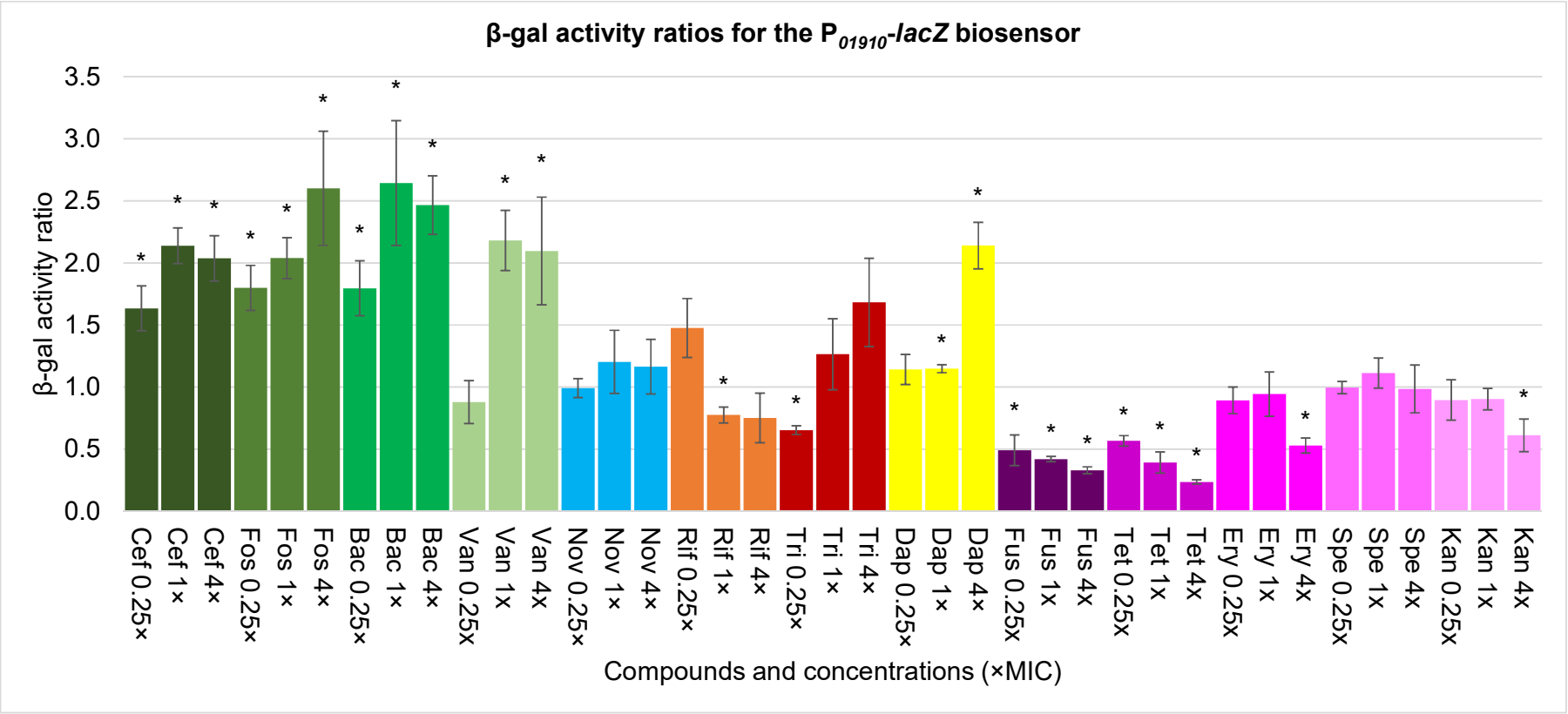


Figure 5.9 β-gal activity ratios for the P_{01910}-*lacZ* biosensor. Cef = cefotaxime, MIC = 1.10 μM; Fos = fosfomycin, MIC = 87.9 μM; Bac = bacitracin, MIC = 45.0 μM; Van = vancomycin, MIC = 1.35 μM; Nov = novobiocin, MIC = 0.204 μM; Rif = Rifampicin, MIC = 0.00972 μM; Tri = triclosan, MIC = 6.91 μM; Dap = daptomycin, MIC = 0.617 μM; Fus = fusidic acid, MIC = 0.484 μM; Tet = tetracycline, MIC = 1.04 μM; Ery = erythromycin, MIC = 0.341 μM; Spe = spectinomycin, MIC = 74.3 μM; Kan = kanamycin, MIC = 1.72 μM.

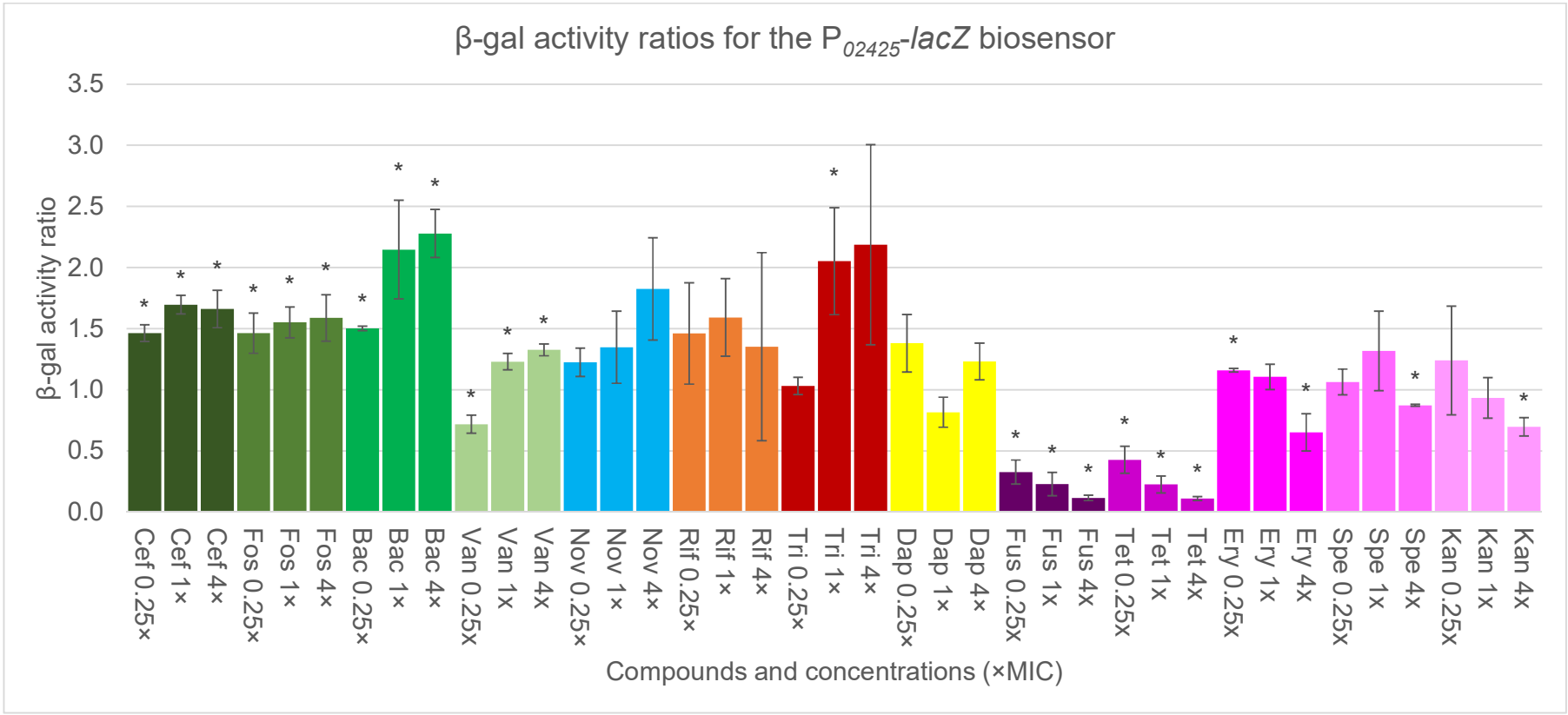


Figure 5.10 β-gal activity ratios for the P_{02425}-*lacZ* biosensor. Cef = cefotaxime, MIC = 1.10 μM; Fos = fosfomycin, MIC = 87.9 μM; Bac = bacitracin, MIC = 45.0 μM; Van = vancomycin, MIC = 1.35 μM; Nov = novobiocin, MIC = 0.204 μM; Rif = Rifampicin, MIC = 0.00972 μM; Tri = triclosan, MIC = 6.91 μM; Dap = daptomycin, MIC = 0.617 μM; Fus = fusidic acid, MIC = 0.484 μM; Tet = tetracycline, MIC = 1.04 μM; Ery = erythromycin, MIC = 0.341 μM; Spe = spectinomycin, MIC = 74.3 μM; Kan = kanamycin, MIC = 1.72 μM.

