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BACTERIAL GENE EXPRESSION INHIBITION WITH ANTISENSE PEPTIDE NUCLEIC ACIDS

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"Theory is when you know everything and nothing works
Practise is when everything works and nobody knows why
In here theory and practise is joined
Nothing works and nobody knows why"

-Unknown

ABSTRACT

Regulated gene expression is widely useful for gene function studies and as a means to control genes important in disease. There are many methods for regulating the expression of genes, and one approach is to use antisense oligonucleotides that bind complementary mRNAs to inhibit translation. A number of nucleic acid analogues and mimics have been developed to improve antisense efficiency.

Peptide nucleic acid (PNA) is a DNA mimic with many interesting properties. PNA has proven effective in a variety of applications and mRNA has been a frequent target with the aim to silence gene expression in a sequence specific manner. However, mRNA targeting antisense PNA has almost exclusively been used on eukaryotic cells and relatively little is known about inhibition of bacterial gene expression.

In this thesis antisense PNA is used to target bacterial genes. The focus is on design features such as antisense length and uptake and also on selection of appropriate target sites and target genes. Moreover, practical applications for antisense PNAs are examined including their use as inhibitors of essential bacterial genes.

In terms of design features, the results show that antisense effects are much improved when a cell penetrating peptide is conjugated to the PNA. Also, relatively short molecules show higher efficiency. It is further demonstrated that the ribosome binding site is most likely to be susceptible to antisense inhibition and that antisense PNA directed to genes within operons may strongly affect the expression of cotranscribed genes.

In terms of practical applications it was observed that an antisense PNA directed to an essential bacterial gene inhibited growth and was bactericidal. Furthermore, the PNA was able to rescue a human cell culture from infection and select for PNA resistant bacteria in a mixed population, indicating that efficient killing effects can be both cell type and strain specific. Also, growth inhibitory properties of antisense PNAs were examined in combination with conventional antimicrobial drugs. Combinations of mRNA specific antisense PNAs and protein specific drugs with shared genetic targets commonly displayed antimicrobial synergy, suggesting that antisense agents can be used to potentiate conventional antimicrobial drugs.

In summary, the results described here provide guidelines for the design of sequence specific antisense PNAs and demonstrate practical uses for antisense agents as inhibitors of bacterial genes.

LIST OF PUBLICATIONS

I. Good L, Awasthi SK, **Dryselius R**, Larsson O, Nielsen PE. Bactericidal antisense effects of peptide-PNA conjugates. Nature Biotechnol. 2001 Apr;19(4):360-4.

II. **Dryselius R**, Nekhotiaeva N, Nielsen PE, Good L. Antibiotic-free bacterial strain selection using antisense peptide nucleic acid. Biotechniques. 2003 Nov;35(5):1060-4.

III. **Dryselius R**, Awasthi SK, Rajarao GK, Nielsen PE, Good L. The translation start codon region is sensitive to antisense PNA inhibition in *Escherichia coli*. Oligonucleotides. 2003;13(6):427-33.

IV. **Dryselius R**, Nekhotiaeva N, Good L. Antimicrobial synergy between mRNA- and protein-level inhibitors. J Antimicrob Chemother. In press.

V. **Dryselius R**, Kulyté A, Nikravesh A, Good L. Polar effects of antisense PNAs in *Escherichia coli*. Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

2'MOE	2'-O-methoxy-ethyl RNA
2'OMe	2'-O-methyl RNA
ACP	acyl carrier protein
Ap^r	ampicillin resistance
CFU	colony forming unit
CPP	cell penetrating peptide
DNA	deoxyribonucleic acid
dsDNA/RNA	double stranded DNA/RNA
FIC	fractional inhibitory concentration
FICI	FIC index
LNA	locked nucleic acid
MIC	minimal inhibitory concentration
MP	methylphosphonates
mRNA	messenger RNA
ON	oligonucleotide
PCR	polymerase chain reaction
PMO	phosphorodiamidate morpholino oligomer
PNA	peptide nucleic acid
PS	phosphorothioates
RBS	ribosome binding site
RISC	RNA induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
rRNA	ribosomal RNA
SD	Shine-Dalgarno
siRNA	small interfering RNA
T_m	melting temperature
tRNA	transfer RNA
UTR	untranslated region

