Entry of antisense oligonucleotides into

*Streptococcus mutans*

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Abstract

Infectious disease accounts for the highest percentage of preventable deaths worldwide and today’s health care systems are predominately reliant upon antibiotics to treat bacterial infections bacterial strains. Serious infection caused by antibiotic resistant bacteria has become a major global healthcare issue and there is a rapidly growing need for the development of new antimicrobials. Antisense oligonucleotides (AS-ODN) target genes in a sequence specific manner and inhibit gene function. However, barriers such as peptidoglycan, cell surface proteins such as teichoic acids and lipopolysaccharide membranes are thought to currently prevent the use of AS-ODN from becoming an effective treatment option for microbial disease as effective antisense inhibition in bacteria requires the delivery of the antisense agent across these bacterial cell barriers. Other reasons such as antisense size, charge and hydrophobicity and the Gram classification of the bacterial strain are also implicated in contributing to uptake difficulty.

*Streptococcus mutans* is frequently implicated as the primary etiological agent in the development of dental caries - Severe dental disease can lead to a number of serious health problems, including cardiac disease and septicaemia. In a previous study done in 2008 the ability of the combined use of zoocin A, a bacteriolytic enzyme, and two targeted PS-ODN sequences (targeted towards *fab-M* and *fba*) to produce a synergistic inhibitory effect upon closely related streptococcal species was examined and showed that a combination of zoocin A and PS-ODN could be used to achieve a dose-dependent inhibitory response upon bacteria that were A) susceptible to zoocin A, and B) contained the PS-ODN target site, and it was concluded that the zoocin A was indeed causing damage to the susceptible bacterial cell walls and thus allowing the PS-ODN entrance to the bacterial cell interior. However the large size of zoocin A precludes its possible use in clinical settings.

The current study examines a further variety of lytic antimicrobial agents for their ability to deliver PS-ODN into *S. mutans* OMZ175 and produce a synergistic inhibitory effect upon growth, viability and target mRNA production. The overall hypothesis of this work was that the combined use of antisense and clinically relevant lytic agents would cause a target specific decrease in bacterial growth. This hypothesis was examined by three different experimental approaches that aimed to examine A) The down regulation of
target mRNA, B) The measurement of intracellular PS-ODN and C) The effects of different lytic agents and PS-ODN’s on growth rates.

A RNA extraction and RT-qPCR method was developed to analyse gene expression levels in *S. mutans* OMZ175, including that of the *fba* target. Whilst a protoplasting protocol was unable to be successfully developed (in order to analyse membrane permeability), radiolabelled $\gamma^{32}$P-AP-ODN was used to determine which antimicrobial delivery mechanism allowed the greatest amount of PS-ODN delivery into *S. mutans* OMZ175 in different growth stages. Results indicated that of all delivery mechanisms tested only zoocin A and penicillin showed positive synergism together with targeted PS-ODN. A significant decrease in *fba* expression was shown for the first four hours post zoocin A + targeted PS-ODN treatment for both lag and exponential phase *S. mutans* OMZ175, which corresponded with the observation that $\gamma^{32}$P ATS2 molecules gained entry into lag and exponential phase *S. mutans* cells over the first 4 hours of combined $\gamma^{32}$P PS-ODN + zoocin A treatment. Penicillin was shown to be able to facilitate the entry and synergistic inhibition of *fba* in lag phase *S. mutans* OMZ175 only, resulting in prolonged suppression upon *fba* expression, corresponding with a greater amount of $\gamma^{32}$P-PS-ODN becoming cell associated compared with that observed for those cells treated with zoocin A. A comparison between zoocin A and penicillin highlights the fact that the different modes of action of each agent result in different amounts of PS-ODN cell association over time. This study suggests that the choice of delivery agent may influence the time point at which PS-ODN intracellular concentration will reach a critical threshold and allow gene suppression to occur and that different modes of action of delivery agents affect the rate at which the PS-ODN is delivered into the bacterial cell. This study also suggests that for *S. mutans* OMZ175, the peptidoglycan layer acts an effective barrier preventing PS-ODN penetration, but that this may vary between bacterial strains due to the variety of bacterial cell components capable of influencing AS-ODN, PNA or PMO penetration.
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List of Abbreviations

\( \gamma^{32} \) P-ATS2  ATS2 antisense sequence with a single \( \gamma^{32} \) P addition

2 x THB  double strength THB

\( 16s \) rRNA  16S RNA gene

\( \lambda \)  lag phase

\( \mu_{\text{max}} \)  maximal growth rate

ANOVA  analysis of variance

APS  ammonium persulphate

AS-ODN  antisense

ATS2  no target control PS-ODN sequence for \( S. \) mutans OMZ175

AU  arbitrary units

BA  blood agar

BDH  British Drug Houses Chemicals Ltd

BHI  brain heart infusion broth

BLIS  bacteriocin-like inhibitory substance

BSA  bovine serum albumin

CAB  columbia blood agar base

CAB + rif  CAB agar with 100 \( \mu \)g/ml rifampicin

CAB + strep  CAB agar with 100 \( \mu \)g/ml streptomycin

DDT  dithiothreitol

dH\textsubscript{2}O  distilled water

DMCC  Department of Microbiology and Immunology culture

collection, University of Otago

EDTA  ethylenediaminetetraacetic acid

\( fba \)  fructose bis-phosphate adolase gene

GRAS  generally regarded as safe

GTF(s)  glucosyltransferase(s)

gyrA  gyrase A gene

His-tag  polyhistidine-tag

HPLC  high-performance liquid chromatography

Hr  hour

IPTG  isophenyl-thio-\( \beta \)-D-galactopyranoside
LB  luria broth
LBA  luria broth agar
LBA + amp  LBA with 100 μg/ml ampicillin
LBA + amp + kan  LBA with 100 μg/ml ampicillin and 25 μg/ml kanamycin
LBA + amp + kan + cm  LBA with 100 μg/ml ampicillin, 25 μg/ml kanamycin and 34 μg/ml chloramphenicol
LBA + cm  LBA with 34 μg/ml chloramphenicol
LBA + kan  LBA with 25 μg/ml kanamycin
M17  M17 broth
Mins  minutes
MHB  Mueller Hinton broth
MIC  minimum inhibitory concentration
MQ water  milli-Q deionised water
NBS  New Brunswick Scientific
NC  no change
OD  optical density at 595 nm
O/N  overnight
PAA  Polyacrylamide-Bis solution
PBS  phosphate buffered saline
PEG  polyethylene glycol
PS-ODN  phosphorothioate oligonucleotide
RM  reversion media
RMS  reversion media stabilised with sucrose
RMR  reversion media stabilised with raffinose
RO water  reverse osmosis water
RT  room temperature
SDS  sodium dodecyl sulphate
SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLS  sodium lauryl sulphate
SM-FBA  PS-ODN sequence targeting S. mutans OMZ175 fba mRNA
TAE buffer  tris-acetate ethylenediaminetetraacetic acid buffer
TEMED  N,N,N’,N’-tetramethylethylenediamine
THA  Todd Hewitt agar
THB  Todd Hewitt broth
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>$T_m$</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TSB</td>
<td>tryptic soy broth</td>
</tr>
<tr>
<td>TV</td>
<td>tryptone- vitamin base</td>
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1. Introduction

1.1 Introduction

Antibiotics were introduced in the global healthcare system a mere seventy years ago and revolutionised the treatment of infectious disease\textsuperscript{350}. Currently infectious disease still accounts for the highest percentage of preventable deaths worldwide and even today health care systems are still predominately reliant upon antibiotics to treat bacterial infections bacterial strains\textsuperscript{239}. As the world faces a future without effective antibiotics to treat these infections due to increasing levels of antibiotic resistance,\textsuperscript{316,317} there is a rapidly growing need for the development of new antimicrobials. A naturally occurring gene regulation mechanism that is thought to exist in all eukaryotic and prokaryotic cells is known as gene silencing, or antisense gene regulation\textsuperscript{282}. Recently researchers have begun to investigate the potential of this gene silencing technique as a possible means of controlling both gene expression and cell viability in various target strains, including pathogenic bacterial strains\textsuperscript{63,93,211,282,328}.

1.2 Antibiotic resistance

Antibiotics are chemotherapeutic agents which kill or inhibit the growth of a microorganism such a bacteria, fungi and protozoa. Antibiotics have many modes of action\textsuperscript{8}, including inhibiting cell wall synthesis\textsuperscript{204}, activating enzymes that destroy the cell wall\textsuperscript{333}, increasing cell membrane permeability and interfering with protein synthesis and nucleic acid metabolism\textsuperscript{66,259}. The discovery of the first antibiotics suitable for systemic use (penicillin and sulphonamides) was heralded as truly miraculous. In the 1940’s, the widespread availability of penicillin and streptomycin led to a dramatic decrease in illness and death from infectious bacterial disease\textsuperscript{8} and in 1967 so high was the confidence in antibiotics that the US surgeon general suggested that through the use of antibiotics it might be possible that infectious disease would become a thing of the past\textsuperscript{297,316}.
Bacteria have developed resistance through the transfer of resistance genes, and mutation\textsuperscript{230,256,350}. DNA encoding resistance mechanisms can be transferred between bacteria through transformation (uptake of naked DNA from another organism), transduction (infection by bacteriophage) and conjugation (transfer of resistance genes on plasmids or transposons)\textsuperscript{245}. The DNA can encode for resistance mechanisms such as beta-lactamases, or efflux pumps which can selectively extrude specific antibiotics\textsuperscript{363}, whilst other multidrug resistant (MDR) pumps expel a number of differing antibiotics\textsuperscript{203}. The current list of resistant bacteria is impressive, from penicillin-resistant \textit{S. aureus} emerging in the 1950’s to methicillin-resistant (MRSA) \textit{S. aureus}\textsuperscript{261} and multi-drug resistant (MDR) \textit{Mycobacterium tuberculosis} in the late 1970’s\textsuperscript{8}. Resistant strains of \textit{Pseudomonas aeruginosa} arose in the 1990’s, together with vancomycin resistant MRSA (VRSA)\textsuperscript{148-151}.

\subsection*{1.2.1 Cost of antibiotic resistance}

Serious infection caused by antibiotic resistant bacteria has become a major global healthcare issue\textsuperscript{8,201}. More than one third of the world’s population is likely currently infected by bacterial pathogens\textsuperscript{235}. Cosgrove and Carmeli (2003) estimated that antibiotic resistant infections are associated with a 1.3 to 2-fold increase in mortality compared to susceptible infections\textsuperscript{74}. In particular, pneumonia and diarrhoeal disease kill approximately 3.8 million children under 5 each year\textsuperscript{254}. Antibiotic resistant pathogens are more expensive to treat, often require longer and more complex treatments, and are associated with greater morbidity\textsuperscript{8,73,74,212,217}. It has been estimated that the in-hospital costs of hospital acquired infections caused by just six common kinds of resistant bacteria were $1.3 billion in 1992\textsuperscript{72}. However, resistance related costs affect not only the health sector. Household income, government tax revenues and total national savings in the U.K. are estimated to fall by at least 0.3%, 0.35% and 2% respectively, due to MRSA alone\textsuperscript{314}. Antibiotic resistance is also jeopardising the achievements of modern medicine. Without effective antimicrobials, the success of treatments such as organ transplants, chemotherapy and surgery are compromised. If we do not act to address this problem of antimicrobial resistance, we may lose quick and reliable treatment of infections that have been manageable since the 1940’s. Drugs of choice will become limited, expensive and
may even become non-existent as there is an expectation that bacteria will develop resistance to all or almost all antibiotics\textsuperscript{239}.

Effective infection control efforts range from simple procedures such as diligence in hand-washing to new materials for use in medical devices that impede the growth of bacteria. But the spread of antibiotic resistance amongst all bacterial strains is predicted to increase rapidly, and there is now an intense focus upon the need for new antimicrobials as the key for the treatment of bacterial infections.

1.3 Discovering new antimicrobials

The development of antibiotic resistance has unfortunately been coupled with insufficient investment in new antibiotic treatments\textsuperscript{259}. Due to decreasing revenue from antibiotics earnings, by 1991, approximately 50\% of large pharmaceutical companies had stopped or reduced funding for infectious disease research\textsuperscript{239,273,317} and in 2004, over 70\% of pathogenic bacteria were estimated to be resistant to at least one of the currently available antibiotics\textsuperscript{88}.

Most of the broad spectrum antimicrobials in development are derivatives of antibiotic classes already in use and therefore they share the same mode of action. Unfortunately, this means that resistance to one can result in resistance to many antibiotics\textsuperscript{275}. Whilst antibiotic development is often based on incremental change that produces new iterations of old chemical structures, it is unlikely that this approach will yield innovative and effective new antimicrobials. A transformational change is urgently required and there is increasing need for antimicrobials with a novel method of action\textsuperscript{259}. In April 2000, linezolid (Zyvox\textregistered, Pharmacia) became the first antimicrobial in 35 years which showed a novel mechanism of action to obtain FDA approval\textsuperscript{305}. An oxazolidinone, linezolid inhibits bacterial protein synthesis by interfering with ribosomal initiation of translation, and is used to treat vancomycin resistant enterococci (VRE) infections. Other novel technologies aimed at preventing or curing infection may be useful to reduce antibiotic dependency. Vaccines have proven invaluable in controlling and eliminating a number of serious bacterial infections. The vaccine for \textit{Haemophilus influenza} type B has drastically reduced the incidence of meningitis through the 1990’s following its introduction\textsuperscript{106}.
Vaccines against other Gram positive and Gram negative bacteria are in the early stages of development\textsuperscript{106}.

The identification of new bacterial targets through the use of genomics is one method available for use in the hunt for new antimicrobials\textsuperscript{156,259,358}. A good understanding of the molecular basis of antibiotic resistance is also important as it allows new strategies to be developed to manage infections and the developments of new treatments. This has led to a new focus on bacterial metabolism and pathogenesis as keys in the search for new, successful antimicrobial treatments\textsuperscript{256}. This method allows the researcher to identify genes of major importance to bacterial viability or pathogenicity and to research genes interactions with one another within the bacterial cell. Theoretically, using this knowledge, an antimicrobial can then be synthesised to interfere with the chosen target\textsuperscript{156}.

1.4 Antisense – a gene regulation mechanism

Antisense gene regulation can be defined as the regulation of the expression of a DNA or RNA target through direct base pairing with complementary DNA or RNA oligonucleotides\textsuperscript{247}. Despite the fact that this mechanism has come to light relatively recently, its importance and abundance is now widely recognised, with thousands of potential antisense sequences being identified in many different species and across all three biological kingdoms\textsuperscript{126}. One example of a naturally occurring antisense mechanism is the \textit{hok/sok} mechanism of the \textit{E. coli} R1 plasmid\textsuperscript{104}. Recently many groups have considered the use of antisense as therapeutic agents for the treatment of human and bacterial disease.

1.4.1 Current antisense usage

Formivirsen (Vitravene, Isis Pharmaceuticals, California, U.S.A) was the first AS-ODN approved for human use by the US Food and Drug Administration (FDA). It is available for the treatment of peripheral cytomegalovirus retinitis\textsuperscript{77,138,211}. Numerous clinical trials are being conducted for the treatment of diseases such as cancer, HIV/AIDS and other viral infections and various autoimmune disorders, with many showing promising
therapeutic potential\textsuperscript{34,120,286,345}. In New Zealand, CoDa therapeutics is currently investigating the use of connexin specific antisense which are proving beneficial in decreasing inflammation and scar formation caused by corneal wounds\textsuperscript{139,291}. Intellectual property has been assigned to CoDa Therapeutics, Inc. USA which has completed a number of clinical trials\textsuperscript{255}. One of the most promising examples is Custirsen (the antisense OGX-011 - OncoGeneX Technologies Inc. Seattle, USA). Custirsen is a second-generation antisense drug currently in phase three trials for treatment of prostate, lung and breast cancers. It is designed to block the production of clusterin, a cell-survival protein that is over-produced in cancer cells and is linked to faster rates of cancer progression\textsuperscript{191,293}. Although these trials have shown the potential for use of antisense as therapeutic agents, application of this technology to the control of microbial pathogenesis has been largely overlooked.

\subsection*{1.4.2 Antisense inhibitory mechanisms}

Antisense sequences are short oligonucleotide sequences designed to target and bind to a corresponding complementary DNA or RNA sequence. There are various ways that antisense oligonucleotides of various chemistries can inhibit gene expression- depending upon the way they have been designed. The simplest approach is antisense binding through Watson-Crick base pairing to mRNA to block translational initiation or elongation, resulting in a decrease in the levels of the corresponding protein\textsuperscript{163}. It is thought the presence of an AS-ODN molecule blocks the ability of the ribosome to bind to the mRNA\textsuperscript{94,127,128}. Steric blocking requires a strongly binding oligonucleotide analogue to ensure competition for the mRNA, and to block other proteins or processes involved in translation. A further mechanism involves antisense binding to RNA followed by the recruitment and activation of RNase-H, which degrades the mRNA target, allowing the freed AS-ODN molecule to bind to a further target\textsuperscript{76}. RNase H is a naturally occurring endoribonuclease that specifically hydrolysates the 3’-O-P phosphodiester bond of an RNA/DNA duplex, but is unable to digest single or double stranded DNA\textsuperscript{77}. It normally cleaves the RNA strand of DNA-RNA hybrids that form during lagging strand synthesis\textsuperscript{76}. This naturally occurring degradation mechanism is often exploited by researchers when targeting specific gene sequences in an organism as AS-ODN that bind to target RNA are able to activate RNase H- causing the target sequence to be
degraded\textsuperscript{84,132,242,282,355}. The mechanism is thought to be enzymatic, and that once RNA cleavage occurs the AS-ODN dissociates from the duplex and is free to bind to another target sequence\textsuperscript{171}. In principle, catalytic turnover allows a lower concentration of oligonucleotides to be used compared to steric blocking AS-ODN’s\textsuperscript{132}.

Antisense can be designed to interfere with transcription of DNA by invading the DNA-DNA bond in the DNA double helix- forming a DNA triplex and thus inhibiting transcription, leading to a decrease in mRNA production\textsuperscript{75,132,184}. Some AS-ODN are able to hybridise to DNA that is exposed during the formation of a locally opened loop which is created by RNA polymerase transcribing the gene sequence, thereby preventing the polymerase from transcribing anymore of the gene\textsuperscript{77,132,368}. AS-ODN can also hybridise to intron/exon junctions- preventing the message from being spliced and processed correctly\textsuperscript{75,132,184}. The majority of studies, in both eukaryotic and prokaryotic systems, have however been aimed at reducing gene expression levels by targeting translational processes such as sterically hindering the ribosome from binding to the RNA\textsuperscript{18,94,128,130,249}, inducing RNA cleavage with Ribonuclease H (RNase H)\textsuperscript{140,226,228,355} and inhibiting ribosomal assembly\textsuperscript{12,75,93}.

\subsection*{1.4.3 Chemistry}

When designing an antisense sequence to target translational processes, the chemistry of the AS-ODN is important, as different chemical structures and bonds can produce AS-ODN with different properties\textsuperscript{93}. Unmodified DNA or RNA phosphodiester oligonucleotides show low bioavailability and can be prone to nuclease attack\textsuperscript{75}. Numerous chemical modifications have been developed in order to increase the stability of the molecule and affinity of the oligonucleotides towards the target sequence. These modifications can also reduce the oligonucleotide’s own toxicity to human and bacterial cells and enables the oligonucleotide to upregulate the cells own natural mechanisms (such as the RNase H enzyme) which can render the antisense effect more potent\textsuperscript{77,163,184}. Each analogue type has particular properties that make them suitable for different applications\textsuperscript{163}.

The first generation antisense agents contain backbone chemical modifications such as Phosphorothioate Oligonucleotides (or PS-ODN), in which one non-bridging oxygen
group of the phosphate linkage is replaced with sulphur\textsuperscript{35,132,163}. The sulphur group renders the conformation of the oligonucleotide far more resistant to nuclease attack. Phosphorothioate linkages are included in all U.S. food and drug administration (FDA) approved oligonucleotides to date\textsuperscript{120,138,265,286,334,345}. The second generation of antisense agents were designed to improve upon these properties, and involve such modifications as substituting the 2’ position ribose with an alkoxy group (2’-O-methyl phosphorothioate)\textsuperscript{75,184}. This improves nuclease resistance but does not permit RNase-H activation\textsuperscript{163}. Third generation antisense agents contain various structural modifications, with locked nucleic Acids (LNA)\textsuperscript{50}, phosphoroamidate morpholino oligonucleotides (PMO)\textsuperscript{69,119,331}, and peptide nucleic acids (PNA)\textsuperscript{18,20,183,185,246} being the most commonly studied forms. PNA are uncharged, with the sugar-phosphate backbone replaced by repeating $N$-(2-aminoethyl)-glycine units linked together by amide bonds. This pseudo-peptide backbone provides great biological stability and protection from nucleases, as well as excellent hybridisation of the PNA to DNA, RNA or other PNA which is partly due to the lack of charge repulsion of its peptide backbone\textsuperscript{247,248}. PNA’s display high selectivity and mismatch discrimination towards their target strand. PNA are the most widely studied antisense type for use against bacterial strains\textsuperscript{94,103,128,183,246,326}. Due their different chemistry, PNA’s and PMO’s are referred to separately from AS-ODN.

1.4.4 PS-ODN

PS-ODN have been widely used in human and bacterial studies due to their good solubility, stability and relatively low price\textsuperscript{184,242,282}. PS-ODN are able to bind to target DNA or RNA using the Watson- Crick base pairing as previously described\textsuperscript{35,75,83,184,282}. They are most commonly used to target RNA due to their ability to activate the cells RNase H enzyme\textsuperscript{35,75,76,282}. When used for their RNase H activating mechanism they are able to target virtually any region of RNA, whereas if they are involved in the steric hindrance of the ribosome they need to target the initial 5’ sequence of the target RNA\textsuperscript{282}. This RNase H activation mechanism is unique to PS-ODN, Phosphodiesters and chimeric PS-ODN/ phosphodiester oligonucleotides\textsuperscript{35,75,76,355}. This catalytic destruction of the RNA through recruitment of a cellular enzyme amplifies the efficacy of the antisense oligonucleotide, and is the main method by which PS-ODNs exert an inhibitory effect upon gene expression\textsuperscript{184,242}. 


1.5 Antisense for gene function studies

Gene regulation studies are essential to basic laboratory science, as they permit researchers to analyse the function of genes and their various gene products. In the post-genomic era, the rapidity with which AS-ODN, PNA or PMO sequences can be designed could dramatically decrease the amount of time needed to determine the essentiality and function of a gene of interest\(^\text{132}\). PNAs conjugated to a Lys-rich peptide have been used successfully to validate essential gene targets in \textit{E. coli} by studying the relationship between the decrease in mRNA expression and decline in growth rate\(^\text{124}\). Choi et al. (2012) used a PNA based probe real-time PCR assay (PNAqPCR) to detect both \textit{M. tuberculosis} and non-tuberculosis causing mycobacteria within the same clinical specimens\(^\text{65}\). Antisense designed to target various regions can also be added exogenously to different bacterial strains, together with an antisense delivery agent suitable for the bacterial strain under investigation, and the effects monitored\(^\text{93,119,128,242,328}\). Artificial antisense RNA can also be designed and cloned into inducible expression vectors. Kaur et al. developed a mycobacterium specific isopropyl-β-D-1-thiogalactopyranoside (IPTG) inducible vector system and monitored the effect of antisense inhibition of several known essential genes in mycobacteria to determine which produced a bacteriostatic or bactericidal effect\(^\text{174}\). Baev et al. developed a system whereby a \textit{S. mutans} strain was capable of chromosomal antisense RNA expression targeted towards the Streptococcus GTP- binding protein (SGP) in order to investigate the physiological role of this protein, and to analyse how it functions under a variety of conditions\(^\text{16}\).

1.6 Bacterial cell wall barriers

Many antimicrobials target proteins or processes within the bacterial cell cytoplasm, and the mechanism by which these antimicrobials pass through the peptidoglycan layer and gain access to these sites is not yet understood, or even well examined\(^\text{292}\). The spatial organisation of peptidoglycan itself is still an unresolved issue. It is still not fully understood how some antimicrobials such as vancomycin or penicillin gain entrance through this layer, whilst other antimicrobials must be coupled to cell-penetrating peptides or encased in liposomes in order to cross the bacterial cell wall\(^\text{91}\). There is a large variability in the structure of bacterial cell walls due to differences in amino acid
sequences, pore size, different types of peptidoglycan cross-links and the absence or presence of secondary modifications in both the glycan strands and peptides (Figure 1.1). High resolution techniques such as atomic force microscopy have revealed high complexity but the true architecture of bacterial cell wall and peptidoglycan layers cannot be thoroughly determined by currently available methods.

Peptidoglycan (or murein) is a continuous covalent macromolecular structure located on the outside of the cytoplasmic membrane of almost all eubacteria. It functions as a protective exoskeleton, providing rigidity and a defined cell shape whilst enabling the bacteria to withstand fluctuations in internal pressure and osmotic changes and acting as a molecular sieve. The main structural feature of bacterial peptidoglycan are linear glycan chains interlinked by short cross-peptides. The glycan chains are composed of alternating units of N-acetylglucosamine and N-acetylmuramic acid. A tetrapeptide, often L-alanine- D-glutamine- L-lysine- D-alanine, is often attached to the carboxyl group of the N-acetylmuramic acid. These building blocks of the bacterial cell wall are synthesised in the bacterial cell interior. In both gram-positive and gram-negative bacteria, the scaffold of the cell wall consists of the cross-linked polymer peptidoglycan (Figure 1.1). Although the basic structure of the peptidoglycan is very similar in Gram positive and Gram negative bacteria, the thickness of the peptidoglycan layer differs. There are only 1 – 3 layers of peptidoglycan in gram-negative cells, compared to at least 10-30 layers in a gram-positive cell. In gram-negative bacteria, the peptidoglycan is covalently attached to the outer membrane via lipoprotein. Gram positive bacteria have covalently linked charged polymers (such as teichoic acid) which are anchored to the cell wall. One of the key markers for the differentiation of bacteria is the Gram-reaction. The gram-positive bacteria are distinguished from the gram-negative bacteria due to the gram-positive bacteria’s ability to hold the dye-iodine complex within their thick layers of peptidoglycan, whereas the gram-negative bacteria are decolourised by the addition of alcohol due to the fact that their layer of peptidoglycan is too thin to withstand decolourisation and thus allows the dye-iodine complex to be removed from their peptidoglycan layer.

Bacterial peptidoglycan is known to contain pores, but the size of these pores and their ability to permit the entrance of different sized proteins into the bacterial cell interior is not well studied. In Bacillus subtilis a pore size of 5-25 nm was determined using atomic force microscopy. A study by Demchick et al. (1996) found that for both E. coli
and *B. subtilis* there were few imperfections in the cell wall, and gave a mean estimate of the pore radius size in *E. coli* as 2.06 nm and as 2.12 nm in *B. subtilis*\(^{89,236,269,346,361}\). The authors calculated that globular, uncharged, hydrophilic proteins between 22-24 KDa should be able to penetrate and diffuse through the peptidoglycan, but that larger globular proteins (50 -100 KDa), whilst able to pass through the bacterial pores, would be unable to do so by simple diffusion\(^{89}\). Penicillin, a small beta-lactam antibiotic is readily able to penetrate the peptidoglycan layer, most likely due to its small size (334 Da) and thus is predicted to easily diffuse through bacterial pores\(^{31,356}\). Other small biocides such as phenols, alcohols, aldehydes and quaternary ammonium compounds are also believed to penetrate the cell wall with ease\(^{189}\). Although the peptidoglycan from both Gram positive and Gram negative bacteria have relatively wide pores enabling diffusion of large, neutral molecules such as proteins\(^{89,300}\), the negative charge caused by the presence of teichoic acids may influence diffusion rates\(^{189,347}\). It is also possible that the polarity of antimicrobial molecules, the presence of side chains on the molecules, the osmotic pressure the bacterial cell is subjected to and the conformation of the peptidoglycan itself also influence antimicrobial uptake into the bacterial cell. Several studies have been conducted investigating the architecture of Gram negative bacteria\(^{89,91,179,186,269,361}\), few have examined Gram positives\(^{89,98}\). It is known that the size of the pores present is influenced by the degree of cross-linking, which changes throughout the life span of a bacterial cell\(^{153}\). Studies on the uptake kinetics of fluorescent nucleic acid binding dyes by different Gram positive bacteria suggest that *B. cereus* has a more permeable cell wall than *E. faecalis* or *S. aureus*, which is possibly linked to the different percentages of glycan chains and cross-links present within these cells peptidoglycan layers\(^{105,348}\).

### 1.6.1 Teichoic acids

De Jonge et al. (1996) found that there was no difference in peptidoglycan structure between vancomycin resistant and sensitive *Enterococcus faecium* strains\(^{82}\) and it is possible that the peptidoglycan structure itself is not the primary agent involved in allowing antimicrobial entrance to the bacterial cell. Teichoic acids are anionic bacterial polysaccharides consisting of glycerol phosphate or ribitol phosphate linked via phosphodiester bonds and are found within the peptidoglycan layers of gram-positive bacteria (teichoic acids) or to the cell membrane (lipoteichoic acids)\(^{180}\). They are linked to
Figure 1.1 Differences in cell wall composition between Gram positive and Gram negative bacteria
peptidoglycan through disaccharide bonds and glycerol-phosphate linkage units\textsuperscript{180} and provide a high density negative charge with the capacity to bind cationic molecules\textsuperscript{347}.

Vancomycin has a molecular weight of 1449.3 g/mol. Its site of action is the terminal D-alanyl-D-alanine moieties of the NAM subunits which are found on the inside of the peptidoglycan layer near the bacterial membrane\textsuperscript{313}. Due to its large size and positive charge, it is unlikely that vancomycin is unable to passively diffuse through the bacterial cell pore, and studies indicate that it may in fact initially bind to the negatively charged teichoic acids. Best and Durham proposed that vancomycin was ‘absorbed’ on to the acidic groups on the cell wall through ionic bonding, and observed that Mg\textsuperscript{2+} and other cations compete with vancomycin for binding sites on the cell wall, and alleviate growth inhibition by vancomycin\textsuperscript{37,38,313}. They proposed that teichoic acids may provide the binding sites. A study by Sieradzki and Tomasz found that the removal of teichoic acids from the \textit{S. aureus} cell wall reduces the binding capacity of vancomycin\textsuperscript{266,307}. The peptidoglycan layer of Gram-negative cell walls are covered with lipoprotein and lipopolysaccharide\textsuperscript{37}. If these layers are removed, the amount of vancomycin binding to the exposed peptidoglycan rapidly increases. The relative insensitivity of Gram negative bacteria to vancomycin has been proposed to be due to the physical shielding of the peptidoglycan by the lipoprotein/ lipopolysaccharide layer, which precludes the attachment of vancomycin in varying degrees\textsuperscript{37}. Several reports of oligonucleotide-mediated gene suppression have been executed in a permabilised \textit{E. coli} mutated to have a cell wall which contains negliable amounts of LPS\textsuperscript{164,277}. These reports also demonstrated a lack of oligonucleotide-mediated gene suppression in \textit{E. coli} strains which possessed full LPS layers. Only when coupled with a cell penetrating agent was the oligonucleotide able to have an inhibitory effect upon the whole \textit{E. coli} strains\textsuperscript{277}. Multiple areas within the bacterial cell wall are therefore postulated to affect bacterial susceptibility to antimicrobials, such as the rate of peptidoglycan precursor synthesis, cell wall thickness, peptidoglycan cross-linking, the bacterial membrane and the amount of muropeptides with deamidated glutamine residues.

\section*{1.7 Antisense delivery mechanisms}
These bacterial barriers (peptidoglycan, bacterial membrane, cell surface proteins such as teichoic acids) are thought to currently prevent the use of AS-ODN, PNA or PMO sequences from becoming an effective treatment option for microbial disease as effective antisense inhibition in bacteria requires the delivery of the antisense agent across these bacterial cell barriers, which can present a problem for relatively large molecules such as AS-ODN, PNA or PMO sequences. Antisense oligomers require assistance to gain entry into bacterial cells because of their relatively high molecular weights and polar characteristics. Recently, antisense oligomers have been conjugated to membrane-penetrating peptides, which are composed of repeating patterns of cationic and nonpolar residues. Peptide-oligomer conjugates are significantly more effective in inhibiting expression of their specific targets in gram negative bacteria than their non-conjugated counterparts. Apparently the membrane-penetrating peptide carries its cargo (the antisense oligomer) across the gram-negative outer membrane after which it traverses the plasma membrane by an unknown mechanism. Good et al. (2001) showed that coupling a delivery peptide (KFFKFFKFK) to PNA greatly increased cell penetration of the PNA into E. coli. An unexpected result from their study was the observation that the conjugate was more membrane active than the free peptide. Although conjugated the two units (the peptide and the PNA) are still able to act with some independence in terms of action. It has been suggested that the conjugate shows greater membrane activity that the free peptide due the possibility that the uncharged PNA residues enhance the amphipathic features of the peptide that help perturb the membrane.

Use of the cell wall permeability agent ethambutol was also found to be necessary in order to achieve an inhibitory effect with PS-ODN when targeted against M. smegmatis. Methods such as electroporation and mutant strains with increased permeability membranes have also been used to study the effects of PS-ODN upon gene regulation and expression, but these systems are not suited to an in vivo situation. However, the combined use of antibiotics to increase cellular permeability and PS-ODN seems to be becoming increasingly popular, with various combinations under study, such as the use of sub-inhibitory concentrations of lysis-inducing antibiotics to ‘soften’ the cell wall as discussed above. These strategies have however only shown slight increases in antisense efficacy, so an optimal delivery system has still not yet been developed. Encapsulation of AS-ODN, PNA or PMO sequences inside liposomes has also opened up new possibilities for cell delivery. Encapsulation greatly lowers the risk of non-specific
inflammatory immune response occurring in vivo. The use of cholesterol-conjugated antisense has been shown to improve pharmacological properties in vitro and in vivo within human cells.

1.8 Antisense as an antimicrobial against Gram negative bacteria

Many research groups when targeting Gram negative bacteria with AS-ODN, PNA or PMO, target the essential gene acpP. AcpP is the scaffold on which fatty acids are synthesised and is essential for lipid biosynthesis. Good et al. attached a short, membrane penetrating peptide (KFFKFFKFFKC) to various PNA’s, including one complementary to acpP, which reduced E. coli viability by 4 orders of magnitude. Tan et al. demonstrated that acpP targeted PNA when used in vivo in a mouse model, was able to significantly reduce bacterial load when targeted towards either an E. coli mutant strain with a defective outer membrane, or when a peptide-PNA conjugate was added targeted towards E. coli K-12 (which possess an intact outer cell membrane). These results demonstrate that the outer membrane presents a significant challenge for the delivery of AS-ODN, PNA or PMO sequences to Gram-negative bacteria, and it is believed that entry of PMO’s into E. coli (a Gram negative) is limited by its presence. Bai et al. (2012) demonstrated that growth of multi-drug resistant strains of various Gram negative bacteria including Shigella flexneri, S. enterica and E. coli, were significantly inhibited when treated with a PNA-peptide conjugate targeted towards an RNA polymerase sigma (70) factor. Their results showed not only the suppression of the target gene, but also the down regulation of associated downstream genes. Both in vitro and in vivo (when the multi-drug resistant strains were intracellularly contained within human gastric mucosal epithelial cells), treatment by the PNA-peptide complex resulted in the complete inhibition of bacterial growth, whilst showing no influence on morphology and growth of the human cells themselves. Jeon et al. found that when fluoroquinolone resistant Campylobacter jejuni is treated with a conjugated KFFKFFKFFK carrier peptide- PNA complex targeted towards the Cmeabc multidrug efflux transporter, C. jejuni demonstrated increased susceptibility towards ciprofloxacin and erythromycin.

The coupling of PMOs to other membrane penetrating peptides, such as RFFRFFRFFXB, has been shown to increase the permeability of AS-ODN, PNA or PMO sequences into E.
coli, S. enterica, Klebsiella pneumonia and Burkholderia multivorans both in pure culture and/or intracellularly. Acyl carrier protein (AcpP) targeted PMO’s have also been shown to reduce viable bacterial counts in mouse peritonitis to a greater extent than in pure cultures of E. coli. It was demonstrated by Tilley et al. that when used in both E. coli and S. enterica mixtures of peptide and PMO had no effect on gene expression in pure cultures, indicating that the peptides were not able to permeabilize the bacterial cell to solutes.

1.9 Antisense as an antimicrobial against Gram positive bacteria

Far less research has been conducted on the use of AS-ODN, PNA or PMO in Gram positive bacteria, due to the higher level of difficulty in enabling the AS-ODN, PNA or PMO sequences to cross the thick peptidoglycan layer that is the Gram positive cell wall. S. aureus has been found to be the causative agent for many human diseases, such as pneumonia, osteomyelitis, meningitis, endocarditis, septicaemia and Toxic Shock Syndrome. It is also a common cause of nosocomial infections worldwide. MRSA is a human pathogen which is causing growing concern worldwide due to its ability to cause invasive infection and high mortality rate. Its ability to resist methicillin based antibiotics is a major factor in its pathogenicity, and there has been an increase in reports of isolated MRSA strains developing multi-drug or vancomycin (intermediate) resistance. Several studies have used antisense – peptide conjugates for the targeting of S. aureus genes in order to obtain growth inhibition in proof of principle studies. Meng et al. have so far showed the most promising use of PS-ODN targeted towards MRSA. They have shown the restoration of oxacillin susceptibility to MRSA and the rescue of mice from lethal sepsis when infected with MRSA through the use of anionic liposome encased PS-ODN targeted towards mec-A, the methicillin resistance gene. Bai et al. (2012) used cell penetrating peptides (KFFKFFKFFK) conjugated with PNA targeted towards the RNA polymerase sigma (70) factor to target and kill MRSA/ vancomycin intermediate S. aureus both in vitro and in vivo. This is the same target that the same group also targeted within multi-drug resistant Gram negative strains and represents a most promising AS-antisense target site within both Gram positive and Gram negative species.
*Mycobacterium tuberculosis* is one of the most successful human pathogens, and estimations suggest that one third of the human population carry these bacteria\(^{122,173}\). Mycobacteria are generally classed as Gram positive due to their lack of an outer cell membrane, and possess a thick unique cell wall rich in mycolic acids\(^{208}\). Triple-layered images have been attributed to an inner layer of mycobacterial peptidoglycan, a further layer of arabinogalactan-mycolic acid complexes and a final negatively charged outer layer composed of polysaccharides, glucans, arabinans and arabinomannas\(^{43,79,173,193}\). This lipid rich outer layer is noted as being highly impermeable, and forms a diffusion barrier which is 100-1,000-fold less permeable to hydrophilic molecules than that of *E. coli*\(^{43,162,231}\). However porins are known to exist within this barrier, and these allow the diffusion of small hydrophilic molecules into the bacterial cell\(^{172,335,336}\). Treatment with a PS-ODN–amikacin moiety, designed to guide the PS-ODN’s to the ribosomes of the *M. tuberculosis* in order to target the glutamine synthetase (*MtGS*) gene, was shown to inhibit biosynthesis of poly-L-glutamate-glutamine and prevent bacterial growth\(^{146}\). However, no greater inhibitory effect was observed by the PS-ODN amikacin moiety compared to PS-ODN alone. Harth et al. (2000) also showed a non-dose dependent synergistic inhibitory effect occurring between mycobacterium targeted antibiotics and the PS-ODN\(^{144,146}\). It is not yet understood how PS-ODN gain access into the *M. tuberculosis* cell without a delivery molecule attached. Peptide- PNA conjugates have also been shown to produce sequence specific inhibition in *M. smegmatis* when targeted towards either a *gfp* reporter gene or the endogenous essential *inhA* gene\(^{183}\). Chitosan-oligodeoxynucleotide nanoparticles targeted towards inositol-1-phosphate synthetase have also been reported as showing increased effectiveness in inhibiting *M. tuberculosis* growth compared to free targeted oligodeoxynucleotides\(^{197}\). It is proposed that interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular constituents\(^{276}\).

The use of thiocationic lipids to encase targeted PS-ODN within liposomes targeted towards several essential *M. tuberculosis* genes has also been investigated\(^{81}\). It was found that a liposome-PS-ODN combination produced a far greater inhibitory effect when compared to that of liposomes, or PS-ODN, alone. The use of thiocationic lipids seems to increase PS-ODN uptake by allowing them to be delivered across the thick cell coat on *M. tuberculosis*\(^{81}\). Meng et al. (2012) also restored fluoroquinolone sensitivity to resistant *E. coli* through the use of an anionic liposome encapsulated phosphorothioate
oligodeoxynucleotide which targeted the gene *acrB*, which encodes for the AcrAB-TolC efflux pump responsible for decreasing intercellular antibiotic concentrations.

1.9.1 *Streptococcus mutans*

*S. mutans* is frequently implicated as the primary etiological agent in the development of dental caries. Dental caries is the most prevalent of all infectious diseases and is a major public health problem in both developed and developing nations. *S. mutans* are acidogenic, aciduric, gram-positive bacteria and their ability to grow and survive under the acid conditions they create, and form biofilms, greatly contributes to their pathogenic potential. Severe dental disease can lead to a number of serious health problems, including cardiac disease and septicaemia. Dental caries are traditionally controlled through topical oral hygiene techniques, such as the mechanical removal of plaque and diet changes including a reduction in sugar intake. Also, chemical agents such as fluoride are commonly used topically as it helps to strengthen and remineralise tooth enamel. Antibiotics are also used to treat severe cases of dental caries and oral disease. The glucosyltransferase B (*gtfB*) gene in *S. mutans* strains is in part responsible for the production of water-insoluble glucans and previous gene inactivation studies have shown that the inactivation of *gtfB* through insertional mutagenesis significantly lowered the sucrose-dependent adherence of the *S. mutans* to the tooth surface, which in turn resulted in a decrease in cariogenicity. Guo et al., in 2006, treated *S. mutans* with PS-ODN antisense oligonucleotides that were specific to *gtfB* gene, and monitored the effects it had upon *gtfB* mRNA transcription, GtfB expression and activity. They found that the anti-*gtfB* PS-ODN had a significantly inhibitory effect upon *gtfB* mRNA transcription, GtfB expression and activity, and upon the ability of the *S. mutans* cells to form biofilms.

In a previous study done in 2008, the ability of the combined use of zoocin A and two targeted PS-ODN sequences (targeted towards *fab-M* and *fba*) to produce a synergistic inhibitory effect upon closely related streptococcal species was examined. In *S. mutans* the enzyme responsible for the generation of monounsaturated membrane fatty acids is termed Fab-M, and Fozo et al. (2004) demonstrated that an inability to produce these fatty acids rendered the organism acid sensitive, which resulted in a decreased growth rate. The *fabM* gene therefore was deemed as a one potential antisense target
site, where the rate of inhibition could be determined through analysis of the growth rate, measurable as culture optical density.

The other PS-ODN target site in this previous study\textsuperscript{95,223} was chosen as it was present not just in \textit{S. mutans}, but many different streptococcal strains. The fructose-1, 6-bisphosphate adolase (FBA) gene was found to have 100\% homology for the first 20 bp sequences among all the bacterial species used in this study whose genome sequence was available on the genome databases. The FBA enzyme is a key metabolic enzyme which catalyses the cleavage of β-fructose-1, 6-phosphate in glycolysis, necessary for cellular metabolism, and was found by Song et al. (2005) to be an essential gene for growth in \textit{Streptococcus pneumoniae}\textsuperscript{315}. The results of this 2008 study showed that a combination of zoocin A and PS-ODN could be used to achieve a dose-dependent inhibitory response upon bacteria that were A) susceptible to zoocin A, and B) contained the PS-ODN target site, and it was concluded that the zoocin A was indeed causing damage to the susceptible bacterial cell walls and thus allowing the PS-ODN entrance to the bacterial cell interior.

\subsection*{1.10 Fructose bis-phosphate adolase gene}

It is a generally held principle that the complete repression of a key metabolic gene will result in bacterial stasis \textsuperscript{315}. It is thought that \textit{S. mutans} OMZ175 is only able to metabolise through glycolysis which is dependent on a functional \textit{fba} gene\textsuperscript{244,364,365}, which would therefore mean that \textit{fba} is essential to \textit{S. mutans} growth and presents a promising AS-ODN target gene. Although the \textit{fba} gene is perceived to be an essential gene for growth, and was regarded as such for the 2008 study which targeted this gene\textsuperscript{95,223}, this has yet to be proved for \textit{S. mutans} OMZ175 as a complete metabolic cycle for this strain has yet to be determined. In this current study, experiments were conducted to determine whether the \textit{fba} gene is essential to growth, and that no other metabolic cycle which does not include use of the \textit{fba} gene can be utilised.

\subsection*{1.11 Stimulation of autolysis by antimicrobials}
Figure 1.2. Reconstruction of specific metabolic pathways and transport mechanisms in *S. mutans*. Based on the annotated genome sequence, extracellular and intracellular sugar metabolism and metabolism of organic compounds are shown.

A variety of lytic antimicrobial agents exist that are active against gram-positive bacteria. Most work to activate autolysis in two possible ways; 1) by the formation of pores in the bacterial cell wall, resulting in the loss of membrane potential, changes in the cells permeability and leakage of intracellular material\textsuperscript{196} and 2), by triggering the sensor of a sensor/regulator autolysis gene such as lytA\textsuperscript{196,250}. Whilst zoocin A may be able to deliver antisense to \textit{S. mutans} OMZ175 \textit{in vitro}\textsuperscript{95,223}, its specificity is limited to various members of the streptococcal species\textsuperscript{5,95}. This has the added benefit of ensuring that even in a mixed bacterial population the antimicrobial will gain entry only to those bacterial species that the zoocin A targets. This helps to prevent unspecific ecological effects, and reduces the concentration of antimicrobial needed for producing a specific effect. However, as well as \textit{S. mutans} there are other pathogenic bacterial strains that are of major importance to human health, such as \textit{S. aureus} and \textit{E. faecalis}. Chua et al. 2008, looked at several endopeptidases and lipophilic compounds, and their ability to act synergistically with PS-ODN’s on different bacterial species, including \textit{S. mutans}, \textit{L. monocytogenes}, \textit{S. aureus} and \textit{B. cereus} and concluded that combinational therapy provides synergistic growth inhibition. A copy of Chua’s report is included on McLeod 2012 Data CD.

\textbf{1.11.1 Lysins}

Zoocin A is a 27 kDa D-alanyl endopeptidase produced by \textit{Streptococcus equi} subsp. \textit{zooepidemicus} 4881\textsuperscript{310,312}. \textit{S. equi} subspecies \textit{zooepidemicus} 4881 is a Lancefield group C streptococcus and is most commonly found in a range of animal hosts\textsuperscript{27,28}. Zoocin A has been found to have a range of activity for various streptococci, including \textit{S. pyogenes}, \textit{S. sobrinus} and \textit{S. mutans}\textsuperscript{95,161,223}. The sensitivity of bacterial strains to zoocin A correlates with the amount of zoocin A bound by the cells. This indicates that susceptibility is determined by the ability to bind to the target\textsuperscript{6}. Akesson et al. suggested that this may be due to the presence of choline in the cell wall\textsuperscript{6}. Zoocin A’s mode of action has been shown to cause a loss of cytoplasmic constituents due to its ability to rupture the cell wall through the separation of the cell membrane from the cell wall\textsuperscript{311}. Gargis et al. determined that zoocin A is a D-alanyl-L-alanine endopeptidase, and thus its activity directly results in peptidoglycan lysis\textsuperscript{117}. Zoocin A has previously been studied in our laboratory and has already been proven useful as an agent to facilitate the entry of small inhibitory molecules such as monolaurin\textsuperscript{194} or hypothiocyanate radicals into Gram-positive cells\textsuperscript{96}. The use of
zoocin A in combination with the antimicrobial lactoperoxidase system has shown synergy between the two agents. This is believed to be due to zoocin A permeablising the bacterial cell wall allowing the hypothiocyantate ion greater access to the cell interior\textsuperscript{96,99}. Zoocin A is not approved for human use, and has been shown to be highly immunogenic in mice and rabbits (unpublished data, Simmonds, R. 2012). Whilst the lytic delivery agent could be used alone to cause bacterial cell death, a combination of cell targeting agents such as delivery agent plus antisense will help to reduce the overall concentration of each agent needed to produce an effect, reducing the likelihood of resistance developing.

1.11.2 Peptides

Bacteriocins are peptides or proteins released extracellularly that have a bactericidal or bacteriostatic effect on bacteria closely related to the producer strain, whilst the producer strain itself is protected by the presence of an immunity factor\textsuperscript{159,270,301}. Lantibiotics are low molecular weight ribosomally synthesized antimicrobial peptides that contain extensive post-translational modifications\textsuperscript{13,158}. Nisin is the best known example of a lantibiotic and is synthesized by some strains of \textit{Lactococcus Lactis} subspecies \textit{lactis}\textsuperscript{158,301}. Nisin is one of the most studied pore forming antimicrobial agents in the world, and holds FDA generally regarded as safe (GRAS) status in the United States as well as being approved for use as a direct food additive in 57 countries\textsuperscript{52,158}. Nisin is an amphipilic polypeptide lantibiotic bacteriocin\textsuperscript{52,147,301,353}. Like most bacteriocins, nisin is predominately active against Gram-positive organisms, but treatment of Gram-negative bacteria with chelating agents allows nisin to penetrate the cell membrane rendering these organisms sensitive such as \textit{B. cereus}, \textit{L. monocytogenes}, Enterococci, Staphylococci and Streptococci\textsuperscript{207}. Nisin has a dual mode of action. The highly positive C-terminus of nisin interacts primarily with the anionic surface of the bacterial cell membrane\textsuperscript{48}, by using lipid II as a docking molecule. With nisin bound to both Lipid II and the cell membrane, pores are formed across the cell membrane, resulting in the loss of intracellular ions and lethally reducing membrane potential\textsuperscript{13}. Nisin also works to produce cellular lysis by breaking down the cell wall at the septum of dividing cells. The positively charged lantibiotic associates with the negatively charged teichoic and lipoteichoic acids, which
displace and activate N-acetyl-L-alanine amidase and N-acetylglucosaminidase enzymes. These enzymes are peptidoglycan hydrolases that can act as autolysins by hydrolyzing the crosslink’s between the glycan’s and peptides present in the cell wall, resulting in the rupturing of the bacterial cell wall. No resistance to nisin has been reported, despite its use for almost 50 years. Such an antimicrobial could also potentially be used to deliver AS-ODN, PNA or PMO sequences into the bacterial cell interior. The dual mode of action of Nisin is concentration dependent. At a low concentration only lipid II is affected, preventing the synthesis of peptidoglycan. At such a concentration it is possible that Nisin will work synergistically with AS-ODN, PNA or PMO sequences to cause an inhibitory effect. A combinational therapy will also help to prevent the development of resistance. Pediocin is a small non-modified peptide, produced by Pediococcus acidilactici, is also believed to stimulate pore formation in gram positives through a highly similar mechanism, although further study is required.

1.11.3 Small chemical molecules

Some small chemical molecules, such as sodium dodecyl sulphate (SDS), have potential to be used as AS-ODN, PNA or PMO sequences delivery agents. SDS is an anionic detergent which is often used to lyse open bacterial cells, and solubilise proteins. However due to its toxicity it is unsuitable for use in vivo.