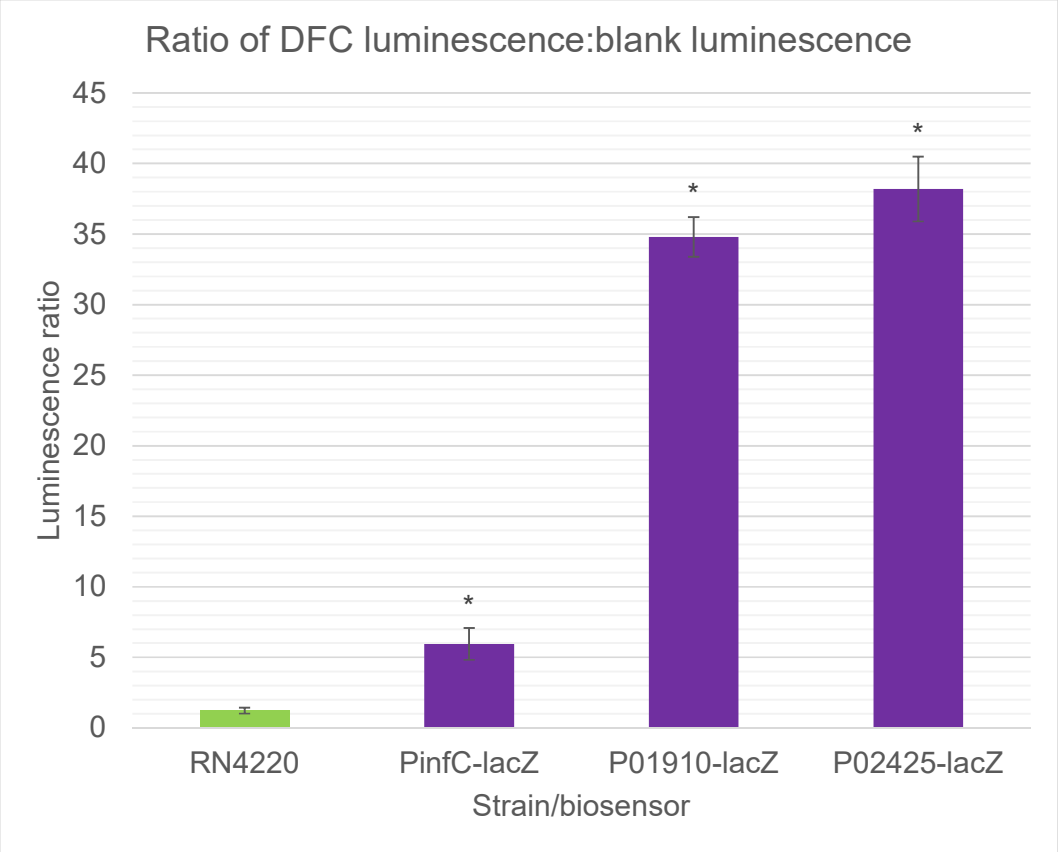


Figure 5.11 Ratio of drug-free control (DFC) luminescence:blank well luminescence for RN4220 and the three PBI biosensors.

5.2.4 Design of resistance constructs

Designs for two separate constructs, each harbouring three genes conferring resistance to common PBIs produced by *Actinomycetes* species, were conceptualised. The backbone vector used was the pRAB11 vector, amplified using the same LO and RO primers that were used in Chapter 4. The inserts to be used in the Gibson assembly strategy were synthesised in two parts, leading to a three-part Gibson assembly for each of the two constructs. This was done because the size of the total desired inserts (~3.2kbp for construct 1 and ~4.0kbp for construct 2) was too big and therefore expensive to synthesise as one complete insert.

PBI Gibson construct 1 contained the resistance genes *sat4*, *aadA* and AAC(6')/APH(2"), conferring resistance to streptothricin, streptomycin and

spectinomycin, and deoxystreptamine aminoglycosides (such as kanamycin), respectively.

PBI Gibson construct 2 contained the resistance genes *tetM*, *cfr* and *ermC*. The *tetM* gene confers resistance to tetracycline, doxycycline and minocycline. The *cfr* confers resistance to many PBIs: pleuromutilins, streptogramin As, oxazolidinones, lincosamides and phenicols. The *ermC* gene confers resistance to compounds in the MLS_B class, which comprises of macrolides, lincosamides, and streptogramin B compounds.

Figure 5.12 illustrates the design of the synthetic constructs used to make the PBI dereplication constructs.

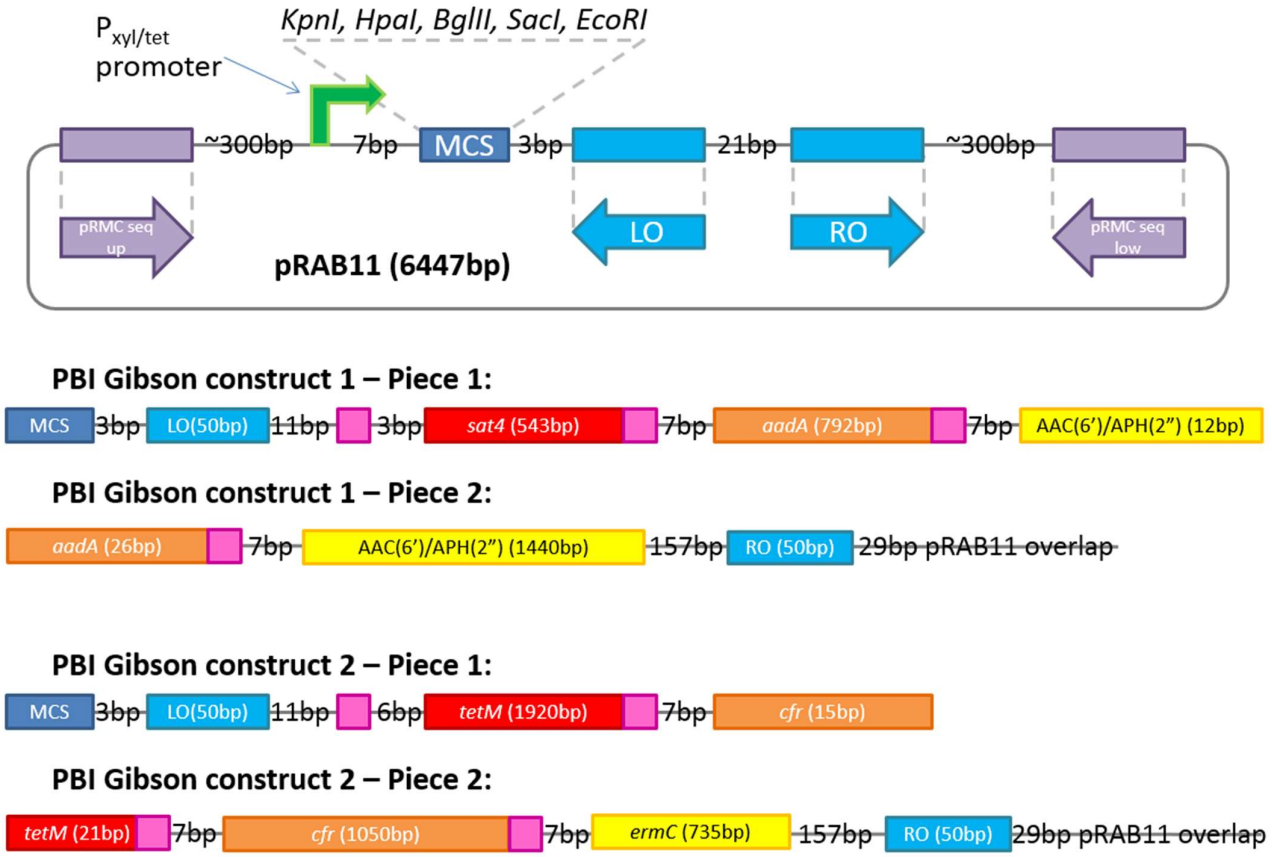


Figure 5.12 The design of the synthetic pieces of the two PBI Gibson constructs, to be assembled with the amplified pRAB11 vector at the top of the figure.

5.2.5 Generation of resistance constructs

Three Gibson reactions were carried out for each of the two constructs and for the insert-free control reaction. Once complete, the reactions were concentrated using Pellet Paint and used to transform XL1-Blue cells using a heat shock procedure. Colony PCRs were performed with a selection of colonies that appeared on the plate the next day and plasmid DNA was extracted from overnight bacterial cultures of colonies that displayed the correct size of band when the colony PCR product was analysed by agarose gel electrophoresis. The plasmid DNA of the suspected correct constructs was transformed into RN4220 cells using electroporation. Colonies from these transformations were used to inoculate fresh TSB to generate bacterial cultures from which MIC values were determined. Anhydrotetracycline (ATc) was used at a concentration of 0.15 µM to induce the expression of the resistance genes driven by the $P_{xyl/tet}$ promoter on pRAB11. It was established in Chapter 4 that this concentration of ATc was optimal for expressing the resistance genes within a biosensor. The constructs in Table 5.3, Table 5.4 and Table 5.5 are listed in the format “GC[construct #].[colony # picked from the plate of the transformation of the Gibson reactions into XL1-Blue cells]”. The GC1.10 strain (RN4220(GC1)) gave the correct phenotype (Table 5.3).

Table 5.3 MIC values to determine which Gibson construct 1 colonies displayed the correct phenotype.

Strain	MIC (μg.mL^{-1})				Correct phenotype?
	Streptothricin (*sat4* gene)	Spectinomycin (*aadA* gene)	Kanamycin (AAC(6')/APH(2") gene)	Fusidic acid (control)	
SH1000	<0.25	32	1	0.25	
GC1.2	[1]	16	>256	0.25	No
GC1.10	4	>1024	>256	0.25	Yes

Table 5.4 MIC values to determine which Gibson construct 2 colonies displayed the correct phenotype.

Strain	MIC (μg.mL^{-1})				Correct phenotype?
	Tetracycline (*tetM* gene)	Linezolid (*cfr* gene)	Erythromycin (*ermC* gene)	Fusidic acid (control)	
SH1000	0.5	1	0.25	0.25	
GC2.2	0.5	1	>256	0.25	No
GC2.3	16	0.5	>256	0.25	No
GC2.4	32	0.5	0.25	0.25	No

Since linezolid does not generally give rise to a large increase in MIC (Long *et al.*, 2006), the MIC values were also determined for GC2.3 (since this was the construct closest to showing the correct phenotype out of the three GC2 colonies tested, see Table 5.4) with tiamulin and florfenicol (Table 5.5). A higher concentration of ATc was also tested with these MICs in case the higher level of expression driven by the promoter outweighed the fitness cost of the biosynthesis of the resistance genes.

Table 5.5 Further MICs for GC2.3 (RN4220(GC2))

Strain and ATc concentration	MIC (μg.mL^{-1})			Correct phenotype?
	Tiamulin (*cfr* gene)	Florfenicol (*cfr* gene)	Fusidic acid (control)	
SH1000	0.5	2	0.25	
GC2.3 0.15 μM ATc	0.25	2	0.25	No
GC2.3 0.4 μM ATc	0.25	2	0.25	No

[1] MIC values for streptothricin were determined after those for kanamycin and spectinomycin, so no value was determined for GC1.2 with streptothricin because it had already displayed the wrong phenotype with spectinomycin.

