The Application of Antisense Silencing for the Characterisation of Essential Gene Stringency and for the Development of Species-Specific Antimicrobials

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Abstract

The emergence of multiple-antibiotic resistance among clinical pathogens has created an urgent requirement for the development of new antibiotics. The current lack of new antibiotics has not only renewed interest in traditional natural product screening approaches, but also prompted efforts to develop alternate antimicrobial strategies.

Antisense RNA based silencing provides a strategy for developing whole cell screening assays, whereby antisense RNA induction leads to target protein depletion and subsequently the increased sensitivity of test organisms to target specific inhibitors. The development of synthetic derivatives to expressed antisense RNA such as peptide Nucleic Acids (PNA), has also been explored for use as bacterial inhibitors. This thesis aims to examine two novel antimicrobial strategies, firstly by comparing mRNA and protein based techniques to evaluate essential gene requirement in bacteria, to identify novel targets for antibiotic screening assays. Secondly, to evaluate the potential use of peptide peptide-PNA’s as antimicrobials capable of targeting individual bacterial species.

To successfully develop either approach requires the identification and validation of suitable gene encoded molecular targets. Essential genes may provide potential candidates, yet a suitable system is necessary for characterisation to enable genes to be ranked, so that the most suitable targets can be prioritized. A disproportionate growth requirement (stringency) is known to exist among essential genes, which provides a means to delineate between essentially required targets, yet is based upon the measurement of mRNA abundance. Due to post-transcription and translation mechanisms, mRNA does not provide a reliable indicator of expressed protein, which represents the ultimate output of gene expression.

This study demonstrates the use of a quantitative proteomics strategy for evaluating essential gene stringency at the protein level, using the *E.coli* gene *fabI*. Using expressed antisense RNA silencing to deplete target protein concentration and to reduce normal growth rate to 50%, absolute protein determinations were used to define a Minimum Protein Level (MPL), for the quantitative characterisation of essential gene stringency. To support the
justification of evaluating gene stringency using expressed protein abundance, the stringency of operon based genes fusA and rplE using antisense RNA silencing was investigated and revealed transcript profiles that contradict the use of Minimum Transcript Level (MTL\textsubscript{50}) previously used to define gene stringency.

Finally, to demonstrate a potential application that would benefit from the characterisation of essential gene stringency, the species-specificity of a peptide-PNA targeting the essential gene ftsZ was evaluated. Exposing a mixed culture of \textit{S.typhimurium} and \textit{E.coli} to a peptide-PNA conjugate, incorporating a 2 base pair mismatch demonstrated the capacity to inhibit translation of ftsZ in \textit{S.typhimurium} but not \textit{E.coli}.

This study highlights how characterising essential genes using the MPL\textsubscript{50} can be used to delineate stringently required gene targets to support antimicrobial screening and the development of species specific antimicrobials. Furthermore, the applications of evaluating gene stringency may be extended further, to provide a tool for standardising genetic components in synthetic biology approaches.
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This thesis was in many ways a collaboration, since it was completed with the extensive support and advice from many individuals, for which I am eternally grateful.

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Lastly I would like to thank my long suffering Maddie (Boobaa) for putting up with the mood swings, frustration and the myriad of difficulties associated with individuals undertaking a PhD.

Ashley Chessher, September 2013
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<thead>
<tr>
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<tr>
<td>°C</td>
<td>Degree Celsius</td>
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<tr>
<td>a, A</td>
<td>Adenine</td>
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<tr>
<td>ACP</td>
<td>Acryl Carrier Protein</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5′-triphosphate</td>
</tr>
<tr>
<td>AUG</td>
<td>Translation start codon</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
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<tr>
<td>BSA</td>
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<td>c, C</td>
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<tr>
<td>cDNA</td>
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<tr>
<td>Cfu</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CoA</td>
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</tr>
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<td>DHF</td>
<td>7,8-dihydrofolate</td>
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<tr>
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<td>Dimethylsulfoxide</td>
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<tr>
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</tr>
<tr>
<td>ESBL</td>
<td>Extended-spectrum β-lactamases</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
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<td>--------------</td>
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</tr>
<tr>
<td>g, G</td>
<td>Guanine</td>
</tr>
<tr>
<td>HGT</td>
<td>Horizontal Gene transfer</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>$^{15}$N</td>
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<tr>
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<td>LC-MS/MS</td>
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<td>Lipopolysaccharide</td>
</tr>
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<tr>
<td>MCS</td>
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<tr>
<td>MDR</td>
<td>Multi-drug Resistance</td>
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<tr>
<td>mg</td>
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<tr>
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<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
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<tr>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MW</td>
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</tr>
<tr>
<td>MRSA</td>
<td>Methicillin Resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>n/a</td>
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</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide Adenine Dinucleotide</td>
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<tr>
<td>ng</td>
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<tr>
<td>nt</td>
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</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PMO</td>
<td>Phosphorodiamidate Morpholino Oligomers</td>
</tr>
<tr>
<td>PNA</td>
<td>Peptide Nucleic Acid</td>
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<tr>
<td>PT</td>
<td>Paired Termini</td>
</tr>
<tr>
<td>PTasRNA</td>
<td>Paired Termini antisense</td>
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<tr>
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<td>Ribosomal RNA</td>
</tr>
<tr>
<td>R</td>
<td>Resistance</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions Per Minute</td>
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<tr>
<td>SD</td>
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<td>t,T</td>
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<td>TCA</td>
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<td>u,U</td>
<td>Uracil</td>
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Chapter 1 Introduction

1.1 Antibiotics overview

1.1.1 Antibiotic origins, function & resistance

The discovery and derivatization of antibiotics from natural products has provided the foundations of modern chemotherapy, while pioneering techniques in isolation, characterisation, purification and systematic screening have contributed to the development of the modern pharmaceutical industry. The study and elucidation of antibiotic modes of action on bacterial physiology, has not only advanced the understanding of cellular mechanisms underlying replication, transcription and translation, but also provided selection tools for cloning strategies (Fabbretti et al., 2011).

The origins of naturally derived antibiotics can be traced to organisms within the plant and animal kingdoms, primarily unicellular bacteria and eukaryotic fungi. Of particular interest are the filamentous gram-positive bacteria belonging to the order Actinomycetales (Lechevalier and Lechevalier, 1967), typified by the genera *Streptomyces*, which exist ubiquitously within the terrestrial environment, fulfilling a saprophytic role (Goodfellow and Williams, 1983). *Streptomyces* are among the most prolific producers of naturally derived antibiotics that have entered clinical practice, with a speculative capability of producing up to 100,000 potential antimicrobial compounds (Watve et al., 2001).

Antibiotics are characterized as low molecular weight compounds (MW<3000) which are synthesized via complex secondary metabolic pathways, divorced from the primary metabolic functions concerned with cell growth (Bérdy, 2005). Secondary metabolic pathways are a consequence of distinct biosynthetic gene clusters that encode metabolic enzymes, regulatory genes and resistance mechanisms, implying functional evolution as a unit (Maplestone et al., 1992). The secondary metabolites produced, possess intricate molecular scaffolds with unique functional group configurations (Silver, 2008). These properties enable specific interactions with molecular targets (cellular components or enzymes) (Figure 1.1), through specific binding sites either on the target surface, or at sites that are exposed following structural re-arrangement, when the target is assembled as part of a macromolecular complex with other cellular constituents.
(Lange et al., 2007). The subsequent binding alters the ordered structure of the target causing impairment or complete abolition of normal function. Essential cellular function is subsequently effected, which depending on the degree of target inhibition, is characterized as either inhibitory (bacteriostatic) or capable of initiating cell death (bactericidal) (Walsh, 2003).

Impaired cellular function and cell death can also occur via a secondary pathway that is activated following primary antibiotic target binding, which initiates a universal mechanism of cell death through oxidative damage (Kohanski et al., 2010). Interaction between the antibiotic and respective cellular target, alters metabolic feedback to the Tricarboxylic Acid Cycle (TCA), through increased oxidation of the reduced form of the co-enzyme nicotinamide adenine dinucleotide (NADH). Hyper-activation of the electron transport chain ensues, causing an increase in the formation of superoxide (O\textsuperscript{2−}), hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and hydroxyl (OH) free radicals, which react readily with enzymes containing iron complexes. Iron is subsequently lost following oxidation and becomes freely available to interact with H\textsubscript{2}O\textsubscript{2} via the Fenton reaction to produce OH radicals, which are highly reactive and capable of readily oxidising DNA and proteins (Imlay, 2003).

The role of antibiotics was deemed to be an evolved survival strategy in the guise of a chemical defence mechanism, for minimising competition from other bacteria (Fajardo et al., 2009) and providing protection against bacterivorous predators such as protozoa and nematodes (Jousset, 2012). This concept was supported by widespread antibiotic production among organisms subject to Darwinian competition, in addition to the structural complexity of natural products encoded by a significant proportion of the genomes of Actinomycetes and the sophisticated interaction between the antibiotic and it’s cognate receptor (Williams et al., 1989). More recently it has been suggested that, antibiotics may fulfil a role as autoregulators in intracellular communication, since they are known to induce secondary metabolism and aerial mycelium formation in Actinomycetes, in addition to cellular differentiation in other species (Beppu, 1992). The dual role of defence and communication has been suggested to be concentration dependant, with antibiotics behaving as autologous signals at low concentrations, whereas at high concentrations they
act in the traditional sense of inhibiting bacterial growth (Fajardo and Martinez, 2008).

### 1.1.2 Antibiotic mode of action

Antibiotics are broadly grouped according to the cellular pathways and targets they affect (Table 1.1) peptidoglycan assembly in cell wall synthesis, disruption of the outer cytoplasmic membranes, protein synthesis machinery such as the ribosome and associated elongation factors, enzymes responsible for replication and transcription and the biosynthesis of folate, tRNA and fatty acids (Lange et al., 2007).

![Cellular targets of current antibiotics in clinical use](image-url)

*Figure 1.1. Cellular targets of current antibiotics in clinical use (adapted from Walsh, 2003).*
<table>
<thead>
<tr>
<th>Pathway</th>
<th>Antibiotic class</th>
<th>Primary Target</th>
<th>Mechanism of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell wall synthesis</td>
<td>β-Lactams and derivatives:&lt;br&gt;Cephalosporins  Carbenems  Monobactems  Glycopeptide</td>
<td>DD-transpeptidases (penicillin binding proteins)&lt;br&gt;Peptidoglycan units</td>
<td>Pencilloyation of the penicillin binding protein active site, preventing the catalysis of peptide bond formation between peptidoglycan units. Binds peptidoglycan units (at D-alanyl-D-alanine-dipeptide), thereby inhibiting transglycolase activity and preventing cross linking of peptidoglycan.</td>
</tr>
<tr>
<td>DNA replication</td>
<td>Quinolones/fluoroquinolones&lt;br&gt;A-subunit of DNA gyrase or A-subunit of Topoisomerase IV</td>
<td>A-subunit of DNA gyrase or A-subunit of Topoisomerase IV</td>
<td>Interferes with the maintenance of chromosome topology, through the inhibition of strand cleavage and rejoining, resulting in double strand breaks and replication fork arrest.</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>Aminoglycosides  Tetracyclines  Macrolides</td>
<td>16S rRNA 30S ribosome  30S ribosome  50S ribosome</td>
<td>tRNA mismatching resulting in mistranslation. Blocks access of aminoacyl t-RNA to the ribosome. Inhibiting the initiation phase of translation and translocation of peptidyl tRNA.</td>
</tr>
<tr>
<td>Transcription</td>
<td>Rifamycins  Sulfonamide</td>
<td>B-subunit of DNA-dependent RNA polymerase  Dihydropteroate synthase</td>
<td>Stable binding to the DNA/RNA tunnel of the B-subunit subsequently blocks RNA elongation. Acts as a competitively inhibitor</td>
</tr>
<tr>
<td>Folate biosynthesis</td>
<td>Trimethoprim</td>
<td>Dihydrololate reductase</td>
<td>Prevents recycling of folate coenzymes 7,8 dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF)</td>
</tr>
</tbody>
</table>
1.1.3 The emergence of antibiotic resistance

Decades of anthropogenic activity in biopharmaceuticals, medicine and agriculture, have been speculated to increase the capacity for bacterial evolution. Antibiotic exposure promotes a transient increase in rates of mutation, recombination and Horizontal Gene Transfer (HGT), which underlie genetic diversity. Over time lineages displaying higher mutation rates predominate, leading to the directional selection of antibiotic resistance among bacteria (Gillings and Stokes, 2012). Although the probability of antibiotic resistance developing was initially thought to be low, subsequent advances in in microbial genetics have increased understanding regarding the precursors to resistance and it’s dissemination amongst other species (Davies, 1994). Within a clinical context, persistent antibiotic use has prompted the emergence of three predominant classes of resistant pathogens; gram positive organisms such as Methicillin Resistant Staphylococcus aureus (MRSA), and gram negative organisms such as multidrug resistant (MDR) Escherichia coli, Enterococcus faecium, Enterobacter cloacae, Extended-spectrum β-lactamases (ESBL) Klebsiella pneumoniae, MDR Actinetobacter baumanii and Pseudomonas aeruginosa. In addition a third class consisting of multidrug resistant (XDR) Mycobacterium tuberculosis has also been observed (Fischbach and Walsh, 2009). It is now accepted that antibiotic exposure creates a Darwinian selection pressure which promotes an adaptive response, through the development of survival mechanisms that enable continued growth in cytotoxic antibiotic concentrations (Wright, 2007). Those microorganisms best adapted for survival persist with the capability of disseminating resistance traits amongst other species (Allen et al., 2010).

Antibiotic resistance mechanisms are considered artefacts arising from a comprehensive and evolving natural reservoir of resistance genes, among pathogenic and environmental bacteria. This compendium encompasses cryptic resistance elements within chromosomes and precursor genes encoding proteins with alternate functions, that serve as prototypes to bona fide resistance mechanisms, collectively referred to as the antibiotic resistome (Wright, 2007). The resistome is thought to have originated from the co-evolution of proteins with alternate biochemical function which undergo convergence, whereby a single functional transcriptional unit develops from the
merging of contiguous genes sets coding for structural, regulatory and resistance elements into a single bio-synthetic cluster (Fischbach, 2009). The source of antibiotic resistance mechanisms has been attributed to Actinomycetes, which require a resistance strategy to compensate for antibiotic production. Indeed antibiotic resistance in Actinomycetes is known to increase in conjunction with synthesis. In *Streptomyces coelicolor*, elucidation of the production of the antibiotic actinorhordin, revealed a coupling between biosynthesis and intracellular export. Pathway intermediates were identified and found to be capable of binding to a transcriptional repressor (ActR), responsible for regulating the expression of a trans-membrane efflux pump (actA). As a result of this mechanism, *S.coelicolor* was primed in preparation for the toxic build-up of antibiotic (Tahlan et al., 2007).

Resistance mechanisms may be classified as either active (stimulated by an evolutionary selective pressure) or passive (a consequence of innate cell function or general adaptive process) (Wright, 2005). In these cases, the expression of intrinsic resistance mechanisms can arise following spontaneous chromosomal mutation or a series of cooperative mutations within genes that dictate the synthesis and structural assembly of the antibiotic target. Mutations may cause structural re-orientation of the active site (target structural mutations), enhance access to the target (target access mutations), or shield the target from antibiotic action (target protection mutations) (Martinez and Baquero, 2000).

A prerequisite for spontaneous mutation is pre-mutagenic damage to chromosomal DNA as a consequence of native replication errors following DNA synthesis, characterised as base substitution, frame-shift, or sequence substitutions. Alternatively, mutations may arise from DNA lesions caused by adverse chemical reactions (Oxidation, methylation, depurination, deamination and hydrolytic decomposition of nucleotide bases), in conjunction with failures in cell repair mechanisms (Maki, 2002). The probability of a spontaneous mutation event creating resistant phenotypes (mutability) is dependent on gene structure, length and surrounding sequences, which may be prone to genetic alteration. Environmental cues such as nutrient limitation are also known to increase the rate of mutation by initiating an adaptive response, involving the expression of contingency genes, linked to the SOS response (Martinez et al.,
Such factors underlie the conditions for mutator phenotypes to arise within bacterial populations, which provide a survival mechanism for bacteria to adapt and respond to environmental changes. Alternatively, resistance genes may be acquired from other species through horizontal gene transfer (Mazodier and Davies, 1991), encompassing the transmission of mobile genetic elements from bacteriophages (transduction), plasmids and conjugative transposons (conjugation) and integration of exogenous DNA within chromosomal DNA (transformation) (Alekshun and Levy, 2007). Ultimately, such mutations support the development of common resistance mechanisms enabling mutant bacteria to compete with wild-type counterparts. The outcome is subject to the relative fitness conveyed by the mutation, which may present a significant biological cost in terms of additional metabolic requirements, arising from the expression and maintenance of resistance genes (Andersson and Levin, 1999). Although resistance is often viewed as reducing fitness, it can be compensated by additional mutations in other chromosomal loci, restoring fitness equivalent to wild-type strains (Martinez and Baquero, 2000).

Despite the extensive documentation of emerging resistance mutations, only three new classes of antibiotic have been developed in the last few decades, consisting of mutilins, lipopetides and oxazolidinones (Fischbach and Walsh, 2009). Efforts at reversing resistance within a clinical context, through reducing antibiotic treatments have had limited effects (Andersson and Hughes, 2010). The primary means to treat resistant strains may lie in the discovery of antibiotics possessing novel modes of action capable of negating established resistance mechanisms.
Table 1.2. Resistance mechanisms associated with current antibiotics.

<table>
<thead>
<tr>
<th>Resistance Mechanism</th>
<th>Mode of action</th>
<th>Antibiotic example</th>
<th>Mutation description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural modification of the molecular target</td>
<td>Alterations in target structure reduce interactions / disrupt recognition sites for antibiotic binding</td>
<td>Quinolones</td>
<td>Mutations in gyRA (Ser83 – Leu), gyRB (Asp426-Asn), which encode the subunits of DNA gyrase and subunit of topoisomerase IV encoded by parC (Ser80-Arg)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aminoglycosides</td>
<td>Mutation in nucleotide A1408G of Helix 44 of decoding site of 30S subunit</td>
</tr>
<tr>
<td>Alteration in cell membrane permeability</td>
<td>Restricts entry of the antibiotic to intracellular targets</td>
<td>Vancomycin</td>
<td>Thickened cell wall in S.aureus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polymyxin</td>
<td>Modification of lipopolysaccharide and lipid A composition in S.enterica by 2 component regulatory system PmrAB</td>
</tr>
<tr>
<td>Expression of active efflux pumps</td>
<td>Removes antibiotic from the cytoplasm and across inner and outer membrane of the cell envelope</td>
<td>Tetracycline</td>
<td>Tet transmembrane efflux transmembrane proteins A-E (gram-) and K, L (gram+) protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Macrolides</td>
<td>MsrA ATP binding cassette (ABC) efflux protein</td>
</tr>
<tr>
<td>Expression of enzymes</td>
<td>Enzymatic modification or degradation of antibiotic structure rendering it inactive</td>
<td>Aminoglycosides</td>
<td>Acetyltransferase(AAC), Nucleotidyltransferases(ANT), Phosphotransferases (APH)</td>
</tr>
<tr>
<td>Overproduction of molecular target</td>
<td>Excess target saturates antibiotic levels, increasing</td>
<td>Isoniazid</td>
<td>Mutations in the promoter region of inhA encoding enoyl transferase in M.tuberculosis</td>
</tr>
</tbody>
</table>
1.1.4 The antibiotic discovery paradigm

The paradigm of antibiotic discovery can be traced to the convergence of two separate approaches; systematic chemical synthesis and natural product discovery. Systematic chemical synthesis aimed to utilise reaction mechanisms to generate variant chemical structures with antibacterial properties. This culminated in the development of the first true antibacterial pronisil rubrum, which demonstrated in vivo activity against streptococcal infection. Further investigation attributed antibacterial activity to the metabolite sulfonilamide, which heralded the process of rational design and screening of antimetabolites leading to the development of antifolates. The origins of natural product discovery began with the serendipitous observation and subsequent isolation of penicillin from the mould Penicillium notatum. This was followed by the discovery of streptomycin, which provided a template for the screening fermentation broths derived from soil Actinomycetes. This became the prevailing approach which facilitated the discovery of the majority of antibiotic classes, referred to as “golden age of antibiotics” (Silver, 2012).

Over recent decades traditional discovery programs within the pharmaceutical industry have declined, due to a loss in profitability associated with developing new antibiotics. This has been attributed to the short duration of antibiotic therapy which generates a lower return on capital invested in comparison to other treatments (e.g.: hypertensives). Profitability has been further exacerbated by the conservative pricing of antibiotics and efforts to limit their prescribing to minimise resistance (Tillotson, 2008). A substantial proportion of the economic costs incurred, have been attributed to the reliability of natural sources and the characterisation necessary to identify active components in often complex extracts (Li and Vederas, 2009). The most significant factor in the decline of traditional antibacterial discovery occurred following the introduction of combinatorial chemistry. Combichem refers to the systematic assembly of diverse chemical structures by the connection of individual components, through biological, chemical or biosynthetic means (Gallop et al., 1994). Using this approach it is possible to construct structurally diverse compounds through the addition of functional groups to a primary scaffold element, often derived from a natural product template (Koehn and Carter, 2005). Unlike traditional natural product libraries, sufficient quantities can be synthesized economically.
and undesirable chemical properties that may cause unwanted side effects can be avoided at the design stage.

The introduction of combichem subsequently prompted a paradigm shift in screening strategies that saw empirical screening from natural sources, superseded by High Throughput Screening (HTS) using synthetic libraries (Li and Vederas, 2009). Yet despite extensive efforts, synthetic libraries have failed to make a significant impact. The evidence for this limited success has been exemplified by the screening of synthetic polyketides libraries, which have demonstrated a hit rate of <0.001% compared to a 0.3% hit rate achieved with natural product libraries (Weissman and Leadlay, 2005). Even the use of genomics to identify and select essential genes for target based screening has failed to deliver new antibiotic entities. In a landmark study, a genomics based approach was used to identify 70 essential genes, which proceeded to generate only 5 leads from subsequent HTS campaigns (Payne et al., 2007). Similar results were identified in 127 antibacterial screening programs covering 69 essential targets (Chan et al., 2004).

Conclusions drawn from these studies revealed the limitations of synthetic libraries, specifically an inherent lack of chemical diversity in terms of steric complexity, unique functional groups, chiral centres, and broad molecular properties associated with natural products. Furthermore, synthetic compounds often lack evolved target specificity, often against subtle features such as protein domains and binding motifs (Koehn and Carter, 2005). The lack of chemical diversity may have arisen from an inherent bias towards compounds that fulfil Lipinski’s rule of 5, a set of parameters associated with solubility and permeability, in terms of molecular weight, Log p and number of hydrogen acceptors and donors (Lipinski et al., 2012). In comparison natural products typically display higher MW and polarity compared to other drug classes (O’Shea and Moser, 2008). With increasing rates of antibiotic resistance and a lack of novel antibiotics in development, there has been a renaissance in natural product discovery.

1.1.5 Possibilities to exploit new sources of antibiotics

Current efforts in natural product discovery have focused on identifying new antibiotic producing microorganisms on the rationale that taxonomic diversity is
a proxy for the chemical scaffold diversity, associated with natural products (Bull et al., 2000). New microorganisms have been sought by surveying previously unexplored geographical locations, where extreme environmental conditions of temperature pH and salinity are encountered. Considerable focus has been expended on the marine environment including sea ice, the ocean subsurface, hydrothermal vents and the ocean floor (Keller and Zengler, 2004).

Marine sediments in particular have shown large diversity of microorganisms in particular Actinomycetes (Stach and Bull, 2005; Pathom-aree et al., 2006). Currently members covering 50 genera of Actinomycetes have been isolated and cultured from marine habitats including the discovery of new genera; Demequina, Lamia, Marinactinospora, Marisediminicola, Miniimonas, Paraoerskivia, Phycicococcus, Phycicola, Salinibacterium, Salinispora, Aciscionella and Serinicoccus (Goodfellow and Fiedler, 2010). Akin with their terrestrial counterparts, marine Actinomycetes possess a significant coding capacity reserved for numerous biosynthetic clusters encoding secondary metabolites (Baltz, 2008). Evidence has emerged suggesting that marine secondary metabolites are synthesised via unique halogenation mechanisms, absent in terrestrial Actinomycetes (Butler, 1998). Marine halogenated metabolites are speculated to possess novel properties which arise from the incorporation of halogen atoms within aromatic, heterocyclic rings and carbon centres, that influence steric effects and electrophilic reactivity of the antibiotic scaffold, thereby dictating biological activity (Vaillancourt et al., 2006). Recently a novel enzymatic pathway that utilises vanadium-dependant haloperoxidases was identified within the biosynthetic gene cluster of a marine Streptomyces (Lane and Moore, 2011). Consequently, natural products derived from rare marine Actinomycetes display desirable characteristics that may be exploited to treat antibiotic resistant pathogens. Screening for such products is therefore a priority for antibiotic discovery programs.

The search for Actinomycete derived natural products has been supported by advances in genomics and chemical biology, which have aided the elucidation of biosynthesis mechanisms, and subsequently defined new biodiscovery approaches (Kurtboke, 2012). The exploitation of whole Actinomycete genomes such as Streptomyces avermitilis (Omura et al., 2001) and use of bioinformatic analyses to interrogate sequences, has provided the basis for numerous
genome mining strategies (Van Lanen and Shen, 2006; Zerikly and Challis, 2009).

One such strategy has been founded on manipulating the synthesis of novel secondary metabolites such as polyketides (PK) and non-ribosomal proteins (NRP). It is known that the synthesis of PK and NRP antibiotics proceeds in an assembly line sequence, where the construction of unique scaffolds arises from the coordinate assembly of monomer units by multi-modular enzymes encoded in biosynthetic clusters. The structural organization of the multimodular protein domains function as a template to direct the order of monomer units within a linear oligomer, while catalytic domains govern the activation and incorporation via covalent tethering (Fischbach and Walsh, 2006). Using experimentally derived data, it has been possible to ascribe product structure to subunit composition thereby enabling characterization of individual subunits (McDaniel et al., 1995). Functional information for individual subunits can be utilized in predicting metabolic products, including their putative physiochemical properties, as exemplified by the polyene marolactam Salinilactam A from Salinispora tropica (Udwary et al., 2007). The re-arrangement of monomer units within the assembly sequence can also enable the synthesis of novel metabolic products. In one example the loading module of Avermectin polyketide synthase was substituted with a cyclohexanecarboxylic loading module, enabling the synthesis of the novel analogue doramectin (Wang et al., 2011).

Alternative genome mining approaches have combined bioinformatics analysis with assay guided fractionation in a genomisotopic approach. Analysis of the genome of Pseudomonas fluorescens Pf-5 revealed three unknown biosynthetic clusters for non-ribosomal peptide synthases, the nucleotide sequence of which enabled the prediction of amino acid assembly of the product. By using a isotopically labelled amino acid, incorporation into the unknown product could be identified by NMR, resulting in the discovery of the novel lipopeptide orfamide A (Gross et al., 2007).

Biosynthetic clusters may also be isolated and expressed in host organisms followed by the purification of proteins, which are then incubated with co–factors in an in vitro reconstitution approach, as performed with the biosynthetic cluster of lantibiotic haloduracin produced by Bacillus halodurans C-125 (McClerren et al., 2006). In cases where biosynthetic gene clusters are not constitutively
expressed or dictated by specific environmental conditions, expression may be induced by modulating transcriptional regulatory factors. In one example, overexpression of the transcriptional activator apdR under an inducible promoter in *Aspergillus nidulans* lead to the identification of aspyridones A and B (Bergmann *et al.*, 2007). Alternatively cryptic biosynthetic clusters can be cloned and expressed in laboratory and industrial strains of *Streptomyces* with characterized metabolomes (Baltz, 2010).

An alternative biodiscovery perspective is to examine microbial communities directly within their ecological habitats in order to provide an assessment of functional diversity. This has been achieved using metagenomics whereby DNA is isolated directly from environmental sources and cloned into a model organism such as *E.coli*. Transformants are then screened for functional activity in a high-throughput assay, in order to assess metabolic potential (Handelsman *et al.*, 1998). The comparison of metagenomic data from microbial communities can not only reveal trends that suggest functional diversity, but also reveal spatio-temporal characteristics. Metagenomic data can therefore reflect environmental factors that promote the distribution and evolution of antibiotic biosynthetic gene clusters that contribute towards speciation and diversification (Kurtboke, 2012). The detection of rare Actinomycetes can also be pursued using taxonomical studies to devise selective isolation procedures. Refinements of Actinomycetes systematics and the development of selective isolation techniques within culture dependant bio-prospecting strategies have also been investigated (Goodfellow and Fiedler, 2010).

In addition to the identification of new taxa, existing Actinomycetes can be evaluated with greater scrutiny to reveal their secondary metabolite producing capacity. It has been documented that a discrepancy exists between the number of compounds produced and potential synthesis capability of many Actinomycetes. An attempt to explore this facet is exemplified by the One Strain Many Compounds (OSMAC) approach, whereby cultivation conditions are systematically modified to influence key steps in biosynthesis and thereby generate diverse metabolic profiles (Bode *et al.*, 2002). Successful biodiscovery requires not only consideration of ecological habitat, selective isolation procedures and bacterial systematics for the identification of rare
Actinomycetes, but also the implementation of an appropriate screening strategy to assess the bioactivity of secondary metabolites that are synthesized (Kurtboke, 2012).

1.1.6 Antibiotic screening approaches

A key requirement for an antibacterial screening assay is the capacity to discriminate between known antibiotics and novel undiscovered compounds. Within the terrestrial biosphere, antibiotic biosynthetic clusters are not distributed uniformly. Random genome sampling has revealed that 1% of Actinomycetes present in the soil produce streptomycin compared to 0.1% that synthesize tetracycline, with other antibiotics isolated at even lower frequencies of $1 \times 10^{-7}$ (Baltz, 2007). Consequently antibiotics that occur at high frequencies are easily isolated, thereby potentially masking the presence of novel antibiotics that exist in low abundance. The consistent rediscovery of known compounds therefore represents a significant technical challenge to screening approaches (Williams, 2009).

Conventional screening strategies have utilised whole cell screens, cell free or modular assays to evaluate inhibitors. Whole cell screening involves seeding a target organism onto media to which a natural product extract is added (Figure 1.2). Antibacterial activity is then assessed by observing zones of inhibition (Mills and Dougherty, 2012). Refinements to this assay format have lead to the development of mechanism based screening that targets pathways such as the cell wall, using hypersusceptible mutant strains to identify β-lactam antibiotics (Gadebusch et al., 1992) and the anucleate blue cell assay to detect Type II topoisomerase inhibitors of DNA synthesis (Oyamada et al., 2006). Cell free assays differ in that a single target or multiple targets encoding enzymes representing a pathway are reconstituted in vitro; inhibition is monitored directly using biochemical assay to examine enzyme kinetics. Modular assays are similar to cell free systems except that a target is identified *in silico*. The target sequence is cloned into *E.coli* or other suitable host organisms, expressed with a Histag and purified. The isolated target is then screened against natural product extracts, and signs of inhibition are evaluated in a binding assay.
### Table 1.3. Evaluation of antibiotic screening approaches.

<table>
<thead>
<tr>
<th>Assay type</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wholecell antibacterial activity</td>
<td>Identifies <em>in vivo</em> antibacterial activity</td>
<td>Unable to discriminate between secondary mechanisms related to cytotoxicity and actual antibacterial activity</td>
</tr>
<tr>
<td></td>
<td>Selects compounds capable of transversing the cell wall</td>
<td>Compounds lacking cell penetration, but, which would otherwise inhibit the target with suitable modification are overlooked</td>
</tr>
<tr>
<td></td>
<td>Enables multi-protein interactions to be studied without the need to reconstitute complex cell systems</td>
<td>Secondary biochemical assay required to identify the mode of action</td>
</tr>
<tr>
<td>Target based cell free systems</td>
<td>High sensitivity</td>
<td>Requires prior knowledge of the target</td>
</tr>
<tr>
<td></td>
<td>Validates mode of action in addition to identifying target</td>
<td>Complex reaction or substrates make enzymatic tests difficult</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Constrained to targets with enzymatic function</td>
</tr>
</tbody>
</table>
Figure 1. Whole cell screening approach to antibiotic discovery.