1.11.4 Beta-lactams

The other major class of autolytic antimicrobials that could potentially be used to deliver AS-ODN, PNA or PMO sequences into the interior of target bacterial cells are the β-lactam antibiotics, and there exists many different varieties approved for systemic use in humans. As bactericidal antimicrobials their most noted mechanism of action is the inhibition of the synthesis of the peptidoglycan layer of bacterial cell walls. Vancomycin is a key member of the glycopeptide group used clinically against Gram-positive pathogens. Vancomycin inhibits the biosynthesis of peptidoglycan by inhibiting transpeptidation through binding to the peptidoglycan monomer pentatpetides. This weakens the cell wall and causes damage to the underlying cell
membrane. Beta-lactam antibiotics such as penicillin inhibit penicillin binding proteins (PBP) involved in peptidoglycan synthesis. Tipper and Strominger were the first to suggest that the binding of penicillin to PBP and the subsequent inhibition of peptidoglycan synthesis resulted in an osmotically and mechanically weak bacterial cell wall. They covalently modify the active site of PBP’s and prevent their activity, resulting in a disruption of cell wall integrity.

However, β-lactams are also noted for stimulating the autolysis of target bacteria. It is believed that antibiotics like penicillin deregulate autolytic gene control. During normal cell growth, autolysin activity is believed to be subject to strong, prolonged down regulation, which occurs independently from transcription of the autolysin. Studies conducted using S. pneumoniae have found a two-component regulatory system labelled vcnR-vcnS, with vncS mutants reported to be resistant to killing by a large range of antibiotics which act upon various target sites. The growth of the mutant cells was inhibited by the presence of antibiotics as effectively as in the wild type cells, illustrating that although penicillin was able to act normally against S. pneumoniae, lysis of these cells was prevented. Novak et al. recently reported that the two component system vcnR-vcnS gene locus encodes a peptide labelled P27, which is exported by the ABC transporter vex. P27 is a dose-dependent death signalling peptide which is constitutively expressed at a low level by logarithmic cells. Once the intracellular concentration of P27 reaches a critical threshold multiple cell death mechanisms are triggered- including the gene encoding the autolysin LytA. It has been found that cells lysed with β-lactams have high and prolonged down-regulation of the vcnR-vcnS genes during the early and mid-exponential phases, causing the upregulation of P27 expression, and thus the stimulation of the cells own autolytic enzymes.

1.12 Bacterial growth assay

It has previously been established that the addition of lytic agents to a bacterial culture can cause a measurable effect upon bacterial growth rates. It has also been established that bacterial growth rates can be monitored to determine the presence of any synergistic inhibitory activity between a cell lytic agent and PS-ODN. Bacterial growth often shows a phase in which the specific growth rate starts at a value of zero and then
increases to a maximal value in a certain period of time$^{24,30,369}$. This lag phase is typically observed as a response of the microbial population to a (sudden) change in the environment, an adjustment period during which bacterial cells modify themselves in order to take advantage of the new environment and initiate exponential growth$^{56,324}$. A hypothesis formulated by Robinson et al. (1998) states that the lag phase is determined by two (hypothetical) qualities, namely, (i) the amount of work that a cell has to perform to adapt to its new environment and (ii) the rate at which it can perform that work$^{287}$. Various factors influence the length of the lag phase ($\lambda$). Typically, bacteria are grown in a pre-culturing environment in order to achieve an appropriate number of cells before inoculation into the test environment under study. Changes in the new test environment compared to the pre-culturing environment can all work to influence $\lambda$. Different media types, the addition of antimicrobial components, changes in temperature, the identity and phenotype of the bacteria under study and the growth stage or physiological history of the cells can all influence lag time duration$^{287}$.

Determining the duration of the lag phase ($\lambda$) and particularly the end-point of $\lambda$ is a point of mathematical conjecture. Many authors emphasise the particular difficulties in estimating the lag phase$^{24,30,222}$. There are several reasons for this difficulty- the first being that the actual definition of the lag phase is purely mathematical and several alternative definitions of the lag phase exist. $\lambda$ is often defined as the time when a horizontal tangent to the curve at time zero intersects with the linear extrapolation of the exponential phase (Figure 1.3). Buchanan and Solberg (1972) used the time for the initial population density to increase twofold as a definition of the lag phase$^{57}$. $\lambda$ has also been defined as the time when bacterial growth acceleration is at its maximum$^{55}$. The second reason for the difficulty in defining $\lambda$ is that there is a lack of physiological understanding of the lag phenomenon and very little biological information about $\lambda$ has been put into the bacterial growth models$^{30}$. When the purpose of models is descriptive or predictive, it is important to incorporate physiological knowledge into the model in order to make it more generally valid.

A variety of different statistical methods exist to model bacterial growth and determine maximal growth rate ($\mu_{\text{max}}$) and the end point of lag phase ($\lambda$). Two methods are commonly used to monitor the growth of a bacterial population. The standard measuring method is the total viable count. For accurate estimations of growth parameters it is recommended that a minimum of 10 data points per growth curve are collected$^{272,325,349}$.
Figure 1.3. A growth curve exhibiting a sigmodal curve\textsuperscript{369}. The $\lambda$ end point and the part of the growth curve exhibiting maximum growth rate are indicated.
and this is time consuming and expensive. A second method based on absorbance or OD measurements of bacterial culture is easy to automate and relatively inexpensive. A key issue however is that the detection threshold typically corresponds to a bacterial concentration of $10^6$ - $10^7$ bacteria/ml. This high concentration can influence the growth of the bacterial inoculum.

Using OD measurements, models of bacterial growth can be fitted to the OD growth data and used to determine statistical points such as $\mu_{\text{max}}$ and $\lambda$. The Baranyi model and the fitting of a sigmoidal growth curve are the two methods most often used to determine $\lambda$.

The Baranyi model describes a progressive acceleration of growth from the lag phase to the exponential phase ending with a logistic inhibition\textsuperscript{24,25}. It can be used to model bacterial growth curves based on both viability and absorbance data. It is often used to determine $\mu_{\text{max}}$ rates and to predict models of food spoilage rates. One drawback to its use is its extremely complicated statistical methods.

Sigmoidal curves have been developed to analyse dose-response and were historically used to describe the increase in the logarithm of the bacterial cell density with time\textsuperscript{369}. Zwietering et al. (1990) evaluated several different sigmoidal curve models and concluded that the modified Gompertz model (modified for bacterial growth description) could be regarded as the best sigmoidal model to describe growth data\textsuperscript{369}. The modified Gompertz model describes the log10 transformed data by an empirical sigmoid curve and is also often used to describe $\lambda$.

### 1.13 Aims and Hypothesis

The overall hypothesis of this work was that the combined use of antisense and lytic agents would cause a target specific decrease in bacterial growth. This hypothesis was examined by three different experimental approaches that aimed to examine A) The down regulation of target mRNA, B) The measurement of intracellular PS-ODN and C) The effects of different lytic agents and PS-ODN’s on growth.
1.13.1 The down regulation of target mRNA

It was hypothesised that the addition of PS-ODN to bacteria in the presence of lytic agents would result in the down-regulation of expression of the target RNA. Whilst the phenotypic effects of the presence of AS-ODN, PNA or PMO sequences can often easily be detected\textsuperscript{24,95,140}, the effect of PS-ODN on the expression levels of the target DNA or RNA can also be determined through the use of RT-PCR. A method allowing the treatment of \textit{S. mutans} OMZ175 with a variety of different PS-ODN delivery methods, the extraction of total RNA from PS-ODN treated bacteria, and the use of quantitative reverse transcriptase PCR using primers specific for the target genes and various controls was developed.

1.13.2 The measurement of intracellular PS-ODN

It was hypothesised that agents that induce autolysis would facilitate the entry of PS-ODN to enter the bacterial cell. The use of the lytic agent zoocin A to deliver PS-ODN into the bacterial cell has previously been examined\textsuperscript{95,223}, with a synergistic inhibitory effect found to exist between the two agents. However, the presence of the PS-ODN inside the target bacterial cell had not been demonstrated. Whilst studies using PS-ODN’s to target various genes in several different bacterial species have already been conducted\textsuperscript{18,19,95,140,146,165,166,183,226,228,246}, it has yet to be determined where in the bacterial cell the PS-ODN end up. This project sought to determine whether or not lytic agents used in combination with PS-ODN to produce a growth inhibitory effect were in fact delivering the PS-ODN within the bacterial cell. This was investigated by use of radioactively labelled oligonucleotides and a detection assay that permitted the detection of PS-ODN’s in the target bacterial cell interior.

1.13.3 Effects of different delivery agents and PS-ODN’s on growth

It was hypothesised that bacteriocins would deliver PS-ODN more effectively than standard laboratory chemicals, but that they would have limited delivery potential as they have a narrow target range. It was hypothesised that a range of lytic agents could
potentially facilitate the entry of PS-ODN into bacterial cells. A simple growth assay system was used to determine the effectiveness of lytic enzymes (zoocin A), pore forming bacteriocins (pediocin and lysostaphin) and secondary metabolites (penicillin and vancomycin) at inhibiting cell growth when used in combination with PS-ODN.
2  Materials and Methods

2.1  Stock Materials and Standard Methods

2.1.1  Stock Solutions and media

All stock solutions were sterilised by autoclaving at 121°C for 15 minutes and stored at room temperature (RT) unless otherwise stated, and all chemicals used were obtained from Sigma Chemical Co., St Louis, USA (Sigma), unless otherwise specified. Milli-Q deionised water (MQ water) used for the preparation of media and reagents was prepared using a Nanopure Diamond Life Science UV/UF Water System according to the manufacturer’s instructions (Barnstead International, Thermo Scientific, Waltham, MA, USA). All stock solutions and media used are listed in Appendix 1.

2.1.2  Bacterial strains

Bacterial strains used in this study are listed in Table 2.1. Stock cultures of all strains were stored in 5 M glycerol at -70°C. Strains not in regular use were subcultured from freezer stock cultures as required. Strains in regular use were subcultured on to agar plates and stored at 4°C for up to one week. *Escherichia coli* strain M15 (pREP4) was grown on LBA supplemented with the appropriate antibiotics and incubated at 37°C for 24 hours. *E. coli* broth cultures were prepared in LB with the appropriate antibiotics and incubated at 37°C with shaking at 200 rpm on an incubator shaker (Innova 44, New Brunswick Scientific (NBS), Edison, New Jersey) for 18 hours. Streptococcal and staphylococcal strains were maintained on BA agar and incubated at 37°C in air supplemented with 5% CO₂. *S. mutans*
Table 2.1: Bacterial strains and Plasmids used in this study

<table>
<thead>
<tr>
<th>Species and Strain</th>
<th>Plasmid</th>
<th>Description</th>
<th>Selection system</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> M15 ZooA1</td>
<td>pREP4 (kanamycin)</td>
<td>Zoocin A producer</td>
<td>100 µg/ml Amp +</td>
<td>(Lai et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>pQE80L ZooA1 (ampicillin)</td>
<td>strain</td>
<td>25 µg/ml Kan</td>
<td></td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em> AR01/DGVS</td>
<td></td>
<td></td>
<td></td>
<td>DMCC</td>
</tr>
<tr>
<td><em>Streptococcus mutans</em> OMZ175</td>
<td></td>
<td></td>
<td></td>
<td>DMCC</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> Oxford</td>
<td></td>
<td></td>
<td></td>
<td>DMCC</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> WSSP-1</td>
<td></td>
<td>MRSA</td>
<td></td>
<td>DMCC</td>
</tr>
<tr>
<td><em>Micrococcus leuteus</em> IL1</td>
<td></td>
<td></td>
<td></td>
<td>DMCC</td>
</tr>
</tbody>
</table>

DMCC = Department of Microbiology and Immunology Culture Collection, University of Otago
OMZ175 and *S. equi* subspecies *zooepidemicus* 4881 were incubated for 24 hours and broth cultures were prepared in THB, while staphylococcal, enterococcal and micrococcal strains were incubated for 48 hours and broth cultures were prepared in BHI. Both THB and BHI overnight cultures were incubated at 37°C in air supplemented with 5% CO₂ for 18 hours. All strains were obtained from the University of Otago, Department of Microbiology and Immunology Culture Collection unless otherwise stated.

2.1.3 **Electrophoresis procedures for DNA**

Agarose gels (1 or 2%) were subject to electrophoresis in 1 x TAE buffer at a constant voltage of 80 V (Power Pac 300, Bio-Rad); in a submarine gel tank (Owl, Separation Systems, Portsmouth, NH, USA). DNA (8 μl sample) was mixed with 2 μl tracking dye and loaded into the gel. A DNA marker (1 Kb plus DNA ladder, Invitrogen Life Technologies, CA, U.S.A) was used in comparison to allow estimation of molecular weight. Electroporation was stopped when the bromophenol blue dye had migrated two thirds of the way down the gel. Gels were then stained in ethidium bromide for 30 minutes and destained in dH₂O for half the staining time. Bands were viewed and photographed using a Gel DOC 2000™ gel documentation system (Bio-Rad) and printed on a Mitsubishi video graphic printer.

2.1.4 **Reagents and gel preparation for SDS-PAGE**

Preparing and running the gel:

A 7.5% resolving gel was prepared as previously described by Guo et al., (2006), using a mixed solution of 2.5 ml 30% acrylamide/bis solution (Bio-Rad), 2.5 ml 0.5 M Tris-HCl, pH 8.8; 100 μl 10% sodium dodecyl sulphate (SDS), and 4.85 ml MQ water. Freshly prepared 10% ammonium per sulphate (APS) (Bio-Rad) (50 μl) and 5 μl of N,N,N’,N’-tetramethylethylenediamine (TEMED, Bio-Rad) was added last. The gel mix was poured into the gel apparatus, overlain with MQ water (to exclude air) and left to set. The overlying MQ
water was removed prior to pouring the stacking gel. A 4% stacking gel was prepared by mixing 330 μl 30% acrylamide/bis solution (Bio-Rad), 630 μl 0.5 M Tris-HCl, pH 6.8, 25 μl of 10% SDS, and 1.5 ml MQ water. TEMED (Bio-Rad) (2.5 μl) and 12.5 μl 10% APS were then added. The stacking gel was poured, a comb inserted to create the wells and the gel allowed to polymerise before storage at 4°C for one hour.

Protein preparations were mixed with an equal volume of 2x SDS-PAGE sample buffer, and boiled for 2 minutes before loading onto the gel. Five microliters of a low MW protein standard (Bio-Rad) was loaded onto the gel for size determination of the protein.

Electrophoresis was performed at a constant voltage of 100 V (Model 250 Power Supply, Bethesda Research Laboratories (BRL), Life Technologies Inc., Gaithersburg, MD, USA) in a Mini-PROTEAN® II electrophoresis cell (Bio-Rad) containing running buffer. Electrophoresis was stopped when the bromophenol blue dye had migrated to the bottom of the gel. The gel was removed from between the glass plates, rinsed briefly in dH2O, and immersed in Commassie Blue stain solution (Bio-Rad) for 2.6 hours. The gel was destained O/N in RO water. Bands were viewed under white light, photographed using a Gel DOC 2000™ gel documentation system (Bio-Rad) and printed on a Mitsubishi video graphic printer.

2.1.5 Confirmation of strain designation by 16S rDNA PCR

A colony PCR to amplify the 16S rDNA gene of S. mutans OMZ175, S. aureus WSSP-1, S. aureus Oxford, E. faecalis AR01/DGVS and E. coli M15 ZooA1 was carried out. Primers are listed in Appendix 2. Taq polymerase and 10 x Taq buffer were obtained from Roche. A reaction mix consisted of 34.5 μl MQ water; 5 μl 10 x Taq buffer; 5 μl 2 mM dNTPs; 1.5 μl forward primer and 1.5 μl reverse primer (final concentrations of 0.3 μM); and 1 μl Taq polymerase. Template DNA was added by plucking a colony with a sterile toothpick and dipping it in the reaction mix prior to the addition of the Taq polymerase. Toothpicks were then struck on CAB and incubated at 37°C for 48 hours. Amplification was performed with a thermocycler (Mastercycler, Eppendorf) using the following parameters: Cycle 1,
denaturation temperature of 95°C for five minutes, annealing temperature of 33°C for 2.5 minutes, and extension temperature of 65°C for three minutes; cycles 2–31, denaturation temperature of 92°C for 30 seconds; annealing temperature of 33°C for 30 seconds, and extension temperature of 65°C for one minute. PCR products were visualised on a 1% agarose gel. Products that were 800 bp in length were purified using a PCR purification kit (Axygen) according to the manufacturer’s instructions. The amount of product was determined using a Nanodrop spectrophotometer (Nanodrop Technologies). Products were then sequenced by the Massey DNA Sequencing Facility (Allen Wilson Centre) and sequencing reactions were prepared according to their instructions. The resulting sequence was analysed using SeqED software (DNASTAR Incorporated). Sequences were compared to published 16S sequences and the strain designation confirmed.

2.1.6 Production of zoocin A

Recombinant zoocin A was produced and purified as previously described by Lai, et al., 2002, using the *E. coli* ZooA1 producer strain and the Ni-NTA affinity purification (Qiagen, Hamburg, Germany).

2.1.7 Protocol for preparation of a 96 well NUNC LCB checkerboard titration plate and determination of viability

Unless otherwise specified, checkerboard titration plates were prepared using ninety-six well Low Cell Binding (LCB) plates (Nagle Nunc International K.K, Tokyo, Japan). The order in which reagents were added to a plate and the volumes of each stayed constant throughout this study, as described by Nekhotiaeva et al., 2004. Each well had a final volume of 200 μl comprised of: 40 μl antisense or THB, 10 μl zoocin A or THB and 150 μl of a 5% bacterial inoculum. Zoocin A and antisense oligonucleotide concentrations varied, with all specified concentrations being their final concentrations. All experiments were performed in triplicate. Dilutions of stock antisense were always prepared in double strength THB (2x THB), whilst
all zoocin A dilutions were made using single strength THB. Eppendorfs used to contain antisense were siliconised, to prevent adhesion of the antisense to the plastic surface. Once prepared, all plates were incubated for either 18 or 24 hours in an Infinite® M200 (Tecan. Trading AG, Switzerland) plate reader at 37°C. Prior to incubation, an initial shaking step (orbital shaking, 25 rpm/sec, 3 sec) was followed by the optical density (OD) of each sample being recorded at 595 nm. The shaking and OD reading was repeated every hour, and the OD of each well for each plate recorded. Bacterial inocula were prepared as a 5% dilution of an O/N culture in fresh THB, unless stated otherwise.

Unless otherwise specified, the viability of bacterial cultures was determined using serial dilutions made in peptone water down to $10^{-8}$. Ten microliters of dilutions $10^{-3}$ to $10^{-8}$ were then spotted five times onto CAB agar. One hundred microliters of dilutions $10^{0}$ to $10^{2}$ were spread onto CAB agar. The dilutions, spot and spread plating of each culture was done in triplicate. Agar plates were incubated at 37°C in air supplemented with 5% CO$_2$ for 48 hours. The total amount of bacteria in the overnight culture was enumerated using both spread and spot plates.

2.2 Development of novel methods

2.2.1 Statistical analysis of growth and growth inhibition

Most mathematical models, such as the Baranyi model apply an end point of the lag phase which corresponds to the point at which the maximal growth rate in the bacterial growth curve is achieved. However, the large number of variable parameters between the different *S. mutans* OMZ175 treatments (untreated, treated with zoocin A, treated with zoocin A and antisense) mean that the F-test method (on which the user friendly version of the Baranyi analysis software is based) is unsuitable for use in this study. Also, as different treatments of *S. mutans* OMZ175 can result in growth at different maximal rates, in order to apply a
consistent measurement, an alternative mathematical basis was used to define the length of the lag phase.

To provide a cut-off point at which it could be stated with confidence that growth was occurring (and thus the end point of the lag phase reached) a measure needed to be determined that would permit consistent application across all treatment cultures to be analysed. Multiple readings of bacterial growth by OD gives rise to a degree of variation in results, so the threshold value must be high enough to avoid misinterpretation during data analysis. After reviewing papers that had analysed the length of the lag phase (Baranyi and Roberts (1994), Juneja et al. (2007), Lopez et al. (2004)) it was decided that we would deem the lag phase, or the recovery phase of treated exponential phase or stationary phase bacterial cultures, to be complete after the OD level had increased to an amount that was 0.1 OD greater than the initial starting OD. For each experiment analysed, modified Gompertz sigmoidal curves were generated to fit the growth curve data generated for each of the triplicate replicates for each bacterial strain under each of the different growth conditions tested. The equation, for a modified Gompertz sigmoidal curve, reads

\[ Y = bottom + \frac{(top - bottom)}{(1 + 10^{(log Ec50 - X)1}} \]

In order to determine at what time point the modified Gompertz sigmoidal curve (fitted to our data points) passed the point of initial OD of the test culture + 0.1 OD, the equation was rearranged to find X (Time), with Y (OD) always equalling the bottom value (starting OD) for each growth curve + 0.1 OD (Figure 2.2).

\[ X = - \log \left( \frac{(top-bottom)}{(Y - bottom) -1} \right) - log Ec50 \]

This enabled the length of time taken for each data set to pass the point of initial starting OD + 0.1 OD to be determined, and thus determine the \( \lambda \) for each growth curve. As each
Figure 2.1: The theoretical growth of *S. mutans* OMZ175 is shown by •, a sigmoidal curve has been fitted to this growth curve using the Prism software™. From the equation generated from this curve, it is possible to determine at what time point (9.77 hours) the growth curve passes the point of initial OD + 0.1 OD.

\[ X = -\log \left( \frac{(\text{top-bottom})}{(Y - \text{bottom}) - 1} \right) - \log \text{Ec50} \]

**X intercept = 9.77 hours after time 0**
treatment type was done in triplicate, the averages and standard deviations of \( \lambda \) for each growth curve and treatment type could be determined, and used in a one-way ANOVA to determine significance. All statistical analyses were performed using Prism Software™ and Microsoft Excel. P values were generated using the Tukey Multiple Comparison Test. Any P value greater than 0.5 was determined to be significantly different. Using this method the length of the lag phase for each growth curve could be determined using a method that applied a constant and consistent cut-off point (initial starting OD + 0.1 OD) across all treatment types, and which did not have to rely on having an equal \( \mu_{\text{max}} \) across all growth curves in order to calculate \( \lambda \).

**2.2.2 Use of minimal media to demonstrate the nature of the fba gene in S. mutans OMZ175**

**2.2.2.1 Tryptone- vitamin base (TV) minimal media**

This consisted of media described by Burne et al., 1999 \(^{58}\) containing 3.5% (w/v) tryptone, 0.04 \( \mu \)g/ml P-amino benzoic acid, 0.2 \( \mu \)g/ml thiamine- HCl, 1 \( \mu \)g/ml nicotinamide, 0.2 \( \mu \)g/ml riboflavin and 0.5% of carbohydrate source (as defined in each experiment). The media was sterilised by autoclaving.

**2.2.2.2 Growth on minimal media**

An O/N culture of *S. mutans* OMZ175 was prepared as above and the resultant cell culture centrifuged at 1.6 \( x \) g for ten minutes, the pellet washed, and then resuspended in an equal volume of saline. These resuspended cells were used to prepare 5% inoculums in either THB or TV. A 96 well NUNC plate was then prepared according to the above protocol, with dH\(_2\)O used in place of PS-ODN and zoocin A. The 96 well NUNC plate was incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.17.
2.2.2.3 Growth in TV supplemented with defined carbon sources

An O/N culture of *S. mutans* OMZ175 was prepared as above and the resultant cell culture centrifuged at 1.6 x g for ten minutes, the pellet washed, and then resuspended in an equal volume of saline. The resuspended cells were used to prepare 5% inoculums in either THB or TV. Inoculums were also prepared in TV containing 0.5%, 1% and 2% sucrose, and also 0.5%, 1%, 2% and 5% pyruvate. A 96 well NUNC plate was then prepared according to the above protocol, with dH₂O used in place of PS-ODN and zoocin A. The 96 well NUNC plate was incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.1.7.

2.2.2.4 Growth in TV supplemented with defined carbon sources

An O/N culture of *S. mutans* OMZ175 was prepared as above and the resultant cell culture centrifuged at 1.6 x g for ten minutes, the pellet washed, and then resuspended in an equal volume of saline. The resuspended cells were used to prepare 5% inoculums in either THB or TV. Inoculums were also prepared in TV containing a final 1% concentration of either: sucrose, α- keto-glutarate, sodium citrate, L-glycine, succinate acid, L- glutamic acid, pyruvate, 2-phosphophenylpyruvate or hexaglycine. The pH of each supplemented TV media was then determined using Whitman® pH strips. A 96 well NUNC plate was then prepared according to the above protocol, with dH₂O used in place of PS-ODN and zoocin A. The 96 well NUNC plate was incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.1.7. After the programme has finished, the pH of the wells containing each of the additional carbohydrates was determined using the above protocol.

2.2.2.5 Growth in TV supplemented with defined carbon sources + yeast
The above experiment was repeated with the addition of a final concentration of 0.1% yeast to each supplemented TV medium. A TV + 0.1% yeast (yeast extract) only control was also included. α- keto-glutarate, succinate acid and 2-phosphophenylpyruvate were not used as carbon sources, and were instead replaced by a further TV volume of medium supplemented by pyruvate a final concentration of 5%.

2.2.2.6 *S. mutans* OMZ175 in TV + carbohydrate + zoocin A

To establish the sub-lethal concentration of zoocin A to be used, *S. mutans* OMZ175 in either the lag or exponential phase was incubated in the presence of zoocin A in microtitre plates (as described section 2.1.7). An O/N culture of *S. mutans* OMZ175 was prepared as above, at time 0 (exponential phase, OD = 3.5) and time 3 hours (lag phase, OD = 0.13). The time 0 cell culture was incubated at 37˚C in air supplemented with 5% CO₂ for 3 hours until the time 3 hour culture was prepared. The resultant cell cultures were centrifuged at 1.6 x g for ten minutes, the pellets washed, and then resuspended in an equal volume of saline. These resuspended cells from both time points were then used to prepare separate 5% inoculums in either THB or TV. TV inoculums for both time points were supplemented with final concentrations of either 0% sucrose + 0% yeast, 0.5% sucrose or 0.5% sucrose + 0.1% yeast. A 96 well NUNC LCB plate was prepared according to the above protocol, the growth of *S. mutans* OMZ175 for each time point and each media type was tested with the addition of zoocin A to a final concentration of either: 100, 20, 10, 5, 2.5, 1, 0.5 AU/ml or 0.02 AU/ml. The 96 well NUNC plate was incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.1.7.

2.2.2.7 Interaction between media and PS-ODN and zoocin A

In order to check that the presence of sucrose or yeast did not affect the solubility of the PS-ODN, a 96 well NUNC plate containing 200 µl volumes of THB or TV + 0.5% sucrose + 0.1% yeast per well was prepared. Wells were then modified such that they contained either media alone, or media with a final concentration of either: 20 mM SM-FBA, 20 mM ATS2,
2.5 AU/ml zoocin A, SM-FBA + zoocin A, or ATS2 + zoocin A. The pH of each combination was determined using the above protocol and the 96 well NUNC plate was incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.1.7.

2.2.2.8 *S. mutans in supplemented media with zoocin A + PS-ODN*

An O/N culture of *S. mutans* OMZ175 and 50 ml 5% inoculums of twice washed exponential and lag phase cultures in TV + 0.5% sucrose + 0.1% yeast were prepared as above; with each growth phase monitored through viable counts, pH and OD until the desired growth phase was reached as described above (section 2.2.2.6). Once the growth phases had been reached, each growth phase was divided into 6 ml amounts and placed into silanized 20 ml glass universals. Each respective growth phase was then tested with the addition of: TV + 0.5% sucrose + 0.1% yeast alone; a sub-lethal concentration of zoocin A appropriate for that growth phase in TV (Results, Table 3.2); 10 μM ATS2; 10 μM SM-FBA; sub-lethal zoocin A + 10 μM ATS2, and sub-lethal zoocin A + 10 μM SM-FBA antisense. For each treatment for each growth phase viable counts were made using the above protocol (section 2.1.7). The pH was also monitored using the protocol previously described. A 96 well LCB NUNC plate was also prepared according to the above protocol. The 96 well NUNC plate was incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.1.7.

2.2.3 *Determining the gene sequences of the RT-PCR target genes in S. mutans OMZ175*

2.2.3.1 *PCR to determine fba, 16s rRNA and gyrA sequences in S. mutans OMZ175*

The primary reason for this research was to investigate the effect that SM-FBA combined with a lytic delivery agent had upon the *fba* gene. SM-FBA is defined as the PS-ODN sequence targeting *S. mutans* OMZ175 FBA mRNA (Dufour *et al.* 2011). The antisense sequences used in this study and their target sequences are listed in Table 2.2.
Table 2.2: Antisense (PS-ODNs) sequences and their target genes

<table>
<thead>
<tr>
<th>Antisense designation</th>
<th>Antisense sequence (5’ – 3’)</th>
<th>Bacterial strain</th>
<th>Gene</th>
<th>Target mRNA sequence (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM-FBA</td>
<td>TCGTGAAACGATTGCCAT</td>
<td><em>S. mutans</em> OMZ175</td>
<td><em>fba</em></td>
<td>ATGGCAATCGTTTCACGA</td>
</tr>
<tr>
<td>SA-FBA</td>
<td>CATTGAAACTAAAGGCAT</td>
<td><em>S. aureus</em> Oxford</td>
<td><em>fba</em></td>
<td>ATGCCTTTAGTTTCAATG</td>
</tr>
<tr>
<td>EF-FBA</td>
<td>TCCTGATACTACTGGCAT</td>
<td><em>E. faecalis</em> AR01/DGVS</td>
<td><em>fba</em></td>
<td>ATGCCAGTAGTATCAGGA</td>
</tr>
<tr>
<td>ATS2</td>
<td>ATCTATATCTAAACCTA</td>
<td>N/A</td>
<td>N/A</td>
<td>None</td>
</tr>
</tbody>
</table>
course of this research three different gene transcripts were investigated, the $fba$ gene, as well as two known reference genes which are both essential and stably expressed in $S.\ mutans$. Both the 16s rRNA and gyrase A genes were chosen as internal controls as they are both constitutively expressed essential genes$^{182,304,321,330}$. Using NCBI BLAST software, a sequence search was conducted to investigate the $fba$, 16s RNA and gyrA genes of $S.\ mutans$ OMZ175.

2.2.3.2 \textit{Determining the sequence of the qPCR target genes}

As the genetic sequence of $S.\ mutans$ OMZ175 was not available, primers for the $fba$, 16s rRNA and gyrA genes in $S.\ mutans$ OMZ175 were designed based upon the equivalent gene sequences in $S.\ mutans$ UA159 using the genome database NCBI BLAST. Three sets of primers (Invitrogen, NZ) for each gene (9 primer pairs in all) were designed using PrimerSelect on the Lasergene software Suite for Sequence Analysis version 8.0.2 (DNASTAR, USA) (Appendix 2). The PCR protocol for each primer set is listed in Appendix 2. Products amplified from $S.\ mutans$ OMZ175 DNA using each primer pair was purified, sequenced, and the sequence identity confirmed using BLAST search. The sequence data from each primer set then allowed the full gene sequence for each target gene to be determined for $S.\ mutans$ OMZ175.

2.2.3.3 \textit{Design of quantitative PCR primers}

Primers for quantitative PCR were designed using the above sequence data and both Primer Express version 2.0 (Applied Biosystems, USA) and the PRISM design software for TAQMAN™, SYBR™. To ensure maximum amplification efficiency, amplicons were designed to be short, to have similar melting temperatures and to avoid sequences consisting of a run of the same nucleotide. Two sets (A and B) of RT-PCR primers were designed for each gene. Primers were produced by Invitrogen, NZ. Primer pairs are listed in Appendix 2.
RT-PCR products of each were then run on an electrophoresis gel to ensure only single products of the correct size were formed. The dissociation curve of each product was determined using the method provided with the ABI prism® 7500 FAST Sequence Detection System Real Time PCR Thermocycler with Sequence Detection Software version 1.4 (Applied Biosystems, USA). In addition, the products generated were sequenced in order to confirm their target sequence.

FBA RT-PCR set A were: forward, 5’-gaaccgccgtgaagtacga-3’ and reverse, 5’-catggaccatactgctactg-3’, designed to amplify to an internal fragment of the coding region from 614-713 bp of the fba gene of S. mutans OMZ175. The primer sequences for 16s rRNA RT-PCR set A were: forward, 5’-tgcgttagctccggcata-3’ and reverse, 5’-cggcgtgctccgcaagagatt-3’, designed to anneal to an internal coding region from the 777-849 bp region of the 16srRNA gene of S. mutans OMZ175. The primer sequences for gyrA RT-PCR set A were: forward, 5’-gacgcaggcgcatatcaag-3’ and reverse, 5’-ccgcaatagtgagacagataccat-3’, corresponding to the 1182-1282 bp region of the gyrase A gene of S. mutans OMZ175.

2.2.4 RNA extraction from S. mutans OMZ175

2.2.4.1 RNA extraction using phenol/ chloroform

Using an O/N culture of S. mutans OMZ175 a 5% inoculum was prepared. Aliquots of different volumes (1, 1.5, 1.7, 2.5, 5 ml) were removed and the total RNA isolated. Aliquots were harvested by centrifugation at 18 x g for 10 min at 4°C, the supernatant removed and the RNA in each sample extracted using TRIzol® following the manufacturer’s instructions. Briefly, to each cell suspension 20 volumes of TRIzol® reagent was added, and the samples allowed to incubate at RT for 5 minutes before being vortexed and chilled on ice. Each sample was then spun at 15 x g for 30 seconds and the resultant supernatant transferred to a fresh eppendorf. Two hundred microliters of chloroform was then added to each sample, mixed vigorously for 15 seconds, incubated for two minutes at RT and centrifuged at 12 x g for 15 minutes at 4°C. The aqueous phase was transferred to a new eppendorf and the RNA precipitated by addition of 500 μL of isopropyl alcohol. The samples were then incubated at
RT for 10 minutes and then centrifuged at 12 x g for 10 minutes at 4°C. The supernatant was removed and the RNA pellet washed in 1 mL of 75% ethanol. Finally, the supernatant was again removed and the RNA pellet dried at RT for 1 hour before being dissolved in RNase-free water. The quality and quantity of the RNA was determined through UV spectrophotometry using a NanoDrop™ with NanoDrop software version 3.0.1 (Thermo Scientific, USA).

2.2.4.2 RNA extraction using phenol/chloroform plus zoocin A

The above protocol detailing the method for the extraction of RNA from *S. mutans* OMZ175 cells was followed using 1.7 ml aliquots, but following centrifugation at 18 x g for 10 min at 4°C, the supernatant was removed and the cell pellets resuspended in 100 mls of either: 0, 2, 4, 6, 8, 10 or 12 AU/ml zoocin A and the cells incubated on ice for 30 seconds. Following this, to each cell suspension 20 volumes of TRIzol® reagent was added, and the above RNA extraction protocol followed.

2.2.5 Development of a protoplasting protocol

2.2.5.1 Subcellular fractionation of *S. mutans* OMZ175

This technique was adopted from that described by Kling et al, 1999; *S. mutans* OMZ175 was inoculated onto a trypticase soy + 5% blood agar plate and incubated O/N at 37°C + 5% CO₂. A 10 ml THB broth was then inoculated from the resultant growth and incubated at 37°C + 5% CO₂ until the OD₆₀₀ reached 0.5. The resultant culture was then placed on ice, and 1 ml aliquots transferred to pre-chilled micro-centrifuged tubes and centrifuged at 14,000 x g for three minutes at 4°C. The supernatant was then removed from the resultant cell pellets and the cell-pellets washed three times in 1 ml amounts ice-cold protoplasting buffer at 14,000 x g at 4°C. The cell pellets were then resuspended in 0.5 ml of ice-cold protoplasting buffer, 170 U mutanolysin added to each tube, and the suspensions incubated for either one, 10 or 30
minutes at 37°C. The resultant cell suspensions were prepared into wet mounts and examined for the formation of protoplasts using dark-field or phase contrast microscopy.

2.2.5.2 Comparing growth on THA and osmotic RMS

An O/N culture of *S. mutans* OMZ175 was diluted 1:10 in fresh THB, and incubated at 37°C for 4 hours. The cells were then washed in saline and resuspended in hypertonic buffer. Ten-fold dilutions were made from this solution in hypertonic buffer and 0.5 ml aliquots were gently mixed with 4.5 ml of soft RMS (20% sucrose) or THA overlay held at 50°C and the mixture gently spread over RM or THA pour plates respectively. A further five ml layer of soft RM or THA was then overlaid and the plates incubated at 37°C for 48-72 hours.

2.2.5.3 Presumptive protoplast formation by sub cellular fractionation

*S. mutans* OMZ175 was treated using the sub cellular fractionation protocol described above in section 2.2.5.1. To examine for the formation of protoplasts ten-fold dilutions were made from these solutions in hypertonic buffer and 0.5 ml aliquots were gently mixed with 4.5 ml of soft RMS (15% sucrose) or THA overlay held at 50°C and the mixture gently spread over RMS (20% sucrose) or THA pour plates respectively. A further five ml layer of soft RM or THA was then overlaid and the plates incubated at 37°C for 48-72 hours.

2.2.5.4 Assay of zoocin A activity in osmotic stabilising buffers

This technique was adopted from that described by Fischetti, Gotschlich and Bernheimer (1971)\(^{107}\). A 5% inoculum of *S. mutans* OMZ175 was prepared as above, and incubated at 37°C + 5% CO\(_2\) until the OD reached 0.35. The resultant cell culture was then divided into 1.5 ml aliquots, and spun at 18 x g for 15 minutes at 4°C. The resultant pellets were washed in saline, and finally resuspended in equal 1.5 ml amounts of hypertonic (sucrose) buffer.
Zoocin A was added to final concentrations of 0, 1, 5, 10, 15 or 20 AU/ml. From each tube three replicate 200 µl volumes were immediately added to a 96 well NUNC plate, and the OD monitored every ten minutes for 120 minutes. Using the remaining volumes of zoocin A treated cells, gram strains were prepared for each zoocin A concentration and observed using an Olympus CH-2 biological microscope (Olympus Corporation, Tokyo, Japan).

2.2.5.5  **Protoplast preparation and reversion using RM + Sucrose (A)**

The method used for protoplast preparation followed that described by Simmonds (1978) which was based upon that initially described by Freimer, Krause and McCarty. The strain to be protoplasted was inoculated into THB and incubated at 37°C for 18 hours. The cells were then diluted 10-fold in THB and incubated for a further four hours at 37°C. The cells from ten ml were then spun down and the cells washed in sterile saline, (0.85% (w/v)), pelleted again and then resuspended in ten ml of hypertonic buffer. Rather than using phage associated lysin (PAL), zoocin A was added to a final concentration of 20 AU/ml and the tubes incubated at 37°C for 30 minutes. The protoplasts were then pelleted at 1.75 x g for 20 minutes in a cooled rotor and very gently resuspended in ten ml of hypertonic buffer containing 20% sucrose. To examine for reversion to bacterial phase growth ten-fold dilutions were made from this solution in hypertonic buffer and 0.5 ml aliquots were gently mixed with 4.5 ml of soft RMS (15% sucrose) or THA overlay held at 50°C and the mixture gently spread over RMS (20% sucrose) or THA pour plates respectively. A further five ml layer of soft RM or THA was then overlayed and the plates incubated at 37°C for 48-72 hours.

2.2.5.6  **Assay of zoocin A activity in hypertonic media with sucrose**

Using the assay of lytic agent activity method as described above, zoocin A was tested for lytic ability in increasing concentrations of sucrose. A 5% inoculum of *S. mutans* OMZ175 was prepared as above, and incubated at 37°C in air supplemented with 5% CO₂ until the OD
reached 0.35. The resultant cell culture was then divided into 1.5 ml aliquots, and spun at 18 x g for 15 minutes at 4°C. The resultant pellets were washed in saline, and then resuspended in equal amounts of hypertonic buffer containing final concentrations of either: 0, 1, 5, 10, 20 or 30% sucrose. The cell suspensions were all adjusted to an OD of 0.4 using further THB + sucrose at the appropriate concentration for each aliquot. The cell suspensions were then divided into aliquots of 1.5 ml. Zoocin A was added to a 1.5 ml aliquot of each sucrose concentration to a final concentration of 32 AU/ml. From each tube, three replicate volumes were immediately added to a 96 well NUNC plate, as well as controls of cells alone in each sucrose concentration without zoocin A, and the OD monitored every ten minutes for 20 hours.

A further 5% inoculum of S. mutans OMZ175 was prepared as above, and incubated at 37°C in air supplemented with 5% CO₂ until the OD reached 0.35. The resultant cell culture was then divided into ten 1.5 ml aliquots, and spun at 18 x g for 15 minutes at 4°C. The resultant pellets were washed in saline, and duplicate pellets were resuspended in hypertonic buffer containing either: 0, 1, 5, 10 or 20% sucrose. Duplicate pellets were also resuspended in either THB or saline. To one aliquot in each sucrose percentage buffer, a final concentration of 32 AU/ml zoocin A was added. Triplicate 200 µl volumes of each aliquot were immediately added to a 96 well NUNC plate and the OD monitored every two minutes for 30 minutes.

2.2.5.7 Protoplast preparation and reversion using RM + Sucrose (B)

The above protoplast preparation and reversion experiment was repeated, this time using RMS media with final sucrose concentrations for the pour plates and soft agar overlay plates being respectively either: 0% and 0%, 15% and 20%, 25% and 30% or 35% and 40%.

2.2.5.8 Assay of lytic activity in hypertonic media with raffinose

A 5% inoculum of S. mutans OMZ175 was prepared as above, and incubated at 37°C in air supplemented with 5% CO₂ until the OD reached 0.35. The resultant cell culture was then
divided into 1.5 ml aliquots, and spun at 18 x g for 15 minutes at 4°C. The resultant pellets were washed in saline, and resuspended in a 15% raffinose containing hypertonic buffer. To triplicate aliquots, a final concentration of 32 AU/ml zoocin A was added. Three x 200 µl amounts of cells both with and without zoocin A were immediately added to a 96 well NUNC plate, and the OD monitored every ten minutes for 20 hours.

2.2.5.9 Comparing growth on THA and RMR media

An O/N culture of *S. mutans* OMZ175 was diluted 1:10 in fresh THB, and incubated at 37°C for 4 hours. The cells were then washed in saline and resuspended in hypertonic buffer containing either; 0, 15, 20, 30 or 40% raffinose. Ten-fold dilutions were made from these solutions in the corresponding hypertonic buffers and 0.5 ml aliquots were gently mixed with 4.5 ml of soft RMR (containing the same percentage of raffinose as the corresponding hypertonic buffer) or THA overlay held at 50°C and the mixture gently spread over RMR or THA pour plates respectively. A further five ml layer of soft RMR or THA was then overlayed and the plates incubated at 37°C for 48-72 hours.

2.2.5.10 Protoplast preparation and reversion using RM and 15 or 20% raffinose

Putative protoplasts were prepared and checked for reversion on 20% raffinose supplemented media using the method described above. Hypertonic buffer and RM media were prepared using raffinose, with pour plates and soft agar overlay prepared as 15 and 20% raffinose respectively.

2.2.5.11 Protoplast preparation and reversion using RM and 30 or 40% raffinose

Putative protoplasts were prepared and checked for reversion on media supplemented with either 30% or 40% raffinose using the method described above. Hypertonic buffer and RM
media were prepared using raffinose, with both pour plates and soft agar overlays prepared with both 30% and 40% raffinose.

2.2.5.12 Assay of zoocin A lytic activity in a range of raffinose concentrations

A 5% inoculum of *S. mutans* OMZ175 was prepared as above, and incubated at 37°C in air supplemented with 5% CO₂ until the OD reached 0.35. The resultant cell culture was then divided into ten 1.5 ml aliquots, and spun at 18 x g for 15 minutes at 4°C. The resultant pellets were washed in saline, and duplicate pellets were resuspended in hypertonic buffer containing either: 0, 10, 20, 30 or 40% raffinose. Duplicate pellets were also resuspended in either THB or saline. To one aliquot in each raffinose percentage buffer, a final concentration of 32 AU/ml zoocin A was added. Triplicate 200 µl volumes of each aliquot were immediately added to a 96 well NUNC plate and the OD monitored every two minutes for 30 minutes.

2.2.6 Demonstrating the entrance of radiolabelled antisense

2.2.6.1 Determining percentage of oligonucleotide recovery

In order to determine if Roche Mini Quick Spin Columns provide 100% recovery of added 18 bp oligonucleotide, 10 µl amounts of either SM-FBA or ATS2 (final concentration 20 mM) (Table 2.2) were added to separate columns Roche Mini Quick Spin Columns. These experiments were done in triplicate. Once spun through the column (following the manufacturers’ instructions), 1 µl volumes from each sample were taken, and the amount of product in each sample determined using a nanodrop spectrophotometer (Nanodrop Technologies, Long Island, U.S.A) and the manufacturer’s instructions. These were then compared to 1 µl volumes of either SM-FBA or ATS2 at the original 20 mM concentration, and the differences compared.
2.2.6.2 Labelling ATS2 with [methyl-\(^3\)H] thymidine

To label ATS2 with [methyl-\(^3\)H] thymidine (Methyl-\(^3\)H thymidine, Amersham, GE Healthcare), using two separate eppendorfs the following were added to each in order- 19 µl of dH\(_2\)O, 10 µl of Terminal Transferase Buffer (Roche), 10 µl of ATS2 (final concentration of approximately 10 picomole DNA ends, calculated using the Promega online biomath calculator to convert micrograms of linear DNA to picomoles of DNA ends (literally how many ‘ends’ of linear DNA sequences there are present)), 5 µl of cobalt (II) chloride (final concentration 2.5 mM), 5 µl of [methyl-\(^3\)H] thymidine, and 1 µl of Terminal Transferase (Roche). The reactions were mixed and centrifuged briefly and then incubated for 1 hour at 37°C before been placed on ice. Five microliters of 0.2 M EDTA (pH 8.0) was then added to each reaction. One labelling reaction was then cleaned up using Roche Mini Quick Spin Columns following the manufacturer’s instructions to remove unincorporated [methyl-\(^3\)H] thymidine. The other reaction was labelled as ‘uncleaned’. Five microliter amounts of both ‘cleaned’ and ‘uncleaned’ [methyl-\(^3\)H] thymidine labelled ATS were spotted and harvested onto a pre-wetted filter mat (Wallac cat# 1450-421) using a Tom Tec Harvester. The filter was placed into a sample bag (Wallac cat# 1450-437) and beta plate scintillation fluid (Wallac cat# SC/9200/4) was added and the bag sealed. Scintillation fluid was distributed evenly without generating bubbles. The sealed bag was placed into a cassette and radioactivity measured in a microbeta reader (Wallac 1450 Microbeta plus), connected to a PC with installed Wallac 50 microbeta windows workstation (version 4.00.001).

This procedure was then repeated using 45 µl volumes of both the ‘cleaned’ and ‘uncleaned’ [methyl-\(^3\)H] thymidine labelled ATS and the amount of radioactivity associated with each reaction type determined.

2.2.6.3 Labelling PS-ODN with \(^{32}\)P and T4 polynucleotide kinase

To label ATS2 or SM-FBA with \(^{32}\)P- ATP (3000Ci/mmol, 10mCi/ml, 250 µCi) (Perkin Elmer), two separate eppendorfs were used and the following added to each in order- 3 µl of ligase buffer (New England Biolabs), 18 µl dH\(_2\)O, 6 µl of either ATS or SM-FBA (final concentration 20 mM), 2 µl of \(^{32}\)P and 1 µl of T4 polynucleotide kinase (New England
The reactions were then incubated for 1 hour at 37°C. To separate the unincorporated $^{\gamma}{^32}P$ from the oligonucleotide salt precipitation was then performed. One hundred microliters of isopropanol was added to each eppendorf, together with a $1/10^{th}$ the volume of 5 M Sodium chloride (pH 4.6), and the reactions incubated at -20°C for 30 minutes. The reactions were then centrifuged at 13 x g for 20 minutes and the supernatant removed. The resultant pellets were then incubated at 70°C for 5 minutes, after which they were run on a polyacrylamide gel (section 2.2.6.4). Once run, an x-ray exposure of the gel was prepared (section 2.2.6.4). The ladder marker used in this study was kindly borrowed from Dr Rita Przybilski, Fineran Laboratory, Microbiology Department, University of Otago. The marker was developed using a Hammerhead ribosome sequence (HHR2) attached to a T7 promoter. When transcribed in vitro in the presence of Mg$^{2+}$ ions, HHR2, which is divalent metal ion dependent, cleaves itself twice, resulting in 62, 50 and 12 bp products. As $^{\gamma}{^32}P$- ATP was included in the transcription reaction, these products are radiolabelled and used as radiomarkers.

### 2.2.6.4 Preparation of polyacrylamide gel

Polyacrylamide gel glass plates and spacers were thoroughly cleaned using distilled water and 95% ethanol, and then assembled. The spacers were placed along the bottom edge and sides of the two plates. The edges of the plates containing the spacers were then sealed using a 20% agarose gel. A 10% polyacrylamide gel was then prepared using 42 g urea, 25 ml of 40% polyacrylamide-Bis solution (PAA) (Bio-Rad), and 2 ml of 50% TAE. Distilled water was then added until a final 100 ml volume was reached, and the solution mixed and heated until fully dissolved. Freshly prepared 20% APS (500 µl) and 100 µl of TEMED was added last. The solution was quickly mixed and loaded into the gel apparatus with care taken that that no bubbles formed. The gel comb was then inserted into the top of the gel frame, and all spaces filled using the polyacrylamide solution. Once set, the bottom gel frame spacer was removed, along with any agarose along the bottom of the gel. All clips were removed from the gel frame, except for one flexible clip on each side of the frame. The polyacrylamide gel tank (Hoefer Scientific Instruments, San Francisco, U.S.A) was then filled with 1x TAE, and
the gel inserted and clipped into position, taking care that no air bubbles formed along the bottom of the gel. The comb was then removed, excess polyacrylamide flushed from the wells, and filled with 1x TAE. Loading dye was prepared by mixing 950 μl formamide, 50 μl of 0.5 M EDTA (pH 8) and 4 μl of bromophenol blue. Fifteen microliters of each sample was then mixed with 5 μl loading dye. Electrophoresis was performed at a constant voltage of 1.5 V/cm. Electrophoresis was stopped when the bromophenol blue dye had migrated two thirds of the way down the gel. When handling a gel containing radioactive samples, care was taken to always keep the gel behind a shield to prevent radiation reaching the user. The gel was then removed from between the two glass plates, and laid on plastic wrap. The well tops were then trimmed off using a sterile blade. If necessary, the gel was cut and trimmed smaller to fit into an X-ray exposure cassette, or inserted as is into a phosphoimaging cassette.

In order to expose X-ray film (Biomax, Kodak scientific imaging film, Eastman Kodak Company, Rochester, U.S.A) a radioactive polyacrylamide gel, the gel was placed in a X-ray exposure cassette (Cronex Quanta III X-ray Cassette, Dupont, Delaware, U.S.A) and the film exposed for 15 minutes. The gel and film were then removed from the cassette and the film developed using Ilford LL29 film developer (Harman technology Ltd. Cheshire, U.K.) following the manufactures instructions (Kodak).

To create a phosphoimage the gel was placed on one side of a cleaned phosphoimaging cassette with a cleaned and blanked phosphoimaging plate on the other (BAS cassette 2040, Fujifilm medical systems, Stanford, U.S.A). The cassette was closed and the phosphoimaging plate exposed to the gel for approximately 15 minutes. The gel was then removed from the cassette, and the exposed phosphoimaging plate read in a Storm phosphoimager (Amersham Biosciences, Uppsala, Sweden) following the manufacturer’s instructions.

### 2.2.6.5 Comparing salt-precipitation and affinity chromatography for purification of labelled PS-ODN

In order to compare the ability of salt precipitation and spin columns to separate unincorporated radiolabel from labelled PS-ODN, two ATS2 radiolabel reactions were
prepared as above. After incubation at 37°C for one hour, one labelling reaction was salt precipitated using the method described above. The other labelling reaction was cleaned up using Roche Mini Quick Spin Columns following the manufacturer’s instructions. Both reactions were then stored at -20°C O/N. The reactions were then run on a polyacrylamide gel and a phosphoimage created as described in section 2.2.6.4.