The GC2.3 plasmid was sequenced and this revealed that a frameshift mutation had occurred due to the omission of a thymine base at base position 57 of the *cfr* gene. The GC2.2 plasmid and the GC2.4 plasmids were also sequenced to verify that this error was not repeated in the faulty *cfr* gene in those Gibson reaction products. The identical error occurring in all three faulty genes would have indicated that the synthetic DNA insert had not been synthesised correctly.

In order to obtain a construct with a functional *cfr* gene, the GC2.3 strain was plated out onto TSA containing sufficient chloramphenicol to maintain the plasmid, along with tiamulin at a concentration of 4×MIC. Colonies obtained from this plate still did not display the correct phenotype, so this process was repeated, this time using 8×MIC and 16×MIC tiamulin. No colonies appeared on the plate containing 16×MIC tiamulin, but there were 8 colonies on the plate containing 8×MIC tiamulin. The MIC values of these colonies with tiamulin were determined and one of the colonies gave an apparent MIC of 64 $\mu g.mL^{-1}$. Unfortunately, when retested, this same strain reverted to having and MIC of 0.5 $\mu g.mL^{-1}$. Consequently, culture from a well in the MIC plate containing 16 $\mu g.mL^{-1}$ tiamulin was used to determine further MICs. MICs were determined both with and without 0.15 μM ATc, and unfortunately the MIC values obtained were 128 $\mu g.mL^{-1}$ in both of these conditions.

5.3 Discussion

5.3.1 RNA-seq data

The paper by (Delgado *et al.*, 2008) describes the fusidic acid stimulon of *S. aureus*, specifically strain SH1000. In theory, this should have sufficed in the place of generating a set of RNA-seq data for the effect of fusidic acid on gene expression for this study. However, the conditions used in this paper were not comparable with investigating gene expression at the early stages of protein biosynthesis inhibition: the concentration of fusidic acid used in the paper was 2 $\mu g.mL^{-1}$ and, even though the incubation time was only 15 minutes and the experiment in this study used an incubation time of 40 minutes, this concentration is still a lot higher than 0.2×MIC, given that the MIC of fusidic acid against SH1000 is 0.125 $\mu g.mL^{-1}$. Additionally, in the reported study

(Delgado *et al.*, 2008), the antibiotic is added to the cells once they had reached an OD_{580} of 1.0 and were therefore likely to be in the stationary phase of cell growth. In contrast, in this project the fusidic acid was added when the cells had reached an OD_{625} of 0.2. This meant that the cells were multiplying at a constant rate as they were in the exponential phase of growth.

The gene *infC* encodes for translation initiation factor IF-3, which is an important component in the initiation of protein biosynthesis in bacteria, and would therefore logically be upregulated in the presence of some PBI antibiotics (Liveris *et al.*, 1993). IF-3 inhibits the premature association of the 30S subunit with the 50S subunit, which provides abundant 30S subunits required for translation initiation. It also improves the rate of the association between the codon on the mRNA and the anticodon on the cognate tRNA at the P-site (Petrelli *et al.*, 2001). This is very similar to the process inhibited by tetracycline, so it is logical that the *infC* gene is upregulated to a greater extent in the tetracycline exposure experiment, compared with its expression in the fusidic acid exposure experiment (Table 5.2).

The gene *infC* is the first gene in the IF3 operon which also includes the *rplT* gene and the *rpmI* gene (Lesage *et al.*, 1990). The *rplT* gene encodes for 50S ribosomal protein L20 and the gene *rpmI* encodes for 50S ribosomal protein L35 (Lesage *et al.*, 1990). None of these genes were upregulated by non-PBI antibiotic conditions in the experiments in the SATMD. Interestingly, the *rplT* gene and the *rpmI* gene were both upregulated when exposed to tetracycline by factors of 3.81 and 4.69, respectively. They were not upregulated in the presence of fusidic acid in this study, but were upregulated by higher concentrations of fusidic acid in a previous study (Delgado *et al.*, 2008). This could indicate that the *infC* gene is upregulated earlier during the inhibition of bacterial protein biosynthesis than the *rplT* and *rpmI* genes. The L20 protein is one of four 50S subunit repressor proteins (Raibaud *et al.*, 2003), and so it would only be useful to express this once translation had been initiated and the 30S subunit could bind to the 50S subunit to form the complete 70S ribosome.

The genes SAOUHSC_00033 and SAOUHSC_00034 were the only two genes in their operon and were not upregulated by non-PBI antibiotics. Although SAOUHSC_00033 is the first gene in the operon, SAOUHSC_00034 appeared

to be upregulated more when exposed to tetracycline and fusidic acid, so the primers were designed to include the first few codons in this gene and the whole of gene SAOUHSC_00033.

The genes SAOUHSC_02241 and SAOUHSC_02243 were the only two genes in their operon, but SAOUHSC_02243 was upregulated in cells where the expression of the *murF* gene was conditional on IPTG concentration and a suboptimal concentration of IPTG was used (Sobral *et al.*, 2007). This transcriptome was used alongside other data on the transcriptome of *S. aureus* following exposure to early stage cell wall biosynthesis inhibitors to identify the genes to be used when generating the P_{gltA}-*lacZ* biosensor and the P_{oppB}-*lacZ* biosensor. However, these conditions are not the same as direct exposure to a CBI antibiotic, and so the gene SAOUHSC_02243 was still considered a viable gene to be used for the PBI biosensor.

The genes SAOUHSC_00182, SAOUHSC_01910 and SAOUHSC_02425 were all the only gene in their operon, and were therefore all viable genes from which to use the promoter and RBS for the PBI biosensors.

The disruption of the codon-anticodon interaction between tRNA and mRNA caused by tetracycline, along with the complex formation between fusidic acid and the ribosome, necessitate the generation of additional ribosomes to overcome the presence of these PBIs. It is therefore not surprising that the expression of gene SAOUHSC_R0004, encoding for 16S rRNA, is upregulated under these conditions. Indeed, a PBI biosensor using the promoter and RBS for this gene should have particularly high specificity for PBIs in general.

The gene SAOUHSC_R0004 was not included in the dataset on *S. aureus* operon prediction. Neither could this gene be identified on the SATMD. It appeared to be in its own operon judging by the graphics of the surrounding genes in the SAOUHSC_R0004 entry on the NCBI website.

The second round of the elimination of genes from the RNA-seq results did, unfortunately, necessitate eliminating some genes with relatively high fold changes in expression. In particular, the genes SAOUHSC_01794 and SAOUHSC_02399 had average fold changes across the tetracycline and fusidic acid data sets of 12.26 and 9.035, respectively, but they were also upregulated when *S. aureus* strain Newman was exposed to vancomycin

(McCallum *et al.*, 2006). In this experiment, Newman cells were grown to an OD_{600} of 0.2, and then 10 μg.mL^{-1} vancomycin (around 5×MIC) was added to the cells, which were incubated for only 5 minutes before the RNA was extracted. Gene SAOUHSC_01794 was upregulated more by fusidic acid (17.29) than tetracycline (7.23) and is equivalent to the *gap* gene in strain Newman, which encodes for type I glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GAPDH protein is primarily considered to play a role in glycolysis, but recent studies have revealed that it binds tightly to RNA and also participates in translation control, although these studies mainly concern translation control in eukaryotes (Zhou *et al.*, 2008). Gene SAOUHSC_02399 was upregulated more by tetracycline (12.0) than fusidic acid (6.07) and is the same as the *glmS* gene in strain Newman and this encodes for glucosamine-fructose-6-phosphate aminotransferase (GFAT). This enzyme catalyses the first step of the hexosamine biosynthetic pathway, which ultimately leads to the formation of UDP-GlcNAc, a key precursor of bacterial peptidoglycan (Kato *et al.*, 2002). It is therefore not surprising that the *glmS* gene is upregulated in the presence of vancomycin, and it is logical that general protein biosynthesis inhibition would also necessitate increased expression of a gene encoding such a crucial component in cell survival.

It was a possibility that there were some genes that were strongly upregulated in only one or two of the three biological replicates, such that the mean fold change was below 2-fold (following the EDGE test) and the gene would not have been included in the initial set of upregulated genes. In order to assess whether this was the case with any of the 2,782 genes in each of the RNA-seq data sets, the fold change was calculated for each of the three biological replicates for fusidic acid and for tetracycline. Any gene for which the mean fold change was below 2-fold, but where one or two of the biological replicates gave a fold change of 3-fold or more, was identified. There were four such genes in the fusidic acid dataset and seven such genes in the tetracycline dataset. For most of these genes, the anomalous >3-fold change in expression was the result of single digit expression values, which gave a P-value much higher than the accepted 0.05 value. The exception to this was the SAOUHSC_01114 gene, which was upregulated 1.93-fold by tetracycline with a P-value of 9.17E-04. The individual fold changes were 1.65 for replicate 1, 3.23 for replicate 2,

and 0.95 for replicate 3. According to the SATMD, SAOUHSC_01114 was upregulated by mupirocin treatment (Anderson *et al.*, 2006; Reiss *et al.*, 2012), but also by suboptimal *murF*, cefoxitin, oxacillin and daptomycin (Kuroda *et al.*, 2007; Muthaiyan *et al.*, 2008; Sobral *et al.*, 2007). Therefore, it is unlikely that this would have been a suitable gene to use for the PBI biosensors after all.

5.3.2 Generation of PBI biosensors

When both ends of an insert exist as blunt ends, the direction in which the insert ligates to the vector, if at all, is random and not directional as it would have been with one cohesive end (*Kpn*I overhang of four bases) and one blunt end (from digestion with *Sma*I). One strategy for obtaining the correct construct from transforming such ligations into the XL1-Blue cells would have been to perform colony PCRs on a large selection of colonies using one of the PBI *lacZ* confirmation primers, along with the corresponding amplifying primer for each insert. However, since there was no native promoter for the *lacZ* gene on the pAJ130 vector, the expression of this gene was dependent on the insert providing a promoter and RBS to drive the expression of *lacZ* and the consequent production of β-galactosidase. The transformations could be plated out on TSA containing the ampicillin selection antibiotic for the vector, along with 40 $\mu g.mL^{-1}$ X-gal. In this case, any colonies containing the desired construct with the insert facing towards the *lacZ* gene would be blue and colonies in which the insert had been inserted facing away from the gene would be white. XL1-Blue cells do not bear a full *lacZ* gene on their chromosome, making them suitable for this application. A diagrammatic explanation of this strategy is given in Figure 5.13.