Using whole cell screening provides a number of advantages over cell free systems (Table 1.3) and prior to the development of synthetic libraries has been the pre-eminent system for evaluating natural products. Rational Drug Design (RDD) represents a further approach that has benefited from advances in biophysical characterization techniques, to enable the derivatization of sufficient structural data from molecular targets. This information can then be used for the construction of putative inhibitors (Donadio et al., 2002).

Considerable effort to overcome the inability of whole cell assays to delineate between cellular targets have been pursued using recombinant organisms as the basis for target-based screening strategies, in which the expression or activity of a specific cellular target is modulated. For example the Keio collection, consisting of approximately 4000 individual essential gene knockouts strains of *E.coli* (Baba et al., 2006), which was subsequently screened to identify mutants with increased susceptibilities to antibiotics (Tamae et al., 2008).
An alternate approach has been to delete chromosomal copies of essential genes in *E. coli*, replacing them with plasmids containing a complementary copy of the gene, under the control of an inducible promoter, to enable gene expression modulation. Using this system it is possible to reduce target protein expression in order to generate hypersensitivity to target specific inhibitors. Screening strains against cognate inhibitors demonstrates increased susceptibility, by decreasing the MIC (DeVito *et al.*, 2002). Conversely strains have been engineered to overexpress essential proteins from inducible plasmids, thereby rendering strains hyposensitive to target protein inhibitors (Xu *et al.*, 2006).

Of the target based assays developed to date many have used known targets for which resistance had already been demonstrated. The discovery of novel antibiotics therefore requires the identification and validation of either molecular targets or components of cellular pathways possessing unique modes of action, not currently subject to resistance mechanisms (Black and Hodgson, 2005). Potential targets should be evaluated for their capacity to form multiple interactions to reduce the likelihood of generating endogenous resistance according to the multi-target hypothesis (Silver, 2007).

### 1.2 Searching for new antibiotics

#### 1.2.1 Identification of novel antibiotic targets

Despite the conserved range of cellular targets exploited by current antibiotics, the number of potential targets may be larger then anticipated, due to the multiple co-factors and accessory components that cooperate with essential targets within biosynthesis pathways (Donadio *et al.*, 2002). A suitable example of this multiple-target approach is the peptidoglycan biosynthesis pathway that culminates in assembly of the bacterial cell wall. Numerous cytoplasmic intermediates have previously been unavailable due to the inability to reconstitute them *in vitro*. Efforts to synthesise key cytoplasmic precursors such as UDPMurNAc-pentapeptide, have aided biochemical characterization of the peptidoglycan pathway necessary for the development of assays and screens for lipid-linked steps. To date screening for Lipid I and II intermediates revealed that MraT a integral membrane translocase, was found to be the target of uridine-based nucleoside antibiotics (Bugg *et al.*, 2011). Additional targets have
been identified in other metabolic pathways including Aminoacyl t-RNA synthetases, Polypeptide deformylase and fatty acid biosynthesis (McDevitt and Rosenberg, 2001), enzymes responsible for NAD(P) biosynthesis (Bi et al., 2011) and component enzymes of cell division (Lock and Harry, 2008).

Irrespective of novelty, there is a requirement for candidate targets to fulfil the following criteria; (i) they must demonstrate an essential function, (ii) the target should be conserved across a range of species for broad-spectrum activity, (iii) the target should be specific to bacteria with no human homolog to limit toxicity and (iv) the target should exist as a single gene copy to minimise potential resistance development. Ideally the target should also display desirable physiochemical properties to enable inclusion into a high-throughput screening format (Brown and Warren, 1998).

In recent years a paradigm shift in the identification of potential antibiotic targets has emerged following advances in genomics and bioinformatics (Bull et al., 2000), specifically following the advent of high-throughput whole genome shotgun sequencing, pioneered with Haemophilus influenzae (Fleischmann et al., 1995). The number of bacterial genomes sequenced has since increased dramatically following the development of new sequencing technologies such as pyrosequencing, DNA ligase-mediated sequencing, and the Illumina platform (McLeod et al., 2012). Accompanying the progress in genome sequencing technologies have been parallel developments in bioinformatics. Algorithm based programs such as TIGR Assembler, Glimmer and Genemark have enabled the assembly of contiguous sequences, identification of open reading frames and revealed putative function of genes on the basis of protein structural motif homology or operon structure (McDevitt and Rosenberg, 2001; McLeod et al., 2012). Critically, bioinformatics programs such as BLAST and FASTA have permitted the development of comparative genomics in silico, enabling the identification of conserved orthologues (homologous genes derived from a common ancestral gene) across bacteria species. Following the genome comparison of H. influenzae and Mycoplasma genitalium, approximately 256 genes with purported essential functions common to both organisms were identified. From this data was established the concept of the minimal genome, a core set of genes required for sustaining cellular existence (Mushegian and Koonin, 1996). Subsequent genome studies have assigned essential functions
to 620 genes in *E.coli* (Gerdes *et al.*, 2003), 478 genes in *H.influenzae* (Glass *et al.*, 2006) 658 genes in *S.aureus* (Forsyth *et al.*, 2002) and 271 genes in *B.subtilis* (Kobayashi *et al.*, 2003b).

Within core gene sets a number of ubiquitous cell functions have been identified consistently, representing elementary cell functions of transcription, translation, replication, membrane transport and energy conversion (Koonin, 2003). From an antibacterial search and discovery perspective, these genes may serve as potential targets since they are evolutionary conserved among closely related bacteria species (Jordan *et al.*, 2002). Critically, the majority of genes identified within minimal gene sets are also proportionally biased towards enzymes (Gao and Zhang, 2011), representing ideal targets for potential inhibitors, since they possess an active site, which is predisposed towards ligand interaction and proves highly amenable for biochemical assays to validate target function (Bumann, 2008).

The pursuit of inhibitors with specific activity for an individual essential gene target has been questioned due to the high frequency of spontaneous resistance mutation ($10^{-6} – 10^{-9}$), that occur in single genes (Silver, 2011). The use of hybrid pharmacophores, combinations of single target inhibitors and structure based drug design have been suggested as potential methods for minimising resistance in individually encoded targets. However, a more viable approach would be to identify a range of essential genes amenable to polypharmacology, whereby a single inhibitor has the capacity to engage with multiple molecular targets (Brötz-Oesterhelt and Brunner, 2008). According to the multi-target hypothesis, endogenous resistance development is minimised where antibiotics bind with multiple molecular targets, since mutations would be required in each gene encoded target to render an antibiotic completely ineffective (Silver and Bostian, 1993).

Indeed the majority of antibiotics discovered using whole cell screening approaches, act on multiple encoded targets or on individual structures assembled from the products of multiple genes (Silver, 2007). The targeting of Penicillin Binding Proteins (PBP’s) by β-lactams and topoisomerase GyrA/ParC subunits by quinolones represents two such examples. More recently it has been suggested that the bacterial proteolytic complex may also prove a suitable target (Raju *et al.*, 2012). Consequently new targets have been proposed
including the MurA ligases (D and E), involved in the catalysis of peptidoglycan, and Lpx acyltransferases (A and D), which are involved in lipidA biosynthesis (East and Silver, 2013).

Defining core gene sets however has proved problematic, with varying estimates that obscure consensus as to the number of essential genes a set comprises (Juhas et al., 2011). A phylogenetically balanced study that analysed 147 bacterial and archaea genomes, found the core set to consist of 38 essential genes (Charlebois and Doolittle, 2004). More recently a comprehensive analysis comparing the genome of M. genitalium to 92 gram negative and 93 gram positive bacteria revealed a core set of 151 genes (Huang et al., 2012). The discrepancy in the core set suggests that not all essential genes are conserved among species (Koonin, 2003). It has been determined that the number of core genes decreases as the number of phylogenetically diverse groups used in comparative analysis increases. This reflects Non-orthologous gene displacement (NOGD), whereby non-orthologous genes replace orthologous counterparts encoding the same essential cellular function. Consequently alternative biochemical pathways arise, which provide a capacity for metabolic redundancy rendering many gene targets dispensable and therefore unsuitable as target candidates for screening (Becker et al., 2006). A known example of this phenomenon is the fabI homologue fabK in Streptococcus pneumonia, which is not subject to the same degree of inhibition by triclosan (Heath and Rock, 2000).

Further discrepancies arise depending on the environmental conditions under which a minimal gene set is defined (Koonin, 2003). The requirements imposed by an organism’s lifestyle and environment conditions may actually abolish the function of some essential genes. Notably the type II fatty acid synthesis pathway can be negated by gram positive pathogens capable of utilising C18 unsaturated fatty acids from human serum (Brinster et al., 2009). Further variability among core gene sets arises from the experimental strategy used to validate gene essentiality. The majority of techniques used are based upon the generation of mutants, grown as clonal populations or as part of mixed populations. Clones grown as part of a mixed population are selected against by competitive outgrowth, thus demonstrating reduced fitness rather than true essentiality which can result in false assignment (Gerdes et al., 2006).
Common experimental techniques for determining gene essentiality are founded upon random mutagenesis and targeted gene disruption (Miesel et al., 2003; McLeod et al., 2012). Random mutagenesis strategies include Transposon Saturation Mutagenesis and variants such as Genomic Analysis and Mapping By *In vitro* Transposition (GAMBIT) (Akerley et al., 1998), Genome scanning (Reich et al., 1999) and Transposon-Mediated Differential Hybridisation (TMDH) (Chaudhuri et al., 2009). A common theme to these techniques is the use of transposons (mobile genetic elements), which are engineered into plasmids containing a conditional replicon and antibiotic selection marker. Once introduced into a cell the transposon inserts randomly in the chromosome with its location mapped by sequencing. Transposition into an essential gene coding sequence disrupts the gene function resulting in cell death thereby confirming essential function (McLeod et al., 2012). Targeted gene disruption techniques involve plasmid insertion mutagenesis, whereby the central sequence of a target gene is cloned into a suicide vector. Integration of the vector via a single crossover recombination event involving the target gene and homologous sequence on plasmid results in allelic replacement of the target gene with a resistance marker (McLeod et al., 2012).

Mutagenesis based techniques pose the risk of generating polar effects, that involve gene disruption arising from non-sense mutations in translation regions, reducing the synthesis of distal proteins in operons (Zipser, 1969). The gradient of polarity is evident due to the distance between the mutant and distal genes, often with strongest effects at the proximal end (Epstein and Beckwith, 1968). Integration of external DNA sequences within a single operon gene may disrupt the transcription of genes downstream to the insertion event that may also be essential, subsequently essentiality may be attributed in error (McLeod et al., 2012). Characteristic of all techniques is the definition of essentiality through abolition of function, whereby all genes that cannot tolerate replacement or disruption are deemed essential. Such techniques are incapable of assessing the contribution to fitness of individual essential genes, which may be conserved due to small advantage presented under some growth conditions. This difficulty has been addressed using gene down regulation, whereby an inducible promoter is inserted upstream of the target gene. One variant technique in particular has proved useful and is founded on natural phenomenon of Antisense RNA based regulation.
1.2.2 Antisense RNA

Despite the recognized and accepted concepts of gene organization, it was suggested that gene regulation could be coordinated by genes that encode a transitory intermediate composed of RNA (Jacob and Monod, 1961). Regulation via RNA was later confirmed in prokaryotes, following investigation into the replication of plasmid colE1 (Tomizawa et al., 1981), where it was determined that a complementary RNA blocks primer RNA formation required by DNA polymerase I to initiate synthesis (Eguchi, 1991). Although many fundamental principles have been ascertained through the study of bacterial plasmids (Simons and Kleckner, 1988), RNA based regulation has also been observed in eukaryotes (Almeida and Allshire, 2005) and plants (Baulcombe, 2004).

RNA based gene regulation has been associated with heterogeneous RNAs that coordinate gene expression through diverse mechanisms and can be classified into four broad groups (Waters and Storz, 2009). Riboswitches are multidomain RNA’s capable of binding metabolites which initiates a global conformational change enabling the riboswitch to bind within the 5’ untranslated region (UTR), where it occludes the Shine Dalgarno sequence (Montange and Batey, 2008). Protein binding small RNA’s possess innate enzyme activity, or antagonize binding (Waters and Storz, 2009). Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) represents the most recent addition to the RNA regulatory family and comprises a defensive system against phages (Sorek et al., 2008). The most significant group consists of small RNAs encoded in cis (or Antisense) and trans form (Waters and Storz, 2009). Trans-encoded RNA’s are transcribed at distant genomic locations in relation to the target mRNA they regulate, in contrast to cis-encoded RNA which are transcribed parallel to their respective target mRNA on the sense DNA strand and therefore share full sequence complementarity (Thomason and Storz, 2011).

Antisense RNA are of particular interest since they represent a subset of small RNA entities (sRNA’s) in prokaryotes, responsible for the coordination of gene expression through mechanisms that influence transcription and translation. Following advances in transcriptome analysis, in particular the use of Deep RNA sequencing (RNA-Seq) (Wang et al., 2009) the number of antisense RNA transcripts identified has increased. In E.coli alone over 1000 antisense
transcripts have been identified (Dornenburg et al., 2010). From this wealth of data has emerged an array of regulatory roles including the inhibition of transposition, repression of toxic protein synthesis, regulation of transcription factors and metabolic enzymes (Thomason and Storz, 2010). Roles in bacterial virulence (Gripenland et al., 2010) and more recently the restriction modification system in prokaryotes have also been observed (Mruk et al., 2011).

The regulatory functions performed by (cis-encoded) antisense RNAs is founded upon complementary hybridization with cognate mRNA sequences at the 5' > 3' end, internally or 3' and 5' untranslated regions (UTRs) (Georg and Hess, 2011). The capacity for target hybridization is attributed to the characteristic secondary structural features of antisense RNA, consisting of a ubiquitous YUNR motif (Y= pyrimidine, R=purine). The motif forms a U-turn loop which serves as a recognition element and provides a scaffold for facilitating helix formation with the target mRNA (Franch et al., 1999). The process of sequence hybridization is thought to proceed via two independent binding pathways, that are initiated by a recognition event between loop structures on both the antisense and target mRNA, before progressing towards the formation of duplex RNA (Brantl, 2007).

A multistep pathway elucidated from the CopT/CopA antisense system of plasmid R1 (Kolb et al., 2000), is initiated with the binding of stem loops to form an unstable kissing complex that is extended via a single stranded region to form a four-helix junction intermediate. The intermediate is transformed into a stable inhibitory complex which converts to a stable RNA duplex. An alternative single step binding pathway was reported for the RNA-IN/RNA out antisense system responsible for regulating the transposition of transposase IS10 (Kittle et al., 1989). In this particular system, an initial interaction occurs between stem loop and single stranded target that permits extension via the loop and stem domain. Following complete RNA duplex formation between antisense RNA and it’s cognate mRNA target, gene expression can be modulated, by altering transcript stability and either transcription or translation efficiency of the target mRNA via distinct mechanisms (Figure 1.3. Mechanisms of antisense RNA inhibition.) (Georg and Hess, 2011).
Figure 1.3. Mechanisms of antisense RNA inhibition.

The stability and half-life of target mRNA is known to decrease following antisense duplex formation through a degradation pathway. In the cyanobacterium *Synechocystis* 6803, the expression of iron stress induced protein (Isia) is known to decrease following over expression of its antisense counterpart IsrR (Iron stress repressed RNA) (Duhring *et al.*, 2006). Conversely stability may also be increased as exemplified with the glutamate decarboxylase transcriptional activator/repressor (*gadX/gadW*) of the *E.coli* acid response system. Glutamate decarboxylase contains an intergenic region between *gadX* and *gadW* that encodes the antisense RNA GadY, complementarily to the 3’ UTR of *gadX*. Overexpression of GadY increases accumulation of *gadX* mRNA which increases expression of downstream decarboxylases (Opdyke *et al.*, 2004).

The alteration in mRNA stability is thought to involve a common degradative pathway that regulates mRNA decay. The process is dictated by the structural organization of prokaryote mRNA transcripts. Bacterial mRNA’s possess a 5’-terminal tri-phosphate, while the 3’-terminus typically forms a stem loop structure, which is subject to processing events that effect stability and initiate
the process of mRNA degradation (Belasco, 2010). The 3’ terminal can be destabilized by polyadenylation by Poly(A)polymerase (Dreyfus and Régnier, 2002), while RNA pyrophosphohydrolase (RppH) is known to convert 5’ triphosphate to 5’ monophosphates (Deana et al., 2008). Both events initiate mRNA degradation via two distinct pathways, involving the RNA degradosome, a multi-protein assembly responsible for mRNA processing and degradation (Carpousis, 2007). The principle component of the degradosome is RNaseE, a multi-domain endoribonuclease, which cleaves polyribosomal RNA to fragments. The C-terminal of RNaseE serves as a molecular scaffold for assembly of accessory proteins including; Polynucleotide phosphorylase (PNP) a endoribonuclease with 3’ activity, RhlB a DEAD-box RNA helicase with ATPase dependent activity for unwinding RNA, and enolase, a glycolytic enzyme which has a purported role as a metabolite sensor (Carpousis, 2007).

Degradation may occur via the 5’-dependant pathway, whereby the 5’ single stranded regions are subject to hydrolysis of 5’-terminal triphosphate to 5’-terminal monophosphate by RppH. The 5’ terminal monophosphate facilitates the binding of RNaseE via subdomains, thereby enabling the correct orientation for initiating internal cleavage (Bouvier and Carpousis, 2011). Alternatively endonucleolytic degradation by RNaseE is initiated by a 5’ independent mechanism that generates multiple fragments which are then subject to polyadenylation by polyA polymerase. This permits 3’ exonucleases such as PNPase, to attempt to remove structural features such as stem loops (Carpousis et al., 1999).

In some cases, antisense RNA exerts its modulatory effects at the level of gene transcription. The hybridization between antisense RNA and it’s cognate mRNA target can initiate the formation of secondary structure which prevents RNA polymerase proceeding, thereby inhibiting transcription. A known example of this mechanism has been observed in Vibrio anguillarum, where transcription of the Iron uptake biosynthesis operon was due to a 427 nucleotide antisense transcript that formed a terminator structure consisting of a stem loop (Stork et al., 2007).

An alternate mechanism of antisense RNA based regulation referred to as transcriptional interference has been noted, which arises from the proximity and transcriptional direction of promoters. Convergent promoters facilitate the
collision of independent RNA polymerases, causing displacement of one polymerase and generating inverse correlations of mRNA and antisense RNA, similar to those observed following analysis of the ubiGmccBA operon in *Clostridium acetobutylicum* (Andre et al., 2008). Alternatively the collision event may stall elongation or promote backtracking of the polymerase (Crampton et al., 2006). If the strength of converging promoters differs, then the weaker promoter may be occluded or RNA polymerase and associated transcriptional apparatus may be displaced (Sitting Duck interference) (Georg and Hess, 2011).

Antisense RNA based regulation also prevails at the post-transcriptional level, where the hybridization of antisense RNA and cognate mRNA target, directly modulates ribosome binding efficiency. In *E.coli* mutation of SymR, a *cis* encoded antisense regulator complementally to the promoter sequence of the SOS-induced antitoxin gene *symE*, causes a differential increase in protein compared to RNA (Kawano et al., 2007). Post-transcriptional control by antisense RNA may be influenced by Hfq, an RNA binding protein, with a multimeric pore structure known to interact with small RNAs by facilitating base-pairing between *cis*-encoded and target mRNA. Roles of Hfq have been postulated in translation repression via binding of sRNA’s that sequester the RBS, thereby preventing ribosome binding and subsequently inhibit protein translation. Conversely antisense RNA binding may also expose translation initiation regions and enhance translation. (Vogel and Luisi, 2011).

### 1.2.3 Antisense RNA quantitative effects

The widespread conservation of antisense RNA based regulation among species, suggests a significant evolutionary role, which complements existing control mechanisms responsible for regulating both transcription and translation (Thomason and Storz, 2010). From a cellular perspective RNA based regulation presents an efficient regulatory mechanism, since the energy costs associated with transcription are considerably less in comparison to the process of protein translation and assembly. In addition, due to speed of synthesis and degradation, mRNA expression can be rapidly modulated unlike protein regulation (Gripenland et al., 2010). Indeed, it was shown that the rate of synthesis is up to two magnitudes faster for antisense RNAs compared to mRNA (Shimoni et al., 2007). Unlike protein based regulation, antisense RNA
has the capacity to minimise temporal fluctuations and filters transient signals in gene expression arising from stochastic events in transcription and translation, thereby minimising surges in protein abundance (Kaern et al., 2005). The regulatory capacity of antisense RNA provides a highly responsive system, capable of adapting to diverse and often sudden stimuli. Consequently antisense regulation is often associated with genes that require expeditious expression in response to environmental changes such as regulators of the cellular stress response. Such genes include the outer membrane porin protein F (ompF) in *E.coli*, which is regulated by the antisense RNA MicF. Expression of MicF causes a reduction in protein expression, thereby limiting extracellular uptake, especially in the presence of antibiotics (Delihas and Forst, 2001). Antisense based regulation is also prevalent where the accumulation of toxic proteins would be detrimental to cell viability. The hok/sok system for regulating plasmid stability by post-segregation killing involves the expression of hok (host killing), a membrane associated toxin that causes irreversible damage to cell membranes and is regulated via a 64 nucleotide antisense RNA sok (suppression of killing). Inhibition occurs indirectly as sok inhibits translation of mok (modulation of killing), which in turn mediates the translation of hok (Thisted and Gerdes, 1992). Metabolism represents further cellular activity requiring a rapid response to variations in nutrient levels, and is represented by such systems such as the lac operon of *E.coli* which is disordinately regulated by a 109 nucleotide antisense RNA Spot 42 (Møller et al., 2002). Virulence is also reliant on antisense regulation for the expression of key proteins, when particular environmental conditions are encountered. In pathogenesis antisense regulation provides the appropriate degree of responsiveness to host immune threats as observed in *Salmonella enterica*, where a 1.2kb antisense RNA AmgR was found to be complementary to MgtC, which encodes an inner membrane protein essential for intracellular survival within macrophages (Lee and Groisman, 2010).

Quantitative studies of antisense RNA regulation have suggested that the equilibrium between the transcription of antisense and target mRNA dictates gene expression via a threshold linear response (Levine et al., 2007; Levine and Hwa, 2008). Through the modulation of gene expression, characteristic expression profiles are generated for both mRNA and antisense transcripts that infer the regulatory mechanism in addition to advocating the physiological
effect. (Lapidot and Pilpel, 2006). The fine modulation and temporal effects of antisense RNA based regulation, have been exploited for numerous experimental purposes (Rasmussen et al., 2007). Primarily, antisense RNA has been developed for gene silencing techniques and applied to the identification of essential genes in both gram positive and negative organisms, such as Staphylococcus aureus (Forsyth et al., 2002), and more recently E.coli (Meng et al., 2012). Adaption of the same antisense RNA silencing strategy has also enabled the delineation of bacteriostatic and bactericidal targets in mycobacteria (Kaur et al., 2009). Antibiotic discovery strategies have also benefited from the implementation of antisense RNA silencing in whole cell target-based assays, to elucidate inhibitor mode of action using a differential assay format (Singh et al., 2007). Success with this approach has been demonstrated, with the identification of novel fatty acid synthesis inhibitors platencin and platensimycin (Wenzel et al., 2011) and the novel protein synthesis inhibitor peptidyltransferase (Bandow et al., 2003).

More recently antisense RNA silencing has been used to evaluate the individual contributions of essential genes in maintaining cell viability. Using a combination of insertion mutagenesis and DNA microarrays revealed that the fitness contribution of essential genes under specified growth conditions varies significantly (Badarinarayana et al., 2001). Further efforts have attempted to quantitate essential gene requirement for cell viability termed stringency, by examining mRNA transcript abundance. In a novel study the titration of E.coli growth rates using antisense RNA silencing of essential gene targets fabl, murA, acpP and ftsZ, established a differential transcript requirement for each gene to maintain cell growth. By defining the minimum transcript level required to maintain 50% cell viability, a hierarchy in stringency was revealed in the order of acpP > ftsZ > fabl > murA (Goh et al., 2009). This concept was supported by a recent study that examined cell tolerance to degradation of essential protein and it’s subsequent effect on cell growth in determining cell vulnerability (Wei et al., 2011).

The concept of quantifying essential gene stringency represents a significant asset to antibiotic discovery strategies; as it would permit the prioritization of essential gene targets for inclusion into screening assays. However, despite the apparent relationship between mRNA reduction and decline in cell growth,
mRNA abundance provides only a surrogate for expressed protein. Functional protein epitomizes the culmination of gene expression, and is viewed as accurate reflection of the physiological state of a bacterial cell. Current estimates of essential gene stringency based upon mRNA abundance may therefore be inaccurate, since the correlation between mRNA and protein expression is often non-linear (Lee et al, 2003). Even across different species, steady state protein abundance displays a higher correlation then corresponding mRNA abundances (Laurent et al., 2010).

The lack of concurrence between mRNA and protein expression is attributed to regulatory mechanisms that operate during transcription and translation (Vogel and Marcotte, 2012). The efficient transcription of mRNA is dependant upon the complementarity of its Shine Dalgarno sequence, which dictates ribosome binding efficiency and therefore translation. The complex secondary structure of RNA represents a further influence since it can either expose or sequester the ribosome binding site under different environmental conditions. At the translational level small antisense RNA affects post-transcription levels via translation inhibition, interference, or degradation of mRNA transcripts, while ribosome density affects translation efficiency. Ensuing translation, protein half life is influenced by factors such as intrinsic stability, rates of degradation, modification by phosphorylation and cell localization (Maier et al., 2009).

Consequently the evaluation of essential gene stringency may be reflected by measuring protein abundance. Protein quantification has previously been performed using western blotting, whereby proteins are transferred from SDS-PAGE gel to an absorbent membrane (e.g.: nitrocellulose) and probed with an antibody specific for the protein under investigation. Quantification is achieved by chemiluminescence using a secondary antibody conjugated to an enzyme, that catalyses the breakdown of a substrate to a coloured product (Kurien and Scofield, 2006). However western blotting lacks quantitative capacity and is incapable of distinguishing between post-translational modifications. Critically only those proteins targeted are subject to analysis, at the exclusion of the global proteome (Mann, 2008). To perform accurate quantification at the protein level requires the use of sensitive techniques such as liquid chromatography tandem mass spectrometry (LC-MS/MS).
1.3 The use of mass spectrometry in antibiotic discovery

1.3.1 Tandem mass spectrometry

Technological advances in LC-MS/MS have permitted global proteomic analyses, enabling the systematic identification of primary sequences, structural characterization of post-translational modifications, elucidation of protein-protein interactions and quantification of expressed proteins in complex matrices (Aebersold and Mann, 2003).

The Proteomics based LC-MS/MS process, initiates with the pre-separation of individual proteins from a whole cell lysate using either 1D or 2D gel electrophoresis, with the aim of removing low molecular weight contaminants capable of interfering with mass analysis. The protein bands of interest are exercised from polyacrylamide gels and subject to destaining and in-gel digestion. During this process samples are reduced to covert disulphide bonds to free sulfhydryl groups which are then alkylated to a S-carboxymethyl derivative, to facilitate disruption of three-dimensional structure and expose cleavage sites for heterophase enzymatic digestion (Shevchenko et al., 2006). The protease trypsin is frequently utilized, since it predicatively cleaves C-terminal residues at arginine and lysine, generating an appropriate mass range of peptides required for fragmentation during LC-MS/MS (Olsen et al., 2004).

According to peptide fragmentation models, the N-terminus is protonated, but the charge is capable of migrating along the backbone by internal solvation, becoming randomly localized on amide bonds. Cleavage along the peptide backbone results in cleavage at either the alkyl carbonyl bond (CHR-CO), peptide amide bond (CO-NH) or amino alkyl bond (NH-CHR). Following fragmentation, the charge can be retained at either the N terminus producing \( a, b, c \) ions or at the C terminus producing \( x, y, z \) ions. The peptides generated by trypsin are typically doubly charged structurally informative \( y \) ions, providing a high degree of predictability with respect to their fragmentation patterns (Covey et al., 1991).

Digested peptides are eluted from the polyacrylamide matrix in solvent and subject to further separation using High Performance Liquid Chromatography (HPLC) to minimise sample complexity. By reducing complexity the dynamic range can be increased to permit low abundance peptides to be detected in the
presence of peptides of higher abundance, thereby increasing the confidence of protein identification. Typically a Reversed Phase (RP-HPLC) format is employed, involving the differential separation of peptides between a stationary (nonpolar matrix) and mobile phase (polar solvent) according to the degree of hydrophobic interaction. During this stage hydrophobic peptides interact with the stationary phase, while hydrophilic peptides are eluted earlier in the mobile phase (Dass, 2007).

Eluted peptides are then introduced into an ion source and converted to charged species by introduction or removal of an electron using electrospray ionization, whereby ions are desorbed under a high electric field (Fenn et al., 1989). During this process the sample flows through a capillary with high potential that generates an electrostatic charge that dispenses solution into charged droplets for transportation, via a pressure gradient to a mass analyser (Dass, 2007). The mass analyser of choice is often the orbitrap mass spectrometer, which has provided an unprecedented level of sensitivity required for high resolution tandem mass spectrometry techniques (Makarov and Scigelova, 2010). Using this form of mass analyser provides the high mass resolution, accuracy (2-5 ppm), mass charge ratio and dynamic range ($10^3$) required for protein characterization and quantification. The orbitrap operates on the principle of dynamic ion trapping in electrostatic fields (Makarov, 2000) and constitutes a central spindle electrode encased by a coaxial outer electrode. Following electrospray ionization, ions are transported through a series of quadropoles that guide, transport and store ions prior to delivery via an optical deflection lens system into the orbitrap. The ions become trapped about the central spindle and undergo harmonic ion oscillations, the frequency of which provides the basis for mass charge values (Hu et al., 2005). The mass charge values of the ions generate a peptide mass fingerprint, which can then be compared to an in silico generated mass within a protein sequence database. The database is constructed from theoretical spectra, derived from peptide fragmentation models. Comparisons between the experimental and theoretical spectra is scored to assess the probability of a match and validated using algorithm based descriptive, interpretative, stochastic probability or statistical and probability models (Sadygov et al., 2004).
1.3.2 Proteomic strategies

The characterization of functional differences between biological systems requires comprehensive proteome coverage, if system-wide variations are to be identified and quantified accurately (Cox and Mann, 2007). To date partial proteome coverage has been achieved for 60% of open reading frames in the minimal genome bacteria Mycoplasma pneumonia (Kühner et al., 2009), which is marginally higher than that of multicellular organisms such as Caenorhabditis elegans (54%) (Schrimpf et al., 2009) and Arabidopsis thaliana 50% (Baerenfaller et al., 2008). The challenges in obtaining complete proteome coverage can be attributed to a number of technical and biological factors (Beck et al., 2011). The amenability of proteins to solubilisation and digestion dictates whether appropriate tryptic peptides and by extension, characteristic ion spectra can be generated. In the event of obtaining quality spectra, identification of proteins by matching the MS dataset to known sequences is dependant on the quality of annotated open reading frames held within genome databases. Inaccuracies may arise due to experimental conditions which may unknowingly influence transcription and translation parameters, thereby altering protein abundance. Furthermore, sequence coverage is subject to a sufficient fraction of peptides being detected, each of which may vary in terms of ionization and fragmentation properties. The number of peptides arising from enzymatic digestion may also be limited by the size of the protein, with smaller proteins generating fewer unique peptides capable of unambiguously identifying the protein under investigation (Beck et al., 2011). In order to provide consequential data, experimental workflows must be consistently reproducible and accurate for reliable quantification across multiple samples. To this end shotgun (discovery), directed and targeted MS-based strategies that differ in terms of how prior information is utilised and analysed, have been introduced (Domon and Aebersold, 2010).