2.2.6.6 Scintillation counting of labelled PS-ODN

Once ATS2 has been labelled and purified using the Roche Mini Spin Columns, the labelling efficiency was determined. Using Millipore 0.45 µM membrane filters, 1 µl volumes of the γ^{32}P ATS2 were dotted on to separate membranes and the membranes placed into separate four ml scintillation vials (Kartell, Kartell Labware Division, Noviglio, Italy). Two milliliters of scintillation fluid (Optiphase Hi-safe 2, Perkin Elmer, Massachusetts, U.S.A) was then added to each vial, and the scintillation of each vial read using the γ^{32}P reading programme on the Quanta Smart scintillation counter by Perkin Elmer for Tri-Carb® liquid scintillation, and the amount of radiolabel incorporated into the ATS2 determined using the method described by Roche for use with the Roche Mini Spin Columns. Briefly, the amount of radioactivity associated with 1 µl of the purified γ^{32}P ATS2 elute from the labelling reaction was determined using a scintillation counter (Quanta Smart, Perkin Elmer). The total number of Counts Per Minute (CPM) of the purified γ^{32}P ATS2 was then divided by the CPM calculated for the amount of γ^{32}P added to the labelling reaction and the amount of associated radioactivity with the known concentration of PS-ODN was then determined. As an end labelling reaction ensures a maximum of one γ^{32}P can be attached to one PS-ODN molecule, the percentage of incorporation could be determined.

2.2.6.7 Protocol for the measurement of uptake of γ^{32}P-ATS2

An O/N culture of *S. mutans* OMZ175 was prepared in THB, and a 5% inoculum prepared and incubated until exponential phase was reached the protocol described in section 2.2.1.
Millipore 0.45 µM membrane filters (MF-Millipore, Massachusetts, U.S.A) were soaked in MilliQ until used. $\gamma^{32}$P ATS2 was diluted 1:10 using MilliQ, and the amount of radioactivity per ml determined using the scintillation counter and the protocol described above.

A checkerboard 96 well LCB NUNC plate was prepared with triplicate wells containing 150 µl of S. mutans OMZ175 in the appropriate growth phase and either 40 µl zoocin A (at the desired concentration) or 40 µl of MQ water; and either 10 µl $\gamma^{32}$P ATS2 (at the desired concentration) or 10 µl MQ water. Ten sets of each experimental condition were prepared and the contents of one complete set removed and placed into individual eppendorfs at 0, 0.5, 1.5, 2, 2.5, 3, 4, 5, 7, 10 and 12 hours. These eppendorfs were then spun at 15 x g for 10 minutes, the supernatant transferred to separate eppendorfs and labelled. The cell pellets were then washed three times with PBS, and each time the supernatant was transferred to separate eppendorfs and labelled. The cell pellets were then resuspended in 200 µl of PBS. The soaked 0.45 µM membrane filters were then transferred onto a Millipore Membrane Filter (1225 sampling manifold, Millipore, Massachusetts, U.S.A) and the top of the filter sealed. Using the resuspended pellets, each 200 µl amount was pipetted onto a separate membrane, and the membrane suction filter switched on. As soon as any visible liquid had passed through the membrane, each membrane was washed three times with 1 ml amounts of PBS. After the final wash, once dry the membranes were transferred using sterile forceps to individual 4 ml scintillation vials (Kartell). This was repeated until all cell pellets had been transferred to membranes. One microliter from each of the separate supernatants from each triplicate of each treatment type (for that time point) was then dotted onto separate individual 0.45 µM membrane filters and each placed into individual 4 ml scintillation vials. Scintillation fluid was then added to each vial, and the scintillation of each vial read using the $\gamma^{32}$P reading programme on the Quanta Smart scintillation counter by Perkin Elmer for Tri-Carb® liquid scintillation.

As well, at the same time separate experiments were set up to record optical density and viability of these treated cells. These two experiments were set up using the above protocols, using the same 5% inoculum created for the above radioactivity experiment, as well as the same zoocin A and ATS2. The one difference being that unlabelled ATS2 was used in these experiments rather than $\gamma^{32}$P-ATS2. Unlabelled ATS2 was used in these experiments due to
the fact that $\gamma^{32}$ P was not allowed outside of the isotope room, so could not be used in the Magellan plate reader for optical density readings. As the viability experiment used a large number of agar plates, permission was not granted for a viability study using $\gamma^{32}$ P-ATS2 in the isotope room, due to the large number of radioactive agar plates that would have been created.

2.2.6.8 Statistical analysis of radiolabel results.

Due to the short half-life of $\gamma^{32}$ P, for each day that $\gamma^{32}$ P-ATP was used the decay rate and changes in concentration of specific activity need to be determined using the Perkin Elmer radioactive decay calculator. This online calculator allows for the exact tracking of each vial of $\gamma^{32}$ P-ATP ordered and allows the calculation of concentration on day of use (mCi/ml) and specific activity on day of use (Ci/mmol). As the specific activity and µM concentration will change constantly due to decay, these calculations must be done for each experiment done on each separate day.

Using this data, the amount of $\gamma^{32}$ P associated with one CPM could be determined for each experiment. Using the percentage of radiolabel incorporation data as described above, the number of $\gamma^{32}$ P atoms associated with the PS-ODN could be determined and therefore the percentage of $\gamma^{32}$ P-ATS2 molecules determined. Using the CPM data generated by the scintillation counter together with the viability data, it was therefore possible to determine the number of ATS2 molecules associated with each CFU.

**Example A:** On the day of use it was calculated that $\gamma^{32}$ P-ATP had a concentration of 2.0209 mCi/ml and a specific activity of 822.0429 Ci/mmol.

Using this knowledge, the amount of $\gamma^{32}$ P-ATP added to that day’s labelling reaction and the amount of $\gamma^{32}$ P-ATP present in the ‘cleaned-up’ $\gamma^{32}$ P-ATS2 can be determined.

**First determine the activity of the $\gamma^{32}$ P-ATP in cpm/M**
The $\gamma^{32}$P-ATP was diluted 1/100 in RO water, and the amount of CPM associated with 1 µl volumes of this dilution determined. Five measurements were taken, and the average used.

\[ 1 \text{ µl of } 1/100 \gamma^{32} \text{P} = 44,460 \text{ cpm} \]

Therefore:

\[ 1 \text{ ml of } \gamma^{32} \text{P} = 4.446 \times 10^9 \text{ cpm} \]

It is known that on the day of use the concentration of $\gamma^{32}$P-ATP was 2.0209 mCi/ml.

Therefore we can state that:

\[ 2.0209 \text{ mCi } \gamma^{32} \text{P-ATP} = 4.446 \times 10^9 \text{ cpm} \]

\[ 2.0209 \text{ Ci } \gamma^{32} \text{P-ATP} = 4.446 \times 10^{12} \text{ cpm} \]

\[ 1 \text{ Ci } \gamma^{32} \text{P-ATP} = 2.2 \times 10^{12} \text{ cpm} \]

It is known that on the day of use the specific activity of $\gamma^{32}$P-ATP was 822.0429 Ci/mmol.

Therefore we can state that:

\[ \text{Ci } \gamma^{32} \text{P-ATP} = \frac{1 \text{ mM}}{822.0429} = 1000 \times 10^{-6} \text{ M} \]

\[ = 1.216 \times 10^{-6} \text{ M} \]

Therefore we can state that:

\[ 1.216 \times 10^{-6} \text{ M } \gamma^{32} \text{P-ATP} = 2.2 \times 10^{12} \text{ cpm} \]

\[ 1 \text{ M } \gamma^{32} \text{P-ATP} = \frac{2.2 \times 10^{12} \text{ cpm}}{1.216 \times 10^{-6}} \]

\[ 1 \text{ M } \gamma^{32} \text{P-ATP} = 1.809 \times 10^{18} \text{ cpm} \]
Second determine the activity of the $\gamma^{32}$P ATS2 in cpm/M

The concentration of cleaned up $\gamma^{32}$P ATS2 was 20 µM / 30 µl. This is a known concentration calculated by using a 200 µM ATS2 stock, and diluting in RO water. This final concentration of 20 µM / 30 µl is equivalent to $6.667 \times 10^{-7}$ M / µl and 1 µl of this preparation gave 146707 cpm.

Therefore: \[
1 \mu l \gamma^{32}P \text{ATS2} = \frac{146707 \text{ cpm}}{1.809 \times 10^{18} \text{ cpm/M}}
\]
\[
= \frac{81098 \text{ M}}{10^{18}}
\]
\[
= 8.11 \times 10^{-14} \text{ M}
\]

So we can now state that 1 µl of $\gamma^{32}$P ATS2 contains $8.11 \times 10^{-14}$ M of $\gamma^{32}$P.

Third determine the amount of label incorporated in each oligonucleotide

We know 1 µl of $\gamma^{32}$P ATS2 is equivalent to $6.667 \times 10^{-7}$ M

Therefore: \[
6.667 \times 10^{-7} \text{ M } \gamma^{32} \text{P ATS2} = 8.11 \times 10^{-14} \text{ M of } \gamma^{32} \text{P}.
\]
\[
6.667 \text{ M } \gamma^{32} \text{P ATS2} = 8.11 \times 10^{-7} \text{ M of } \gamma^{32} \text{P}.
\]
\[
1 \text{ M } \gamma^{32} \text{P ATS2} = 1.216 \times 10^{-7} \text{ M of } \gamma^{32} \text{P}.
\]

\[
1 \text{ M } \gamma^{32} \text{P-ATS2} = \frac{1 \gamma^{32} \text{P}}{1.216 \times 10^{-7} \text{ M}}
\]
\[
8.224 \times 10^{6} = 1 \gamma^{32} \text{P}
\]

So we can now state that 1 in $8.224 \times 10^{6}$ molecules of $\gamma^{32}$P ATS2 are labelled with an atom of $\gamma^{32}$P.
Determination of the numbers of ATS2 entering each *S. mutans* OMZ175 cell.

1) **Determination of the number of CPM per cell pellet for each treatment**

Treated cells were passed through a membrane, and the total CPM associated with these cells determined. By washing both the cells and membrane, only the $\gamma^{32}P$ ATS2 associated with the inside of the *S. mutans* OMZ175, regardless of treatment, was counted. Figure B shows that only those cells treated with zoocin A and $\gamma^{32}P$ ATS2 contain $\gamma^{32}P$.

2) **Calculation of the number of cpm per cfu for each timepoint**

The calculation to determine the number of CPM per CFU is as follows:

$$\text{CPM per CFU} = \frac{x \text{ CPM/ml}}{y \text{ CFU/ml}}$$

For Example A, at 3 hours past treatment addition, for those *S. mutans* OMZ175 cells treated with zoocin A + $\gamma^{32}P$ ATS2 there was found to be a viability count of 1018328674 CFU/ml (9.00788 Log CFU/ml) and a CPM/ml of 28689.

$$\text{CPM per CFU} = \frac{28689 \text{ CPM/ml}}{1018328671 \text{ CFU/ml}}$$

$$= 2.817 \times 10^{-5}$$

3) **Calculation of the number of molecules of ATS2 per cfu**

For Example A, it was calculated that there were $2.187 \times 10^{-5}$ CPM per CFU at 3 hours past treatment addition. Also we know from our previous calculations that on the day 1 M $\gamma^{32}P$ gave rise to $1.809 \times 10^{18}$ cpm.

Therefore: if 1 M $\gamma^{32}P$ ATS2 = $1.809 \times 10^{18}$ cpm.

$$2.187 \times 10^{-5} \text{ cpm} = 1.209 \times 10^{-23} \text{ M } \gamma^{32}P \text{ ATS2}$$

Therefore: there are $1.209 \times 10^{-23}$ M (or 7.28 molecules) of $\gamma^{32}P$ ATS2 per cfu.
Since we previously calculated that for every ATS2 labelled with a $\gamma^{32}$P radiolabel there are $8.224 \times 10^6$ ATS2 without a $\gamma^{32}$P radiolabel, therefore:

$$(1.209 \times 10^{-23} \text{ M}) \times (8.224 \times 10^6) = 9.942816 \times 10^{17}$$

Therefore there are $59,875,637.95 \ (5.98 \times 10^6)$ molecules of ATS2 per cfu.

This calculation was performed for each separate triplicate value recorded for each treatment type for each scintillation experiment. Thus a mean and standard deviation for the number of ATS2 molecules present per CFU could be generated for each time point and for each treatment type.

2.3  Use of zoocin A to facilitate entry of PS-ODN to S. mutans OMZ175

2.3.1  Establishment of the sub-lethal zoocin A concentration for S. mutans OMZ175

2.3.1.1  Establishment of growth of S. mutans OMZ175

A 10 ml overnight of S. mutans OMZ175 was used to prepare a five percent inoculum of S. mutans OMZ175 in fresh THB which was then incubated at 37°C in air supplemented with 5% CO₂. Each hour, for 24 hours, ten-fold serial dilutions to $10^{-8}$ were made in PBS, and the viability of these bacterial cultures determined using spread and spot plate techniques detailed in section 2.1.7. A growth curve was also carried out in a 96 well NUNC LCB plate using the technique described in section 2.1.7, with each well having 50 μl THB added in place of antisense and zoocin A. The length of the lag phase, and the points at which the exponential and stationary phases were reached were also determined.
2.3.1.2 Determining the sub-lethal concentration of zoocin A for \textit{S. mutans} OMZ175 at different growth phases

To establish the sub-lethal concentration of zoocin A to be used, each \textit{S. mutans} OMZ175 growth phase was incubated in the presence of zoocin A in microtitre plates (as described above). An O/N culture of \textit{S. mutans} OMZ175 was prepared and 5% sub-cultures prepared and incubated at 37°C until the desired OD was reached for each growth phase. These were labelled as stationary (OD of 0.7), exponential (OD of 0.3) and lag phase (OD of 0.13) respectively. Subsequent growth in each growth phase was monitored using the programme previously described in section 2.1.7. Viable counts were taken of each culture by the removal of 200 µl amounts every hour, followed by ten-fold serial dilutions to $10^{-8}$ in PBS, and the viability of these bacterial cultures determined using spread and spot plate techniques detailed in section 2.1.7.

Once the desired growth phases were reached, the cultures were divided into 15 ml amounts in silanized 20 ml glass universals, with THB and zoocin A then added to each growth phase to achieve zoocin A concentrations between 10 AU/ml and 0.02 AU/ml. A THB alone control was also included for each growth phase. For each growth phase, at each zoocin A concentration, ten-fold serial dilutions to $10^{-8}$ were made in PBS at 30 minutes, 1, 2, 4, 6, 8, 10, 12, 14, 16, 24 and 48 hours past zoocin A inoculation. A time zero control was also included for each growth phase. Viability counts of these bacterial cultures were then determined using the method detailed in section 2.1.7.

Once the desired growth phases were reached, a 96 well NUNC LCB plate was also prepared according to the above protocol detailed in section 2.1.7, with 150 µl of each sub-culture added to each well. Each growth phase culture was grown with THB alone and zoocin A concentrations between 10 AU/ml and 0.02 AU/ml. The 96 well NUNC plate was then incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.1.7. The zoocin A concentration selected for the exponential phase as sub-lethal was one that showed lytic activity, resulting in a significant (P <0.001) drop in optical density to the level of lag phase density without decreasing the OD of the culture at 18 hours in comparison to the untreated control. Statistical analysis of results was done using the method described in section 2.2.1.
2.3.2 **Strain inhibition by targeted and control antisense**

2.3.2.1 **Design of targeted antisense for *S. mutans* OMZ175**

The targeted antisense was first designed by Dufour et al. (2011). In brief the sequences of potential target genes were first found using genome databases such as NCBI BLAST, and primers were designed specifically to amplify the start sequences of each gene. Before the antisense sequences were designed, PCR followed by DNA sequencing was used to check the nucleotide sequence of each target gene in each targeted bacterial strain were similar. For the FBA target genes in *S. mutans* OMZ175, *S. aureus* Oxford and *E. faecalis* AR01/DGVS PS-ODN’s complimentary to bases 1-18, starting at the mRNA translational start sites, were synthesized by Sangon (Table 2.2).

2.3.2.2 **Design of control antisense.**

A control sequence was designed by the random generation of 18 bp sequences and BLAST searching each against the *S. mutans* UA159 genome sequence in order to ensure there were no matches. This control sequence was first designed and tested by Dufour et al., 2011. The sequence selected was chosen at random except for the requirement that its nucleotide ratio matched those of the target sequence, and was designated ATS2 control PS-ODN (Table 2.2).

2.3.2.3 **Synergistic effect of zoocin A and SM-FBA on *S. mutans* OMZ175**

An O/N culture of *S. mutans* OMZ175 and 50 ml 5% inocula of stationary, exponential and lag phase cultures were prepared as described above (section 2.3.1.2). Once the growth phase of each culture had been reached, 6 ml volumes were placed into silanized 20 ml glass universals. Each respective growth phase was then tested with the addition of: THB alone; a sub-lethal concentration of zoocin A appropriate for that growth phase (Results, Table 4.2);
10 μM ATS2; 10 μM SM-FBA; sub-lethal zoocin A + 10 μM ATS2 and sub-lethal zoocin A + 10 μM SM-FBA antisense. For each treatment for each growth phase ten-fold serial dilutions to 10^-8 were made in PBS at times 0, 1, 2, 4, 5, 6, 7, 9, 13, 15, 16, 20 and 24 hours past zoocin A inoculation. A time zero control was also included for each growth phase. The viability of these bacterial cultures was determined using the methods described in section 2.1.7. Once the desired growth phases were reached a checkerboard 96 well NUNC LCB plate was prepared, and the bacterial growth monitored, according to the protocol described in section 2.1.7, with each growth phase being tested with: THB alone, a sub-lethal concentration of zoocin A appropriate for that growth phase (Results, Table 4.2), 10 μM ATS2, 10 μM SM-FBA, sub-lethal zoocin A + 10 μM ATS2 and sub-lethal zoocin A + 10 μM SM-FBA antisense.

2.3.2.4 Dose response of zoocin A and SM-FBA on lag phase S. mutans OMZ175

A 96 well NUNC LCB checkerboard titration plate was prepared, and the bacterial growth monitored, according to the protocol described in section 2.1.7 with the following exceptions. SM-FBA was added to each well at either 0 or 10 μM final concentration and zoocin A to 0, 0.02, 0.05, 0.1, 0.2, 0.3 or 0.4 AU/ml.

A further 96 well NUNC LCB checkerboard titration plate was prepared, and the bacterial growth monitored, according to the protocol described in section 2.1.7. Each well received a final concentration of either: 0, 1, 10, 20 or 40 μM SM-FBA; and zoocin A to final concentrations of 0.2 AU/ml.

2.3.3 RNA extraction from S. mutans OMZ175

2.3.3.1 RNA isolation
For each qPCR experiment, an O/N culture of *S. mutans* OMZ175 was made and a 5% inoculum prepared as described above. Using 1.7 ml aliquots, the RNA of each treated *S. mutans* OMZ175 culture was extracted using the phenol/ chloroform + zoocin A protocol described in section 2.2.4.2.

The RNA was then further purified and treated for DNase contamination by use of RNeasy mini columns (Qiagen, Germany), following the manufacturer’s instructions. In brief, each RNA sample was adjusted to a 100 µl volume using RNase-free water. Then 350 µl of buffer RLT was added to each sample and mixed well before the addition of 250 µl of absolute ethanol. Each sample was then transferred to an RNeasy Mini spin column and the column centrifuged at 8,000 x g for 15 seconds on the flow-through discarded. In order to eliminate genomic DNA contamination an on-column DNase digest was performed (Qiagen, Germany). Three hundred and fifty microliters of buffer RW1 was added to each column and the column centrifuged at 8000 x g for 15 seconds and the flow-through discarded. A DNase I incubation mix (comprising of 10 µl DNase stock solution mixed with 70 µl of buffer RDD) was added to each column membrane and incubated at RT for 15 minutes before repeating the previous buffer RW1 wash step. The column was then washed with 500 µl of buffer RPE, the column centrifuged at 8,000 x g for 15 seconds and the flow-through discarded. This step was then repeated using a 2 minute centrifugation step. The RNeasy mini spin column was placed in a new collection tube and centrifuged at 12,000 x g for 1 minute to eliminate any buffer carry over. Fifty microliters of RNase-free H2O was added directly onto the column membrane and the column was centrifuged at 8,000 x g for 1 minute in order to elute the RNA. The RNA was quantified by UV spectrophotometry as described above (section 2.2.4.1). The RNA was checked for DNA contamination using the set A *S. mutans* OMZ175 RT-PCR primers and PCR protocol designed above (see Appendix 2). In brief, one microliter amounts of either DNase treated RNA, or non-DNase treated RNA were used as the template for each PCR reaction, using the set A RT-PCR primer sets designed for each of the three genes of interest. FBA RT-PCR set A were: forward, 5’-gaacgccgctgaagtacga-3’ and reverse, 5’-catggaccataccctcactcg-3’, designed to amplify an internal fragment of the coding region from 614-713 bp of the *fba* gene of *S. mutans* OMZ175. The primer sequences for 16s rRNA RT-PCR set A were: forward, 5’-tgcgttagctccggcata-3’ and reverse, 5’-cgtggtagaagccaggatt-
3’, designed to anneal to an internal coding region from the 777-849 bp region of the 16srRNA gene of \textit{S. mutans} OMZ175. The primer sequences for GyrA RT-PCR set A were: forward, \textit{5’-gacgcaggcgcatatcaag-3’} and reverse, \textit{5’-ccgcaatagtgagacagataccat-3’}, corresponding to the 1182-1282 bp region of the gyrase A gene of \textit{S. mutans} OMZ175. The resulting products were then visualised on an 1.5% agarose gel as described above.

2.3.3.2 \textit{Conversion of RNA to cDNA}

The RNA samples were then converted to cDNA by reverse transcription using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, USA) following the manufacturer’s instructions. An equal amount of total RNA from each treatment type, at each time point from within each growth phase analysed was used to produce comparable transcript levels of cDNA for RT-PCR analysis. RNA (100 ng) was mixed with 1 µl of 50 ng/µl random hexamer primers and 1 µl of 10 mM dNTP mix, and made up to 10 µl with RNase-free H$_2$O. This mixture was heated to 65°C for 5 minutes then incubated on ice for 1 minute. To this mixture was added 2 µl of 10x reverse transcriptase buffer, 4 µl of 25 mM MgCl$_2$, 2 µl of 0.1 M dithiothreitol (DTT), 1 µl of 40 U/µl RNase OUT and 1 µl of 200 U/µL SuperScript III. The sample was then incubated for 10 minutes at 25°C, followed by 50 minutes at 50°C. The reaction was terminated by heating the sample at 85°C for 5 minutes, and then chilled on ice. To each sample 1 µl of RNase H was added and the sample incubated at 37°C for 20 minutes. The cDNA preparations were stored at -20°C until needed. This RNA isolation and the subsequent RT-PCR were repeated in three experimental separate replicates, in order to produce triplicate experimental values.

2.3.4 \textit{RT-PCR}

2.3.4.1 \textit{Quantitative real-time PCR Assay}

Using the qPCR primers designed in section 2.2.3.3 to amplify up the three genes of interest (\textit{fba}, 16s rRNA and \textit{gyrA}) (section 2.2.3.2) real-time quantitative PCR were performed using the ABlolute™ QPCR SYBR® Green Low ROX Mix (ABgene, UK), and carried out in
MicroAmp® Fast Optical96-well plates (Applied Biosystems, USA). The reactions were set up in a final volume of 25 µL. RT-PCR primer sets A were used to amplify each of the three genes of interest (Appendix 2) as described above in section 2.3.3.1. Individual reactions contained 6.25 µl of ABsolute™ QPCR SYBR® Green Low ROX Mix, forward and reverse primers at a final concentration of 100 µM, 1 µl of a known amount of cDNA (100 ng for exponential phase experiments, 10 ng for lag phase experiments) and were made up to 25 µl with DNase-free water. All samples were assayed in duplicate to ensure assay reproducibility. No template controls with 1 µl of DNase-free water substituted for the sample cDNA were included for each primer pair to ensure that contamination did not occur.

The qRT-PCR reactions were carried out in an ABI prism® 7500 FAST Sequence Detection System Real Time PCR Thermocycler with Sequence Detection Software version 1.4 (Applied Biosystems, USA). The qRT-PCR protocol used was run in the 7500 Standard mode with the cycle profile as follows: 1 cycle at 95°C for 15 mins to activate the DNA polymerase, followed by 40 cycles of 95°C for 15 sec to denature the double stranded DNA and 60°C for 1 min for annealing and extension of the primers. Fluorescence was detected during the annealing and extension step of each cycle. The critical threshold cycle (Ct) was defined as the cycle at which the amplification generated fluorescence became detectable above the background noise. After the last amplification cycle, a dissociation protocol was performed as follows: One cycle of 95°C for 15 sec, followed by 60°C for 1 min. This dissociation was done to ensure that only single products of the correct size were being generated by the different primer pairs and that no products were produced in the no template control samples.

2.3.4.2 Statistical analysis of RT-qPCR data

Using RT-PCR, followed by analysis by the $2^{-\Delta\Delta Ct}$ method, a one-way analysis of variance (ANOVA) was performed using the $2^{-\Delta\Delta Ct}$ values to compare all time points and treatment types P values were generated using the Tukey multiple comparison test in order to establish whether pairs of $2^{-\Delta\Delta Ct}$ values were statistically different or not. All statistical analyses were performed using Prism Software™ and Microsoft Excel.
The ΔΔCt method\textsuperscript{200,303} was used to establish relative expression levels of the transcripts of interest. Based on the mathematics of real time PCR, the ΔΔCt method has been applied to calculate relative quantity of particular gene transcripts. A simple mathematical equation normalises ΔCt values to reference gene ΔCt values, thereby accounting for variation in cDNA concentrations. Normalising to an endogenous reference provides a method for correcting results for differing amounts of input RNA\textsuperscript{200}. The $2^{-\Delta\Delta\text{Ct}}$ method uses data generated as part of the real time PCR experiment to perform this normalisation function. The formula then compares these normalised samples to an appropriate control to generate a fold change ratio. Baseline values for each amplification curve and the threshold value for each set were set manually. The Ct values and the ΔRn values (background subtracted raw fluorescence data) for each sample were exported to a Microsoft Excel spreadsheet.

If the PCR efficiency of the sample is not 100% this may invalidate the results, and the ΔΔCt method assumes a PCR efficiency of 100%. To adjust for this, the individual amplification efficiencies of one particular primer pair for each set of primers on a plate were averaged to give a reliable amplification efficiency adjustment\textsuperscript{290}.

For these experiments the ΔRn values were analysed using LinReg PCR Software version 7.5 using linear regression to determine the PCR efficiency of each individual sample\textsuperscript{279}. This determines if any samples have not been amplified efficiently due to the presence of PCR inhibitors etc. Then all the individual PCR efficiencies for a particular primer pair were averaged to give a final PCR efficiency for that primer pair. This compensates for differences in the amplification efficiency of different primer pairs. The mean PCR efficiency was subsequently incorporated into the ΔΔCt equation for data analysis. Results were normalised between samples using the 16s rRNA expression level in order to generate ΔCt values. The ΔCt value describes the difference between the Ct value of the target gene and the Ct value of the corresponding endogenous reference housekeeping gene.

16sRNA was chosen as the normalising gene as it has been found to be a suitable gene to use by previous studies\textsuperscript{304,321,330}. The calibrator sample used to generate the ΔΔCt values for each treatment type for each individual repeat was the zoocin A only treated control at each time point, allowing the calculation of the fold change of expression when treated with antisense. The ΔΔCt method allows the comparison of the expression of each target transcript between
the different treatment groups, and allows the calculation in the average fold change for the control group being 1 (i.e. no change) and the expression levels of the different treatment/outcome groups given as a fold change relative to the average of the control group.

Statistical analysis for these results was carried out using GraphPad Prism Software version 5 (GraphPad Software, Inc.) and using the ΔCt or ΔΔCt values rather than the fold-change values, due to the fact that fold-change values do not follow a normal distribution. ΔCt and ΔΔCt values are effectively log transformed values and did not deviate significantly from a normal distribution. The results from each experimental set for each treatment type and time point were analysed using the one-way ANOVA with Tukey’s multiple comparison test as a post test. Significance is expressed as either \( p<0.05 \), \( p<0.01 \) or \( p<0.001 \).

### 2.3.4.3 Determination of the effect of zoocin A on gene transcription

An O/N culture of *S. mutans* OMZ175 was made and a 5% inoculum prepared as described above (section 2.1.7). The culture was divided into two aliquots, with one aliquot receiving an addition of zoocin A to a final concentration of 500 AU/ml. The two treatments were incubated at 37°C in air supplemented with 5% CO₂, with samples removed at 0, 0.5, 5 and 16 hours after treatment addition for RNA extraction and RT-qPCR, following the protocol described above. A 96 well NUNC LCB checkerboard plate was also prepared using the two cultures after zoocin A had been added although no further treatment was added to either culture. The 96 well NUNC plate was incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.1.7.

### 2.3.4.4 RNA isolation and RT-qPCR for lag or exponential phase *S. mutans* OMZ175

Fifty millilitres of a 5% inoculum of an overnight culture of *S. mutans* OMZ175 in THB was prepared. The culture was either used immediately (lag phase *S. mutans* OMZ175) or else incubated at 37°C until exponential phase (0.35 OD) was reached. Using either the lag phase or exponential phase cells, the culture was separated into six separate 8 ml aliquots and
treated with either: THB alone, a sub-lethal zoocin A concentration appropriate to the growth phase alone (Results, Table 4.2), 10 μM SM-FBA alone, 10 μM ATS2 alone, sub-lethal zoocin A + 10 μM SM-FBA or sub-lethal zoocin A + 10 μM ATS2. Samples were removed at 0, 0.5, 5 and 16 hours after treatment addition for RNA extraction and RT- qPCR, following the above protocol.

A 96 well NUNC LCB plate was also prepared using the above cultures after the various treatments had been added to either the lag or exponential phase S. mutans OMZ175. Two hundred microliters of each culture and treatment was added to triplicate wells. The 96 well NUNC plate was incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.1.7.

2.3.5 Effect of zoocin A upon S. mutans OMZ175

2.3.5.1 Determining the effect of PS-ODN upon the target gene sequence

In order to determine whether the presence of SM-FBA or ATS2 selected for mutations in the target fba gene, or the two control gyrA and 16s rRNA genes, a 5% inoculum of S. mutans OMZ175 was prepared and incubated at 37˚C in air supplemented with 5% CO₂ until exponential phase was reached using the above protocol. This inoculum was then divided into six equal volumes. To each, either a final concentration of 20 mM SM-FBA, 20 mM ATS2 or an equal volume of MilliQ was added. As well, to each a final sub-lethal concentration of zoocin A (Results, Table 4.2) was added, or an equal volume of MilliQ was added. A 1 ml time zero sample was taken from each treatment type, centrifuged at 18 x g for ten minutes, and the DNA extracted using the QIAGen Blood and Tissue Kit, following their protocol for Gram positive bacteria. A lysis step was added before the kit was used, which consisted of the addition of 100 μl of stock zoocin A (> 2000 AU/ml) to each cell pellet for 30 seconds, before the first treatment step. The remaining cultures were all incubated at 37˚C in air supplemented with 5% CO₂ until they reached an OD of 0.6. When each separate culture reached an OD of 0.6, the DNA was extracted from it using the above protocol.
A PCR of each of the three genes of interest was performed using the reaction mix and programme described in Tables 2.3 and 2.4. All reagents for PCR were obtained from Novagen (Novagen, Darmstadt, Germany). All primers used, and amplification conditions are given in Tables 2.3 and 2.4. Only primer pair 1 was used for 16sRNA and GyrA genes. Primer pairs 1 and 2 were used for the FBA gene.

A reaction mix comprised: 35.8 μl MQ water, 5 μl of x10 Taq buffer, 5 μl of 2 mM dNTP, 0.2 μl of Taq polymerase, 1.5 μl primer 1 (10 μM), 1.5 μl primer 2 (10 μM) and 100 ng of template (Table 2.3) Amplification was performed using the following parameters: Cycle 1, denaturation temperature of 95°C for two minutes; cycle 2, 94°C for two minutes; cycles 3-32, 94°C for 15 seconds; annealing temperature as defined in Appendix 2 for 30 seconds, and extension temperature of 72°C for the times defined in Appendix 2, cycle 33, denaturation temperature 94°C for 30 seconds, annealing temperature as defined in Appendix 2 for 30 seconds and elongation temperature of 72°C for 1 minute. The PCR product was purified using a QIAGEN PCR purification kit (QIAGEN) according to the manufacturer’s instructions, and product size determined by running 8 μl of product on a 2% agarose gel. The amount of product was determined using a nanodrop spectrophotometer (Nanodrop Technologies, Long Island, U.S.A) and the manufacturer’s instructions. Products were then sent for sequencing to the Massey DNA Sequencing Facility (Allen Wilson Centre, Massey University, Palmerston North, New Zealand), and the sequence analysed using SeqED software (DNASTAR Incorporated).

2.3.5.2 Determining whether repeated exposure to zoocin A results in resistance

A 5% inoculum was prepared and the culture incubated at 37°C and its growth monitored until exponential phase (0.35 OD) was reached. A 96 well NUNC LCB plate was then prepared according to the above protocol, with 150 μl of the exponential phase culture added to each well. One triplicate had the cells incubated with THB alone, whilst four sets (A, B, C and D) of triplicate wells were incubated in the presence of 2 AU/ml zoocin A. The 96 well NUNC plate was then incubated in the Infinite® plate reader using the programme previously described. When the cells treated with zoocin A began to recover, and their OD reached an
average of 0.3, 10 μl of the cell culture was removed from the well sets B, C and D, and a second fresh 10 μl volume of zoocin A added to give a final concentration of 2 AU/ml zoocin A per well. The 96 well NUNC plate was then incubated in the Infinite® plate reader using the programme previously described. This procedure was repeated twice, first with fresh zoocin A added to sets C and D, then finally with fresh zoocin A added only to D. The 96 well NUNC plate was incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.1.7.

### 2.3.5.3 Determining if repeated exposure to zoocin A and PS-ODN results in resistance

A 5% inoculum was prepared and the culture then incubated at 37°C and its growth monitored until exponential phase (0.35 OD) was reached. A 96 well NUNC LCB plate was then prepared according to the above protocol, with 150 μl of the exponential phase culture added to each well. As a control, wells were prepared with the cells incubated in the presence of THB only, SM-FBA only (10 mM) or ATS2 only (10 mM). To test for the development of resistance, wells were also prepared in which the exponential phase *S. mutans* OMZ175 cells received one, two, three or four doses of zoocin A (2 AU/ml). For cells that received just a single dose of zoocin A, wells containing either zoocin A alone, zoocin A + SM-FBA (10 mM), or zoocin A + ATS2 (10 mM) were set up. For wells in which multiple doses of zoocin A were to be delivered, each well was initially set up containing a single dose of zoocin A, or zoocin A + SM-FBA (10 mM), or zoocin A + ATS2 (10 mM). The 96 well NUNC plate was incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.1.7.

When any well containing cells treated with zoocin A or zoocin A + PS-ODN, began to recover, and the OD of the triplicate reached an average of 0.35, the second, third or fourth dose of zoocin A or zoocin A + PS-ODN was added as appropriate. This was done by removing 50 μl from each of the respective wells, and replacing it with 50 μl containing either zoocin A or zoocin A + PS-ODN as appropriate. The 96 well NUNC plate was incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.1.7.
2.3.6 Demonstrating the entrance of PS-ODN into S. mutans OMZ175

2.3.6.1 Uptake of $\gamma^{32}$P-ATS2 by exponential phase S. mutans OMZ175

An O/N culture of S. mutans OMZ175 was prepared in THB and a 5% inoculum prepared and incubated until exponential phase was reached. Using the protocol described in section 2.1.7, a checkerboard 96 well LCB NUNC plate was prepared using 40 µl zoocin A (1.4 AU/ml final concentration) and 10 µl (20 mM final concentration) $\gamma^{32}$ P-ATS2 and the experiment conducted following the novel protocol described above in section 2.2.6.7. Corresponding optical density and viability experiments were also conducted using the protocol described in section 2.1.7. This experiment was repeated twice, with each repeat done on a separate day using separate cultures. Statistical analysis was done using the novel protocol described in section 2.2.6.7.

2.3.6.2 Uptake of $\gamma^{32}$P-ATS2 by lag phase S. mutans OMZ175

An O/N culture of S. mutans OMZ175 was prepared in THB and a 5% inoculum prepared. S. mutans OMZ175 was then used in the lag phase for the following experiment. Using the protocol described above (Novel methods section- radiolabelling section) a checkerboard 96 well LCB NUNC plate was prepared using 40 µl zoocin A (0.2 AU/ml final concentration) and 10 µl (20 mM final concentration) $\gamma^{32}$ P-ATS and the experiment conducted following the novel protocol described above in section 2.2.6.7. Corresponding optical density and viability experiments were also conducted using the above novel protocol section 2.1.7. This experiment was repeated twice, with each repeat done on a separate day using separate cultures. Statistical analysis was done using the novel protocol described in section 2.2.6.8.

2.4 Use of alternative agents to facilitate entry of PS-ODN to gram-positive bacteria
2.4.1 Determination of the length of the lag phase for all other strains used

The identity of all strains used in the following experiments were confirmed using the 16S PCR protocol described in section 2.1.6. Following the above protocol in section 2.2.1 for determining the length of the lag phase for *S. mutans* OMZ175, 5% inocula of each strain (Table 2.3) were prepared and diluted ten-fold in 0.1% peptone water to $10^8$ as described in section 2.1.7. One hundred microliters of each dilution for each strain type was spread on an appropriately labelled CAB plate, and incubated as described in Table 2.3 before colonies were counted. A growth curve of each bacterial strain was also carried out using a 96 well NUNC plate which was incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.1.7. Each well having 50 µl of the appropriate broth (Appendix 2) added in place of antisense and zoocin A.

2.4.2 PCR screening for FBA and ATS2 binding sites

Using NCBI BLAST software, a sequence search was conducted to investigate the first 18 bp of the putative *fba* gene present in *E. faecalis* AR01/DGVS. This gene has been found to be an essential gene for *Streptococcus pneumoniae* (Song et al. 2005) and *S. mutans* OMZ175 (Dufour et al. 2011). A PCR that spanned the first 18 bp of the start region of the gene was performed using the reaction mix and programme described below and in Appendix 2. All reagents for PCR were obtained from Novagen (Novagen, Darmstadt, Germany). All primers used are given in Appendix 2. A reaction mix consisted of: 35.8 µl MQ water, 5 µl of x10 Taq buffer, 5 µl of 2 mM dNTP, 0.2 µl of Taq polymerase, 1.5 µl each of forward primer (10 µM) and its corresponding reverse primerpair (10 µM) and 100 ng of template (Table 2.3). Amplification was performed as described by Nekhotiaeva et al., (2004), using the following parameters: Cycle 1, denaturation temperature of 95°C for two minutes; cycle 2, 94°C for two minutes; cycles 3-32, 94°C for 15 seconds; annealing temperature of 45°C for 30 seconds, and extension temperature of 72°C for 80 seconds; cycle 33, denaturation temperature 94°C for 30 seconds, annealing temperature 45°C for 30 seconds and elongation
Table 2.3: Growth conditions for each bacterial strain used in this study

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Media type</th>
<th>Incubation</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> M15 ZooA1</td>
<td>Luria broth (LB)</td>
<td>24</td>
<td>37</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em> AR01/DGVS</td>
<td>LB</td>
<td>24</td>
<td>37</td>
</tr>
<tr>
<td><em>Streptococcus mutans</em> OMZ175</td>
<td>Todd Hewitt broth (THB)</td>
<td>48</td>
<td>37</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> Oxford</td>
<td>Mueller Hinton broth (MH)</td>
<td>24</td>
<td>37</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> WSSP-1</td>
<td>MH</td>
<td>24</td>
<td>37</td>
</tr>
<tr>
<td><em>Micrococcus leuteus</em> IL1</td>
<td>LB</td>
<td>24</td>
<td>37</td>
</tr>
</tbody>
</table>
temperature of 72°C for 80 seconds. The PCR product was purified using a QIAGEN PCR purification kit (QIAGEN) according to the manufacturer’s instructions, and product size determined by running 8 μl of product on a 2% agarose gel. The amount of product was determined using a nanodrop spectrophotometer (Nanodrop Technologies, Long Island, U.S.A) and the manufacturer’s instructions. Products were then sent for sequencing to the Massey DNA Sequencing Facility (Allen Wilson Centre, Massey University, Palmerston North, New Zealand), and the sequence analysed using SeqED software (DNASTAR Incorporated). Sequences were aligned and an 18 bp region starting at the ATG start codon of the fba gene for E. faecalis was identified. A PS-ODN antisense oligonucleotide complimentary to the corresponding mRNA sequence was designed and called E. faecalis FBA specific PS-ODN (EF-FBA) (Appendix 2) and ordered from Sangon. Using the consensus sequence for S. aureus from PubMed an antisense sequence for the fba gene in S. aureus Oxford was designed (SA-FBA).

2.4.3 Use of other inhibitory agents as antisense delivery agents

Alternative delivery agents were also tested for their ability to deliver PS-ODN into S. mutans OMZ175. A list of molecules tested is given in Table 2.4.

2.4.4 Establishment of sub-lethal pediocin concentrations for S. mutans OMZ175

A 0.1 mg/ml solution of pediocin (Sigma) in 100 mM sodium acetate (pH = 5) was prepared. An O/N culture of S. mutans OMZ175 and a 5% inoculum was prepared as described above. A 96 well NUNC LCB plate was prepared according to the above protocol with pediocin in place of zoocin A. Using pediocin stock, triplicate wells each received pediocin concentrations of either: 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, 1.9, 0.97, 0.00488 or 0 μg/ml. The 96 well NUNC plate was then incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.1.7.
Table 2.4: Alternative delivery agents tested for synergism with antisense

<table>
<thead>
<tr>
<th>Type of molecule</th>
<th>Name</th>
<th>MW</th>
<th>MOA</th>
<th>Reported as lytic (Ref)</th>
<th>Reported in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lytic enzyme</td>
<td>Zoocin A</td>
<td>27 kDa</td>
<td>endopeptidase</td>
<td>Simmonds 1997&lt;sup&gt;312&lt;/sup&gt;</td>
<td>This study</td>
</tr>
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<td></td>
<td>Lysostaphin</td>
<td>25 kDa</td>
<td>endopeptidase</td>
<td>Browder 1965&lt;sup&gt;54&lt;/sup&gt;</td>
<td>Chua (2008)</td>
</tr>
<tr>
<td></td>
<td>Lysozyme</td>
<td>14.7 kDa</td>
<td>glycoside hydrolase</td>
<td>Blake 1967&lt;sup&gt;46&lt;/sup&gt;</td>
<td>Chua (2008)</td>
</tr>
<tr>
<td></td>
<td>Mutanolysin</td>
<td>23 kDa</td>
<td>endoacetylmuramidase</td>
<td>Calandra 1980&lt;sup&gt;59&lt;/sup&gt;</td>
<td>Chua (2008)</td>
</tr>
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<td>Lipids</td>
<td>Monolaurin</td>
<td>274.4 g/mol</td>
<td>lipophilic/lysogenic</td>
<td>Oh, 1993&lt;sup&gt;252&lt;/sup&gt;</td>
<td>Chua (2008)</td>
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<td></td>
<td>(lauricidin)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peptide antibiotics</td>
<td>Nisin</td>
<td>34000 g/mol</td>
<td>Pore-former</td>
<td>Henning 1986&lt;sup&gt;147&lt;/sup&gt;</td>
<td>This study and Chua (2008)</td>
</tr>
<tr>
<td></td>
<td>Pediocin</td>
<td>16.5 kDa</td>
<td>Pore-former</td>
<td>Asaduzzaman&lt;sup&gt;13&lt;/sup&gt;</td>
<td>This study and Chua (2008)</td>
</tr>
<tr>
<td>Chemical antibiotics</td>
<td>Penicillin</td>
<td>334.3 g/mol</td>
<td>B- lactam</td>
<td>Novak 2000&lt;sup&gt;250&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>Vancomycin</td>
<td>1449.2 g/mol</td>
<td>B- lactam</td>
<td>Sinha 1968&lt;sup&gt;313&lt;/sup&gt;</td>
<td>This study</td>
</tr>
</tbody>
</table>
2.4.5 Establishment of sub-lethal nisin concentrations for S. mutans OMZ175 and E. faecalis AR01/DGVS

O/N cultures of S. mutans OMZ175 and E. faecalis AR01/DGVS were prepared, a 5% inoculum, and an exponential phase culture of each were prepared as described in section 2.3.1.2. A 96 well NUNC LCB plate was prepared according to the above protocol with the exception that each well containing S. mutans OMZ175 received a final concentration of either: 0, 0.15, 0.3, 0.6, 1.2, 2.5, 5.0, or 10.0 mg/ml of nisin in place of zoocin A. An HCl only control was also included. For E. faecalis, the nisin concentrations tested were doubling dilutions of nisin from 25 mg/ml to 0.15 mg/ml. The 96 well NUNC plate was then incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.1.7.

2.4.5.1 Nisin as an PS-ODN delivery agent in S. mutans OMZ175

An O/N culture of S. mutans OMZ175, a 5% inoculum, and an exponential phase culture were prepared as described above in section 2.2.1. A 96 well NUNC LCB plate was prepared according to the above protocol with the bacterial strain being tested with: THB alone, 1.2 mg/ml nisin, 20 μM SM-FBA, 20 μM ATS2, 1.2 mg/ml nisin + 20 μM SM-FBA, 1.2 mg/ml nisin + 20 μM ATS2. The 96 well NUNC plate was then incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.1.7.

2.4.5.2 Titration of differing nisin concentrations with PS-ODN

It was necessary to determine whether nisin and PS-ODN interact with each other, resulting in a reduction in the inhibitory effect of either agent. The spot test used was similar to that used for determining the titre of zoocin A (Section 2.1.7). The 1 mg/ml nisin stock was serially diluted two-fold seven times in 0.02 M HCl. Each dilution was then divided into two aliquots, to one of which SM-FBA was added to a final concentration of 20 μM. Twenty microliters of each dilution was spotted onto a CAB agar plate and spots were allowed to
absorb. A lawn of *M. leuteus* was swabbed onto each plate and the plates were incubated at 37°C in air supplemented with 5% CO$_2$ for 24 hours. The diameter of the zone of inhibition of each spot was then recorded and compared.

### 2.4.5.3 Titration of nisin with increasing PS-ODN

Using the spot test as described above (section 2.1.7) and the concentration of nisin deemed to cause the smallest measurable zone of inhibition of *M. leuteus* (0.0312 mg/ml) (Results, Table 5.4), the ability of nisin to inhibit *M. leuteus* when titrated with increasing concentrations of SM-FBA was determined. A two-fold dilution of the 1 mg/ml nisin stock down to 0.0312 mg/ml was prepared using 0.02 M HCl and the resultant 0.0312 mg/ml dilution divided into equal aliquots. Using a 100 µM solution of SM-FBA, these aliquots then received final concentrations of either; 0, 1, 5, 10, 20 or 50 µM SM-FBA. Twenty microliters of each nisin dilution, and also of each 0.0312 mg/ml nisin dilution containing a defined amount of SM-FBA, was spotted onto a CAB agar plate and spots were allowed to absorb. A lawn of *M. leuteus* was swabbed onto each plate and plates were incubated at 37°C in air supplemented with 5% CO$_2$ for 24 hours. The diameter of the zone of inhibition of each spot was then recorded and compared.

### 2.4.6 Beta-lactams as an PS-ODN delivery agent

#### 2.4.6.1 Establishment of sub-lethal penicillin or vancomycin concentrations for *S. aureus* Oxford and WSSP-1, *S. mutans* OMZ175 and *E. faecalis* AR01/DGVS

A 96 well NUNC LCB checkerboard titration plate was prepared as described previously with wells containing either lag phase *S. mutans* OMZ175, *S. aureus* WSSP-1, *S. aureus* Oxford or *E. faecalis* AR01/DGVS. These strains were incubated in the appropriate media listed in Table 2.3. Either: 0, 12.5, 25, 50 or 100µg/ml of penicillin or 0.1, 1.0, 10, 25, 50 or 100µg/ml vancomycin were added to wells for each bacterial strain. Both *S. aureus* strains
were also incubated in the presence of ten-fold dilutions of vancomycin from 1- 0.0001 mg/ml. The 96 well NUNC plate was then incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.1.7.

2.4.6.2 Establishment of a sub-lethal penicillin concentration for S. aureus Oxford and S. mutans OMZ175

A 96 well NUNC LCB checkerboard titration plate was prepared as described previously with either exponential phase S. mutans OMZ175 in TSB or S. aureus Oxford in MH added with either 0, 0.0025, 0.0005, 0.01, 0.02, 0.03 or 0.04 µg/ml penicillin. The 96 well NUNC plate was then incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.1.7.

2.4.6.3 Establishment of sub-lethal penicillin concentrations for exponential and phase S. mutans OMZ175 in unbuffered TSB

To establish the sub-lethal concentration of penicillin to be used, exponential phase S. mutans OMZ175 was incubated in the presence of penicillin in microtitre plates using the method described in section 2.4.6.1. An O/N culture of S. mutans OMZ175 was used to prepare 5% subcultures which were incubated at 37°C until the exponential phase was reached. This was determined using the method described in section 2.2.1.

A 96 well NUNC LCB plates was prepared according to the above protocol, with 150 µl of exponential phase culture added to each well. Each culture was grown with penicillin added at concentrations of either: 500, 250, 125, 62.5, 31.25, 15, 10, 5, 2.5, 1, 0.5, 0.1, 0.05, 0.025 and 0 µg/ml. The 96 well NUNC plate was then incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.1.7.

2.4.7 Use of buffered media for penicillin
2.4.7.1 Preparation of TSB with potassium phosphate buffer

A 96 well NUNC LCB checkerboard titration plate was prepared as described previously with lag phase cells grown in either TSB, or in TSB buffered with 10, 5, 2.5, 1, 0.5, 0.1, 0.05 or 0.025 M potassium phosphate buffer (pH 7.22). A second 96 well NUNC LCB checkerboard titration plate was prepared with the lag phase cells grown in either TSB, Antibiotic media number three, either TSB or Antibiotic media number three each buffered with 0.05M tris citrate buffer, or TSB buffered with 0.05 M potassium phosphate buffer (pH 7.22). The 96 well NUNC plate was then incubated in the Infinite® plate reader and the OD monitored using the programme previously described in section 2.1.7.

2.4.7.2 The buffering capacity of 0.05 M potassium phosphate in TSB

A 96 well NUNC LCB checkerboard titration plate was prepared as described previously with lag phase cells added to TSB buffered with 0.05 M potassium phosphate buffer (pH 7.22). The plate was incubated at 37°C in air supplemented with 5% CO2 for 48 hours. At hours 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 24 and 48 three lots of 200 µl amounts were removed, and using pH strips (Whatman®) the pH determined and recorded.

2.4.7.3 Exponential phase S. mutans OMZ175 growth in buffered TSB

S. mutans OMZ175 was grown O/N in TSB, and a 5% inoculum in TSB buffered with 0.05 M potassium phosphate buffer (pH 7.22) was prepared. A 96 well NUNC LCB checkerboard titration plate was prepared and the OD monitored as described previously in section 2.1.7.