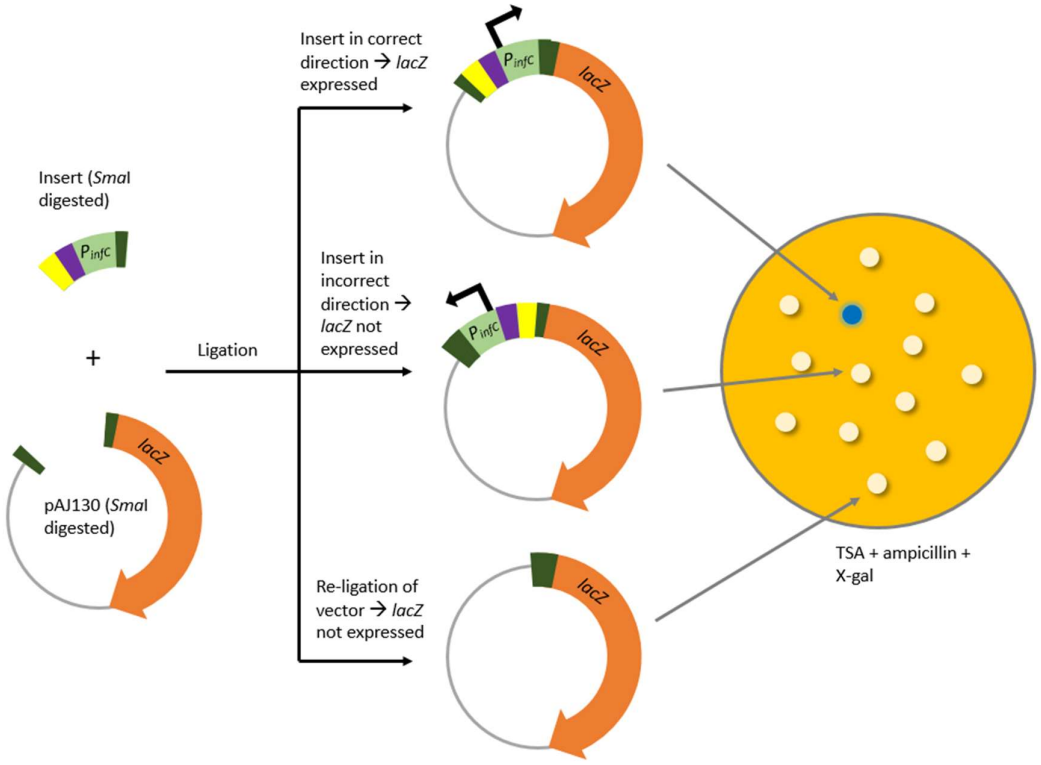


Figure 5.13 The strategy for detecting the correct orientation of the insert using blunt-end cloning after digesting the insert and the vector with only *Sma*I. Dark green = *Sma*I blunt ends. Purple = *Kpn*I restriction site. Yellow = random eight bases originally intended to aid the digestion of the promoter region amplicon using the *Kpn*I enzyme – these still gave a blunt end due to using Phusion DNA polymerase.

5.3.3 Testing the PBI biosensors using the β-glo assay

The lack of response of the PBI biosensors to exposure to either fusidic acid or tetracycline runs contrary to the results expected based on the RNA-seq data. It was verified from the sequencing data that, for the P_{infC}-*lacZ*, P_{01910}-*lacZ* and P_{02425}-*lacZ* biosensors, the promoter region insert ran into the section of the *lacZ* gene from the pMC1871 plasmid in frame. The *lacZ* gene itself was clearly expressed from the promoters on the XL1-Blue transformation plates because blue colonies were produced in a strain of *E. coli* that did not bear a native *lacZ* gene, as illustrated by the vast majority of the colonies showing no blue colour. In addition to this, Figure 5.11 illustrates that the *lacZ* gene was being expressed to produce functional β-galactosidase based on the level of expression from the P_{infC}, P_{01910} and P_{02425} promoters in the drug-free controls. Therefore, there was no issue with the pAJ130 vector itself.

When the P_{infC}-*lacZ*, P_{01910}-*lacZ* and P_{02425}-*lacZ* biosensors were tested with a further ten antibiotics, it became clear that the β-gal activity of the biosensors was not increased by antibiotics targeting any of the biosynthetic pathways represented. Furthermore, it seemed that for inhibitors of cell wall biosynthesis, DNA biosynthesis, and fatty acid biosynthesis, along with antibiotics affecting cell membrane integrity, there was a general trend of increasing β-gal activity ratios with increasing concentration of antibiotic. In contrast, increasing concentrations of inhibitors of RNA biosynthesis and inhibitors of protein biosynthesis resulted in decreasing β-gal activity ratios. The β-gal activity ratios for tetracycline and fusidic acid were particularly low compared to the ratios for the other test antibiotics.

These trends suggest that a biosensor that solely uses a promoter-reporter system that relies on the formation of a protein (such as β-galactosidase) may not be an appropriate way of detecting inhibitors of protein biosynthesis, or indeed inhibitors of RNA biosynthesis. A recent review discussed many of the biosensors that have been designed specifically to detect translation inhibitors (Osterman *et al.*, 2016). Many of these biosensors use either β-galactosidase or luciferase as the reporter gene product to give a measurable output in their assays. The biosensors that were designed to detect translation inhibitors in general tended to generate either false positive or false negative results for some of their test compounds (Bianchi and Baneyx, 1999; Galluzzi and Karp, 2003; Lampinen *et al.*, 1995; Melamed *et al.*, 2014; Urban *et al.*, 2007).

Biosensors that gave more specific responses were those that were designed to detect specific classes of PBIs. For example, biosensors for detecting members of the tetracycline class were generated by cloning a tetracycline-sensitive promoter and a tetracycline repressor upstream of genes encoding for the luciferase enzyme (Kurittu *et al.*, 2000). In the presence of tetracycline, the drug binds to the repressor, which causes it to dissociate and activate the expression of the *luc* or *lux* genes. These biosensors were capable of detecting as little as 1.25 ng of the following members of this antibiotic class: tetracycline, oxytetracycline, chlortetracycline, doxycycline, demeclocycline, methacycline and minocycline. Another example of biosensors that detect specific classes of PBIs is the work described by Möhrle *et al.*, (2007). This also involved a repressor-based system – macrolide antibiotics would bind to MphR, leading to

their dissociation from the promoter of the *mphA* gene, resulting in the expression of the *luxCDABE* operon driven by the *mphA* promoter. Having verified that this biosensor could specifically detect 12- and 14-member macrolides, plus semi-synthetic macrolides like clarithromycin and azithromycin, the biosensor was then used to screen crude extracts of several *Actinomycetes* strains that were known producers of macrolide antibiotics. The biosensor was able to detect erythromycin, picromycin and oleandomycin from their respective producing organisms.

One of the disadvantages with a lot of the reported biosensors is that they rely on an antibiotic selection marker to maintain the plasmid on which the promoter-reporter system is situated, resulting in some antibiotics potentially being missed during screening. This issue was addressed in the paper by Melamed *et al.*, (2014), where the antibiotic resistance marker was negated by using a tryptophan auxotroph strain of *E. coli*. In this paper, 14 biosensors were generated, again using the *luxCDABE* operon to produce a light signal. One of these biosensors was induced in the presence of tetracycline, oxytetracycline and chloramphenicol, but was unable to detect the aminonucleoside PBI, puromycin.

Osterman *et al.*, (2012) reported the generation of a biosensor in which the expression of the cerulean fluorescent protein was regulated by a mutated tryptophan attenuator *trpL*. The expression of the signal gene was rendered independent of the tryptophan concentration by replacing two tryptophan codons with two alanine codons. When the biosensor was not exposed to translation inhibitors, translation of the mutated leader peptide resulted in transcription termination before the reporter gene could be expressed. When the biosensor was exposed to a PBI, the ribosome would stall and the attenuation of the mutated *trpL* gene would be reduced, resulting in the production of the fluorescent protein. This was the first reported case of ribosome stalling being used as the basis for screening for translation inhibitors. Unfortunately, this biosensor did give false negative results for tetracycline and clindamycin, but on the other hand it was induced correctly by 9 other PBIs. It was also used to verify the mode of action of the novel PBI, amicoumacin A (Polikanov *et al.*, 2014).

It may be that a strategy of having a suite of biosensors or promoter-reporter systems that detect specific classes of PBIs could be less likely to result in false positive or false negative results. The tetracycline and the macrolide biosensors described above have a high specificity for their intended inducing class of compounds (Kurittu *et al.*, 2000; Möhrle *et al.*, 2007). Tetracyclines have an affinity for the TetR repressor protein that is around 1000× that of their affinity for their binding site on the ribosome, and this contributed to the exquisite sensitivity the tetracycline biosensors had for members of the tetracycline class (Möhrle *et al.*, 2007). This meant that they could detect tetracyclines at concentrations much lower than that which would inhibit the biosynthesis of the protein encoded for by the reporter gene. The results of the individual RNA-seq experiments (*i.e.* the separate tetracycline and fusidic acid datasets) could be useful in identifying promoter regions to use for class-specific biosensors of this sort, although these would not be as useful for high-throughput screening of natural products as biosensors that might reveal genuinely novel PBIs.

5.3.4 Generation of resistance constructs

The GC1.10 strain (RN4220(GC1)) displayed sufficient increases in the MIC of relevant compounds compared with the MICs of the same compounds with SH1000. Despite the reality that it was not possible to generate functional PBI biosensors during this study, this construct will prove useful in generating a PBI biosensor strain in the future that does not respond to all known aminoglycosides.