The shotgun approach utilizes ionized peptides arising from electrospray ionization (precursor ions) to generate an initial mass spectrum known as a survey scan. Data-dependant analysis (DDA) using heuristics is used to perform product ion scanning, whereby precursor ion signal intensity is used to determine m/z ratio and subsequent peptide mass. A suitable precursor ion is then selected for fragmentation by Collision Activated Dissociation (CAD), that
generates fragments via collision with inert atoms (Hunt et al., 1986). The resulting fragment-ion masses form the product-ion spectra are used for protein identification, while the precursor ion signal intensity is used as the basis for quantification. Since no prior protein knowledge is required, the shotgun approach is suited to open discovery. The method however suffers from bias towards the selection of precursor ions, which represent the most abundant proteins present in a complex sample. Furthermore different samples of peptide ions in each experiment arise from the heuristics used. A variation to this approach is a directed proteomics strategy which entails two independent LC-MS analyses. An initial LC-MS/MS run generates survey scans to identify precursor ions relating to a peptides of interest, and records their mass spectrum characteristics (elution time, m/z ratio, charge), which are formed into a master inclusion list as part of the data dependant analysis. The LC-MS/MS is repeated in product ion mode using the inclusion list to limit CAD to specific fragments identified in the survey scan. Further improvements can be obtained by adopting a targeted proteomics strategy, which requires the use of triple quadruple instruments operating in Selective Reaction Monitoring (SRM) mode. Unlike previous strategies target proteins are preselected and their precursor ion m/z, retention time and unique fragment ions formed by CAD are defined to form a selected reaction monitoring (SRM) transition, which functions as assay parameters for the protein of interest (Picotti and Aebersold, 2012). Ultimately the aim of each strategy is achieve consistent detection of individual or subsets of protein.

### 1.3.3 Mass spectrometry quantification strategies

The accurate differential (relative) or quantitative analysis of protein is paramount and requires the implementation of suitable methods that compare individual peptides between experiments. Quantitation is not integral to the mass spectrometric process, since instrument response is dependant upon the physiochemical properties of proteolytic peptides, which are often influenced by matrix effects (Patterson and Aebersold, 2003). A range of techniques have been introduced based on the isotope dilution principle, in which isotope labelled peptides serve as proxies for their native equivalents. Since isotope labelled peptides possess the same physiochemical properties as native peptides, they generate the same chromatographic and mass spectrometric
profiles. However due to the difference in mass, a differentiation between experimental samples can be made.

Differential (Relative) quantification aims to examine fold changes in protein expression between experimental conditions and can be determined using label free methods (Spectral counts and MS ion intensity) and stable isotope labelling techniques. Spectral counting is founded on an observed linear relationship between protein abundance and the number of precursor ions selected for MS/MS fragmentation (Liu et al., 2004). The generation of MS/MS spectra associated with a particular peptide can therefore be counted and compared relative to the spectral counts of the same peptide under different experimental conditions, enabling relative quantification. Variations to this approach have lead to the estimation of protein expression using the Protein Abundance Index (PAI), derived from the ratio of sequenced peptides and total number of predicted tryptic peptides of a protein (Rappsilber et al., 2002) and absolute Protein Expression profiling (APEX) (Lu et al., 2007).

Alternatively the peak area or ion intensity of all identifiable tryptic peptides including their associated parameters of elution time and m/z ratio can be integrated over a time chromatographic scale. The subsequent data generates an extracted ion chromatogram (XIC) profile that is subsequently normalized to the peak area of an internal protein standard (Bondarenko et al., 2002). Although both techniques provide cost-effective approaches to compare changes in protein expression for multiple experiments, across higher dynamic range, accuracy is unreliable. Systematic and non systematic variations relating to ionization efficiency, sample preparation and instrumental drift are emulated in data, requiring normalization to correct for variation. Furthermore, the linearity of response for each protein is problematic due to variations in saturation effects for individual proteins (Bantscheff et al., 2007; Xie et al., 2011).

Stable isotope labelling provides an alternative to label free methods, by providing greater accuracy for relative quantification. Conceptually, the approach involves the incorporation of low abundance heavy isotopes ($^{13}$C, $^{15}$N $^{18}$O) into proteins and peptides, where they function as a label. Differences in ion signal intensities for heavy and light forms of the peptide are therefore taken to reflect quantitative changes in protein abundance (Becker, 2008). Heavy isotopes may be incorporated into proteins metabolically during growth on
labelled media, exemplified using the Stable Isotope Labelling with Amino acids in Cell culture (SILAC) approach, where culture media deficient in essential amino acids is supplemented with isotope labelled equivalents (Ong et al., 2002).

A variation of this approach is to introduce heavy isotopes of $^{18}$O directly into peptides via the C-terminal of proteins following protease digestion. Alternatively isotope containing tags may be introduced via chemical modification of specific amino acid residues. This approach is utilised by Isotope-coded Affinity tags (ICAT), which react with sulphhydral groups of cysteine residues (Gygi et al., 1999); isobaric Tags for Relative and Absolute Quantification (iTRAQ) which link a carbonyl and reporter group (Wiese et al., 2007) and Tandem Mass tags (TMT), which react with amine groups (Thompson et al., 2003).

Despite providing adequate means to examine changes in protein expression, precise determination of actual protein molecules is not possible without reference to a standard of pre-determined quantity. The requirement for accurate quantification of protein per se, has been met by the development of the Absolute Quantification strategy (AQUA) (Kirkpatrick et al., 2005). In this approach, the target protein under investigation is initially analysed by LC-MS/MS to identify a suitable unique tryptic peptide representative of the target protein, for use as an internal standard (Figure 1.4). The standard is synthesised with an appropriate heavy label and evaluated using LC-MS/MS to determine parameters of retention time and product ion intensities. A quantified amount of standard is then introduced to the sample during tryptic digestion and analysed by LC-MS/MS. Quantification is performed by integrating peaks for light and heavy ions corresponding to the sample and standard respectively and analysing the ratio of the peak area, which over a linear range enables extrapolation of the sample concentration. Using this strategy has enabled the quantification of low abundance yeast proteins including phosphorylated forms (Gerber et al., 2003).
The use of isotope labelled peptides as internal standards has been developed further to enable multiplex protein quantification, using artificially constructed proteins known as quantification concatamers (QconCAT), which can contain multiple proteolytic peptides of the target protein (Brun et al., 2007). A further augmentation has been the development of Protein Standard Absolute Quantification (PSAQ), which utilises a full length isotope labelled protein equivalent to the target protein under investigation. (Brun et al., 2009).

### 1.4 Gene silencing using synthetic antisense structures

Antisense based silencing has not been constrained exclusively to sequences derived biologically. Attempts to create synthetic compounds capable of binding DNA and RNA in a sequence specific manner have culminated in the development of numerous nucleic acid analogues. Initial efforts comprised dideoxynucleotide bases interconnected by various chemical linkers such as phosphotriester, methylphosphonate, phosphorothiolate, and carbamate (Summerton and Weller, 1997). Subsequent improvements in target specificity
and biostability were achieved through the structural alterations of dideoxynucleotides to generate non-ionic backbones. The derivatization of morpholine subunits from ribonucleosides, formed the basis for phosphorodiamidate morpholino oligonucleotides (PMO’s), with significantly improved properties for biological applications (Stirchak et al., 1989). Alternate substitutes for nucleobases precipitated in the synthesis of Peptide Nucleic Acids (PNA) (Nielsen et al., 1991). Structurally, PNA’s are comprised of noncyclic peptides (polyamides of N-2-aminorthyl glycine linked via carbonyl linkers to nucleobases), in substitution of the sugar phosphate backbone (Lundin et al., 2006). This arrangement creates structural flexibility with a neutral charge that negates electrostatic repulsion, thereby increasing hybridization efficiency and sequencing specificity with nucleic acid counterparts (Good and Nielsen, 1997). The high affinity, discriminatory capacity and biostability of PNAs, has made them pre-eminent for antisense applications where short sequences are encountered (Summerton, 2006).

Recognition between PNA and complementary sequences elicits the formation of duplex structures via Watson-Crick hydrogen bonding, when the PNA is composed of purine /pyrimidine nucleobases. If the PNA composition exclusively incorporates homopyrimidines, then triplex structures are assembled via Watson-Crick and Hoogsteen hydrogen bonds (Larsen et al., 1999). To date, complexes representing triplex, triplex Invasion, duplex and double duplex invasion are been observed (Nielsen, 2010). The formation of these alternate complexes, contributes to variant silencing mechanisms. Triplex forming PNA’s significantly distort RNA structure that precipitates steric hindrance, causing inhibition of both translation initiation and ribosome elongation, contrary to duplex structures which prevent translation initiation (Knudsen and Nielsen, 1996) and possibly ribosome read-through (Kulyté et al., 2005). In contrast to expressed antisense, translation inhibition of mRNA appears to be the primary silencing mechanism, since PNA complexes show a significant degree of biostability towards RNaseH (Knudsen and Nielsen, 1996), thereby preventing RNase mediated mRNA degradation.

The biological stability PNA’s have exhibited in bacterial extracts (Demidov et al., 1994), and in addition to their hybridization properties, PNA’s makes them a novel research tool for synthetic RNA silencing applications in bacteria (Good
Central to this development have been efforts to address bacterial entry and accumulation of PNAs to an effective intracellular concentration. These factors are critical since growth inhibition is attributed to PNA accumulation and slow efflux resulting in potent antibactericidal activity (Nikravesh et al., 2007).

The primary obstacle opposing cellular entry is the bacterial membrane, which in gram negative bacteria comprises a cell wall consisting of an outer membrane, a thin peptidoglycan layer, a layer of periplasm, and a plasma membrane (Beveridge, 1999). The outer membrane consists of lipopolysaccharide (LPS), which provides an effective barrier due to its rigid lipid interior and strong interaction between the peptidoglycan layers. The cellular accumulation of PNA is opposed by transmembrane porins within the outer membrane, which possess the capacity to remove certain peptides (Nikaido, 2003). However in a recent study, one such membrane transport protein Smba was speculated to be involved in actively transporting peptide PNA conjugates into cells (Ghosal et al., 2013).

The significance of cellular entry and accumulation was previously evaluated by exposing *E.coli* strains A19 and D22 deficient in cell walls and specific membrane pumps AcrAB and Emr to a PNA targeting the lac repressor to enable a correlation between susceptibility and β-galactosidase activity following permeabilisation. Although the absence of membrane pumps showed little effect on activity, the lack of LPS was found to be a significant factor in increasing β-galactosidase activity, suggesting increased membrane permeabilisation promotes greater susceptibility (Good et al., 2000a).

Consequently numerous strategies have been deployed to improve delivery of PNA’s across the membrane. Initial attempts utilized microinjection, electroporation or co-transfection with cationic lipids (Koppelhus and Nielsen, 2003). A pioneering approach has been to conjugate PNA’s to a synthetic cell wall permeabilising peptide KFFKFFKFFK possessing cationic, hydrophobic and amphiphilic properties (Good et al., 2001b). The presence of a peptide promotes uptake across the membrane by receptor independent mechanism, through interaction with complementarity groups in the LPS (Vaara and Porro, 1996). Divalent cation binding sites of the LPS in particular, may have a role in compromising membrane integrity through the formation of transient rents that
enable PNA entry (Hancock, 1997). The effectiveness of this strategy was demonstrated by conjugating permeabilising peptide to a PNA targeting the essential gene acpP in *E. coli*, resulting in growth inhibition (Good *et al.*, 2001b). Further evidence was obtained using chromogenic reporter systems to monitor permeabilisation of both the outer and inner membrane, which showed an increase in flourescence in the presence of PNA peptide conjugates compared to free PNA (Eriksson *et al.*, 2002). Although the majority of studies examining PNA-conjugates have utilised to gram negative bacteria, their effectiveness has also been established in gram positive species such as *S. aureus*, where the targeting of essential genes also causes growth inhibition (Nekhotiaeva *et al.*, 2004).

Due to the specificity and bactericidal activity displayed *in vitro* and *in vivo*, there has been intense interest into developing PNA’s into species specific antimicrobials, that avoid compromising natural microbiota defences (Good and Stach, 2011). Alternative PNA based approaches have focused on re-introducing antibiotic susceptibility using PNA’s to target resistance mechanisms. One study demonstrated that PNA targeting of the multidrug efflux transport in CmeABC in *Campylobacter jejuni*, was found to increase sensitivity to ciprofloxacin and erythromycin (Jeon and Zhang, 2009a). Addition roles for PNA’s have also been explored including targeted gene repair, whereby PNAs are constructed with a corrected sequence of a mutated gene. The formation of triplex PNA structure with the target gene sequence perturbs the regular helical structure, initiating DNA repair mechanisms that result in homologous recombination. Subsequently the repaired sequence is exchanged with the mutated gene (Vasquez *et al.*, 2001). A viable demonstration of this potential was shown by single base pair modification of β-globin intron using pseudocomplementary PNAs (Lonkar *et al.*, 2009). Further roles for PNA’s have been demonstrated such as artificial restriction DNA cutters that enable high fidelity site selective scission of DNA (Miyajima *et al.*, 2009), probes for microarrays (Brandt and Hoheisel, 2004), and investigation of microbial communities (Hatamoto *et al.*, 2010).
1.5 Project outline

The central aim of this project is to investigate the application of antisense silencing in two distinct roles. Firstly, as a technique for determining the stringency of essential genes in *E. coli* and how stringency differs between individually transcribed and operon based genes. Secondly as a species specific silencer, by evaluating the capacity of synthetic antisense sequences to discriminate between essential gene homologues and thereby enable selective growth inhibition of a specific organism.

Expressed antisense RNA silencing using inducible paired termini vectors, has been employed to determine essential gene stringency, as defined by a Minimum Transcript Level (MTL$_{50}$) (Goh *et al.*, 2009). Determinations of essential gene stringency have been limited to individually transcribed genes (Goh *et al.*, 2009), which are not subject to the complex regulation associated with operon based genes (Ames and Martin, 1964). This raises the question of whether the MTL$_{50}$ is applicable to operon based genes. Furthermore, stringency is based upon the relationship between growth rate decline and mRNA abundance, which is assumed as a proxy for expressed protein. However post-transcriptional and translation regulation mechanisms, account for uncorrelated abundancies between mRNA and protein (Maier *et al.*, 2009). Consequently measurements of mRNA abundance may be unsuitable for determining essential gene stringency requirements. Determinations of gene stringency would therefore have more relevancy if the relationship between growth rate decline and protein abundance was examined. From this a Minimum Protein Level (MPL$_{50}$) could be derived to replace MTL$_{50}$, as a more accurate measure of gene stringency. A method capable of accurately quantifying expressed protein following gene silencing is therefore essential.

Historically, protein quantification has been performed using biochemical assays (Sapan *et al.*, 1999), immunoassays and more recently protein microarrays (MacBeath, 2002). However such techniques lack sensitivity and are subject to cross reactivity, which complicates quantification (Patterson and Aebersold, 2003). Recent developments in the quantitative capacity of LC-MS/MS provide a number of advantages over such methods. LC-MS/MS provides a greater sensitivity over a large dynamic range, enabling low abundance proteins to be detected. This is crucial for antisense silencing
experiments, where target protein may be depleted to unpredictable levels. Crucially, LC-MS/MS permits the identification of individual proteins from complex matrices, such as whole cell lysate. If required parallel identification and quantification of more then one protein can also be accomplished, which supports the evaluation of operon based genes, where the monitoring of more then one protein may be required. Furthermore LC-MS/MS possess the capacity to discriminate between proteins subject to subtle post-translation modifications that can be used to estimate rates of degradation and synthesis. (Bantscheff et al., 2007; Bronsema et al., 2012). The viability of determining protein abundance has been demonstrated with LC-MS/MS using label free methods a modified protein abundance index method (Ishihama et al., 2005), revealing proteins to exist over a dynamic range from 100 to 10^5 copies per cell (Ishihama et al., 2008).

In this study, an absolute quantification strategy using LC-MS/MS that incorporates the use of a stable isotope (^{15}N) labelled protein internal standard for absolute quantification, will be used to examine protein expression in E.coli subject to antisense silencing. Although LC-MS/MS strategies that use protein internal references for absolute quantification are not widely employed, they have nonetheless been demonstrated in bacteria such as Leptospira interrogans (Malmström et al., 2009).

The essential E.coli genes fabl and murA have been selected as candidate targets for developing protein internal standards, since both genes are individually transcribed and have been previously evaluated with regards to gene stringency. Furthermore, both genes have well characterised functions and are subject to inhibition by antibiotics with known modes of action. In E.coli fabl encodes the enzyme enoyl-acyl carrier protein reductase, which functions as part of the dissociated (type II) fatty acid synthase system, that regulates membrane lipid synthesis (Magnuson et al., 1993). The broad spectrum biocide triclosan (trichloro derivative of 2-hydroxy-phenyl ether) specifically targets Fabl in E.coli, in addition to gram positive bacteria, fungi and mycobacteria (Saleh et al., 2011). Triclosan inhibits lipid synthesis, through direct targeting of fabl as demonstrated in mutants, which show an approximate 300-fold increase in resistance (McMurry et al., 1998). The established mode of action arises from irreversible binding of triclosan to the enoyl substrate site, leading to an
increase in affinity for NAD+ (Heath et al., 1999). Triclosan binds non-covalently via face to face stacking of the phenol ring and nicotinamide rings hydrogen bonding. Consequently a stable ternary complex is formed that is incapable of catalysis between phenolic hydroxyl and hydrolysis of 2’ nicotinamide robose, causing enzyme inhibition. The binding efficiency of triclosan is enhanced by its ability to induce a closed conformation of a flexible loop structure located above the enoyl active site (Qiu et al., 1999).

The essential gene murA encodes enolpyruvyl transferase, which catalyses’ the transfer of an enolpyruvate moiety from phosphoenolpyruvate (PEP) to uridine diphosphate. This represents the initiating step in the synthesis of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) glycan units that comprise peptidoglycan, of the bacterial cell wall (Lovering et al., 2012).

The antibiotic fosfomycin ((1R,2S)-1,2-epoxypropylphosphonic acid) inhibits MurA through the formation of covalent interactions between a thiol group of cytesine115 residue within the active site of the enzyme, which prevents a conformational change in the enzyme required for the dissociation of the product UDP-N-glucosamine (UNAG) (Eschenburg et al., 2005).

Once validated, LC-MS/MS will be applied to investigating gene stringency in operon based genes in E.coli by examining fusA and rplE. The essential genes fusA which is encoded in the str operon (Dean et al., 1981b) and rplE encoded in the spc operon (Cerretti et al., 1983). The essential gene fusA encodes elongation factor G (EF-G), a translational GTPase that binds to the ribosome complex and induces a conformational change involving hydrolysis of GTP, which initiates translocation of tRNA and mRNA (Rodnina et al., 1997). A functional role has also been determined in ribosome recycling, where EF-G in conjunction with the essential protein Ribosome Recycling Factor (RRF) induces dissociation of the 70S ribosome into its component subunits in preparation for next round of protein synthesis (Hirokawa et al., 2005). In E.coli rplE encodes the 5S rRNA ribosome accessory protein L5, which has been demonstrated to be essential for cell survival (Korepanov et al., 2007). The L5 protein is one of 21 accessory proteins that in conjunction with 16S rRNA constitute the small ribosome subunit (30S), that interacts with tRNA and mRNA (Steitz, 2008) The exact role of rplE was unknown until recently, when it was
revealed that the L5 protein forms a prominent part of the central protuberance (CP) of the large ribosomal subunit (Korepanov et al., 2012).

In addition to quantifying expressed protein for the purposes of measuring gene stringency, the use of LC-MS/MS can also be applied to support the development of an antisense RNA based screening assay for detecting novel target specific inhibitors. To date antisense based target assays have demonstrated potential, with the discovery of novel antibiotics such as platensimycin a FabF inhibitor (Wang et al, 2006), platencin a FabH /F inhibitor (Wang et al, 2007) and Phaeosphenone, which targets the encoded product of rpsD (Zhang et al, 2008). By sensitizing E.coli to target specific inhibitors and then measuring expressed protein abundance at particular MIC’s, would enable suitable levels of IPTG induction required for antisense silencing to be defined. Consequently assay sensitivity could be optimised for screening target specific compounds that are synthesized in low abundance.

Furthermore the knowledge of the expressed protein abundance following antibiotic exposure could be used generate a characteristic proteomic signature (Brotz-Oesterhelt et al., 2005). This data can then be used to identify candidate cell targets for antibiotic screening and infer potential modes of action. In addition, characterisation of proteomic signatures provides a basis for constructing reference compendia for the purposes of identifying novel antimicrobial compounds (Freiberg et al., 2004). Using candidate targets fabI and murA, this study intends to use a validated LC-MS/MS approach to measure protein abundance in E.coli at different inhibitory concentrations of triclosan and phosphomycin, which aims to provide sufficient data for a mathematical model for estimating assay sensitivity.

An alternate to using expressed antisense RNA from plasmids, for the purpose of evaluating essential gene stringency or fine tuning the sensitivity of screening, are peptide PNA’s. The capacity of peptide PNA conjugates to induce gene silencing, has been evaluated against expressed antisense RNA and found to be highly comparable (Goh et al., 2009). In addition, the inherent stability of PNA’s in vivo, has facilitated their development as species-specific antimicrobials in an effort to address antibiotic resistance (Good and Stach, 2011). Indeed bactericidal activity has previously been demonstrated against individual gene targets in E.coli (Good et al., 2001b). To date however
no effort has been made to ascertain if PNA’s can be designed to differentiate between a genes in separate bacterial species. This study intend to evaluate species-specificity, by designing a PNA that targets an essential gene homologue (ftsZ) in *Salmonella typhimurium*, when grown in a mixed microbial culture with *E.coli*. 
1.6 Specific aims and objectives

1. Identify essential genes for silencing using bioinformatics strategy.
2. Construct antisense RNA expressing strains for *E.coli* essential genes *fusA* and *rplE*.
3. Validate target specificity of antisense RNA constructs by constructing over expression vectors to be used in a transcomplementation strategy.
4. Delineate whether bactericidal or bacteriostatic inhibition occurs following silencing of essential genes *fusA* and *rplE* using viable counts.
5. Design and validate a QPCR assay for the relative quantification of mRNA abundance, of *fusA*, *rplE*, *fabI*, and *murA*.
6. Determine essential gene stringency of *fusA* and *rplE*, using expressed antisense RNA silencing to achieve a titration of growth rate in *E.coli*, and measure associated mRNA abundance. From this data MTL$_{50}$ values for *fusA* and *rplE* will be derived.
7. Synthesise heavy labelled protein standards for *fabI* and *murA* and evaluate suitable tryptic peptides for identification and quantification of respective proteins.
8. Quantitate heavy labelled standards using a standard curve constructed from commercial prequantified peptide using LC-MS/MS.
9. Determine gene stringency of *fabI*, using expressed antisense RNA silencing to achieve a titration of growth rate *E.coli*. Protein abundance will be measured using LC-MS/MS and FabI molecules per cell will be calculated to derive a MPL$_{50}$ value for measuring gene stringency.
10. Assess discriminatory capacity of species-specific PNA in mixed bacterial culture.

1.7 Thesis Outline

Chapter 1. Introduction

This Introduction describes the significant features of antibiotics and the challenges posed by multidrug resistance. New strategies for antibiotic discovery are outlined, with particular reference to the use of antisense RNA and quantitative LC-MS/MS. An argument is presented for the assessment of essential gene stringency based upon expressed protein.

Chapter 2. Methods and Materials
The materials and methods details the construction of antisense RNA and transcomplementation expression vectors, in addition to an LC-MS/MS quantitative assay, using a $^{15}$N labelled internal protein standard, for the evaluation of essential gene stringency. In addition a method used to assess the species specificity of a PNA in a mixed culture of *S.typhimurium* and *E.coli* is also described.

Chapter 3. Results

The experimental findings regarding target specificity of antisense RNA expression constructs, their validation by transcomplementation, and characterization of inhibitory action of essential gene targets is presented. Analysis of essential gene stringency using mRNA abundance of candidate genes is given. This chapter also reports the validation of a quantitative LC-MS/MS assay for measuring protein molecules per cell following expressed antisense silencing, and subsequent deriving of the MPL50 value as a measure of essential gene stringency. The analysis of species-specific PNA effects on bacteria in a mixed culture is also presented.

Chapter 4. Discussion

An evaluation of the experimental findings obtained in this study is made. Explanations to account for the observed data including anomalies are proposed, with support from appropriate literature.

Chapter 5. Conclusions and Future Work

This chapter draws on the discussion to frame the findings of the study in the wider context of antibiotic discovery, in addition to extending the scope of the research to support applications in other fields of biological enquiry. Recommendations to address the limitations highlighted during the course of this study are made.

Appendix

Additional experimental detail is presented where appropriate.

References

Literature used to provide a theoretical background is presented.
Chapter 2 Methods & Materials

2.1 Selection of essential gene targets for silencing

A hierarchy of gene stringency (Jem .Stach, personal communication) was generated by obtaining transcript abundance, presence in an operon and number of protein-protein interactions for the essential protein. Transcript abundance for *E. coli* K12 genes was averaged from microarray experiments conducted in LB media at 37°C (RNA extracted at mid-exponential phase, *n* = 50 (Glasner *et al.*, 2003). Presence of the gene in an operon, and location within the operon was obtained from the RegulonDB database (Gama-Castro *et al.*, 2008), and the number of protein interactions was taken from the experimental dataset of the Bacteriome.org database (Su *et al.*, 2008). For each category, the essential gene was ranked, in the case of location within an operon; weighting was assigned prior to ranking, such that genes that were present at the start of large operons, containing other essential genes downstream, were scored highly. Weightings of 20:4:2 were applied for the number of essential genes downstream, the number of genes in the operon, and the number of genes downstream, respectively. The weighted score for each gene was calculated as sum product of these categories, multiplied by the sum of the weights for each category. The weights were arbitrarily assigned considering the mechanism of antisense gene silencing i.e. silencing of the first gene in an operon is more likely to affect genes downstream. The estimated transcript number and number of protein interactions, were given simple rank scores (higher numbers in each giving a higher rank scores). Finally, for each essential gene an overall rank score was applied by weighting (6:6:3) for transcript copy number, number of interactors and presence in an operon, respectively. The final weightings were arbitrarily applied considering that genes with high transcriptional abundance, producing products which are highly interactive, are more likely to be stringently required. Weighting were also applied such that the final rankings for genes *acpP, fabI, ftsZ* and *murA* were consistent with previous reported *MTL*50 values (Goh *et al.*, 2009). The genes *rplE* and *fusA* were chosen for further study, as they were predicted to be stringently required (ranked 1 and 8, respectively) and were also present in operons. This latter fact was considered important for assessing the utility of the *MTL*50 score for genes present in operons, where downstream affects will likely
result from antisense RNA silencing. Nucleotide sequences complementary to the ribosome binding region of mRNA transcribed for fusA (-93 +86) and rplE (-95 +60) were identified and cloned into plasmid pHN678, which contains flanking inverted repeats that form secondary structure in the form of dsRNA termini. The termini increase the stability of antisense transcripts thereby improving silencing efficiency (Nakashima et al., 2006). To validate the specificity of silencing constructs, transcomplementation plasmids expressing the entire ORF of fusA and rplE were also constructed using pBAD (Guzman et al., 1995). For the purposes of comparison, an evaluation was made against antisense RNA expression vectors and associated transcomplementation vectors for essential gene targets acpP, ftsZ, fabl, and murA (kindly supplied by Shan Goh, Royal Veterinary College, U.K).

2.1.1 Bacterial growth conditions and strains

Bacterial growth was performed in liquid and on solid media using Muller Hinton Broth (MHB) (Melford) for both E.coli and S.typhimurium. Growth conditions were 37°C, with aeration at 200-250 rpm for broth cultures. Where maintenance of plasmids was required, media was supplemented with antibiotics (Melford) at a final concentration of 30µg/ml chloramphenicol, 100µg/ml ampicillin, 50µg/ml kanamycin. Detailed recipes or growth media and buffer solutions can be found in Appendix: A. All strains used in this study are listed in Table 2.1.

2.1.2 Bacterial genomic DNA and plasmid extraction

Both antisense and complete ORF sequences for fusA, rplE, fabl and murA were amplified from genomic DNA, which was isolated from E.coli-K12 using the GenElute™ Bacterial Genomic DNA kit (Sigma). Briefly 1.5 ml of overnight bacterial culture was harvested by centrifugation at 16,000 x g for 2 minutes. The supernatant was removed and pelleted cells were resuspended in 180 µl Lysis Solution T with 20 µl of RNase A and incubated for 2 minutes at room temperature. After incubation, 20 µl of proteinase K solution was added and samples were incubated for 30 minutes at 55°C. A volume of 200 µl of Lysis Solution C was added to samples which were then incubated at 55°C for a further 10 minutes. GenElute Miniprep Binding columns were prepared by addition of 500 µl of Column Preparation solution. Columns were centrifuged at
12,000 x g for 1 minute and the flow through was discarded. Prior to loading, 200 µl of ethanol (95-100%) was added to lysate and vortexed, before loading onto columns and centrifuged at 6,500 x g for 1 minute. The spin column was placed in a fresh collection tube and 500 µl of Wash Solution I was added, samples were then centrifuged at 16,000 x g for 3 minutes. The spin column was placed in a fresh collection tube and a further 500 µl of Wash Solution I was added, samples were centrifuged at 6,500 x g for 1 minute. The column was placed in a fresh collection tube and centrifuged at 16,000 x g for 1 minute. DNA was eluted by addition of 200 µl of Elute Solution followed by an incubation of 5 minutes at room temperature and centrifugation at 16,000 x g for 1 minute. Genomic DNA was quantified in triplicate using a NanoDrop™ (Thermo Scientific).

Plasmid DNA was extracted using the QIAprep® Spin kit (Qiagen). Briefly 5 ml of overnight bacterial culture was pelleted by centrifugation at 2000 x g for 5 minutes. Pelleted cells were resuspended in 250 µl Buffer P1 containing RNase A, to which 250 µl of Buffer P2 was added and the sample mixed by inversion. Buffer N3 (350 µl) was added and samples were centrifuged at 17,900 x g for 10 minutes. The supernatant was added to a QIAprep spin column and centrifuged at 17,900 x g for 1 minute. The flow through was discarded and 500 µl of Buffer PB was then added to the spin column. Samples were centrifuged at 17,900 x g for 1 minute, and the flow through was discarded. A second wash using 750 µl of Buffer PE was added to the spin column, prior to centrifugation at 17,900 x g for 1 minute. The wash through was removed and the sample was centrifuged at 17,900 x g for 1 minute. Plasmid DNA was eluted addition of 30 µl of Buffer EB directly to the centre of the spin column, which was incubated at room temperature for 1 minute, prior to centrifugation at 17,900 x g for 1 minute. Plasmid DNA was quantified in triplicate using a NanoDrop™ (Thermo Scientific).

2.1.3 Primer preparation and annealing temperature optimization

To ensure optimal amplification of antisense and ORF sequences, a primer validation study was performed. Lyophilized Primers (Sigma/Eurogentec) (Table 2.4 and Table 2.5), were re-dissolved to a concentration of 100 µM, and then diluted to a working concentration of 10 µM in molecular grade water (Melford). Initial gradient Polymerase Chain Reaction (PCR) was performed by preparing
a 25µl reaction, consisting of 2 x PCR master Mix (0.05 units/µl Taq DNA polymerase, 4 mM MgCl₂, 0.4 mM each of dATP, dCTP, dGTP, dTTP) (Fermentas), diluted with molecular grade water to a final concentration of 1 x and mixed with forward and reverse primers at a final concentration of 0.2 µM. A total amount of 100ng of template DNA was added. The thermal Gradient was devised by using a range of 3°C either side of the average calculated primer pair Tm. The PCR thermal profile was as follows: denaturing 95°C for 2 minutes, 30 cycles of denaturing at 95°C for 30 seconds, annealing temperature for 30 seconds, extension at 72°C for 2 minutes and a final extension of 72°C for 5 minutes. All gradient PCR were performed on a DYAD™ DNA Engine (MJ Research). Optimal primer annealing temperatures were evaluated by visual inspection of amplified product using gel electrophoresis (see methods section: 2.1.4).