2.4.7.4 Establishment of sub-lethal penicillin concentrations for lag, exponential and stationary phase S. mutans OMZ175 in buffered media
An O/N culture of *S. mutans* OMZ175 in TSB was used to prepare a 5% inoculum in TSB buffered with 0.05 M potassium phosphate buffer and lag, exponential and stationary phase cultures were prepared as described above (section 2.2.1). A 96 well NUNC LCB checkerboard titration plate was prepared as described previously. Concentrations of penicillin for lag phase were 0.01, 0.02, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.75, 2.5, 5, 8 and 10 µg/ml. The exponential phase cells were treated with penicillin at concentrations of 0.025, 0.05, 0.1, 0.5, 1, 2.5, 5 and 10 µg/ml whilst stationary phase cells were treated with penicillin at concentrations of 2, 10, 20, 100, 250 and 500µg/ml. The results were monitored by examining both the optical density and viability of each treatment, for each growth phase, using the protocols described in section 2.1.7.

2.4.7.5 Synergism between penicillin and PS-ODN when treating lag, exponential and stationary phase *S. mutans* OMZ175 in buffered media.

An O/N culture of *S. mutans* OMZ175 in TSB, a 5% inoculum into 0.05 M potassium phosphate buffered media (section 2.4.7.3) and lag, exponential and stationary phase cultures were prepared as described above (section 2.2.1). The sub lethal concentrations of penicillin determined for each growth phase in buffered media (Results 5.5.1) were then used in conjunction with both SM-FBA and ATS2 to check for synergism between the two. Briefly; lag, exponential and stationary phase cultures were all divided into 6 ml amounts and these placed into separate silanized 20 ml glass universals. Each growth phase was then tested with the addition of: TSB with 0.05 M potassium phosphate; the sub-lethal concentration of penicillin determined for that growth phase (Results 5.5.1), 10 µM ATS2; 10 µM SM-FBA; sub-lethal penicillin + 10 µM ATS2 and sub-lethal penicillin + 10 µM SM-FBA. For each treatment ten-fold serial dilutions to $10^{-8}$ were made in PBS at times 0, 2, 4, 6, 8, 10, 12, 14, 16, 24 and 48 hours post penicillin inoculation. The viability of the treated cultures for each time point was determined using the method described in section 2.1.7.

A checkerboard 96 well NUNC LCB plate was also prepared according to the above protocol with the lag phase culture being tested with: THB alone, a sub-lethal concentration of
penicillin (Results 5.5.1), 10 μM ATS2, 10 μM SM-FBA, sub-lethal concentration of penicillin + 10 μM ATS2 and sub-lethal penicillin + 10 μM SM-FBA. The 96 well NUNC plate was then incubated in the Infinite® plate reader and the OD monitored using the programme previously section 2.1.7.

2.4.7.6 Dose response for S. mutans OMZ175 and penicillin

A 96 well NUNC LCB checkerboard titration plate was prepared for exponential phase S. mutans OMZ175 as described above in section 2.4.7.4 with the following exceptions. SM-FBA was added to each well at either 0 or 10 μM final concentration and penicillin to either: 0, 0.05, 0.1 or 0.2 μg/ml. The 96 well NUNC plate was then incubated in the Infinite® plate reader and the OD monitored using the programme previously section 2.1.7.

A further 96 well NUNC LCB checkerboard titration plate was prepared according to the above protocol with each well receiving a final concentration of: 0, 1, 5, 10, 20 or 40 μM SM-FBA; and penicillin to a final concentration of 0.1 μg/ml. The 96 well NUNC plate was then incubated in the Infinite® plate reader and the OD monitored using the programme previously section 2.1.7.

2.4.7.7 Multiple dose response to penicillin and PS-ODN of exponential S. mutans OMZ175

A 5% inocula was prepared using the protocol described above, and the culture then incubated at 37°C and its growth monitored until exponential phase (0.35 OD) was reached. A 96 well NUNC LCB plate was prepared according to the above protocol, with 150 μl of the exponential phase culture added to each well. As controls, wells were prepared with the cells incubated in the presence of TSB only, SM-FBA (10 mM) or ATS2 (10 mM). To test for the development of resistance, duplicate wells were prepared which contained penicillin alone (0.1 μg/ml), penicillin + SM-FBA (10 mM) or penicillin + ATS2 (10 mM). The 96 well NUNC plate was then incubated in the Infinite® plate reader and the OD monitored using the programme previously section 2.1.7.
After 6 hours of incubation, a fresh dose of penicillin (0.1 µg/ml), or penicillin + either PS-ODN was added to the second set of duplicate wells that had been prepared. This was done by removing 50 µl from each of the respective wells, and adding in a fresh 50 µl containing either penicillin or penicillin + either PS-ODN as appropriate, with the 50 µl volume made up as described in the above 96 well plate protocol. The 96 well NUNC plate was then incubated in the Infinite® plate reader and the OD monitored using the programme previously section 2.1.7.

2.4.8 RNA isolation and RT-qPCR for lag phase or exponential phase S. mutans OMZ175 treated with penicillin

Fifty millilitres of a 5% inoculum of an overnight culture of S. mutans OMZ175 in THB was prepared. The culture was either used immediately (lag phase S. mutans OMZ175) or else incubated at 37°C until exponential phase (0.35 OD) was reached. Using either the lag phase or exponential phase cells, the culture was separated into six separate 8 ml aliquots and treated with either: THB alone, a sub-lethal penicillin concentration appropriate to the growth phase alone (Results section 5.5.1, Table 5.10). 10 µM SM-FBA alone, 10 µM ATS2 alone, sub-lethal zoocin A + 10 µM SM-FBA or sub-lethal zoocin A + 10 µM ATS2. Samples were removed at 0, 0.5, 5 and 16 hours after treatment addition for RNA extraction and RT-qPCR, following the above protocols described in section 2.3.3.1 and section 2.3.4.1.

A 96 well NUNC LCB plate was also prepared using the above cultures after the various treatments had been added to either the lag or exponential phase S. mutans OMZ175. Two hundred microliters of each culture and treatment was added to triplicate wells. The 96 well NUNC plate was then incubated in the Infinite® plate reader and the OD monitored using the programme previously section 2.1.7.

2.4.9 Demonstrating the entrance of PS-ODN into S. mutans OMZ175 treated with penicillin
Fifty millilitres of a 5% inoculum of an overnight culture of *S. mutans* OMZ175 in THB was prepared. The culture was either used immediately (lag phase *S. mutans* OMZ175) or else incubated at 37°C until exponential phase (0.35 OD) was reached. For either growth phase, using the novel protocol described above in section 2.3.1.2 a checkerboard 96 well LCB NUNC plate was prepared using a sub-lethal penicillin concentration appropriate to the growth phase alone (Results section 5.5.1, Table 5.10) and 10 µl (20 mM final concentration) γ³² P-ATS2 and the experiment conducted following the novel protocol described above in section 2.1.7. Corresponding optical density and viability experiments were also conducted using the protocols described in section 2.1.7. This experiment was repeated twice for each growth phase, with each repeat done on a separate day using separate cultures. Statistical analysis was done using the novel protocol described in section 2.2.1.
3 Results: Development of novel methods

3.1 Development of novel methods

In order to compare AS-ODN delivery rates between different delivery molecules, and to monitor the effects of PS-ODN upon gene expression, various new methods needed to be developed during the course of this study. Whilst the study by Guo et al. examined the ability of targeted PS-ODN to prevent gftB protein production, no study has yet examined the effect of PS-ODN inhibition upon targeted gene expression in *S. mutans* OMZ175, or monitored the rate of PS-ODN delivery into the bacterial cell. qRT-PCR has frequently been used to determine the effects of AS-ODN, PNA or PMO delivery into other targeted bacterial strains. Before this method could be used in this study, the sequence of the target gene in *S. mutans* OMZ175 and its role in metabolism had to be determined, as well as an optimized RNA extraction method created for *S. mutans*. Before the rate of PS-ODN delivery into the bacterial cell could be determined, an efficient PS-ODN labelling protocol needed to be established.

3.2 Production of zoocin A

A heavy band corresponding in size to the molecular weight of zoocin A was seen in the load, wash 2 and elute 1 and 2 samples (Figure 3.1). It was not present in the load through or the first washes samples, but was present in the wash 2 and elute 1 and 2 samples (lanes 5 and 6). Zoocin A activity was present in the wash 2 fraction at greater than 2048 AU/ml and in the elute 1 sample at 1024 AU/ml. The SDS-PAGE gel was deliberately overloaded to allow visualisation of contaminants present in low amounts. No contaminants were observed.

3.3 Role of *fba* as an essential metabolic gene
Figure 3.1: Expression and purification of zoocin A on an SDS Page gel. Lane M, protein standard; lane 1, load; lane 2, load through 1; lane 3, load through 2; lane 4, wash 1; lane 5, wash 2; lane 6, elute 1. Molecular weight markers supplied by Pharmacia. (Pharmacia LKB Biotech, Piscataway, NJ, USA). Twenty microliters of each crude cell prep sample was loaded onto the gel. The gel was deliberately overloaded in order to show contaminates. The gel was stained using Biosafe Coomasie blue stain (BioRad) and destained in RO water overnight.
It was necessary to show that the glycolytic $fba$ was essential for the growth of $S.\ mutans$ OMZ175, and that in the absence of functional $fba$ no other metabolic cycle could be utilised. This ensures that any surviving bacteria, or those that recover from growth inhibition do so due to the lifting of repression of the $fba$ gene, rather than through the utilisation of alternative metabolic pathways. In order to determine whether $S.\ mutans$ OMZ175 was able to utilise any other energy source other than sugars, experiments were designed to compare the inhibitory effect of FBA mRNA targeted PS-ODN against $S.\ mutans$ OMZ175 grown in THB and minimal media supplemented only with sucrose. If the amount of inhibition between the two systems was similar, this would indicate that for $S.\ mutans$ OMZ175, the glycolytic pathway and expression of $fba$ are essential for growth.

3.3.1 Growth of $S.\ mutans$ OMZ175 in minimal TV media

First it needed to be determined which supplements would allow $S.\ mutans$ OMZ175 to grow in minimal TV media. Unsupplemented minimal TV media was unable to support the growth of $S.\ mutans$ OMZ175 (Table 3.1). TV media was able to support the growth of $S.\ mutans$ OMZ175 when supplemented with sucrose, but not when supplemented with pyruvate (Table 3.1). The growth of $S.\ mutans$ OMZ175 in TV + sucrose (either: 0.5, 1 or 2%) showed a significantly greater time lag time (time taken to reach the initial OD value + 0.1) compared to that in THB. When TV media was supplemented with 1% sucrose and 0.1% yeast, no significant difference in lag time was observed for $S.\ mutans$ OMZ175 growth when compared to growth in THB. Viability studies showed that $S.\ mutans$ OMZ175 grown in TV + sucrose + 0.1% yeast was able to achieve a final viable count similar to those achieved when grown in THB (Figure 3.2). All other supplements failed to support the growth of $S.\ mutans$ OMZ175 to grow in TV media both alone and when added in conjunction with 0.1% yeast extract (Table 3.1).

3.3.2 Determination of the sub-lethal concentrations of zoocin A for $S.\ mutans$ OMZ175
Table 3.1: Effect of different media upon *S. mutans* OMZ175 growth

<table>
<thead>
<tr>
<th>Growth Medium</th>
<th>Lag time (Hours) ± SD</th>
<th>Statistical significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>THB</td>
<td>5.9 ± 0.06</td>
<td>N/A</td>
</tr>
<tr>
<td>TV</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>TV + supplement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5% sucrose</td>
<td>7.2 ± 0.19</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>1% sucrose</td>
<td>7.1 ± 0.22</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>2% sucrose</td>
<td>7.3 ± 0.49</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.5% pyruvate</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>1% pyruvate</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>2% pyruvate</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>5% pyruvate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% α-ketoglutarate</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>1% sodium citrate</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>1% L-glycine</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>1% succinate acid</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>1% glutamic acid</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>1% 2-phosphophenylpyruvate</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>1% hexaglycine</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>0.1% yeast</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TV + 0.1% yeast + supplement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% sucrose</td>
<td>6.08 ± 0.19</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>1% pyruvate</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>5% pyruvate</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>1% α-ketoglutarate</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>1% sodium citrate</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>1% L-glycine</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>1% succinate acid</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>1% glutamic acid</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>1% 2-phosphophenylpyruvate</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>1% hexaglycine</td>
<td>NG</td>
<td>†</td>
</tr>
</tbody>
</table>
*All growth compared to that of *S. mutans* OMZ175 in THB

† Lack of growth in these tests meant that a growth curve could not be fitted so the data could not be statistically analysed

NG = No growth after 18 hours

The standard deviation is the standard error between the three triplicates done for each experiment.
Figure 3.2. Changes in the viability of *S. mutans* OMZ175 over time grown in minimal media supplemented with sucrose and/or yeast extract. Growth of *S. mutans* OMZ175 over time in the presence of: THB; TV; TV + 1% sucrose; TV + 1% sucrose + 0.1% yeast extract. The error bars represent the standard error between the three triplicates done for each experiment.
For *S. mutans* OMZ175 grown in sucrose/yeast supplemented TV media, it was necessary to determine the concentration of zoocin A required to give a sub-lethal effect, so that the effect of other antimicrobial agents used in combination with zoocin A could be determined (Methods 2.2.2). For lag phase *S. mutans* OMZ175 the concentration of zoocin A defined as sub-lethal was determined as that which gave rise to a substantial (between 3 and 6 hour) increase in the lag phase, yet did not reduce the final culture OD. A zoocin A concentration of 2.5 AU/ml significantly increased (P = 0.001) the lag phase of *S. mutans* OMZ175. Concentrations at 10 AU/ml or greater prevented growth completely. For exponential phase *S. mutans* OMZ175 cells a zoocin A concentration of 10 AU/ml was found to significantly increase the length of the recovery phase. Concentrations at 20 AU/ml or greater prevented growth completely. Stationary phase *S. mutans* OMZ175 was unaffected by the presence of up to 100 AU/ml zoocin A. The minimum inhibitory concentration (MIC) of zoocin A for each *S. mutans* OMZ175 growth stage was defined as the lowest zoocin A concentration to cause complete inhibition of visible (OD) growth over a 48 hour period. For *S. mutans* OMZ175 grown in TV media supplemented with sucrose/yeast, for either growth stage, the inhibition by zoocin A was found to be significantly less than the inhibition caused by zoocin A when *S. mutans* OMZ175 was grown in THB. This may be due to the fact that the presence of sucrose stimulates the production of extracellular polysaccharide (EPS) by *S. mutans*, which thickens the cell wall, helping to prevent the lytic activity of zoocin A.

### 3.3.3 Interaction between minimal media, antisense and zoocin A

No precipitation or increase in OD was observed when SM-FBA or ATS2 were added to TV + 0.5% sucrose + 0.1% yeast media, either alone or in conjunction with zoocin A. The addition of either PS-ODN to the supplemented TV media resulted in a lowering of the pH from 6.5 to 6 (Table 3.2). The addition of zoocin A either to sucrose/yeast supplemented TV media alone, or together with either PS-ODN, resulted in an increase of pH.

### 3.3.4 Effect of zoocin A and PS-ODN upon *S. mutans* OMZ175 in minimal media
Table 3.2: pH of media, with or without supplementation of zoocin A and/ or PS-ODN

<table>
<thead>
<tr>
<th>Addition to media</th>
<th>Media type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>THB</td>
</tr>
<tr>
<td>None</td>
<td>6.5</td>
</tr>
<tr>
<td>Zoocin A (2.5 AU/ml)</td>
<td>7.5</td>
</tr>
<tr>
<td>SM-FBA</td>
<td>6</td>
</tr>
<tr>
<td>ATS2</td>
<td>6</td>
</tr>
<tr>
<td>Zoocin A + SM-FBA</td>
<td>7</td>
</tr>
<tr>
<td>Zoocin A + ATS2</td>
<td>7</td>
</tr>
</tbody>
</table>
When either lag or exponential phase *S. mutans* OMZ175 were combined with the appropriate amount of zoocin A plus 10 µM SM-FBA, a far greater inhibitory effect upon both lag and exponential phase cells was shown compared to that for zoocin A alone (Table 3.3, Figure 3.3). When 10 µM ATS2 was combined with zoocin A, the inhibitory effect on growth of *S. mutans* OMZ175 in either growth stage was found to be no greater than that of zoocin A alone. When 10 µM of either: SM-FBA or ATS2 were added to *S. mutans* OMZ175 in either growth phase without zoocin A, no significant inhibitory effect was observed. The pH level of the media showed a slower decline in pH when zoocin A plus 10 µM SM-FBA was added (Figure 3.4) than was observed for *S. mutans* OMZ175 was treated with zoocin A alone. The pH level reflects the metabolic activity of the bacteria present and the corresponding amount of acid produced. These results demonstrate that the addition of 1% sucrose + 0.1% yeast to TV media resulted in a *S. mutans* OMZ175 growth and viability rate statistically similar to that of cells grown in THB. Whilst there is a significant difference in the amount of zoocin A required to produce a sub-lethal effect upon *S. mutans* OMZ175 when grown in TV media compared to THB (most likely due to the ability of sucrose to stimulate EPS production by *S. mutans*), the addition of SM-FBA and a sub-lethal concentration of zoocin A to both systems resulted in a statistically indistinguishable inhibition rates between the two – indicating that the recovery and growth of SM-FBA/zoocin A treated *S. mutans* OMZ175 grown in THB is not due to the utilisation of an alternative metabolic pathway.

### 3.4 Determining the gene sequences of the RT-PCR target genes in *S. mutans* OMZ175

In order to determine the effect that antisense was having upon RNA levels, a qRT-PCR method needed to be developed that would allow the determination of *S. mutans* OMZ175 FBA mRNA, 16s mRNA and GyrA mRNA expression levels. Development of a qRT-PCR method allowed the effect of different treatments on the expression of *S. mutans* OMZ175 FBA mRNA to be determined. Firstly, the sequences of the genes of interest in *S. mutans* OM175 were determined, and a protocol developed for optimum RNA extraction.
Table 3.3: The synergistic effect of zoocin A and SM-FBA upon *S. mutans* OMZ175 in supplemented TV media

<table>
<thead>
<tr>
<th>Zoocin A concentration (AU/ml) plus antisense (10 μM) in growth phase:</th>
<th>Lag or recovery time (Hours) ± SD</th>
<th>Statistical significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lag</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 + 0</td>
<td>6.6 ± 0.28</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + SM-FBA</td>
<td>6.9 ± 0.12</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0 + ATS2</td>
<td>6.7 ± 0.22</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>2.5 + 0</td>
<td>9.5 ± 0.42</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>2.5 + SM-FBA</td>
<td>15.4 ± 0.47</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>2.5 + ATS2</td>
<td>9.9 ± 0.65</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td><strong>Exponential</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 + 0</td>
<td>0.3 ± 0.07</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + SM-FBA</td>
<td>0.4 ± 0.13</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0 + ATS2</td>
<td>0.4 ± 0.04</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>10 + 0</td>
<td>1.5 ± 0.37</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>10 + SM-FBA</td>
<td>5.9 ± 0.29</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>10 + ATS2</td>
<td>1.4 ± 0.15</td>
<td>P &gt; 0.05</td>
</tr>
</tbody>
</table>

* All zoocin A and PS-ODN only values compared to the 0 + 0 value. All zoocin A + PS-ODN values are compared to their corresponding zoocin A only controls.

The standard deviation is the standard error between the three triplicates done for each experiment.
Figure 3.3. Effect of zoocin A and antisense upon *S. mutans* OMZ175 OD and viability in minimal media. Viability of lag phase (panel A) or exponential phase (panel B) *S. mutans* OMZ175 grown in minimal TV media supplemented with sucrose and yeast. Growth of *S. mutans* OMZ175 over time in the presence of: TV; 10 µM SM-FBA; 10 µM ATS2; 10 AU/ml zoocin A; 10 AU/ml zoocin A + 10 µM SM-FBA; 10 AU/ml zoocin A + 10 µM ATS2. The error bars represent the standard error between the three triplicates done for each experiment.
Figure 3.4. Effect of zoocin A and antisense upon the pH of *S. mutans* OMZ175 containing media. pH of lag (panel A) or exponential (panel B) phase *S. mutans* OMZ175 in minimal TV media supplemented with sucrose and yeast. pH over time in the presence of: ○ TV; □ 10 µM SM-FBA; ▼△▼ 10 µM ATS2; □ 10 AU/ml zoocin A; ▣ 10 AU/ml zoocin A + 10 µM SM-FBA; ▣ 10 AU/ml zoocin A + 10 µM ATS2.
3.4.1 Primer design for qPCR target genes

The amplification of the *S. mutans* OMZ175 fba, gyrA and 16s rRNA genes worked well for all primer sets, except for the Fwd GyrA 02 and Rev GyrA 02 primer set (Appendix 2), which failed to produce a product (Figure 3.5). The sequences obtained for *S. mutans* OMZ175 genes fba, 16s rRNA and gyrA are available on the data CD accompanying this thesis (McLeod data CD 2012). From these sequences (McLeod data CD 2012) two sets of qPCR primers were designed for each gene (Appendix 2). Both A and B qPCR primer sets for the *S. mutans* OMZ175 fba, gyrA and 16s rRNA genes gave clear single bands of the expected size, and sequence data showed each amplified the correct sequence (Figure 3.6). The product generated by 16s rRNA RT-PCR primer set B showed poor dissociation-indicative of multiple products. All dissociation curves are saved onto the data CD. The qPCR data for all other primer sets showed that the products produced by each demonstrated good dissociation. qPCR primer set A for each gene (fba, gyrA and 16s rRNA) was then chosen for further use in the qPCR studies.

3.5 RNA extraction from *S. mutans* OMZ175

3.5.1 RNA extraction with or without zoocin A

To minimise the high cost of antisense, it was desirable that as low a volume of bacterial cells as possible be used for these experiments. As well, treatment of *S. mutans* OMZ175 with zoocin A, or zoocin A and targeted PS-ODN dramatically decreased the number of bacterial cells available to extract RNA from- thus a protocol for optimum RNA extraction from *S. mutans* OMZ175 needed to be developed. Extraction of RNA from *S. mutans* OMZ175 using phenol/chloroform without the addition of zoocin A resulted in a poor amount of RNA been recovered. A five ml culture volume resulted in a RNA yield of only 29.35 ng/µl (Table 3.4). Streptococci have thick peptidoglycan layers that make up their cell well, which can make the lysis of such bacteria, and thus the extraction of DNA or RNA difficult. The addition of a zoocin A step to this recovery dramatically increased the amount of RNA recovered, due to
Figure 3.5. Amplification of qPCR target genes using PCR primers in order to determine the target gene sequence. Lane M, marker (Invitrogen, 100 bp DNA ladder); Lane 1, product formed by: 16s RNA Fwd/Rev set 01; Lane 2, 16s RNA Fwd/Rev set 02; Lane 3, 16s RNA Fwd/Rev set 03; Lane 4, FBA Fwd/Rev set 01; Lane 5, FBA Fwd/Rev set 02; Lane 6, FBA Fwd/Rev set 03; Lane 7, GyrA Fwd/Rev set 01; Lane 8, Gyr A Fwd/Rev set 02; Lane 9, GyrA Fwd/Rev set 03. Poor quality gel due to re-scanned image of a scanned gel photograph.
Figure 3.6. Amplification of qPCR target genes by qPCR primers. Lane M, marker (Invitrogen, 100 bp DNA ladder); Lane 1, product formed by: 16s rRNA RT-PCR Fwd/Rev Set A; Lane 2, 16s rRNA RT-PCR Fwd/Rev Set B; Lane 3, FBA RT-PCR Fwd/Rev Set A; Lane 4, FBA RT-PCR Fwd/Rev Set B; Lane 5, GyrA RT-PCR Fwd/Rev Set A; Lane 6, GyrA RT-PCR Fwd/Rev Set B. Poor quality gel due to re-scanned image of a scanned gel photograph.
Table 3.4: RNA extracted from *S. mutans* OMZ175 using phenol/chloroform

<table>
<thead>
<tr>
<th>Volume of cells (ml)</th>
<th>Amount of RNA extracted (ng/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5</td>
</tr>
<tr>
<td>1.5</td>
<td>5.7</td>
</tr>
<tr>
<td>1.7</td>
<td>4.1</td>
</tr>
<tr>
<td>2.5</td>
<td>6.3</td>
</tr>
<tr>
<td>5</td>
<td>29.4</td>
</tr>
</tbody>
</table>
the lytic nature of zoocin A upon *S. mutans* OMZ175. The addition of a zoocin A lysis step resulted in far greater RNA yields. A 1.7 ml culture volume was chosen as it allowed optimal RNA extraction in single eppendorf volumes. As the concentration of zoocin A added to each 1.7 ml amount of cells increased, so did the amount of RNA extracted (Table 3.5). A 1.7 ml volume treated with 8 AU/ml zoocin A resulting in a RNA yield of 521.7 ng/µl. 8 AU/ml zoocin A was then used in all RNA extraction experiments which used 1.7 ml volumes of cells.

### 3.6 Development of a protoplasting protocol

The determination of where within the bacterial cell antisense is located once up-taken, required the fractionation of treated bacteria. Unfortunately fractionation methods are often imperfect, and some cross-contamination between fractions is almost unavoidable. The most precise method of partial fractionation available for *S. mutans* at present is that of protoplasting followed by separation of the cytoplasmic contents from membranes. Protoplasts are bacterial cells which have had their peptidoglycan layer removed and are bound by a cytoplasmic membrane only and are therefore spheres irrespective of the shape of the parent bacterium.

#### 3.6.1 Sub-cellular fractionation of *S. mutans OMZ175*

The formation of protoplasts was unable to be observed directly, as neither dark-field, nor phase contrast, microscopy allowed the visualisation of cells.

#### 3.6.2 Protoplast formation on osmotic media

One of the prime requirements for the handling of protoplasts is an efficient buffer for protoplast maintenance during experimental manipulation. In this study a reversion medium was
Table 3.5: Amount of RNA extracted from *S. mutans* OMZ175 with a zoocin A lytic step included in the extraction protocol

<table>
<thead>
<tr>
<th>Amount of zoocin A (AU/ml)</th>
<th>Amount of RNA extracted (ng/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>32.4</td>
</tr>
<tr>
<td>2</td>
<td>85.1</td>
</tr>
<tr>
<td>4</td>
<td>230.7</td>
</tr>
<tr>
<td>6</td>
<td>321.1</td>
</tr>
<tr>
<td>8</td>
<td>521.7</td>
</tr>
<tr>
<td>10</td>
<td>519.8</td>
</tr>
<tr>
<td>12</td>
<td>508.3</td>
</tr>
</tbody>
</table>
used to promote the reversion of protoplasts to bacterial phase growth. This reversion medium was essentially a growth medium capable of supporting the rapid growth of streptococci, to which sugar was added as a stabilising agent. The basic principle of this technique is that protoplasts cannot reform their cell walls and re-grow without sugar being present. The sugar acts as a hypertonic buffer, preventing the osmotic pressure from bursting the protoplast and allowing the bacterial cell time to reform its peptidoglycan layer.

One idea was to monitor the formation of presumptive protoplasts through the use of OD. Zoocin A was added to whole *S. mutans* OMZ175 bacterial cells held in hypertonic buffer, and the cells monitored for a slight loss in OD (indicating removal of peptidoglycan) rather than a large loss of OD (indicating cell lysis).

### 3.6.2.1 Comparing growth rate on THA, RMS and RMR media

Untreated *S. mutans* OM175 was found to grow in equal numbers upon both 20% RMS (sucrose) agar, 20% RMR (raffinose) agar and THA (Table 3.6) indicating that neither 20% sucrose or raffinose significantly alters *S. mutans* OMZ175 growth compared with that on THA.

### 3.6.2.2 Presumptive protoplast formation by sub cellular fractionation

Whilst *S. mutans* OMZ175 cells subjected to sub-cellular fractionation using the method described by Kling 1989\(^{177}\) grew upon THA agar, after treatment none grew upon the RMS media. The treated *S. mutans* OMZ175 that grew upon the THA agar displayed an irregular appearance when compared to the untreated *S. mutans* OMZ175 grown upon THA.

### 3.6.2.3 Assay of zoocin A activity in sucrose containing media

Gram stains of zoocin A treated cells in hypertonic (sucrose) buffer revealed gram negative cocci which were predominantly in singular units, in comparison with the normal gram
Table 3.6: Plating efficiency of *S. mutans* OMZ175 in the presence of osmotic stabilising agents.

<table>
<thead>
<tr>
<th>Plating medium</th>
<th>Viable count</th>
</tr>
</thead>
<tbody>
<tr>
<td>THA</td>
<td>6.20 x 10^7</td>
</tr>
<tr>
<td>RMS (20%)</td>
<td>5.58 x 10^7</td>
</tr>
<tr>
<td>RMR (20%)</td>
<td>9.5 x 10^7</td>
</tr>
</tbody>
</table>
positive cocci in chains and clusters. However, the sugars present in the hypertonic buffer made it difficult to achieve regular staining as the sugar itself also seemed to absorb the stains. The cocci were not of a regular shape, but slightly swollen with bulges. That zoocin A had an effect on peptidoglycan layers was indicated by the observation that as the zoocin A concentration decreased from 20 AU/ml to 0 AU/ml, the number of gram positive cocci observed increased, whilst the number of gram negative cocci decreased and the shape of the cocci became more regular as the concentration of zoocin A decreased. For the *S. mutans* OMZ175 cells treated with between 5-20 AU/ml of zoocin A, an immediate drop in OD was observed (Figure 3.7). The addition of 10 and 20 AU/ml zoocin A caused an OD decrease significantly greater than that caused by 5 AU/ml zoocin A. All sucrose concentrations failed to show any major differences in the time taken for zoocin A to show a lytic effect upon *S. mutans* OMZ175 cells (Figure 3.8). Due to the lack of growth no sigmoidal curve could be fitted to the data. This graph indicates that it is probable that increasing concentrations of sucrose are failing to provide buffering protection, and thus it is doubtful protoplasts are forming. Note all measurements began with the same OD of cells for each treatment (0.35). However lysis by zoocin A often occurred before the measurements could begin.

3.6.2.4 Protoplast reversion efficiency using RMS

A value for the efficiency with which protoplasts reverted to cellular phase growth was obtained from a comparison of the number of colonies yielded on RMS compared to THA. The RMS was assumed capable of supporting the growth of colonies formed by cells already in the walled state and of colonies formed by protoplasts initially growing as L-form colonies prior to their reversion to cell-wall containing bacteria.

If we assume that the presumptive protoplasts are represented by the difference between treated and non-treated when plated on the THA, it can be calculated that there was 99.3% putative protoplast formation (Table 3.7). However, these missing cells might be either true protoplasts or completely dead and non-viable *S. mutans* OMZ175 cells. The counts upon RMS show that there is a 31.6% putative protoplast formation in RMS. However, no formation of protoplast revertants was found upon RMS from which we can conclude that the
Figure 3.7. Changes in OD over time caused by the addition of zoocin A to exponential phase *S. mutans* OMZ175 incubated in 30% hypertonic buffer (sucrose). *S. mutans* OMZ175 growth over time in the presence of: ▲ 0 AU/ml zoocin A; ▬ 1 AU/ml zoocin A; ▴ 5 AU/ml zoocin A; ▼ 10 AU/ml zoocin A; ○ 15 AU/ml; □ 20 AU/ml zoocin A.
Figure 3.8. Changes in the OD of exponential phase *S. mutans* OMZ175 suspended in increasing sucrose concentrations with (panel A) or without (panel B) zoocin A treatment. *S. mutans* OMZ175 growth over time in the presence of: ← 0% sucrose; → 1% sucrose; ▲ 5% sucrose; ▼ 10% sucrose; ▼ 20% sucrose. The error bars represent the standard error between the three triplicates done for each experiment.
Table 3.7: Protoplast formation and reversion efficiencies of zoocin A treated *S. mutans* OMZ175 in RMS

<table>
<thead>
<tr>
<th>Treatment Medium</th>
<th>Treatment</th>
<th>Before treatment Viable count of (CFU/ml)</th>
<th>After treatment</th>
<th>Putative protoplast formation (A) (THA)</th>
<th>Reversion (B) (RMS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sucrase</td>
<td>none</td>
<td>5.8 x 10^7</td>
<td>6.0 x 10^8</td>
<td>5.4 x 10^8</td>
<td>99.3%</td>
</tr>
<tr>
<td>(THA)</td>
<td>zoocin A</td>
<td>5.8 x 10^7</td>
<td>4.4 x 10^6</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td>6.01 x 10^7</td>
<td>3.8 x 10^8</td>
<td>1.3 x 10^8</td>
<td>31.6%</td>
</tr>
<tr>
<td>20%</td>
<td>zoocin A</td>
<td>6.01 x 10^7</td>
<td>2.6 x 10^8</td>
<td>0</td>
<td>0%</td>
</tr>
</tbody>
</table>

\(A\) Protoplast formation efficiency is calculated from a comparison of the original cell viability on THA to that cell viability, also on THA, after incubation with zoocin A. If per ml the original number of viable cells is A, and the number of viable cells post zoocin A incubation is B, then the number of protoplasts produced per ml, C, is given by the equation, \(A - B = C\), and the protoplast formation efficiency (as a percentage), is given by the equation \(C \times 100/A\).

\(B\) Protoplast reversion efficiency is the frequency with which protoplasts plated on RM give rise to colonial growth. Assuming the number of viable cells per ml yielded on RM to be D, and then the protoplast reversion efficiency (as a percentage) is given by the equation \(D \times 100/C\).
zoocin A treated cells were either killed or were unable to revert upon 20% sucrose RMS. Thus protoplast formation was unable to be confirmed.

3.6.2.5 Assay of lytic activity in hypertonic media with increasing concentrations of sucrose

All *S. mutans* OMZ175 cells held in a hypertonic sucrose buffer, no matter the sucrose concentration of the buffer, showed an immediate decrease in OD when treated with final concentrations of 32 AU/ml zoocin A. The amount of lysis caused by the addition of zoocin A decreased as the amount of sucrose increased. Due however to the lack of growth of those *S. mutans* OMZ175 cells treated with zoocin A, no sigmoidal curve could be fitted to the data. However, even in 20% sucrose + zoocin A, the OD dropped to below 0.2 OD after five minutes of incubation (Table 3.8). This indicates that sucrose is not providing adequate buffering of *S. mutans* OMZ175 once the peptidoglycan has been removed.

3.6.2.6 Protoplast reversion efficiency on increasing RMS concentrations

If we assume that the presumptive protoplasts are represented by the difference between treated and non-treated when plated on the THA, it can be calculated that there is 99.5% putative protoplast formation. These might be either protoplasts or completely dead and non-viable *S. mutans* OMZ175 cells. Counts show between a 32.5% putative protoplast formation for *S. mutans* OMZ175 cells held in 10% RMS, and a 37.1% putative protoplast formation for those cells held in 40% RMS. However, no formation of protoplast revertants was found upon RMS of any percentage concentration. A comparison between each sucrose concentration is given in Table 3.9. From this information it can be concluded that the zoocin A treated cells were either killed or were unable to revert upon sucrose RMS. Thus protoplast formation was unable to be confirmed.

It is possible that the ability of zoocin A to act upon the peptidoglycan layer of *S. mutans* OMZ175 had been affected by the presence of high sucrose concentrations. Sucrose
Table 3.8: Lytic activity (osmotic shock) of zoocin A upon *S. mutans* OMZ175 in sucrose containing hypertonic media

<table>
<thead>
<tr>
<th>Added sucrose concentration (%) with or without zoocin A (32 AU/ml)</th>
<th>Lag time (hours) ± SD†</th>
<th>Statistical significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 +0</td>
<td>1.3 ± 0.21</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + zoocin A</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>1 + 0</td>
<td>1.3 ± 0.52</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>1 + zoocin A</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>5 + 0</td>
<td>1.4 ± 0.37</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>5 + zoocin A</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>10 + 0</td>
<td>1.5 ± 0.23</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>10 + zoocin A</td>
<td>NG</td>
<td>†</td>
</tr>
<tr>
<td>20 + 0</td>
<td>2.4 ± 0.14</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>20 + zoocin A</td>
<td>NG</td>
<td>†</td>
</tr>
</tbody>
</table>

† Lack of growth in the zoocin A treated tests means that a growth curve could not be fitted so the lag time could not be determined

* Each sucrose concentration was compared to that of 0% sucrose concentration

NG = No growth after 18 hours

The standard deviation is the standard error between the three triplicates done for each experiment.
Table 3.9: Protoplast formation and reversion efficiency of *S. mutans* OMZ175 in increasing sucrose concentrations

<table>
<thead>
<tr>
<th>Treatment medium sucrose %</th>
<th>Treatment</th>
<th>viable count of (CFU/ml)</th>
<th>efficiency of Putative protoplast formation</th>
<th>Reversion efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before treatment</td>
<td>After treatment</td>
<td>THA</td>
<td>RMR</td>
</tr>
<tr>
<td>0%</td>
<td>None</td>
<td>$6.1 \times 10^7$</td>
<td>$5.8 \times 10^8$</td>
<td>$4.2 \times 10^8$</td>
</tr>
<tr>
<td></td>
<td>Zoocin A</td>
<td>$6.1 \times 10^7$</td>
<td>$3.01 \times 10^6$</td>
<td>0</td>
</tr>
<tr>
<td>10%</td>
<td>None</td>
<td>$7.06 \times 10^7$</td>
<td>$4.7 \times 10^8$</td>
<td>$5.4 \times 10^8$</td>
</tr>
<tr>
<td></td>
<td>Zoocin A</td>
<td>$7.06 \times 10^7$</td>
<td>$3.2 \times 10^8$</td>
<td>0</td>
</tr>
<tr>
<td>20%</td>
<td>None</td>
<td>$4.9 \times 10^7$</td>
<td>$9.4 \times 10^8$</td>
<td>$9.8 \times 10^8$</td>
</tr>
<tr>
<td></td>
<td>Zoocin A</td>
<td>$4.9 \times 10^7$</td>
<td>$5.1 \times 10^8$</td>
<td>0</td>
</tr>
<tr>
<td>30%</td>
<td>None</td>
<td>$6.1 \times 10^7$</td>
<td>$8.01 \times 10^8$</td>
<td>$3.7 \times 10^8$</td>
</tr>
<tr>
<td></td>
<td>Zoocin A</td>
<td>$6.1 \times 10^7$</td>
<td>$4.2 \times 10^8$</td>
<td>0</td>
</tr>
<tr>
<td>40%</td>
<td>None</td>
<td>$7.4 \times 10^7$</td>
<td>$6.2 \times 10^8$</td>
<td>$2.8 \times 10^8$</td>
</tr>
<tr>
<td></td>
<td>Zoocin A</td>
<td>$7.4 \times 10^7$</td>
<td>$3.9 \times 10^8$</td>
<td>0</td>
</tr>
</tbody>
</table>
stimulates the production of Extracellular PolySaccharide (EPS) in *S. mutans*, and it is possible that its production was preventing zoocin A from having a lytic effect. However, it is likely that if EPS production had occurred, this would have been observed as an increase in OD, whereas cells held in sucrose solutions, once treated with zoocin A, showed an immediate decrease in OD. For that reason, raffinose was then used as the osmotic stabilising agent, as it does not stimulate EPS production in *S. mutans* OMZ175.

### 3.6.3 Use of raffinose as a protoplasting buffer

*S. mutans* OMZ175 cells held in a hypertonic raffinose buffer (30%) showed an immediate decrease in OD when treated with final concentrations of 32 AU/ml zoocin A. This indicated that raffinose was allowing zoocin A to have a lytic effect upon the *S. mutans* OMZ175 cells. Due however to the lack of growth of those *S. mutans* OMZ175 cells treated with zoocin A, no sigmoidal curve could be fitted to the data.

#### 3.6.3.1 Comparing growth of *S. mutans* OMZ175 on THA and RMR media

*S. mutans* OM175 was found to grow in equal numbers upon both RMR media supplemented with either 20 or 30% raffinose and THA media (Table 3.10). Problems arose when using high concentrations of raffinose. At 40% raffinose concentration, the raffinose began to crystallise out of revertant agar. Even the storage of raffinose containing agar in a 37°C incubator failed to prevent crystallisation. However, from the number of colonies that could be counted on 40% RMR media, there appeared to 97% less growth of *S. mutans* OMZ175 compared to that on THA.

#### 3.6.3.2 Protoplast preparation and reversion using RMR
Table 3.10: Plating efficiency of *S. mutans* OMZ175 in the presence of increasing raffinose concentrations

<table>
<thead>
<tr>
<th>Percentage of raffinose</th>
<th>Viable count</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (THA)</td>
<td>4.9 x 10^7</td>
</tr>
<tr>
<td>20</td>
<td>3.8 x 10^7</td>
</tr>
<tr>
<td>30</td>
<td>7.1 x 10^7</td>
</tr>
<tr>
<td>40</td>
<td>1.1 x 10^6</td>
</tr>
</tbody>
</table>
When *S. mutans* OMZ175 was held in a hypertonic buffer containing 0, 20 or 30% raffinose, treatment with zoocin A resulted in a two-log decrease in the number cells, compared to the untreated control (Table 3.11). If we assume that the presumptive protoplasts are represented by the difference between treated and non-treated when plated on the THA, it can be calculated that there is 98.2% putative protoplast formation. These might be either protoplasts or completely dead and non-viable *S. mutans* OMZ175 cells. Counts show between a 96.5 – 99.8 % putative protoplast formation for *S. mutans* OMZ175 cells held in either: 20% or 30% RMR respectively. However, no formation of protoplast revertants was found upon either RMR agar concentration from which we can conclude that the zoocin A treated cells were either killed or were unable to revert upon RMS. Thus protoplast formation was unable to be confirmed. RMR media containing 40% raffinose could not be used to determine if any revertants had formed, due to the precipitation of raffinose through the media.

### 3.6.3.3 Assay of zoocin A lytic activity in a range of raffinose concentrations

All raffinose concentrations tested failed to show any major differences in the time taken for zoocin A to show a lytic effect upon *S. mutans* OMZ175 cells (Figure 3.9). Due to the lack of growth of *S. mutans* OMZ175 cells treated with zoocin A, no sigmoidal curve could be fitted to the data. The graph however indicates that it is probable that increasing concentrations of raffinose are failing to provide buffering protection, and thus it is doubtful that protoplasts are forming. Note that all measurements began with the same OD of cells for each treatment. However lysis by zoocin A often occurred before the measurements could begin. Whilst it was determined that zoocin A is able to cause a lytic effect in 30% raffinose containing media (unlike in 30% sucrose containing media), due to the inability to prevent raffinose crystallisation at higher concentrations it was then decided to no longer pursue the protoplasting idea.

It is possible that the presence of high sucrose/raffinose concentrations destroys protoplasted *S. mutans* OMZ175 by osmotic pressure, as when used in solid media the cells are incubated in its presence for over 24 hours. It also seems that non-protoplasted cells are not easily
Table 3.11: Protoplast formation and reversion efficiency of *S. mutans* OMZ175 in increasing RMR concentrations

<table>
<thead>
<tr>
<th>Treatment medium raffinose</th>
<th></th>
<th>viable count of (CFU/ml)</th>
<th></th>
<th>efficiency of Putative protoplast formation</th>
<th>Reversion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>before treatment</td>
<td>After treatment</td>
<td>THA</td>
<td>RMR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>None</td>
<td>2.1 x 10^8</td>
<td>3.02 x 10^8</td>
<td>1.4 x 10^8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zoocin A</td>
<td>2.1 x 10^8</td>
<td>5.4 x 10^6</td>
<td>0</td>
<td>98.2</td>
</tr>
<tr>
<td>20%</td>
<td>None</td>
<td>1.5 x 10^8</td>
<td>2.8 x 10^8</td>
<td>9.5 x 10^8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zoocin A</td>
<td>1.5 x 10^8</td>
<td>9.8 x 10^6</td>
<td>0</td>
<td>96.5%</td>
</tr>
<tr>
<td>30%</td>
<td>None</td>
<td>6.1 x 10^8</td>
<td>9.5 x 10^8</td>
<td>2.3 x 10^8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zoocin A</td>
<td>6.1 x 10^8</td>
<td>2.3 x 10^6</td>
<td>0</td>
<td>99.8%</td>
</tr>
</tbody>
</table>

A Protoplast formation efficiency is calculated from a comparison of the original cell viability on THA to that cell viability, also on THA, after incubation with zoocin A. If per ml the original number of viable cells is A, and the number of viable cells post zoocin A incubation is B, then the number of protoplasts produced per ml, C, is given by the equation, $A - B = C$, and the protoplast formation efficiency (as a percentage), is given by the equation $C \times 100/A$.

B Protoplast reversion efficiency is the frequency with which protoplasts plated on RM give rise to colonial growth. Assuming the number of viable cells per ml yielded on RM to be D, and then the protoplast reversion efficiency (as a percentage) is given by the equation $D \times 100/C$. 
Figure 3.9. OD change overtime of exponential phase *S. mutans* OMZ175 suspended in increasing raffinose concentrations with (panel A) or without (panel B) zoocin A in order to demonstrate occurrence of osmotic shock. *S. mutans* OMZ175 growth overtime in the presence of: ○ 0% raffinose; □ 10% raffinose; ▲ 20% raffinose; ▼ 30% raffinose; ◀ 40% raffinose.
affected by the presence of either sugar, as *S. mutans* OMZ175 shows equal rates of survival upon THA, RMS or RMR agar plates. It is possible that this peptidoglycan layer plays a significant role in protecting the cells from osmotic imbalance.

### 3.7 Demonstrating the entrance of radiolabelled PS-ODN into *S. mutans* OMZ175

In order to determine the entrance of PS-ODN into the *S. mutans* OMZ175 cell, it was decided to attempt to attach a radiolabel to the non-targeted ATS2 molecule. Once labelled, experiments could be designed to monitor its entrance into the *S. mutans* OMZ175 cell under different treatment conditions.

#### 3.7.1 Determining percentage of oligonucleotide recovery from the treatment column

It was determined that no significant difference existed between the concentrations of Roche Mini Quick Spin Column treated oligonucleotides, and those before treatment. It was therefore concluded that approximately 100% recovery of 18 bp oligonucleotides was achieved from the Roche Mini Quick Spin Columns, as stated by the manufacturer.

#### 3.7.2 Labelling ATS2 with [methyl-\(^{3}\text{H}\)] thymidine

Five microliters samples of [methyl-\(^{3}\text{H}\)] thymidine labelled ATS2 either ‘cleaned’ using the Roche Mini Quick Spin column (and therefore will not contain any unincorporated \(^{3}\text{H}\)) , or non-cleaned (not put through the column and therefore likely to still contain any unincorporated \(^{3}\text{H}\)) failed to give any result higher than those considered as ‘background’ (below 100 activity counts per minute (CPM) of radioactivity as measured by the Wallac 1450 Microbeta plus) (Table 3.12). Each unit is an activity count; any reading under 20 units is considered background reading. Counting of 45 µl of the ‘non-cleaned’ labelled ATS2 sample provided a 124 unit reading. Due to the low levels of labelling efficiency, the inability
Table 3.12: Units of radioactivity associated with microliter amounts of ATS2 after labelling reaction terminal transferase and [methyl-\(^3\)H]

<table>
<thead>
<tr>
<th>ATS2 treatment type</th>
<th>Activity counts per minute</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 µl</td>
</tr>
<tr>
<td>Column cleaned</td>
<td>4</td>
</tr>
<tr>
<td>Non-cleaned</td>
<td>44</td>
</tr>
<tr>
<td>Control (dH(_2)O)</td>
<td>12</td>
</tr>
</tbody>
</table>
of terminal transferase to label PS-ODN chemistry and the low radioactivity of the labelling molecule, it was decided that [methyl-$^3$H] thymidine was unsuitable for use as a PS-ODN radiolabel marker.

### 3.7.3 Labelling PS-ODN with $\gamma^{32}$P and T4 Polynucleotide Kinase

X-ray exposure film showed a single smudgy band at the bottom of the polyacrylamide gel for each radiolabelled PS-ODN chemistry, indicating that T4 polynucleotide kinase (New England Bio labs) was suitable for allowing the attachment of $\gamma^{32}$P to ATS2 as it allowed the attachment of a radiolabel to the PS-ODN. The phosphoimage (Figure 3.10) taken after salt-precipitation showed two bands for the $\gamma^{32}$P ATS2 treated with salt precipitation, at approximately 18 and 1 bp in size. Only one band at approximately 18 bp in size was observed for $\gamma^{32}$P ATS2 treated using the Roche Mini Quick Spin Columns. This indicated that the on-column purification, unlike salt precipitation, was able to remove all unincorporated radiolabel. Using the method supplied by Roche Mini Spin Columns it was determined that 13.2% of the $\gamma^{32}$P added to the labelling reaction was incorporated into the PS-ODN oligonucleotides.

#### 3.7.3.1 Scintillation counting of labelled PS-ODN and statistical analysis of results

Three types of test were included for each radiolabelled antisense uptake experiment done. These tests determined the (A) CPM per cell treatment per time point using the $\gamma^{32}$P reading programme on the Quanta Smart scintillation counter by Perkin Elmer for Tri-Carb® liquid scintillation, (B) the viability per cell treatment per time point and (C) the OD per cell treatment per time point. Using this CPM data, and the corresponding viability data (CFU), the number of ATS2 molecules associated with each CFU could be determined.
Figure 3.10. Scanned phosphoimage illustrating the radioisotope labelling of ATS control PS-ODN with T4 polynucleotide kinase and $\gamma^{32}$P and the removal of unincorporated $\gamma^{32}$P achieved through the use of different methods. Lane 1, $\gamma^{32}$P ATS2 purified using Roche® column; Lane 2, $\gamma^{32}$P ATS2 purified using salt precipitation; Lane 3, marker. The marker was developed using a Hammerhead ribosome sequence (HHR2) attached to a T7 promoter. When transcribed in vitro in the presence of Mg$^{2+}$ ions, HHR2, which is divalent metal ion dependent, cleaves itself twice, resulting in 62, 50 and 12 bp products. As $\gamma^{32}$P-ATP was included in the transcription reaction, these products are radiolabelled and used as radiomarkers. The circle indicates the faint but detectable presence of radioactive molecules in Lane 2. Loading dye was prepared by mixing 950 µl formamide, 50 µl of 0.5 M EDTA (pH 8) and 4 µl of bromophenol blue.
4 Results: Use of the bacteriolytic enzyme zoocin A to facilitate entry of PS-ODN to \textit{S. mutans} OMZ175

4.1 Introduction

Previous studies had indicated that zoocin A is suitable to deliver PS-ODN into \textit{S. mutans} OMZ175 and other closely related strains\textsuperscript{95}. This study also demonstrated that bacterial strains which contain the PS-ODN target genes, but which were resistant to zoocin A, remained unaffected by the presence of a combination of zoocin A and SM-FBA. Those bacterial cells which were sensitive to zoocin A, but did not contain the SM-FBA target site, also remained unaffected. This indicates that both the target specificity of the delivery molecule and the PS-ODN itself are important in ensuring an antisense mediated inhibitory effect. Further study however was required into the effects of PS-ODN delivery on gene expression within the target bacterial cell, and determination of the rate of PS-ODN delivery by zoocin A to the susceptible \textit{S. mutans} OMZ175. It was important to evaluate whether different physiological states of \textit{S. mutans} OMZ175 affected the ability of zoocin A and PS-ODN’s to cause inhibition. The aim of this chapter was to determine whether the addition of zoocin A and a PS-ODN targeted to the \textit{fba} gene resulted in inhibition of the mRNA of this gene, whether these effects were gene specific, and whether these effects were comparable to those found through measurement of phenotypic characteristics.