The GC2.3 strain (RN4220(GC2)) displayed the correct phenotype for the *tetM* gene and the *ermC* gene. The fact that adding selection pressure to the GC2.3 strain did not result in a mutation generating a genuinely functional *cfr* gene suggested that the tiamulin was not present on the plate at a sufficient concentration for this method to work by the time the colonies formed. The “mutated” strain that appeared to give a much higher MIC for tiamulin (64 $\mu g.mL^{-1}$) not only gave an even higher MIC upon being retested (128 $\mu g.mL^{-1}$), but gave this MIC both with and without ATc. This suggested that the resistance to tiamulin was being conferred by something other than a functional *cfr* gene controlled by the $P_{xyl/tet}$ promoter.

It has been reported that the stepwise acquisition of mutations in the *rplC* gene can result in the MIC of tiamulin with *S. aureus* RN4220 increasing >256-fold, compared to the wild type RN4220 strain (Gentry *et al.*, 2007). The *rplC* gene encodes for the ribosomal protein L3, which extends towards the binding site of tiamulin, the PTC (Wilson and Nierhaus, 2005). Incidentally, this gene was also upregulated 2.52-fold in the presence of tetracycline, according to the RNA-seq data. It is likely that the decreased susceptibility to tiamulin observed in the GC2.3 strain may be due to mutations in the *rplC* gene on the chromosome, rather than the selection of a functional *cfr* gene present on a plasmid. If this method is repeated, it may be more successful in selecting for a functional *cfr* gene if a streptogramin A compound, such as virginamycin M1, is used as the antibiotic to exert selection pressure on the gene. Hopefully, streptogramin As would not succumb to resistance conferred by mutations in a gene in the chromosome of RN4220 in the way that tiamulin did. An alternative solution would be to use site-directed mutagenesis to add a thymine base insertion in order to repair the frame-shift mutation (Sambrook *et al.*, 1989b), or to simply repeat the Gibson assembly reaction with the synthetic DNA pieces required to generate PBI Gibson construct 2.

Chapter 6 General discussion, conclusions and future work

The introduction of the sulphonamide drugs and of penicillin allowed infections caused by pathogens such as *Staphylococcus* and *Streptococcus* to be controlled for the very first time (Brown and Wright, 2016; Fleming, 1929; Wright *et al.*, 2014). Yet, by 1945, Fleming himself warned people in his Nobel Lecture about the potential dangers of antibiotic resistance emerging as a consequence of underdosing and overuse, not long after penicillin was introduced for clinical use to the general public (Fleming, 1945). Resistance to penicillin arose at around the same time, in the form of a β-lactamase enzyme that irreversibly cleaved the β-lactam ring of penicillin (Kirby, 1944). During the "Golden Age" of antibiotic discovery, several different classes of antibacterial compound were discovered, many of them being isolated from natural product producers such as soil *Actinomycetes* bacteria (Conly and Johnston, 2005; Davies, 2006). These discoveries in the 20th century gave us unprecedented control over infections such as rheumatic fever, syphilis, and pneumonia, in addition to enabling medical interventions such as cancer chemotherapy and organ transplants that would be too risky to perform had a method to prevent infections during invasive surgery or immune system suppression not been available (Wright, 2010). However, the discovery of novel antibiotics has not kept up with the rise of resistance to those drugs, and so we are facing an imminent post-antibiotic era at the current rate of drug discovery (Brown and Wright, 2016; Wright, 2015).

The time-intensive nature of continuing to screen for natural products, coupled with the increasing likelihood of re-isolating known compounds to which there was already widespread resistance, led to pharmaceutical companies abandoning the screening platforms that had been successfully initiated by Selman Waksman (Silver, 2011). Unfortunately, target-based drug discovery has failed to deliver novel antibiotics due to several reasons, but mostly because compounds typically found in large libraries manufactured for high-throughput screening cannot penetrate the bacteria cell wall (Lewis, 2013). Consequently, no novel antibiotic scaffold has been discovered since the 1980s (Silver, 2011).

The prospect of returning to screening natural product extracts in the hope of reversing this trend and discovering novel antibiotics has been increasing in popularity recently, particularly given that around 80% of all clinically used antibiotics are derived from natural products (Brown and Wright, 2016; Butler and Buss, 2006; Gullo *et al.*, 2006; Lewis, 2013; Peláez, 2006; Silver, 2008; Watve *et al.*, 2001). However, the inefficiency of natural product extract screening necessitated innovative strategies for tackling the issue. Re-isolating known compounds is a huge bottleneck in productivity for natural product screening and, in addition to this, traditional empirical screening can easily fail to detect compounds present in a complex mixture at sub-inhibitory concentrations. Using a promoter-reporter system within a whole-cell biosensor is one solution to this conundrum that has been gaining in popularity (Fischer *et al.*, 2003; Osterman *et al.*, 2016; Silver, 2011). However, many of the biosensors that have been generated are not specific enough, meaning they return false positive or false negative results (Osterman *et al.*, 2016). The work in this thesis offers a contribution to the field of using whole-cell biosensors to detect inhibitors of specific bacterial biosynthetic processes.

Chapter 3 described work that built on earlier unpublished work from the O'Neill lab and on work reported by Blake *et al.*, (2009). Initially, the specificity of the P_{gltA}-*lacZ*, P_{oppB}-*lacZ* and P_{murZ}-*lacZ* biosensors was further validated by demonstrating that β-chloro-D-alanine, cloxacillin, friulimicin and aztreonam induced all three biosensors in the full scale MUG assay, and that polymyxin B, EDTA and actinonin did not induce the biosensors. Including the validation done prior to this study by other members of the O'Neill lab, these three cell wall biosynthesis inhibitor (CBI) biosensors have now been validated using 23 CBIs, 13 protein biosynthesis inhibitors (PBIs), 5 DNA biosynthesis inhibitors, 1 RNA biosynthesis inhibitor, 1 fatty acid biosynthesis inhibitor, and 9 membrane damaging compounds. This represents more stringent validation than has typically been attempted for other published biosensors.

With the validation of the biosensors complete, the P_{murZ}-*lacZ* biosensor was used to investigate the mode of action of two compounds from the literature: rhodomyrtone and clomiphene. Rhodomyrtone was reported to inhibit the DAP biosynthesis pathway and clomiphene was initially reported to inhibit the cell wall biosynthesis enzyme, undecaprenyl diphosphate synthase (UppS) (Farha

et al., 2015; Sianglum *et al.*, 2012). It was revealed in a later paper that clomiphene was an uncoupling compound and was therefore a membrane damaging compound (Feng *et al.*, 2015). Neither of these compounds induced the biosensor, implying that neither of them inhibit peptidoglycan synthesis directly.

The P_{murZ}-*lacZ* biosensor was used to screen three libraries totalling nearly 4,000 purified compounds that comprised of FDA-approved drugs and other general bio-active compounds, using the β-glo assay. The biosensor was induced by the 46 CBIs present in the collection, and was not induced by either the 129 non-CBI antibiotics, nor by any of the other compounds that were not antibiotics. Any compounds that had given a false positive or false negative result were retested from external sources and the expected result was obtained. This screening allowed the P_{murZ}-*lacZ* biosensor to be subjected to a large number of positive and negative controls internal to the library and highlighted just how much more specific this biosensor is for CBIs than other similar biosensors.

The P_{murZ}-*lacZ* biosensor was also used to demonstrate that it could detect CBIs produced directly from their producing organism on an agar-based assay. This biosensor was also used to screen around 300 natural product extracts. Of the 192 extracts from the NCI Open Repository that were screened using the P_{murZ}-*lacZ* biosensor in the β-glo assay, 19 of these extracts induced the P_{murZ}-*lacZ* biosensor, suggesting that this biosensor is capable of detecting unpurified CBIs from a crude natural product extract. The O'Neill lab currently possesses another ~34,000 microbial extracts from the NCI Open Repository, and so one clear path for future work based on the work in this chapter would be to continue to use the P_{murZ}-*lacZ* biosensor to screen this collection using the β-glo assay in order to identify all of the extracts that could potentially contain novel CBIs. Given that this collection is primarily provided to groups screening for novel cancer therapeutics, this work may well be the initial stages of the first attempt to screen this vast collection of microbial extracts for novel antibacterial drugs.

The dual biosensors, Z0 and Z1, were generated in an attempt to create biosensors that could detect CBIs at lower concentrations than any of the

single biosensors (the P_{gltA}-*lacZ*, P_{oppB}-*lacZ* and P_{murZ}-*lacZ* biosensors). Unfortunately, the strategy of transforming a plasmid-based promoter-reporter system into a strain harbouring a chromosome-based promoter-reporter system simply resulted in an increase in the background level of β-galactosidase activity.

One possible strategy that could be used to revive the idea of using a dual biosensor would be to construct a system based on an AND logic gate. This would hopefully increase the sensitivity of the dual biosensors and facilitate their use as screening tools to reliably identify CBIs produced at low concentrations by their producing organisms. Such a system could be based on an α-complementarity system, in which one promoter could control the expression of *lacZ* with the fragment encoding the N-terminal amino acids 11–41 deleted, producing the C-terminal ω-protein. A second promoter could then control the expression of the *lacZ* fragment encoding the missing α-protein. Thus, a functional β-galactosidase enzyme could only form if both promoters were activated, improving the signal:noise ratio (Figure 6.1). For this sort of system to offer genuinely higher sensitivity over biosensors such as the P_{murZ}-*lacZ* biosensor, the two promoters would need to be from separate regulons, in order to ensure that there are two separate signals contributing to the apparent increase in β-gal activity.