1 INTRODUCTION

Living organisms mainly store information as deoxyribonucleic acid (DNA). A cell may contain one or more double stranded DNA (dsDNA) molecules, usually packaged as chromosomes, which together constitute the genome of the organism. DNA has a chain-like structure composed of a large number of covalently linked building blocks called nucleotides. Nucleotides contain a phosphoric acid molecule, a sugar and either of four organic bases called adenine (A), cytosine (C), guanine (G) and thymine (T) (Figure 1). Limited stretches of such nucleotides constitute genes.

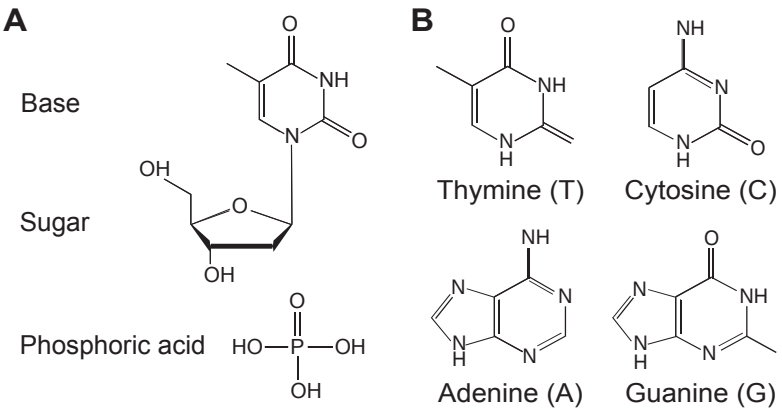


Figure 1. Composition of DNA monomers. A. A DNA nucleotide consists of three distinct parts; a phosphoric acid molecule, a sugar and an organic base (in this case a thymine). **B.** The four different organic bases in DNA are thymine (T), cytosine (C), adenine (A) and guanine (G).

A gene is usually assigned to one or more functions in a cell, although, it is not the gene itself that is the executor of the function(s). Instead, the dsDNA of the gene is transcribed into a single stranded ribonucleic acid (RNA) molecule which is like a complementary casting of the template DNA region. As for DNA, RNA consists of four nucleotides; however, in RNA the T nucleotides are replaced by uracils (U). Moreover, RNA molecules have a ribose sugar backbone instead of deoxyribose that is found in DNA (Figure 2). The RNA molecule is usually further translated into a chain of amino acids that constitute a protein. This process is called translation because it is the order and kind of nucleotides that determine the order and kind of amino

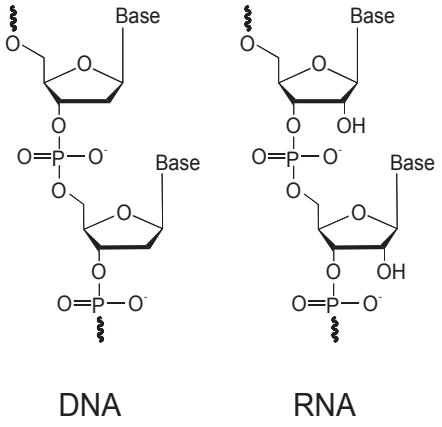


Figure 2. Chemical structure of DNA and RNA. The sugar ring in DNA is a deoxyribose whereas it is a ribose in RNA. Also, the organic base in DNA represents either A, C, G or T, whereas it in RNA represents A, C, G or uracil (U).