4.2 Establishment of growth of \textit{S. mutans} OMZ175.

As gene expression can vary significantly depending on the physiological state of the cell it was necessary to determine the length of the lag phase, and at what point exponential and stationary phases were reached (Methods 2.3.1.1). Using a 5\% inoculum, \textit{S. mutans} OMZ175 has an initial concentration of approximately $10^7$ cfu/ml. The lengths of each growth phase of \textit{S. mutans} OMZ175 were determined statistically. The lag phase was deemed to occur between the values of 0.0 – 0.35 OD, the exponential phase was between 0.35 – 0.7 OD and the stationary phase to occur for at OD levels above 0.7. The length of time of the lag phase
(initial OD + 0.1 OD) was consistent between experiments (SD < 0.1 hours) (Table 4.1). It took approximately 5 hours to reach exponential phase, with a viability count of $10^8$ cfu/ml at an OD reading of 0.35. It took approximately 8 hours to reach stationary phase, with a viability count of $10^{10}$ cfu/ml at an OD reading of 0.7 OD.

4.3 Determining the sub-lethal concentration of zoocin A for *S. mutans* OMZ175 at different growth phases

For all three growth phases (lag, exponential, stationary) it was necessary to determine the concentration of zoocin A required to give a sub-lethal effect. Doing this enabled the lowest possible concentration of zoocin A to be used such that when combined with other antimicrobial agents the inhibitory effect of the combination could be distinguished from that of zoocin A alone (Methods 2.3.1.2).

For lag phase *S. mutans* OMZ175, the addition of zoocin A to final concentration of 0.2 AU/ml was found to significantly increase (P= 0.001) lag phase (Table 4.2, Figure 4.1) in comparison to untreated cells. Furthermore, following an initial rapid decrease in viability to $10^5$ cfu/ml, the treated culture recovered achieving a final density of $10^{10}$ cfu/ml that was not significantly different (P= 0.005) from the untreated control (Figure 4.1, Table 4.3). Use of zoocin A at 0.4 AU/ml or greater, drastically increased lag times, and the treated cultures failed to achieve a final culture density comparable to untreated controls. The term ‘sub-lethal’ was adopted to describe that concentration of zoocin A able to significantly increase the lag phase of the *S. mutans* OMZ175 culture, without compromising its final OD. For exponential phase *S. mutans* OMZ175, the ‘sub-lethal’ zoocin A concentration was found to be 1.4 AU/ml (Figure 4.2, Tables 4.2 and 4.3). The highest concentration tested (30 AU/ml) did not significantly reduce the viability or optical density levels for stationary phase (Figure 4.3, Tables 4.2 and 4.3), and no sigmoidal curve could be fitted to the treatments, so statistical analysis was not possible.
Table 4.1: Length of incubation time at 37 °C to achieve the start of each growth phase of *S. mutans* OMZ175 in THB

<table>
<thead>
<tr>
<th>Growth Phase</th>
<th>Sample taken at time (hours)</th>
<th>cfu/ml ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag</td>
<td>0.0 ± 0.0</td>
<td>5.9 ± 0.04 x 10^7</td>
</tr>
<tr>
<td>Exponential</td>
<td>5 ± 0.17</td>
<td>9.7 ± 0.19 x 10^7</td>
</tr>
<tr>
<td>Stationary</td>
<td>8 ± 0.12</td>
<td>10.1 ± 0.16 x 10^10</td>
</tr>
</tbody>
</table>

The standard deviation is the standard error between the three triplicates done for each experiment.
Table 4.2: Zoocin A concentrations required to produce a sub-lethal effect upon *S. mutans* OMZ175 in each growth phase

<table>
<thead>
<tr>
<th>Growth phase</th>
<th>Zoocin A MIC (AU/ml)</th>
<th>Sub-lethal Zoocin A Concentration (AU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>Exponential</td>
<td>5</td>
<td>1.4</td>
</tr>
<tr>
<td>Stationary</td>
<td>&gt;20</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 4.1. Determining the sub-lethal concentration of zoocin A for lag phase *S. mutans* OMZ175 through the monitoring of OD (panel A) and viability (panel B) of lag phase *S. mutans* OMZ175 in the presence of: THB; 0.02 AU/ml zoocin A; 0.05 AU/ml zoocin A; 0.1 AU/ml zoocin A; 0.2 AU/ml zoocin A; 0.3 AU/ml zoocin A; 0.4 AU/ml zoocin A; 0.5 AU/ml zoocin A. Arrow indicates the time of treatment addition. The error bars represent the standard error between the three triplicates done for each experiment.
Figure 4.2. Determining the sub-lethal concentration of zoocin A for exponential phase *S. mutans* OMZ175 through the monitoring of OD (panel A) and viability (panel B) of exponential phase *S. mutans* OMZ175 in the presence of: THB; 0.5 AU/ml zoocin A; 1 AU/ml zoocin A; 2 AU/ml zoocin A; 3 AU/ml zoocin A; 4 AU/ml zoocin A. Arrow indicates the time of treatment addition. The error bars represent the standard error between the three triplicates done for each experiment.
Figure 4.3. Determining the sub-lethal concentration of zoocin A for stationary phase *S. mutans* OMZ175 through the monitoring of OD (panel A) and viability (panel B) of stationary phase *S. mutans* in the presence of: THB; 2 AU/ml zoocin A; 4 AU/ml zoocin A; 10 AU/ml zoocin A; 20 AU/ml zoocin A; 30 AU/ml zoocin A. Arrow indicates the time point of treatment addition. The error bars represent the standard error between the three triplicates done for each experiment.
Table 4.3: Effect of increasing zoocin A concentrations upon the length of the lag phase or recovery time of *S. mutans* OMZ175 in different growth stages

<table>
<thead>
<tr>
<th>Zoocin A concentration (AU/ml)</th>
<th>Lag or recovery time (Hours) ± SD †</th>
<th>Statistical significance *</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lag</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5.9 ± 0.06</td>
<td>N/A</td>
</tr>
<tr>
<td>0.02</td>
<td>6.9 ± 0.08</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.05</td>
<td>7.6 ± 0.66</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.1</td>
<td>8.9 ± 0.11</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.2</td>
<td>10.1 ± 0.37</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.3</td>
<td>14.9 ± 0.34</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.4</td>
<td>21.1 ± 0.12</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.5</td>
<td>NG †</td>
<td>-</td>
</tr>
</tbody>
</table>

| **Exponential**               |                                   |                           |
| 0                             | 0.6 ± 0.18                        | N/A                       |
| 0.5                           | 0.7 ± 0.18                        | P > 0.05                  |
| 1                             | 2.7 ± 0.10                        | P > 0.05                  |
| 2                             | 4.2 ± 0.09                        | P < 0.001                 |
| 3                             | 8.2 ± 0.13                        | P < 0.001                 |
| 4                             | 17.6 ± 0.28                       | P < 0.001                 |

† No sigmoidal curve could be fitted so statistical analysis was not possible.

*All lag phase treatments were compared to lag phase with no zoocin A treatment. All exponential phase treatments were compared to exponential phase with no zoocin A treatment.

NG = No growth after 24 hours

The standard deviation is the standard error between the three triplicates done for each experiment.
4.4 Synergistic effect between SM-FBA, ATS2 and zoocin A

4.4.1 Use of zoocin A and PS-ODN upon the growth of S. mutans OMZ175

It was necessary to show what inhibitory effect, if any, was produced by a combination between zoocin A and the SM-FBA sequence against S. mutans OMZ175 in all three growth stages (lag, exponential and stationary) (Methods 2.3.2.3). When S. mutans OMZ175 cells in each growth phase were combined with the appropriate amount of zoocin A (Table 4.2) plus 10 µM SM-FBA, a far greater inhibitory effect for each separate growth phase was observed compared to that for zoocin A alone (Figures 4.4, 4.5 and 4.6, Table 4.4). No corresponding inhibition was observed for stationary phase cells. When 10 µM ATS2 was combined with zoocin A, the inhibitory effect on growth of S. mutans OMZ175 in any of the three growth stages was found to be no greater than that of zoocin A alone. When 10 µM of either: SM-FBA or ATS2 were added to S. mutans OMZ175 in any of the three growth phases without zoocin A, no significant inhibitory effect was observed.

4.4.2 Titration of SM-FBA on growth of S. mutans OMZ175

It needed to be determined if SM-FBA alone had an inhibitory effect upon the growth of S. mutans OMZ175, or whether the lytic agent zoocin A was required in order for the antisense sequence to produce an inhibitory effect (Methods 2.3.2.4) In the absence of zoocin A, SM-FBA (1 µM- 40 µM) had no significant effect upon S. mutans OMZ175 growth rate (Figure 4.7, Table 4.5). When combined with 0.2 AU/ml zoocin A, as SM-FBA concentration increased, so did the length of the lag phase. The length of the lag phase increased proportionately with SM-FBA concentration ($R^2 = 0.9959$)

4.4.3 Titration of zoocin A on growth of S. mutans OMZ175

It was necessary to determine the lowest concentration of zoocin A required, when combined
Figure 4.4. Effect of zoocin A and PS-ODN upon lag phase *S. mutans* OMZ175 OD (panel A) or viability (panel B) treated with a sub-lethal concentration of zoocin A and/or antisense. Lag phase *S. mutans* OMZ175 over time in the presence of: ◊ THB; ◆ 0.2 AU/ml zoocin A; ○Δ● 10 µM ATS2; ♦ 0.2 AU/ml zoocin A + 10 µM ATS2; □ 10 µM SM-FBA; ◐ 0.2 AU/ml zoocin A + 10 µM SM-FBA. Arrow indicates the time point of treatment addition. The error bars represent the standard error between the three triplicates done for each experiment.
Figure 4.5. Effect of zoocin A and PS-ODN upon exponential phase *S. mutans* OMZ175 OD (panel A) or viability (panel B) treated with a sub-lethal concentration of zoocin A and/or antisense. Exponential phase *S. mutans* OMZ175 over time in the presence of: THB; 1.4 AU/ml zoocin A; 10 µM ATS2; 1.4 AU/ml zoocin A + 10 µM ATS2; 10 µM SM-FBA; 1.4 AU/ml zoocin A + 10 µM SM-FBA. Arrow indicates the time point of treatment addition. The error bars represent the standard error between the three triplicates done for each experiment.
Figure 4.6. Effect of zoocin A and PS-ODN upon stationary phase *S. mutans* OMZ175 OD (panel A) or viability (panel B) treated with a sub-lethal concentration of zoocin A and/or antisense. Stationary phase *S. mutans* OMZ175 over time in the presence of: ◊ THB; ○ 20 AU/ml zoocin A; •△• 10 µM ATS2; ★ 20 AU/ml zoocin A + 10 µM ATS2; □ 10 µM SM-FBA; ★★★ 20 AU/ml zoocin A + 10 µM SM-FBA. Arrow indicates the time point of treatment addition. The error bars represent the standard error between the three triplicates done for each experiment.
Table 4.4: Synergistic inhibitory effect of zoocin A and antisense on the length of the lag phase or recovery time on *S. mutans* OMZ175 in different growth stages

<table>
<thead>
<tr>
<th>Zoocin A concentration (AU/ml) plus antisense (10 μM)</th>
<th>Lag time (Hours) ± SD</th>
<th>Statistical significance‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 + 0</td>
<td>4.5 ± 0.12</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + ATS2</td>
<td>4.5 ± 0.07</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0 + SM-FBA</td>
<td>4.6 ± 0.09</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0.2 + 0</td>
<td>6.8 ± 0.10</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.2 + ATS2</td>
<td>6.9 ± 0.14</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0.2 + SM-FBA</td>
<td>19.3 ± 0.17</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Exponential</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 + 0</td>
<td>0.8 ± 0.39</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + ATS2</td>
<td>0.8 ± 0.54</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0 + SM-FBA</td>
<td>0.8 ± 0.26</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>1.4 + 0</td>
<td>5.4 ± 0.72</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>1.4 + ATS2</td>
<td>5.4 ± 0.66</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>1.4 + SM-FBA</td>
<td>17.2 ± 0.41</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

‡ For each growth phase; the values for zoocin A, ATS2 and SM-FBA alone, were compared to that of the 0 + 0 control; and the values for zoocin A + PS-ODN were compared to the zoocin A only control. No sigmoidal curve could be fitted to the stationary phase data so statistical analysis was not possible.

The standard deviation is the standard error between the three triplicates done for each experiment.
Figure 4.7. Titration of an increasing antisense concentration (SM-FBA, 0-20 mM) against lag phase *S. mutans* OMZ175 with a constant zoocin A concentration (0.2 AU/ml).
Table 4.5: The synergistic effect of SM-FBA and zoocin A upon lag phase *S. mutans* OMZ175 growth

<table>
<thead>
<tr>
<th>SM-FBA concentration (μM) plus zoocin A (AU/ml)</th>
<th>Lag time (Hours) ± SD‡</th>
<th>Statistical significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 + 0</td>
<td>5.9 ± 0.15</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + 0.2</td>
<td>10.1 ± 0.37</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>1 + 0</td>
<td>5.7 ± 0.18</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>5 + 0</td>
<td>6.01 ± 0.122</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>10 + 0</td>
<td>5.8 ± 0.29</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>20 + 0</td>
<td>5.9 ± 0.24</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>40 + 0</td>
<td>6.1 ± 0.17</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>1 + 0.2</td>
<td>10.7 ± 0.39</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>5 + 0.2</td>
<td>12.8 ± 0.18</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>10 + 0.2</td>
<td>15.2 ± 0.23</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>20 + 0.2</td>
<td>19.2 ± 0.27</td>
<td>P &lt; 0.001‡</td>
</tr>
<tr>
<td>40 + 0.2</td>
<td>NG</td>
<td></td>
</tr>
</tbody>
</table>

*0.2 AU/ml zoocin A and all SM-FBA alone concentrations compared to 0 +0 control. All zoocin A + antisense compared to 0.2 AU/ml zoocin A only concentration.

† No sigmoidal curve could be fitted so statistical analysis was not possible.

NG = No growth after 18 hours

The standard deviation is the standard error between the three triplicates done for each experiment.
with SM-FBA, to produce inhibition of the growth of *S. mutans* OMZ175, so that the lowest concentration of zoocin A required could always be used (Methods 2.3.2.4). As zoocin A concentration increased there was an increase in the length of the lag phase of *S. mutans* OMZ175 (Figure 4.8, Table 4.6). When zoocin A was combined with 10 µM SM-FBA, the length of the lag phase was significantly increased from that of the zoocin A alone. The length of the lag phase increased proportionately with zoocin A (plus 10 µM SM-FBA) concentration ($R^2 = 0.9879$). Combining 0.4 AU/ml zoocin A and 10 µM SM-FBA resulted in total inhibition of *S. mutans* OMZ175 growth during the 24 hour incubation period.

### 4.5 Gene expression quantitative PCR protocol

Quantitative real-time PCR was used to profile the expression level of the targeted *fba* gene. As a significant synergistic effect between zoocin A and SM-FBA had been observed at the phenotypic level (growth inhibition) for both lag and exponential phase *S. mutans* OMZ175 cells, their effect at the genotypic level was investigated using qPCR. To measure the amount of the *fba* transcript in the treated cells, a quantitative RT-PCR was developed.

#### 4.5.1 RNA isolation

Extraction of RNA from *S. mutans* OMZ175 was done following the method described in Methods 2.2.4.2. An on-column DNase digestion was shown to eliminate contamination by genomic DNA, as no DNA bands were found to be present in any of the reactions run using DNase treated RNA as a template (Figure 4.9). DNA bands were produced however for those wells using non-DNase treated RNA as a template, indicating the presence of contaminating DNA in the RNA sample. The total amount of RNA run in each well was approximately 7 ng.
Figure 4.8. Titration of an increasing concentration of zoocin A (0 – 0.4 AU) against *S. mutans* OMZ175, with a constant SM-FBA concentration (10 μM).
Table 4.6: The synergistic inhibitory effect of zoocin A and SM-FBA upon lag phase *S. mutans* OMZ175 growth

<table>
<thead>
<tr>
<th>Zoocin A concentration (AU/ml) plus SM-FBA (μM)</th>
<th>Lag time (Hours) ± SD</th>
<th>Statistical significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 + 0</td>
<td>5.9 ± 0.06</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + 10</td>
<td>5.9 ± 0.12</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0.02 + 0</td>
<td>6.9 ± 0.07</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.05 + 0</td>
<td>7.8 ± 0.22</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.1 + 0</td>
<td>8.9 ± 0.11</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.2 + 0</td>
<td>10.1 ± 0.37</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.3 + 0</td>
<td>14.9 ± 0.34</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.4 + 0</td>
<td>21.1 ± 0.12</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.02 + 10</td>
<td>7.7 ± 0.09</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.05 + 10</td>
<td>9.1 ± 0.08</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.1 + 10</td>
<td>11.9 ± 0.07</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.2 + 10</td>
<td>15.2 ± 0.23</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.3 + 10</td>
<td>23.1 ± 0.43</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.4 + 10</td>
<td>NG</td>
<td></td>
</tr>
</tbody>
</table>

*All zoocin A and SM-FBA alone values compared to the 0 + 0 control. All zoocin A + SM-FBA values are compared to their corresponding zoocin A only controls.

† No sigmoidal curve could be fitted so statistical analysis was not possible.

NG = No growth after 18 hours

The standard deviation is the standard error between the three triplicates done for each experiment.
Figure 4.9. PCR amplification of *S. mutans* OMZ175 extracted RNA both before and after DNase treatment done to show elimination of genomic DNA. Lane M, Marker (Invitrogen 100 bp DNA ladder). Template of DNase treated *S. mutans* OMZ175 RNA: lanes 1-3. Lane 4, blank. Template of Non DNase treated RNA lanes: 5-7. Product formed by: 16s rRNA RT-PCR Fwd/Rev Set A; Lanes 1 and 5, FBA RT-PCR Fwd/Rev Set A; Lanes 2 and 6, GyrA RT-PCR Fwd/Rev Set A; Lanes 3 and 7. Primer pairs and PCR protocol listed in Appendix 2. The total amount of extracted RNA run in each well was approximately 7 ng. Poor quality gel due to re-scanned image of a scanned gel photograph.
4.5.2 Determining the effect of zoocin A upon gene expression levels

No significant difference fold change in expression levels for fba, 16s rRNA or gyrA were observed for S. mutans OMZ175 treated with zoocin A compared to cells alone (Table 4.7) (Methods 2.3.4.3). The ct values determined for each of the three genes showed no significant difference between the cell only control, and those treated with zoocin A.

4.5.3 FBA mRNA expression in S. mutans OMZ175 treated with zoocin A and PS-ODN

Previous research has shown that qPCR is a viable method that allows the expression levels of genes to be closely monitored, and can be used to demonstrate the down regulation of gene expression\(^{200,303}\). Gene expression from either zoocin A and/or PS-ODN treated S. mutans OMZ175 were analysed by using the zoocin A only treatment as the calibrator to enable the calculation of the fold-change in gene expression upon PS-ODN addition (Methods 2.3.4.2). The zoocin A only treatment samples were directly compared to each other, and also to the S. mutans OMZ175 cells only control. For the zoocin A treated samples, the average ΔCt value of the S. mutans OMZ175 only control was used as a calibrator and hence all data for the zoocin A only treated cells is expressed as the fold change from the average no-treatment cells only control value.

Significant differences in FBA mRNA expression were observed for both lag and exponential phase S. mutans OMZ175, when the cells had been treated with a combination of both zoocin A and SM-FBA at both 30 mins and 5 hours after treatment addition (Figure 4.10). Thirty minutes after addition of zoocin A and SM-FBA, a 37.354 ± 16.942 fold decrease in FBA mRNA expression in comparison with those cells treated with zoocin A alone was observed for lag phase S. mutans OMZ175 (Figure 4.10), whilst in comparison treated exponential phase S. mutans OMZ175 cells showed an average fold decrease of 1067.86 ± 230.95 in FBA mRNA expression (Figure 4.10). Five hours after addition of zoocin A and SM-FBA, a 522.241 ± 30.05 fold decrease in FBA mRNA expression in comparison with those cells treated with zoocin A alone was observed for lag phase S. mutans OMZ175 (Figure 4.10),
Table 4.7. Ct values at different time points for the three genes of interest present in *S. mutans* OMZ175 after zoocin A addition

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Time point after treatment addition</th>
<th>ct Value</th>
<th>Statistical significance *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cells only</td>
<td>Zoocin A treated</td>
</tr>
<tr>
<td><em>fba</em></td>
<td>0</td>
<td>17.92</td>
<td>17.97</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>18.02</td>
<td>17.80</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>17.80</td>
<td>17.96</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>17.76</td>
<td>18.03</td>
</tr>
<tr>
<td><em>gyrA</em></td>
<td>0</td>
<td>21.89</td>
<td>21.66</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>21.95</td>
<td>21.57</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>21.78</td>
<td>21.80</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>21.63</td>
<td>21.63</td>
</tr>
<tr>
<td><em>16s rRNA</em></td>
<td>0</td>
<td>7.97</td>
<td>7.97</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>8.12</td>
<td>8.04</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>8.06</td>
<td>8.14</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>8.03</td>
<td>8.16</td>
</tr>
</tbody>
</table>

*P value- Tukey multiple comparison test- between cells only and zoocin A at same time point*
Figure 4.10. Fold change in FBA mRNA expression levels in zoocin A and antisense treated lag (panel A) or exponential (panel B) *S. mutans* OMZ175 in the presence of a variety of agents. FBA mRNA expression levels of *S. mutans* OMZ175 over time in the presence of: THB; 0.2 or 1.4 AU/ml zoocin A; 10 µM ATS2; 0.2 or 1.4 AU/ml zoocin A + 10 µM ATS2; 10 µM SM-FBA; 0.2 or 1.4 AU/ml zoocin A + 10 µM SM-FBA. The error bars stand for the standard error between the three triplicates done for each experiment.
Whilst in comparison exponential phase *S. mutans* OMZ175 showed an average fold decrease of 1902.4 ± 131.26 in FBA mRNA expression (Figure 4.10). The raw data is available on the enclosed data CD McLeod 2012.

In every other case the mRNA expression level of the gene under consideration did not differ significantly from that of its comparator. Neither control, 16s rRNA mRNA or gyrA mRNA showed any significant inhibition or increase in expression level for any treatment type.

96 well NUNC checkerboard titrations also showed that when combined with zoocin A, 10 µM SM-FBA has a greater inhibitory effect upon the growth of either lag or exponential phase *S. mutans* OMZ175, than did zoocin A alone (see Table 4.4). When 10 µM ATS2 was combined with zoocin A, the inhibitory effect on the growth of either lag or exponential phase *S. mutans* OMZ175 was found to be no greater than zoocin A alone. These results match those found previously (see above, McLeod 2008 and Dufour et al., 2011). When 10 µM of either: SM-FBA or ATS2 was added to either lag or exponential phase *S. mutans* OMZ175 without zoocin A, no significant effect on transcription was found, with the exception of the five hour time point for FBA mRNA expression in lag phase cells treated with ATS2 only (Figure 4.10) where a 2.229 ± 0.76 fold difference was observed. However, since no other time point or treatment type for either lag or exponential phase cells showed any significant decrease in FBA mRNA and the result expression does not correlate with any viability, OD or radioactivity results, it is likely it is an artefact of experimental error. All other $2^{-ΔΔCt}$ values found for ATS2 only treated *S. mutans* OMZ175 at the five hour time point were between the range of 1.5 – 0.7.

### 4.6 Effect of zoocin A upon *S. mutans* OMZ175

#### 4.6.1 Effect of zoocin A or PS-ODN upon *S. mutans* OMZ175 gene sequences

PCR of the *fba*, *gyrA* and 16s rRNA genes from cells treated with either zoocin A, or PS-ODN alone, or a combination of zoocin A and PS-ODN yielded single bands when run on agarose gels, and sequencing of the PCR products showed no mutations.
4.6.2 Screening for resistance to zoocin A

It was necessary to determine whether repeat exposure to zoocin A together with targeted or non-targeted PS-ODN resulted in the development of resistance. Exponential phase cells were initially treated with zoocin A (Table 4.8, Figure 4.11) or zoocin A and PS-ODN (Table 4.9). Once treated cells recovered to their initial (before treatment) OD, the treatment was repeated cells were repeated up to four times. No significant difference in the time taken to reach initial pre-treatment OD was observed between identical treatments.

4.7 Demonstrating the entrance of $^{32}\text{P}$-ATS2 into S. mutans OMZ175

4.7.1 Uptake of radiolabeled antisense by lag and exponential phase S. mutans OMZ175 treated with zoocin A

Only the lag or exponential phase S. mutans OMZ175 cells treated with a combination of zoocin A and $^{32}\text{P}$-ATS2 showed any significant uptake of $^{32}\text{P}$-ATS2 greater than that found for non zoocin A treated control cells (Figures 4.12, 4.13, 4.14 and 4.15). The S. mutans OMZ175 cells treated with both zoocin A and $^{32}\text{P}$-ATS2 showed a sharp increase in the amount of radioactivity per cell over the first 4 hours of treatment, followed by a rapid decrease in the amount of cell associated radioactivity over the next three hours (Figures 4.12, 4.14). This was shown in both repeats of each experiment. The optical density and viability experiments showed expected results- matching the results shown in previous experiments (section 4.4).

Using the statistical analysis described in Methods 2.2.6.8, it was determined that for lag phase S. mutans OMZ175 an average of $5.958 \pm 5.12 \times 10^6$ molecules of ATS2 per cell had entered zoocin A and $^{32}\text{P}$-ATS2 treated cells half an hour after treatment addition, whilst those cells treated with $^{32}\text{P}$-ATS2 alone had an average of $41,939 \pm 7805$ molecules of ATS2 associated (Figure 4.14). Exponential phase S. mutans OMZ175 treated with zoocin A and $^{32}\text{P}$-ATS2 had an average of $3.62 \times 10^7 \pm 8.3 \times 10^6$ molecules of ATS2 associated with them half an hour after treatment addition, whilst those cells treated with $^{32}\text{P}$-ATS2 alone
Table 4.8: Multiple sub-inhibitory zoocin A doses repeatedly used upon exponential phase S. mutans OMZ175

<table>
<thead>
<tr>
<th>Zoocin A dose (1.4 AU/ml)</th>
<th>Lag time (Hours) ± SD†</th>
<th>Statistical significance *</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.4 ± 0.25</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>4.9 ± 0.32</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>3</td>
<td>5.3 ± 0.76</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>4</td>
<td>5.2 ± 0.41</td>
<td>P &gt; 0.05</td>
</tr>
</tbody>
</table>

† The initial OD value was read at the time point after the final zoocin A dose was added to the media as sigmoidal curves could not be fitted to any but the final data set for each treatment type.

*P value- Tukey multiple comparison test- between the mean time taken to reach the initial OD value + 0.1 OD for a single dose of zoocin A, compared to each additional dose.

The standard deviation is the standard error between the three triplicates done for each experiment.
Figure 4.11. Screening for *S. mutans* OMZ175 resistance to zoocin A. Multiple additions of zoocin A to exponential phase *S. mutans* OMZ175 over time in the presence of: solid line: zero; dashed line: one dose; dashed line with diamonds: two doses; dashed line with circles: three doses; or dashed line with triangles: four doses. The error bars stand for the standard error between the three triplicates done for each experiment.
Table 4.9. Growth of *S. mutans* OMZ175 with multiple sub-inhibitory zoocin A and PS-ODN doses

<table>
<thead>
<tr>
<th>Zoocin A concentration (AU/ml) plus antisense (10 μM) to <em>S. mutans</em> OMZ175</th>
<th>Lag time (Hours) ± SD†</th>
<th>Statistical significance *</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Single Dose</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 + 0</td>
<td>4.1 ± 0.04</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + ATS2</td>
<td>3.9 ± 0.15</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0 + SM-FBA</td>
<td>3.9 ± 0.18</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>1.4 + 0</td>
<td>5.1 ± 0.24</td>
<td>P &gt; 0.01</td>
</tr>
<tr>
<td>1.4 + ATS2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose 1</td>
<td>4.7 ± 0.18</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Dose 2</td>
<td>4.2 ± 0.28</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Dose 3</td>
<td>4.4 ± 0.09</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Dose 4</td>
<td>4.7 ± 0.11</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>1.4 + SM-FBA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose 1</td>
<td>14.7 ± 0.34</td>
<td>N/A</td>
</tr>
<tr>
<td>Dose 2</td>
<td>15.3 ± 0.44</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Dose 3</td>
<td>14.5 ± 0.13</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Dose 4</td>
<td>13.3 ± 1.02</td>
<td>P &gt; 0.05</td>
</tr>
</tbody>
</table>

† The initial OD value was read at the time point after the final treatment dose was added to the media as sigmoidal curves could not be fitted to any but the final data set for each treatment type.

*P value- Tukey multiple comparison test. All zoocin A + ATS2 dose responses compared to 1.4 + 0. The first dose of 1.4 + SM-FBA compared to a single dose of zoocin A alone. Doses 2, 3 and 4 compared to the respective dose 1 sample.

The standard deviation is the standard error between the three triplicates done for each experiment.
Figure 4.12. Changes in the number of ATS2 molecules (x 10^7) per CFU over time of exponential phase *S. mutans* OMZ175 treated with zoocin A + ATS2. Exponential phase *S. mutans* OMZ175 following treatment with: • γ^{32} P-ATS2; – zoocin A + γ^{32} P-ATS2. The error bars stand for the standard error between the three triplicates done for each experiment.
Figure 4.13. The relationship between cellular growth and ATS2 dilution rates over time for exponential *S. mutans* OMZ175 treated with zoocin A + ATS2. Exponential phase *S. mutans* OMZ175 following treatment with zoocin A + ATS2.  ■, the amount of ATS2 present per CFU;  ●, the number of viable bacterial cells. The error bars stand for the standard error between the three triplicates done for each experiment.
Figure 4.14. Changes in the number of ATS2 molecules ($x \times 10^7$) per CFU over time of lag phase *S. mutans* OMZ175 treated with zoocin A and ATS2. Lag phase *S. mutans* OMZ175 following treatment with: %32$^{\gamma}$ P-ATS2; - zoocin A + %32$^{\gamma}$ P-ATS2. The error bars stand for the standard error between the three triplicates done for each experiment.
Figure 4.15. The relationship between cellular growth and ATS2 dilution rates for lag phase *S. mutans* OMZ175 treated with zoocin A + ATS2. Lag phase *S. mutans* OMZ175 following treatment with zoocin A + ATS2. □, the amount of $\gamma^{32}$P-ATS2 present per CFU; ●, the number of viable bacterial cells per CFU/ml. The error bars stand for the standard error between the three triplicates done for each experiment.
had an average of $62,477 \pm 23,650$ molecules of ATS2 associated (Figure 4.12). Four hours after treatment, it was determined that there was an average of $5.652 \pm 3.91 \times 10^7$ cell associated molecules of ATS2 per cell for lag phase *S. mutans* OMZ175 treated with zoocin A + $\gamma^{32}$ P-ATS2, whilst exponential phase cells given the same treatment had $1.01 \times 10^8 \pm 0.073 \times 10^8$ cell associated molecules of ATS2 per CFU. Lag phase cells treated with $\gamma^{32}$ P-ATS2 alone showed $62,073 \pm 22,140$ cell associated molecules of ATS2 per CFU, whilst exponential phase cells showed $20,491 \pm 1,635$.

In order to determine whether the dilution of $\gamma^{32}$ P-ATS2 in *S. mutans* OMZ175 was due to growth, and the division of $\gamma^{32}$ P-ATS2 molecules between daughter cells, the number of ATS2 molecules per CFU, and the total number of viable cells was compared (Figure 4.13, Figure 4.15). The time 4 hour time point after treatment was added to the *S. mutans* OMZ175 cells was chosen as the starting point of both graphs as at this time point zoocin A is deemed to no longer be effective, and cellular replication begins, allowing the relationship between cellular division and ATS2 dilution to be determined. It was found that as the number of viable cells increased approximately 1000 fold from $10^3$ to $10^6$, so the number of ATS2 molecules per CFU decreased approximately 1000 fold from $10^8$ to the $10^5$, indicating that loss of ATS2 molecules is caused primarily by cell division.

These experiments demonstrate that a small amount of $\gamma^{32}$ P-ATS2 immediately becomes associated with the *S. mutans* OMZ175 cells, regardless of whether the cells are treated with $\gamma^{32}$ P-ATS2 alone, or $\gamma^{32}$ P-ATS2 combined with zoocin A. It was demonstrated that lag phase *S. mutans* OMZ175 cells treated with $\gamma^{32}$ P-ATS2 alone showed between $41,123 \pm 8060$ and $76,893 \pm 12,360$ molecules of $\gamma^{32}$ P-ATS2 associated with each CFU (Figure 4.14, Figure 4.15). Exponential phase *S. mutans* OMZ175 cells treated with $\gamma^{32}$ P-ATS2 alone showed similar results, with between $18,993 \pm 605$ and $62,477 \pm 23,650$ molecules of $\gamma^{32}$ P-ATS2 associated with each CFU (Figures 4.12, 4.14).

Both lag and exponential phase *S. mutans* OMZ175 treated with zoocin A + $\gamma^{32}$ P-ATS2 showed immediate large increases in the amount of $\gamma^{32}$ P-ATS2 associated with the bacterial cells compared to those of *S. mutans* OMZ175 + $\gamma^{32}$ P-ATS2 alone. Uptake of $\gamma^{32}$ P-ATS2 was shown to increase for the first four hours of treatment, after which there was a rapid decrease in the amount of $\gamma^{32}$ P-ATS2 present inside each bacterial cell (Figures 4.12, 4.14).
At four hours after treatment, the greatest amount of $\gamma^{32}$P-ATS2 was found within both those cells, $5.652 \pm 3.91 \times 10^7$ and $1.01 \times 10^8$ molecules of $\gamma^{32}$P-ATS2 per cell for lag phase and exponential phase cells respectively. These time points correspond to the time points at which the expression levels of FBA mRNA expression were most repressed.
5 Results: Use of alternative agents to facilitate entry of PS-ODN to Gram-positive bacteria

5.1 Introduction

The emergence of antibiotic resistant bacteria has re-stimulated the search for new antimicrobials. Despite antisense being a targeted and promising approach to inhibit the growth of specific pathogens, an effective delivery system is needed to deliver PS-ODN across the cell wall. Diffusion barriers such as peptidoglycan layers and lipopolysaccharide membranes, combined with the relatively large size and the net negative charge of unmodified PS-ODN’s, can limit antisense uptake. Whilst techniques such as electroporation do work in in vitro studies they are not feasible for in vivo usage. The primary challenge is to achieve sufficient cellular uptake of the antisense, and its delivery to the site of action, the bacterial cytoplasm. Hence, ‘lytic’ antimicrobials that disrupt the peptidoglycan layer and/or cytoplasmic membrane could facilitate the movement of antisense molecules across the bacterial cell wall. Whilst the preceding work has shown that zoocin A could be used to deliver PS-ODN into S. mutans OMZ175 in vivo it is unlikely that zoocin A could be used systemically in vivo due to its high immunogenicity. Therefore, the search for other lytic, lysogenic, or pore-forming agents active against Gram-positive bacteria (most of which are already approved for in vitro use) became the main focus of this section. Their ability to facilitate the uptake of antisense molecules by S. mutans and other pathogenic Gram-positive bacterial strains was monitored using methods described above which measure growth inhibition.

5.2 Confirmation of bacterial species by 16S PCR

To confirm the identity of bacterial species obtained from the culture collection (Department of Microbiology and Immunology, University of Otago), the 16s genes of S. mutans
OMZ175, *S. aureus* WSSP-1, *S. aureus* Oxford, *E. faecalis* AR01/DGVS and *E. coli* M15 ZooA1 were amplified by colony PCR. All bacterial strains yielded a single product, 800 bp in size. The sequences of these PCR products were compared to published 16S sequences and in each case sequence data supported the bacterial species identification.

### 5.2.1 Confirmation of the length of the lag phase for all bacterial species

It was necessary to determine the average length of the lag phase for each bacterial strain in order to determine average incubation times (Methods 2.4.1). Using a 5% inoculum, all strains had an initial concentration of approximately $10^7$ cfu/ml. (Table 5.1). The length of time of the lag phase (initial OD + 0.1 OD) was consistent between experiments (SD < 0.1 hours), although different strains had different lag phase times (Table 5.1).

### 5.3 PCR screening for FBA gene and sequencing

In order to design an appropriate antisense sequence targeted towards each strains fba gene, all bacterial strains were analysed for the presence of their FBA gene. DNA extracted from *E. faecalis* AR01/DGVS amplified by PCR primers listed in Appendix 2 yielded three approximately 1200 bp amplicons (Figure 5.1) which together with the *E. faecalis* AR01/DGVS genome sequences available on NCBI Blast were assembled into a single contig. An *E. faecalis* fba specific PS-ODN (EF-FBA) was designed from the *E. faecalis* AR01/DGVS contig to complement the first 18 bp of the *E. faecalis* AR01/DGVS fba gene (Table 2.2). Using the consensus sequence from *S. aureus* from PubMed an antisense sequence for the fba gene in *S. aureus* Oxford was designed and designated SA-FBA (Table 2.2).

### 5.4 Use of other inhibitory agents as antisense delivery agents
Table 5.1: Viability counts of each bacterial strain used and length of each lag phase

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>cfu/ml of 5% inoculum</th>
<th>Lag Phase (hours ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus mutans</em> OMZ175</td>
<td>4.8 x 10^6</td>
<td>5.9 ± 0.14</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em> AR01/DGVS</td>
<td>5.2 x 10^6</td>
<td>1.7 ± 0.25</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> Oxford</td>
<td>1.1 x 10^6</td>
<td>4.1 ± 0.13</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> WSSP-1</td>
<td>6.2 x 10^6</td>
<td>2.9 ± 0.18</td>
</tr>
</tbody>
</table>

The standard deviation is the standard error between the three triplicates done for each experiment.
Figure 5.1. Amplification of the FBA gene of *E. faecalis* ARO1/DGV3. Lane M; marker (Invitrogen 1 KbDNA Marker), Lanes 4, 5, 6 and 7; products formed by the amplification of primer pairs Fwd:FBA/Rev:FBA 1f/1r, 2f/2r, 3f/3r and 4f/4r respectively. All primer pairs used are listed in Appendix 2. Poor quality gel due to re-scanned image of a scanned gel photograph.
5.4.1 Effect of pediocin upon S. mutans OMZ175

The lytic agent pediocin was investigated for its ability to produce inhibition of growth of S. mutans OMZ175. Addition of pediocin to the growth medium failed to significantly inhibit growth of S. mutans OMZ175 at all concentrations tested.

5.4.2 Determination of sub-lethal concentration of nisin and synergism with PS-ODN

A nisin concentration of 0.6 mg/ml or more increased the lag phase S. mutans OMZ175, and a final concentration of 1.25 mg/ml was chosen as the sub-lethal concentrations against S. mutans OMZ175 (Table 5.2). A concentration of 0.75 mg/ml nisin was chosen as sub-lethal concentration for E. faecalis AR01/DGVS (Table 5.3).

When nisin with a final concentration of 1.2 mg/ml was combined with 20 μM SM-FBA, the length of the lag phase of S. mutans OMZ175 (4.21 ± 0.20) was shortened relative to use of nisin alone (6.23 ± 0.17) (Table 5.2). ATS2 used in combination with nisin had no significant inhibitory effect on growth of S. mutans OMZ175 (6.27 ± 0.21) compared to that of nisin alone. Use of SM-FBA or ATS2 alone had no significant inhibitory effect upon S. mutans OMZ175 growth. As SM-FBA was observed to be acting together with nisin resulting in a negatively synergistic result for S. mutans OMZ175, a combination of antisense and nisin was not trialled against E. faecalis.

5.4.2.1 Titration of antisense with differing amounts with nisin

As the combination of SM-FBA + nisin appeared to reduce the ability of nisin to inhibit the growth of S. mutans OMZ175, it was necessary to determine whether SM-FBA alone had an inhibitory effect upon the viability (Methods 2.4.5.3). Using the spot test with M. leuteus IL1 as an indicator organism, in the absence of nisin, SM-FBA (1 μM- 40 μM) had no significant effect upon M. leuteus growth (Tables 5.4, 5.5). In comparison, use of nisin alone gave strong
Table 5.2: Synergistic effect between nisin and antisense upon *S. mutans* OMZ175 growth

<table>
<thead>
<tr>
<th>Nisin concentration (mg/ml) and antisense (20 µM)</th>
<th>Lag time (Hours) ± SD †</th>
<th>Statistical significance *</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 + 0</td>
<td>0.4 ± 0.34</td>
<td>N/A</td>
</tr>
<tr>
<td>0.15 + 0</td>
<td>0.9 ± 0.30</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.3 + 0</td>
<td>1.2 ± 0.12</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.6 + 0</td>
<td>2.7 ± 0.19</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>1.2 + 0</td>
<td>6.2 ± 0.17</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>2.5 + 0</td>
<td>NG</td>
<td>-</td>
</tr>
<tr>
<td>5 + 0</td>
<td>NG</td>
<td>-</td>
</tr>
<tr>
<td>10 + 0</td>
<td>NG</td>
<td>-</td>
</tr>
<tr>
<td>0 + SM-FBA</td>
<td>0.7 ± 0.17</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0 + ATS2 control</td>
<td>0.6 ± 0.21</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>1.2 + SM-FBA</td>
<td>4.2 ± 0.20</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>1.2 + ATS2</td>
<td>6.3 ± 0.21</td>
<td>P &gt; 0.05</td>
</tr>
</tbody>
</table>

† Reagents added to exponential phase *S. mutans* OMZ175.

* Nisin only, and antisense alone treatments were compared to the 0 + 0 control. Nisin plus antisense was compared to the 1.2 + 0 treatment.

NG = No growth after 24 hours

The standard deviation is the standard error between the three triplicates done for each experiment.
Table 5.3: Effect of increasing nisin concentration upon *E. faecalis* AR01/DGVS growth

<table>
<thead>
<tr>
<th>Nisin concentration (mg/ml)</th>
<th>Lag time (Hours) ± SD†</th>
<th>Statistical significance *</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.7 ± 0.21</td>
<td>N/A</td>
</tr>
<tr>
<td>0.37</td>
<td>4.2 ± 1.62</td>
<td><em>P &lt; 0.001</em></td>
</tr>
<tr>
<td>0.75</td>
<td>8.2 ± 0.13</td>
<td><em>P &lt; 0.001</em></td>
</tr>
<tr>
<td>1.55</td>
<td>11.6 ± 0.17</td>
<td><em>P &lt; 0.001</em></td>
</tr>
<tr>
<td>3.12</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td>6.25</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td>12.5</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>NG†</td>
<td>-</td>
</tr>
</tbody>
</table>

† No sigmoidal curve could be fitted so statistical analysis was not possible.

* Nisin treatments compared to the 0 nisin control.

NG = No growth after 24 hours

The standard deviation is the standard error between the three triplicates done for each experiment.
Table 5.4: Effect of increasing concentrations of nisin together with 20 µM SM-FBA upon S. mutans OMZ175 growth using the spot test assay

<table>
<thead>
<tr>
<th>Nisin concentration (mg/ml) and antisense (20 mM)</th>
<th>Diameter (mm) ± SD †</th>
<th>Statistical significance *</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 + 0</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>0.0078 + 0</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>0.015 + 0</td>
<td>7 ± 2</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.031 + 0</td>
<td>15 ± 3</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.062 + 0</td>
<td>24 ± 1</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.12 + 0</td>
<td>30 ± 2</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.25 + 0</td>
<td>33 ± 3</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.5 + 0</td>
<td>35 ± 4</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>1 + 0</td>
<td>38 ± 3</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.0078 + 20</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>0.015 + 20</td>
<td>2 ± 1</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0.031 + 20</td>
<td>5 ± 2</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.062 + 20</td>
<td>11 ± 3</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.12 + 20</td>
<td>14 ± 2</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.25 + 20</td>
<td>18 ± 3</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.5 + 20</td>
<td>22 ± 2</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>1 + 20</td>
<td>25 ± 4</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

* Analysed used one–way ANOVA followed by Tukeys multiple comparison test. All nisin only concentrations are compared to that of the 0 + 0 control. Nisin plus antisense was compared to the equivalent nisin alone concentration.

The standard deviation is the standard error between the three triplicates done for each experiment.
Table 5.5: Effect of increasing concentrations of SM-FBA together with 31.2 µg/ml nisin upon *S. mutans* OMZ175 growth using the spot test assay

<table>
<thead>
<tr>
<th>Nisin concentration (31.2 µg/ml) and antisense (µM)</th>
<th>Diameter (mm) ± SD†</th>
<th>Statistical significance *</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 + 0</td>
<td>0 ± 0</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + 1</td>
<td>0 ± 0</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + 2</td>
<td>0 ± 0</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + 5</td>
<td>0 ± 0</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + 10</td>
<td>0 ± 0</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + 15</td>
<td>0 ± 0</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + 20</td>
<td>0 ± 0</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + 30</td>
<td>0 ± 0</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + 40</td>
<td>0 ± 0</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + 50</td>
<td>0 ± 0</td>
<td>N/A</td>
</tr>
<tr>
<td>31.2 + 0</td>
<td>15 ± 5</td>
<td>N/A</td>
</tr>
<tr>
<td>31.2 + 1</td>
<td>8 ± 3</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>31.2 + 2</td>
<td>8 ± 4</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>31.2 + 5</td>
<td>7 ± 2</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>31.2 + 10</td>
<td>6 ± 4</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>31.2 + 15</td>
<td>6 ± 2</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>31.2 + 20</td>
<td>5 ± 2</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>31.2 + 30</td>
<td>4 ± 3</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>31.2 + 40</td>
<td>4 ± 2</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>31.2 + 50</td>
<td>3 ± 2</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

* Analysed using one–way ANOVA followed by Tukeys multiple comparison test. Antisense plus nisin was compared to the equivalent antisense concentration alone.

The standard deviation is the standard error between the three triplicates done for each experiment.
inhibition of the indicator lawns, with zone size ranging from 0 mm for no nisin, to 38 mm for 1 mg/ml nisin (Table 5.4, Figure 5.2). For all nisin concentrations greater than 0.25 mg/ml, no further significant increase in inhibition size was shown, even when the nisin concentration had doubled, indicating a possible saturation effect. The addition of a 1 µM concentration of SM-FBA to 31.2 µg/ml nisin to decrease the zone of inhibition from 15 mm to 8 mm. When a 20 µM concentration of SM-FBA was added to a 31.2 µg/ml nisin concentration, the zone of inhibition size decreased further to 5 mm. When increasing concentrations of nisin were added to a constant 20 µM SM-FBA concentration, zone of inhibition size decreased significantly between identical nisin concentrations, with the size of the zone of inhibition decreasing from 38 mm for a 1 mg/ml nisin concentration alone, to 25 mm for 1 mg/ml nisin + 20 µM SM-FBA (Figure 5.2).

5.5 Antibiotics as an antisense delivery agent

5.5.1 Establishment of sub-lethal penicillin or vancomycin concentrations for S. aureus Oxford and WSSP-1, S. mutans OMZ175 and E. faecalis AR01/DGVS

In order to investigate whether antibiotics could be used in combination with antisense agents to produce a growth inhibitory effect, the antibiotics penicillin and vancomycin were studied (Methods 2.4.6). Addition of vancomycin to S. mutans OMZ175 and E. faecalis AR01/DGVS prevented both bacterial strains from reaching their maximum OD levels, but failed to cause a drop in OD or increase in inhibition that could indicate lysis of cells (Table 5.6). Growth of S. mutans OMZ175 in TSB increased the length of the lag phase, compared to growth in THB. S. aureus Oxford and S. aureus WSSP-1 were highly susceptible to the effects of vancomycin; even 0.001 µg/ml vancomycin completely inhibit the growth of either bacterium (Table 5.6). No lytic effect was observed in either strain following the addition of vancomycin. However, the growth curves produced in the presence of vancomycin upon by both S. aureus strains indicated lack of lysis, so no further intermediate vancomycin concentrations were investigated.
Figure 5.2. The size of the inhibition zone of *S. mutans* OMZ175 to dose response treatments of increasing nisin + SM-FBA concentrations. Ability of nisin and/or SM-FBA to inhibit the growth of *S. mutans* OMZ175 measured using the spot test assay. Panel A: Titration of different amounts of SM-FBA against *S. mutans* OMZ175, with a constant nisin concentration (31.2 µg/ml). Panel B: Titration of different amounts of nisin against *S. mutans* OMZ175, with a constant SM-FBA concentration (20 µM).
Table 5.6. Inhibition of growth and increase in the length of the lag phase by vancomycin upon *S. aureus* WSSP-1, *S. aureus* Oxford, *S. mutans* OMZ175 and *E. faecalis* AR01/DGVS

<table>
<thead>
<tr>
<th>Vancomycin concentration (µg/ml) (mg/ml for <em>E. faecalis</em> AR01/DGVS)</th>
<th>Lag time (Hours) ± SD†</th>
<th>Statistical significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. aureus</strong> WSSP-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.8 ± 0.32</td>
<td>N/A</td>
</tr>
<tr>
<td>0.0001</td>
<td>0.5 ± 0.27</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0.001</td>
<td>0.7 ± 0.34</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0.01</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td>0.1</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td>12.5</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>NG†</td>
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</tr>
<tr>
<td>50</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td>100</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td><strong>S. aureus</strong> Oxford</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.4 ± 0.38</td>
<td>N/A</td>
</tr>
<tr>
<td>0.0001</td>
<td>2.5 ± 0.58</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0.001</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td>0.01</td>
<td>NG†</td>
<td>-</td>
</tr>
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<td>0.1</td>
<td>NG†</td>
<td>-</td>
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<td>12.5</td>
<td>NG†</td>
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<td>25</td>
<td>NG†</td>
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</tr>
<tr>
<td>50</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td>100</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td><strong>S. mutans</strong> OMZ175</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>8.1 ± 0.18</td>
<td>N/A</td>
</tr>
<tr>
<td>1</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td>12.5</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td>100</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td><strong>E. faecalis</strong> AR01/DGVS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.7 ± 0.24</td>
<td>N/A</td>
</tr>
<tr>
<td>3.125</td>
<td>NG†</td>
<td>-</td>
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<tr>
<td>6.25</td>
<td>NG†</td>
<td>-</td>
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<td>12.5</td>
<td>NG†</td>
<td>-</td>
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<td>25</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td>200</td>
<td>NG†</td>
<td>-</td>
</tr>
</tbody>
</table>
† No sigmoidal curve could be fitted so statistical analysis was not possible.

* All strains were compared to the 0 vancomycin concentration for that strain
- Indicates that sigmoidal curves could not be fitted to the growth curves due to the lack of OD change overtime

NG = No growth after 18 hours

The standard deviation is the standard error between the three triplicates done for each experiment.
Addition of penicillin to the growth medium failed to inhibit growth of *S. aureus* WSSP-1 (Table 5.7). A penicillin concentration of 0.0195 µg/ml or 0.078 µg/ml was found to produce an inhibitory effect upon *S. aureus* Oxford and *S. mutans* OMZ175 respectively (Table 5.7). However, the inhibitory effect produced against *S. aureus* Oxford failed to cause a drop in OD or increase in lag phase that could indicate lysis of cells. The growth curves produced in the presence of penicillin by both *S. aureus* strains were atypical of sub-inhibitory agents, so no further intermediate penicillin concentrations were investigated. The inhibitory effect produced by penicillin against *S. mutans* OMZ175 was similar to that produced by zoocin A on susceptible strains. At 0.2 µg/ml, the length of the lag phase of *S. mutans* OMZ175 was significantly increased (P = 0.001) compared to that of uninhibited *S. mutans* OMZ175, but the strain was eventually still able to grow to its own maximum OD level. The addition of penicillin to *E. faecalis* AR01/DGVS prevented the bacteria from reaching their maximum OD levels, but failed to cause a drop in OD or increase in inhibition that could indicate lysis of cells (Table 5.7).