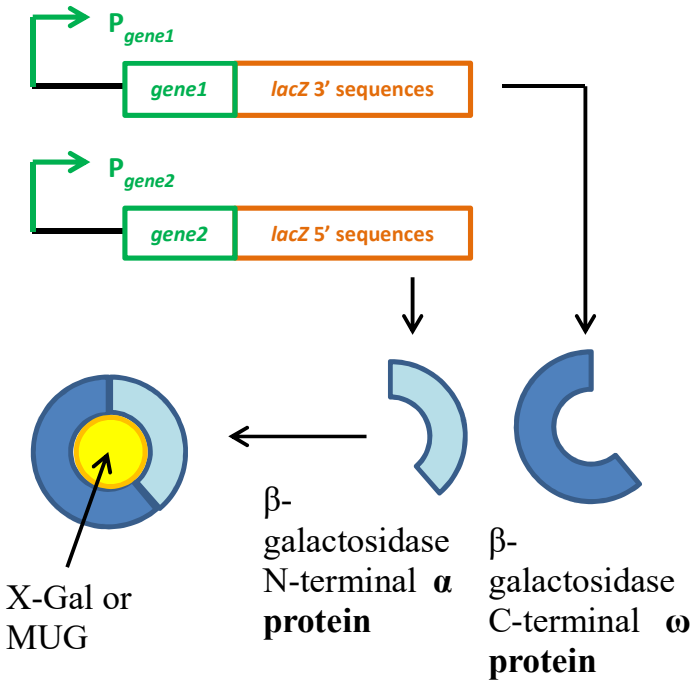


Figure 6.1 Outline of how a biosensor system based on α-complementarity could work.

Increasing the sensitivity of the P_{murZ}-*lacZ* biosensor would be particularly advantageous in the further screening of the NCI plates, given that these natural product extracts are complex mixtures and any novel CBI could be present at a relatively low concentration.

The elusive novel antibacterial compound scaffolds that modern natural product screening efforts aim to find are likely to be present in bacteria at a frequency of less than 1 in 10^7, and previously isolated compounds will be present at much higher frequencies (Baltz, 2007). Given that there may be as many as 10^{26} *Actinomycetes* species present in soil across the world, there will be a demand for a robust method for eliminating known compounds from any high-throughput screening of natural product extracts.

Cox *et al.*, (2017) recently provided a possible solution to this by generating an antibiotic resistance platform (ARP) that incorporated over 40 resistance genes, covering 15 classes of antibiotics. Cubist Pharmaceuticals also developed two Gram-negative strains containing several resistance genes to prioritise which

extracts to focus their attention on in their screen of around 8 million *Actinomycetes* strains (Gullo *et al.*, 2006). The work in chapter 4 combined this sort of strategy with the specificity of the P_{murZ}-*lacZ* biosensor to generate the first CBI biosensor (the P_{murZ}-*lacZ*(derep) biosensor) that is simultaneously capable of dereplicating several types of CBI: β-lactams, phosphonic acids and bacitracin. In addition, supplementing the growth media successfully repressed the β-gal activity ratio for a fourth type of CBI: D-cycloserine. This strategy could be incredibly useful for high-throughput screening of natural products when used in combination with the P_{murZ}-*lacZ* biosensor in a primary screen of a whole library to determine which natural product extracts contain CBIs and not membrane damaging compounds or antibiotics that inhibit other pathways. The P_{murZ}-*lacZ*(derep) biosensor plus the D-alanine supplementation could then be used to distinguish between extracts that contain common compounds from those extracts that might contain a genuinely novel antibacterial drug scaffold. Any hits from the screening of the NCI Open Repository plates can be processed through a secondary screen using this dereplication strategy. Any microbial extract that still induces the P_{murZ}-*lacZ*(derep) biosensor in the presence of D-alanine can then be ordered again from NCI in a larger quantity for further analysis and extraction.

Chapter 5 describes an attempt to apply the same strategy used for creating the P_{murZ}-*lacZ* biosensor and the P_{murZ}-*lacZ*(derep) biosensor to generating a biosensor capable of specifically detecting inhibitors of bacterial protein biosynthesis. Several translation inhibitor biosensors were made when DNA microarray was the standard technique used for measuring the read counts of each gene in a genome. However, RNA-seq is more readily available now and so this technique was used to identify the genes to use to create the biosensors in this chapter. With this being a more accurate technique than DNA microarray, it was hoped that the transcriptional profile attained using RNA-seq would be a closer representation of the genuine response of the SH1000 cells to being exposed to either tetracycline or fusidic acid, which should have lead to biosensors with excellent specificity for PBIs. Unfortunately, that did not turn out to be the case, although this RNA-seq data is at least novel in that the only fusidic acid transcriptional profile currently in the SATMD is for when a much higher concentration of fusidic acid was used. There is also not currently any

data in the SATMD describing the transcriptional signature following exposure to tetracycline.

The three PBI biosensors that were generated (the P_{infC}-*lacZ*, P_{01910}-*lacZ* and P_{02425}-*lacZ* biosensors) were tested in the β-glo assay with a total of 13 antibacterial compounds targeting a range of bacterial biosynthetic processes. The general trend observed, other than a lack of specificity for inhibitors of any particular pathway, was that increasing concentrations of either RNA biosynthesis inhibitors or PBIs led to decreasing β-gal activity ratios. The opposite trend was observed for the other 7 antibacterial compounds that did not fall into these categories.

These observations imply that depending on the biosynthesis of a protein (such as β-galactosidase) to generate a measurable signal is not a viable solution for detecting either PBIs or RNA biosynthesis inhibitors. PBI biosensors reported in the literature tend to be insufficiently specific for PBIs in general to be useful in high-throughput screening (Bianchi and Baneyx, 1999; Galluzzi and Karp, 2003; Lampinen *et al.*, 1995; Melamed *et al.*, 2014; Osterman *et al.*, 2016; Urban *et al.*, 2007). More reliable biosensors have been reported, but only for the purpose of detecting specific classes of PBIs, such as tetracyclines and macrolides (Kurittu *et al.*, 2000; Möhrle *et al.*, 2007). The nature of the promoters used in these class-specific biosensors allow them to be sensitive enough to detect that particular class at concentrations low enough to allow for the signalling protein to be synthesised in sufficient amounts. However, detecting derivatives of known classes is of limited utility, given that resistance to those derivatives is likely to arise rapidly and that the whole point of focussing on screening natural products is to uncover antibacterial compounds with genuinely previously unknown scaffolds.

Although the above examples suggest that biosensors amenable to high-throughput screening may be restricted to utilising protein biosynthesis to generate a measurable signal, it may be that a system could still be made with this in mind where the addition of a PBI does not cause a lack of functionality in the biosensor. For example, consider a biosensor that incorporated one reporter gene whose expression is regulated by a constituitive promoter and another copy of that reporter gene regulated by the promoter section of a gene

upregulated in the presence of PBIs. In the presence of a PBI, the biosynthesis of the protein encoded for by the reporter genes would be inhibited to an equal extent across both promoter-reporter systems, but the gene regulated by the promoter for the gene upregulated by PBIs would be relatively induced compared with the reporter gene regulated by the constituitive promoter.

Overall, then, this thesis provides a valuable contribution to the field of using whole-cell biosensors for the detection of novel antibiotics. A biosensor that is highly specific for detecting CBIs, both purified compounds and compounds produced directly from an organism, was engineered to generate a novel biosensor that incorporated resistance genes as a way of dereplicating several CBIs when the biosensor is used in a secondary screen of natural products. Several microbial extracts were identified that induced the main CBI biosensor. These were part of a natural product extract collection unlikely to have been used previously for screening for novel antibacterial compounds. Unsuccessful attempts to generate novel PBI biosensors highlighted potential limitations of using a promoter-reporter system that relies on the formation of a protein to emit a signal. The RNA-seq data generated could still be useful for generating other PBI biosensors with a different design. This advance in knowledge will assist with screening natural products for novel antibiotics, for which interest is increasing in the face of a lack of success of synthetic chemistry and target-based drug design. Hopefully, this work may lead to a novel antibacterial drug being discovered in a natural product screen, which would be incredibly beneficial in postponing the post-antibiotic era.

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Chapter 7 Appendices

7.1 Appendix A – Primers

Table 7.1 List of primers used in this study.

Primer name	**Primer sequence** (GGTACC = *Kpn*I restriction site; CCCGGG = *Sma*I restriction site)
pRAB11 LOb	CGTTGTAAAACGACGGCC
pRAB11 ROb	CGTTACCCAACTTAATCGCCT
pRMC seq U	ATTACGTAAAAAATCTTGCC
pRMC seq L	GTGAAAACCTCTGACACATG
pZAB S1 U	AAGAGGCTAAACCTCATATGAATTT
pZAB S1 L	CACGACTTCAAGAGCTTTTTCTA
pZAB S2 U	AACAGGGTATGAGAATTTGCAA
pZAB S2 L	AACAATAACTGCGGAAGCTATT
pZAB S3 U	GCGGTATCCTCATGTTTTTAAC
pZAB S3 L	GGGTAACGATAATCCCAGAATA
infC U	gcttaggtGGTACCGATATAAAACGAAAAAAATTCCAT
infC L	cacttggaCCCGGGGATTTGAGTTTGATCTTTTGCTAT
00034 U	tattaggtGGTACCGATGAAGTATGGAACCATTGATAT
00034 L	cacttggaCCCGGGATTAATCATTTTTTTATCATAATTCATAGT
00182 U	cgttagcaGGTACCGATAATCCAATTTCCTCTTTTAAA
00182 L	cacttggaCCCGGGATAAAGTATTTTATAGATAAAGCTTGTCAA
01910 U	tattcggtGGTACCTCCTTCTGTAACAGACTCTGAAGT
01910 L	cacttggaCCCGGGTTTGTCAATTTTAGTTGTTTCAGT
02243 U	tattcggtGGTACCTTGAATTTTTTCTTTTTCACTAAA
02243 L	cacttggaCCCGGGCGCTATTAAAACACGTTTTTTATT
02425 U	taatagatGGTACCGAATAATTTGCAATAAAAATGCTA
02425 L	cacttggaCCCGGGATTATCCTTTTTTACTGTCATTGC
R0004 U	tattatgtGGTACCTGTTTTAAATGCCTATATCCG

Primer name	**Primer sequence** (GGTACC = *Kpn*I restriction site; CCCGGG = *Sma*I restriction site)
R0004 L	cacttggaCCCGGGGCCAGGATCAAACTCTCCATA
PBI conf U	AACAAATAGGGGTTCCGC
PBI conf L	ATCGGCCTCAGGAAGATC
GC2 seq1 U	AAATCGCTGGAAGCCC
GC2 seq1 L	CCCGTGGTGACCTGATAG
GC2 seq2 U	CTATCAGGTCACCACGGG
GC2 seq2 L	TAATTCAAGGGTAAAATGGCC

7.2 Appendix B – MUG assay NIH screen

Table 7.2 β-gal activity ratios for the NIH Clinical Collection screened with the P_{murZ}-*lacZ* biosensor using the MUG assay.