2.1.4 Agarose gel electrophoresis

Analysis of PCR products was performed by agarose gel electrophoresis to ensure correct size of amplified products and ascertain the optimal primer annealing temperature. Agarose gels were prepared by dissolving an appropriate amount of agarose (Invitrogen) to achieve a final concentration of 0.5-2% as required for optimal resolution of DNA fragments, in 10 x Tris-borate-EDTA buffer (1.3 M TRIS, 450 mM boric acid, 25 mM EDTA.Na₂ in H₂O) (Sigma), diluted to 1 x concentrate. Agarose gels were stained with ethidium bromide (Sigma) at a final concentration of 0.5µg/ml. Gels were ran at 8v/cm for 1 hour and photographed using a Uvitec gel doc systems with Essential v12.6 imaging software (UVITECH). Samples were prepared by mixing 5-10 µl of DNA with 6 x loading dye (Fermentas) to achieve 1 x final concentration. For size estimation 5 µl of 100Kb ladder and Hind III digest (Fermentas) were used as required.

2.1.5 Cloning of antisense and ORF sequences into pHN678 and pBAD plasmids

Antisense sequences for essential genes, fusA and rplE and the ORF of target genes fabI, murA, fusA and rplE were amplified from E.coli K12 genomic DNA, using sequence specific primers (Table 2.4). Antisense sequences were designed with restriction sites for Xhol and Ncol, to permit placement of PCR
insert in reverse orientation in the multiple cloning site (MCS) of pHN678, in order to generate antisense transcript. Transcomplementation vectors were designed with restriction sites for XhoI and EcoRI (fabI, murA, rplE) and XhoI and ScaI (fusA). PCR amplification of target sequences was performed using Phusion® High fidelity DNA polymerase (New England Biolabs). A 50µl PCR reaction was prepared with final concentrations of 1 x reaction buffer, 0.2 µM primers with exception of fabI (1 µM), 200 µM dNTP, 0.02 units and 4 µl cDNA template. Thermal cycling was performed on 9700 geneamp thermal cycler (Applied Biosystems) using the following conditions: 98°C 30 seconds, 98°C for 10 seconds, optimized annealing temperature for 15 seconds, 72°C 15 seconds, and final extension of 72°C for 1 minute. Subsequent products were purified using QIAquick PCR purification kit (Qiagen) and quantified using a NanoDrop™ Spectrophotometer and associated software ND1000 v3.2.1 (Thermo Scientific). Confirmation of PCR products was performed by agarose gel electrophoresis (see methods section: 2.1.4).

2.1.6 Restriction digest of PCR inserts and plasmids

To prepare PCR inserts for cloning into their respective vectors, double restriction digests were performed using XhoI and NcoI for antisense sequences fusA and rplE. Amplicons of complete ORF sequences were double digested with XhoI and EcoRI (fabI, murA, rplE) and XhoI and ScaI (fusA) in addition to pBAD. Restriction digests were performed according to manufacturers’ instructions (Fermentas) and consisted of 10 x Tango buffer (330 mM Tris-acetate, 100 mM Mg-Acetate, 660 mM K-acetate, 1 mg/ml bovine serum albumin (BSA)) diluted to a final 2 x concentration. For double digests, 10 units of appropriate restriction enzyme (Fermentas) was added per 1µg of DNA, molecular grade water was then added to bring the final reaction volume up to 40 µl. Samples were incubated at 37°C for 2 hours followed by a thermal inactivation of restriction enzymes by incubating at 80°C for 20 minutes. Digested products were purified using QIAquick PCR purification kit (Qiagen) and quantified in triplicate using a NanoDrop™ (Thermo Scientific).

2.1.7 Ligation of PCR inserts into plasmids

Digested antisense and ORF sequences were ligated into pHN678, and pBAD respectively. Sticky-end ligation was performed using 100 ng of linear vector
DNA according to manufacturers’ instructions. The molar ratio of insert DNA was calculated using the Ligations: Molar ratio of insert to vector calculator (Promega). Vector and insert DNA were mixed in a ratio of 1:3 with 10 x T4 DNA ligase buffer (Fermentas) (400 mM Tris-HCl, 100 mM MgCl₂, 100 mM DTT, 5 mM ATP), which was diluted to 1 x concentration. For each reaction, 5 units of T4 DNA ligase (Fermentas), was added. Molecular grade water was then added to bring final reaction volume to in a 20 µl. Samples were incubated for 10 minutes at 22°C followed by thermal inactivation of T4 ligase by incubating at 65°C for 10 minutes. Confirmation of ligation insertion was performed by restriction digestion and agarose gel electrophoresis (see methods section: 2.1.4).

2.1.8 Transformation of antisense and transcomplementation plasmids into E.coli

Ligated plasmids containing pHN678 or pBAD with antisense sequences or complete ORF sequences respectively were introduced into E.coli by chemical transformation. For each plasmid, 5 µl of ligated product was added to one vial of One Shot TOP10 Chemically competent E.coli (Invitrogen). The competent cells were then incubated on ice for 30 minutes, prior to heat shocking for 30 seconds at 42°C without shaking. Competent cells were then left on ice to recover for 2 minutes. A total volume of 250 µl of pre-warmed SOC medium (see Appendix: A) was added to each vial, which were subsequently incubated at 37°C for 1 hour with aeration. A suitable volume of transformants (100 µl) was pipetted onto pre-warmed LB agar containing an appropriate antibiotic and incubated at 37°C overnight. Transformants carrying the correct plasmid with insert were screened using colony PCR with plasmid specific primers for pHN678 and pBAD respectively (Table 2.4).

2.2 Evaluation of antisense specificity of fusA and rplE constructs

To examine the specificity of antisense RNA constructs, antisense growth assays were prepared according to the procedure used in a previous evaluation of essential gene targets (Goh et al., 2009). A series of IPTG (Melford) concentrations were prepared to induce antisense RNA expression from pHN678 vectors, to generate a titration in E.coli growth rate. IPTG concentrations were prepared in molecular grade water at 10 x concentration,
20 µl of which was pipetted in triplicate into a 96 well micro plate (Sarstedt). Overnight cultures of *E.coli* were standardized by OD<sub>550</sub> readings to 2 x 10<sup>4</sup> cfu/ml, using a LibraS12 Spectrophotometer (Biochrom). Standardized culture volumes of 180 µl were added to respective wells, to bring the final assay volume to 200 µl. The 96 well microplate was incubated in a Powerwave HT plate reader (Biotek Instruments Inc) at 37°C with agitation every 5 seconds and readings at 550nm taken every 5 minutes for a period of 24 hours. Bacterial growth curves were plotted using Gen5 software v1.04.5 (BioTek). Raw absorbance data corresponding to the exponential phase of *E.coli* growth was exported to Excel (Microsoft). A trendline was applied and the gradient of the growth curve recorded. Relative growth rate was derived by normalizing the trendline gradient of induced cells to the trendline gradient of uninduced controls.

2.3 Validation of antisense specificity by transcomplementation

To validate the target specificity of antisense constructs, rescue assays were performed by transforming both antisense and corresponding transcomplementation vectors into *E.coli* TOP10. A matrix of IPTG and L-arabinose induction concentrations were assessed to determine the level of induction required to demonstrate the rescue of bacterial growth rate, in accordance with a previous study (Goh *et al*., 2009). A dilution series of IPTG and L-arabinose (Melford) were prepared at 20 x concentration, 10 µl of each substrate was added to respective wells, prior to the addition of 180 µl standardised culture in MH media supplemented with 30µg/ml chloramphenicol and 100 µg/ml ampicillin. Bacterial growth curves were plotted using Gen5 software v1.04.5 (BioTek). Raw absorbance data corresponding to the exponential phase of *E.coli* growth was exported to Excel (Microsoft). A trendline was applied and the gradient of the growth curve recorded. Relative growth rate was derived by normalizing the trendline gradient of induced cells to the trendline gradient of uninduced controls.
2.4 Delineation of the inhibitory effects of silencing essential gene targets fusA and rplE

To examine the inhibitory effects of antisense RNA silencing on growth, viable cell counts were performed in the presence of 50 µM IPTG, which was double the concentration used to silence growth in the examination of specificity. As per the procedure for antisense and rescue assays, overnight bacterial cultures were standardized by OD\textsubscript{550} readings to 2 x 10\textsuperscript{4} cfu/ml. A 50 ml volume of MH broth containing chloramphenicol (30µg/ml) was inoculated and then incubated at 37°C with aeration. Sample volumes of 100 µl were removed every hour over a period of 8 hours, serially diluted (10\textsuperscript{-5} and 10\textsuperscript{-6}), plated onto MH agar (in triplicate) and incubated 37°C overnight. A viable colony count was then performed on plates containing between 30 and 300 colonies.

2.5 Evaluation of essential gene stringency by quantitative assessment of mRNA transcripts

To maintain consistency with a previous assessment of essential gene stringency (Goh et al., 2009), QPCR was used to provide relative quantification of mRNA abundance in \textit{E.coli}, following silencing of essential gene targets fusA and rplE. Normalisation was performed relative to two reference genes; 16S rRNA which forms part of the 30S subunit (Shajani et al., 2011) and zipA which encodes an inner membrane protein involved in the formation of septal ring structure during cell division (Hale and De Boer, 1997). Both genes had previously been selected following microarray analysis of the \textit{E.coli} transcriptome (Shan Goh personal communication).

2.5.1 Isolation and clean-up of total RNA

\textit{E.coli} was induced with IPTG and harvested once the OD\textsubscript{550} of the control (uninduced) culture reached 0.1, as it had previously been determined that optimal correlation between mRNA abundance and relative growth rate occurred at this point in the growth curve (Goh et al., 2009). Total RNA was extracted from \textit{E.coli} using PureLink™ RNA Mini Kit including on-column DNAse treatment (Life Technologies), in accordance with manufacturer’s protocol for isolating bacterial cell RNA. Briefly, bacterial cells were harvested at
500 x g for 5 minutes and resuspended in 100µl of lysoyme solution (Sigma) containing 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mg lysoyme from chicken egg white. In addition 0.5 µl of 10% SDS and 350 µl of lysis buffer containing 1% 2-mercaptoethanol was also added. Samples were homogenized by repeated expulsion through a 21 gauge needle. Cell lysate was centrifuged at 12,000 x g for 2 minutes to remove cellular debris, prior to addition of 250 µl 100% ethanol to precipitate RNA. Samples were vortexed and transferred to a spin column with collection tube and centrifuged at 12,000 x g for 15 seconds. The spin column containing bound RNA was placed in a fresh collection tube to eliminate carryover and DNase treatment was performed. The spin column was washed with 350 µl of Wash buffer I and centrifuged at 12,000 x g for 15 seconds, prior to the addition of 80 µl of DNase solution, containing 1 x DNase I reaction buffer and resuspended DNase I (~3 U/µl). Samples were incubated at room temperature for 15 minutes prior to the addition of 350 µl Wash buffer I and centrifugation at 12,000 x g for 15 seconds. The spin column was placed in a fresh collection tube and 500 µl of Wash buffer II was added. Samples were centrifuged at 12,000 x g for 15 seconds and the spin column was removed and placed in a fresh collection tube. Samples were centrifuged at 12,000 x g for 1 minute and the spin column was transferred to a clean 1.5ml microcentrifuge tube. To elute the RNA, 30 µl of RNase/DNase free water was added to the center of the column, which was then incubated at room temperature for 1 minute prior to centrifugation at 12,000 x g for 2 minutes. Additional RNA purification was performed using TRI reagent (Molecular Research Centre, INC). Briefly 1 ml of TRI reagent was added to the eluted RNA samples and left at room temperature for 5 minutes. Following dissociation of nucleoprotein complexes, 0.1 ml of 1-bromo-3-chloropropane (BCP) (Sigma) was added, samples were mixed and stored at room temperature for 15 minutes. Samples were centrifuged at 12,000 x g for 15 minutes at 4°C. The aqueous phase containing RNA was removed and precipitated with 0.5 ml of isopropanol at room temperature for 10 minutes before being centrifuged at 12,000 x g for 8 minutes at 4 °C. RNA was washed with 1 ml of 75% ethanol and centrifuged at 12,000 x g for 5 minutes at 4°C. The RNA pellet was air-dried for 5 minutes prior to solubilisation in 50 µl RNase-free water.
2.5.2 cDNA synthesis of extracted RNA

The conversion of RNA template to cDNA was performed using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). A 20µl reaction was prepared consisting of 5 x reaction buffer (250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM MgCl₂, 50 mM DTT), diluted to 1 x concentration. In addition, 20 units Ribolock RNase inhibitor, 1 mM dNTP mix, 5 µM random hexamer and 200 units of M-MuLV Reverse Transcriptase were added. RNA template was standardized to 100 ng for each reaction. Reactions were prepared in two steps; initially molecular grade water, random hexamers and RNA template were heated to 65°C for 5 minutes to remove secondary structure, prior to addition of the remaining reaction components. cDNA synthesis was performed by incubating samples at 25°C for 5 minutes, 42 °C for 1 hour, then 70°C for 5 minutes to terminate reverse transcriptase activity. Genomic DNA contamination was assessed using reverse transcriptase negative and blank reactions.

2.5.3 Relative quantification of mRNA transcripts by QPCR

QPCR assay validation was performed according to established guidelines (Nolan et al., 2006). Briefly primer matrices consisting of concentrations 0.1 – 0.3 µM for all primers with exception of fabI (1-2 µM) were prepared and analysed in duplicate. Optimal primer concentrations for forward and reverse primers were selected according to the primer pair displaying the lowest Cₜ (Cycle threshold) value, which signifies the number of cycles required for the fluorescent signal to exceed background level. Reaction efficiency was assessed using duplicate reactions of six fold serial dilutions of target amplicon, with efficiency calculated from standard curve slopes according to the equation $E = 10^{[(1/slope) - 1] × 100}$. Samples were prepared in final volumes of 25 µl consisting of 2 x Maxima® SYBR Green qPCR Master Mix (Fermentas) diluted to 1 x concentration and 2 µl of cDNA synthesis reaction or 5 µl positive control DNA. Analysis was performed on a Chromo 4 (DYAD) using Opticon Monitor v 3.1.32 (Biorad). Relative quantification was performed using the $\Delta \Delta C_t$ method (Livak and Schmittgen, 2001).
Table 2.1 Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Function</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.coli TOP10</td>
<td>F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ-</td>
<td>Host Strain for plasmids</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>E.coli AS-fabI</td>
<td>As TOP10, pHN682</td>
<td>Expression of antisense fabI RNA</td>
<td>(Nakashima et al., 2006)</td>
</tr>
<tr>
<td>E.coli AS-acpP</td>
<td>As TOP10, pHN682</td>
<td>Expression of antisense acpP RNA</td>
<td>(Goh et al., 2009)</td>
</tr>
<tr>
<td>E.coli AS-murA</td>
<td>As TOP10, pHN682</td>
<td>Expression of antisense murA RNA</td>
<td>(Goh et al., 2009)</td>
</tr>
<tr>
<td>E.coli AS-ftsZ</td>
<td>As TOP10, pHN682</td>
<td>Expression of antisense ftsZ RNA</td>
<td>(Goh et al., 2009)</td>
</tr>
<tr>
<td>E.coli Ash01</td>
<td>As TOP10, pHN678-rplE</td>
<td>Expression of antisense rplE RNA</td>
<td>This study</td>
</tr>
<tr>
<td>E.coli Ash02</td>
<td>As TOP10, pHN678-fusA</td>
<td>Expression of antisense fusA RNA</td>
<td>This study</td>
</tr>
<tr>
<td>E.coli Ash03</td>
<td>As TOP10, pBAD-rplE</td>
<td>Overexpression of rplE</td>
<td>This study</td>
</tr>
<tr>
<td>E.coli Ash04</td>
<td>As TOP10, pBAD-fusA</td>
<td>Overexpression of fusA</td>
<td>This study</td>
</tr>
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<td>E.coli Ash05</td>
<td>As TOP10, pHN678-rplE, pBAD-rplE</td>
<td>Transcomplementation rescue of rplE antisense</td>
<td>This study</td>
</tr>
<tr>
<td>E.coli Ash06</td>
<td>As TOP10, pHN678-fusA, pBAD-fusA</td>
<td>Transcomplementation rescue of fusA antisense</td>
<td>This study</td>
</tr>
<tr>
<td>E.coli Ash07</td>
<td>As TOP10, pHN678, pBAD-HisA</td>
<td>Transcomplementation control</td>
<td>This study</td>
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<tr>
<td>E.coli AG1 ME5305</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17(rk-mk+) supE44 relA1</td>
<td>Overexpression of FabI in minimal media</td>
<td>National Bioresource Project</td>
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Table 2.2 Paired termini antisense expression plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristic</th>
<th>Features</th>
<th>Location and length</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>pHN678</td>
<td>IPTG-inducible promoter (<em>Ptrc</em>) with paired termini (PT flanking MCS CamR)</td>
<td><em>E. coli</em> stabilized antisense expression vector</td>
<td>N/a</td>
<td>(Nakashima et al., 2006)</td>
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<tr>
<td>pHN682</td>
<td>fabI antisense insert</td>
<td>Inducible expression of fabI antisense RNA</td>
<td>-74 to +68 of fabI (160 nucleotides)</td>
<td>(Nakashima et al., 2006)</td>
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<tr>
<td>pHNA</td>
<td>acpP antisense insert</td>
<td>Inducible expression of acpP antisense RNA</td>
<td>-42 to +85 of acpP (127 nucleotides)</td>
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<td>pHNM</td>
<td>murA antisense insert</td>
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<td>-54 to +78 of fabI (130 nucleotides)</td>
<td>(Goh et al., 2009)</td>
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<td>pHNZ</td>
<td>ftsZ antisense insert</td>
<td>Inducible expression of ftsZ antisense RNA</td>
<td>-53 to +76 of fabI (129 nucleotides)</td>
<td>(Goh et al., 2009)</td>
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<td>pHN678-rplE</td>
<td>rplE antisense insert</td>
<td>Inducible expression of rplE antisense RNA</td>
<td>-95 +60 of rplE (155 nucleotides)</td>
<td>This study</td>
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<td>pHN678-fusA</td>
<td>fusA antisense insert</td>
<td>Inducible expression of fusA antisense RNA</td>
<td>-93 +86 of fusA (179 nucleotides)</td>
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Table 2.3 Over expression vectors used in this study

<table>
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<th>Plasmid</th>
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<th>Source</th>
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<td>Invitrogen</td>
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<td>Complete ORF rplE</td>
<td>Inducible expression of rplE</td>
<td>N/a</td>
<td>This study</td>
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<tr>
<td>pBAD-HisA-fusA</td>
<td>Complete ORF fusA</td>
<td>Inducible expression of fusA</td>
<td>N/a</td>
<td>This study</td>
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<td>pGlo</td>
<td>Complete ORF Green Fluorescent Protein (GFP)</td>
<td>Inducible expression of GFP</td>
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<td>Biorad</td>
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<tr>
<td>pDs-Red Express</td>
<td>Complete ORF Red Fluorescent Protein (RFP)</td>
<td>Inducible expression of RFP</td>
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<tr>
<td>Primer Name</td>
<td>Target Amplified</td>
<td>Sequence</td>
<td>Amplicon size (Bp)</td>
<td>Purpose</td>
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<td>-------------</td>
<td>-----------------</td>
<td>----------</td>
<td>-------------------</td>
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<tr>
<td>fusA-XF1</td>
<td>Antisense of fusA</td>
<td>CGTCCTCGAGGTCGATGCGCAGACGATA</td>
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<td>Cloning of fusA antisense into pHN678, resulting in pHN678-fusA</td>
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<td>fusA-XR2</td>
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<td>TGTCTCGAGCCCTTGTGAGTTTAGTACC</td>
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<td>Cloning of rplE antisense into pHN678, resulting in pHN678-rplE</td>
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<tr>
<td>rplEr</td>
<td>rplE</td>
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<td>pHN678 f.</td>
<td>pHN678 Multiple cloning site</td>
<td>CTGCAGGGTCGATAATTTACTGCA</td>
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<tr>
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Table 2.5 QPCR primers used in this study

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<th>Primer Name</th>
<th>Target Amplified</th>
<th>Sequence</th>
<th>Amplicon size (Bp)</th>
<th>Purpose</th>
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<td>fabI QPCR Forward</td>
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<td>fabI QPCR Reverse</td>
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<td>CCGCTTGGTGAATGCTGT TA</td>
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</table>
2.6 Evaluation of essential gene stringency by protein quantification

To address the limitation of using mRNA abundance as a proxy for protein expression in determining essential gene stringency, a method for absolute quantification of expressed protein was developed. Central to this approach is the use of an LC-MS/MS assay incorporating a pre-quantified heavy labelled ($^{15}$N) protein as an internal standard for the quantification of expressed protein following expressed antisense RNA silencing of essential gene targets.

2.6.1 Development of a $^{15}$N labelled protein internal standard

To validate the LC-MS/MS approach, the *E.coli* essential genes fabI and murA were selected as targets, since both have previously been evaluated for essential gene stringency. To enable complete labelling of the target protein, a metabolic labelling strategy was implemented. *E.coli* expressing pBAD-fabI and pBAD-murA were grown in liquid M9 minimal media supplemented with $^{15}$N ammonium chloride (Cambridge Isotope Laboratories Inc) as the sole nitrogen source (see Appendix: A). Due to difficulties in cultivating these particular strains of *E.coli* with ampicillin as a selection agent, an alternative strain was sought. *E.coli* clones overexpressing His-tagged FabI and MurA from the ASKA collection (A Complete Set of *E.coli* K-12 ORF Archive), were obtained from the National Bioresource Project-*E.coli*, (National Institute of Genetics, Japan). Suitable growth was achieved in M9 minimal media supplemented with chloramphenicol using both these strains. Bacterial cultures were grown to an OD$_{550}$ of 0.5, prior to the induction of protein expression by the addition of IPTG to a final concentration of 1 mM. Bacterial cultures were then grown for approximately 12 hours prior to harvesting.

2.6.2 Extraction of $^{15}$N labelled protein internal standard from *E.coli* Lysate using IMAC

Following protein expression in minimal media, His-tagged FabI and MurA were extracted from *E.coli* using Immobilised Metal Affinity Chromatography (IMAC), in conjunction with a nickel column (Invitrogen). All reagents used in the preparation of IMAC buffers were sourced from Sigma. Approximately 250 ml of bacterial culture was centrifuged in a sorvall RC5C Refrigerated Superspeed Centrifuge (Dupont) at 10,000 x g for 10 minutes. To each pellet 10 ml of Lysis buffer (20 mM Tris-Cl pH 7.6, 1 M NaCl, 0.1 mM PMSF, 20 mM Imidazole, 2%
Triton-X-100, 150 mg lysozyme) was added. The sample was then sonicated for 5 minutes at amplitude of 10 microns using a Soniprep 150 (MSE (UK) Ltd). Lysed samples were centrifuged at 20,000 x g for 10 minutes to remove cellular debris prior to the lysate being loaded onto a Nickel column (GE Healthcare Life sciences) coupled with AKta Prime (GE Healthcare Life sciences). Bound protein was washed and removed from the column using an elution buffer (20 mM Tris-Cl pH 7.6, 1 M NaCl, 0.1 mM PMSF, 300 mM Imidazole, 2% Triton-X-100). Binding and elution of his-tagged FabI and MurA was monitored by U.V spectrometry on a trace in PrimeView 5.0 (Amersham Biosciences).

2.6.3 SDS-PAGE of extracted $^{15}$N labelled protein internal standards

Extracted protein was evaluated for approximate size using denaturing SDS-PAGE. All reagents used in the preparation of polyacrylamide gels were obtained from Melford, with the exception of 30% Acrylamide/0.8% Bisacrylamide which was sourced from Sigma. Poly-acrylamide gels (12-20% concentration) were prepared using Tris-Cl pH 8.8, containing final concentrations of 0.1% SDS, 0.375 M Tris-Cl pH 8.8 (separating gel), (0.375 M Tris-Cl pH 6.8, for the stacking gel) 0.05% ammonium persulfate and 0.03% TEMED. A 5 x Tris-glycine running buffer was prepared (0.025M Tris, 0.192 M glycine and 0.1 M SDS) and used at 1 x concentration. Gels were photographed using Image-Scanner (Amersham Biosciences and Labscan v5.0 (GE Healthcare Life Sciences)). Protein samples were prepared in 30 µl volumes using 5 x SDS Page loading dye (Fermentas) and 20 x Reducing agent (Fermentas) at final 1 x concentration. Samples were heated at 97°C for 4 minutes to denature protein samples, prior to loading. For size estimation of proteins, 5 µl of Page Ruler Pre-stained Protein Ladder (Fermentas) was used.

2.6.4 Purification and concentration of $^{15}$N labeled protein internal standards

To remove contaminants associated with the IMAC elution buffer and thereby prevent interference with downstream LC-MS/MS analysis, extracted $^{15}$N labelled protein (FabI and MurA) was purified using an acetone precipitation protocol (New England Biolabs). Briefly, 100 µl of $^{15}$N labelled protein was mixed with 400 µl of ice-cold acetone (Sigma), vortexed and incubated for 1 hour at -20°C. The protein extract was centrifuged at 13,000 x g for 10 minutes.
and the supernatant was decanted. The protein pellet was then redissolved in 100 µl of molecular grade water. A small volume of redissolved protein (80 µl) was mixed with 120 µl of 5 x SDS loading buffer, 370 µl of molecular grade water and 30 µl of 20 x reducing agent. The protein sample was heated to 97°C for 3 minutes and placed in a sonibath (VWR) for 5 minutes, followed by a further heating step of 97°C for 3 minutes. The sample was centrifuged at 16,000 x g for 10 minutes and the supernatant was removed to a fresh tube. Protein purity was confirmed by SDS-PAGE, using a 12% polyacrylamide gel.

2.6.5 Antisense induction for protein and RNA quantification

An overnight culture of As-fabI was standardized by OD_{550} readings to approximately 2 x 10^4 cfu/ml in a final volume of 200 ml in MH broth containing chloramphenicol (30µg/m). An IPTG dilution series was prepared using final concentrations of 50, 55 and 70 µM corresponding to growth inhibition of approximately 75%, 50% and 25% respectively. Cultures were incubated at 37°C with aeration until the OD_{550} of a control culture reached 0.1. Bacterial cells were harvested at 12,000 x g for 5 minutes, resuspended in 21 ml fresh MH broth which was subsequently split into two 10 ml aliquots for protein and RNA isolation (see methods section: 2.5.1) for analysis by QPCR respectively. The Protein aliquot was centrifuged and resuspended in 1.5 ml MH broth and sonicated at 10 microns for 45 seconds five times.

2.6.6 Protein digestion and SDS-gel extraction from E.coli lysate

For LC-MS/MS, there is a requirement for protein to be digested into its respective peptides, to render it amenable to electrospray ionization. Consequently E.coli lysates were separated by SDS-PAGE to reduce sample complexity prior to LC-MS/MS analysis. All reagents with the exception of trypsin were purchased from Sigma. Proteins were exercised from SDS-PAGE gels using a scalpel and placed into a 1.5 ml microcentrifuge tube containing 140 µl of 200 mM ammonium bicarbonate (NH4HCO3) pH 7.8. Gel pieces were destained in a 37°C incubator for 15 minutes. Samples were centrifuged at 10,000 rpm for 10 seconds, followed by removal of the supernatant, 140 µl of 200 mM NH4HCO3 pH 7.8/acetonitrile (MeCN) 4:6 was then added, followed by the addition of 140 µl of 50mM NH4HCO3 pH 7.8. Samples were incubated at 37°C for 30 minutes. Following incubation, the supernatant was removed after a
brief centrifugation at 10,000 x g for 10 seconds after which 140µl of MeCN was added, followed by incubation at room temperature for 5 minutes. The MeCN was removed following brief centrifugation and the entire destaining procedure was repeated until no coomassie dye was visible. The gel pieces were reduced by addition of 50 µl 10 mM DTT in 100 mM NH₄HCO₃ and incubated at 56°C for 1 hour. Samples were briefly centrifuged and the supernatant was removed and 50 µl of 50 mM iodacetamide was added. Samples were incubated in the dark for 30 minutes. The supernatant was removed and gel pieces were washed with 200 µl 100 mM NH₄HCO₃ pH 7.8 and incubated at 37°C for 15 minutes with shaking. The supernatant was removed and 200 µl 50 mM NH₄HCO₃/MeCN (50:50 v/v) was added, followed by incubation at 37°C for 15 minutes with shaking. Following removal of the supernatant, 140 µl of MeCN was added followed by incubation at 37°C for 5 minutes with shaking. The MeCN was removed following centrifugation and 140 µl of fresh MeCN was added until gel pieces turned white. Gel pieces were then digested with trypsin solution consisting of 10 µl of 0.5 µg/µl trypsin (Promega), 250 µl 50 mM NH₄HCO₃ and 1 mM CaCl₂. A 30 µl volume of digest solution was added to the gel pieces followed by incubation at room temperature for 5 minutes. A 30 µl addition of 50 mM NH₄HCO₃ was then added to the gel pieces which were then incubated at 37°C overnight. Peptides were extracted by a 10 µl addition of 5% trifluoroacetic acid (CF₃CO₂H), vortexed and then centrifuged at 10,000 x g for 10 seconds. The supernatant was removed to a fresh (non-autoclaved) 1.5.ml microcentrifuge tube. Gel pieces were covered with 2% CF₃CO₂H acid/60% MeCN solution, vortexed and centrifuged again followed by collection of the supernatant. A 20 µl volume of 100% MeCN was then added to the gel pieces, which were subsequently incubated at room temperature for 5 minutes until changing from colourless to white, at which point supernatant was removed. Extracted peptide solution was concentrated by in a speed vac (Eppendorf) and resuspended in 10 µl 1% CF₃CO₂H, 5% MeCN and stored at -80°C.

2.6.7 Evaluation of representative tryptic peptides to use for the identification and quantification of ¹⁵N labelled protein internal standard and for the assessment of matrix effects

To evaluate the potential of matrix effects, a post-extraction spike method was employed (Matuszewski et al., 2003). A known volume (2 µl) of purified Fabl
internal standard was spiked, prior to in-gel digestion with trypsin, into replicate samples of different volumes (10µl, 20µl, 30µl) of *E.coli* TOP10 cell lysate. The cell lysate originated from *E.coli* silenced for *fabI* expression to achieve a titration of the relative growth rate. Induction concentrations of IPTG (50 µM 55 µM and 65µM) were examined and chosen to achieve an approximate 25%, 50% and 75% reduction in the relative growth rate respectively. Subsequent LC-MS/MS analysis identified 30 tryptic peptides which were evaluated according to established criteria (Han and Higgs, 2008). The absolute area under each extracted ion chromatogram corresponding to the $^{15}$N labelled protein internal standard spike and unlabelled species from the *E.coli* lysate were compared, to ascertain if the signal of the $^{15}$N labelled standard suffered significant variation.

### 2.6.8 Quantification of FabI Internal Standard

The FabI internal standard was quantified using a standard curve, constructed from commercially synthesized and pre-quantified peptide analogues of EGAELAFTYQNDK and ILVTGVASK peptides (JPT). Both peptides were redissolved in RNase/DNase free water (Melford) and serially diluted to generate individual standard curves spanning 6 orders of magnitude (0.5-50,000 fmol). Serial dilutions of each peptide were spiked into trypsin digests (in triplicate) containing a 2 µl volume of FabI internal standard and analysed by LC-MS/MS (see methods section: 2.6.9). The absolute area under each extracted ion chromatogram corresponding to the $^{15}$N labelled protein internal standard and the unlabelled peptide analogue were averaged. Standard curves for each peptide were generated in Sigma plot and a straight line equation fitted. The concentration of the $^{15}$N labelled internal standard was then determined from the straight line equation.