### 5.6 Penicillin and its ability to deliver antisense to *S. mutans* OMZ175

#### 5.6.1 Determination of sub-lethal concentration of penicillin for exponential phase *S. mutans* in unbuffered TSB

Because penicillin was found to induce a sub-lethal inhibition of *S. mutans* OMZ175 when applied to lag phase cells, it was of interest to determine if it had a similar effect on cells in other growth phases, so that the lowest possible concentration of penicillin could be used in combination with other antimicrobial agents (Methods 2.4.6.3). Penicillin was found to be unable to cause a lytic effect upon exponential phase *S. mutans* OMZ175 in TSB media alone for all concentrations tested (0- 500 µg/ml)

#### 5.6.1.1 Use of buffered media for penicillin and the growth of *S. mutans* OMZ175
Table 5.7: Inhibition of growth by penicillin upon exponential phase *S. aureus* WSSP-1, *S. aureus* Oxford, *S. mutans* OMZ175 and *E. faecalis* AR01/DGVS by penicillin

<table>
<thead>
<tr>
<th>Penicillin concentration (µg/ml) (for E. faecalis AR01/DGVS mg/ml)</th>
<th>Lag time (Hours) ± SD†</th>
<th>Statistical significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. aureus WSSP-1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.8 ± 0.75</td>
<td>N/A</td>
</tr>
<tr>
<td>12.5</td>
<td>0.9 ± 0.12</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>25</td>
<td>1.1 ± 0.18</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>50</td>
<td>1.4 ± 0.81</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>100</td>
<td>1.9 ± 0.19</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td><strong>S. aureus Oxford</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>2.9 ± 0.42</td>
<td>N/A</td>
</tr>
<tr>
<td>0.05</td>
<td>2.8 ± 0.61</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0.1</td>
<td>3.1 ± 0.39</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0.2</td>
<td>3.1 ± 0.21</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0.4</td>
<td>3.2 ± 0.76</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0.8</td>
<td>3.8 ± 1.72</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. mutans OMZ175</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>8.1 ± 0.18</td>
<td>N/A</td>
</tr>
<tr>
<td>0.05</td>
<td>8.4 ± 0.39</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0.1</td>
<td>8.4 ± 0.27</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0.2</td>
<td>9.8 ± 0.14</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.4</td>
<td>10.2 ± 0.21</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.8</td>
<td>17.1 ± 0.65</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>E. faecalis AR01/DGVS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.7 ± 0.24</td>
<td>N/A</td>
</tr>
<tr>
<td>12.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
† No sigmoidal curve could be fitted so statistical analysis was not possible.

* All strains were compared to the 0 penicillin concentration for that strain.

- Indicates that sigmoidal curves could not be fitted to the growth curves due to the lack of OD change overtime

NG = No growth after 18 hours

The standard deviation is the standard error between the three triplicates done for each experiment.
It was found that in buffered media, *S. mutans* OMZ175 was capable of growth in TSB media buffered with a potassium phosphate concentration of 0.1 M or less (Table 5.8). *S. mutans* OMZ175 was unable to grow in TSB media buffered with a concentration of potassium phosphate greater than or equal to 0.5 M. *S. mutans* OMZ175 was unable to grow in either TSB or Antibiotic media number three when buffered with 0.1 M tris-citrate buffer (Table 5.8). The buffering capacity of 0.05 M potassium phosphate was found to prevent the large drop in pH caused by the growth of *S. mutans* OMZ175 (Figure 5.3). In unbuffered TSB, the pH value dropped from 7.5 to 4.5, ten hours after media inoculation with *S. mutans* OMZ175. In TSB buffered with 0.05 M potassium phosphate, after 10 hours of incubation, the pH had fallen to only 6.5.

### 5.6.1.2 Addition of penicillin to *S. mutans* OMZ175 in buffered TSB

Penicillin at a concentration of 0.8 µg/ml, added to lag phase *S. mutans* OMZ175 in buffered media, significantly inhibited growth (P = 0.001), reducing the viable count to 5.2 x 10^5. Although the OD of penicillin treated cells did not recover to the same level as untreated cells, the final viable count of the penicillin treated cells (9.5 x 10^{10} cfu/ml) was not significantly different from that of untreated cells (9.3 x 10^{10}) (Table 5.9, Figure 5.4). For exponential phase *S. mutans* OMZ175 cells a penicillin concentration of 5 µg/ml significantly reduced viability to 6.23 x 10^6 cfu/ml but still allowed the bacterial cells to recover and grow back to their initial cell number of 8.9 x 10^8 (Table 5.9, Figure 5.5). However, all concentrations of penicillin failed to induce lysis occurring in exponential phase cells- the optical density of each treated culture remained static- indicating non-bacteriolytic cell death (Table 5.9, Figure 5.6). Concentrations of 10 µg/ml prevented growth completely. The highest penicillin concentration tested 500 µg/ml did not significantly reduce the viability or optical density levels for stationary phase cells (Table 5.9, Figure 5.6).

### 5.6.1.3 Synergism between penicillin and PS-ODN on *S. mutans* OMZ175 in buffered media
Table 5.8: The growth and length of the lag phase of *S. mutans* OMZ175 in TSB with various buffers

<table>
<thead>
<tr>
<th>Media type + buffer</th>
<th>Lag time (Hours) ± SD†</th>
<th>Statistical significance *</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSB + 0</td>
<td>4.2 ± 0.32</td>
<td>N/A</td>
</tr>
<tr>
<td>TSB + 0.025 M potassium phosphate</td>
<td>4.1 ± 0.21</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>TSB + 0.05 M potassium phosphate</td>
<td>4.1 ± 0.16</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>TSB + 0.1 M potassium phosphate</td>
<td>3.9 ± 0.84</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>TSB + 0.5 M potassium phosphate</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>TSB + 1 M potassium phosphate</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>TSB + 2.5 M potassium phosphate</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>TSB + 5 M potassium phosphate</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>TSB + 10 M potassium phosphate</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>TSB + 0.05 M tris- citrate</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>Antibiotic 3 + 0</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>Antibiotic 3 + 0.05 M tris- citrate</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>Antibiotic 3 + 0.05 M potassium phosphate</td>
<td>NG †</td>
<td>-</td>
</tr>
</tbody>
</table>

† No sigmoidal curve could be fitted so statistical analysis was not possible.

* Analysed using one–way ANOVA followed by Tukeys multiple comparison test. Antisense plus nisin was compared to the equivalent antisense concentration alone

NG = No growth after 24 hours

The standard deviation is the standard error between the three triplicates done for each experiment.
Figure 5.3. pH over time of lag phase *S. mutans* OMZ175 grown in buffered media. *S. mutans* OMZ175 grown in the presence of: – TSB; – TSB + 0.05 M potassium phosphate. The error bars stand for the standard error between the three triplicates done for each experiment.
Table 5.9: Growth and length of the lag phase / recovery time of *S. mutans* OMZ175 with increasing concentrations of penicillin

<table>
<thead>
<tr>
<th>Growth phase and penicillin concentration (µg/ml)</th>
<th>Lag or recovery time (Hours) ± SD†</th>
<th>Statistical significance *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>4.6 ± 0.31</td>
<td>N/A</td>
</tr>
<tr>
<td>0.05</td>
<td>4.4 ± 0.28</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0.1</td>
<td>4.2 ± 0.21</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0.2</td>
<td>4.4 ± 0.23</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0.4</td>
<td>4.5 ± 0.36</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0.6</td>
<td>8.4 ± 0.75</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.8</td>
<td>14.3 ± 0.58</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>2</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>Exponential</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.9</td>
<td>N/A</td>
</tr>
<tr>
<td>0.025</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>0.05</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>0.1</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>0.5</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>2.5</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>Stationary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>NG †</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>100</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>250</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>500</td>
<td>NG †</td>
<td>-</td>
</tr>
</tbody>
</table>

† Due to lack of growth, a sigmoidal curve was unable to be fitted, thus statistical analysis was not possible.

*Penicillin concentrations compared to the 0 µg/ml penicillin concentration for the appropriate growth phase. NG = No growth after 24 hours. The standard deviation is the standard error between the three triplicates done for each experiment.
Figure 5.4. OD (panel A) and viability (panel B) changes over time of lag phase *S. mutans* OMZ175 treated with penicillin in 0.05 M Potassium phosphate buffered media. Growth of *S. mutans* OMZ175 over time in the presence of: ■ TSB + 0.05 M Potassium phosphate (1); ■ 2 µg/ml penicillin (2); ○ 0.8 µg/ml penicillin (3); △••• 0.6 µg/ml penicillin (4); △ 0.4 µg/ml penicillin (5); 0.2 µg/ml penicillin (6); 0.1 µg/ml penicillin (7); 0.05 µg/ml penicillin (8). Arrow indicates the time point of treatment addition. The number beside the graph indicates the corresponding treatment type. The error bars stand for the standard error between the three triplicates done for each experiment.
Figure 5.5. OD (panel A) and viability (panel B) changes over time of exponential phase S. mutans OMZ175 treated with penicillin in 0.05 M Potassium phosphate buffered media. Growth of S. mutans OMZ175 over time in the presence of: --- TSB + 0.05 M Potassium phosphate (1); ■ 10 µg/ml penicillin (2); ○ 5 µg/ml penicillin (3); •Δ• 2.5 µg/ml penicillin (4); – – – 1 µg/ml penicillin (5); ◊ 0.5 µg/ml penicillin (6); ☐ 0.1 µg/ml penicillin (7); ○ 0.05 µg/ml penicillin (8); ▼ 0.025 µg/ml penicillin (9). Arrow indicates the time point of treatment addition. The number beside the graph indicates the corresponding treatment type. The error bars stand for the standard error between the three triplicates done for each experiment.
Figure 5.6. OD (panel A) and viability (panel B) changes over time of stationary phase S. mutans OMZ175 treated with penicillin in 0.05 M Potassium phosphate buffered media. Growth of S. mutans OMZ175 over time in the presence of: - TSB + 0.05 M Potassium phosphate (1); - 2 µg/ml penicillin (2); - 10 µg/ml penicillin (3); - 20 µg/ml penicillin (4); - 100 µg/ml penicillin (5); - 250 µg/ml penicillin (6); - 500 µg/ml penicillin (7). Arrow indicates the time point of treatment addition. The number beside the graph indicates the corresponding treatment type. The error bars stand for the standard error between the three triplicates done for each experiment.
It was necessary to show what inhibitory effect, if any, was produced by a combination between penicillin and SM-FBA upon \textit{S. mutans} OMZ175 in all three growth stages (lag, exponential and stationary) (Figures 5.7, 5.8 and 5.9). When \textit{S. mutans} OMZ175 in the lag phase was combined with the appropriate amount of penicillin plus 10 µM SM-FBA, a far greater inhibitory effect was shown than for penicillin alone (Figure 5.7, Table 5.10). Exponential phase cells incubated with penicillin or penicillin plus SM-FBA showed a decrease in viability, but a corresponding decrease in OD was not observed (Figure 5.8, Table 5.10). No further inhibition was observed for exponential and stationary phase cells treated with a combination of 10 µM SM-FBA plus penicillin compared to that of penicillin alone (Figures 5.8, 5.9, Table 5.10). Due to the fact that the OD of exponential and stationary phase cells did not increase over time, the results were unable to be statistically analysed using the same method applied to the other results of this study. When 10 µM ATS2 was combined with penicillin, the inhibitory effect on growth of \textit{S. mutans} OMZ175 in any of the three growth stages was found to be no greater than that of penicillin alone (Figures 5.7, 5.8, 5.9, Table 5.10). When 10 µM of either: SM-FBA or ATS2 were added to \textit{S. mutans} OMZ175 in any of the three growth phases without penicillin, no significant inhibitory effect was observed.

\textbf{5.6.1.4 Effect of the titration of SM-FBA upon the growth of \textit{S. mutans} OMZ175}

It needed to be determined if SM-FBA alone had an inhibitory effect upon the growth of \textit{S. mutans} OMZ175, or whether the β-lactam penicillin was required in order for the antisense sequence to produce an inhibitory effect (Methods 2.4.7.6). In the absence of penicillin, SM-FBA (1 µM- 40 µM) had no significant effect upon \textit{S. mutans} OMZ175 growth rate (Figure 5.10). When combined with 0.1 µg/ml penicillin, as SM-FBA concentration increased, so did the length of the lag phase. The length of the lag phase increased proportionately with SM-FBA concentration ($R^2 = 0.93$ value)
Figure 5.7. OD (panel A) or viability (panel B) changes over time of lag phase S. mutans OMZ175 treated with a sub-inhibitory concentration of penicillin and/ or antisense. Lag phase S. mutans OMZ175 over time in the presence of: THB (1); 0.8 µg/ml penicillin (2); 10 µM SM-FBA (3); 10 µM ATS2 (4); 0.8 µg/ml penicillin + 10 µM SM-FBA (5); 0.8 µg/ml penicillin + 10 µM ATS2 (6). Arrow indicates the time point of treatment addition. The number beside the graph indicates the corresponding treatment type. The error bars stand for the standard error between the three triplicates done for each experiment.
Figure 5.8. OD (panel A) or viability (panel B) changes over time of exponential phase *S. mutans* OMZ175 treated with a sub-inhibitory concentration of penicillin and/ or antisense. Exponential phase *S. mutans* OMZ175 over time in the presence of: – THB (1); – 5 µg/ml penicillin (2); – 10 µM SM-FBA (3); – 10 µM ATS2 (4); – 5 µg/ml penicillin + 10 µM SM-FBA (5); – 5 µg/ml penicillin + 10 µM ATS2 (6). Arrow indicates the time point of treatment addition. The number beside the graph indicates the corresponding treatment type. The error bars stand for the standard error between the three triplicates done for each experiment.
Figure 5.9. OD (panel A) or viability (panel B) changes over time of stationary phase *S. mutans* OMZ175 treated with a sub-inhibitory concentration of penicillin and/or antisense. Stationary phase *S. mutans* OMZ175 over time in the presence of: • THB (1); ○ 500 µg/ml penicillin (2); ⬇ 10 µM SM-FBA (3); → 10 µM ATS2 (4); ▼ 500 µg/ml penicillin + 10 µM SM-FBA (5); ●Δ● 500 µg/ml penicillin + 10 µM ATS2 (6). Arrow indicates the time point of treatment addition. The number beside the graph indicates the corresponding treatment type. The error bars stand for the standard error between the three triplicates done for each experiment.
Table 5.10: Growth and length of the lag phase / recovery time of *S. mutans* OMZ175 in the presence of penicillin and antisense

<table>
<thead>
<tr>
<th>Penicillin concentration (µg/ml) plus antisense (10 µM) to <em>S. mutans</em> in different growth phases</th>
<th>Lag or recovery time (Hours) ± SD</th>
<th>Statistical significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lag</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 + 0</td>
<td>9.2 ± 0.52</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + ATS2</td>
<td>9.1 ± 0.14</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0 + SM-FBA</td>
<td>8.9 ± 0.34</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0.8 + 0</td>
<td>16.9 ± 0.35</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0.8 + ATS2</td>
<td>17.2 ± 0.24</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0.8 + SM-FBA</td>
<td>NG †</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td><strong>Exponential</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 + 0</td>
<td>1.9 ± 0.31</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + ATS2</td>
<td>1.8 ± 0.38</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0 + SM-FBA</td>
<td>1.8 ± 0.31</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>5 + 0</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>5 + ATS2</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>5 + SM-FBA</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td><strong>Stationary</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 + 0</td>
<td>NG †</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + ATS2</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>0 + SM-FBA</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>250 + 0</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>250 + ATS2</td>
<td>NG †</td>
<td>-</td>
</tr>
<tr>
<td>250 + SM-FBA</td>
<td>NG †</td>
<td>-</td>
</tr>
</tbody>
</table>

*All penicillin and PS-ODN alone values compared to the 0 + 0 control of the appropriate growth phase. All penicillin + PS-ODN values are compared to their corresponding penicillin only control.

†No sigmoidal curve could be fitted to the stationary phase data so statistical analysis was not possible. NG = No growth after 24 hours. The standard deviation is the standard error between the three triplicates done for each experiment.
Figure 5.10. Growth inhibition of *S. mutans* OMZ175 caused by the titration of increasing amounts of penicillin (0-6 mM), with a constant SM-FBA concentration (10 μM).
5.6.1.5  Effect of the titration of penicillin upon the growth of S. mutans OMZ175

It was necessary to determine the lowest concentration of penicillin required, when combined with SM-FBA, to produce a significant inhibition of S. mutans OMZ175 growth, so that the lowest concentration of penicillin required could always be used (Methods 2.4.7.6). As the penicillin concentration increased there was an increase in the length of the lag phase of S. mutans OMZ175 (Figure 5.10, Table 5.11). When penicillin was combined with 10 µM SM-FBA, the length of the lag phase was significantly increased from that of the PS-ODN alone. The length of the lag phase increased proportionately with penicillin concentration. A R² value was unable to be determined as only two values were able to be fitted to the graph, due to the lack of growth of S. mutans OMZ175 when treated with higher concentrations (Table 5.11). Combining 0.1 µg/ml penicillin and 10 µM SM-FBA resulted in total inhibition of S. mutans OMZ175 growth during the 24 hour incubation period.

5.6.1.6  Multiple exposure of S. mutans OMZ175 to penicillin and PS-ODN

It was necessary to determine whether repeat exposure to penicillin resulted in the development of resistance, or enhanced penicillin’s ability to lyse S. mutans OMZ175 (Figure 5.11). Due to the bacteriostatic nature of penicillin on exponential phase S. mutans OMZ175, a repeated dose of penicillin, given 6 hours after the addition of the initial penicillin treatment, produced no further lysis of S. mutans OMZ175, and therefore the results are unable to be statistically analysed using the method described above. Measured optically, penicillin when combined with either SM-FBA or ATS2, again showed no further inhibition compared to that penicillin alone, even when a second dose was given. PS-ODN alone did not result in any changes in the growth rate of S. mutans OMZ175.

5.7  FBA mRNA expression in S. mutans OMZ175 treated with penicillin and PS-ODN

Significant differences in FBA mRNA expression levels were observed, when lag phase S.
Table 5.11: The synergistic effect of SM-FBA and penicillin upon *S. mutans* OMZ175 growth and length of the lag phase

<table>
<thead>
<tr>
<th>SM-FBA concentration (mM) plus penicillin (µg/ml)</th>
<th>Lag time (Hours) ± SD†</th>
<th>Statistical significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 + 0</td>
<td>4.9 ± 0.53</td>
<td>N/A</td>
</tr>
<tr>
<td>0 + 0.05</td>
<td>6.2 ± 0.39</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>0 + 0.1</td>
<td>10.6 ± 0.66</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>0 + 0.2</td>
<td>13.9 ± 1.68</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>10 + 0</td>
<td>5.2 ± 0.32</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>10 + 0.05</td>
<td>17.2 ± 0.09</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>10 + 0.1</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td>10 + 0.2</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td>1 + 0</td>
<td>4.8 ± 0.10</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>5 + 0</td>
<td>5.4 ± 0.42</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>10 + 0.1</td>
<td>4.7 ± 0.59</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>20 + 0</td>
<td>4.9 ± 0.45</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>40 + 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 + 0.1</td>
<td>11.4 ± 0.24</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>5 + 0.1</td>
<td>16.1 ± 5.96</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>10 + 0.1</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td>20 + 0.1</td>
<td>NG†</td>
<td>-</td>
</tr>
<tr>
<td>40 + 0.1</td>
<td>NG†</td>
<td>-</td>
</tr>
</tbody>
</table>

† A sigmoidal curve was unable to be fitted due to lack of growth, this statistical analysis could not occur.

*All treatments containing no SM-FBA were compared to the 0 + 0 control. Those containing 10 mM SM-FBA were compared to the 10 mM SM-FBA + 0 penicillin control. All treatments with increasing amounts of penicillin only were compared to the 0 + 0 control. All treatments containing 0.1 µg/ml penicillin and increasing amounts of SM-FBA were compared to the 0 + 0.1 µg/ml penicillin control.

NG = No growth after 24 hours. The standard deviation is the standard error between the three triplicates done for each experiment.
Figure 5.11. Inhibition of exponential phase *S. mutans* OMZ175 with multiple additions of penicillin and antisense. Growth of *S. mutans* OMZ175 over time in the presence of: 

1. TSB + 0.05 M potassium phosphate (1);
2. 5 µg/ml penicillin (2);
3. 10 mM SM-FBA (3);
4. 5 µg/ml penicillin + 10 mM SM-FBA (4);
5. 5 µg/ml penicillin (2 doses) (5);
6. 5 µg/ml penicillin (2 doses) + 10 mM SM-FBA (6);
7. 10 mM ATS2 (7);
8. 5 µg/ml penicillin + 10 mM ATS2 (8);
9. 5 µg/ml penicillin (2 doses) + 10 mM ATS2 (9).

The error bars stand for the standard error between the three triplicates done for each experiment.
mutans OMZ175 cells had been treated with a combination of both penicillin and SM-FBA at 30 mins, 5 hours and 16 hours after treatment addition (Figure 5.12). No significant difference in FBA mRNA expression was observed for exponential phase S. mutans OMZ175 regardless of treatment type. Thirty minutes after addition of penicillin and SM-FBA, a 5.728 ± 0.23 fold decrease in FBA mRNA expression in comparison with those cells treated with penicillin alone was observed for lag phase S. mutans OMZ175 (Figure 5.12), whilst in comparison treated exponential phase S. mutans OMZ175 cells showed non-significant fold change of 0.686 ± 0.16 in FBA mRNA expression (Figure 5.12). Five hours after addition of penicillin and SM-FBA, a 656.890 ± 192.40 fold decrease in FBA mRNA expression in comparison with those cells treated with penicillin alone was observed for lag phase S. mutans OMZ175 (Figure 5.12), whilst in comparison exponential phase S. mutans OMZ175 showed a non-significant fold change of 0.949 ± 0.08 in FBA mRNA expression (Figure 5.12). Sixteen hours after addition of penicillin and SM-FBA, a 640.563 ± 186.8 fold decrease in FBA mRNA expression in comparison with those cells treated with penicillin alone was observed for lag phase S. mutans OMZ175 (Figure 5.12) whilst in comparison exponential phase S. mutans OMZ175 showed a non-significant fold change of 1.136 ± 0.13 in FBA mRNA expression (Figure 5.12). The raw data is included on McLeod 2012 Data CD.

No significant difference in the fold expression levels was found for lag or exponential phase S. mutans OMZ175 at any time point, for any treatment type, for the mRNA expression levels of either 16s rRNA and Gyr A.

5.8 Uptake of radiolabeled antisense by lag and exponential phase S. mutans OMZ175 treated with penicillin

These experiments demonstrate that an amount of $\gamma^{32}$ P-ATS2 immediately becomes associated with the S. mutans OMZ175 cells, regardless of whether the cells are treated with $\gamma^{32}$ P-ATS2 alone, or $\gamma^{32}$ P-ATS2 combined with penicillin. It was demonstrated that lag phase S. mutans OMZ175 cells treated with $\gamma^{32}$ P-ATS2 alone showed between 21,756 ±15,279 and 84,808 ± 89,607 molecules of $\gamma^{32}$ P-ATS2 associated with each CFU (Figures
Figure 5.12. Fold change in fba expression levels over time for *S. mutans* OMZ175 treated in the lag phase (panel A), *S. mutans* OMZ175 treated in the exponential phase (panel B). Expression levels over time in the presence of: THB; 0.8 or 5 µg/ml penicillin; 10 µM SM-FBA; 10 µM ATS2; 0.8 or 5 µg/ml penicillin + 10 µM SM-FBA; 0.8 or 5 µg/ml penicillin + 10 µM ATS2. The error bars stand for the standard error between the three triplicates done for each experiment.
5.13, 5.14). Exponential phase *S. mutans* OMZ175 cells treated with $\gamma^{32}$ P-ATS2 alone showed similar results, with between 8,419 ± 543 and 50,388 ± 25,684 molecules of $\gamma^{32}$ P-ATS2 associated with each CFU (Figures 5.15, 5.16).

Only lag phase *S. mutans* OMZ175 treated with zoocin A + $\gamma^{32}$ P-ATS2 showed immediate increases in the amount of $\gamma^{32}$ P-ATS2 associated with the bacterial cells compared to those of *S. mutans* OMZ175 + $\gamma^{32}$ P-ATS2 alone. Increased uptake of $\gamma^{32}$ P-ATS2 was shown to occur for the first five hours of treatment, after which there was a rapid decrease in the amount of $\gamma^{32}$ P-ATS2 present inside each bacterial cell. At five hours after treatment, the greatest amount of $\gamma^{32}$ P-ATS2 was found within lag phase cells ($2.73 \pm 1.82 \times 10^8$). This time point corresponds to the time point at which significant decreases in the expression levels of *fba* were observed (656.890 ± 192.40 fold decrease). Whilst there is also significant repression of growth and *fba* expression (640.563 ± 186.8 fold decrease) at 16 hours after treatment with penicillin + SM-FBA, radioactivity experiments were conducted using ATS2, the corresponding data showed a lower level of ATS2 associated with each CFU at 12 hours after treatment ($5.29 \pm 2.19 \times 10^5$). This is presumed to be due to the fact that ATS2 does not work synergistically with penicillin, and thus growth recovery and subsequent dilution of the PS-ODN molecules per CFU would occur at an earlier time point compared to that of SM-FBA + penicillin treated cells.

A comparison between zoocin A and penicillin highlights the fact that the different modes of action of each agent result in different amounts of PS-ODN cell association over time (Table 5.12). Those cells treated with zoocin A show a greater amount of cell associated $\gamma^{32}$ P-ATS2 30 minutes after treatment ($5.958 \pm 5.12 \times 10^6$ molecules of $\gamma^{32}$ P-ATS2 per CFU), compared to those treated with penicillin ($44,804 \pm 11,703$). Not until four hours after treatment addition does the number of $\gamma^{32}$ P-ATS2 associated with penicillin treated cells ($2.73 \pm 1.82 \times 10^8$) become greater than those associated with those cells which are zoocin A treated ($5.652\pm 3.91 \times 10^7$). Whilst the ability of penicillin to allow PS-ODN bacterial cell access is perhaps slower than that of zoocin A over the first four hours of incubation, those lag phase cells treated with penicillin + SM-FBA show growth inhibition (Figure 5.7, Table 5.10) and *fba* repression (Figure 5.12) for +16 hours after treatment. Those lag phase cells treated with zoocin A + SM-FBA show growth inhibition (Figure 4.4) and *fba* suppression (Table 4.10) at
Figure 5.13. Number of ATS2 molecules (x10^7) per CFU of lag phase *S. mutans* OMZ175 CFU treated with penicillin + ATS2. Lag phase *S. mutans* OMZ175 following treatment with: γ^32 P-ATS2; penicillin+ γ^32 P-ATS2. The error bars stand for the standard error between the three triplicates done for each experiment.
Figure 5.14. The relationship between cellular growth and ATS2 dilution rates for lag phase S. mutans OMZ175 treated with penicillin + ATS2. Lag phase S. mutans OMZ175 following treatment with zoocin A + ATS2. – , the amount of ATS2 present per CFU; – – , the number of viable bacterial cells. The error bars stand for the standard error between the three triplicates done for each experiment.
Figure 5.15. Number of ATS2 molecules (x10^5) per CFU of exponential phase *S. mutans* OMZ175 CFU treated with penicillin + ATS2. Exponential phase *S. mutans* OMZ175 following treatment with: γ<sup>32</sup> P-ATS2; penicillin+ γ<sup>32</sup> P-ATS2. Due to graph constrictions, the time zero time points for figure B were unable to be added. The error bars stand for the standard error between the three triplicates done for each experiment.
Figure 5.16. The relationship between cellular growth and ATS2 dilution rates for exponential S. mutans OMZ175 treated with penicillin + ATS2. Exponential phase S. mutans OMZ175 following treatment with zoocin A + ATS2; the amount of ATS2 present per CFU; the number of viable bacterial cells. The error bars stand for the standard error between the three triplicates done for each experiment.
Table 5.12: Comparison of the ability of zoocin A and penicillin to allow ATS2 molecules to associate with lag phase S. mutans OMZ175

<table>
<thead>
<tr>
<th>Growth (hours)</th>
<th>ATS2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zoocin A</td>
</tr>
<tr>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>0.5</td>
<td>5.9 ± 5.12 x 10^6</td>
</tr>
<tr>
<td>1</td>
<td>1.6 ± 1.38 x 10^7</td>
</tr>
<tr>
<td>2</td>
<td>7.1 ± 2.59 x 10^7</td>
</tr>
<tr>
<td>4</td>
<td>5.7 ± 3.91 x 10^7</td>
</tr>
<tr>
<td>5</td>
<td>89.7 ± 8.35 x 10^6</td>
</tr>
<tr>
<td>7</td>
<td>28.9 ± 2.48 x 10^6</td>
</tr>
<tr>
<td>10</td>
<td>2.8 ± 2.06 x 10^6</td>
</tr>
<tr>
<td>12</td>
<td>3.8 ± 2.43 x 10^5</td>
</tr>
</tbody>
</table>

The standard deviation is the standard error between the three triplicates done for each experiment.
four hours after treatment, but show significant growth recovery and non-suppressed *fba* expression at 16 hours.
6 Discussion

6.1 The need for new antimicrobials

Throughout history, bacterial pandemics have decimated whole populations, and today it is only through public health measures and the development of vaccines and antibiotics that bacterial disease is kept in check. Antibiotic resistance rates continue to rise however, and there is a pressing need for the development of new, targeted, antimicrobials. Antisense oligonucleotides represent a gene repression technology, which through the use of sequence specific oligonucleotides, can target genes in a variety of different bacterial strains. The problem of delivering these oligonucleotides across the bacterial cell wall is one key issue preventing their use as antimicrobial agents. Their use in combination with a bacterial cell permeabilising agent to aid delivery is one possible solution. The aims of this study were three fold: The development of methods to measure the amount and effect of PS-ODN’s in S. mutans OMZ175; the use of these methods to demonstrate whether zoocin A facilitates the entrance of PS-ODN’s into S. mutans OMZ175; and the use of these methods to determine if other molecules also facilitate the entrance of PS-ODN’s into S. mutans OMZ175.

6.2 The use of exogenous antisense

The choice of an essential gene as the antisense target allows the researcher to read the effect of the inhibition as a direct correlation to growth rate and viability. One previous study by Sato et al\textsuperscript{298} used antisense sequences which when expressed from introduced plasmids in S. mutans targeted the essential streptococcus G protein (SPG). A further study by Baev et al\textsuperscript{16} used the same antisense target, but rather than express the antisense sequence from an introduced plasmid, integration vectors carrying the SGP antisense transcript were used to ensure that the antisense sequences were expressed from the host S. mutans genome. However, there is currently only one published work other than the authors own in which antisense has been exogenously added to S. mutans cells. Guo et al\textsuperscript{140} treated S. mutans with
PS-ODN targeted towards gtfB, a gene involved in sucrose-dependent adherence of S. mutans to the tooth surface. They found the targeted antisense had a significant inhibitory effect upon gtfB transcription and expression. Guo et al. were the first researchers to demonstrate that exogenous antisense could be added to S. mutans and result in a genotypic and phenotypic change.

6.3 Choice of antisense target

It was necessary to establish that the fructose-1, 6-bisphosphate adolase gene (fba) was essential for growth in S. mutans OMZ175. The fba gene was reported by Song et al. (2005) to be an essential gene in S. pneumoniae and PCR and sequence analysis (Dufour et al. 2008) determined there was high sequence homology between S. mutans fba genes. fba is a key component of the glycolytic pathway in streptococcal metabolism, as illustrated in Figure 1.2. Attempts to disrupt the FBA gene through insertional mutagenesis in a variety of bacterial strains such as E. coli, Pseudomonas aeruginosa, Giardia lamblia and M. tuberculosis have been unsuccessful, suggesting that the FBA gene is essential to the viability of these bacterial strains. However it may be that this gene is simply essential for bacterial growth and that a knockout of this gene results in the inability to metabolise and thus prevent growth - thus creating bacterial cell stasis, rather than being essential for bacterial viability. This would mean that the bacterial cells were in a survivable but non-culturable state, and that fba is not an essential gene - as essential genes are absolutely required for the survival of an organism. Disruption of genes that are not essential for viability, but are required for bacterial metabolism, may result in a bacterial cell with suspended metabolism, unable to grow, but survives in a state of stasis.

To confirm that expression of fba was essential for growth of S. mutans OMZ175 experiments were designed to determine which substrates permitted growth. By comparing growth in a minimal media supplemented with specific substrates, to growth in THB, it was possible to determine which substrates were metabolised by S. mutans OMZ17. Sugar is metabolised through the glycolytic pathway, and when present as the sole carbohydrate it
requires a complete glycolytic pathway for its metabolism, which ensures that the \textit{fba} gene is essential for metabolism. No substrate present in THB (or any other substrate tested) except glucose, permitted the growth of \textit{S. mutans} OMZ175 assuring us that this strain did not possess metabolic pathways allowing it to metabolise in the absence of a functional glycolytic pathway. Therefore, the growth of \textit{S. mutans} OMZ175 in THB requires a functional \textit{fba} gene. Comparison of growth in both THB and minimal media supplemented with glucose, and comparison of inhibition caused by the presence of zoocin A and SM-FBA, showed no significant differences between the two systems, confirming that \textit{S. mutans} in THB is unable to metabolise compounds which do not require a functional \textit{fba} and that without a function FBA mRNA, the bacterial cell will enter a state of stasis. It is possible that the lack of functional \textit{fba} also has an effect on bacterial viability, as the viability of cells treated with delivery agent + SM-FBA decreases further than those treated with just the delivery agent alone. This decrease in viability indicates that the \textit{fba} gene may well be essential for viability in \textit{S. mutans}. However further insertional knockout studies are needed in order to clarify the true role and essential nature of \textit{fba} in \textit{S. mutans} cells. In this study however it is clear that the \textit{fba} gene is essential for cellular growth and replication - which ensures that the recovery and growth of zoocin A/ SM-FBA treated \textit{S. mutans} OMZ175 is due to the lifting of \textit{fba} repression, rather than the utilisation of alternative carbohydrates or metabolic pathways. When combined with a lytic delivery agent, it is important to acknowledge that the immediate death of treated \textit{S. mutans} is due to the action of the delivery agent (penicillin or zoocin A- which are known bacteriocidal agents), and when combined with an FBA targeted PS-ODN, results in immediate decrease in bacterial viability due to the action of the delivery agent, and continued repression of growth due to the action of the PS-ODN.

\textbf{6.4 Determining the gene sequences of the RT-PCR genes}

Real-time PCR (qRT-PCR) was used to analyse gene expression levels in this study, as it is a sensitive, reproducible and high-throughput method able to show subtle changes in relative quantities of mRNA\textsuperscript{62,279,290}. During this research the levels of three different gene transcripts
were determined, the *fba* gene, and two normalisation genes -16s rRNA and *gyrA*- both of which are essential, stable and relatively constantly expressed in *S. mutans* and neither of which contain the SM-FBA target site\(^62\). For accurate data interpretation a stable normalizer is mandatory, and constitutively expressed housekeeping genes such as 16s rRNA are widely used for this purpose\(^62,304,321\). A reliable internal control should show minimal changes, whereas a gene of interest might change over the course of an experiment, and it is considered prudent to normalize the data using a normalization factor based on multiple normalisation genes\(^200,303\). The purpose of normalisation is to control several variables such as different amounts and quality of starting material, variable enzymatic efficiencies of retro transcription from RNA to cDNA, or differences between cells in overall transcriptional activity\(^200,303\).

In this study, for each treatment type and each time point monitored, no significant differences were found in the fold-level expression values of either control genes. 16s RNA is a commonly used normalisation gene and although it is abundantly expressed, this high expression level has not been found to affect the accuracy of qRT-PCR\(^62\). Senadheera et al.\(^304\) used 16s rRNA and *gyrA* to normalise their qRT-PCR experiments using *S. mutans* UA159, as they found that the expression of both control genes did not fluctuate within their cDNA samples when monitoring growth kinetics, oxidative stress tolerance and biofilm formation effects upon *S. mutans* UA159 *gtfB/C* expression rates. Many other researchers have also used 16s rRNA and/or *gyrA* as internal standards for qRT-PCR in *S. mutans*, finding no significant difference in expression under the various conditions or samples tested\(^182,321,330\).

### 6.5 RNA extraction

The use of zoocin A as a *S. mutans* digestion agent allowed for a far greater yield of RNA compared to standard digestion agents. Initially we extracted RNA from *S. mutans* OMZ175 using \(N\)-acetylmuramidase (mutanolysin)\(^59\) and B-1,4-\(N\)-acetyl-muramidase (lysozyme)\(^140,199,306\). Once suspended with the digestion agents, the digested cells were added to a phenol/chloroform solution mix, RNA extracted and then purified by use Qiagen RNeasy mini column kits. Use of either phenol/chloroform and/or the Qiagen RNeasy mini columns
are standard RNA extraction protocols for *S. mutans*. Use of zoocin A to digest *S. mutans* OMZ175, followed by use of phenol/chloroform and the Qiagen RNeasy mini column kit, resulted in more than 1000-fold increase in yield, suggesting that the commonly used mutanolysin/lysozyme mix is far from optimal for use with *S. mutans*. It is probable that zoocin A more efficiently digests the cell wall of *S. mutans* due to its mode of action. Zoocin A is a D-alanyl-L-alanine endopeptidase that hydrolyses the peptidoglycan cross-link between the junction of the D-alanine of the stem peptide and the first L-alanine of the cross bridge. In contrast, lysozyme (a glycoside hydrolase) catalyses the hydrolysis of the 1,4-beta-linkages between N-acetylmuramic acid (NAM) and N-acetyl-D-glucosamine (NAG) residues in peptidoglycan. Mutanolysin (an endo-N-acetylmuramidase) cuts at the β1-4 glycosidic linkage between NAM and NAG present in streptococci peptidoglycan. It is possible that the *S. mutans* OMZ175 cell wall possess fewer cross-links than NAM-NAG links. Therefore cleavage of these cross-links by zoocin A would cause a greater lytic effect compared to lytic cleavage between NAM-NAG units by the same concentration of lysozyme or mutanolysin. *S. mutans* is also known to possess large quantities of teichoic acids (TA) and lipoteichoic acids (LTA) upon the bacterial cell surface. It is possible that these interact and prevent the function of lysozyme, but do not interfere with the function of an endopeptidase. We therefore recommend the use of zoocin A, over that over lysozyme or mutanolysin, to lyse *S. mutans*. The use of a PCR protocol designed to bind to any contaminating DNA present in the RNA sample illustrated that for *S. mutans* OMZ175 RNA extraction, a DNase treatment is necessary to eliminate the presence of any contaminating DNA. No RNA bands appeared on the agarose gel due to the low concentration of RNA (approximately 7 ng) added to each well. A 1 µg concentration is usually the minimum concentration required for RNA visualisation.

### 6.6 Tracking molecules within a bacterial cell

In order to determine the amount of PS-ODN gaining entry to a bacterial cell, and to permit investigation of its intracellular localisation an attempt was made to create a method that would allow the determination of the amount of antisense that gained access to the bacterial...
cell interior, and to determine where in the bacterial cell the antimicrobial ends up. This is an area in which there has been minimal research. Researchers in this area have concentrated on finding methods that allow a bacterial cell to be divided into its individual components, such as peptidoglycan, membrane, and cytoplasm \cite{251,260,268}. These methods rely on size exclusion and centrifugation techniques to separate the different cell components on the basis of sedimentation rate – the speed at which it drifts down the centrifuge tube \cite{44,177}. This technique has future potential for allowing the study of each individual component without interference from other bacterial cell components. If developed correctly, a cellular fractionation technique would allow the accurate determination of the localization of specific proteins within the cell, or the localisation of antimicrobials within the cell. Whilst various studies have fractionated streptococcal species \cite{4,45,177}, published methods of sub-cellular fractionation do not result in 100% complete fractionation of cellular material, and combined with the use of a hot radio-label, this incomplete fractionation could yield unreliable results due. An initial attempt to fractionate \emph{S. mutans} OMZ175 was performed using the sub-cellular fractionation technique described by Kling \cite{177}, but this method failed to produce an observable reaction short of using Electron Microscopy (EM) for the validation of each step and for every attempt, and so this method was taken no further.

**6.7 Development of a protoplasting protocol**

Whilst various non-whole cell methods are available such as hybridisation or pulse-chase \cite{101}, which can be used to determine whether the PS-ODN will bind specifically to the target DNA and monitor the entrance of antisense into the bacterial cell, but these techniques do not allow the exact location of the PS-ODN within the bacterial cell to be determined, do not keep the bacterial cell intact for further study, and are only poorly quantitative. Whilst some proteins and other substrates are believed to be able to enter the bacterial cell through pores and passive diffusion \cite{91,178}, there has not yet been any reported effective uptake of PS-ODN by any bacterial strain, without the use of a combined antisense delivery system. As peptidoglycan is often referred to in research as the main barrier preventing AS-ODN, PNA or PMO entrance to the bacterial cell \cite{63,128,226}, it was suggested that stripping the
peptidoglycan off \textit{S. mutans} OMZ175 and converting them into protoplasts, could provide a system with which to determine whether the peptidoglycan layer truly acts as one of the major barriers to AS-ODN, PNA or PMO entry. Protoplasted cells would also allow the rate of passive diffusion of AS-ODN, PNA or PMO across the \textit{S. mutans} OMZ175 cell membrane to be determined. It is known that in Gram-negative \textit{E. coli} the outer LPS membrane layer acts as an efficient barrier to AS-ODN diffusion\textsuperscript{131} and is had been shown using a liposomal model of the plasma membrane, that AS-ODN, PNA or PMO do not readily penetrate a membrane barrier\textsuperscript{357}. As a protoplast is classified as a functional cell without a peptidoglycan layer, the membrane should still be polarised and active transport across the membrane of substrates needed for metabolism should still occur. It is important that any diffusion across the bacterial cell membrane is determined using a membrane which is as physiologically close to a whole live bacterial cell membrane as possible, in order to rule out any possible changes in AS-ODN, PNA or PMO diffusion which may occur due to the membrane state.

Bacterial protoplasts were first observed by Lederberg in 1956, and since then many bacterial strains have been reported as capable of forming protoplasts\textsuperscript{59,109,258,274}. The method used for protoplast preparation was based upon that initially described by Freimer et al.\textsuperscript{113} who reported the successful generation of protoplasts using a bacterial reversion medium to which osmotic stabilising agents could be added. Although Freimer used sodium chloride as the osmotic stabilising agent, it was not used in this study due to the difficulty of growing \textit{S. mutans} at a high salt concentration and the moderately high loss of viability produced. In this study the medium was stabilised by the addition of either raffinose or sucrose. Differences between these two agents were noted, as the presence of high concentrations of sucrose decreased zoocin A lytic function, whilst raffinose proved to be difficult to use in solid media at high concentrations. It is possible that the ability of zoocin A to act upon the peptidoglycan layer of \textit{S. mutans} OMZ175 was affected by the presence of high sucrose concentrations. Sucrose stimulates the production of extracellular polysaccharide (EPS) in \textit{S. mutans}, and it is possible that its production prevented effective zoocin A activity. For that reason, raffinose was then used as the osmotic stabilising agent, as it does not stimulate EPS production in \textit{S. mutans} OMZ175. In this study no protoplast formation was observed in either sucrose or raffinose containing media. In previous experiments by Simmonds\textsuperscript{309} and Lowry\textsuperscript{206}, it was shown that \textit{Streptococcus pyogenes} protoplast reversion frequencies were higher on raffinose
than sucrose, and that the higher the percentage of raffinose, the greater the recovery of protoplasts. Due to solubility limitations, concentrations over 30% raffinose could not be tested in this study. Several studies have found that the addition of up to 25% gelatine to the reversion media can promote a high reversion frequency of protoplasts of group D streptococci\textsuperscript{134,175} and \textit{Bacillus} spp. strains\textsuperscript{111,141,359}. However the reports showed great variation in the reversion frequencies found between gelatine batches which resulted in inconclusive comparison between studies\textsuperscript{85,176}. Agar concentrations of 2.0-2.5% have also been found to promote reversion of \textit{Bacillus} protoplasts\textsuperscript{100,176} and it has been suggested that similar concentrations may aid reversion of group A streptococcal protoplasts\textsuperscript{219}. King and Goode\textsuperscript{176} however found no quantitative recovery of streptococcal colonies when higher agar concentrations were used. As small (60 x 15 mm) agar plates were used in this study for bacterial reversion agar plates (due to the high cost of raffinose), a 1.5% agar concentration was used, as higher agar concentrations failed to allow the agar plates to be poured correctly before agar setting occurred. \textit{S. mutans} cells were observed to change from a Gram-positive appearance to a Gram-negative appearance upon the addition of zoocin A to the media, indicating that zoocin A worked effectively to strip the peptidoglycan from the bacterial cells. However, as no revertants were formed upon the osmotically stabilised media, this indicates that the osmotic stabilisation process was unable to protect the protoplasted cells from complete lysis. The fact that increasing raffinose concentrations failed decrease the rate in loss of OD for zoocin A treated \textit{S. mutans} OMZ175 also indicates that even a high concentration of osmotic stabilising agents were unable to prevent complete lysis of \textit{S. mutans} OMZ175. The reversion media used also contained a number of other osmotic stabilising agents such as peptone, NaCl, magnesium chloride and tris-HCL, all of which are also regarded as osmotic stabilising agents. Although the osmotic stabilising conditions which were used in this study have been successful in allowing the recovery of protoplast revertants for other streptococcal strains, they were unable to allow revertant formation for \textit{S. mutans} OMZ175. It is possible that a higher concentration of raffinose was required for osmotic stabilisation, but due to sugar saturation higher concentrations were unable to be reached in this study.
6.8 Characteristics of antisense

There are several key characteristics of AS-ODN, PNA or PMO which may prevent rapid bacterial cell penetration. The charge, size and hydrophobicity of the AS-ODN, PNA or PMO molecules may influence their ability to permeate the bacterial cell. The negatively charged, polar SM-FBA used in this study has a molecular weight of 5.77 KDa, which is within the size of protein molecules that are able to pass through the calculated pore size of B. cereus and E. coli\(^89\). Demchick et al. after calculating that the mean pore radius size in E. coli was 2.06 nm stated that uncharged proteins under 24 KDa should be able to diffuse through the peptidoglycan layer\(^89\). However even when used a 40 mM concentration in this study, no antimicrobial effect by SM-FBA was observed unless used in combination with zoocin A.

Other studies which have used PS-ODN’s targeted towards S. mutans\(^140\) or other bacterial strains such as M. tuberculosis\(^146\), E. coli\(^226\) or S. aureus\(^246\) have also shown either the inability of poly-anionic polar PS-ODN’s to permeate the bacterial cell wall, or that only a weakly inhibitory effect is produced. Only when encapsulated within liposomes (artificially prepared vesicles composed of one or more lipid bilayers) or combined with a cell-penetrating agent does the PS-ODN seem to effectively gain entrance into most bacterial strains\(^18,103,228,249\). It is possible that the PS-ODN’s negative charge affects its ability to diffuse through the peptidoglycan pores, or possibly mediates its interactions with the teichoic acids present on the bacterial cell surface. However, it has been shown that unattached and un-encapsulated PS-ODN’s are able to enter M. tuberculosis and cause a weak inhibitory effect. This may be due to an as yet unknown interaction between the PS-ODN molecules and the M. tuberculosis cell surface.

6.8.1 Charge

Liposomes help encased molecules access the lipid membrane layer\(^218\). In order to reach the lipid membrane of Gram positives, liposomes must first penetrate the peptidoglycan layer. It is possible that by encasing the AS-ODN, PNA or PMO within liposomes, any AS-ODN, PNA or PMO charge is ‘hidden’ from the bacterial cell, allowing for greater ease of AS-
ODN, PNA or PMO penetration. However, charge does not seem to be the sole factor for liposome penetration, as charged liposomes have also been used to deliver AS-ODN, PNA or PMO into the bacterial cell interior. Meng et al. (2009) have shown the restoration of oxacillin susceptibility to MRSA and the rescue of penicillin treated mice from lethal sepsis when infected with MRSA through the use of anionic liposome encased PS-ODN targeted towards the \textit{mec-A} resistance gene present in MRSA\textsuperscript{228}. Interestingly, the neutrally charged PNA also often shows no effective antimicrobial effect unless coupled with a highly charged carrier peptide\textsuperscript{18,128}. Carrier peptides are thought to increase antisense penetration into the bacterial cell through a three step process. First the cationic peptide is electrostatically attracted to the membrane surface. Second, the membrane is then disrupted and finally penetration occurs\textsuperscript{116,209}. Cationic cell penetrating peptides are thought to be attracted to the negatively charged LPS layer of Gram-negative bacteria. By competing for divalent cation binding sites within the LPS layer, sugar moiety crosslinks between the LPS layer would be disrupted leading to reduced outer membrane integrity\textsuperscript{116}. Bai et al. (2012) used cell penetrating peptides (KFFKFFKFFK) conjugated with PNA targeted towards the RNA polymerase sigma (70) factor to target and kill MRSA/ vancomycin intermediate \textit{S. aureus}, both \textit{in vitro} and \textit{in vivo}\textsuperscript{18}. It is possible that these highly charged carrier peptides also help the antisense molecules to penetrate the bacterial peptidoglycan layer through an unknown mechanism.

Guo et al., treated \textit{Streptococcus mutans} with PS-ODN antisense oligonucleotides that were specific to the \textit{gtfB} gene\textsuperscript{140} and clearly showed that \textit{S. mutans} gene expression could be affected by the introduction of PS-ODN, either alone, or in combination with a membrane transfection agent. PS-ODN alone reduced target gene expression, but exhibited a significantly weaker inhibitory effect compared to the combination of free antisense PS-ODN’s with the cationic polymer SoFast\textsuperscript{TM}. It has been argued that the presence of positively charged polymers can neutralize the negatively charged PS-ODN’s, which would enable them to transverse through bacterial peptidoglycan pores\textsuperscript{64}. Such an effect would also help them to permeate the lipid membrane, as due to their polar and polyanionic nature, PS-ODN’s theoretically should be unable to passively cross a lipid bilayer\textsuperscript{108,320}. It is unlikely that the ability of antisense to gain entrance to the bacterial cell interior is solely determined by charge. Guo et al., also transformed the \textit{S. mutans} in liquid media into competent cells
before the addition of PS-ODN. Guo et al., also used *S. mutans* in biofilms, and *S. mutans* cells in biofilms show natural competence due to a quorum sensing mechanism. Competence allows the *S. mutans* cells to uptake extracellular DNA from its environment. PS-ODN’s are a close chemical similarity to DNA, and it is likely that Guo et al., were able to show that PS-ODN was able to have an effect upon gene expression without the use of a delivery molecule due to the competent state of their *S. mutans*. In this current study, the *S. mutans* OMZ175 cells used were not in a competent state, and thus unable to take up exogenous DNA from their environment. This is a possible reason as to why this study showed that PS-ODN used without any delivery molecule was unable to have an inhibitory effect upon *S. mutans* growth. As well, AS-ODN, PNA or PMO’s have various different peptide backbone modifications, and it is possible these somehow play an as yet undetermined role in preventing passage of the antimicrobial through the bacterial cell wall.

### 6.8.2 Size

Most AS-ODN, PNA or PMO sequences targeted towards bacteria are between 16-20 bp in length, as at the genomic level, any sequence of 17 residues is expected to occur only once\(^7\). When AS-ODN, PNA or PMO’s are coupled to cell penetrating peptides their size dramatically increases. Whilst it would seem probable that an increase in size would lead to a corresponding decrease in the rate of AS-ODN, PNA or PMO entrance into bacterial cells, instead the opposite seems to occur. Attachment to highly charged carrier peptides is thought to help increase antisense penetration across the bacterial membrane\(^126,246\). Whether attachment of AS-ODN, PNA or PMO to carrier peptides influences the rate of peptidoglycan penetration has not yet been studied. However, based on current knowledge of the peptidoglycan layer, it seems counterintuitive that attachment of an AS-ODN, PNA or PMO to a charged large protein would lead to an increase in peptidoglycan permeabilisation.