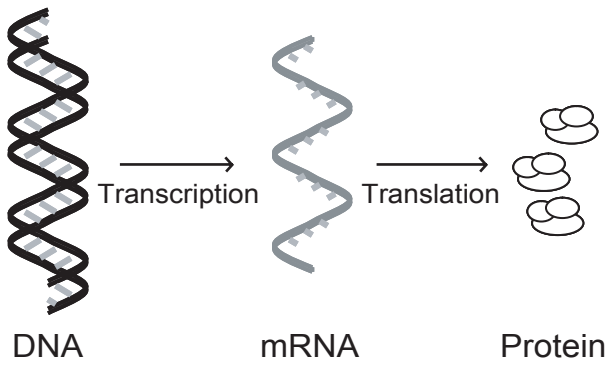
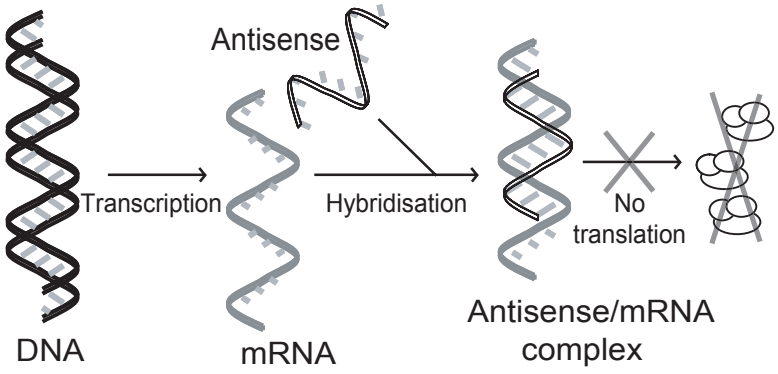


Figure 3. The Central Dogma as it was described in 1958. The unidirectional genetic flow is illustrated, where DNA is transcribed into a messenger RNA (mRNA), which is further translated into a protein.

acids in the protein product. Figure 3 summarises the unidirectional flow of information from DNA to protein presented by Crick in 1958 as "The Central Dogma" (1). Historically, only the protein in this series of events was ascribed the executive power of a specified function in the cell. However, as knowledge about cellular function advance, RNA molecules are assigned an increasing amount of important regulatory functions.

It is only recently that small non protein coding RNAs have been detected and recognised as important and diverse regulators of gene expression. Today, regulation by small RNAs is thought to be a common feature in all organisms and one important class is antisense RNA. In the typical case, an antisense RNA exerts its regulation by complementary hybridisation to a target RNA (a sense RNA). Hybridisation is due to interactions between the bases of the antisense and its target where A-nucleotides bind to U-nucleotides and G-nucleotides bind to C-nucleotides. For example, the RNA stretch 5'-GGUAUCCC-3' binds/hybridises to the complementary 3'-CCAUAGGG-5' stretch. The effects of such complementary binding are diverse; however, a common scenario for antisense regulation involves binding to an mRNA target molecule in a manner that leads to inhibition of translation (see Figure 4).

Figure 4. Model for antisense inhibition of translation. The antisense molecule interfere by hybridising to the mRNA. Antisense binding leads to inhibition of translation that results in that no protein is produced.



Knowledge about the regulatory properties of RNA has led to the development of a number of nucleic acid analogues and mimics designed to regulate gene expression. Such analogues and mimics, also called antisense agents, usually possess

higher stability and improved target binding properties than the naturally occurring antisense RNAs. Antisense agents can be designed to target and regulate any gene of choice, a property that makes them suitable both for studies of gene function and as regulators of disease causing genes. This thesis focuses on the nucleic acid mimic peptide nucleic acid (PNA) and how it can be used as an antisense agent to down-regulate gene expression in bacteria. Antisense properties and design features have been examined as well as practical applications for PNA as a gene specific inhibitor.

1.1 NATURAL ANTISENSE

In addition to transfer RNA (tRNA), ribosomal RNA (rRNA) and messenger RNA (mRNA) a great number of small noncoding RNAs are present in cells. Although it is thought that only a fraction of these have been detected and analysed, they are known to display a wealth of different regulatory properties (2-10). A major group of small noncoding RNAs are the antisense RNAs that execute their regulatory function through base pairing.