### 2.6.9 LC-MS/MS Analysis of FabI protein in silenced samples

LC-MS/MS was performed by the North East Proteome Facility (NEPAF) using an in-house protocol. Peptides were concentrated on a Pepmap C-18 trap column (300 µm ID x 5 mm) and separated on a Pepmap C18 reversed phase column (Dionex, UK) (3 µm particles, 75 µm ID x 250 mm), using a linear gradient over 42 min from 96% A (0.05% formic acid), 4% B (0.05% formic acid, 80% acetonitrile) to 35% A, 65% B and a flow rate of 300 nl/min. Using the
software package; Xcalibur 2.0. Intact peptides were detected between \( m/z \) 400 and \( m/z \) 1,600 in the Orbitrap XL (Thermofisher) at a resolution of 30,000. Internal calibration was performed using the ion signal of \((\text{Si}(\text{CH}_3\text{O})_6)^+\) at \( m/z \) 445.120025 as a lock mass. Parts per million mass accuracy on an Orbitrap mass spectrometer (via lock mass injection into a C-trap). Maximum ion accumulation time allowed on the LTQ Orbitrap was 1s for all scan modes. Automatic gain control was used to prevent over-filling of the ion trap. Collision induced dissociation (CID) spectra of the top 5 peptide ions (rejection of singly charged precursors) were acquired between \( m/z \) 400 and \( m/z \) 1,600 at normalized collision energy of 35. Dynamic exclusion was set with a repeat count of 1, a repeat time of 30s and an exclusion time of 3 min. The chromatography feature was enabled with a correlation area ratio of 1.0. Activation Q was set to 0.25 with 30 ms activation time.

2.6.10 Whole cell screening assays using triclosan and phosphomycin

To examine the potential of defining appropriate levels of sensitivity in antisense based whole cell screening assays using MPL\(_{50}\) Values, an MIC liquid assay was performed. The target specific antibiotics for \( \text{fabI} \) (triclosan) and \( \text{murA} \) (phosphomycin) were selected as proxies for natural compounds, to examine the level of antisense silencing required to reduce the MIC. MIC assays were performed as per antisense assays (see methods section 2.2.2). A dilution series of triclosan (Sigma) and phosphomycin (Sigma) were prepared in 100% Dimethyl sulfoxide (DMSO) (Sigma) and water respectively, based upon published MIC values for triclosan (Bailey \textit{et al.}, 2009) and phosphomycin (Takahata \textit{et al.}, 2010). To each well of a 96 well assay plate, 10 \( \mu l \) of respective antibiotic and 10 \( \mu l \) of and IPTG were added, prior to the addition of 180 \( \mu l \) standardised culture of strains \textit{As-fabI} or \textit{As-murA}.

2.7 Assessment of target specificity using PNA

To investigate whether peptide conjugate PNA with antisense sequences could discriminate between gene homologues, and silence an essential gene target in different bacterial species, a mixed microbial assay was performed.
2.7.1 Design of Species-specific PNA

The design of species specific PNA was performed using Artemis (Rutherford et al., 2000), according to established rules (Good and Nielsen, 1997). Gene homologues from Salmonella enterica serovar Typhimurium LT2 (Knuth et al., 2004) and Escherichia coli DH10B (Durfee et al., 2008b) were identified by BLAST (Altschul et al., 1990) from the Database of essential genes (Zhang and Lin, 2009b). Using Artemis, a 20 base pair sequence (-10 to +10) relative to the start codon of essential gene homologues were identified and aligned in Clustal X version 2 (Larkin et al., 2007) to determine base pair mismatches. A 2 base pair cut-off was assigned to identify potential PNA binding sites. A custom perl script was used to identify -5 to +5 bases relative to the start codon for all against comparison (McGinnis and Madden, 2004b), using BLAST to identify essential genes with TIR’s amenable to the design of PNA’s. From the generated list, a 10 base pair sequence (aacataatct) targeting the TIR of ftsZ was identified (PNA Se002). To enable PNA’s to transverse the bacterial cell wall, a short peptide (KFFKFFKFFK) was conjugated to the PNA. The peptide sequence is has previously been shown to increase uptake and target specificity (Good et al., 2001b).

2.7.2 Transformation of S. typhimurium and E.coli with reporter gene plasmids

To highlight phenotypic variation arising from the effects of PNA silencing of ftsZ, Salmonella typhimurium and E.coli were transformed with pGlo expressing Green Fluorescent Protein (Bio-Rad) and pDs-Red expressing red fluorescent protein (Clontech) respectively. Salmonella typhimurium LT2 strains JR501 was obtained from the Salmonella Genetic Stock Centre, (University of Calgary, U.S.A). E.coli TOP10 was obtained from Invitrogen. Transformation of S. typhimurium with pGlo was based on an adapted protocol for the transformation of enteric bacteria (Tsai et al., 1989). An overnight culture of S. typhimurium LT2 JR501, was diluted 1/20 in SOB medium (see Appendix: A) and then incubated at 37°C with aeration until the OD$_{550}$ reached mid log phase (0.5-0.6). A 50 ml aliquot of the culture was chilled and cells were harvested by centrifugation at 10,000 x g for 10 minutes. Harvested cells were washed with 50 ml of cold (4°C) 0.1 M MgCl$_2$ and then resuspended in 25 ml 0.1 M CaCl$_2$ and incubated on ice for 20 minutes. Cells were centrifuged at 10,000 x g for 10
minutes and resuspended in 5 ml of cold 0.1 M CaCl$_2$, which was dispersed as 0.2 ml aliquots into thin wall 15 ml tubes (Falcon). Upon addition of plasmid DNA, cells were incubated on ice for 20 minutes prior to heat shocking for 2 minutes at 42°C without shaking. A total volume of 250 µl of pre-warmed SOC medium was added to Transformants, which were then incubated at 37°C for 1 hour with aeration. A suitable volume of transformants (100 µl) was pipetted onto pre-warmed LB agar containing ampicillin and incubated at 37°C overnight. *E.coli* TOP10 was transformed with pDs-Red as per previous *E.coli* transformations (see methods section: 2.1.8)

### 2.7.3 Mixed microbial assay

Overnight cultures of *E.coli* Top 10 harbouring pDs-Red and *Salmonella typhimurium* LT2 JR501, containing pGlo, were incubated overnight in low salt LB media containing ampicillin. Overnight cultures were diluted in fresh low salt LB containing 0.2% L-arabinose (Sigma) and standardized by OD$_{550}$ readings to approximately 2×10$^4$ cfu/ml. Equal volumes were then combined to produce a mixed culture. Prior to use, PNA's (Panagene, Korea) were heated at 55°C for 10 minutes as per manufacturer’s instructions and added to a final concentration of 2.5 and 1.25 µM, in a 96 well tissue culture plate (Sarstedt). The well volume was made up to 100 µl with low salt LB containing 0.2% L-arabinose, prior to the addition of 100 µl of mixed bacterial culture. The 96 well plate was sealed with breathable self-adhesive film (Starlab) and incubated in a Powerwave HT plate reader (BioTek) at 37°C with medium intensity shaking. After 6 hours the plate was removed and 10 µl samples were removed and harvested by centrifugation, prior to re-suspension in 1 x PBS (Sigma). Microscope slides were prepared by addition of 2 µl of sample to a 1% agarose pad (Levin, 2002a) and heat fixing for Fluorescent Microscopy. Images were captured using a Leica DMRB microscope with Aqua EXI bioimaging camera (Qimiaging). and processed using Image pro plus software (MediaCybernetics).

### 2.8 Statistics

All statistical analyses used in this study were performed using Minitab (Minitab Inc). Before the selection of statistical analysis methods, data was assessed for normality using a Kolomogorov-Smirnov test prior to the use of parametric tests.
(T-test). Where data did not confirm to a normal distribution non-parametric statistical analyses (Mann-Whitney U test) were undertaken.
Chapter 3 Results

3.1 Evaluation of gene silencing of *fusA* and *rplE*

3.1.1 Specificity of expressed antisense RNA constructs

The assessment of essential gene stringency requires exclusive silencing of a target gene, to generate a reduction in growth rate. Determining the specificity of expressed antisense RNA sequences is therefore necessary to ensure no additional genes are inadvertently targeted. Antisense sequences of approximately 101-152 nucleotides complementary to the ribosome binding region of mRNA for essential genes *fusA* and *rplE*, were cloned into a paired termini antisense expression vector (pHN678) (Nakashima et al., 2006) and transfected into *E. coli* (TOP10) to create strains Ash01, and Ash02 (see methods section 2.1.5-2.18). The growth rate of each strain was titrated down using a range of IPTG concentrations to induce increased expression of antisense RNA. The growth rates of silenced clones were measured relative to uninduced controls to obtain relative growth rate (see methods section: 2.2).

Both antisense RNA expression constructs demonstrated significant growth inhibition of *E. coli* (2 sample T-test n =3 P <0.005), compared to controls containing empty constructs (Figure 3.1). Greater silencing efficiency was displayed for *rplE*, as evident from the early decrease in the relative growth rate compared to *fusA*. The specificity of both *fusA* and *rplE* constructs were compared to expressed antisense RNA constructs targeting essential genes *acpP*, *ftsZ*, *fabI* and *murA* (Goh et al., 2009). The relative growth rates of *acpP*, *ftsZ*, *fabI* and *murA* (Figure 3.2) were comparable to those obtained previously. However a greater level of silencing efficiency was demonstrated for *fusA* and *rplE* over a low IPTG induction range, with growth inhibition occurring at 20-25 µM compared to *acpP* (80 µM), *ftsZ* (60 µM), *fabI* (70 µM), and *murA* (50 µM). Antisense RNA expression constructs for *fusA* and *rplE* demonstrate the capacity to reduce bacterial growth, which suggests specificity for their respective essential gene targets in *E. coli*. 
Figure 3.1 Relative growth rate of *E. coli* following expressed antisense RNA silencing of essential gene targets *fusA* and *rplE*.

Growth rate titration was achieved by induction with increasing concentrations of IPTG to increase the expression of antisense RNA and measuring OD$_{550}$. Raw OD$_{550}$ data corresponding to the exponential phase of growth was plotted and a trendline applied to obtain the rate of growth from the straight line equation $y = mx + c$. Relative growth rate was determined by normalising the growth rate of induced samples to uninduced controls. Graph shows average relative growth rate with standard error bars ($n=3$).
Figure 3.2 Relative growth rates of *E. coli* following expressed antisense RNA silencing of essential gene targets *acpP*, *ftsZ*, *fabI*, and *murA*.

Growth rate titration was achieved by induction with increasing concentrations of IPTG to increase the expression of antisense RNA and measuring OD$_{550}$. Raw OD$_{550}$ data corresponding to the exponential phase of growth was plotted and a trendline applied to obtain the rate of growth from the straight line equation $y = mx + c$. Relative growth rate was determined by normalising the growth rate of induced samples to uninduced controls. Graph shows average relative growth rate with standard error bars (n=3).
3.1.2 Validation of antisense construct specificity by transcomplementation

The validation of target specificity of expressed antisense RNA constructs was attempted using a transcomplementation strategy. This approach aims to rescue cells from growth inhibition induced by antisense RNA silencing, through over-expression of the complete open reading frame (ORF) for each target gene from a separate plasmid. Target specificity can then be confirmed following the rescue of clones from growth inhibition and restoration of normal growth. The entire ORF’s for fusA and rplE were cloned into pBAD-HisA containing an L-arabinose inducible promoter to generate strains E.coli Ash03 and Ash04 (see methods section 2.1.1-2.1.9). The rescue constructs were extracted and transfected with their corresponding expressed antisense RNA constructs to create E.coli strains, Ash05 and Ash06 respectively. Strains was grown in the presence of IPTG and L-arabinose to induce expression of both antisense RNA and corresponding episomal copy of the target genes, to determine appropriate levels of induction required to demonstrate rescue of growth rate (see methods section 2.3).

Growth inhibition of E.coli, following expressed antisense RNA silencing of rplE at 25 µM IPTG, was prevented following gene complementation using 2% L-arabinose (Figure 3.3). However this was deemed statistically insignificant (Mann Whitney, P=0.104, n=7). Difficulties were encountered with the transcomplementation of fusA, for which suitable levels of induction for expressed antisense RNA and gene overexpression could not be determined. Subsequently no significant rescue of growth rate following silencing of fusA (Mann Whitney, P=0.734, n=7) was observed with L-arabinose induction (Figure 3.5). To investigate the potential influence of metabolic load associated with expression of two plasmids, strain Ash07 containing empty expression vectors, was examined using identical rescue conditions for both rplE (Figure 3.4) and fusA (Figure 3.6). Induction with IPTG and L-Arabinose concentrations did not significantly affect the growth rate of E.coli using the same rescue conditions for rplE (Mann Whitney, P=0.610, n=7) or fusA (Mann Whitney, P=0.710, n=7). Higher OD₅₅₀ readings were observed in the control strains. In summary using transcomplementation validated target specificity of rplE but not for fusA.
Figure 3.3 Transcomplementation of expressed antisense RNA silencing effects of the essential gene rplE in E.coli.

Growth rate of E.coli strain Ash05 following rplE over-expression, induced with 2% L-arabinose. Antisense RNA expression was induced with 25 µM IPTG. Graph shows bacterial growth curves derived from average OD$_{550}$ readings with standard error bars (n=3).
Figure 3.4 Transcomplementation control mimicking the expression conditions used for the rescue of *rplE*.

Expression of *E.coli* strain Ash07 was induced with 25 µM IPTG and 2% L-arabinose as used in the rescue of *E.coli* strain Ash05. Graph shows bacterial growth curves derived from average OD$_{550}$ readings with standard error bars (n=3).
Figure 3.5 Transcomplementation of expressed antisense RNA silencing effects of the essential gene fusA in *E. coli*.

Growth rate of *E. coli* strain Ash06 following fusA over-expression, induced with 0.001% L-arabinose. Antisense RNA expression was induced with 25 µM IPTG. Graph shows bacterial growth curves derived from average OD$_{550}$ readings with standard error bars (n=3).
Figure 3.6 Transcomplementation control mimicking the expression conditions used for the rescue of *fusA*.

Expression of *E.coli* strain Ash07 was induced with 25 μM IPTG and 0.001% L-arabinose as used in the rescue of *E.coli* strain Ash06. Graph shows bacterial growth curves derived from average OD$_{550}$ readings with standard error bars (n=3).
3.1.3 Delineating inhibitory effects caused by gene silencing of fusA and rplE

To ascertain whether the effects of expressed antisense RNA silencing of fusA and rplE promoted a bactericidal or bacteriostatic effect on cell growth, a survival assay was performed (see methods section 2.4). Silencing of fusA and rplE was induced using 50 µM IPTG with viable cell counts performed over 8 hours. Prior to the assay, it was postulated that silencing of fusA would cause a bacteriostatic effect on cell growth, in accordance with inhibitory effects observed in S.aureus by the target specific antibiotic fusidic acid (Howden and Grayson, 2006). Similarly silencing of rplE was also expected to cause a bacteriostatic effect, based upon the inhibitory action exerted by the majority of antibiotic classes that target the ribosome, with the exception of aminoglycosides (Kohanski et al., 2010).

Both fusA and rplE silenced strains exhibited significant growth inhibition rather then cell death following gene silencing. A significant reduction in viable cell numbers was observed upon silencing for both fusA (Mann Whitney U Test, P=0.0118, n= 9) and rplE (Mann Whitney U Test, P = 0.0009 n=9). In the case of rplE (Figure 3.7) the viable number of cells initially decreased prior to increasing after 8 hours. For fusA (Figure 3.8) the number of viable cells remained relatively static throughout the 8 hour growth period. The growth effects of gene silencing of both rplE and fusA were determined to be bacteriostatic, since cell number decline was below the >3 log reduction of the final inoculum required for the demonstration of bactericidal activity. One particular observation was that growth curves generated for uninduced antisense RNA expression of fusA and rplE displayed an exponential increase with a plateau at approximately 4 hours. This anomaly was not observed with both the uninduced and induced control plasmid, where no difference in viable cell count was observed (Figure 3.9), following induction (Mann Whitney U Test, P=0.8946, n=9)
Figure 3.7 Delineation of growth effects following expressed RNA silencing of the essential gene rplE in *E.coli*.

Antisense RNA expression was induced using 50 μM IPTG *E.coli* strain Ash01 and viable cell counts were performed over 8 hours. Graph shows average viable cell count with standard error bars (n=3).
Figure 3.8 Delineation of growth effects following silencing of the essential gene *fusA* in *E.coli*.

Antisense RNA expression was induced in *E.coli* strain Ash02 using 50 µM IPTG and viable cell counts were performed over 8 hours. Graph shows average viable cell count with standard error bars (n=3).
Figure 3.9 Viable cell counts of *E.coli* expressing empty pHN678 construct.

As per previous experimental conditions 50 µM IPTG was used to induce expression of empty antisense expression plasmid in *E.coli* strain Ash03 and viable cell counts were performed over 8 hours. Graph shows average viable cell count with standard error bars (n=3).
3.1.4 Evaluation of gene stringency of fusA and rplE using Mean Transcript Level 50 (MTL\textsubscript{50})

To enable comparisons of stringency among essential genes, a Mean Transcript Level (MTL\textsubscript{50}) value, representing the amount of mRNA required to maintain 50\% of relative growth has been proposed, which reflects the relationship between transcript abundance and growth rate decline. (Goh et al., 2009). The MTL\textsubscript{50} of rplE and fusA was initially evaluated by performing a titration of growth rates of \textit{E.coli} strains Ash01 and Ash02, using increasing concentrations of IPTG, to promote expressed antisense RNA expression and measuring the corresponding mRNA abundance by performing relative quantification with QPCR, (see methods section 2.5). Examination of the relative growth rate and corresponding mRNA abundance for rplE and fusA (Figure 3.10 and Figure 3.11 respectively), reveals an increase in mRNA abundance with relative growth rate decline. This is contrary to the expectation that mRNA abundance would decline with relative growth rate, due to antisense mediated degradation of mRNA transcripts.

A reduction in growth rate was observed following silencing of rplE yet mRNA abundance initially decreased then remained stable, prior to an increase in abundance at the lowest relative growth rate. Silencing of fusA, caused the relative growth rate and mRNA abundance to initially decline, followed by a large increase in mRNA abundance at the lowest relative growth rate. No significant correlation between relative growth rate decline and mRNA abundance, following silencing for either rplE ($r_s$ = -0.02) or fusA ($r_s$ = -0.6) was observed.

To compare the stringency of rplE and fusA against previously validated targets, the MTL\textsubscript{50} value was determined by plotting relative growth rate against relative mRNA abundance (Figure 3.12 and Figure 3.13 respectively). The MTL\textsubscript{50} reveals an inverse trend to previously investigated gene targets, whereby mRNA abundance increases as relative growth rate declines. The number of rplE transcripts required to maintain 50\% cell viability increases to approximately 2.4 relative value (or 240\% of a normal cell growth). For fusA the 50\% decrease in growth rate was not achieved, but indicates that a large number of transcripts are present, approximately 1.5 relative value (or 150\% of a normal cell growth).
Figure 3.10 Relative growth rate and mRNA abundance profiles following expressed antisense RNA silencing of the essential gene *rplE* in *E.coli*.

Transcript and relative growth rate profile for *E.coli* strain Ash01. Total RNA was isolated from cultures, which were harvested once the OD$_{550}$ of the uninduced control had reached 0.1. mRNA abundance was determined by QPCR using *E.coli* genes 16S and ZipA as endogenous references for relative quantification by ΔΔCt method. Graph shows average relative growth rate and average relative mRNA abundance with standard error bars (n=3).
Figure 3.11 Relative growth rate and mRNA abundance profiles following expressed antisense RNA silencing of the essential gene \textit{fusA} in \textit{E.coli}.

Transcript and relative growth rate profile for \textit{E.coli} strain Ash02. Total RNA was isolated from cultures, which were harvested once the OD$_{550}$ of the uninduced control had reached 0.1. mRNA abundance was determined by QPCR using \textit{E.coli} genes 16S and ZipA as endogenous references for relative quantification by ΔΔCt method. Graph shows average relative growth rate and average relative mRNA abundance with standard error bars (n=3).
Figure 3.12 MTL_{50} for essential gene \textit{rplE}.

The MTL_{50} was calculated by plotting the mean relative mRNA abundance against its corresponding mean relative growth rate. This enables stringency of essential genes to be determined by examining the number of transcripts required to maintain 50% relative growth rate.
Figure 3.13 MTL$_{50}$ for essential gene $fusA$.

The MTL$_{50}$ was calculated by plotting the mean relative mRNA abundance against its corresponding mean relative growth rate. This enables stringency of essential genes to be determined by examining the number of transcripts required to maintain 50% relative growth rate.
3.2 Development of LC-MS/MS protein quantification method

3.2.1 $^{15}$N labelled protein internal standard preparation for LC-MS/MS

The evaluation of stringency in essential gene targets fusA and rplE in this study, suggests that mRNA may be limited as a surrogate for expressed protein, and therefore inappropriate as a measure of gene essentiality. Indeed correlations between mRNA and protein abundance are known to vary widely, due to numerous post-transcriptional and post-translational mechanisms that operate within prokaryotes (Maier et al., 2009).

To circumvent the use of mRNA for determining essential gene stringency, an LC-MS/MS assay was developed to provide absolute quantification of target protein, following expressed antisense RNA silencing. Development of the assay required the synthesis of a heavy labelled ($^{15}$N) target protein for use as an internal standard. The use of a fully labelled protein enables an initial LC-MS/MS analysis to identify the most suitable tryptic peptides, possessing the same physiochemical properties as their native equivalent, but distinguishable by mass difference for the purpose of quantifying protein in experimental samples. The essential genes fabI and murA were selected as candidate targets for use as internal standards, due to their extensive characterization and previous evaluation as stringently required genes (Goh et al., 2009).

As an alternative to commercially synthesized heavy labelled peptides, fully $^{15}$N labelled proteins were generated from E.coli MG1 AE5305 obtained from the ASKA collection (Kitagawa et al., 2005a). Using a complete stable isotope labelled form enabled correction for variability in the analytical workflow, and has been show to be preferable choice compared to other forms of internal standard (Bronsema, 2013). Candidate proteins were overexpressed in minimal media containing $^{15}$N labelled NH$_4$ as the sole nitrogen source (see methods section 2.6.1), extracted from whole cell E.coli lysate by Immobilised metal affinity chromatography (see methods section 2.6.2) and subject to acetone precipitation to remove contaminants (see methods section 2.6.4). Purified $^{15}$N labelled FabI and MurA (Figure 3.14) revealed both target proteins (27.9 and 44.8 kDa respectively), in addition to high molecular weight contaminants. Contamination was more prevalent for MurA, which revealed numerous bands in the range of 100 – 130 kDa, including two low molecular weight bands in the
predicted range of MurA. Unlike FabI which resides in the cytosol, MurA is a membrane associated protein; therefore contaminating bands were likely to have been accessory proteins that co-eluted. The identity of each protein was confirmed by LC-MS/MS (see results section 3.2.2).

Figure 3.14 Purification of $^{15}$N labelled FabI and MurA protein from E.coli whole cell lysate.

E.coli MG1 ME5305 overexpressing His-tagged FabI and MurA was grown in minimal media until mid log phase and then induced with 1 mM IPTG and grown over 24 hours prior to harvesting. Extracted protein was eluted from a nickel column over an imidazole gradient generating 10 eluted fractions. A protein ladder (first lane) was used to confirm approximate protein size.
Both FabI and MurA protein elutes were subsequently concentrated and purified further using Amicon filters with appropriate molecular weight ranges. However SDS-PAGE of filtered protein revealed the presence of warped bands indicative of high salt concentration (Figure 3.15 A), that could detrimentally effect electrospray ionization efficiency. Both proteins were therefore subjected to an additional purification step using acetone precipitation (Figure 3.15 B), which subsequently removed contaminants. Following purification, the yield of FabI and MurA appeared to be reduced. This however was not deemed to affect the quantification strategy as both protein batches would be quantified independently using a standard curve, prior to use as internal standards (see methods section: 2.6.8). This would ensure known quantities of internal standard would be spiked into experimental samples.
Figure 3.15 Purification and concentration of $^{15}$N labelled FabI and MurA.

(A) Contamination in $^{15}$N FabI and MurA, following initial clean-up after elution from IMAC. High molecular weight bands corresponding to co-eluted contaminants were observed for MurA (B) Acetone precipitation of $^{15}$N FabI and MurA, revealing the removal of contaminants. Purified protein was pooled and 10 µl aliquots were ran on a 15% polyacrylamide gel. A protein ladder (first lane) was used to confirm protein size.
3.2.2 Confirmation of complete $^{15}$N labelling of protein internal standards

For reliable quantification, the heavy labelling of an internal protein standard requires complete and stable $^{15}$N incorporation to a level approaching 95%, as a minimum for quantitative analysis (Huttlin et al., 2008). The use of $^{15}$N is particularly suited as a heavy label, since peptides contain fewer types of nitrogen compared to carbons. This has the effect of reducing the complexity of isotopic envelopes in $^{15}$N labelled peptides, which subsequently reduces the distribution of the $m/z$ signal thereby increasing the signal to noise ratio. This subsequently increases confidence in identification and quantification (Huttlin et al., 2008). Purified FabI and MurA samples were digested with trypsin and analysed by LC-MS/MS to determine the relative degree of $^{15}$N incorporation. The degree of $^{15}$N incorporation in FabI was assessed by submitting the raw LC-MS/MS data to the gpm (Global Protein Machine), a publically available system, comprising a data analysis server, user interface and a relational database, containing experimentally generated peptide mass data (Craig et al., 2004). Within this system, raw tandem mass spectra is compared to spectra derived from the same peptide sequences using X! Tandem, a class B open source search engine. An algorithm performs a two step calculation, initially a ‘survey’ is performed whereby experimentally derived spectra are compared to the protein sequences, under the assumption that complete peptide cleavage occurs. Peptides identified with statistically significant matches are then re-matched taking into consideration potential chemical modifications (e.g.: oxidation). To ascertain the level of incorporation the gpm search was initially performed with the assumption of no $^{15}$N incorporation (i.e. the search engine would only identify peptide sequences with spectra relating to native unlabelled protein sequences specific for FabI or MurA). The gpm search (Figure 3.16) did not match any spectra corresponding to native FabI or MurA, with spectra generated from $^{15}$N labelled internal standards, or any other E.coli proteins. The only positive identifications made were keratin and bovine trypsin, which represent typical contaminants arising from sample preparation. Due to financial constraints, a decision was made to proceed with developing an LC-MS/MS assay for fabI only.
Figure 3.16 GPM Results for unlabelled FabI and MurA.

No hits for native (unlabelled) FabI and MurA were obtained when gpm search parameters were modified to assume the absence of $^{15}$N incorporation. This supports complete incorporation of $^{15}$N in both FabI and MurA.
The gpm search was repeated by altering the search parameters to accommodate for $^{15}$N incorporation (Figure 3.17) in peptides specific to FabI and MurA proteins. The probability of peptide identification was ranked according to a logE score, which represent the base -10 logarithm of the probability that a peptide identification is a random match. Log E scores of -251.6 and -220.4 were obtained for MurA and FabI respectively, indicating that the chance of false positive identification is low (for reference a logE score of -3 corresponds to 1 in 1000 chance). The logE scores for $^{15}$N labelled FabI and MurA correspond to approximately 1 in $1 \times 10^{2000}$ chances that identification has been made at random, which represents a statistically significant probability of identification.

To provide direct evidence of complete $^{15}$N labelling, characteristic peptide sequences for FabI (ILVTGVASK and SMLNPGSALLTLSYLGAER) and MurA (MGAHAIESNTVICHGVEK and LQGEVTISGAK), were selected to confirm the degree of $^{15}$N incorporation. Incomplete labelling of each protein would be confirmed by the presence of mass spectra corresponding to unlabelled peptides. The mass spectrum for peptide ILVTGVASK (Figure 3.18) revealed an isotopic envelope corresponding to $^{15}$N labelled pseudo-molecular ion for the doubly charged peptide, with a m/z of 449.27 and no trace of the unlabelled native protein with a m/z of 444.27. The mass spectrum for SMLNPGSALLTLSYLGAER (Figure 3.19) also revealed a characteristic isotopic envelope corresponding to a $^{15}$N labelled pseudo-molecular 3+ ion for the doubly charged peptide, with a m/z of 677.9 and the absence of corresponding unlabelled peptide. For MurA the mass spectrum of MGAHAIESNTVICHGVEK revealed the presence of a $^{15}$N labelled pseudomolecular 3+ ion with a m/z of 702.9 (Figure 3.20). The mass spectrum of LQGEVTISGAK (Figure 3.21) revealed the presence of a $^{15}$N labelled pseudomolecular 2+ ion with m/z of 558.2. No unlabelled species were observed for either peptide. The absence of unlabelled species ($^{14}$N) indicates that complete labelling (i.e. every $^{14}$N atom is replaced by $^{15}$N) was achieved, as expected in unicellular systems grown over eight generations (Snijders et al., 2005). The complete $^{15}$N incorporation of FabI and MurA validates their use as internal standards for absolute quantification of cellular protein, since the high level incorporation permits an optimal signal to noise ratio, which aids identification and permits accurate quantification.
The gpm search parameters were adjusted to assume complete $^{15}$N labelling of FabI and MurA, which revealed significant log (e) scores, indicating a significant probability that labelled protein internal standards were fully labelled.
Figure 3.18 Mass spectrum of tryptic peptide ILVTGVASK used for establishing the level of $^{15}$N incorporation in FabI.

The red arrow indicates where a completely unlabelled ($^{14}$N labelled) peptide would be observed for peptide ILVTGVASK.
Figure 3.19 Mass spectrum of tryptic peptide SMLNPGSALLTSYLGAER used for establishing the level of $^{15}$N incorporation in FabI.

The red arrow indicates where a completely unlabelled ($^{14}$N labelled) peptide would be observed for peptide SSMLNPGSALLTLSYLGAER.
Figure 3.20 Mass spectrum of tryptic peptide MGAHAEIESNTVICHGVEK used for establishing the level of $^{15}$N incorporation in MurA.

The red arrow indicates where a completely unlabelled ($^{14}$N labelled) peptide would be observed for peptide MGAHAEIESNTVICHGVEK.
Figure 3.21 Mass spectrum of tryptic peptide LQGEVTISGAK used for establishing the level of $^{15}$N incorporation in MurA.

The red arrow indicates where a completely unlabelled ($^{14}$N labelled) peptide would be observed for peptide LQGEVTISGAK.
3.2.3 Selection of peptides for quantification of protein internal standard by LC-MS/MS

The strategy for evaluating essential gene stringency in this study incorporates the use of absolute quantification of cellular protein using LC-MS/MS. Quantification is achieved by comparing the ratio of peak areas of extracted ion chromatograms for native peptide to an internal standard of known amount. It is critical that tryptic peptides representative of both labelled and unlabelled protein generate an equivalent response during electrospray ionization. Ideally heavy labelled and native peptides should be equivalent in elution times, generate characteristic spectra and be consistently detected in both $^{15}$N labelled and unlabelled forms over orders of magnitude. Furthermore, the detection of peptides should be resilient to potential effects arising from the sample matrix. A key requirement of LC-MS/MS method validation is therefore to assess for matrix effects that can alter the instrument response through the suppression or enhancement of electrospray ionization. Such effects are associated with endogenous components of sample (biological) matrices that co-elute and compete with peptides or interfere with ionization, specifically the efficiency of droplet formation (Van Eeckhaut et al., 2009).

To evaluate any potential matrix effects associated with whole cell *E.coli* lysate, a post-extraction spike method was employed (see methods section 2.6.7). A known volume of internal standard was spiked prior to in-gel digestion with trypsin into replicate samples of cell lysate. The cell lysate originated from *E.coli* silenced for fabI expression to achieve a titration of relative growth rate. Induction concentrations of IPTG were examined and chosen to achieve an approximate 25%, 50% and 75% reduction in the relative growth rate. LC-MS/MS analysis identified 30 tryptic peptide sequences indicative of FabI, which were evaluated according to established criteria (Han and Higgs, 2008). Suitable peptides were selected based on optimal length, (7-30 residues), lack of extreme hydrophillic or hydrophobic regions and absence of residues susceptible to chemical modification, that could alter $m/z$ values thereby complicating identification and quantification. Peptide sequences containing specific residues were avoided particularly those containing methionine (subject to oxidation), asparagine (can be glycooslytaed or deaminated), glycine or glutamic acid (undergo cyclization to pyroglutamic acid), or peptides with
internal trypsin cleavage sites. The absolute areas of extracted ion chromatogram peaks for selected peptides, identified in both $^{15}$N labelled and unlabelled FabI, were determined and converted to a Light/Heavy ratio. An arbitrary cut-off value of 0.15 was used to identify peptides displaying the lowest L/H ratio (approximately 1:1). Four candidate peptides were subsequently determined as suitable for quantification standards that could be used to quantitate the FabI Internal standard (Figure 3.22).
Figure 3.22 Tryptic peptides identified for the quantification of FabI internal standard.