### 6.8.3 Hydrophobicity
It is known that PS-ODN’s are more hydrophobic than phosphodiesters$^{3,86}$. This hydrophobicity may lead to increased non-specific interactions with other hydrophobic molecules. Increased hydrophobicity amongst antimicrobial molecules is usually associated with decreased ability to translocate across the bacterial membrane, and the hydrophobic molecules interact and ‘stick’ within the lipid rich membrane interior. Hydrophobic molecules also often display poor water solubility. PS-ODN’s however display good solubility properties, and have been shown to be able to cross the bacterial cell membrane without the use of a carrier peptide$^{140,146}$. Varying levels of hydrophobicity are found between different antisense chemistries$^{86}$, and the true effect of hydrophobicity upon their ability to penetrate the bacterial cell has not yet been thoroughly examined.

6.9 Cell membrane and Peptidoglycan layer

This study has shown that peptidoglycan barrier presents the major barrier to PS-ODN entry for *S. mutans* OMZ175. Neither of the two key PS-ODN delivery agents used in this study (zoocin A or penicillin) are known to cause an effect upon the bacterial membrane. Each agent’s mode of action is centred solely upon the peptidoglycan layer. When combined with either agent, PS-ODN entry into the bacterial cell was shown to increase significantly, dependent upon the ability of the delivery agent to produce an effect upon *S. mutans* OMZ175 in different growth stages. These results, together with those of a previous study (McLeod 2008, Dufour 2011) indicate that for certain *S. mutans* strains, such as *S. mutans* OMZ175, *S. mutans* UA159, *S. gordonii*, *S. salivarius* and *S. pyogenes*, the peptidoglycan layer represents the major barrier preventing PS-ODN access to the bacterial cell interior. There is however no consensus of opinion of whether it is the peptidoglycan layer or the bacterial membrane which represents the greatest barrier to AS-ODN, PNA or PMO entry. It is quite possible that both layers act to inhibit AS-ODN, PNA or PMO entrance in different ways for different bacterial strains, and it is obvious from the research available that different bacterial strains, both Gram positive and Gram negative, show clearly different abilities to uptake AS-ODN, PNA or PMO, and that dependent on the bacterial strain targeted, different AS-ODN, PNA or PMO delivery mechanism are required. Some bacterial strains require AS-
ODN, PNA or PMO delivery mechanism targeted towards penetrating the bacterial membrane\textsuperscript{226,246}, whilst others require use with agents which help to permeate the bacterial peptidoglycan layer\textsuperscript{146,281}.

Diffusion across the lipid membrane by PNA is generally attributed to their neutral charge, but is also noted to be extremely slow\textsuperscript{357}. Good et al. (2000) and Eriksson et al. (2002) found that neutral PNA molecules demonstrated very slow entrance in to \textit{E. coli}. However faster penetration and target gene specific effects were observed if the PNA was used in conjunction with an \textit{E. coli} mutant which showed defects in in LPS layer resulting in a weaker LPS layer\textsuperscript{131}, when used in conjunction with LPS permeabilising compounds\textsuperscript{131} or when conjoined to highly charged carrier peptides which are attracted to the negatively charged lipids present on the bacterial cell surface\textsuperscript{103}. Combined use of PNA with antibiotics which blocked peptidoglycan formation was found to not increase PNA effects\textsuperscript{131}. It was concluded that for Gram negative \textit{E. coli}, that the outer membrane LPS layer, and not the peptidoglycan layer, presented the greatest barrier to AS-ODN, PNA or PMO diffusion\textsuperscript{103}.

Nekhotiaeva et al. (2004) demonstrated that peptide-PNA conjugates also work well at reducing target gene expression in Gram positive \textit{S. aureus}. However this conjugate was designed to increase passage across the bacterial membrane, and no research was done on whether the peptide-PNA conjugate showed increased or decreased passage across the bacterial peptidoglycan. It is possible that these highly charged carrier peptides also help the PNA molecules to penetrate the bacterial peptidoglycan layer through an unknown mechanism.

Harth et al. (2000) used cell wall softening antibiotics which affect membrane metabolism of \textit{M. tuberculosis}, which at high concentrations cause the bacterial cell to lyse. A combination of these antibiotics and PS-ODNs were studied. However, whilst they observed greater growth inhibition caused by the antibiotic- PS-ODN combination compared to that of antibiotics alone, cultures treated with PS-ODN alone showed no significance difference in growth inhibition compared to those treated with the antibiotic \textendash{} PS-ODN combination\textsuperscript{146}. This suggests that for \textit{M. tuberculosis}, PS-ODN’s are able to enter the bacterial cell and cause a target specific inhibitory effect without the use of either a cell wall or cell membrane permeabilising agent, or PS-ODN carrier. Although the effects of antisense PS-ODN’s on \textit{M.}}
*tuberculosis* were found by Harth et al. (2000) to be highly consistent, they were also shown to be relatively weak compared to standard anti *M. tuberculosis* agents. The authors concluded that this is possibly due to inefficient uptake across the mycobacterial cell wall\(^{146}\), and it is possible that this is due to the fact that although membrane penetrating agents were investigated for their ability to improve PS-ODN access to the bacterial cell interior, a cell wall peptidoglycan permeabilising agent was not included in this study. A study by Rapaport et al. (1996) showed that greater PS-ODN growth inhibition effects were achieved in *Mycobacterium smegmatis* when PS-ODN’s targeted towards the aspartokinase gene were combined with the cell wall disrupting agent ethambutol (compared to ethambutol alone)\(^{281}\).

The addition of charged carrier peptides to AS-ODN, PNA or PMO molecules directly affects their size and charge, and the literature is inconclusive with regards to which charge and which AS-ODN, PNA or PMO delivery mechanism best allows AS-ODN, PNA or PMO delivery. Theoretically, whilst neutral PNA molecules should be able to diffuse through peptidoglycan pores and lipid membranes, the polyanionic and polar nature of PS-ODN molecules should prevent their ability to diffuse simply through the bacterial membrane and peptidoglycan pores\(^{108}\). Whilst liposomes should help to increase AS-ODN, PNA or PMO penetration across lipid membranes\(^{190}\), it is not known whether encapsulation within liposomes increases AS-ODN, PNA or PMO penetration across peptidoglycan, or the mode by which such a mechanism would operate. The literature available illustrates that both PNA and PS-ODN molecules show greatly varying degrees of bacterial cell penetration, depending on the bacterial strain against which they are targeted, and the delivery mechanism to which they are coupled.

### 6.10 Demonstrating the entrance of radiolabelled antisense

When examining the entrance of either AS-ODN, PNA or PMO or other antimicrobials into human cells, fluorescence label tracer molecules are frequently used\(^{125,294}\). However, this labelling technique is not often applicable to bacteria due mainly to their size exclusion of such large molecules. The use of fluorescent reporter genes in Gram positives such as *S. aureus* or Gram negatives such as *E. coli* has been shown to be able to successfully indicate
whether sequence specific gene repression has occurred\textsuperscript{216}. However, when a fluorescent \textit{gfp} reporter gene was used in \textit{S. mutans} UA159, it was found that the thick peptidoglycan layer associated with \textit{S. mutans} effectively blocked successful measurement of the reporter\textsuperscript{95}. Whilst probes such as biotin or digoxygenin can be attached to oligonucleotides, these probes are usually used upon extracted DNA or mRNA samples, rather than whole cells, again due to size exclusion\textsuperscript{14,33}.

Radiolabels have been used to determine the uptake and distribution of PS-ODN in the intracellular parasites \textit{Schistosoma mansoni}\textsuperscript{327} and \textit{Leishmania amazonensis}\textsuperscript{232,280} and in human cells\textsuperscript{278,329}. This technique is also becoming widely used in bacteria for antisense studies\textsuperscript{77,277}. It was determined that the use of a radiolabel would be the least intrusive method of adding a tracer molecule to PS-ODN which is then introduced to \textit{S. mutans}. Substitution of a radioactive label in place of its ‘cold’ analogue is considered to be the best choice for pharmacokinetic studies\textsuperscript{35}. Isotopic sulphur is often used to label PS-ODN’s, and the sulphur present in the phosphodiester backbone can be substituted with \textsuperscript{35}S. This method of labelling must be included during the actual synthesis of the PS-ODN oligonucleotides. The sulphur backbone cannot be modified to include \textsuperscript{35}S once synthesis is complete. Various companies such as Panagene\textsuperscript{®} synthesise PS-ODN’s and offer the addition of radioactive isotopes to their synthesis. Due to their high costs, custom made radiolabelled PS-ODN were not used in this study. \textsuperscript{14}C was considered undesirable due to its higher costs, low emission levels (as whole cell extracts were used in this study) and difficulty in incorporating the label due to the chemical structure of the PS-ODN molecule. Initially \textsuperscript{3}H tritium was used to label the PS-ODN’s used in this study, but was abandoned due to low labelling efficiency and low emission levels. Also the \textsuperscript{3}H tritium was unsuitable for labelling of a DNA molecule, as a phosphorous is needed to attach to the oligonucleotide backbone (such as methyl 3H thymide 5’triphosphate). Isotopic phosphorous was chosen for use in this study due to its high emission rate, and the fact that a successful labelling method which utilised the addition of a phosphorous atom to the phosphodiester backbone was available\textsuperscript{2}. T4 polynucleotide kinase catalyses the transfer and exchange of the radiolabelled phosphorous from the \textit{y}-position on the ATP to the 5’-hydroxyl terminus of polynucleotides. It was thought preferable that a labelling method be used that could also attach a radiolabel to a variety of classes of antisense which possess phosphodiester backbones. Due to the difference in chemistry, it is unlikely
however that such a method could be used to label PNA’s, as they possess a pseudo-peptide backbone which does not contain any reactive hydroxyl groups necessary for the transfer of a radiolabel. Use of the T4 polynucleotide kinase labelling kit together with $\gamma^{32}$p resulted in the labelling of ATS2, and purification with the Roche mini spin column allowing the complete removal of all unincorporated $\gamma^{32}$p molecules. The use of $\gamma^{32}$p was favourable, as due to the chemical structure of the PS-ODN molecule the incorporation of a further phosphorous molecule was possible. A labelling efficiency of approximately 1 in $10^6$ was achieved. Although this seems a low labelling efficiency, this was not unexpected as sulphur-oligonucleotides show poor kinase ability together with T4 polynucleotide kinase and $\gamma^{32}$p ATP$^{308}$. Despite this low labelling efficiency, the high emission levels of $\gamma^{32}$p made this label preferable for use. Although developed independently within the Microbiology Department, University of Otago, this method closely resembles the method used by Stazic et al. which was published in 2011 during the course of this study$^{319}$.

6.11 The effect of bacterial growth, and the dilution of PS-ODN

Another question that must be addressed is that of PS-ODN clearance. The ability to recruit RNase H theoretically means that the PS-ODN is able to be ‘recycled’ within a bacterial cell, and bind multiple targets over time$^{76}$. However, some bacteria can produce drug efflux pumps which specifically export antibiotics from the cell and thereby prevent it from producing an antimicrobial effect$^{152,271}$. Good et al. (2000) determined that the presence of the Acr and Emr drug efflux pumps did not affect $E. \text{coli}$ susceptibility towards PNA’s. As yet, no bacterial resistance to AS-ODN, PNA or PMO’s has been found. In this study, through the use of growth assays and target gene examination using DNA sequencing, no gene mutations or resistance development towards either zoocin A or targeted PS-ODN were found to occur after a four dose treatment.

Increased uptake of $\gamma^{32}$ P-ATS2 was shown to occur during the first four to five hours after combined delivery agent (zoocin A or penicillin) and $\gamma^{32}$ P-ATS2 treatment, after which a rapid decrease in the amount of $\gamma^{32}$ P-ATS2 present inside each bacterial cell was observed (with the exception of exponential phase $S. \text{mutans}$ OMZ175 treated with penicillin). This
window of increased $\gamma^{32}$ P-ATS2 uptake corresponds to the time frame where the lytic delivery agent actively prevented cellular growth. After this window of lytic activity has passed, $\gamma^{32}$P-ATS2 molecules were found to be no longer able to gain access to the bacterial cell interior. Zoocin A activity in the media was found to be near to nothing once bacterial growth resumes, indicating all zoocin A was either bound to the bacterial cells, or inactivated by this time.

It is assumed that the presence of zoocin A or penicillin and the corresponding permeability of the bacterial cell wall consequently increases antisense oligonucleotide penetration into the cell- which in turn causes the repression of target $fba$ expression. The presence of a bacteriolytic agent will result in a sudden decrease in growth and viability due to cell death, and the repression of a gene essential for cellular metabolism will result in corresponding bacterial stasis. Optical density and viability studies support this statement, with an immediate decrease in cellular viability occurring after treatment with either the delivery agent + PS-ODN, or the delivery agent alone. This is then followed by significant periods of bacterial stasis, before growth recovery (if any) occurs. The qPCR results also support this statement, with a rapid decrease in $fba$ expression between zero and four to five hours shown for both lag phase and exponential phase $S.\ mutans$ OMZ175 treated with zoocin A or penicillin (with the exception of exponential phase $S.\ mutans$ OMZ275 treated with penicillin). For both stages of cell growth, at four to five hours after zoocin A + SM-FBA treatment addition, $fba$ expression was at its lowest point, with a $522.241 \pm 30.05$ decrease in expression for lag phase $S.\ mutans$ OMZ175 treated with zoocin A + SM-FBA, or a $1902.4 \pm 131.26$ decrease in $fba$ expression for those in the exponential phase. For lag phase $S.\ mutans$ OMZ175 treated with penicillin + SM-FBA, $fba$ expression was decreased $656.890 \pm 192.40$ fold. At the time points at which FBA mRNA levels were significantly decreased compared to their controls, bacterial stasis was observed through optical density and viability measurements. Only when FBA mRNA levels returned to normal (compared to the control), did bacterial stasis cease and growth recovery occur.

With the exception of exponential phase $S.\ mutans$ OMZ175 treated with penicillin + $\gamma^{32}$ P-ATS2, once zoocin A/ or penicillin + $\gamma^{32}$ P-ATS2 treated cells had reached stationary phase, the amount of radiolabel present inside each bacterial cell had decreased by approximately
2.5 logs. For instance lag phase cells treated with zoocin A + $\gamma^{32}$ P-ATS2, show a decrease from $5.652 \pm 3.91 \times 10^7$ ATS2 molecules per CFU to $3.829 \pm 2.43 \times 10^5$. This decrease in the number of ATS2 molecules associated with each CFU corresponds (for each growth stage) to an equal log increase in the number of viable cells per ml. This suggests that the loss of $\gamma^{32}$ P-ATS2 per CFU was due to cellular dilution as a result of cellular replication, and corresponding division of $\gamma^{32}$ P-ATS2 between daughter cells over time, rather than any $\gamma^{32}$ P-ATS2 loss due to cellular leakage or $\gamma^{32}$ P-ATS2 diffusion out of the bacterial cells. Whether loss of $\gamma^{32}$ P-ATS2 from actively growing cells is due to cellular leakage or $\gamma^{32}$ P-ATS2 diffusion could be tested by the monitoring of zoocin A + $\gamma^{32}$ P-ATS2 treated cells in $\gamma^{32}$ P-ATS2 free media. If treated S. mutans OMZ175 cells were washed and placed in $\gamma^{32}$ P-ATS2 free media, after $\gamma^{32}$ P-ATS2 uptake had been completed, measurement of the radioactivity in the supernatant over time would determine whether leakage of $\gamma^{32}$ P-ATS2 occurred from S. mutans OMZ175 cells.

Label stability was important for this study and while it is assumed that in these experiments the $\gamma^{32}$ P-ATS2 counts were directly proportional to the PS-ODN concentration, breakdown of PS-ODN inside the cell cannot be entirely discounted. However, PS-ODN’s have been synthesised to show greater resistance to DNA nucleases which are present in bacterial cells$^{21,77,123}$. A study by Harth showed that PS-ODN’s have a half-life of around 23-25 days when incubated together with M. tuberculosis$^{144}$. They found that after 14 days incubation with the bacterial strain, the PS-ODN remained intact and chemically unchanged. Assuming the same holds true for S. mutans, it is unlikely that significant intracellular PS-ODN degradation by cell nucleases occurred over the 24 hours that these experiments were conducted. Due to this long ‘half-life’ within the bacterial cell a lower concentration of PS-ODN will be required to produce a long term antimicrobial effect compared to that of antibiotics which can be exported or degraded by the bacterial cells. The development of these growth assay, qRT-PCR and radiolabel methods could in future be used for the comparison of different AS-ODN, PNA or PMO delivery systems and their efficacy between different bacterial strains.
6.12 The use of zoocin A.

The aim of the second section of this study was to determine whether zoocin A facilitate the entrance of PS-ODN’s into *S. mutans* OMZ175. A study done previously (McLeod, 2008) observed that when targeted PS-ODN was combined with sub-lethal concentrations of zoocin A, a clear synergistic effect occurred. It was concluded that zoocin A was able to facilitate the entry of antisense oligonucleotides into zoocin A sensitive bacterial cells. However, this assumption was based solely on observed OD and viability data, without the use of more sensitive tests such as qRT-PCR to monitor gene expression. Thus one purpose of this study was to prove that inhibition of cell growth was the result of inhibition of FBA gene.

6.12.1 Susceptibility to zoocin A

It is assumed that the presence of zoocin A and the corresponding permeability of the bacterial cell wall, which zoocin A is known to produce on susceptible strains\(^6,95,187\), consequently increases antisense oligonucleotide penetration into the cell- which in turn causes the repression of target RNA expression. Due to its essential nature, it is assumed that complete repression of the *fba* gene would result in cell stasis, and combined with a bacteriocidal delivery agent, will result in decreased cell growth and viability.

RT-qPCR results showed a decrease in *fba* expression over first 4 hours of treatment for those lag and exponential phase *S. mutans* OMZ175 cells treated with the SM-FBA/zoocin A combination. This was matched by the observation that \(\gamma^{32}\)P ATS2 molecules gained entry into lag and exponential phase *S. mutans* cells over the first 4 hours of combined \(\gamma^{32}\)P ATS2 + zoocin A/penicillin treatment. Assuming that the observed \(\gamma^{32}\)P ATS2 cell association levels would be similar to SM-FBA cell association levels, it is apparent that there seems be a threshold value of \(\gamma^{32}\) P-ATS2 per CFU required before an effect upon *fba* expression is observed, and that the phase of growth the bacterial cell is in may influence the ability of \(\gamma^{32}\) P-ATS2 to permeate the cell. A significantly lower level of *fba* suppression was achieved after a 30 minute incubation time with zoocin A for lag phase cells (-37.354 ± 16.94) compared to those in the exponential phase (-1067.8 ± 230.95), and correspondingly, a lower
number of $\gamma^{32}$ P-ATS2 molecules were found to be cell associated with lag phase ($5.958 \pm 5.12 \times 10^6$) than exponential phase ($36.2 \pm 8.3 \times 10^6$) cells. Four hours after treatment addition, those *S. mutans* OMZ175 cells which had been in the lag phase when treatment was began continued to show a significantly lower level of *fba* repression (-522.241 ± 30.05) compared to those cells which had been in the exponential phase when treatment was begun (-1902.4 ± 131.26). Again, a lower number of $\gamma^{32}$ P-ATS2 molecules were found to be associated with lag phase ($5.652 \pm 3.91 \times 10^7$) than exponential phase cells ($101 \pm 7.29 \times 10^6$). No significant repression of the *fba* gene was observed 16 hours after treatment for both those *S. mutans* OMZ175 cells treated in the lag phase (1.446 ± 0.82), or in the exponential phase (0.948 ± 0.37), and both treatment types also demonstrated lower levels of $\gamma^{32}$ P-ATS2 cell association (3.829 ± 2.43 x $10^5$ and 1.63 ± 1.47 x $10^5$ respectively). It seems that cell associated PS-ODN levels below $10^6$ are unable to bring about a measurable change in *fba* expression. It is possible that variations between the amounts of zoocin A added between the two treatment groups is the primary reason for the difference in *fba* repression levels and levels of $\gamma^{32}$ P-ATS2 cell association. It is also possible that structural differences between the bacterial cells in different growth phases contributed to these differences. As the bacterial cell matures, it is possible that the glycan chain length, the number of peptidoglycan layers and/ or the peptide cross-link saturation is altered, which may alter zoocin A’s ability to function, or which may influence the ability of polyanionic ATS2 molecules to interact with the peptidoglycan layer.

How the PS-ODN becomes associated with the bacterial cell is as yet unknown, but it is possible that the PS-ODN is interacting with the teichoic acids present on the cell surface, or interacting with/ diffusing through the peptidoglycan or membrane layers. Whilst a small number of $\gamma^{32}$ P-ATS2 molecules are associated with cells which were treated with $\gamma^{32}$ P-ATS2 alone (Figures 4.12, 4.14), without the presence of zoocin A (and SM-FBA in the place of ATS2), no corresponding decrease in *fba* expression was observed, with *fba* expression not significantly altered for SM-FBA only treated cells in either the lag or exponential phase (Figure 4.10). Due to the fact that this study was unable to produce protoplasts, this study was unable to establish where in the bacterial cell the $\gamma^{32}$ P-ATS2 became associated, but it is hypothesised that these $\gamma^{32}$ P-ATS2 molecules were unable to gain access to the bacterial cell cytoplasm, and thus were trapped within the bacterial peptidoglycan layer.
A concentration of zoocin A (30 AU/ml) calibrated to give a lethal effect against log-phase cells had no such effect upon stationary phase cells, and no synergistic effect was observed between zoocin A and SM-FBA when added to S. mutans OMZ175 in the stationary phase. It is a well-known phenomenon that the growth phase of a bacterial cell and its metabolic state can drastically affect its susceptibility to antimicrobials\textsuperscript{31,230,333}. If a bacteria cell is in a resting state, and no longer replicating (stationary phase) they are often far less susceptible to antimicrobials\textsuperscript{152,181,337}. Zoocin A is a domain-structured protein containing a N-terminal catalytic domain (CAT) and a C-terminal target recognition domain (TRD)\textsuperscript{187}. The catalytic domain must locate to the target structure before catalytic cleavage can take place and it is believed that the peptidoglycan layer is the primary binding site\textsuperscript{6}. It is previously documented phenomenon that zoocin A does not cause the lysis of stationary phase cells\textsuperscript{95}. This may in part be due to the lack of peptidoglycan replication occurring. The peptidoglycan layer at the septum of dividing cells will often only be a few layers thick, whilst the peptidoglycan layers of a stationary phase cell may be many layers thick\textsuperscript{339,346}. Consequently, far higher amounts of zoocin A are required to produce hydrolysis of the peptidoglycan\textsuperscript{187} of stationary phase cells. If not enough zoocin A is added to the stationary phase cells to cause a measurable lytic effect, it is unlikely that the PS-ODN will be able to gain entrance to the bacterial cell. qRT-PCR results also show that \textit{fba} transcript levels remained unaffected by the presence of zoocin A or a combination of zoocin A and PS-ODN in stationary phase cells, with no significant differences in transcript levels found. As the cells are in the stationary phase, transcription of \textit{gyrA}, 16s rRNA and \textit{fba} is likely to be minimal and it is unlikely that even if SM-FBA was able to permeate the stationary phase cell that any repressive effect could be measured. Due to this conclusion, corresponding radiolabel experiments were not conducted using stationary phase cells.

### 6.13 Importance of target specificity

The PS-ODN used in this study was designed to bind to the ATG region of the FBA gene RNA. PS-ODN work through the up regulation of the RNase H mechanism which results in the degradation of the target mRNA in the PS-ODN/mRNA duplex, and the steric hindrance
of ribosome binding due to the PS-ODN/RNA complex\textsuperscript{26,282}. A control mismatched PS-ODN (ATS2) was used in this study to check for any nonspecific effects caused by the presence of PS-ODN. All studies conducted using antisense as a means of gene inhibition in bacterial cells, no matter the species, include mismatched antisense sequences in order to prove that the inhibition effect shown by the targeted antisense sequence is due to the specificity of the antisense to its target, rather than the physical presence of antisense within the bacterial cell interior\textsuperscript{94,140,249}. The ATS2 sequence was chosen on the basis that no complementary match to it was found to be present in the \textit{S. mutans} UA159 genome sequence (NCBI BLAST). Care was taken to ensure that both the control and target PS-ODN sequences contained no more than a 65\% G: C content. It has been reported that the G: C content of antisense oligonucleotide sequences can influence the ability of antisense oligonucleotides to bind to target sequences, and that sequences with a G: C content greater than 65\% may bind nonspecifically to the genome resulting in non-specific inhibitory effects\textsuperscript{75,267}. This is most likely due to the intrinsically high stability of G:C pairs, as they utilise three hydrogen bonds compared to A:T pairs which utilise only two hydrogen bonds. As no full genome sequence has yet been determined for \textit{S. mutans} OMZ175 it was determined through PCR in a previous study that this strain also does not contain an ATS2 binding site\textsuperscript{95}. This present study has shown that γ\textsuperscript{32}P-ATS2 is able to penetrate the bacterial cell without causing inhibitory effects in \textit{S. mutans} OMZ175. ATS2 showed a complete lack of any significant inhibitory activity for lag, exponential and stationary phase \textit{S. mutans} OMZ175, and when combined with zoocin A no further significant inhibition was observed when compared to that of zoocin A alone. We know that the inhibition of \textit{S. mutans} OMZ175 with PS-ODN was sequence specific as only the combination of SM-FBA / zoocin A affected the gene expression of \textit{fba}, highlighting the specificity of this PS-ODN complex. The treatment of lag, exponential and stationary phase \textit{S. mutans} OMZ175 with ATS2 alone, or in combination with zoocin A, did not result in any significant differences in the gene expression of \textit{fba}, 16s rRNA and \textit{gyrA}. The presence of zoocin A alone, SM-FBA or ATS2 alone also did not result in the alteration in gene expression levels.

\textbf{6.14 Ability of ATS2 to penetrate the bacterial cell}
The question of whether the lack of inhibition of gene expression by AST2 is due to the possible lack of bacterial cell penetration by this PS-ODN has been accounted for in this study. The mismatched antisense sequence was used for all antisense radio-label experiments in this study, as using an antisense sequence which is known to have no significant effect upon bacterial gene expression or growth rate meant that these variables could be removed from the experiment. Using $\gamma^{32}$P-ATS2, the mismatched PS-ODN was been shown to be just as capable of penetrating lag and exponential phase *S. mutans* OMZ175 bacterial cells when used in combination with zoocin A.

### 6.14.1 Dose-dependency

The results of this study further confirmed that the inhibition of *S. mutans* OMZ175 by zoocin SM-FBA is dose dependent. Dose dependence is *prima facia* evidence that the inhibition effect observed in *S. mutans* OMZ175 is caused by the presence of the SM-FBA rather than any other molecules present in the experimental procedure. Dose dependence is also of vital importance in clinical therapy. It indicates that the greater the concentration of drug administered, the greater the clinical effect should be. In this study the *fba* gene was chosen as a target as it is an essential metabolic gene, where inhibition results in cell stasis and repressed cell growth$^{263,315}$. Thus, inhibition of FBA expression allowed us to directly measure the effect of *fba* antisense oligonucleotides though analysis of growth rate and viability, as well as further methods such as qRT-PCR. When combined with a sub-lethal concentration of zoocin A, the use of SM-FBA resulted in an inhibitory effect on *S. mutans* OMZ175 and the degree of inhibition was found to be dose dependent. Whilst the zoocin A concentration remained constant, as the concentration of SM-FBA increased there was a positive linear correlation with increased lag phase ($R = 0.9959$). This dose dependent inhibition effect was also observed when the SM-FBA concentration was kept stable, and the zoocin A concentration increased ($R = 0.9879$). The lack of saturation of growth inhibition observed with increasing SM-FBA concentration in the media indicates that there are multiple copies of *fba* mRNA in the *S. mutans* OMZ175 cells, and that the greater the concentration of SM-FBA added the greater the number of inhibited target mRNA sites.
As zoocin A levels were increased the corresponding increase in lag phase inhibition that was observed could be due in part to two factors. 1) That there was an increase in the permeability of the bacterial cell wall and a consequent increase in antisense oligonucleotide penetration into the cell. 2) That the increased concentration of zoocin A (a lytic agent) is itself increasing the lag phase. Whilst this study has not determined which of these two factors is responsible for this increase in lag phase, it is most probable that it is due to a combination of both. In order to determine the true nature of the inhibitory effect of the zoocin A/PS-ODN combination, the length of the lag phase of bacterial cells grown in the presence of zoocin A only was subtracted from the length of the lag phase of bacterial cells grown in the presence of a combination of both antimicrobial agents. The resultant lag phase times still correlate positively with zoocin A concentration ($R^2 = 0.9879$) suggesting the former as the most likely explanation.

### 6.15 Protein stability and turnover

The FBA protein is essential for metabolism in *S. mutans* OMZ175\(^70\). Only when this protein ceases to exist at a level required by the cell would any corresponding decrease in growth and viability occur\(^210\). The fact that a significant decrease in FBA mRNA levels was observed after 30 mins using qRT-PCR (with either zoocin A or penicillin as the PS-ODN delivery agent) and that this corresponding decrease in FBA mRNA levels is mirrored by a corresponding decrease in viability and growth indicates that in *S. mutans* OMZ175 the overall protein turnover of FBA must be rapid. Without the corresponding mRNA transcript, overall protein levels will only produce an effect on growth rate once the level of that protein available to the cell decreases to a level below that required by the cell for growth\(^210\). Whilst some bacterial cell death can be attributed to the action of the delivery agent, greater decreases in cell viability were observed for those cells treated with a combination of delivery agent and SM-FBA, compared to those treated with delivery agent alone, indicating that action by the SM-FBA itself is responsible for some reduction in growth and viability levels.

Whilst expression levels and turnover rate of FBA protein in *S. mutans* is unknown, studies have been conducted examining FBA expression or turnover rate in *S. aureus*, *E. coli*\(^143\), *S.*
cerevisiae \textsuperscript{343} and \textit{P. aeruginosa}\textsuperscript{23}. It has been found that \textit{E. coli} have an FBA protein turnover level of $850 \pm 35 \text{ min}^{-1}\textsuperscript{143}$, whilst \textit{S. aureus} was found to have a turnover rate between $472-836 \pm 30 \text{ min}^{-1}\textsuperscript{61}$. The total number of FBA protein molecules per cell for \textit{E. coli} and \textit{S. aureus} were not reported, but for \textit{P. aeruginosa} it was reported to be $2.4 \mu \text{ mol/ g}$ of wet cell weight\textsuperscript{23}. This equates to $14 \times 10^{17}$ copies of FBA protein per gram of wet cell.

Whilst an exact weight of pseudomonas has not yet been determined, the ‘average’ weight of an \textit{E. coli} bacterial cell (chosen to represent the average weight of a bacterial cell) has been proposed to range from $10^{-12}$ to $10^{-13}$ of a gram\textsuperscript{143}. If $12 \times 10^{17}$ copies of FBA protein exist in either $10^{12}$ or $10^{13}$ bacterial cells, this will result in between $14,000 – 140,000$ copies of FBA protein present per cell.

With a turnover rate estimated at $800 \text{ min}^{-1}$, this would indicate that $24,000$ FBA proteins are lost after 30 minutes. If there are only $14,000$ copies of the protein within the cell, this turnover rate is extremely significant, with over $170\%$ FBA protein turnover occurring within the first 30 minutes. Even if the upper limit of $140,000$ copies of FBA protein is present with in the cell, there would still be a $17\%$ decrease in FBA protein 30 minutes after treatment addition. This decrease may still be able to produce a serious effect on viability if the enzyme acts as a rate limiting step within the metabolic cycle\textsuperscript{210}.

Although these figures are taken from FBA levels which occur in different bacterial strains, evidence suggests that FBA protein turnover is rapid within \textit{S. mutans} OMZ175. FBA mRNA levels were found to be significantly decreased 30 minutes after treatment (approx. $5 – 30$ fold reduction in FBA mRNA levels) and this corresponds with a noted decrease in bacterial viability at 30 minutes which indicates that in \textit{S. mutans} OMZ175 a fast FBA protein turnover rate is likely to be occurring. It is possible that FBA acts as the rate limiting step for \textit{S. mutans} metabolism, and thus any reduction in protein levels has an immediate impact on overall metabolism and growth rates. This explanation would also explain why treated \textit{S. mutans} OMZ175 are slow to recover from treatment with delivery agent + SM-FBA.

It must also be noted that the assay used means that FBA mRNA levels at 30 minutes cannot be directly compared to cellular viability at 30 minutes. Thirty minutes after treatment addition (delivery agent + SM-FBA) the sample was tested, and the FBA mRNA levels measured and the amount of antisense associated with each cell determined. It has been
determined that 30 minutes after treatment addition a significant amount of antisense is associated with each cell, and that a significant decrease in FBA mRNA levels has occurred. A further sample is removed 30 minutes after treatment and plated upon agar in order to determine bacterial viability. Any SM-FBA present inside these *S. mutans* OMZ175 cells will still working to inhibit FBA mRNA production even once the cells have been plated. Thus the viability count taken 24 hours after the cells were initially plated may not directly correlate to the viability count at 30 minutes. Due to the viability assay type, an artefact of this assay means that the *S. mutans* OMZ175 cells could be held in stasis or die due to the presence of SM-FBA at any time point before colony formation, and thus there would be no colony to count. It is therefore possible that the viability levels of the treated *S. mutans* OMZ175 cells was much higher immediately following 30 minutes of treatment, compared to that found 24 hours after agar plate incubation. This would help to explain why such a significant decrease in viability has been attributed to only 30 minutes incubation with the treatment type. It is therefore possible that even if SM-FBA is having a slight effect (17%) upon FBA protein levels after 30 minutes that such a large decrease in cellular viability has been noted.

The presence of delivery agent + ATS2 (scrambled control) produces no effect upon mRNA FBA levels, and no greater effect upon cell growth rates and viability that that produced by the delivery agent itself. This indicates that this effect upon bacterial metabolism is caused by the presence of a targeted PS-ODN (targeted towards the FBA gene), rather than any unspecific effect created by the presence of PS-ODN alone.

It is also possible that *S. mutans* OMZ175 has a greater turnover rate of FBA protein compared to that of *E. coli* or *S. aureus*, as *S. mutans* OMZ175 is completely dependent upon sucrose metabolism (and therefore a functional FBA gene), whilst *E. coli* and *S. aureus* possess other metabolic pathways which don’t require a functional FBA gene for metabolism. If a greater turnover rate of FBA exists in *S. mutans* OMZ175, this again would help to explain why such a quick effect was observed on cellular growth and viability.

### 6.16 Development of resistance
Repeated exposure to zoocin A, and or zoocin A/ PS-ODN combinations did not cause any significant increase or decrease in inhibition levels of either lag or exponential phase *S. mutans* OMZ175 compared to those found for a single exposure of the treatment. The presence of zoocin A alone, SM-FBA alone, ATS2 or a combination of PS-ODN/ zoocin A did not result in the mutation of any of these genes of interest. This indicates that resistance to these antimicrobial zoocin A/ PS-ODN combinations was not apparent after 4 repeat exposures. No research paper has yet reported the ability of a bacterial strain to mutate the target gene as a direct response to targeted antisense addition. This is also a factor of clinical importance. The ability to give multiple doses of the same AS-ODN, PNA or PMO treatment without the bacterial strain either adapting to its presence or mutating to gain resistance means that a course of treatment can be developed which will provide a more effective outcome compared with that of an antibiotic, especially that of an antibiotic to which the bacterial strain has previously been exposed. Lower doses of the AS-ODN, PNA or PMO will be able to be given as the bacteria will not develop resistance, which will lead to better patient outcomes- as antimicrobials can sometimes cause undesirable side effects such as liver toxicity\(^8^0\). The ability to give multiple doses at the same concentration also makes it more likely that the bacterial infection will be able to be treated with a single antisense type, rather than combinations\(^2^4^1\). Combinational antibiotic therapy is often used as a method to avoid the development of antibiotic resistance, but the use of multiple antibiotics increases cost. It is also more likely that the length of patient hospital stays due to bacterial infections will reduce when treated with targeted AS-ODN, PNA or PMO, as the treatment can be more effective, thereby lowering costs to the public health systems\(^7^4,1^9^5,2^2^1\).

### 6.17 Significance of zoocin A delivery

These results indicate that zoocin A does indeed facilitate the entry of PS-ODN into *S. mutans* OMZ175. Whilst other studies have used carrier peptides\(^1^8,2^4^6\), permeabilising agents such as So-Fast\(^1^4^0\), anti- mycobacterial drugs\(^1^4^5,1^4^6\), liposomes\(^2^2^7\) or plasmid encoded antisense sequences\(^1^6\) to deliver antisense into the bacterial interior, this study has shown that a bacterial produced endopeptidase also holds great delivery potential. The above listed
agents possess greatly varied modes of action and often show their AS-ODN, PNA or PMO delivery potential upon a limited number of bacterial strains. Depending of the bacterial strain targeted, different AS-ODN, PNA or PMO delivery mechanisms offer different delivery potential. Zoocin A for example is limited by is its narrow host range acting only upon the peptidoglycan of a closely related group of streptococci. A further limitation of zoocin A is its suspected immunogenicity which would limit zoocin A’s use in \textit{in vivo} (Simmonds, per. comm. 2012). Although zoocin A is a two domain enzyme (N-terminal catalytic domain, responsible for peptidoglycan hydrolysis and a C-terminal wall-binding domain responsible for cell targeting) it is unlikely that the N-terminal by itself could be used to deliver PS-ODN to the \textit{S. mutans} cell alone in an attempt to reduce immunogenicity. The N-terminal domain alone shows a greatly reduced level of efficacy (Lai \textit{et al.} 2002). This reduction in efficacy would require a far greater concentration of the zoocin A fragment in order to produce a lytic effect, therefore increasing possible immunogenicity.

However, zoocin A was primarily used for proof of principle that a peptidoglycan permeabilising agent helps to increase rates of PS-ODN delivery into bacteria. In order to apply antisense technology which demonstrates a broader spectrum, different delivery mechanisms must be investigated that would allow antisense delivery into a wide range of pathogenic bacterial strains. The use of multiple delivery agents would greatly reduce the chance of resistance to the delivery agent developing, as lower concentrations of each delivery agent would be required.

6.18 Other cell permeating delivery agents

Despite antisense being a targeted and promising approach to inhibit growth of specific pathogens, an effective delivery system is needed to deliver PS-ODN across the cell wall. Liposomes have been used to encapsulate PS-ODN’s and deliver them either into human cell interiors\textsuperscript{188} or bacterial cells\textsuperscript{228}. PS-ODN’s have also been coupled to cholesterol or other molecules to improve uptake into human cells\textsuperscript{18,41}. Whilst zoocin A has been shown to allow targeted delivery of PS-ODN into \textit{S. mutans} OMZ175, due to zoocin A target specificity, if AS-ODN, PNA or PMO is to be delivered exogenously to further bacterial strains, other
effective antisense delivery systems will need to be investigated. As well, it is possible that zoocin A, whilst allowing antisense entrance into *S. mutans* OMZ175, is not the most efficient delivery agent available. To examine this, the third aim of the experiment was to test various cell permeating polypeptides or antibiotics as potential delivery molecules for their ability to deliver PS-ODN into *S. mutans* OMZ175 and other potentially pathogenic bacterial strains.

### 6.18.1 Nisin and pediocin as antisense delivery molecules

Both nisin and pediocin are amphiphilic polypeptides, with nisin containing a net positive charge $^{353}$. Nisin is active against related Gram-positive bacteria such as *Bacillus cereus* $^{158}$, *Listeria monocytogenes* $^{207}$, Enterococci $^{192}$, Staphylococci $^{1}$ and Streptococci $^{170}$, whilst pediocin is active against many strains of Gram-positive bacteria including *L. monocytogenes* $^{39,192,262}$. Pediocin failed to inhibit the growth of *S. mutans* OMZ175 at all concentrations tested. This could be due to resistance of *S. mutans* OMZ175 towards pediocin $^{36,102}$, pediocin’s own narrow antimicrobial spectrum $^{40,159}$ or the use of concentrations too low to have an effect on bacterial growth $^{36,135}$.

*S. mutans* OMZ175 and *E. faecalis* AR01/DGVS were inhibited in sub-lethal fashion by nisin concentrations of 1.25 mg/ml and 0.7 mg/ml respectively. When nisin combined with 20 μM of SM-FBA, or ATS2, was added to *S. mutans* OMZ175 culture, the amount of inhibition caused by both combinations of nisin + PS-ODN was significantly less than the amount of inhibition seen for just nisin alone. This was an unexpected result, and suggested that nisin and PS-ODN were interacting together to neutralise the effect of nisin on *S. mutans* OMZ175.

Growth of the indicator organism *Micrococcus leuteus* IL1 with nisin and increasing concentrations of antisense, showed that as the amount of antisense present increased, so the amount of inhibition decreased. This indicates that the PS-ODN is able to inhibit nisin effectively, with even a 1 μM concentration of SM-FBA significantly decreasing the inhibitory effect of nisin. The ability by which SM-FBA is able to inhibit nisin activity is unknown, but it can be speculated that perhaps an interaction between the positively charged
nisin and negatively charged SM-FBA occurred. PS-ODN’s, due to their overall negative charge have been reported to bind in a length- and sequence specific manner to a number of human cell factors, such as fibroblast, platelet and epidermal growth factors\textsuperscript{288}. It has previously been noted that lysozyme and PS-ODN’s also interact in a way which decreases the ability of lysozyme to inhibit growth\textsuperscript{223}. Research has shown lysozyme binds to many types of large molecules in solution, and complexes between lysozyme and negatively charged molecules or DNA mimics can result in precipitation\textsuperscript{198,322}. It is also known that nisin activity can decrease in the presence of anionic substances\textsuperscript{71,90}. Therefore, it is possible that nisin as a cationic peptide with a positive charge of net two\textsuperscript{169}, can bind to a poly-negatively charged PS-ODN\textsuperscript{2} - preventing the nisin from inhibiting cell growth. No other studies have yet been conducted using nisin and AS-ODN, PNA or PMO combinations, and it would be interesting to determine whether this inhibitory effect is limited only to PS-ODN’s, or whether neutral antisense chemistries such as PNA’s also exhibit such an effect.

A further possibility could relate to the ability of AS-ODN, PNA or PMO to non-specifically bind to DNA, RNA or protein. Studies have shown that as the G: C ratio of an antisense sequence increases, the possibility of non-specific binding by the antisense also increases\textsuperscript{29,320}. It was observed both in this study, and a further corresponding study\textsuperscript{67}, that for those PS-ODN’s tested in combination with nisin, the greater the G: C content of the PS-ODN, the more marked the reduction in inhibitory effect compared to that of the corresponding nisin control. This suggests that the higher the G: C content of the PS-ODN the greater its binding to nisin. The nisin used in this study is commercially prepared, and contained 2.2% nisin balanced with 77.5% denatured milk solids and NaCl. Therefore it is possible that the milk solids were bound by the PS-ODN molecules, reducing their inhibitory activity.

\section*{6.18.2 Antibiotics as antisense delivery molecules}

The concept of whether an agent will lyse a bacterial cell is significant. If an agent is able to cause the destruction of the peptidoglycan layer leading to the lysis of the bacterial cell at a certain dose, then it is highly probable that at lower concentrations that agent is able to
produce a non-lytic permeabilising effect upon the bacterial cell. The antibacterial action of penicillin, vancomycin and many other antimicrobials results from their ability to inhibit the synthesis of the peptidoglycan layer of bacterial cell walls leading to a loss of structural integrity. These types of antibiotics may also stimulate autolysis of target bacteria by deregulation of autolytic gene control\textsuperscript{31,196,264}. Therefore the use of such antibiotics presents a method of inducing cell permeabilisation which has already been extensively studied and is already approved for \textit{in vivo} use. Tipper and Strominger\textsuperscript{332} were the first to suggest that the binding of penicillin to penicillin-binding proteins (PBP’s) and the subsequent inhibition of peptidoglycan replication resulted in an osmotically and mechanically weak bacterial cell wall\textsuperscript{31}.

Permeabilising agents such as SoFast or the anti-mycobacterium drugs amikacin, ethambutol or polymyxin B have been trialed for their ability to improve AS-ODN, PNA or PMO delivery into bacterial cells with limited success\textsuperscript{140,146}. Penicillin works best on actively growing cells, and has been shown to act synergistically with streptomycin\textsuperscript{234}. When streptomycin resistant \textit{E. coli} were treated with a combination of penicillin and radioactive streptomycin, it was shown that the streptomycin was able to enter the bacterial cell in much greater amounts than when the cells were treated with streptomycin alone. It is thought that this phenomenon is due to the cell wall permeabilising effect of the beta-lactam\textsuperscript{234}. This study attempted to determine whether penicillin, or another cell permeabilising antibiotic vancomycin, would act synergistically together with targeted PS-ODN.

\textbf{6.18.3 The importance of the growth phase for penicillin interaction}

With penicillin or vancomycin added to exponential phase \textit{S. mutans} OMZ175, \textit{S. aureus} Oxford, \textit{S. aureus} WSSP-1 or \textit{E. faecalis} AR01/DGVS, in either; THB, MH or LB broth respectively, either no significant difference in growth inhibition was observed between the addition of the antibiotic and the no- antibiotic control, or else no further OD increase (growth) occurred. This lack of growth was not accompanied by an immediate decrease in OD, indicative of cell lysis, and was followed by growth recovery. Reports of non-lytic penicillin killing are found throughout the scientific literature\textsuperscript{154,204,220,237}, although the
mechanism behind such an effect is as yet largely unknown. These findings are consistent with the findings in this study, in which the presence of penicillin resulted in non-lytic death of *S. mutans* OMZ175 in the exponential phase. The SOS response proposed by Miller\(^{230}\) proposes that bacteria treated with penicillin may stay viable but will not replicate in the presence of penicillin – thereby producing static OD readings. However, Bayles\(^{31}\) proposed that the bactericidal effect of penicillin causes an immediate loss in viability, but the lysis may occur either after a substantial lag period, or not at all. Either theory puts forth the idea that penicillin may often not cause bacterial lysis, which corresponds to our observed results.

Researchers have proposed that the amount of acid produced by some bacterial strains during growth lowers the pH of the media to such an extent that the antibiotic is no longer able to function\(^{137}\). Other studies have suggested that low pH works indirectly upon the acid producing bacterial cell- which may result in the suppression of the activity of enzymes involved in autolytic processes in the cell. Such effects have been termed ‘pH dependent antibiotic tolerance’\(^{154,204,344}\). Some research teams have managed to overcome this non-lytic cell death effect by using buffered media\(^{133,154,204}\). In this study, a potassium phosphate buffer was found to permit growth of *S. mutans* OMZ175 in TSB, and to have an adequate buffering effect on media pH during growth when compared to TSB alone.

For lag phase *S. mutans* OMZ175 treated with penicillin the results indicate that the inhibition of *S. mutans* OMZ175 by penicillin SM-FBA is dose dependent, which further suggests that the inhibition effect observed in *S. mutans* OMZ175 is caused by the presence of the SM-FBA rather than any other molecules present in the experimental procedure. Whilst the SM-FBA concentration remained constant, as the concentration of penicillin increased there was a positive linear correlation with increased lag phase (R = 0.93).

That the addition of penicillin or penicillin + PS-ODN to stationary phase *S. mutans* OMZ175 produced no significant inhibitory effect is unsurprising as penicillin has frequently been reported to only function on dividing bacterial cells\(^{68,333}\). Penicillin works by inhibiting the penicillin binding proteins (PBP’s) involved in peptidoglycan synthesis\(^{68}\) and once bacterial cells enter the stationary phase peptidoglycan synthesis no longer occurs and therefore one would predict that in stationary phase cells penicillin would not enhance entry of PS-ODN’s
to the cell cytoplasm. This conclusion was supported by the observations that the addition of penicillin or penicillin + PS-ODN also showed no effect upon gene transcript levels.

6.18.3.1 The ability of penicillin to deliver PS-ODN into lag phase cells

The qRT-PCR results in this study demonstrated a decrease in fba expression for lag phase S. mutans OMZ175 cells treated with the SM-FBA /penicillin combination over time with a 37.354 ± 16.94 decrease in fba expression after a 30 minute incubation, and a 522.241 ± 30.05 decrease four hours after treatment addition. This was matched by the observation that γ^{32}P-ATS2 molecules gained entrance into lag phase cells interior during the first 5 hours of penicillin treatment (5.652 ± 3.91 x 10^7 ATS2 molecules per CFU four hours after treatment addition). Penicillin treated lag phase S. mutans OMZ175 show that a greater number of ATS2 molecules become cell associated (2.73 ± 1.82 x 10^8) whilst penicillin is actively inhibiting growth (and causing a permeabilising effect) compared to those cells without penicillin treatment (30,741 ± 14,200). These results indicate that penicillin can weaken the bacterial peptidoglycan sufficiently to allow enough PS-ODN into the bacterial cell to have a measurable effect upon target RNA transcript levels. Once growth recovery begins, a rapid loss in the amount of γ^{32} P-ATS2 per CFU is demonstrated for both treatment added in the lag phase, or in the exponential phase, with an approximate 2.5 - 3 log decrease in γ^{32} P-ATS2 per CFU over time, which corresponds to an approximate 2.5 - 3 log increase in the number of viable cells per ml (Figure 5.14, 5.16). This is believed to be due to the fact that once penicillin is unable to produce an effect upon peptidoglycan synthesis, the bacterial peptidoglycan is repaired and γ^{32} P-ATS2 molecules are no longer able to gain access to the bacterial cell interior. This further supports the suggestion that any loss of γ^{32} P-ATS2 per CFU is due to cellular dilution due to cellular replication, rather than loss due to cellular leakage or diffusion.

6.18.3.2 The effect of penicillin upon the exponential phase cells
Different results were however observed for exponential phase *S. mutans* OMZ175 held in buffered TSB. Whilst the viability of the exponential phase cells could be reduced upon the addition of penicillin to the media, no corresponding decrease in OD occurred. No significant increase or decrease in bacterial cell density was observed after addition of penicillin, and this together with the viability data indicated that penicillin was having a non-lytic killing effect upon the *S. mutans* OMZ175 cells. This is the same type of effect that has been frequently observed when non-buffered media has been used\textsuperscript{133,154,204}. That this effect has been observed when using buffered media is slightly puzzling. However, *S. mutans* OMZ175 is a known acid producing strain, and during the exponential phase would be producing high amounts of acid compared to a bacterial strain with lower rates of acid production. It is possible that due to this high acid production, despite the presence of buffering salts in the medium, the surface close to the bacterial cell wall remained at a low pH affecting the penicillin function. As well, such a high acid production rate may have resulted in suppression of genes involved in the autolysis process, as proposed by other research groups\textsuperscript{31,133,344}, thus preventing cell lysis. Such a result indicates that further research is needed in this area to determine the exact mechanism by which acid production affects the ability of penicillin to function and cause cell lysis. Until this is done, and a solution found, penicillin does not present a feasible antisense delivery option into exponential phase bacterial cells *in vitro*. *In vivo* it is likely that the ability of the bacterial strain to produce acid would be of much lower significance. In general bacterial growth rates in infection are considered to be much slower than in *in vitro*, which may result in a lower metabolic rate which may in turn reduce acid production. The production of highly acidic conditions within dental plaque, caused by the formation of biofilms often incorporating acid producing strains such as *S. mutans* might however represent a challenge. One other hypothesis which could shed light on this problem of acid/penicillin interaction was proposed by Tylewska et al. in 1981\textsuperscript{338}. He proposed that penicillin decreases the bacterial cell surface hydrophobicity which results in a net increase in the cells negative electric charge. He found that this affects the adhesion of *S. pyogenes* to pharyngeal epithelia cells, but if true, this effect could also lead to the *S. mutans* OMZ175 bacterial cell repelling the negatively charged antisense, diminishing interaction between the two.
That when used in the exponential phase of *S. mutans* OMZ175 growth penicillin causes a non-lytic killing effect is further supported by the observed result that exponential phase *S. mutans* OMZ175 cells showed no greater uptake of $\gamma^{32}$P-ATS2 over time compared to those cells treated with $\gamma^{32}$P-ATS2 alone. It was determined that an average of 15,435 ± 9,807 molecules of ATS2 per cell had entered the penicillin + $\gamma^{32}$P-ATS2 treated exponential phase *S. mutans* OMZ175 cells within the first 30 minutes of treatment, whilst 8,419 ± 543 molecules of $\gamma^{32}$P-ATS2 per CFU were associated with those cells treated with $\gamma^{32}$P-ATS2 alone. Four hours after treatment, it was determined that there was an average of 24,344 ± 14,008 molecules of $\gamma^{32}$P-ATS2 per CFU for those cells treated with penicillin + $\gamma^{32}$P-ATS2, and 14,459 ± 813 molecules of $\gamma^{32}$P-ATS2 per CFU associated with those treated with $\gamma^{32}$P-ATS2 alone.