Plate	Antibiotics that inhibit cell wall synthesis	β-gal activity ratio	Antibiotics that do not inhibit cell wall synthesis	β-gal activity ratio
1875	Pipericillin	4.0	Minicycline	0.3
			Norfloxacin	1.1
			Ofloxacin	0.5
			Ethambutol	1.2
			Rifampicin	0.6
			Trimethoprim	1.5
			Nalidixic acid	1.1
			Chloroxine	1.0
			Ethionamide	1.3
			Hexachlorophene	0.6
			Isoniazid	1.1
			Triclosan	0.9
			Nitrofurantoin	0.9
			Oxytetracycline	0.3
1974	Cefazolin	2.7	Pyrazinamide	1.0
	Cefoxitin	2.4	Sulfisoxazole	1.0
	Pfizerpen (penicillin G)	3.4	Sulfamethoxazole	1.1

Plate	Antibiotics that inhibit cell wall synthesis	β-gal activity ratio	Antibiotics that do not inhibit cell wall synthesis	β-gal activity ratio
	Nafcillin	3.2	Sulfacetamide	1.0
			Spectinomycin	1.1
			Demeclocycline	0.3
2073	Oxacillin	6.7	Doxycycline	0.6
	Cefuroxime	7.4	Lincomycin	1.1
	Ampicillin	8.4	Spectinomycin	1.0
	Amoxicillin	1.6	Chloramphenicol	0.7
	D-cycloserine	1.0		
2172	Cefotaxime	7.3	Gatifloxacin	0.3
	Dicloxicillin	5.9	Rifapentine	0.6
	Penicillin V	4.2	Sulfamylon	0.9
			Azithromycin	0.9
			Rifabutin	0.8
2275	D-cycloserine	0.7	Metronidazole	0.9
2302	Cefalexin	1.4	Levofloxacin	0.6
	Cefatrizine	1.5	Clarithromycin	1.0
	Meropenam	1.1	Rufloxacin	0.9
	Mecillinam	1.0	Kitasamycin	0.9

Plate	Antibiotics that inhibit cell wall synthesis	β-gal activity ratio	Antibiotics that do not inhibit cell wall synthesis	β-gal activity ratio
			Cefpodoxime proxetil[1]	1.6
2333			Pazufloxacin	0.6
			Moxifloxacin	0.6
			Oligomycin C	0.9
			Rifabutin	0.4
			Pefloxacin mesylate	0.8
2378			Rifapentine	0.6
			Rifaximin	0.6
			Linezolid	0.4
2387	Cefaclor	2.7	Dactinomycin	0.3
	Cefixime	5.2	Encroflaxacin	0.6
	Cefdinir	5.1	Rolitetracycline	0.9
2418			Telithromycin	0.6

[1] Cefpodoxime proxetil is the prodrug form of cefpodoxime, and so it would not have been expected to induce the biosensor as it existed on the plate.

7.3 Appendix C – β-glo assay screen of the NIH, Microsource Spectrum and Tocris libraries

Table 7.3 β-gal activity ratios for the NIH Clinical Collection, the Microsource Spectrum library and the Tocris Totalscreen library screened with the P_{murZ}-*lacZ* biosensor using the β-glo assay.

Antibacterial compound	β-gal activity ratio	Collection	Plate	Well	β-gal activity ratio from external source	Conditions for external source β-gal activity ratio
Antibiotics that inhibit cell wall biosynthesis **(n = 46 (+ 5 inactive prodrugs))**						
Amoxicillin	1.0	Spectrum	504	3D	17.6±8.4	10 μM. Also induces at 12.0±8.6 with 1 μM.
Ampicillin	8.7	NIH	2073	9F		
Azlocillin	9.9	Spectrum	503	4B		
Aztreonam	0.7	Spectrum	502	7H	9.3±1.5	1×MIC (MIC >256 μg.mL^{-1} or 588 μM)
Bacampicillin	1.2	Spectrum	509	4F		This form is a prodrug and would therefore not necessarily be expected to induce the biosensor.
Bacitracin	0.9	Spectrum	504	5E	5.5±0.4	0.25×MIC (MIC = 90.0 μM) (MUG assay, induction threshold ≥2-fold)
Carbenicillin	1.5	Spectrum	504	4G	20.8±2.1	10 μM
Cefaclor	3.0	NIH	2387	6B		
Cefadroxil	1.3	Spectrum	504	7G	10.0±1.0	10 μM
Cefalexin	1.7	NIH	2302(B)	2A	10.7±1.4	4×MIC. Result for 1×MIC (11.5 μM) = 2.5±0.3
Cefalonium	3.7	Spectrum	516	6G		
Cefalosporin C	1.3	Spectrum	516	4E	3.9±0.7	1×MIC (66.8 μM)
Cefalothin	4.5	Spectrum	504	9G		
Cefamandole	2.0	Spectrum	502	6E	8.9±0.7	10 μM
Cefapirin	1.6	Spectrum	504	10G	22.7±7.7	10 μM

Antibacterial compound	β-gal activity ratio	Collection	Plate	Well	β-gal activity ratio from external source	Conditions for external source β-gal activity ratio
Cefatrizine	9.2	NIH	2302(C)	3G		
Cefazolin	5.6	NIH	1974	5C		
Cefdinir	15.1	Spectrum	511	5E		
Cefditorin pivoxil	5.0	Spectrum	511	5H		This form is a prodrug and would therefore not necessarily be expected to induce the biosensor.
Cefixime	3.4	NIH	2387	10C		
Cefmetazole	1.0	Spectrum	502	7E	21.0±4.2	10 µM
Cefonicid	1.0	Spectrum	512	10C	5.4±0.3	10 µM
Cefoperazone	2.2	Spectrum	502	8E	5.8±0.5	10 µM
Cefotaxime	18.6	NIH	2172	4B		
Cefoxitin	3.7	NIH	1974	5F		
Cefpodoxime proxetil	1.6	NIH	2302(A)	7G		This form is a prodrug and would therefore not necessarily be expected to induce the biosensor.
Cefprozil	0.8	Spectrum	502	9H	8.8±0.3	10 µM
Cefradine	1.0	Spectrum	504	11G	2.0±0.03	1×MIC (22.9 µM) (MUG assay, induction threshold ≥2-fold)
Cefsulodin	1.3	Spectrum	509	6B	4.9±0.2	10 µM
Ceftazidime	0.8	Spectrum	513	7B	4.9±0.4	1×MIC (MIC = 16 $\mu g.mL^{-1}$ or 25.1 µM)
Ceftibuten	6.9	Spectrum	511	4B		
Ceftriaxone	0.9	Spectrum	509	9F	5.7±0.5	10 µM
Cefuroxime	10.1	NIH	2073	8G		

Antibacterial compound	β-gal activity ratio	Collection	Plate	Well	β-gal activity ratio from external source	Conditions for external source β-gal activity ratio
Cloxacillin	2.5	Spectrum	505	8B	6.4±0.8	4×MIC (MIC = 0.273 μM) (MUG assay, induction threshold ≥2-fold)
D-cycloserine	1.2	NIH	2073	10E	9.2±1.6	4×MIC (MIC = 32 μg.mL^{-1} or 313 μM)
Dicloxacillin	29.1	NIH	2172	3C		
Fosfomycin	1.2	Spectrum	509	7B	6.8±0.3	100 μM
Hetacillin	2.1	Spectrum	506	3D		This form is a prodrug and would therefore not necessarily be expected to induce the biosensor
Mecillinam	1.8	NIH	2302(C)	7C	7.6±0.8	8×MIC (MIC = 16 μg.mL^{-1} or 49.2 μM). Result for 4×MIC = 2.7±0.4
Meropenam	1.5	NIH	2302(C)	4D	18.8±1.1	10 μM. Also induces at 12.6±1.4 with 1 μM.
Metampicillin	0.7	Spectrum	515	4A		This form is a prodrug and would therefore not necessarily be expected to induce the biosensor.
Methicillin	1.7	Spectrum	506	2H	3.3±0.7	4×MIC (MIC = 9.94 μM) (MUG assay, induction threshold ≥2-fold)
Moxalactam	1.0	Spectrum	501	5C	4.0±0.4	1×MIC (MIC = 8 μg.mL^{-1} or 14.2 μM)
Nafcillin	21.1	Spectrum	501	7C		
Oxacillin	11.3	NIH	2073	2B		
Penicillin G	10.2	Spectrum	507	3A		
Penicillin V	53.6	NIH	2172	3G		
Piperacillin	5.3	NIH	1875	5G		
Teicoplanin	4.0	Spectrum	512	11G		
Ticarcillin	3.6	Spectrum	512	8F		

Antibacterial compound	β-gal activity ratio	Collection	Plate	Well	β-gal activity ratio from external source	Conditions for external source β-gal activity ratio
Vancomycin	1.7	Spectrum	508	11B	6.0±0.3	10 μM
Compounds that adversely affect cell membrane integrity **(n = 17)**						
Berberine	0.9	Spectrum	516	2E		
Chlorhexidine	1.9	Spectrum	508	8H		
Chlorixine	0.2	NIH	1875	9C		
Chloroxylenol	0.9	Spectrum	505	3A		
Clofoctol	1.8	Spectrum	515	5A		
Cloxyquin	0.7	Spectrum	505	9B		
Colistimethate sodium	1.3	Spectrum	505	11B		
Colistin sulfate	1.0	Spectrum	513	6C		
Curcumin	1.0	Spectrum	525	11D		
Gramicidin	0.5	Spectrum	506	7C		
Hexachlorophene	0.4	NIH	1875	10B		
Palmatine	1.0	Spectrum	517	3H		
Parachlorophenol	0.9	Spectrum	501	3F		
Polymyxin B	0.9	Spectrum	507	4B		
Salinomycin	0.2	Spectrum	516	8F		
Tomatine	1.5	Spectrum	517	10H		
Tyrothricin	1.2	Spectrum	508	7B		
Inhibitors of DNA synthesis **(n = 57)**						
2-aminobenzenesulfonamide	1.4	Spectrum	518	3F		
Aminosalicylate sodium	0.8	Spectrum	504	10C		