The Light/Heavy ratio for four tryptic peptides specific to FabI were generated from extracted ion chromatograms and plotted against the relative growth rate of *E.coli*, which was titrated using different concentrations of IPTG to achieve increased antisense RNA silencing of *fabI*. Graphs show average peptide L/H ratio with standard error bars (n=3). Peptide VNAISAGPIR was discounted from analysis due to substantial variability in the L/H peptide ratio obtained for 50% relative growth rate.
For quantitation of the $^{15}$N labelled internal standard, two tryptic peptides EGAELAFTYQNDK and ILVTGVASK were selected due to their similarity in L/H ratio’s across all relative growth conditions. Significant variation was displayed for VNAISAGPIR at 50% relative growth, hence it was discarded from selection with ASLEANVR.

In order to provide accurate quantitation of the FabI internal standard, the mass spectra and elution characteristics for respective peptide ions was determined. Since these characteristics are specific to each peptide ion, they can be used to confirm that extracted ion chromatogram peaks are assigned to the correct peptide ion, which serves a proxy for both the internal standard and the protein under investigation.

Evaluation of EGAELAFTYQNDK revealed spectra corresponding to both unlabelled and $^{15}$N labelled tryptic peptides (Figure 3.23 and Figure 3.24 respectively). A pseudomolecular 2+ ion was identified with a $m/z$ of 743.35 for the unlabelled peptide and 751.32 for the $^{15}$N labelled counterpart (Figure 3.25). Extracted chromatograms revealed an elution time of 16.85 minutes (Figure 3.26 A and B). Similarly ILVTGVASK also revealed characteristic spectra for unlabelled and $^{15}$N labelled forms of the peptide (Figure 3.27 and Figure 3.28). The pseudomolecular 2+ ion was identified with a $m/z$ of 444.28 and 449.27 for the unlabelled and $^{15}$N labelled counterpart respectively (Figure 3.29). Extracted ion chromatograms revealed a peptide elution time of approximately 15 minutes (Figure 3.30 A and B). Due to the consistent detection of tryptic peptides EGAELAFTYQNDK and ILVTGVASK in all relative growth conditions, both peptides were selected for incorporation as template profiles into LC-MS/MS data analysis software, for subsequent identification and quantitation of the $^{15}$N labelled FabI internal standard.
Figure 3.23 LC-MS/MS spectra for unlabelled EGAELAFTYQNDK.

The spectra for peptide EGAELAFTYQNDK revealed eight individual m/z peaks that could be used to confirm identity of the native (14N) peptide.
Figure 3.24 LC-MS/MS spectra for $^{15}$N labelled peptide EGAELAFTYQNDK.

The spectra for $^{15}$N labelled EGAELAFTYQNDK revealed eight individual m/z peaks that could be used to confirm identity. A noticeable shift in m/z values was observed compared to the spectra for the native peptides.
Figure 3.25 LC-MS/MS characteristics of peptide EGAELAFTYQNDK.

Zoomed MS spectra showing characteristic isotopic envelope of native and $^{15}$N labelled EGAELAFTYQNDK with m/z of 743.35 and 751.32 respectively.
Figure 3.26 LC-MS/MS characteristics of peptide EGAELAFTYQNDK.

Extracted ion chromatogram of native (A) and $^{15}$N labelled (B) EGAELAFTYQNDK peptide with a co-elution time of 16.85 minutes. **Relative abundance represents the ion intensity**
Figure 3.27 LC-MS/MS spectra for unlabelled and $^{15}$N labelled peptide ILVTGVASK.

The spectra for peptide ILVTGVASK revealed six individual $m/z$ peaks that could be used to confirm identity of the heavy labelled peptide.
Figure 3.28 LC-MS/MS spectra for $^{15}$N labelled peptide ILVTGVASK.

The spectra for peptide ILVTGVASK revealed seven individual $m/z$ peaks that could be used to confirm identity of the heavy labelled peptide. A noticeable shift in $m/z$ values is observed compared to spectra for corresponding unlabelled peptides.
Figure 3.29 LC-MS/MS characteristics of peptide ILVTGVASK.

Zoomed MS spectra showing characteristic isotopic envelope of native and $^{15}$N labelled EGAELAFTYQNDK with $m/z$ of 444.28 and 449.27 respectively.
Figure 3.30 LC-MS/MS characteristics of peptide ILVTGVASK.

Extracted ion chromatogram of native (A) and $^{15}$N labelled (B) peptide ILVTGVASK with a co-elution time of approximately 15 minutes.
3.3 Quantification and assessment of gene stringency using protein abundance

3.3.1 Quantification of $^{15}$N labelled Fabl protein internal standard

Prior to utilizing the $^{15}$N labelled fabl as an internal standard, quantification of the purified protein was performed using commercially synthesized (pre-quantified) peptide analogues of EGAELAFTYQNDK and ILVTGVASK (see methods section 2.6.8). Both peptides were serially diluted to generate individual standard curves spanning 6 orders of magnitude, to enable extrapolation of the amount of internal standard. Serial dilutions of each peptide were spiked into trypsin digests containing a known volume of Fabl internal standard and analysed by LC-MS/MS.

Insufficient data was obtained for both peptides corresponding to spiked amounts in the lower range of the standard curve (0.5 and 5 fmol). Consequently standard curves were generated over 4 orders of magnitude (50 – 50,000 fmol). Standard curves for EGAELAFTYQNDK and ILVTGVASK (Figure 3.31 A and B respectively), generated by linear regression displayed good correlation ($r^2 = 0.99$). Using the linear regression equations $y = 7\times10^{-6}x - 765.71$ and $y = 4\times10^{-6}x + 113.66$, the amount of internal standard was calculated as 675 pmol for EGAELAFTYQNDK and 4547 pmol for ILVTGVASK. The average amount of $^{15}$N Fabl internal standard was subsequently calculated as 2.61 nmol.
Figure 3.31 Tryptic peptide standard curves for quantification of $^{15}$N FabI internal standard.

(A) Standard curve for tryptic peptide EGAELAFTYQNDK. (B) Standard curve for tryptic peptide ILVTGVASK. Standard curves were constructed from 10-fold serial dilution of peptides ranging from 50-50,000 fmol. The average amount of $^{15}$N FabI was calculated using linear regression. Standard curves show average amount of peptide with standard error bars (n=3).
3.3.2 Quantification of FabI protein per cell following gene silencing of fabI in E.coli

To evaluate the differential growth requirement of the essential gene fabI, the growth rate of E.coli strain As-fabI was titrated down using expressed antisense RNA silencing. Concentrations of IPTG (50, 55 and 65 µM) were used to achieve approximate growth reductions of 25%, 50% and 75% (see methods section: 2.6.5). For comparative purposes the amount of FabI synthesised following overexpression in E.coli MG1 ME5305 was investigated using IPTG induction at 0.5 and 1 mM.

Transcript abundance was measured by QPCR and protein was quantified by LC-MS/MS. The peak area derived from extracted ion chromatograms for peptides EGAELAFTYQNDK and ILVTGVASK was used to generate ratios of unlabelled: \(^{15}\)N labelled peptide. The amount of protein was obtained by multiplying the calculated ratios for each peptide by the amount of internal standard spiked into tryptic digests. The calculated amount was converted to protein molecules by multiplying by Avogadro’s number and corrected for viable cell counts to provide an estimation of FabI molecules per cell (Figure 3.32).

The number of FabI molecules per cell decreased with declining growth rate, following silencing of fabI. A 3-fold decrease in FabI molecules per cell was observed as the relative growth rate decreased to approximately 75% of normal growth. A smaller decrease in FabI molecules per cell (1.2 fold) was observed as the growth rate declined to approximately 50% of normal growth. A 2-fold decrease in FabI molecules per cell occurred as the relative growth rates dropped to approximately 25% of the normal growth rate.

When overexpressed the amount of FabI per cell increased by approximately 100-fold in comparison to both wild-type E.coli and uninduced controls (Figure 3.33). The number of FabI molecules per cell increased slightly (1.2-fold) when the concentration of IPTG was doubled from 0.5 to 1 mM. Interestingly the number of FabI molecules per cell in the antisense control (uninduced) was 3.5-fold higher then wild-type E.coli and 5-fold higher when compared to the ASKA control (uninduced). Both wild-type E.coli and the ASKA control revealed a similar basal expression of FabI.
Figure 3.32 FabI protein molecules per cell following expressed antisense silencing of the cognate essential gene.

IPTG induction was used to titrate down the relative growth rate of \textit{E.coli As-fabI} to approximately 75\% 50\% and 25\% of normal growth. Protein was measured by comparing the extracted Ion chromatogram peak areas of light and $^{15}$N labelled forms of tryptic peptides EGAELAFTYQNDK and ILVTGVASK. Protein amounts were corrected by viable cell counts. Graph shows average protein molecules per cell with standard error bars (n=3).
Figure 3.33 FabI protein molecules per cell in *E.coli* MG1 ME5305 and Wild-type *E.coli*.

FabI overexpression was induced with 0.5 and 1 mM IPTG. Protein abundance was measured by comparing the extracted Ion chromatogram peak areas of light and $^{15}$N labelled forms of tryptic peptides EGAELAFTRYQNDK and ILVTGVASK. Protein amounts were corrected by viable cell counts. Graph shows average protein molecules per cell with standard error bars (n=3).
Both mRNA and protein abundance following gene silencing of *fabI* was evaluated to identify any correlation between the decrease in either mRNA or protein, with the observed decline in relative growth rate (Figure 3.34). No significant correlation (Pearson product correlation, n = 4, P >0.05) was observed. The abundance of *fabI* transcripts appeared to initially increase prior to a large spike at approximately 50% of normal relative growth rate, after which mRNA abundance declined. When evaluated over the induction range of IPTG, the decline in protein abundance followed the decrease in growth rate (Figure 3.35), although again, no significant correlation was observed (Pearson product correlation, n = 4, P>0.05). The decline in relative growth rate and mRNA abundance also revealed no significant correlation (Pearson product correlation, n = 4, P>0.05) (Figure 3.36).
Figure 3.34 mRNA transcript and protein abundance in E.coli following expressed antisense RNA silencing of the essential gene fabI.

Relative mRNA abundance and protein molecules per cell were plotted against corresponding relative growth rate. Pearson product correlation revealed no significant correlation (n = 4, P>0.05) between mRNA and protein abundance. Graph shows average protein molecules per cell and mRNA abundance with standard error bars (n=3).
Figure 3.35 Relative growth rate correlation with FabI molecules per cell following expressed antisense RNA silencing of the essential gene fabI in E.coli.

Growth rate titration of E.coli strain As-fabI was achieved with increasing concentrations of IPTG (50, 55 and 65 µM). Pearson product correlation revealed no significant correlation ($n = 4$, $P>0.05$) revealed between FabI molecules per cell or relative growth rate decline. Graph shows average protein molecules per cell plotted with average relative growth rate against IPTG induction with standard error bars ($n=3$).
Figure 3.36 Relative mRNA abundance correlation with FabI per cell following expressed antisense RNA silencing of the essential gene fabI in E.coli.

Growth rate titration of E.coli strain As-fabI was achieved by induction with increasing concentrations of IPTG (50, 55 and 65 µM). No significant correlation was revealed between protein molecules per cell or growth rate decline. Pearson product correlation revealed no significant correlation (n = 4, P>0.05). Graph shows average protein per cell plotted with average relative growth rate against IPTG induction with standard error bars (n=3).
3.3.3 $MTL_{50}$ and $MPL_{50}$ for evaluating stringency of the essential $E.coli$ gene $fabI$

In this study the stringency of the essential $E.coli$ gene $fabI$ has been assessed using the concept of an $MTL_{50}$ value (Figure 3.37). An $MTL_{50}$ of 5 relative value or 500% of normal growth was determined for $fabI$, suggesting a large number of mRNA transcripts are required to maintain cell viability, and thus provides a measure of how stringency required the gene is.

An alternative measure to $MTL_{50}$ values for determining gene stringency can be obtained by substituting relative mRNA abundance for protein molecules per cell. Relative growth rate can then be expressed as a function of protein molecules per cell to create a Minimum Protein Level ($MPL_{50}$), whereby the number of protein molecules required for maintaining cell viability at 50% growth can be estimated. Application of this measure to $fabI$ reveals a $MPL_{50}$ value of approximately $7.5 \times 10^2$ molecules per cell (Figure 3.38).
Figure 3.37 Determining MTL\textsubscript{50} value for the essential gene \textit{fabI}.

Average relative mRNA was plotted against corresponding average relative growth rate to generate the MTL\textsubscript{50}. This enables an estimation of the amount of mRNA required at the point where growth is reduced by 50% (y=0.5). This value represents the MTL\textsubscript{50} which serves as a measure of gene stringency. Due to the increase in relative mRNA abundance at approximately 50% and 75% of normal growth rate, no linear relationship could be established; hence MTL\textsubscript{50} could not be estimated in this case.
Figure 3.38 Determining MPL$_{50}$ value for the essential gene *fabI*.

Average protein molecules per cell were plotted against corresponding average relative growth rate to generate the MPL$_{50}$. The resulting curve enables an estimation of the protein required at the point where growth is reduced by 50% (y=0.5). This value represents the MPL$_{50}$ which provides an alternative measure of essential gene stringency.
3.3.4 Antisense RNA based whole-cell screening assays using triclosan and phosphomycin

In addition to identifying essential gene targets, MPL\textsubscript{50} values could be utilised for the optimisation of antisense RNA based whole cell screening assays. Appropriate levels of IPTG induction for antisense RNA expression required to achieve a specific level of sensitivity could therefore be defined. This is considered critical requirement for assays when screening for low abundance natural products (Baltz, 2008).

As the initial phase of this experiment, the level of antisense RNA expression required to achieve a decrease in the MIC of target specific antibiotics was investigated. Having found a level of IPTG induction required to decrease the MIC for example 2-fold, cellular protein would then be quantified using the quantitative LC-MS/MS protocol incorporating the FabI protein internal standard.

The MIC of strains sensitized for FabI (Figure 3.39) and MurA (Figure 3.40) were determined by liquid growth assay (see methods section: 2.6.10). The FabI inhibitor triclosan and MurA inhibitor phosphomycin were used since both compounds directly inhibit their respective encoded targets. Sub-inhibitory concentrations of each antibiotic were used based on previously determined MIC's for the susceptibility of wild-type \textit{E.coli} to triclosan (Bailey \textit{et al}., 2009) and phosphomycin (Takahata \textit{et al}., 2010).

Expressed RNA silencing of \textit{fabI}, resulted in a significant difference between relative growth rates at each triclosan concentration (Kruskal-Wallis, H= 13.705, 3 df, P<0.05). At the highest level of IPTG induction (110 \textmu M) an approximate 7 fold increase in triclosan sensitivity was observed. In the case of murA silencing, no significant difference was observed between relative growth rates at selected phosphomycin concentrations (Kruskal-Wallis, H= 0.628, 3 df, P>0.05).
Figure 3.39 Triclosan sensitivity in *E.coli* following expressed antisense RNA silencing of *fabI*.

IPTG was used to induce antisense RNA expression in order to sensitize *E.coli* As- fabI to sub-inhibitory concentrations of triclosan at 0.0339, 0.0078 and 0.0156 µg/ml. This was intended to demonstrate how MIC changes with increased gene silencing. Graph shows average relative growth rates with standard error bars (n=3).
Figure 3.40. Phosphomycin sensitivity in *E.coli* following expressed antisense RNA silencing of *murA*.

IPTG was used to induce antisense RNA expression in order to sensitize *E.coli* As-*murA* to sub-inhibitory concentrations of phosphomycin at 0.0156, 0.0313 and 0.0625 µg/ml. As with the assay for triclosan, this was intended demonstrate how MIC changes with increased gene silencing. Graph shows average relative growth rates with standard error bars (n=3).
3.4 Species-specificity of peptide-PNA conjugates

3.4.1 Evaluation of PNA to discriminate between bacterial species in a mixed culture

The capacity of peptide PNA conjugates to induce gene silencing has been assessed directly with expressed antisense RNA from plasmids and found to be highly comparable (Goh et al., 2009). In addition to defining gene stringency, the inherent stability of PNA’s in vivo has facilitated research into their development as species-specific antimicrobials, in an effort to address antibiotic resistance (Good and Stach, 2011). The use of MPL_{50} values could therefore be applied to in the context of PNA’s to ascertain sufficient dose concentrations required to achieve bactericidal activity. Indeed bactericidal activity has previously been demonstrated against individual gene targets in *E.coli* (Good et al., 2001b). To date however, the capacity for PNA’s to promote bactericidal activity in an individual species within a mixed culture has not been evaluated. To examine the discriminatory potential of PNA’s further, a bioinformatics approach (see methods section: 2.7.1), was employed to identify the +5 to -5 translation initiation region (TIR) of essential genes conserved between *S.typhimurium* and *E.coli*. A total of 93 orthologous genes were identified, 47 of which possessed TIR’s with >2 base-pair mismatches necessary for effective silencing (Dryselius et al., 2006a). The essential gene *ftsZ* was selected for further investigation. In the majority of prokaryotes *ftsZ* encodes an FTPase that functions as a cytoskeletal element, analogous to the mammalian homologue tubulin in the regulation of cytokinesis (Bi and Lutkenhaus, 1991; De Boer et al., 1992). Following chromosome replication, FtsZ self-assembles into protofilaments that form a Z-ring structure via isodesmic mechanisms. Once assembled the Z-ring localizes to the interior of the cytoplasmic membrane at the midcell, where it is stabilised by FtsA and ZipA and recruits other cell division proteins in the formation of a division septum (Margolin, 2005).

Mutations that reduce the expression of *ftsZ* cause a characteristic change in cell morphology, whereby highly elongated cells are formed (Bi and Lutkenhaus, 1992). Therefore PNA inhibition of *ftsZ* would enable phenotypic differentiation between *S.typhimurium*, which would be expected to form elongated cells compared to *E.coli*, where normal cell size would be maintained thereby allowing microscopic observation of PNA specificity within a mixed
culture. To provide contrast, *E.coli* AC01 and *S.typhimurium* AC02 were transfected with L-arabinose inducible plasmids, expressing DS-red and Green Fluorescent Protein (GFP) respectively. Mixed cultures were prepared, induced with L-arabinose and treated with 1.25 µM of Peptide-PNA Se0002. Samples were taken after 6 hours incubation and viewed using fluorescent microscopy (Figure 3.41). The phenotype of *E.coli* AC01 treated with Se0002 was consistent with untreated controls, indicating the absence of gene silencing. In contrast *S.typhimurium* AC02 displayed cell elongation, indicating a failure in cellular division associated with the inhibition of *ftsZ*. These results demonstrate the capacity for PNA’s to discriminate between species in a mixed culture.

![Figure 3.41](image)

Figure 3.41 Effects of species-specific PNA essential gene *ftsZ* on *E.coli* and *S.typhimurium*.

Mixed cultures of *E.coli* AC01 expressing DS-Red and *S.typhimurium* AC02 expressing GFP were treated with 1.25 µM Peptide PNA Se0002 targeting *ftsZ* and observed with fluorescent microscopy after 6 hours incubation. A filamentous phenotype associated with *ftsZ* silencing was observed for *S.typhimurium* AC02, but not *E.coli* AC01.
Chapter 4 Discussion

4.1 Introduction
The threat of increasing antibiotic resistance has created an urgent requirement for the development of targeted screening strategies for identifying novel antibiotics. Critical for such assays is the identification of suitable molecular targets that sustain cellular growth. Essential genes have previously been identified as potential candidates using various genetic strategies. However, only recently has a concerted effort been made to ascertain the minimally required amount of gene expression necessary to sustain bacterial growth. A differential growth requirement (stringency) has been observed among essential genes. To date stringency has been assessed using mRNA abundance to represent expressed protein, yet issues have been raised concerning the large variation in mRNA-protein correlations for many genes. The lack of correlation between mRNA and protein is attributed to distinct post-transcriptional and post translational processing mechanisms, but also gene stochasticity. This study aimed to evaluate the stringency of essential gene targets in *E. coli*; firstly by examining operon based targets *fusA* and *rplE* and secondly; developing an LC-MS/MS strategy for measuring stringency at the protein level, using the essential gene *fabI* to validate the approach.

4.2 Specificity of antisense constructs
The concept of antisense RNA based regulation in prokaryotes has been adapted to provide practical tools for studying gene expression (Rasmussen *et al.*, 2007). Typical strategies involve the plasmid based expression of natural RNA transcripts within cells. Gene silencing occurs through a *trans*-mechanism, whereby expressed antisense RNA hybridizes to cognate mRNA, inducing transcript degradation and steric hindrance, and subsequently reducing the amount of translated protein. Using this strategy two essential gene targets in *E. coli*; *fusA* encoding elongation factor G (EF-G), a translational GTPase involved in protein synthesis (Rodnina *et al.*, 1997) and *rplE* encoding the 5S rRNA ribosome accessory protein L5 (Korepanov *et al.*, 2007), in addition to previous gene targets *fabI*, *murA*, *ftsZ* and *acpP*, have been evaluated. Growth assays revealed that expressed antisense RNA silencing of both essential gene targets *fusA* and *rplE*, causes a significant decline in cell growth rate. Notably a
reduction in growth rate was achieved at lower levels of IPTG induction, in comparison to previously assessed essential genes (Goh et al., 2009).

Experimental conditions may have exerted significant influence in this study since the specificity of fusA and rplE was examined using MH media compared to LB media used in the assessment of fabI, murA, ftsZ and acpP (Goh et al., 2009). In LB media carbon sources are limited and promote a change in the physiological state of E.coli, which switches metabolism to utilize amino acids causing a diauxic growth profile (Sezonov et al., 2007). In comparison, the use of MH media may support a higher rate of growth and subsequent antisense expression, resulting in higher silencing efficiency. A comparison between fabI, murA, ftsZ and acpP grown in MH and LB media however, revealed similar growth profiles over the same induction range of IPTG. An additional source of variability may arise from exogenous small RNA’s present in complex growth media, that arise from either the environment or as a by-product of RNA biogenesis from cells grown in the media. It has been determined that the presence of such exogenous sRNA’s exerts a significant effect on protein profiles (Pavankumar et al., 2012). Whether the presence of such entities would significantly enhance or suppress the effects of expressed antisense silencing is unknown. To validate any potential effects of exogenous sRNA’s would require growth profile comparisons of each strain using media pre-treated with RNase to prevent extraneous interference, ideally supported by transcript analyses.

With regards to the difference in growth rate to previously examined essential gene targets, It is more plausible that the low level of IPTG induction required for growth inhibition of fusA and rplE compared to acpP, ftsZ, fabI or murA, can be ascribed to antisense sequence design. Silencing efficiency is dependent upon mRNA turnover, efficiency of translation initiation and accessibility of the paired termini-antisense sequence to mRNA (Nakashima and Tamura, 2009). Such factors could be assessed using an array of bioinformatics frameworks that incorporate structural fluctuation and the transition state mRNA undergoes according to environmental conditions to predict silencing efficiency (Johnson and Srivastava, 2013). Critically a BLAST alignment of the fusA antisense sequence reveals an alignment with the 3’ translated region of rpsG, which encodes the 30S ribosomal subunit protein S7. Similarly the rplE antisense sequence is complementary to the 3’ translated region of the 50S ribosomal
subunit protein L24. Consequently off target effects may have occurred, resulting in reduced transcription of *rpsG* and *rplX*.

Despite the significant growth inhibition which indicated a high degree of specificity for *fusA* and *rplE* paired constructs, speculation regarding gene stringency should be avoided in the absence of transcript or protein data.

### 4.3 Validation of *fusA* and *rplE* expressed antisense RNA specificity

Transcomplementation strategies are an established method to validate target specificity and mechanisms of action in gene silencing studies, since the expression of a complete ORF accounts for all transcript variations (Goh *et al.*, 2009). Although the specificity of *rplE* could be validated by transcomplementation, cell growth in *fusA* silenced *E.coli* could not be rescued. Currently no published data exists regarding the transcomplementation of either *rplE* of *fusA* in *E.coli*, following expressed antisense silencing. However transcomplementation has been used successfully to validate the specificity of expressed antisense RNA constructs for *acpP*, *ftsZ*, *tabl* and *murA* (Goh *et al.*, 2009).

The overexpression of *fusA* appeared to contribute towards further growth reduction with increasing concentrations of L-arabinose. Furthermore, growth curves exhibited an extended lag phase in addition to a final cell density that was considerably lower compared to *rplE*. The absence of rescue may reflect a number of phenomena associated with gratuitous protein expression. The overexpression of certain genes is known to be toxic and detrimentally affects the growth rate of *E.coli*, due to a decrease in protein synthesis and accumulation of heat shock proteins (Dong *et al.*, 1995). In addition the perturbation of normal cellular protein concentrations can disrupt the homeostasis of protein-protein interactions, thereby altering the rate of protein degradation and post-translational modification. Consequently protein half-life can be extended culminating in an accumulation of extraneous protein, which localizes at critical intracellular sites and disturbs normal cell functions (Koller *et al.*, 2000). Excessive protein expression can induce an internal starvation response, resulting in a depletion of nutrients which through an unknown
mechanism, initiates the destruction of rRNA and ribosomes, thereby reducing cellular capacity for protein synthesis (Kurland and Dong, 1996). The breakdown of rRNA also increases competition between the mRNA species of clones and native proteins for functional ribosomes, contributing towards further growth reduction (Dong et al., 1995). Although not examined further, microscopic examination of E.coli under these conditions would confirm if protein aggregation was occurring during transcomplementation of fusA. This would suggest if production was excessive and prompt the redesign of transcomplementation plasmid, possibly to incorporate a weaker promoter to reduce expression.

Alternatively the reduced growth rate could be due to the metabolic burden associated with the simultaneous expression of plasmids with different selection markers. This state arises as a consequence of excess requirements for cell resources (energy and biosynthetic precursors), associated with the transcription and translation of encoded genes (Glick, 1995). Previous studies have reported growth defects arising from the expression of two strong promoter gene cassettes from multicopy plasmids that increase the metabolic burden on cells promoting plasmid loss (Anthony et al., 2009). Furthermore plasmid maintenance can alter the intracellular concentrations of cAMP-crP, disrupting the transcription of many host regulons (Diaz Ricci and Hernández, 2000). However rplE did not reveal a similar trend using the same plasmids and selection marker genes, which suggests that fusA itself, may have been contributory factor in the failure to rescue cell growth.

Although the fusA encoded protein functions as a translational GTPase in protein synthesis, a secondary role has been suggested as a molecular chaperone in facilitating the stabilization and folding of denatured proteins (Caldas et al., 2000). This purported role has been supported by increases amounts of FusA in response to exogenous stressors in E.coli (Han et al., 2008). Consequently, the excess of FusA synthesized following induction may exceed a critical level required for protein synthesis. Excess FusA may therefore have been available to function inappropriately as a chaperone and bind non-specifically with cytosol proteins to detrimental effect. This could be evaluated by examining cell lysate via SDS-PAGE or by using western blotting to detect increased levels of FusA.
The inability to validate antisense function by transcomplementation in the case of fusA may reflect a disruption in protein homeostasis, by altering protein-protein interactions and stoichiometry. Such changes may promote the misfolding and aggregation of numerous cellular proteins and alteration in protein turnover. Where excessive synthesis occurs, chaperone proteins and proteases that constitute the intracellular quality control system can no longer compensate (Mogk et al., 2011). Alternatively fusA expression for cell viability may be dependant upon a fine equilibrium between mRNA transcripts and translated protein. This could be attributed to the IPTG and L-arabinose based expression systems, which may lack the capacity to provide sufficiently refined induction ranges to maintain optimal expression. Indeed cross talk has been observed between $P_{Bad}$ and $P_{Lac}$ promoters through an undefined mechanism, which was found to be alleviated through directed evolution of the arabinose transcriptional activator AraC (Sung et al., 2007). Incidentally the detrimental effects on cellular growth observed from transcomplementation would suggest that fusA may be a stringently required target. Due to the discrepancies observed with transcomplementation however, in particular with fusA, this technique may be limited to the validation of expressed antisense specificity of individually transcribed genes.

### 4.4 Growth inhibitory effects of silencing rplE and fusA in E.coli

Essential gene targets that display bactericidal activity upon inhibition are desirable due to the reduced prospects of resistance developing and increased therapeutic efficacy (Stratton, 2003). Therefore characterizing the activity of molecular targets is a pre-requisite for the selection of suitable targets as part of antibiotic screening assay development.

The inhibitory effects of silencing both fusA and rplE in this study were determined to be bacteriostatic, since viable cell counts did not show a 3 log reduction in cfu/ml, representing the benchmark for bactericidal activity. The bacteriostatic nature of fusA is supported by the documented activity of known inhibitors such as fusidic acid in sensitive E.coli strains (Harvey et al., 1966). To date there is no known antibiotic that specifically targets rplE, however, inhibition following the silencing of ribosomal genes rplJ and rpsL, (encoding 50S ribosomal protein L10 and 30S ribosomal subunit protein S12 respectively) in M. smegmatis, have been characterized as bacteriostatic (Kaur et al., 2009).
Further support is evident in the mechanism of macrolide antibiotics, which are characteristically bacteriostatic and have a speculated capacity to interact with the L5 subunit (Schlünzen et al., 2003). Bacteriostatic inhibition arising from targeting of the ribosome has been linked to the inhibition of the in vitro folding capacity of the 23S rRNA, preventing the peptidyl transferase reaction rather then ribosome destruction (Chattopadhyay et al., 1996). This is especially prevalent with antibiotics that target the 50S subunit exclusively (Chattopadhyay et al., 1999), where the L5 ribosome protein encoded by rplE is assembled.

Despite the specificity and rapid decline in growth rate observed following silencing of fusA and rplE, the experimental findings characterize both genes as bacteriostatic targets. Whether the use of expressed antisense provides an accurate characterization of target inhibition is unclear, since inhibition of the same gene targets by different entities can cause variable effects. In the case of antibiotics, chloramphenicol is bacteriostatic in E.coli, but bactericidal in H.influenza. Furthermore, the inhibitory effects of PNA’s on gene targets are bactericidal, despite bacteriostatic inhibition observed with target specific antibiotics.

The bactericidal activity observed with ribosome targeting has been attributed to the mistranslation and misfolding of membrane proteins which initiates a stress response. Antibiotic induced conformational changes in the ribosome, causes tRNA mismatching resulting in mistranslated proteins, which are bound to chaperone proteins and directed towards the membrane. At this stage defective proteins undergo translocation across the inner membrane which activates the envelope two-component stress response sensor CpxA. CpxA phosphorylates CpxR which in turn activates the stress response proteins including the redox responsive two-component transcription factor ArcA which upregulates both metabolic and respiratory systems leading to oxidative stress (Kohanski et al., 2008). In contrast antisense mediated inhibition reduces target mRNA abundance through decay or steric hindrance, neither of which initiates a secondary pathway capable of initiating oxidative stress. Consequently the use of antisense silencing may not be predictive of the mode of action utilised by target specific inhibitors. Although the silencing of one essential gene may be insufficient to generate bactericidal activity, the silencing of two essential genes simultaneously may prove synergistic. Dual silencing of the ackA-pta operon in
*E. coli*, demonstrated increased silencing compared to individual expression (Nakashima and Tamura, 2009). Therefore essential genes within the same operon may be assessed in tandem to determine if bactericidal activity is evident.
4.5 Evaluation of stringency in essential genes *rplE* and *fusA*

Evaluating the stringency of essential genes supports gene characterization, network analyses and the identification of suitable molecular targets for antibiotic screening assays. This study has evaluated the stringency of essential genes *rplE* and *fusA* in *E.coli*, using expressed antisense RNA silencing to titrate down the growth rate and measure the associated transcript abundance. Gene silencing of both *rplE* and *fusA* although causing a significant decline in growth rate, failed to cause a significant decrease in mRNA abundance. In *E.coli* expressing antisense *rplE*, the transcript abundance initially increased upon induction and remained stable as relative growth rate decreased. Silencing of *fusA* revealed an initial decrease in mRNA abundance, prior to a large increase in mRNA at the lowest growth rate. The standard error for relative growth rate showed significant variation, especially for two data points in *fusA*. This may reflect variation in cDNA synthesis despite batch reactions being performed. These results contradict previous determinations of essential gene stringency for *fabI*, *murA*, *acpP* and *ftsZ*, where mRNA abundance decreased in parallel with growth rate (Goh *et al.*, 2009).