1.1.1 Antisense RNA

Antisense RNAs were first discovered in 1981 when two independent studies demonstrated antisense controlled regulation of copy numbers for the plasmids ColE1 (11) and R1 (12). Since these early findings many other antisense controlled mechanisms have been detected of which most regulate plasmids, transposones and other accessory elements (3, 10, 13). The first chromosomally expressed antisense RNA was detected by Mizuno and co-workers in 1984 (14). They found that a non-translated transcript (named micRNA for mRNA interfering complementary RNA) was involved in repression of *ompF* gene expression. The micRNA (later named *micF*) appeared to inhibit translation of the essential outer membrane protein OmpF by binding complementary to *ompF* mRNA. The cellular role of *micF* was shown to be as an inverse regulator to create a balanced expression between OmpF and another outer membrane protein, OmpC.

Following the identification and characterisation of *micF* as an antisense RNA, many other chromosomally encoded RNAs have been detected. One is DicF that binds to and silences the expression from *ftsZ* mRNA, thereby inhibiting cell division (3). Another example of an antisense RNA that inhibits translation is Spot 42, which negatively regulates *galK* expression from the *gal*-operon and thereby also decouples

the expression of GalK and GalE (15). Interestingly, some antisense RNAs are able to regulate the expression of two or more genes. One such example is *oxyS* that acts both with a direct antisense mechanism by blocking the ribosome binding site (RBS) of *flhA* (16) and indirectly on the sigma factor *rpsO* by binding to and sequestering its protein regulator Hfq (17). Another antisense RNA that regulates more than one gene is DsrA, which can act both as a repressor and an activator of gene expression. DsrA represses translation of the transcriptional modulator *hns* by blocking the RBS. Also, it activates *rpoS* mRNA translation by resolving a stemloop structure that otherwise renders the RBS inaccessible (18).

As can be understood from these few examples, antisense RNA mediated regulation is very diverse. Nevertheless, the majority of antisense RNAs that are known today act as selective gene silencers either by blocking translation or by inducing degradation of the target molecule. A more recently discovered kind of antisense mechanism is RNA interference (RNAi), where double stranded RNA (dsRNA) induces sequence specific gene silencing.

1.1.2 RNAi

RNAi was first described in the worm *Caenorhabditis elegans* by Fire and co-workers in 1998. They observed that long dsRNA targeted to mRNA from several genes were at least two orders of magnitude more potent in expression inhibition than was observed with either a sense or an antisense strand used alone (19). The mechanism behind this strong sequence specific effect has now been partly explained. The first step seems to involve processing of the dsRNA to shorter fragments of ~21-23 nucleotides, called short interfering RNAs (siRNAs). Further, the strands of the siRNAs are separated from each other and one is integrated into an RNA-induced silencing complex (RISC) (20). Finally, the target RNA is recognised and cleaved by the RNA loaded RISC in a sequence specific manner (21). The cellular role of this gene silencing mechanism is both as a regulator of endogenous gene expression (22), and as a defence against RNA viruses and transposable elements (23-25).

Although an interesting and widely applied technique for both gene functional studies and therapeutics development, RNAi is not directly applicable to bacteria as they lack the required enzymes. Therefore, RNAi will only be briefly discussed in this thesis.

1.2 ANTISENSE TECHNOLOGY USING NATURAL NUCLEIC ACIDS

The finding that antisense oligonucleotides (ONs) could regulate gene expression in a sequence specific manner gave hope for antisense applications in both gene functional studies and in the development of therapeutics that specifically silence expression from disease causing genes. Indeed, a first notion that small ONs could be used to specifically inhibit gene expression was presented by Zamecnik and Stephenson in 1978, who also suggested possible therapeutic applications (26, 27). However, their experiments were performed with a DNA ON *in vitro*.

It soon became apparent that externally added DNA and RNA ONs were largely inefficient *in vivo* as they suffer from both high nuclease susceptibility and relatively inefficient cellular uptake. As a consequence, *in vivo* antisense effects from DNA/RNA can only be expected to be very transient. However, for longer-term or even permanent antisense effects, vectors that express antisense RNA can be introduced into cells (28). Moreover, use of inducible regulation of the antisense transcripts produced by such vectors enable a tuneable inhibition that is especially useful in bioengineering for optimisation of protein expression (29). A tuneable inhibition also allows functional studies on the many essential bacterial genes that can otherwise only be studied with difficulty (30). A drawback, though, is that construction of efficient vectors can sometimes be complicated and not all species or strains are amenable to this kind of genetic modifications. Also, there is little therapeutic use for such recombinant antisense RNA expressing strains. As an alternative, that circumvent both stability problems and restricted applicability, a number of chemically modified nucleic acid analogues and mimics have been developed.