Antibacterial compound	β-gal activity ratio	Collection	Plate	Well	β-gal activity ratio from external source	Conditions for external source β-gal activity ratio
Ciprofloxacin	1.2	Spectrum	510	8E		
Clofazimine	1.1	Spectrum	513	2D		
Dapsone	1.6	Spectrum	505	4D		
Enoxacin	0.9	Spectrum	504	4H		
Enrofloxacin	0.7	Spectrum	513	11G		
Flumequine	1.0	Spectrum	508	2H		
Furaltadone	0.5	Spectrum	515	10D		
Furazolidone	1.0	Spectrum	506	9B		
Gatifloxacin	0.8	NIH	2172	3H		
Gemifloxacin	1.0	Spectrum	503	10F		
Levofloxacin	1.7	NIH	2302(C)	3F		
Lomefloxacin	0.8	Spectrum	502	5E		
Mafenide	0.9	Spectrum	501	2A		
Metronidazole	1.2	NIH	2275	4D		
Moxifloxacin	0.9	NIH	2333	3F		
Nalidixic acid	1.0	Spectrum	523	3F		
Nitrofurantoin	0.4	Spectrum	501	4D		
Norfloxacin	0.7	NIH	1875	4A		
Novobiocin	0.8	Spectrum	501	3E		
Ofloxacin	1.1	Spectrum	502	9E		
Orbifloxacin	0.9	Spectrum	515	3A		
Oxolinic	0.8	Spectrum	502	10D		

Antibacterial compound	β-gal activity ratio	Collection	Plate	Well	β-gal activity ratio from external source	Conditions for external source β-gal activity ratio
Pazufloxacin	0.6	NIH	2333	3A		
Pefloxacine	0.7	Spectrum	511	11F		
Phthalylsulfacetamide	1.1	Spectrum	516	4G		
Phthalylsulfathiazole	0.9	Spectrum	502	6D		
Pipemidic acid	0.3	Spectrum	514	9H		
Piromidic acid	0.4	Spectrum	514	6G		
Rufloxacin	0.6	NIH	2302(B)	11F		
Sarafloxacin	1.0	Spectrum	511	5G		
Succinylsulfathiazole	1.1	Spectrum	502	7D		
Sulfabenzamide	1.4	Spectrum	507	3F		
Sulfacetamide	1.5	Spectrum	507	4F		
Sulfachlorpyridazine	0.9	Spectrum	502	6A		
Sulfadiazine	1.5	Spectrum	507	5F		
Sulfadimethoxine	0.7	Spectrum	502	7A		
Sulfadoxine	0.9	Spectrum	502	2H		
Sulfaguanidine	1.1	Spectrum	516	2H		
Sulfamerazine	1.5	Spectrum	507	6F		
Sulfameter	0.8	Spectrum	502	6B		
Sulfamethazine	1.2	Spectrum	507	7F		
Sulfamethizole	1.3	Spectrum	507	8F		
Sulfamethoxazole	1.1	NIH	1974	3C		
Sulfamethoxypyridazine	1.0	Spectrum	502	7B		

Antibacterial compound	β-gal activity ratio	Collection	Plate	Well	β-gal activity ratio from external source	Conditions for external source β-gal activity ratio
Sulfamonomethoxine	0.7	Spectrum	502	9A		
Sulfanilamide	1.1	Spectrum	516	4D		
Sulfanilate	1.4	Spectrum	510	6C		
Sulfanitran	1.3	Spectrum	510	11C		
Sulfaphenazole	0.2	Spectrum	514	8A		
Sulfapyridine	0.9	Spectrum	507	10F		
Sulfaquinoxaline	0.9	Spectrum	502	11H		
Sulfathiazole	1.4	Spectrum	507	2G		
Sulfisoxazole	1.0	NIH	1974	3B		
Tosufloxacin	0.9	NIH	2302(C)	7B		
Trimethoprim	0.9		508	10A		
Inhibitors of RNA synthesis **(n = 6)**						
Dactinomycin	0.05	NIH	2387	9H		
Rifabutin	0.5	NIH	2172	6B		
Rifampicin	0.1	NIH	1875	6B		
Rifampin	0.2	Spectrum	507	3E		
Rifapentine	0.6	NIH	2172	3F		
Rifaximin	0.4	NIH	2378	9B		
Inhibitors of protein synthesis **(n = 44)**						
Apramycin	0.9	Spectrum	511	4F		

Antibacterial compound	β-gal activity ratio	Collection	Plate	Well	β-gal activity ratio from external source	Conditions for external source β-gal activity ratio
Azithromycin	2.5	NIH	2172	5B		
Bekanamycin	0.9	Spectrum	508	9F		
Capreomycin	0.9	Spectrum	504	11F		
Chloramphenicol	1.6	Spectrum	504	5H		
Chlortetracycline	0.02	Spectrum	505	7A		
Clarithromycin	1.1	NIH	2302(B)	9D		
Clindamycin	1.1	Spectrum	505	4B		
Demeclocycline	0.06	Spectrum	505	7D		
Dibekacin	0.4	Spectrum	516	11A		
Dihydrostreptomycin	1.2	Spectrum	505	7F		
Dirithromycin	0.7	Spectrum	503	7C		
Doxycycline	0.1	NIH	2073	2C		
Enoxolone	1.0	Spectrum	516	6E		
Erythromycin	1.6	Spectrum	516	5B		
Florfenicol	0.8	Spectrum	513	6D		
Fusidic acid	0.02	Spectrum	506	11B		
Geneticin	1.1	Spectrum	525	9D		
Gentamicin	1.3	Spectrum	506	4C		
Kanamycin A	1.0	Spectrum	506	2G		
Kitasamycin	1.8	NIH	2302(B)	11C		
Lincomycin	1.4	NIH	2073	3C		
Linezolid	0.1	NIH	2378	4C		

Antibacterial compound	β-gal activity ratio	Collection	Plate	Well	β-gal activity ratio from external source	Conditions for external source β-gal activity ratio
Meclocycline	0.1	Spectrum	502	3F		
Minocycline	0.03	NIH	1875	3F		
Mupirocin	0.4	Spectrum	512	10G		
Neomycin	1.0	Spectrum	506	7H		
Netilmicin	0.9	Spectrum	512	2D		
Oleandomycin	0.6	Spectrum	516	9D		
Oxytetracycline	0.003	NIH	1875	11H		
Paromomycin	1.0	Spectrum	509	8H		
Ribostamycin	0.5	Spectrum	516	10F		
Rolitetracycline	1.1	NIH	2387	3G		
Roxithromycin	1.1	Spectrum	510	2C		
Sisomicin	1.7	Spectrum	507	8E		
Spectinomycin	1.2	Spectrum	509	9E		
Spiramycin	0.9	Spectrum	509	8A		
Streptomycin	1.7	Spectrum	507	11E		
Telithromycin	1.1	Spectrum	511	7F		
Tetracycline	0.9	NIH	1974	3G		
Thiamphenicol	0.9	Spectrum	509	5G		
Tilmicosin	1.4	Spectrum	511	9C		
Tobramycin	1.5	Spectrum	507	7H		
Tylosin	1.2	Spectrum	511	4G		

Antibacterial compound	β-gal activity ratio	Collection	Plate	Well	β-gal activity ratio from external source	Conditions for external source β-gal activity ratio
Inhibitors of fatty acid synthesis **(n = 4)**						
Ethionamide (prodrug)	1.2	Spectrum	506	8A		
Isoniazid	1.0	Spectrum	506	7F		
Pasiniazid	1.0	Spectrum	515	8F		
Triclosan	0.2	NIH	1875	10F		
Compounds listed by Microsource as antibacterial with unknown or miscellaneous mode of action **(n = 28)**						
Alexidine	1.0	Spectrum	514	6H		
Arsanilic acid	0.9	Spectrum	512	3F		
Asarinin (-)	1.0	Spectrum	524	8G		
Avocadyne	0.9	Spectrum	525	9F		
Benzoylpas	0.9	Spectrum	512	3A		
Benzyl isothiocyanate	0.9	Spectrum	516	5H		
Captan	0.9	Spectrum	516	2G		
Carbadox	0.9	Spectrum	513	10G		
Conessine	1.2	Spectrum	524	6D		
Ethambutol	0.7	Spectrum	506	6A		
Garlicin	1.2	Spectrum	525	10B		
Gentian violet	0.8	Spectrum	506	5C		
Merbromin	1.5	Spectrum	516	2D		
Methenamine	0.1	Spectrum	506	11G		
Monensin	0.1	Spectrum	509	6D		

Antibacterial compound	β-gal activity ratio	Collection	Plate	Well	β-gal activity ratio from external source	Conditions for external source β-gal activity ratio
Natamycin	0.3	Spectrum	512	11D		
Nithiamide	1.2	Spectrum	512	7B		
Nitromide	0.9	Spectrum	506	9H		
Oligomycin C	0.5	Spectrum	2333	8B		
Paeonol	1.3	Spectrum	523	9B		
Patulin	0.8	Spectrum	521	6A		
Physcion	1.4	Spectrum	519	11B		
Piperine	0.8	Spectrum	508	9G		
Protionamide	0.6	Spectrum	514	10C		
Pyrazinamide	0.3	Spectrum	507	3D		
Pyrithione zinc	0.2	Spectrum	505	4G		
Roxarsone	1.0	Spectrum	507	4E		
Tramiprosate	0.2	Spectrum	517	6A		