For direct comparisons of stringency with previously validated essential targets, MTL	extsubscript{50} values were calculated. An inverse trend for both *fusA* and *rplE* was observed, whereby expressed antisense silencing causes an increase in transcription, resulting in higher target mRNA abundance. The transcript abundance required to maintain a 50% growth rate was higher then the level for normal growth rate. This finding initially suggests that *rplE* and *fusA* are not stringently required for cell viability. However, the apparent increase and stability of mRNA abundance could be ascribed to partial degradation of mRNA, resulting in non-functional transcripts that were still viable templates for amplification during QPCR. If true, then a reduction in growth rate would be expected, while mRNA abundance remains high. The location of amplified regions used by primers in QPCR would likely play a significant role. If amplified regions were located at the 5' end of the mRNA transcript, then the possibility of viable template persisting is likely, since enzyme mediated destruction of antisense bound transcripts occurs primarily from the 3'end (Condon, 2007).

Unlike *fusA* and *rplE*, previously assessed essential genes were individually transcribed (Goh *et al.*, 2009). In contrast both *rplE* and *fusA* are located within
ribosomal operons, which are subject to an autogenous feedback mechanism from genes encoding ribosomal subunit proteins (Yates et al., 1980). The spc operon encodes rplE in addition to nine other genes encoding sequential subunits; rplN (L14), rplX (L24), rplE (L5), rpsN (S14), rpsH (S8), rplF (L6), rplR (L18), rpsE (S5), rpmD (L30), and rplO (L15), (Cerretti et al., 1983). Within this operon the ribosomal protein S8, functions as a translational repressor (Dean et al., 1981a), through binding in proximity to the translation initiation region of rplE, inhibiting its translation and that of distal proteins. Additionally S8 also governs the expression of proximal proteins (L14 and L24) via a retro-regulation mechanism, involving exonuclease degradation following translation repression of L5 (Mattheakis et al., 1989). These regulatory mechanism may account for the discoordinate expression of mRNA, which has been observed following mutations in the start codon region of rplE, which reduce the synthesis of distal proteins 20-fold, yet exhibit little effect on mRNA synthesis. In addition overproduction of the S8 repressor causes a 3-fold reduction in protein synthesis, but not mRNA synthesis (Mattheakis and Nomura, 1988). A separate ribosomal operon str encodes both ribosomal protein subunits; rpsL (S12), rpsG (S7), elongation factors fusA (EF-G) and tufA (EF-Tu). Within this operon, S7 acts as a translational repressor and regulates the synthesis of EF-G and EF-Tu by translational coupling (Dean et al., 1981b), and S12 by retro regulation (Saito and Nomura, 1994).

Unlike individual genes, the transcription of polycistrionic mRNA and subsequent translation is discoordinated (Dryselius et al., 2006a). Evidence For this phenomena is derived from a study of the lac operon in E.coli using targeted PNA’s to the structural genes lacZ, lacY and lac, which revealed a directed inhibitory effect. PNA mediated inhibition of lacZ reduces expression of downstream lacY and lacA, yet inhibition of lacY does not affect the expression of lacZ, only lacA (Dryselius et al., 2006a). A further discrepancy was observed with the number of lacZ transcripts that were 3-4 fold higher, due to cleavage by RNaseE within an intergenic region between lacZ and lacY, resulting in increased stability of lacZ transcripts and decay of lacA (Li and Altman, 2004).

The lack of decline in mRNA abundance observed following gene silencing of fusA and rplE, may reflect a disturbance in normal feedback regulation within each operon due to translational coupling of distal genes. For rplE in particular,
the regulation of r-protein expression is founded on the interaction between rRNA and mRNA, due to structural features that recognize homologous sequences. A single subunit can therefore coordinately regulate the synthesis of other subunits via translational feedback (Nomura et al., 1980). In the context of these results a reduction in rplE encoded L5 protein may alter the stoichiometry of ribosomal subunits, consequently reducing repressor mediated effects of the S8 regulatory protein. Parallel examination of both mRNA and protein abundance would aid in elucidating these effects. Indeed disproportionate reductions of mRNA and protein have been observed for genes within operons (Dryselius et al., 2006a).

The lack of consistent mRNA decline with increased antisense RNA silencing may also reflect the inherent heterogeneous nature of isogenic (genetically identical) cell populations. Within these populations, sub-populations exist that exhibit variations in physiologically state, manifesting as in growth rate, cell age, metabolic state or stage in the cell cycle. These variations arise due to intracellular differences in resources and cell machinery, which are amplified by cellular cascade mechanisms (Lidstrom and Konopka, 2010). These differences underlie the phenomena of gene stochasticity (noise), whereby the expression of genes occurs at irregular intervals and in short burst of transcription and translation to generate variable numbers of proteins (McAdams and Arkin, 1997). Early studies of gene stochasticity identifying key causes, which were attributed to Intrinsic noise; associated with the expression of a gene sequence, and extrinsic noise arising from the interactions of other cellular components (Elowitz et al., 2002). From an evolutionary perspective, these stochastic events have been speculated to function as a survival strategy, that confers a selective advantage, by priming regulatory pathways for the onset of sudden environmental changes (Fraser and Kærn, 2009). Subsequent studies into gene stochasticity have lead to the development of fluorescent based techniques that permit the measurement of mRNA in individual bacterial cells (Golding et al., 2005). Such techniques represent an alternative to QPCR and provide a more direct and visible means of quantifying transcript abundance.

From an experimental perspective, the abnormally high MTL$_{50}$ values could also reflect the narrow range of IPTG used to induce silencing. For fusA and rplE, this ranged from 5 – 25 µM in comparison to wider range of inducer
concentrations for \textit{fabl}, \textit{murA}, \textit{ftsZ}, \textit{acpP} (Goh \textit{et al.}, 2009). In retrospect, both
\textit{rplE} and \textit{fusA} represent poor targets and highlight the limitations of assessing
gene stringency based upon mRNA abundance, especially for gene targets
encoded within operons with regulatory feedback mechanisms.

Recently a method for evaluating operon genes in isolation was developed to
address such discoordinate expression. A dynamic transcriptional control on/off
switch was constructed using the Lambda repressor, which was inserted into
the chromosomal position of the \textit{lac} repressor. A plasmid expressing \textit{fusA} under
control of the lambda promoter was then used for constitutive expression. The
addition of IPTG, subsequently enabled gene expression to be switched on and
off (Min \textit{et al.}, 2012). In summary the determined MTL$_{50}$ values for \textit{fusA} and
\textit{rplE} suggest neither gene is stringently required for cell viability, based on the
present definition.

These results emphasize the complexities of evaluating operon based essential
genes due to translation coupling and regulatory feedback mechanisms.
Consequently the MTL$_{50}$ value may be limited as a measure of gene stringency
and supports the case for evaluating essential genes at the level of protein
expression.
4.6 Evaluation of gene stringency in essential gene fabI by proteomics

Previous measures of essential gene stringency have assumed that relative mRNA transcripts are directly related to the amount of translated protein. However a lack of correlation between mRNA and protein due to gene organization and regulatory mechanisms is well documented. Consequently the direct measurement of expressed protein provides the most appropriate determination of cell physiology and therefore gene stringency. This study aimed to address the limitations of the MTL\text{50} value used for measuring essential gene stringency. An LC-MS/MS assay was devised to quantify the abundance of fabI protein in \textit{E.coli} in parallel with mRNA abundance, following expressed antisense RNA silencing.

The LC-MS/MS assay revealed a significant decrease in the abundance of FabI following gene silencing; however no correlation with mRNA abundance was observed. Indeed relative mRNA abundance increased when the relative growth rate reached 50\% of normal growth. Although transcriptome and proteome analyses often reveal divergent profiles (Jayapal \textit{et al.}, 2008), the silencing of \textit{fabI} in a previous study did show an overall decline in mRNA abundance with IPTG induction ranging from 20 to 80 µM. At induction concentrations above 80 µM however, mRNA abundance was seen to increase marginally (Goh \textit{et al.}, 2009). The lack of definitive decrease in mRNA abundance observed in this study may reflect the narrow range of IPTG concentrations used to induce antisense expression (50 – 65 µM). Over this range, decreases in mRNA abundance may be obscured by inherent noise associated with stochastic gene expression (Raj and van Oudenaarden, 2008). However the observed growth reduction suggests expressed antisense is functioning. As in the case of \textit{rplE} and \textit{fusA}, those transcripts detected in QPCR analyses may be non functional, yet provide viable templates for amplification, generating mRNA profiles.

The disproportionate correlation between mRNA and protein abundance may also reflect the dynamic relationship between their respective rates of synthesis and degradation (Vogel and Marcotte, 2012). The synthesis of mRNA is dictated by factors that effect recruitment of RNA polymerase and associated transcription factors to the transcription start site, such as chromosome structure and promoter strength (Balleza \textit{et al.}, 2009). The stability of mRNA
transcripts is governed by RNaseE and the length of mRNA. Although mRNA turnover contributes to mRNA-protein correlation, the greatest influence is attributed to post-transcriptional and post-translational mechanisms that regulate protein turnover (Maier et al., 2009). The secondary structure of transcribed mRNA, strength of the Shine Dalgarno sequence, codon bias and ribosome density all contribute to translational efficiency (Maier et al., 2009). Following translation mRNA-protein ratios are influenced further by protein half-life, which is dictated by protein stability and post-translational processes (Maier et al., 2009). Consequently examining mRNA and protein turnover may provide a more comprehensive assessment of gene stringency. Attempts at quantifying mRNA and protein turnover have been attempted in the minimal genome bacterium *Mycoplasma pneumonia*, where protein abundance ranged over three orders of magnitude (Maier et al., 2011). Furthermore the ratio of mRNA:protein varied during the growth phase and was highly uncorrelated for genes existing within operons. Notably, a higher correlation was observed if operons were short and genes were not situated at the 3’ end (Maier et al., 2011). However a global analysis of mRNA decay and abundance of 4288 predicted mRNA’s in *E.coli*, suggested that generalizations concerning the decay of transcripts was limited in applicability. This conclusion arose from the finding of an inverse relationship between transcript abundance and stability. A similar inverse trend was found following analysis in *M.tuberculosis*, where a global stabilization of transcripts was observed in response to cellular stress (Rustad et al., 2013).

An alternative to the use of QPCR would be to employ RNA sequencing to quantify expression of silenced genes. The approach utilizes deep sequencing technologies based on the conversion of mRNA to cDNA which is ligated to adapters and sequenced. RNA sequencing displays a high level of sensitivity and is not susceptible to background interference resulting in strong signal:noise ratios. Furthermore, unlike QPCR RNA sequencing does not require normalization of data sets (Wang et al., 2009). The quantitative capacity of RNA sequencing has been demonstrated, and shows strong correlation with both QPCR (Nagalakshmi et al., 2008) and label--free LC-MS/MS methods (Fu et al., 2009).
A comparison of FabI molecules per cell between *E.coli* As-fabl, AG1 ME5305 and a wild-type control revealed a significant difference in protein abundance. The number of FabI molecules per cell was higher in the antisense control, which may be evidence of high basal promoter (*Ptrc*) activity associated with the paired termini vector in the absence of IPTG (Nakashima and Tamura, 2009). To date limited published data exists regarding the protein abundance of *fabI* quantified using LC-MS/MS following antisense expressed silencing. In *E.coli* a value 12,500 FabI copies per cell has been determined (Ishihama *et al.*, 2008). A basal expression level of 12,000 copies per cell has also been determined in *S.aureus* (Slater-Radosti *et al.*, 2001). These estimations are approximately 5-fold higher then this study’s observations in the *E.coli* As-fabl control (2561 FabI molecules per cell) and approximately 17 fold higher then the basal level observed in wild-type *E.coli* (718 FabI copies per cell). An accurate comparison is limited however, since abundance measurements in *E.coli* was determined using a linear ion trap rather then an orbitrap mass spectrometer, resulting in faster scan cycles and increased m/z range. The use of additional protein fractionation steps to increase identification coverage, in addition to the use of an emPAI approach for quantitative analysis (Ishihama *et al.*, 2008), would also account for the variation observed with this study. With regards to *S.aureus*, genetic differences such as the presence of homologues and the semi-quantification of protein abundance using western blotting, also limit a direct comparison (Slater-Radosti *et al.*, 2001).

The quantification of the $^{15}$N FabI internal standard revealed a significant difference in the fmol amount after extrapolation from independent standard curves generated for EGAELAFTRYQNDK and ILVTGVASK. Experimental error is a feasible explanation due to the low volumes of standard spiked into pre-trypsin digests. However, steps were introduced to limit potential inaccuracies, such as the preparation of serial dilutions at fixed ratio rather then fixed interval (Han and Higgs, 2008). Further variation in the quantitative analysis may be accounted for by the effects of $^{15}$N incorporation. Absolute quantification using a $^{15}$N labelled internal standard is reliant upon the isotope dilution principle. This concept is based on the assumption that physiochemical properties between light and heavy labelled peptides are equivalent. The concept also assumes that the effects of isotope incorporation on protein structure and function are
negligible. This notion has been disputed following a recent study of $^{15}$N labelling on protein expression in *E.coli* where incorporation of $^{15}$N was found to effect the expression of over 40 cytoplasmic proteins. Critically the proteins affected included key metabolic enzymes, which accounted for significantly reduced growth rates (Filiou et al., 2012). The direct incorporation of a heavy isotope may result in decreased enzyme function due to conformational changes in key residues such as the active site. It is known that $^{15}$N can alter structural integrity of proteins, through changes in peptide backbone torsion, side-chain orientation of residues and hydrogen bonding between neighbouring peptides (Xu and Case, 2002). This phenomenon has been validated by studies of copper metallothioneins in *S.cerevisiae*, which show aberrant spectroscopic profiles, indicating the distortion of normal protein architecture with $^{15}$N labelling (Hartmann et al., 2003).

The incorporation effects of $^{15}$N may therefore influence the quantification process in particular the tryptic digestion step, as trypsin has been found to cleave light proteins at a faster rate compared to heavy labelled proteins (Konopka et al., 2012). False L:H ratios could therefore be generated compromising accurate quantification of protein abundance. Despite this risk, the effects of $^{15}$N incorporation have been limited to physiochemical properties of peptides, such as chromatographic retention time and found to exert no influence on quantification accuracy (Webhofer, 2013). Nonetheless, incorporation effects on trypsin digestion should be evaluated as part of the LC-MS/MS validation. Ideally this would be incorporated as part of the internal standard validation process and would be evaluated by analysing tryptic digests of equal quantities of $^{15}$N labelled and unlabelled FabI protein over varying incubation times. LC-MS/MS could then be used to examine the degree of variance in the L:H ratio, which would be expected to be equal if the effects of $^{15}$N incorporation were negligible.

The absence of correlation between transcripts and expressed FabI in *E.coli* as demonstrated in this study, highlight the limitations of using mRNA as a direct proxy for expressed protein. The obtained results demonstrate the capability of an LC-MS/MS strategy incorporating a fully $^{15}$N labelled internal standard, for measuring protein concentration per cell. From the quantitative data, essential gene stringency has been established using the MPL$_{50}$ value to define the
absolute growth requirement of the essential gene *fabI*. These findings therefore support the argument for quantifying expressed protein, which provides a more accurate reflection of essential gene stringency.
4.7 Species specificity of peptide-PNA conjugates in mixed bacterial cultures

Alternative strategies for the treatment of bacterial infections have been pursued to address the limitations posed by the development of multiple antibiotic resistance mechanisms. Gene silencing using peptide-PNA conjugates demonstrates the potential to discriminate between gene sequences, since they can be designed to exploit the degeneracy of the genetic code, thereby allowing gene silencing to be used in a discriminatory capacity.

This study has examined this potential of peptide-PNA conjugates as a species-specific antimicrobial, by designing a PNA sequence incorporating a 2 base-pair mismatch, to delineate the effects of silencing of the essential gene ftsZ in S.typhimurium and E.coli. Treatment of mixed cultures with PNA revealed no effect on E.coli, however elongated cells of S.typhimurium were observed. This phenotype is consistent with mutations that reduce expression of ftsZ causing a characteristic change in cell morphology to form highly elongated cells (Bi and Lutkenhaus, 1992). Further validation is provided by similar results in previous silencing studies of ftsZ using peptide-PNA conjugates (Goh et al., 2009). The results confirm that a peptide-PNA conjugate with sequences incorporating a 2 base-pair mismatch were sufficient to selectively bind to and inhibit translation of ftsZ thereby interrupting cell division causing cell elongation in S.typhimurium but not E.coli.
Chapter 5 Conclusion and Future Work

The sustained threat of multiple antibiotic resistance has invoked interest in empirical target based screening assays, for the discovery of novel antibiotic compounds. An integral component of target based strategies is the selection of an encoded molecular target, such as essential genes which provide suitable candidates. Defining essential genes has been previously founded on systematic gene deletion studies, where cessation of cell growth in the absence of a gene defines essentiality. The aim of this study was to expand on previous findings that a differential growth requirement or stringency exists among essential genes and is reflected in both the transcriptome and proteome profiles of E.coli, following gene silencing.

The unpredictable effects of silencing operon based genes presents a significant limitation with the antisense silencing strategy for evaluating gene stringency, as observed in this study with divergent transcript and protein profiles. Consequently, alternative approaches have been sought to provide temporal gene regulation. The development of inducible protein degradation systems represents one such example. This strategy is founded upon adaptor mediated proteolysis, which regulates protein turnover in prokaryotes. The central component is the protease complex ClpXP consisting of an ATPase (ClpP) and a peptidase (ClpX), which recognises and binds to unstructured peptide sequences at the N or C terminus of proteins that function as degradation tags (degrons) (Baker and Sauer, 2012). A known degron tag is the short peptide SsrA which is translated and added to incomplete proteins, following ribosome stalling, due to the absence of stop codons or premature transcription termination (Keiler et al., 1996). Adapting this process enabled an assessment of antibiotic inhibition required to inactivate encoded gene products (termed vulnerability) (Wei et al., 2011). In this study, phage homologous recombination was used to insert a sequence encoding a modified SsrA tag fused to a protecting peptide, into chromosome regions encoding individual antibiotic targets in M.smegmatis. A virus-specific protease targeting the protecting peptide was put under the control of a tetracycline promoter and also integrated. Expression of the viral protease removed the protecting peptide and exposed the SsrA tag to ClpXP mediated degradation of individual target proteins causing an increase in antibiotic sensitivity. This strategy also enabled
the differentiation between bactericidal and bacteriostatic antibiotic targets (Wei et al., 2011).

More recently an alternative inducible protein degradation system has also been described in *E. coli*. Strains were constructed using homologous recombination to insert a proteolytic system consisting of a promoter (*lac*) driven ClpA gene for inducible degradation and constitutive promoter driven ubiquitin protease. A modified tryptophan promoter was combined with the *trpR* gene encoding tryptophan repressor and replaced the tryphanonanse *tnaA* gene. The ORF for *ubi4* (target for ubiquitin protease) was then inserted upstream of the target gene. This system enabled biphasic control of target protein and also delineation between bactericidal and bacteriostatic activity (Yoshida et al., 2012).

Unlike expressed antisense RNA silencing, inducible protein degradation systems are capable of maintaining basal expression levels, while avoiding directional effects associated with operon silencing, including the disruption of feedback regulation. Protein half-life can also be strictly regulated to prevent extraneous accumulation and potential toxicity. The experimental approach in this study could therefore be improved by combining inducible protein degradation with a quantitative LC-MS/MS strategy, to provide a comprehensive determination of gene stringency.

Whether using antisense RNA or protein degradation systems for studying gene stringency, consideration should be given towards the unknown consequences of silencing a single gene on the expression of other genes, either directly or through feedback regulation. Due to the lack of knowledge concerning how cellular pathways interact, a pre-requisite to determining gene stringency, would therefore be to validate the effects of silencing strategies on the global transcriptome and proteome of model organisms such as *E. coli*.

An intended aim of evaluating essential gene stringency was to facilitate the pre-screening of genes to enable the prioritization of targets for natural product screening assays and for designing species specific PNA’s (Goh et al., 2009). Further applications have since become apparent in the emerging research field of synthetic biology, which aims to combine investigative biology techniques with engineering concepts such as standardization, decoupling and abstraction
This multidisciplinary approach is envisaged to facilitate the rationale design and systematic construction of a library of compatible modular devices, which can be assembled to form functional metabolic pathways and networks. This would support the elucidation of a minimal genome chassis, and ultimately the development of synthetic organisms (Purnick and Weiss, 2009).

In order to facilitate the assembly of complex cell systems, there is a requirement for a library of characterized and standardized elements to enable interchangeability between modules (Güttinger, 2013). With regards to the construction of a minimal genome, a pre-requisite would be the characterization of minimal gene sets for cell viability. Previous efforts to define minimal genes have generated inconsistent results, due to inherent limitations in the experimental techniques used. Transposon mutagenesis overestimates the number of essential genes by considering those that slow growth, but do not stop it as essential. Antisense RNA silencing is also limited to genes that can be inhibited by sufficiently expressed levels of antisense inhibitor and does account for redundant genes (Moya et al., 2009). Consequently misinterpretations arise as evident from genome reductions of up to 30% which do not decrease cell viability (Fehér et al., 2007), despite many deleted genes having previously been characterized as essential (Gerdes et al., 2003).

Given these discrepancies, the evaluation of essential gene stringency would support characterization efforts and enable the creation of an essentiality threshold. Knowledge of stringently required genes would therefore minimise biological complexity through the removal of non-stringent genes from minimal gene sets, facilitating the construction of a minimal genome chassis and supporting the classification of modular functionality (Esvelt and Wang, 2013). Furthermore, essential gene stringency would aid the development of in silico modelling especially for metabolic reconstitutions (Chavali et al., 2012) and genome scale engineering (Esvelt and Wang, 2013).
References


Ishihama, Y., Oda, Y., Tabata, T., Sato, T., Nagasu, T., Rappsilber, J. and Mann, M. (2005) 'Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of
sequenced peptides per protein', *Molecular and Cellular Proteomics*, 4(9), pp. 1265-1272.


Lundin, K.E., Good, L., Strömberg, R., Gräslund, A. and Smith, C.I.E. 56 (2006) 'Biological Activity and Biotechnological Aspects of Peptide Nucleic Acid' Hall,


Appendix A: Growth Media & Buffer compositions

Luria-Bertani (LB) Agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
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<td>Yeast Extract</td>
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<td>Sodium Chloride</td>
<td>5.0 g/L</td>
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<tr>
<td>Agar</td>
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Muller Hinton Broth

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SOB medium

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<td>Potassium chloride</td>
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<tr>
<td>Magnesium Chloride</td>
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SOC Medium

As SOB medium with the addition of 20 ml of 1 M glucose to provide a final 20 mM concentration.

M9 Minimal media

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<td>Iron Sulphate</td>
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Appendix B: Publications associated with this study

Mondhe, M., Ashley Chessher, A., Good, L., Stach, J.E.M (2012). **Species-selective killing of bacteria by "smart" antimicrobial peptide-PNAs.** (In review at time of print).

Species-selective killing of bacteria by "smart" antimicrobial peptide-PNAs

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Running title: Species-specific antibacterials

Keywords: species-specific, species-selective, antibiotic, antimicrobial, antisense, peptide-nucleic acid, narrow-spectrum, microbiome

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Broad-spectrum antimicrobials kill indiscriminately, a property that can lead to negative clinical consequences and an increase in the incidence of resistance. Species-specific antimicrobials that could selectively kill pathogenic bacteria without targeting other species in the microbiome could limit these problems. The pathogen genome presents an excellent target for the development of such antimicrobials. In this study we report the design and evaluation of species-selective peptide nucleic acid (PNA) antibacterials. Selective growth inhibition of B. subtilis, *E. coli*, *K. pneumoniae* and *S. enterica* serovar *Typhimurium* in axenic or mixed culture could be achieved with PNAs that exploit species differences in the translation initiation region of essential genes. An *S. Typhimurium*-specific PNA targeting *ftsZ* resulted in a cell elongation phenotype that was not observed in *E. coli*, providing phenotypic evidence of the selectivity of PNA-based antimicrobials. Analysis of the genomes of *E. coli* and *S. Typhimurium* gave a conservative estimate of >150 PNA targets that could potentially discriminate between these two closely related species. This work provides a basis for the development of a single class of antimicrobial with a tuneable spectrum of activity.

Introduction.

Treatment of bacterial infections with antimicrobial drugs has been clinically effective for over six decades. However, the rise of antimicrobial resistance threatens to limit options for the treatment of life-threatening microbial diseases (Jayaraman, 2009). Arturo Casadevall noted that antimicrobial therapy ‘is the only branch of medicine where therapeutic options were better in the mid-20th century than at the beginning of the 21st century (Casadevall, 2006)’. A critical factor behind the rise in resistance is the spectrum of activity of the antibacterial agent; the development of broad-spectrum agents (effective against a number of microbial species) enabled empirical usage (i.e. without the use of diagnostic identification of the pathogen) and rapid treatment of fulminate microbial infections. However, it is this broad-spectrum of activity that is partly responsible for the prevalence of multi-drug resistant bacterial pathogens. Also, the human body plays host to hundreds of microbial species, collectively termed the microbiome (Eckburg et al., 2005), the majority of which are beneficial to health.
Thus, the use of non-specific broad-spectrum antimicrobial agents will have unintended detrimental affects on the microbiome, which can result in colonization of pathogenic microbes. Examples of such opportunistic infections include *Clostridium difficile*-associated diarrhea, antibiotic-associated colitis and candidiasis (Fowler et al., 2007; Casadevall, 2009). Perhaps of greater concern is the link between the microbiome and the normal development of the immune system; there are indications that the use of broad-spectrum agents may increase the risk of diseases such as asthma, eczema, rhinoconjunctivitis and breast cancer (Velicer et al., 2004; Foliaki et al., 2009). Furthermore, broad-spectrum antimicrobial treatment selects for resistance mechanisms in non-target species that are readily transferred to pathogenic species (Hoban, 2003; Yates et al., 2006). The impact of broad-spectrum antimicrobial agents on the gut microflora was highlighted in a recent study by Jakobsson et al (Jakobsson et al., 2010): the authors reported that treatment of *Helicobacter pylori* infection with the macrolide antibiotic clarithromycin, resulted in perturbation of the gut microflora associated with an increase in level of the macrolide antibiotic resistance gene *erm* (B). These effects were observable four years after antibiotic exposure.

Recent findings demonstrating the importance of the microbiome in host health, have led to a growing interest in the use of microbiota-targeted therapies that can eliminate individual strains of single species (Lemon et al., 2012), for example, through targeting of drugs to microbial genes (Holmes et al., 2012). The increasing incidence of multi-drug resistant bacteria, coupled to declining discovery of novel broad-spectrum antimicrobial compounds, will likely make the development of species-specific antimicrobials a necessity; simultaneously providing the market conditions required for such a change in emphasis within drug development companies. Improved on-site diagnostics should soon enable pathogen/drug pairing in clinics. The development and implementation of any species-specific antimicrobial possess significant clinical challenges: nevertheless, the approach, alongside immunotherapy aimed at improving host responses, is predicted to deliver the “Third Age of antimicrobial therapy (Casadevall, 2006; Casadevall, 2009; Then and Sahl, 2010)”.

Technologies for species-specific pathogen inhibition include immunotherapy, radioimmunotherapy, anti-virulence agents, and phage therapy
(Then and Sahl, 2010, and references therein). Growing interest in narrow-spectrum antimicrobial compounds is evident in recent studies that have developed species-specific antimicrobial screens (Granieri et al., 2009) and through the identification of species-selective antimicrobial compounds (Qiu et al., 2003; Qiu et al., 2005; Eckert et al., 2006a; Eckert et al., 2006b; Yamaichi et al., 2009; Srinivas et al., 2010). Theoretically, the DNA sequence of the genome itself provides the ultimate species-specific target; antimicrobial compounds that bind to nucleic acids in a sequence-specific manner, can be used to define the spectrum of activity. Natural products such as netropsin and distamycin bind to adenine and thymine rich DNA sequences in the minor groove of DNA (Turner and Denny, 2000), with synthetic compounds that recognise all four bases having been described (Dervan, 2001). An alternative approach to targeting specific sequences in the chromosome is silencing of essential genes using antisense mechanisms. Exogenously delivered antisense DNA oligonucleotides, designed to bind to specific mRNA sequences, have been demonstrated to be effective against bacterial targets (White et al., 1997; Harth et al., 2000). Furthermore, DNA mimics such as peptide nucleic acid (PNA) and phosphorodiamidate morpholino (PMO) have uncharged backbone structures that enable delivery across negatively-charged bacterial cell barriers, and offer superior hybridization and stability properties when compared to modified DNA oligonucleotides (Good et al., 2000b). Attachment of carrier peptides to PNAs/PMOs can enhance cell permeation and uptake and improve antisense effects (Good et al., 2001a). PNAs and PMOs targeted to the translation initiation region (TIR) of essential mRNAs are bactericidal and have been successfully applied to a number of different species (Good and Nielsen, 1998; Nekhotiaeva et al., 2004; Kurupati et al., 2007; Shen et al., 2009). Bactericidal PNAs/PMOs are typically 10 bp in length and are more sensitive to target mismatches than equivalent DNA oligonucleotides, properties that make them highly suited to species discrimination.

In this study we tested the hypothesis that the selective binding properties of peptide PNA antimicrobials can be exploited to selectively target certain species in mixed culture based on sequence differences in the translation initiation region of essential genes. We report, for the first time, that
peptide-PNAs can form the basis of a single class of antimicrobial with a tuneable spectrum of activity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A list of the strains used in this study is given in Table 1. All strains were grown in Miller's modified Luria broth (MMLB; Sigma-Aldrich, UK) with constant shaking (200 rpm) at 37°C. For mixed-culture growth, MMLB was inoculated with 1 x 10^4 CFU/ml; the proportion of each species needed to give reproducible species counts after 16 h of growth at 35°C was experimentally determined (Table S1 in the supplementary material).

Design of species-specific peptide PNAs: General guidelines for the design of antibacterial peptide-PNAs are described elsewhere (Good and Nielsen, 1998; Good et al., 2000b; Good et al., 2001a; Good, 2002). Criteria used for the design of species-specific peptide-PNAs are described in the results section. The Database of Essential Genes (Zhang and Lin, 2009a) and BLAST (Altschul et al., 1990) were used to identify any essential gene homologues present in all four species used in this study. The Artemis program (Rutherford et al., 2000) was used to extract twenty base-pairs (-10 + 10 bases relative to the start codon) of the TIRs from the genome sequences of Bacillus subtilis (Kobayashi et al., 2003a), Escherichia coli DH10B (Durfee et al., 2008a), Klebsiella pneumoniae and Salmonella enterica serovar Typhimurium LT2 (Knuth et al., 2004) (GenBank accession numbers: AL009126; CP000948; CP000647; and AL513382 respectively). The 20 bp TIRs from gene homologues were aligned in Clustal X version 2.0 (Larkin et al., 2007) and the number of base-pair mismatches between species was determined. The predicted thermal stability (Tm) of PNA/DNA duplexes was determined according to formula of Giesen et al. 1998 (Giesen et al., 1998). A genomic analysis of the possible binding sites of the PNAs within their target species was conducted in Artemis, using a cut-off of greater than 2 bp mismatches. Secondly, to comprehensively examine the number of potential antibacterial PNAs that could be used to discriminate
between two closely related species, a semi-automated method was employed: genome sequences were used to identify the start codon positions of essential genes from *E. coli* DH10B and *S. Typhimurium* LT2. A custom PERL script was used to extract the -5 - + 5 bases relative to the start codon of each gene. The 10 bp sequences were used for an all-against-all comparison using standalone BLAST (Altschul *et al*., 1990; McGinnis and Madden, 2004a) to identify the TIRs of essential genes that were amenable to the design of species specific PNAs. The peptide-PNAs used in this study and their properties are listed in Table 2.