1.3 ANTISENSE TECHNOLOGY USING NUCLEIC ACID ANALOGUES AND MIMICS

The early application for short antisense ONs as specific gene inhibitors demonstrated by Zamecnik and Stephenson together with a growing awareness of antisense RNA controlled regulation in cells generated an intense interest in developing synthetic antisense ONs with improved chemical stability. From the mid-1980s a boom in creating synthetic nucleic acid analogues started. However, it soon became apparent that to maintain antisense binding properties only certain kinds of backbone stabilising modifications were successful. Also, cell uptake of the ONs was a major issue (31). Another important property that became evident was whether the ONs were RNase H

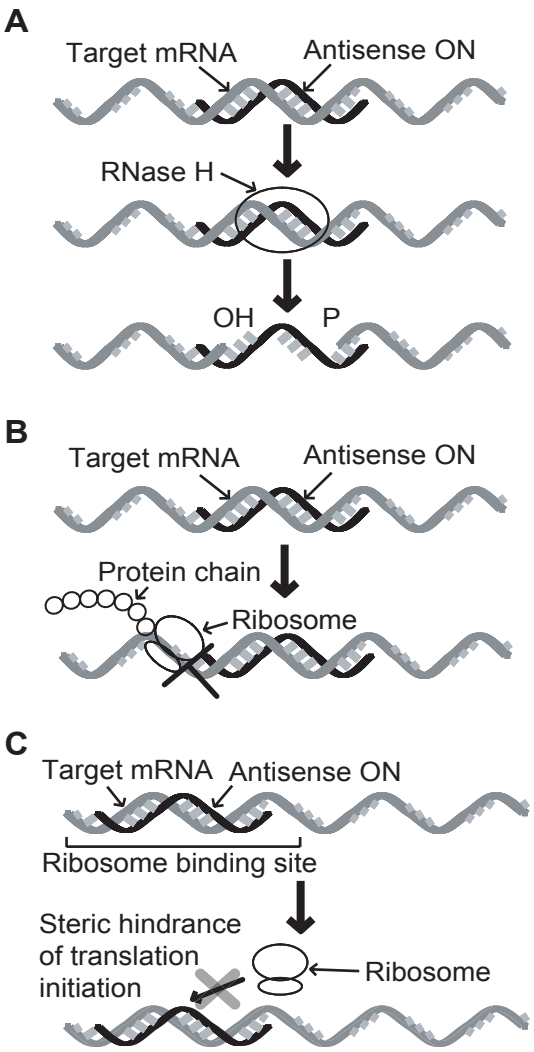


Figure 5. Different mechanisms of antisense inhibition. A. RNase H mediated cleavage. Hybridisation of an antisense ON to a target mRNA is recognised by the cellular nuclease RNase H. The nuclease cleaves the mRNA part of the complex and generates 5'-phosphate and 3'-hydroxy termini. **B.** Translational arrest. The antisense ON hybridise to the coding region of the mRNA and functions as a road-block for the elongating ribosome. **C.** Sterical hindrance of translation initiation. The antisense ON hybridise upstream of or at the translation start site and thereby sterically hinder the ribosome to access the mRNA and initiate translation.

inducing or acting through sterical hindrance (Figure 5). The distinction between these two mechanisms is important when considering gene expression inhibition. RNase H is a ubiquitous enzyme that specifically hydrolyses the RNA moiety in RNA/DNA duplexes (32), (Figure 5A). Interestingly, many synthetic antisense agents (especially those structurally similar to DNA) are able to induce RNase H cleavage of the target RNA. Such ONs can therefore be directed to almost any accessible site along the mRNA molecule to inhibit production of a functional protein. Moreover, ONs inducing RNase H cleavage are catalytic and may be repeatedly recycled as substrates (33). In contrast, ONs that do not activate RNase H are thought to sterically block translation from the target mRNA. Sterically hindering antisense ONs can either arrest an elongating ribosome (Figure 5B) or, more commonly, block its initiation (Figure 5C). However, it is not known whether sterically hindering antisense ONs can act catalytically. A majority of the antisense ONs that have reached clinical trials act through RNase H cleavage (34).