These results are unsurprising. When exponential phase *S. mutans* OMZ175 is treated with penicillin + PS-ODN, OD and viability results show that no synergism between the two agents exists, and that no lytic effect is caused by the presence of penicillin (Figure 5.8). If penicillin is unable to permeabilise the bacterial cell wall, then the $\gamma^{32}$P ATS2 molecules are unable to enter the bacterial cells. In order for the antisense to gain entrance into the bacterial cell interior, the delivery agent must ‘permeabilise’ the bacterial cell wall to make ‘pores’ through which the antisense can gain access. If penicillin is unable to function properly due to the production of acid by the *S. mutans* OMZ175, this may result in non-lytic killing of the bacterial cell, decreasing the viability of the bacterial cell, but without affecting the permeability of the bacterial cell wall, thus inhibiting antisense from gaining entrance to the bacterial cell. The gene expression experiments in this study support this claim, as no significant difference was found in the expression of *fba* in exponential phase *S. mutans* OMZ175 treated with penicillin which supports the idea of the pH dependent antibiotic hypothesis.

This is further supported by the radiolabelling experiment results, which show that exponential phase *S. mutans* OMZ175 cells treated with $\gamma^{32}$P-ATS2 alone show a similar amount of ATS2 uptake as those cells treated with $\gamma^{32}$P-ATS2 alone during the corresponding zoocin A experiments, further indicating that there is a maximum amount of $\gamma^{32}$P-ATS2 that can associate with each CFU, without the use of permeating agents. The
comparison of growth to the number of ATS2 molecules present per CFU, further demonstrates this fact (Figures 5.14, 5.16). Whilst cell numbers increase over time once penicillin has ceased to have an effect upon bacterial viability, for exponential phase S. mutans OMZ175 treated with penicillin the amount of ATS2 associated with each CFU remains a constant.

As the number of ATS2 molecules associated with each cell remains steady for exponential phase S. mutans OMZ175 treated with penicillin + $\gamma^{32}$ P-ATS2, as well as indicating that ATS2 molecules are not present within the bacterial cell interior, this also indicates that as cell replication occurs, further free ATS2 molecules present in the media must interact and associate with each new cell formed, to an approximate maximum of $10^5$ molecules per CFU.

That penicillin is unable to facilitate PS-ODN entrance into the exponential phase S. mutans OMZ175 cell is further illustrated by the fact that fba expression remained unaffected when the cells were treated with either SM-FBA alone, or a combination of penicillin and SM-FBA. Assuming that the number of $\gamma^{32}$ P-ATS2 molecules which gain access to the bacterial cell interior are comparable to the number of SM-FBA molecules which gain access, this result indicates that both ATS2 and SM-FBA are unable to gain access into S. mutans OMZ175 in the exponential phase treated with penicillin, whilst the permeabilising effect of penicillin upon lag phase S. mutans OMZ175 does allow greater PS-ODN cell association as has been shown for ATS2.

6.18.3.3 E. faecalis and S. aureus

Combinations of targeted PS-ODN, together with vancomycin or penicillin, showed no synergistic inhibitory effect upon either E. faecalis AR01/DGVS or S. aureus Oxford or WSSP-1. This is most likely due to the inability of these delivery agents to correctly ‘permeabilise’ the bacterial cell wall, thus denying the PS-ODN access to the bacterial cell interior. It is also possible that the fructose bis-phosphate adolase gene is not essential for the growth of either strain. Whilst the metabolism of both strains has been the topic of research$^{284,295,323}$, no studies into the nature of fba has yet been conducted for either strain.
6.19 Comparison between zoocin A and penicillin PS-ODN delivery

It is known that penicillin acts both directly upon the bacterial cell wall, through the inhibition of peptidoglycan synthesis\textsuperscript{264} and indirectly by deregulating autolytic gene control\textsuperscript{196}. However, it seems likely that zoocin A facilitates entry of PS-ODN’s by acting directly against cell peptidoglycan. A comparison between zoocin A and penicillin highlights the fact that the different modes of action of each agent result in different amounts of PS-ODN cell association over time. Zoocin A, as a direct lytic agent was expected to produce a rapid lytic effect which in turn would allow the immediate increase of cell associated PS-ODN\textsuperscript{311}, and the corresponding repression of \textit{fba} expression. As penicillin first has to induce autolysis within the target bacterial cell\textsuperscript{237,250}, it was expected that compared to zoocin A, penicillin would be slower in allowing PS-ODN to become cell associated, and thus there would be an observable delay effect in \textit{fba} repression. The data generated in this study supports such a statement.

Zoocin A demonstrates the ability to allow a greater amount of PS-ODN to become cell associated (36.2 ± 8.3 x 10^6), and a greater decrease in \textit{fba} expression (-37.354 ± 16.94 fold decrease) 30 minutes after treatment of lag phase \textit{S. mutans} OMZ175, than penicillin (44,804 ± 11,703 and -5.728 ± 0.23 respectively). At 4 hours after addition, both agents + PS-ODN showed higher amounts of PS-ODN entry compared to that of 30 minutes, with penicillin allowing a greater amount of ATS to enter/ become associated with/ the bacterial cell (2.73 ± 1.82 x 10^8 molecules of ATS2 per CFU) compared with zoocin A (5.652 ± 3.91 x 10^7). Over time the addition of penicillin + SM-FBA results in a far greater growth suppression effect than that of zoocin A, with increased \textit{fba} suppression over time. Such a result reveals that choice of delivery agent may influence the time point at which antisense intracellular concentration will reach a critical threshold and allow gene suppression to occur and that different modes of action of both delivery agents affect the rate at which the PS-ODN is delivered into the bacterial cell. Such an effect could greatly influence gene suppression studies and could also influence the ability of delivery agents to provide an effective synergistic response \textit{in vivo}. Further studies are recommended for all AS-ODN, PNA or PMO.
delivery agents in order to determine which delivery agents provide the greatest degree of cell delivery, and over which time points. Furthermore, the penicillin mediated uptake of PS-ODN into S. mutans cells and the SM-FBA mediated inhibition of cell growth provides prima facia evidence that strong synergistic interactions between PS-ODN’s and clinically relevant antibiotics is possible.

6.20 Conclusions

There is currently no standard method for the optimal delivery of AS-ODN, PNA or PMO into bacterial strains. This is primarily due to the fact that this technology is so very new, and that further research is needed. The research reports available so far have shown that many different mechanisms are able to allow AS-ODN, PNA or PMO into a variety of bacterial strains\(^7\). They have also illustrated that each delivery mechanism is often effective upon only certain strains, and that significant differences between AS-ODN, PNA or PMO delivery mechanisms exist when different antisense chemistries or bacterial strains are used. It appears that for some strains, such as Gram negative \(E. coli\)^\(^{94,331}\), \(S. enterica\)^\(^{233}\), \(K. pneumoniae\)^\(^{185}\) or even Gram positive \(S. aureus\)^\(^{18}\) that the cell membrane acts as the major barrier preventing AS-ODN, PNA or PMO penetration. However, for other strains such as \(M. smegmatis\)^\(^{146}\) or \(S. mutans\) OMZ175\(^{95}\) it appears that the bacterial cell wall peptidoglycan layer acts as the primary barrier preventing AS-ODN, PNA or PMO entrance. It is also possible that the surface proteins present on each bacterial cell can interfere with AS-ODN, PNA or PMO entrance by as yet undetermined mechanisms.

6.20.1 Cell surface barriers

This study has shown that for \(S. mutans\) OMZ175 the peptidoglycan layers (or possibly the peptidoglycan surface decoration molecules such as teichoic acids) present the major barrier to PS-ODN entry, and that the membrane itself does not present a major barrier to PS-ODN entry. It is likely that zoocin A is causing limited (the cells are able to recover) but significant
damage to the \textit{S. mutans} OMZ175 peptidoglycan layer, which is resulting in a far greater level of PS-ODN penetration, compared to untreated \textit{S. mutans} OMZ175 cells. That PS-ODN’s are entering the cell is demonstrated by this study’s qRT-PCR results, which demonstrate a target specific decrease in RNA levels, and the use of radiolabelled $\gamma^{32}$ P-ATS2 to directly measure label entry into the \textit{S. mutans} OMZ175 cell. Theoretically, peptidoglycan should be porous enough to allow a molecule the size of the 18 bp PS-ODN used in this study to diffuse through unaided\textsuperscript{229}, and PS-ODN’s due to their polyanionic nature should not be able to diffuse through the bacterial membrane\textsuperscript{108}. Some studies however have shown that PS-ODN’s are able to diffuse unaided through the bacterial cell wall\textsuperscript{140}, whilst others have shown that the attachment of highly charged carrier peptides\textsuperscript{18,103}, or encasement within liposomes\textsuperscript{226}, enable PS-ODN’s to permeate the bacterial cell barrier. Most studies however have not focused upon how the AS-ODN, PNA or PMO penetrates the bacterial peptidoglycan layer, although some researchers have clearly demonstrated that for some bacterial strains at least, the bacterial peptidoglycan layer presents a significant barrier to AS-ODN entry\textsuperscript{95,183}.

Most of the AS-ODN, PNA or PMO delivery systems discussed in this study result in enhanced antisense efficacy compared to AS-ODN, PNA or PMO alone. However, different antisense delivery mechanisms result in different levels of AS-ODN, PNA or PMO efficacy, with different bacterial strains showing different results and different AS-ODN, PNA or PMO efficacy rates even when treated with the same AS-ODN delivery system. It is possible that a variety of different bacterial cell components prevent AS-ODN, PNA or PMO delivery and that these may vary between bacterial strains. The development of this radiolabelling method used in this study means that many different cell permeabilising technologies can be compared across different bacterial cell types, and further pharmacokinetic studies and their antisense delivery potential determined. This will allow the determination for each different bacterial cell type the mode of antisense delivery which permits the greatest delivery of AS-ODN, PNA or PMO to the cell interior.

\textit{6.20.2 The bacterial membrane}
Neither zoocin A or penicillin is known to interact with the bacterial membrane. Whilst other studies have shown the bacterial membrane to act as the major barrier to AS-ODN, PNA or PMO entry, and that PNA’s have greatly increased inhibitory effects upon mutant bacterial strains with weakened cell membranes\textsuperscript{129,130}, in this study, the peptidoglycan layer was shown cause the greatest inhibition to PS-ODN entry. Whilst the membrane may have been involved in inhibiting PS-ODN penetration, due to the fact that a successful protoplasting protocol was unable to be developed, the amount of membrane inhibition was unable to be determined.

6.20.3 Peptidoglycan

Zoocin A as an endopeptidase is able to cause specific, limited, but significant damage to the \textit{S. mutans} peptidoglycan layer\textsuperscript{187}. Streptococcal strains sensitive to zoocin A have peptidoglycan with di- or tri-alanine cross-links\textsuperscript{117}. By cleaving at the D- alanine residues present in the cross-linking peptides present within the peptidoglycan layer, the cross-links are cleaved open, results in larger pores in the peptidoglycan being formed, as the NAM-NAG chains no longer form an interlocked arrangement. Instead cleavage by zoocin A results in an opened peptidoglycan layer, with single chains of NAM-NAG units running in parallel within the peptidoglycan, but without interlocking cross-links present between each chain. Normally \textit{S. mutans} possess a tight cell wall, as there are only two or three alanine’s are present within the cross-links between the NAM-NAG units (compared with the five glycine’s present within the cross-links which make up the \textit{S. aureus} cell wall)\textsuperscript{117}. Only a small amount of hydrolysis by zoocin A would be necessary to result in the formation of a large number of pores within the \textit{S. mutans} peptidoglycan layer. This compares to the different mechanism of the lytic agent lysozyme, which is often used to lyse streptococcal cells. Lysozyme cuts between the NAM and the NAG units, but does not interfere with the cross-linking peptide\textsuperscript{175}. These cross-links hold the NAM-NAG units in place, despite the join between the NAM and the NAG themselves having been destroyed. Such a mechanism requires a far greater concentration of the lytic agent to be delivered to the cell in order to produce a lytic effect.
A previous study has examined the ability of two peptidoglycan targeted lytic agents, mutanolysin and lysozyme to work synergistically with PS-ODN to produce a targeted inhibitory effect upon *S. mutans* OMZ175 and other closely related streptococcal strains\(^{223}\). Whilst both agents were able to cause a growth inhibitory effect upon targeted strains, mutanolysin and antisense combined failed to produce any inhibitory effect greater than that caused by antisense alone, indicating that mutanolysin was unable to cause a permeabilising effect upon the peptidoglycan layer which would permit PS-ODN entrance. This may be due to mutanolysin's mode of action which cuts at the β1-4 glycosidic linkage between MurNAc and GlcNAc present in streptococci peptidoglycan (between the NAM-NAG units)\(^{59}\). Again, as with lysozyme, such cleavage would not interfere with the cross-linking peptides present in the bacterial peptidoglycan layer, and it is possible that any pore opening caused by the action of mutanolysin would be too small to allow PS-ODN entrance into the bacterial cell.

A combination of lysozyme and PS-ODN resulted in the immediate formation of a precipitate and no further inhibitory effect upon lysozyme susceptible strains were observed. No others studies have been reported involving the use of lysozyme and PS-ODN, or any other AS-ODN chemistry, so such a result was unexpected. Several PS-ODN sequences were used in this study and all when combined with lysozyme resulted in precipitate formation, indicating that precipitation is not a result of a sequence specific interaction, but rather is one that is common to all PS-ODN. It has been reported that lysozyme binds to many types of large molecules in solution and complexes between lysozyme and DNA can also result in precipitation\(^{198,322}\). PS-ODN acts as a DNA mimic so it is likely that a similar complex was formed between the lysozyme and DNA.

It is possible that the *S. mutans* OMZ175 cell wall possess fewer cross-links than NAM-NAG links. Therefore cleavage of these cross-links by zoocin A would cause a greater lytic effect compared to lytic cleavage between NAM-NAG units by the same concentration of lysozyme or mutanolysin. Such a reason would explain why zoocin A was found to be so much more efficient at releasing RNA from the *S. mutans* OMZ175 cells than either lysozyme or mutanolysin, or a combination of both.

Other researchers however have not found the peptidoglycan layer to be a notable barrier to AS-ODN, PNA or PMO entry into the bacterial cell\(^{18,140}\). It is possible that the peptidoglycan
layers present did limit AS-ODN entry into the cell, but the rate of peptidoglycan inhibition was undetermined. If the peptidoglycan structure of the *S. mutans* OMZ175 is however already completely porous to small molecules such as PS-ODN, then it is unlikely that opening the peptidoglycan layer up further will make much difference except to increase the rate of AS-ODN, PNA or PMO uptake into the bacterial cell, and make the bacterial cell membrane more accessible to AS-ODN, PNA or PMO molecules. Whilst the size, charge and hydrophobicity of the AS-ODN, PNA or PMO molecules may effect diffusion through the peptidoglycan layers, it is also possible that there are other structures present on the bacterial cell surface, whose action is also affected by the presence of peptidoglycan destroying agents, and whose presence interferes with AS-ODN, PNA or PMO entrance.

### 6.21 Cell surface proteins

This study demonstrated that PS-ODN alone is unable to permeate the bacterial cell or produce an inhibitory effect when used alone with *S. mutans* OMZ175 whereas Guo et al. were able to show that a weak inhibitory effect was produced through the use of PS-ODN alone upon *S. mutans* GS-5\(^{140}\). It is possible that this difference in the ability of PS-ODN to penetrate is due to differences between either the peptidoglycan structure or cell surface molecules for each mutans strain. Differences between cell surface proteins present upon the surface of streptococcal strains has been proposed to be one reason for different susceptibilities between strains to various antimicrobials\(^{199}\). Teichoic acids are covalently attached to peptidoglycan through glycosidic bonds, and reach out beyond the peptidoglycan cell surface\(^ {266}\). They provide a high density negative charge to the cell surface\(^ {347}\), and it’s possible that this charge could repel negatively charged PS-ODN. Lipoteichoic acids are attached through a lipid anchor present in the cell membrane\(^ {180}\). It has been observed that some bacterial strains, such as *S. aureus*, have a cell surface consisting primarily of teichoic acid, with only small amounts of peptidoglycan present on surface\(^ {340}\). It has been found that teichoic acids interact with vancomycin\(^ {307}\), and it is possible that the teichoic or lipoteichoic acids present on the bacterial cell surface prevent effective PS-ODN entrance into the bacterial cell. The mode of action of both zoocin A and penicillin results in the destruction of
peptidoglycan, and as more peptidoglycan is lost from the cell surface, so the levels of surface proteins will be decreased. It is possible that the indirect loss of surface proteins such as teichoic acids from the cell surface, rather than the direct loss of peptidoglycan due to the action of zoocin A or peptidoglycan is responsible for the increase of PS-ODN entrance into treated S. mutans.

Whilst the use of antisense as a targeted antimicrobial is still in the initial investigation stages, results from this study and others indicate that the ability of antisense to specifically target specific genes provides this technology with a clear advantage over standard antibiotics. The ability to target specific sequences provides the AS-ODN, PNA or PMO an ability to affect gene expression only in targeted bacteria strains, unlike antibiotics, which select due to cell wall composition.

Currently there is no gold standard method available for the delivery of antisense into bacterial cells and each delivery mechanism must be tailored for the chemistry of the AS-ODN, PNA or PMO it will be used with and the bacterial strain it is targeting. In this study, due to the presence of the cell wall peptidoglycan barrier (and/or the possible interaction of cell surface proteins with the PS-ODN), PS-ODN alone were found to be unable to exert any inhibitory effect upon target site containing bacterial strains. When combined with the permeabilising and peptidoglycan destroying agent’s zoocin A or penicillin, PS-ODN were able to gain entrance to lag phase S. mutans OMZ175 in a dose-dependent manner, and affect target gene expression. Zoocin A also permitted the entrance of PS-ODN to exponential phase S. mutans OMZ175, and the combination of zoocin A + SM-FBA also affected target gene expression. The entrance of the PS-ODN was able to be monitored through the use of a radio-label which enabled the molar concentration of PS-ODN inside the bacterial cell to be determined, and this technique was found to be effective for measuring the delivery potential of both zoocin A and penicillin. During this study a RT-qPCR method to determine target gene transcript levels and a method to radiolabel PS-ODN in order to determine and quantify PS-ODN delivery into S. mutans OMZ175 were developed. Various molecules with possible PS-ODN delivery potential were also studied for their ability to deliver PS-ODN. The three experimental objectives of this study were therefore achieved, and the hypothesis of this
study which proposed that antisense molecules can be used to inhibit the growth of Gram-positive \textit{S. mutans} OMZ1755 was proved correct.

\section*{6.22 Future directions}

It would have been preferable if the protoplasting protocol used in this study had been successful, allowing the determination of whether the \textit{S. mutans} OMZ175 bacterial membrane acted as a barrier to PS-ODN entrance into the bacterial cell. However RT-qPCR and growth results in this study illustrate that even if the membrane does provide a barrier to PS-ODN entrance, a concentration of PS-ODN able to cause significant levels of inhibition was able to enter the bacterial cell and disrupt target RNA transcription if the peptidoglycan layer was disrupted by the presence of zoocin A or penicillin. It would be interesting to combine the use of cationic carrier peptides together with zoocin A or penicillin, in order to determine if the combined use of both bacterial peptidoglycan and membrane permeating agents results in greater PS-ODN efficacy. Such a method could be used to test further bacterial strains and antisense chemistries. If a successful protoplasting method could be developed, \textit{in vitro} this would mean that the need for delivery agents is no longer required. Without cell wall barrier, researchers could easily test different antisense chemistries and target sites in the protoplasted bacteria. This would simplify gene function and expression studies, and provide a method by which potential AS-ODN, PNA or PMO target genes could easily be evaluated for their ability to effect bacterial metabolism etc.

In future, further qPCR experiments could be included in order to determine the minimum concentration of $\gamma^{32}$ P-ATS2 molecules required to produce a significant inhibitory effect upon \textit{fba} expression. If protoplasting protocols improve, it would be possible to determine how many $\gamma^{32}$ P-ATS2 molecules are able to reach the \textit{S. mutans} OMZ75 cell cytoplasm for those cells treated simply with $\gamma^{32}$ P-ATS2, and to determine which layer of the bacterial cell wall, teichoic acids, peptidoglycan, or membrane acts as the major barrier preventing their entry. The results gained in this study demonstrate that for \textit{S. mutans} OMZ175, the major barrier was either the teichoic acids or peptidoglycan layers, as both of these are effected by
the presence of zoocin A, rather than the bacterial membrane, which zoocin A is not known to interfere with.

In order to determine whether PS-ODN entry into the treated *S. mutans* OMZ175 cell is due to the direct effect of zoocin A upon the peptidoglycan layer, or the indirect effect upon cell surface teichoic acids, teichoic acids could be purified and their ability to interact with PS-ODN determined. Rate of PS-ODN entry into mutant strains which do not possess teichoic acids present on their cell surface could be compared to rates of entry into strains possessing teichoic acids. If the peptidoglycan layer for each cell type is intact, and PS-ODN show greater entrance into those mutant strains which do not possess teichoic acids upon their cell surface, this would indicate that teichoic acids play an important role in determining AS-ODN, PNA or PMO entrance. It might also be possible to remove all teichoic acids from the bacterial cell surface using a proteolytic enzyme such as trypsin, leaving the peptidoglycan layer intact.

It is unlikely that the encapsulation of AS-ODN, PNA or PMO within liposomes would result in increased permeation through the bacterial peptidoglycan layer. Liposomes consist of one or more concentric lipid bilayers which enclose an internal aqueous volume and help to avoid immune reactions developing towards the PS-ODN chemistry. It is possible that liposome encapsulated AS-ODN, PNA or PMO is able to cause greater inhibitory effects compared to that of AS-ODN, PNA or PMO alone due to both increased membrane penetration, together with the shielding effects of the liposome itself. The liposomes may prevent the AS-ODN, PNA or PMO charge from interacting with the bacterial cell surface proteins, which possibly prevent un-encapsulated AS-ODN, PNA or PMO from diffusing through the peptidoglycan layer. Further research as to whether for each bacterial strain the membrane, the peptidoglycan, or the cell surface molecules such as teichoic acids act as the major inhibitor of AS-ODN, PNA or PMO penetration could also help to shed further light as to why different AS-ODN, PNA or PMO delivery systems produce different results for different bacterial strains. If the specific cell components preventing AS-ODN, PNA or PMO diffusion could be identified for each bacterial strain, this would lead to new delivery systems allowing greater efficacy to be developed.
This study demonstrated that nisin is not suitable for use in combination with PS-ODN. It is believed that this may be due to the electrostatic interaction between the positively charged nisin molecule, and the negatively charged PS-ODN. To determine this, this experiment could be repeated with the use of neutral antisense molecules such as LNA’s or PNA’s. If interaction with nisin is dependent upon charge, these neutral molecules should not interact with the nisin molecule. The use of radiolabelled nisin molecules could be used to give credibility to the theory that the positively charged nisin binds to negatively charged PS-ODN. When nisin binds to cells alone, nisin should be found at its site of action- the cell wall. A combination of nisin and negatively charged PS-ODN’s should result in nisin being found only in the media- if binding to the PS-ODN’s does indeed prevent nisin from been able to bind to and penetrate the cell wall.

PS-ODN’s are the most common form of antisense studied currently, due to their low immunogenicity. However, other antisense chemistry’s, such as LNA or PNA show higher levels of stability and are more resistant to degradation. However, due to their cost, these chemistries were not used for this study. The search for and development of novel, low cost, high stability antisense types are on-going. The strain of bacteria targeted must be taken into consideration when choosing which type of antisense chemistry to use. Whilst this study achieved targeted, temporary inhibition using PS-ODN targeted towards the FBA mRNA of S. mutans OMZ175, PS-ODN targeted towards the same target of S. aureus was unable to produce an inhibitory effect. If fact, S. aureus grew significantly faster in the presence of PS-ODN, a phenomenon which has been noted previously (Chau 2008). It is likely that the DNase production by S. aureus led to the degradation of the PS-ODN molecules which S. aureus subsequently metabolised. Bai et al. 2012 and Meng et al. 2009 were able to achieve targeted, temporary inhibition of S. aureus using PNA’s targeted towards essential genes. It is likely that the unique chemistry of the PNA’s renders them resistant to DNase attack. However, if PNA’s had been used in this study it is likely that a far greater concentration of PNA would have been required compared to that required of PS-ODN’s. This is due to the fact that PS-ODN’s recruit RNase H, which can allow the PS-ODN to be ‘recycled’ within the cell and bind to further target sites. PNA’s however are unable to be ‘recycled’ within the cell, and thus a greater concentration would be required to produce a similar inhibitory effect.
The choice of antisense target remains a key issue concerning the use of AS-ODN, PNA or PMO as an antimicrobial. The high degree of target specificity means that gene sequences are able to be targeted which are unique to a single bacterial species, or ubiquitous throughout a bacterial genus. The need to prevent the loss of ‘good’ bacterial microflora requires that the gene sequences chosen as the antisense target site are found only in ‘bad’ pathogenic bacterial strains. It must also be remembered that the AS-ODN, PNA or PMO target site must not be present within the human genome, in order to prevent any possible unspecific binding to human gene sequences.

More work is clearly required before antisense technology becomes more widespread. The limiting factor to their use in bacteria is the need for appropriate delivery solutions to allow the AS-ODN, PNA or PMO to gain access to the bacterial cell interior. Zoocin A has a narrow spectrum range, and penicillin, whilst approved for in vivo use, seems only to be able to cause a ‘permeabilising’ effect under certain situations. The use of the radiolabel technique used in this study could be used to compare various delivery systems, and determine which delivery mechanism provides the greatest amount of AS-ODN, PNA or PMO delivery into the bacterial cell under study. Until an antisense delivery system has been fully developed, tested and found to be both effective and safe, in vivo use of AS-ODN, PNA or PMO to treat an in vivo bacterial infection remains unlikely. Topical applications of AS-ODN, PNA or PMO are most likely to be the first developed, as shown by the first use of antisense as a topical application to treat ocular CMV infection. Zoocin A remains a possible agent for use against dental cavity causing strains[^194], both alone and together with PS-ODN, whilst penicillin may have the potential to be used as a in vivo delivery system under certain circumstances. As well as highly selective therapeutic agents, AS-ODN, PNA or PMO oligonucleotides also show a great potential as a molecular biology investigative tool.
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Appendix 1

Stock solutions
Filter sterilised stock solutions:

Unless otherwise specified, the following stock solutions were prepared in RO water, filter sterilised using a 0.22 μm pore size filter (Millipore Corporation, Bedford, MA, USA) and stored at -20°C until use.

2-Phosphophenylpyruvate (100 mg/ml)

α-keto-glutarate (146.11 MW) (250 mg/ml)

Ampicillin (349.10 MW) (100 mg/ml)

Ethidium bromide (394.31 MW) (10 mg/ml)

The stock solution was stored in the dark at 4°C.

Hexaglycine (360.32 MW) (12.5 mg/ml)

Isopropyl-β-D-thiogalactoside (238.2 MW) (0.1 M)

(IPTG, Roche Molecular Biochemicals, Mannheim, Germany)

Kanamycin (483.51 MW) (25 mg/ml)

Lauricidin (274.4 MW)
Lauricidin (Seolim Corporation, Seoul, Korea) was dissolved in 95% ethanol to form stock solution with a concentration of 100 mg/ml.

*L-glutamic acid* (147.13 MW) (125 mg/ml)

*L-glycine* (157.19 MW) (250 mg/ml)

*Lysostaphin* (500 U/mgP) (100 mg/ml)

*Lysozyme* (14.3 kDa) (100 mg/ml)

*Mutanolysin* (23 kDa) (200 mg/ml)

*Nicotinamide* (122.12 MW) (10 mg/ml)

*Nisin* (Nisaplin®) (3354.07 MW)

Nisin (2.5%, balanced with sodium chloride and denatured milk solids) was mixed with 0.05% of HCl to form a stock solution of 100 mg/ml.

*P-amino benzoic acid* (312.28 MW) (10 mg/ml)

*Pediocin* (16.5 kDa)

Pediocin PA-1 was dissolved in a 100 mM sodium acetate solution to form a stock solution of 0.5 mg/ml.
Penicillin (356.37 MW) (200 mg/ml)

Peptone water

1 g Bacto peptone (DIFCO, Fort Richard Laboratories Ltd, Auckland, New Zealand) and 0.5 g Sodium chloride (Scharlau) were dissolved in 1L R/O water.

Pyruvate (110.04 MW) (250 mg/ml)

Riboflavin (376.36 MW) (10 mg/ml)

Streptomycin (728.69 MW) (100 mg/ml)

Sodium citrate (294.10 MW) (250 mg/ml)

Succinate acid (867.61 MW)(125 mg/ml)

Sucrose (342.30 MW) (250 mg/ml)

Thiamine-HCl (337.27 MW) (10 mg/ml)

Urea (60.06 MW) (0.5 M)

Urea (Bio-Rad Laboratories, Hercules, CA, USA)
Vancomycin (1485.71 MW) (200 mg/ml)

Yeast extract (20%)  
10 g of yeast extract (DIFCO, Fort Richard Ltd.) was dissolved in 50 ml of R/O water and autoclaved.

Other stock solutions:

Unless otherwise specified the following stock solutions were prepared in RO water, sterilised by autoclaving (15 minutes/ 121°C) and stored at RT until use.

Ethylenediaminetetraacetic acid, (292.24 MW) pH 8.0 (0.5 M)

Magnesium chloride (95.21 MW) (2 M)

Phosphate buffered saline

Phosphate buffered saline (PBS) consisted of 140 mM NaCl (58.44 MW) (Scharlau Chemie, S.A., La Jota, Barcelona, Spain), 1 mM KH₂PO₄ (136.09 MW) (BDH), 10 mM Na₂HPO₄ (141.96 MW) and 3 mM KCl (74.55 MW) dissolved in RO water. The pH was adjusted to 7.2 and the solution autoclaved.

Potassium dihydrogen orthophosphate (136.08 MW) (1 M)

Potassium dihydrogen orthophosphate (British Drug Houses Chemicals Ltd, (B.D.H) Laboratory, Chemicals Division, Poole, Dorset, U.K.).
**Di-potassium hydrogen orthophosphate** (174.2 MW) (1 M)

Di-potassium hydrogen orthophosphate (BDH).

**1 M Potassium phosphate buffer**

One millilitre amounts of 1 M potassium dihydrogen orthophosphate (see above) was added slowly to the 200 ml 1 M di-potassium hydrogen orthophosphate solution (see above), whilst the pH of the solution was monitored using an MP220 pH meter (Mettler Toledo, Greifensee, Switzerland). The pH was adjusted to 7.22, and the solution autoclaved.

**Sodium acetate** (82.03 MW) (0.2 M)

**5 M Sodium chloride** (58.44 MW) (pH 4.6) (5 M)

Sodium chloride (Scharlau)

**30% Sodium hydroxide**

NaOH (39.997 MW) (Merck) (6.0 g) was dissolved in MQ water to a final volume of 20 ml.

**Antisense stocks**

PS-ODN stock (125 OD) was sourced from Sangon, Shanghai, China and prepared according to the manufacturer’s instructions. All antisense stocks were stored at -20°C.

**Radioactive labelling stocks**
T4 polynucleotide kinase and its buffer were sourced from New England Biolabs (New England Biolabs, Ipswitch, Massachusetts, USA). The radioactive isotope $\gamma^{32}$P (3000 Ci/mmole, 10 mCi/ml, 250 Ci) was sourced from Perkin Elmer (Perkin Elmer, Waltham, Massachusetts, USA). Fresh isotope was ordered for each experiment due to the 14 day half-life of $\gamma^{32}$P.

**Protoplasting buffer agents:**

*50% Sucrose solution*

For use in the protoplasting solution the sucrose solution was made up as a stock solution at 50% (w/v) in RO water and sterilised by autoclaving.

*Pepstatin A (685.89 MW)*

Pepstatin A was dissolved in methanol to form a stock solution with a concentration of 10 mM and stored at -20°C.

*Phenylmethyl sulfonyl fluoride (PMSF) (174.19 MW)*

PMSF was dissolved in isopropanol to form a stock solution with a concentration of 10 mM and stored at -20°C.

*Benzamidine (156.61 MW)*

Benzamidine was dissolved in MQ water to form a stock solution with a concentration of 1 M and stored at -20°C.

*Iodoacetic acid (185.95 MW)*
Iodoacetic acid was dissolved in 1 M sodium hydroxide (NaOH) to form a concentration of 1 M. This solution was made fresh for every day of use.

*Potassium Phosphate* (136.09 MW)

Potassium phosphate (KPO₄) was dissolved 900 mls MQ water to form a stock solution with a concentration of 1 M. The pH was adjusted to 6.2 and the final volume adjusted to 1 L using MQ water.

*Protoplasting Buffer*

The protoplasting buffer was adopted from Kling et al., 1999. In this study the protoplasting buffer consisted of 40% sucrose, 0.1 M potassium phosphate, 0.029 M pepstatin A, 1.25 mM benzamidine, 0.05 mM PMSF and 1.25 mM iodoacetic acid.

*Osmotic stabilising agents:*

For use in the preparation of both buffers and agar the sucrose and raffinose were both made up as stock solutions at 60% (w/v) in RO water and sterilised by autoclaving.

*Hypotonic buffer*

Both the hypotonic and hypertonic buffers used were adopted from Calandra, Nugent and Cole. In this study the hypotonic buffer used consisted of 0.85% (w/v) NaCl, 0.001 M 2-mercaptoethanol (78.13 MW) and 0.25 M Tris-HCl (pH 8). This was sterilised by autoclaving.

*Hypertonic buffer*
This consisted of 20% (v/v) hypotonic buffer, 0.1 M magnesium chloride (95.21 MW) and 30% (w/v) sucrose or raffinose. It was found necessary to prepare the magnesium chloride as a 2M stock solution, because the complete buffer was autoclaved a precipitate formed.

**DNA gel electrophoresis:**

**50x Tris-acetate EDTA buffer, pH 8.0 (TAE)**

Tris base (Applichem gmbH, Darmstadt, Germany) (121.14 MW) (24.2 g) was dissolved in 60 ml of MQ water. Glacial acetic acid (60.05 MW) (5.71 ml) and 10 ml 0.5 M EDTA (pH 8.0) were added, and the volume made up to 100 ml with MQ water.

**Agarose gel**

Agarose (SeaKem LE agarose, Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) was added to 90 ml of 1 x TAE buffer to form a 1 or 2% solution. Gels were prepared as required.

**Tracking dye**

Bromophenol blue (669.96 MW) (0.25%), 0.025 g xylene cyanol (538.61 MW) and 1.5 g ficoll 400 were dissolved in 10 ml RO water.

**SDS-PAGE and polyacrylamide gel casting reagents:**

**30% acrylamide/bis solution**
Acrylamide/N,N’-methylene-bis-acrylamide premix (40%) (Bio-Rad Laboratories Hercules, CA, USA) (50 g) was dissolved in MQ water to a final volume of 100 ml. The solution was stored at 4°C for up to one month.

1.5 M Tris-HCl, (121.14 MW) pH 8.8

Tris base (Applichem) (18.17 g) was dissolved in MQ water. The pH was adjusted to 8.8 with 1 M HCl and the volume made up to 100 ml with MQ water. The solution was stored at 4°C.

0.5 M Tris-HCl, (121.14 MW) pH 6.8

Tris base (Applichem) (6.0 g) was dissolved in MQ water. The pH was adjusted to pH 6.8 with 1 M HCl and the volume made up to 100 ml with MQ water. The solution was stored at 4°C.

10% SDS (288.372 MW)

Sodium dodecylsulphate (SDS) (BDH) (10 g) was dissolved in MQ water to a final volume of 100 ml.

10% Ammonium persulphate (228.18 MW)

Ammonium persulphate (APS) (Bio-Rad) (100 mg) was dissolved in MQ water to a final volume of 1 ml. The solution was made fresh as required.

2x SDS-PAGE sample buffer
SDS (BDH) (2.0 g), 0.5 g Sucrose (Bio-Rad) and 0.2 ml bromophenol blue were dissolved in 10 ml 0.5 M Tis-HCl (pH 6.8) and made up to a final volume of 50 ml. Before each use, 100 μl β-mercaptoethanol was mixed with 900 μl of the above solution.

Running buffer
Tris base (Applichem) (6.0 g), 28.8 g glycine (75.07 MW) and 2.0 g SDS were dissolved in 2 L MQ water.

Expression and purification of recombinant protein:

Lysis buffer
Lysis buffer solution consisted of 50 mM NaH$_2$PO$_4$ (119.98 MW) (BDH), 300 mM NaCl (Scharlau) and 10 mM imidazole (68.08 MW) dissolved in MQ water. The pH was adjusted to 8.0 and the solution was autoclaved.

Wash buffer
Wash buffer solution consisted of 50 mM NaH$_2$PO$_4$ (BDH), 300 mM NaCl (Scharlau) and 20 mM imidazole dissolved in MQ water. The pH was adjusted to 8.0 and the solution was autoclaved.

Elution buffer
Elution buffer solution consisted of 50 mM NaH$_2$PO$_4$ (BDH), 300 mM NaCl (Scharlau) and 250 mM imidazole dissolved in MQ water. The pH was adjusted to 8.0 and the solution was autoclaved.
Media

Commercial media used in this study were prepared according to the manufacturer’s specifications, and sterilised by autoclaving: Todd-Hewitt broth (THB), Todd-Hewitt agar (THA), Columbia blood agar base (CAB), Brain Heart Infusion broth (BHI), M17 broth (M17), Mueller Hinton broth (MHB), (DIFCO, Fort Richard Laboratories Ltd.). CAB plates containing antibiotics were prepared by supplementing CAB with 100 μg/ml rifampicin (CAB + Rif) or 100 μg/ml streptomycin (CAB + Strep). Blood agar (BA) was prepared by adding 5% (v/v) defibrinated sheep blood (New Zealand Venous Supplies, Tuakau, New Zealand) to CAB. LB agar was prepared by the addition of 14 g agar to 1 L of LB. Agar plates were stored at 4°C for a period of up to two weeks before use.

Todd-Hewitt Agar (THA)

Todd-Hewitt agar consisted of 3% (w/v) THB with either 1.5% (w/v) Davis Bacteriological Agar, (DIFCO) when used for pour plates, or 0.5% (w/v) Davis agar, when used as a soft agar overlay. The media was sterilised by autoclaving.

Reversion media (RM)

The medium used was adopted from that of Wyrick and Rogers (1973), Fodor, Hadlaczky and Alfoldi (1975) and Simmonds (1978)111,309,359. The effectiveness of this medium relies on the presence of an osmotic stabilising agent to prevent protoplast lysis and to assist their reversion to complete cell walled state. Initially sucrose was used as the osmotic stabilising agent, but due to its apparent inability to allow revertants, raffinose (Merck) was substituted in at a later date. RM stabilised with sucrose is referred to as RMS and with raffinose as RMR. The media consisted of 3% (w/v) THB, 1% (w/v) proteose peptone number three, 0.3% (w/v) NaCl, 10% (v/v) sterile horse serum, 0.5% or 1.5% (w/v) Davis agar for soft agar overlays and pour plates respectively, and 20% or 15% (w/v), (sucrose or raffinose) for soft agar overlays and pour plates respectively. Both the sterile horse serum and either
the sucrose or raffinose were added to the basic medium held at 50°C after it had been sterilised by autoclaving.
Appendix 2

PCR Primer sequences and target sites used in this study
Table 1: PCR Primer sequence and target sites used in this study

<table>
<thead>
<tr>
<th>Primer Designation</th>
<th>Sequence</th>
<th>Target site/ Description or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S fwd</td>
<td>GGACTAHAAGGGTATCTAAT</td>
<td>Weisburg et al., 1991$^{351}$</td>
</tr>
<tr>
<td>16S rev</td>
<td>AGAGTTTGATCMTGG</td>
<td>Weisburg et al., 1991</td>
</tr>
</tbody>
</table>

PCR Primer sequence and target sites for 16s rRNA, *fba* or *gyr A* of *S. mutans* UA159

<table>
<thead>
<tr>
<th>Fwd 16sRNA 01</th>
<th>CGCCGCGTGAGTGAAGAAGAG</th>
<th>Binds 405 to 420 bp downstream of the ATG of 16s RNA gene of <em>S. mutans</em> OMZ175</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rev 16s RNA 01</td>
<td>GCGGCTGGCCCCCTAAAAG</td>
<td>Binds 1456 to 1473 bp downstream of the ATG of 16s RNA gene of <em>S. mutans</em> OMZ175</td>
</tr>
<tr>
<td>Primer Set</td>
<td>Oligonucleotide Sequence</td>
<td>Binds Range</td>
</tr>
<tr>
<td>------------</td>
<td>--------------------------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>Fwd 16s RNA 02</td>
<td>AGACACGGCCCAGACTCCTACG</td>
<td>332 to 353 bp downstream of the ATG</td>
</tr>
<tr>
<td>Rev 16s RNA 02</td>
<td>TCCAGCCGCACCTTCGATAC</td>
<td>1519 to 1539 bp downstream of the ATG</td>
</tr>
<tr>
<td>Fwd 16s RNA 03</td>
<td>AGACACGGCCCAGACTCCTACG</td>
<td>332 to 353 bp downstream of the ATG</td>
</tr>
<tr>
<td>Rev 16s RNA 03</td>
<td>ACCCAATCATCCATCCCACCTTA</td>
<td>1480 to 1498 bp downstream of the ATG</td>
</tr>
<tr>
<td>Fwd FBA 01</td>
<td>GTAGAATCAATGGGAATCACTGTA</td>
<td>649 to 672 bp downstream of the ATG</td>
</tr>
<tr>
<td>Rev FBA 01</td>
<td>TCTTCAACCCTTCTGTAATAG</td>
<td>37 to 59 bp downstream of the ATG</td>
</tr>
<tr>
<td>Fwd FBA 02</td>
<td>GTAGAATCAATGGGAATCACTGTA</td>
<td>649 to 672 bp downstream of the ATG</td>
</tr>
<tr>
<td>Rev FBA 02</td>
<td>TTCTTCAACCCTTCTGTAATAG</td>
<td>37 to 59 bp downstream of the ATG</td>
</tr>
<tr>
<td>Fwd FBA 03</td>
<td>GTAGAATCAATGGGAATCACTGTA</td>
<td>649 to 672 bp downstream of the ATG</td>
</tr>
<tr>
<td>Rev FBA 03</td>
<td>TTCTTCAACCCTTCTGTAATAG</td>
<td>37 to 59 bp downstream of the ATG</td>
</tr>
<tr>
<td>DNA Sequence</td>
<td>Target Site</td>
<td>Description</td>
</tr>
<tr>
<td>-------------------</td>
<td>------------------------------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>Fwd GyrA 01</td>
<td>TTTGCAGTTCGGCCGTATTCAGG</td>
<td>Binds 1751 to 1773 bp downstream of the ATG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>of GyrA gene of <em>S. mutans</em> OMZ175</td>
</tr>
<tr>
<td>Rev GyrA 01</td>
<td>GGCCCCGATTTCACAACAGG</td>
<td>Binds 649 to 669 bp downstream of the ATG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>of GyrA gene of <em>S. mutans</em> OMZ175</td>
</tr>
<tr>
<td>Fwd GyrA 02</td>
<td>TTTAGCGGTGATATTGACAGTTTT</td>
<td>Binds 1337 to 1361 bp downstream of the ATG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>of GyrA gene of <em>S. mutans</em> OMZ175</td>
</tr>
<tr>
<td>Rev GyrA 02</td>
<td>TCCGCAATAGTGAGACAGATACC</td>
<td>Binds 2042 to 2065 bp downstream of the ATG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>of GyrA gene of <em>S. mutans</em> OMZ175</td>
</tr>
<tr>
<td>Fwd GyrA 03</td>
<td>CAGCGCTTTGAGACCCATTGGTA</td>
<td>Binds 1932 to 1954 bp downstream of the ATG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>of GyrA gene of <em>S. mutans</em> OMZ175</td>
</tr>
<tr>
<td>Rev GyrA 03</td>
<td>TTTATTATTGAAGTCCGCGTGAT</td>
<td>Binds 913 to 937 bp downstream of the ATG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>of GyrA gene of <em>S. mutans</em> OMZ175</td>
</tr>
</tbody>
</table>

qPCR primer sequences and target sites and sequences for *S. mutans* OMZ175

<table>
<thead>
<tr>
<th>DNA Sequence</th>
<th>Target Site</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBA RT-PCR Fwd A</td>
<td>GAACCGCGGTGAAGTACGA</td>
<td>Binds 614-633 bp downstream of the FBA ATG start</td>
</tr>
<tr>
<td></td>
<td></td>
<td>codon.</td>
</tr>
<tr>
<td>FBA RT-PCR Rev A</td>
<td>CATGGACCATACCCAGCTAATG</td>
<td>Binds 694-713 bp downstream of the FBA ATG start</td>
</tr>
<tr>
<td></td>
<td></td>
<td>codon.</td>
</tr>
<tr>
<td>FBA RT-PCR Fwd B</td>
<td>CGTCACCAATGATACCGTCTTC</td>
<td>Binds 534-556 downstream of the FBA ATG start</td>
</tr>
<tr>
<td></td>
<td></td>
<td>codon.</td>
</tr>
<tr>
<td>Primer Set</td>
<td>Sequence</td>
<td>Binds</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------------------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>FBA RT-PCR Rev B</td>
<td>AAAGGCTCGCAAGTTTTGTA</td>
<td>613-635 downstream of the FBA ATG start codon</td>
</tr>
<tr>
<td>16s rRNA RT-PCR Fwd A</td>
<td>TCGGTTAGCTCCGTCATA</td>
<td>849-877 bp downstream of the 16s RNA ATG start codon</td>
</tr>
<tr>
<td>16s rRNA RT-PCR Rev A</td>
<td>CGTGGGTCAGCGAACAGATT</td>
<td>757-777 downstream of the 16s RNA ATG start codon</td>
</tr>
<tr>
<td>16s rRNA RT-PCR Fwd B</td>
<td>TTCATGGAGGCGAGTTGCA</td>
<td>1322-1340 downstream of the 16s RNA ATG start codon</td>
</tr>
<tr>
<td>16s rRNA RT-PCR Rev B</td>
<td>GCTACAATGGTCGGAACAGGA</td>
<td>1240 downstream of the 16s RNA ATG start codon</td>
</tr>
<tr>
<td>GyrA RT-PCR Fwd A</td>
<td>GACGCAGGCGCATATCAAG</td>
<td>1182-1200 bp downstream of the GyrA ATG start codon</td>
</tr>
<tr>
<td>GyrA RT-PCR Rev A</td>
<td>CCGCAATAGTGAGACAGATACCAT</td>
<td>1259-1282 bp downstream of the GyrA ATG start codon</td>
</tr>
<tr>
<td>GyrA RT-PCR Fwd B</td>
<td>TTGTGATAACACGTCTGGTTAGC</td>
<td>1221-1246 downstream of the GyrA ATG</td>
</tr>
<tr>
<td>GyrA RT-PCR Rev B</td>
<td>CCGGCTTTGGAACGTGATAA</td>
<td>1304-1322 downstream of the GyrA ATG</td>
</tr>
</tbody>
</table>
### E. faecalis AR01/DGVS fba PCR primers and target sites and sequences

<table>
<thead>
<tr>
<th>Primer Designation</th>
<th>Sequence</th>
<th>Binding site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fwd: FBA1f</td>
<td>GCCAGTAGTATCAGGAGCAGAAT</td>
<td>Binds 2 bp downstream of the fba start codon</td>
</tr>
<tr>
<td>Rev: FBA1r</td>
<td>GAACCGAACCAATCAATGTGT</td>
<td>Binds 840 bp downstream of the fba start codon</td>
</tr>
<tr>
<td>Fwd: FBA2f</td>
<td>AGCACGTAAAGGCGGGGTATG</td>
<td>Binds 37 bp upstream of the fba start codon</td>
</tr>
<tr>
<td>Rev: FBA2r</td>
<td>GCGTTGCTTTTAGCGAATGAT</td>
<td>Binds 720 bp downstream of the fba start codon</td>
</tr>
<tr>
<td>Fwd: FBA3f</td>
<td>ATGAAGATGAACCAACGGATTTTT</td>
<td>Binds 300 bp upstream of the fba start codon</td>
</tr>
<tr>
<td>Rev: FBA3r</td>
<td>TAGTTACCCGCCAGATTTATG</td>
<td>Binds 134 bp downstream of the fba start codon</td>
</tr>
<tr>
<td>Fwd: FBA4f</td>
<td>GTCTTAACGGGAAAACCTGGAG</td>
<td>Binds 179 bp upstream of the fba start codon</td>
</tr>
<tr>
<td>Rev: FBA4r</td>
<td>ATAGTGTCAGGTCATCGTAAAGT</td>
<td>Binds 232 bp downstream of the fba start codon</td>
</tr>
</tbody>
</table>

^ A binding site is the target nucleotide to which the 5’nucleotide of the primer binds
<table>
<thead>
<tr>
<th>Construct</th>
<th>Annealing Temperature</th>
<th>Extension Time (Seconds)(^{a})</th>
<th>Primer 1 (10 μM)</th>
<th>Primer 2 (10 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. mutans</em> FBA 1</td>
<td>47</td>
<td>80</td>
<td>Fwd FBA 01</td>
<td>Rev FBA 01</td>
</tr>
<tr>
<td><em>S. mutans</em> FBA 2</td>
<td>45</td>
<td>80</td>
<td>Fwd FBA 02</td>
<td>Rev FBA 02</td>
</tr>
<tr>
<td><em>S. mutans</em> FBA 3</td>
<td>47</td>
<td>80</td>
<td>Fwd FBA 03</td>
<td>Rev FBA 03</td>
</tr>
<tr>
<td><em>S. mutans</em> 16s RNA 1</td>
<td>50</td>
<td>80</td>
<td>Fwd 16sRNA 01</td>
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<tr>
<td><em>S. mutans</em> 16s RNA 2</td>
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<td>80</td>
<td>Fwd 16sRNA 02</td>
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<td><em>S. mutans</em> 16s RNA 3</td>
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<td>Fwd 16sRNA 03</td>
<td>Rev 16sRNA 03</td>
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<tr>
<td><em>S. mutans</em> GyrA 1</td>
<td>50</td>
<td>80</td>
<td>Fwd GyrA 01</td>
<td>Rev GyrA 01</td>
</tr>
<tr>
<td><em>S. mutans</em> GyrA 2</td>
<td>47</td>
<td>80</td>
<td>Fwd GyrA 02</td>
<td>Rev GyrA 02</td>
</tr>
<tr>
<td><em>S. mutans</em> GyrA 3</td>
<td>47</td>
<td>80</td>
<td>Fwd GyrA 03</td>
<td>Rev GyrA 03</td>
</tr>
</tbody>
</table>

\(^{a}\)Calculated as 20 seconds per Kb for predicted product size