Antimicrobial susceptibility and peptide-PNA minimal inhibitory concentration (MIC) testing. Strains were tested with twenty different antibiotic disks (Oxoid, UK) representing the major classes of antimicrobial compounds. Tests were done according to the standardized disc susceptibility testing method of the British Society for Antimicrobial Chemotherapy (Andrews, 2001; Andrews, 2009). The minimum inhibitory concentration (MIC) of the peptide-PNA conjugants were determined using a method modified from Hacek *et al* (Hacek *et al*., 1999) and Friedman *et al* (Friedman *et al*., 2006): Peptide-PNA conjugants, obtained as lyophilized powder (Panagene, Korea), were dissolved in ddH2O. MIC assays were performed in an ultra low-bind (Costar, UK) polystyrene 96-well plate format in a final volume of 150 µl MMLB. An extended gradient of peptide-PNA concentrations was created by combining five sets of twofold serial dilutions from four starting concentrations (10, 4.8, 3.2 and 3 and 2 µM); giving 55 final peptide-PNA concentrations which extended over five rows of the 96-well plate. All cultures were incubated at 35°C for 16 h without shaking or agitation. Each peptide-PNA MIC calculation was performed in triplicate, each replicate representing a different starting colony. Mixed-culture experiments were conducted as above with a 1 x 104 CFU/ml starting inoculum. For growth curve analysis, 200 µl cultures were grown in a BioTek PowerWave HT spectrophotometer, under constant agitation at 37°C in a 96-well plate covered with a breathable film. Growth (OD550) was monitored every 5 mins, each experiment was performed in triplicate.
Microscopy. Cells prepared for fluorescence imaging were grown in a BioTek PowerWave HT spectrophotometer as above. After six hours 10 μl of culture was removed, washed and resuspended in 1 X PBS by centrifugation (13,000 x g). Cells were applied to an agarose pad (Levin, 2002b) and viewed using an epifluorescence Leica DMRB microscope. An EXi Aqua CCD camera (QImaging) and Image Pro Plus (MediaCybernetics) were used for image acquisition and processing.

Species identification. In mixed-culture experiments, colonies on MMLB plates were identified using a combination of phenotypic and genotypic properties. In order to rapidly identify species post peptide-PNA treatment, we designed a species-specific PCR-based identification method. Primer sets for peptide deformylase (def) were designed that yielded different sized amplicons for each species: *B. subtilis* (352 bp), *E. coli* (394 bp), *K. pneumoniae* (231 bp) and *S. Typhimurium* (280 bp) (see Table S2). All colonies were picked from the plates with the most countable dilution (30-50 cfu per plate) and used directly for colony multiplex PCR in 10 μl reaction volumes according to the simplified method of (Menossi et al., 2000). Colonies were identified using standards prepared from pure cultures. Furthermore, in the three-species mixed-culture experiments, colonies were identified using the colony PCR protocol with 16S rRNA gene primers. The sequences of both strands of the resulting amplicons were determined with the BigDye (version 3.1) cycle sequencing kit and a 3730 DNA analyzer (Applied Biosystems, UK). Species identity was confirmed using the SEQMATCH function of the Ribosomal Database Project (Cole et al., 2009).

RESULTS

Peptide-PNA mediated growth inhibition and species-selectivity. The four bacterial species used in this study were chosen as they have all been reported to be susceptible to antisense antibiotics (Good et al., 2001a; Tilley et al., 2006; Kurupati et al., 2007; Shen et al., 2009). The parameters used in the design of
species-selective peptide-PNAs resulted in a number of potential gene targets that could be used for species-selective growth inhibition. The following criteria were used for the design of species-selective peptide PNAs: 1) target gene is essential and homologues are present in all four species used in this study; 2) the translation initiation region (TIR) of the mRNA had at least two base-pair differences between species (see below); 3) the TIR sequence was amenable to the design of peptide-PNAs with low melting temperatures; 4) where possible, off-target sites within and between species were not in the TIRs of essential genes; and 5) evidence that gene silencing of the target and/or inhibition of its cognate protein is growth inhibitory. We have previously shown that murA and ftsZ are good targets for peptide-PNA mediated growth inhibition (Goh et al., 2009), both genes were identified in this study as potential targets for species-selective peptide-PNAs, and thus, were selected for further study. Two base-pair mismatches were selected, as PNAs with one base-pair mismatch will bind to the target, but with reduced affinity (Good et al., 2001a); peptide-PNAs with one base-pair mismatch to their target sites have approximately 33% increase in MIC (Liam Good, unpublished). This is in agreement with the design parameters suggest by Dryselius et al. (Dryselius et al., 2003) in which they suggest 1-bp mismatches within the TIR of off-target genes should be avoided.

The peptide-PNAs designed in this study were assayed for their antibacterial activity against both target and non-target species. The previously reported En108 peptide-PNA (called Ec108 in (Goh et al., 2009)) was used as a control to test the feasibility of species-selectivity at a broad taxonomic level; *E. coli*, *K. pneumoniae* and *S. Typhimurium* (Gram-negative, Enterobacteriaceae) have identical acpP TIRs and thus all three species should be susceptible to En108, while the acpP TIR of *Bacillus subtilis* (Gram-positive, Bacillaceae) has six base-pair mismatches and should be resistant to En108. Antibacterial assays with En108 proved this to be the case; En108 had an MIC of 1.2, 0.4 and 0.3 μM for *E. coli*, *K. pneumoniae* and *S. Typhimurium* respectively (Table 2), and had no detectable antibacterial activity against B. subtilis at concentrations of up to 20 μM (data not shown). Similarly, the species-selective PNAs for *B. subtilis*, *K. pneumoniae* and *S. Typhimurium* were only antibacterial to the intended species (Fig. 1A). The *E. coli*-selective Ec1000 was unexpectedly cross-reactive
with S. Typhimurium (discussed below). Table 3 shows the analysis of potential binding sites within the genomes of the target species. Of note is the difference in MIC between *E. coli*, *K. pneumoniae* and *S. Typhimurium* when treated with the acpP-targeting En108; the MIC of this PNA was 3 and 4 fold less in *K. pneumoniae* and *S. Typhimurium*, respectively (Table 2). Analysis of the binding sites of En108, Kp0001 and Se0001 in the genomes of these species revealed that En108 likely binds in the TIR region of other essential genes in *K. pneumoniae* (*mukF* and *ribH*) and *S. Typhimurium* (*yhhM*) each with a 1 bp mismatch. This could account for the decreased MIC in these species, however the relationship is not straightforward as Se0001 is predicted to bind in the TIR of at least three other genes determined to be essential in *E. coli* and *S. Typhimurium* (*hemK*, *Int* and *rluA*) and has an MIC equivalent to that of En108 in *E. coli* (Table 2). Furthermore, there is no obvious relationship between the MIC of a peptide-PNA and the number of off-targets in the genome of the target species, including those that bind in the TIRs of both essential and non-essential genes (Table 3). Reasons for the possible differences between the MICs of the different peptide-PNAs are discussed below.

Use of species-selective PNAs in mixed culture. To test the selectivity of peptide-PNAs in mixed culture, we first used reciprocal treatment in two-species culture (Fig. 1B). When mixed cultures of *E. coli* and *K. pneumoniae* were treated with 3.2 μM of *E. coli*-selective Ec1000, after 16 hrs of incubation only *K. pneumoniae* was detectable. Untreated control cultures maintained both species throughout the incubation period. Reciprocal treatment of the same mixed culture with 3.2 μM of *K. pneumoniae*-specific Kp0001 showed equivalent selectivity. Species-selective growth inhibition was also observed in mixed culture of *K. pneumoniae* and *S. Typhimurium* treated with 3.2 μM Kp0001 or 2.0 μM Se0001 (Fig. 1B). *S. Typhimurium* was successfully removed from mixed culture with *E. coli* when treated with 2.0 μM Se0001, but reciprocal removal of *E. coli* could not be achieved with Ec1000 (see above). Three-species mixed culture of *B. subtilis*, *K. pneumoniae* and *S. Typhimurium* was used to test the possibility of specifically targeting either one or two species, with a single peptide-PNA or a combination of two. Kp0001 and Se0001 both at 4.5 μM were successfully applied to the three-species mixed culture; only *B. subtilis* and *S. Typhimurium* could be detected after 16 hrs incubation with
Kp0001, and only *B. subtilis* and *K. pneumoniae* after Se0001 treatment (Fig. 2). Treatment of the three-species culture with 3.5 μM En108 resulted in the expected selective growth inhibition of both enteric species, with only *B. subtilis* detectable after after 16 hrs. Combined use of Kp0001 and Se0001, at 4.5 μM each was also able to specifically remove *K. pneumoniae* and *S. Typhimurium* from the mixed culture, however there was a significant difference in the final *B. subtilis* CFU count between the En108 and dual Kp0001/Se0001 treated cultures. This indicates that, while both Kp0001 and Se0001 had no affect on *B. subtilis* at concentrations of > 4.5 μM, combined use of the PNAs may have a weak synergistic antibacterial effect (Stach and Good, 2011). Mixed culture experiments with four species were not attempted in this study, however the potential of peptide-PNAs as species-selective antibacterial compounds is highlighted by a comparison of all possible species combinations for the four species tested in this study, alongside the antibiotic spectrum of the peptide-PNAs and twenty known antibiotics (Fig. S1). Only peptide-PNAs are capable of species-selective growth inhibition for the three Gram-negative species. For these species, there are six possible outcomes for species-selective antibacterial treatment of two-species mixed cultures. Treatment with the twenty antibiotics assessed in this study (Table S3), could theoretically achieve four of these outcomes (all combinations except those requiring inhibition of *E. coli* or *K. pneumoniae* in combination with *S. Typhimurium*). Use of peptide-PNAs Ec1000, Se0001 and Kp0001 enabled five of the possible outcomes; unexpected cross-reactivity of Ec1000 prevented selective inhibition of *E. coli* in combination with *S. Typhimurium*. However, it is very likely that evaluation of other *E. coli*-selective peptide PNAs would be able to rectify this result (see below). Furthermore, of the known antibiotics, only streptomycin could select between *E. coli* and *S. Typhimurium*. This would not be the case for most strains of *E. coli* as strain DH10B has an rpsL mutation that confers resistance to streptomycin. Unlike the peptide-PNAs, in mixed culture experiments with three species (Fig. 2), no combination of the known antibiotics would be able to selectively kill any of the Gram-negative species tested without killing *B. subtilis*.

Peptide-PNA mediated discrimination of *E. coli* and *S. Typhimurium*. The observation that Ec1000 was antibacterial to the non-target *S. Typhimurium* led us to use a comprehensive genomic analysis to determine the number of
potential targets that could be used to design peptide-PNAs that would discriminate between these two closely related species. For the purposes of defining potential discriminatory target sites, we allowed for 1 bp mismatch difference between the target site of *S. Typhimurium* and *E. coli*; while 1 bp mismatch is not likely to be sufficient to prevent binding, differences in gene silencing activity may be sufficient, in some cases, to enable the development of peptide-PNAs that would be selectively antibacterial to *S. Typhimurium*. Three separate BLAST analyses were done: 1) TIR sequences of genes described as essential in *S. Typhimurium* against the TIRs of *E. coli* essential genes; 2) TIRs of *S. Typhimurium* genes described as essential in *E. coli*, against TIRs of *E. coli* essential genes and 3) the TIRs of *E. coli* essential genes against TIR sequences of genes described as essential in *S. Typhimurium* (Tables S4-6). Using the essential genes of *S. Typhimurium* from the DEG database, and the -5 to +5 TIR region we identified 113 genes that could serve as targets for peptide-PNAs that would be selectively antibacterial to *S. Typhimurium* over *E. coli* (Table S4). Of these, 68 genes had orthologues not identified as essential in *E. coli*, 34 did not have orthologues in *E. coli* and of the 11 genes that had essential orthologues in *E. coli*, 5 of these had TIR sequences with > 2 bp mismatches. The fact that peptide-PNA Se0001, was selectively antibacterial for *S. Typhimurium*, and designed to target a gene not reported to be essential in *S. Typhimurium* indicates that orthologues of genes that are identified as essential in *E. coli* are likely to be essential in *S. Typhimurium*. Furthermore, *E. coli* genes have been identified as essential by failure to construct a specific knockout, whereas those of *S. Typhimurium* were identified by trapping lethal insertions (Knuth et al., 2004). We applied the bioinformatic screening technique described above to identify peptide-PNAs that would discriminate between *S. Typhimurium* and *E. coli* using the essential genes of *E. coli* as the query sequences. This analysis identified a further 93 orthologous genes that could potentially act as targets for discriminatory peptide-PNAs (Table S5). Of these, 47 genes had TIRs with > 2 bp mismatches between the two species, from which *ftsZ* was chosen for further study. This target was chosen for the reasons given above, and because gene silencing of *ftsZ* should result in a cell filamentation phenotype that would enable microscopic evaluation of the specificity of the peptide-PNA in mixed culture. DsRed-labelled *E. coli* AC01, and GFP-labelled *S. Typhimurium* AC02 were exposed to peptide-PNA Se0002
at concentrations \( \leq 5 \, \mu M \). \textit{E. coli} AC01 was unaffected by Se0002 at all concentrations tested, whereas \textit{S. Typhimurium} growth was inhibited at 1.25 \( \mu M \) (0.5 \( \times \) MIC), with a lag phase ca. 7.5 hrs longer than that of the untreated sample (Fig. 3a). Growth of \textit{S. Typhimurium} observed in Se0002-treated cultures after 10.5 hrs was not due to the generation of spontaneous resistance mutants, as samples of cells taken after 14 hrs of incubation, passaged into fresh media containing the same concentration of Se0002, exhibited identical growth kinetics to the parent culture. Growth is more likely due to the effective concentration of Se0002 falling below the minimum inhibitory concentration, caused by the peptide-PNA accumulating in non-growing cells, adsorbing to the plastic of the well, or proteolysis of the carrier peptide. Mixed cultures of \textit{E. coli} AC01 and \textit{S. Typhimurium} AC02 prepared as above, treated with 1.25 \( \mu M \) of Se0002 were sampled after 6 hrs of growth and were observed by fluorescence microscopy (Fig. 3b). As predicted, \textit{E. coli} AC01 treated with Se0002 had an identical phenotype to untreated controls, whereas \textit{S. Typhimurium} AC02 cells displayed a distinct filamentous phenotype only upon treatment with Se0002. This phenotype is consistent with previous studies using anti-\textit{ftsZ} peptide-PNAs \textit{in E. coli} (Goh et al., 2009). These results, and those of the mixed culture experiments detailed above, prove that it is possible to employ antisense-based molecules as species-selective antimicrobial agents in mixed culture.
The aim of this study was to test the hypothesis that peptide-PNAs can be applied as species-selective antimicrobial compounds. We demonstrate, for the first time, the design and application of species-selective antisense antibacterials. Antibacterial peptide-PNAs evaluated against B. subtilis, E. coli, K. pneumoniae and S. Typhimurium, both in single and mixed cultures, with the exception of Ec1000, displayed detectable antibacterial activity against the intended species only. Peptide-PNA treatment of mixed cultures enabled selective growth inhibition that, in theory, could not be achieved using the twenty antibiotics evaluated in this study. The current requirement for narrow-spectrum antimicrobial agents will likely be met by both small molecule approaches, where examples already exist (Then and Sahl, 2010), and by novel approaches such as the use of antisense agents. Our findings suggest that PNAs are good candidates for narrow-spectrum antimicrobials. For species-selectivity, 16S rRNA would appear to be a logical candidate. However, while peptide-PNAs targeted to 16S rRNA have been demonstrated to be bactericidal and sequence selective (Good and Nielsen, 1998; Hatamoto et al., 2009), prior to this study, species selectivity has not been observed and may be difficult to achieve due to sequence conservation within functional regions of 16S rRNA. Currently, while the mode of action of peptide-PNA antimicrobials is well understood (Good and Nielsen, 1998; Good et al., 2000b; Good et al., 2001a; Good, 2002), differences in uptake of the peptide-PNA (see below), species sensitivity and the affect of non-target binding remain important areas for future experimentation.

The observation that En108 has different activity in three closely related species is intriguing from the perspective of improving the antibacterial activity of peptide-PNAs. Analysis of the binding sites of En108 in the genomes of the three species provides a possible explanation: En108 will bind (allowing for 1 bp mismatch) to the TIRs of off-target essential genes in both, K. pneumoniae and S. enterica, and only the intended target in E. coli. Antisense-based antimicrobials have been shown to have greater growth inhibitory activity when the expression of multiple essential genes is simultaneously inhibited (Harth et
al., 2000). While the efficacy of gene silencing with mismatched PNAs may be reduced, it is reasonable to suggest that silencing of more than one essential gene will lead to lower MICs. En108 will bind to two off-target essential genes in K. pneumoniae, and one in S. Typhimurium, yet has a lower MIC in the later. This may be explained by mechanistic differences in the gene silencing itself (binding efficacy, Tm differences caused by mismatches, and location of the off-target relative to TIR) or differences in the stringency of requirement for the target and off-target genes, i.e. a small reduction in the mRNA pool of the two off-targets in K. pneumoniae may be better tolerated than that of the off-targets in S. Typhimurium (Goh et al., 2009). Differences in the susceptibility of the three species to En108 may also be explained by uptake efficiency: The transporter protein SbmA has recently been identified as required for peptide-PNA uptake in E. coli (A. Ghosal, J.E.M. Stach, A. Vitali and P.E. Nielsen, submitted for publication). Mutations in the sbmA gene affect uptake kinetics, and as E. coli shares 86% and 92% similarity to its orthologues in K. pneumoniae and S. Typhimurium respectively, the observed difference in sensitivity to En108 in these three species may be explained by differences in SbmA-mediated uptake.

The finding that PNA Ec1000, designed to silence the murA gene of E. coli, was antibacterial to S. Typhimurium was unexpected. The parameters described for the design of species-selective PNAs (see Materials & Methods) should theoretically prevent binding of PNAs to the TIRs of essential genes in non-target species. A study by Dryselius et al. (Dryselius et al., 2003) demonstrated that the TIR of a gene is most sensitive to antisense PNA gene silencing. However, some PNAs that bind intragenically were shown to significantly elevate gene expression. Furthermore, PNAs can repress co-transcribed genes; a PNA binding to the TIR of a nonessential gene, may affect the transcription of an essential downstream gene to a varying degree depending on transcript stability (Dryselius et al., 2006b). Thus, the unexpected antibacterial activity of Ec1000 in S. Typhimurium may be due to such events. An analysis of the binding sites (allowing for 1 bp mismatch) for Ec1000 in S. Typhimurium shows that it binds intragenically to a number of essential genes (alaS, fusA, hemE and rplF) that are reported to be toxic upon overproduction (Kitagawa et al., 2005b). Also, Ec1000 binds (1 bp mismatch) in the intergenic region of rpsN
and rplE, genes that are located in a ribosomal protein operon. Thus, it is possible that translation of the transcript rplNXE-rpsNH-rplFR-rpsE-rpmD-rplO-secY-rpmJ could be repressed for genes downstream of rpsE. The predicted ribosomal binding site for rpsN is 25 bp upstream of the start codon, with the Ec1000 binding site 12 bp upstream, explaining why it was missed in the initial screen for undesirable off-target binding sites. In S. Typhimurium, Ec1000 can form a duplex in the TIRs of six genes: pipC, a pathogenicity island-encoded protein; pepP, a metallopeptidase; mrcA, a murein transglycosylase/transpeptidase; STM1127, a putative transcriptional regulator; STM3527, a hypothetical protein and STM4051, a putative outer membrane protein. None of these genes are reported to be essential in S. Typhimurium or the other species used in this study. It is also possible that the Ec1000 PNA may bind to a non-coding RNA. Non-coding RNAs are well documented as regulatory elements in S. Typhimurium (Vogel, 2009), and while we could identify no potential binding between Ec1000 and known non-coding RNAs in S. Typhimurium (Pfeiffer et al., 2007) there may be as yet unidentified non-coding RNAs that are transcribed from, or interact with, intergenic Ec1000 binding sites; disruption of a RNA antitoxin is one possible mechanism that would lead to bactericide (Faridani et al., 2006). It is unlikely that small elevations in essential gene expression, partial silencing of non-essential genes, or disruption of non-coding RNAs could be effective alone, but in combination they may be growth inhibitory. The elucidation of the mechanism responsible for Ec1000-induced growth inhibition in S. Typhimurium is required for the continued development of species-selective antibacterial PNAs; understanding of the mechanism will enable the design parameters of peptide-PNAs to be modified to exclude likely off-target effects and/or identification of new targets for genesilencing antimicrobials. Comparative genomics between sensitive and resistant species, qRT-PCR (Goh et al., 2009) and the introduction of point mutations within putative targets could be applied to identify the cause of the off-target selectivity in S. Typhimurium. While the unexpected activity of Ec1000 prevented its use as an E. coli species-selective peptide-PNA, our in-silico analysis, and the identification of E. coli-specific TIRs (Table S6) suggest that finding targets that are amenable for discriminating E. coli from closely related species is readily achievable. There are 46 targets, in the -5 to +5 TIR region of essential genes in E. coli, that have >2 bp mismatches with orthologs in S.
Typhimurium (Table S5). The in silico approach was applied in the successful design of Se0002, and thus a peptide-PNA raised to the same target in E. coli, will likely discriminate E. coli for S. Typhimurium. Furthermore, the number of potential species-selective targets between these two strains is likely an underestimate as we confined our search to a single target region (-5 to +5 of the TIR) and peptide-PNAs with gene silencing activity have been described based on a target region at least twice this size (Dryselius et al., 2003).

As the peptide-PNA mediated growth inhibition is dependent on the silencing of an essential gene or genes, a better understanding of which genes are essential in the specific pathogen, in a relevant (i.e. in host) environment, will facilitate in the design of peptide-PNAs. For example, the silencing of genes that are essential for survival or virulence in the host (Fields et al., 1986) or antimicrobial resistance factors, as peptide-PNAs silencing of such genes can reestablish the antimicrobial sensitive phenotype in resistant strains (Good and Nielsen, 1998; Jeon and Zhang, 2009b). Discovery of synthetically lethal gene combinations in bacteria (Typas et al., 2008) will also broaden the number of targets available for the design of species-selective peptide-PNAs.

As peptide-PNAs are used within environments of increasing species diversity, it is clear that there will be a higher the likelihood of non-target species activity, as a consequence of identical target regions or off-target binding (as is likely the case for Ec1000 in S. Typhimurium). Nevertheless, relative to currently used antibiotics, PNAs provide greater opportunity for the design of narrow-spectrum antimicrobials where the primary target is dictated by the nucleic acid sequence. The availability of in-depth microbiome sequencing will enable the assessment (in terms of spectrum of activity) of PNA-based antimicrobial therapy in an animal model to be assessed; aid in the determination of conditions where such strategies may provide alternative or complementary therapy (Knight, 2010), and in the design of species-selective PNAs through exhaustive prediction of binding sites in all species of the microbiome.

While examples of bacterial resistance to peptide-PNAs have not been published, it is reasonable to assume that it may arise through point mutation of the PNA binding site. In this case, activity would be restored by simple modification of the PNA sequence, whereas point mutations that lead to
structural changes in the binding site of proteins and ribosomes require chemical modification of the antimicrobial based on structure activity relationships that are not straightforward to predict. Resistance due to prevention of uptake (as above), degradation of the peptide carrier, or efflux remain possible, and as such, characterization of peptide-PNA resistance mutants and the evaluation of alternative PNA carriers, remain priority areas for the development of antimicrobial PNAs.

Narrow-spectrum antimicrobial therapy has likely been hindered by the need to treat infections quickly without waiting for identification of the pathogen. However, with the advent of cheap sequencing and rapid diagnostics, the benefits of species-selective or narrow spectrum antimicrobials may improve patient outcome and ameliorate the development of antimicrobial resistance. Antimicrobials that act at the gene sequence level, such as PNAs, have great potential in the development of what Casadevall has termed them ‘Third-Age antimicrobials’ (Casadevall, 2006).
FIG. 1. Species-selective antibacterial peptide-PNAs in two-species mixed culture. *E. coli* (dark grey), *K. pneumoniae* (white) and *S. Typhimurium* (light grey). All cultures were incubated for 16 hrs. A) axenic cultures of the species were treated with *E. coli*- specific Ec1000 at 3.2 µM, *K. pneumoniae*-specific Kp0001 at 3.2 µM and *S. Typhimurium*-specific Se0001 at 2.0 µM. Asterisks indicate species-selective growth inhibition of *E. coli*, *K. pneumoniae* and *S. Typhimurium* respectively. B) Two-species mixed cultures treated with peptide-PNAs as above. The control cultures show the relative proportion of the two species without treatment, the two treatments to the left of the control represent the same mixed culture treated with a peptide-PNA. Black arrows indicate non species-selective growth inhibition of *S. Typhimurium* by Ec1000.
FIG. 2. Species-selective antibacterial peptide-PNAs in three-species mixed culture. *B. subtilis* (dark grey), *K. pneumoniae* (white) and *S. Typhimurium* (light grey) in mixed culture were separately treated with Ec108 at 3.5 μM, Kp0001 or Se0001 at 4.5 μM or by combined treatment of Kp0001 and Se0001 both at 4.5 μM. All cultures were incubated for 16 hrs. Selective inhibition of either *K. pneumoniae* or *S. Typhimurium* individually or together, achieved with the peptide-PNAs, could not theoretically be achieved with any combination of the twenty known antimicrobial compounds tested in this study.

FIG. 3. *S. Typhimurium*-selective growth inhibition. Peptide-PNA Se0002 was designed to target the -5 to +5 region of the translational initiation region (TIR) of *ftsZ* in *S. Typhimurium*. Se0002 has 2 base pair mismatches in the TIR of *ftsZ* in *E. coli*. (A) Growth curve analysis of Se0002 in pure culture. *E. coli* growth in the presence of 1.25 μM Se0002 (solid line) was identical to that of untreated controls (not shown). *S. Typhimurium* growth was inhibited in the presence of 1.25 μM of Se0002 (dotted line) relative to the untreated control (dashed line). Growth in the treated samples after 10 hrs was not due to resistance (see text for details). (B) Mixed cultures of GFP-labeled *S. Typhimurium* AC02 and DsRed-labeled *E. coli* AC01 were treated with 1.25 μM Se0002; and imaged by fluorescence microscopy after 6 hrs of incubation. The
filamentous growth phenotype was only observed in *S. Typhimurium* AC02 and is consistent with silencing of *ftsZ* expression.
Table 1. Bacterial strains used in this study. *American Type Culture Collection* *b* *Salmonella Genetic Stock Center*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Genotype</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em> susp subtilis 168</td>
<td>ATCC(^a) 23857</td>
<td>trpC2</td>
<td>Genome sequenced strain</td>
</tr>
<tr>
<td><em>Escherichia coli</em> DH10B</td>
<td>Invitrogen</td>
<td>F endA1, recA1, galE15, galK16, napG, rpsL, ΔlacX74, Φ80lacZΔM15, araD139, Δ(ara,leu)7697, mcrA, Δ(mrr-hsdRMS-mcrBC), λ</td>
<td>Genome sequenced strain, parent of <em>E. coli</em> AC01</td>
</tr>
<tr>
<td><em>E. coli</em> AC01</td>
<td>This study</td>
<td>As above, pDsRed-Express2</td>
<td>Expression of DsRed fluorescent protein</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> subsp. pneumoniae</td>
<td>ATCC 700721</td>
<td>n/a</td>
<td>Genome sequenced strain</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> serovar Typhimurium LT2</td>
<td>SGSC(^b) 1412</td>
<td>n/a</td>
<td>Genome sequenced strain</td>
</tr>
<tr>
<td><em>S. Typhimurium</em> LT2 substr JR501</td>
<td>SGSC 1593</td>
<td>hsdSA29, hsdSB121, hsdL6, metA22, metE55.1 trpC2, ilv-452, H1-b, H2-e,n,x (cured of Fels 2), fla-66, nml, rpsL120, xyl-404, galE719</td>
<td>Restriction-deficient, modification-proficient cloning strain of <em>S. Typhimurium</em> LT2, parent of <em>S. Typhimurium</em> AC02</td>
</tr>
<tr>
<td><em>S. Typhimurium</em> AC02</td>
<td>This study</td>
<td>As above, pGFPuv</td>
<td>Expression of green fluorescent protein</td>
</tr>
</tbody>
</table>
Table 2. Properties of peptide-PNAs used in this study.

*a* Code refers to predicted species-specificity Bs = *B. subtilis*, Ec = *E. coli*, En = Enterobacteriaceae, Kp = *K. pneumonia*, Se = *S. enterica* Typhimurium

*b* Indicates if specificity based on bioinformatic prediction was observed.

*c* Target is shown as positions of nucleotides relative to the start codon.

*d* Thermal stability of PNA/DNA duplex.

*e* Ec1000 lacked predicted specificity, see text for details.

<table>
<thead>
<tr>
<th>Name</th>
<th>Target</th>
<th>Sequence</th>
<th>Minimum Inhibitory Concentration (µM)</th>
<th>Expected specificity</th>
<th>Target site</th>
<th>$T_m$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>B. subtilis</em></td>
<td><em>E. coli</em></td>
<td><em>K. pneumonia</em></td>
<td><em>S. Typhimurium</em></td>
<td></td>
</tr>
<tr>
<td>Bs000 1</td>
<td>ftsZ</td>
<td>(KFF) caacatgcta</td>
<td>4.0</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>yes</td>
</tr>
<tr>
<td>En108</td>
<td>acpP</td>
<td>(KFF) ctcatactct</td>
<td>&gt;10</td>
<td>1.2</td>
<td>0.4</td>
<td>0.3</td>
<td>yes</td>
</tr>
<tr>
<td>Ec100 0</td>
<td>murA</td>
<td>(KFF) ccattagtt</td>
<td>&gt;10</td>
<td>2.4</td>
<td>&gt;10</td>
<td>3.2</td>
<td>noe</td>
</tr>
<tr>
<td>Kp000 1</td>
<td>murA</td>
<td>(KFF) tccattgatt</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>2.5</td>
<td>&gt;10</td>
<td>yes</td>
</tr>
<tr>
<td>Se000 1</td>
<td>murA</td>
<td>(KFF) tccattattg</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>1.2</td>
<td>yes</td>
</tr>
<tr>
<td>Se000 2</td>
<td>ftsZ</td>
<td>(KFF) aacataactct</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>2.5</td>
<td>yes</td>
</tr>
</tbody>
</table>
Table 3. PNA binding site analysis in target species.

<table>
<thead>
<tr>
<th>Species</th>
<th>PNA</th>
<th>No. off targets*</th>
<th>Off Targets within TIR(^b) of gene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. essential (gene)(^c)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>En108</td>
<td>128</td>
<td>0</td>
</tr>
<tr>
<td><strong>K. pneumoniae</strong></td>
<td>En108</td>
<td>143</td>
<td>2 (mukF(^{a,e}), ribH(^{a}))</td>
</tr>
<tr>
<td><strong>S. Typhimurium</strong></td>
<td>En108</td>
<td>138</td>
<td>1 (yhhM(^a))</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>Ec1000</td>
<td>201</td>
<td>2 (Inf(^{a,e}), rpsN(^{a}))</td>
</tr>
<tr>
<td><strong>S. Typhimurium</strong></td>
<td>Se0001</td>
<td>320</td>
<td>5 (aspC(^a), hemK(^{a,e}),</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Inf(^{a}), nuoL(^a), purD(^a),</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>rluA(^a))</td>
</tr>
<tr>
<td><strong>S. Typhimurium</strong></td>
<td>Se0002</td>
<td>311</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>K. pneumoniae</strong></td>
<td>Kp0001</td>
<td>157</td>
<td>3 ispA(^{a,e}), rpsN(^{a}),</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>topB(^a)</td>
</tr>
<tr>
<td><strong>B. subtilis</strong></td>
<td>Bs0001</td>
<td>104</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) includes sites with ≤ 1 base-pair mismatch with PNA  
\(^b\) Translation Initiation Region  
\(^c\) identified by BLAST searching of the Database of Essential Genes  
\(^d\) essential in E. coli  
\(^e\) essential in S. Typhimurium  
\(^f\) essential in other prokaryotes  
ND Not determine