1.3.1 Methylphosphonates

The first modified ONs to be synthesised were the methylphosphonates (MP). These biologically stable ONs have one of the nonbridging oxygens replaced by a

methyl group at the phosphor in the phosphodiester backbone (35) (Figure 6). This modification gives a noncharged, highly nuclease resistant molecule that does not induce RNase H mediated cleavage (36). However, the lack of charge reduces both solubility and cellular uptake. Also, target affinity is relatively poor as the usual helical conformation of nucleic acid duplexes is destabilised (37).

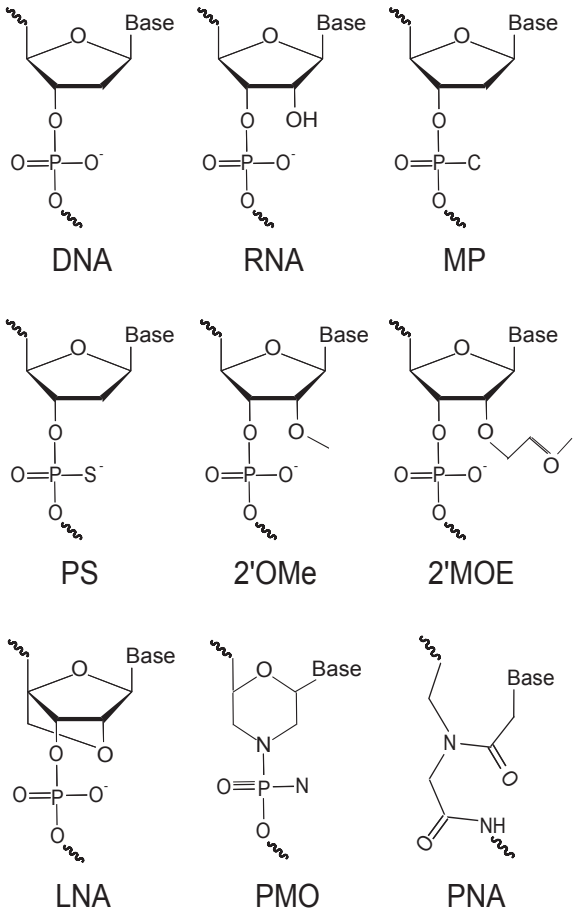


Figure 6. Structures of DNA, RNA and some commonly applied antisense chemistries. MP = methylphosphonate; PS = phosphorothioate; 2'OMe = 2'-O-methyl RNA; 2'MOE = 2'O-methoxy-ethyl RNA; LNA = locked nucleic acid; PMO = phosphorodiamidate morpholino; PNA = peptide nucleic acid.

1.3.2 Phosphorothioates

Phosphorothioate (PS) ONs is a group of early synthetic analogues that has shown more promising antisense properties (38). These ONs also show increased (but far from complete) nuclease stability due to a modification at the phosphodiester backbone. In PS ONs, the nonbridging oxygen is substituted by a sulphur atom (Figure 6). This modification maintains a negatively charged backbone, does not impair molecular stability and it does not seem to interfere with specificity for the target, although a slight decrease in melting temperature (T_m) has been reported for PS/RNA duplexes (33, 39). Also, similar to DNA, PS ONs support RNase H dependent cleavage of the target RNA (29, 37, 38, 40). These beneficial antisense properties have made PS the most widely used antisense chemistry. Several PS ONs have reached clinical therapeutic trials, and one (Vitravene^{TM}) designed to inhibit cytomegalovirus induced retinitis, has reached the market (38).

However, there are some properties of PS that interfere with specificity. The backbone show high affinity for several cellular proteins including growth factors, serum proteins and many heparin binding molecules (37, 38). Also, PS ONs that

contain CpG motifs can show strong immune stimulatory activities by inducing several cytokines and chemokines (40). Although specificity is impaired, immune stimulation can be positive from a therapeutic point of view as it might synergise with the antisense effects in disease treatment (38). Moreover, binding to plasma proteins allow PS ONs to remain longer in circulation and enhance their tissue distribution (41).

1.3.3 2'-O modified ONs

PS ONs suffer from 3'-end degradation by exonucleases that slightly interfere with their stability. Such degradation can be prevented by placing functional groups at the 2' position of the ribose sugar (42). This kind of modification has generated a large group of antisense agents (38, 39), and perhaps the best studied are 2'-O-methyl (2'OMe) and 2'-O-methoxy-ethyl (2'MOE) RNA (Figure 6).

2'OMe and 2'MOE are generally less toxic than PS and they also display enhanced affinity for RNA. However, they are not able to induce RNase H activity (43, 44). Nevertheless, the increased nucleolytic stability of 2'-modified ONs has proven useful for synthesising gapmers, where a core of RNase H competent DNA or PS nucleotides is flanked by more nuclease resistant nucleotides. The use of this technology has demonstrated great improvements in antisense effects and 2'-OMe/DNA, 2'-MOE/DNA and 2'-MOE/PS chimeras are under clinical investigation (38, 45). A benefit with RNase H inducing gapmers compared to other RNase H inducing ONs is that problems with unspecific cleavage can be largely reduced. RNase H requires only partial hybridisation by as few as six to eight nucleotides to be activated, meaning that an RNase H competent ON of for example 15 bases contains as many as 10 short overlapping sequences that are all capable of inducing RNase H (43, 46). Therefore, gapmers containing a length limited core of RNase H competent nucleotides can significantly enhance antisense specificity.

1.3.4 Locked nucleic acid

Another synthetic nucleotide commonly used for producing chimeric gapmers is locked nucleic acid (LNA). LNA was first described in 1998 (47, 48) and is a ribonucleotide that contains a methylene bridge between the 2'-oxygen and the 4'-carbon on the ribose (Figure 6). Characteristics for LNA ONs are high nuclease stability, low toxicity, high solubility and – most prominently – an extremely high affinity for DNA and RNA (43-45). Indeed, an LNA monomer introduced into an antisense DNA may increase T_m by 1-8°C when hybridised to DNA and by 2-10°C when hybridised to RNA (49, 50). LNA

does not induce RNase H and it is therefore common to introduce stretches of DNA or other RNase H competent nucleotides into the sequence. Kurreck and co-workers demonstrated that an LNA/DNA chimera with eight consecutive DNA nucleotides showed strongly improved stability, higher binding affinity and more efficient RNase H activating properties than a corresponding 2'-OMe/DNA chimera (44).

1.3.5 Phosphorodiamidate morpholino oligomers

Phosphorodiamidate morpholino oligomers (PMOs) were developed by Summerton and Weller as antisense agents that should act by blocking translation through steric hindrance (51). In PMOs, the sugar ring is replaced by a morpholino moiety and a phosphorodiamidate linkage is applied instead of phosphodiesters (Figure 6). This results in an uncharged molecule that is unable to induce RNase H (52, 53). The uncharged backbone has low affinity for proteins and cellular components other than RNA and this is thought to generate fewer non-antisense effects and lower toxicity (54). Moreover, PMOs show complete resistance to nucleases and demonstrate relatively high affinity and specificity for their targets (52, 53). These properties have enabled drug developers to bring several PMOs into clinical trials (54). Among the most commonly used antisense chemistries, PMOs belong to the few that use steric hindrance for gene expression inhibition. Another example of an antisense agent that acts through steric hindrance is peptide nucleic acid (PNA). The following sections will concentrate on the properties of PNA and discuss its potential as a specific inhibitor of gene expression.

1.3.6 Peptide nucleic acid

Peptide nucleic acid (PNA) is a nucleic acid mimic with a completely altered backbone composed of N-(2-aminoethyl)glycine units with the organic bases attached to the central amine with an acetyl linker (Figure 6). PNA was designed to bind the major groove of dsDNA in a sequence specific manner to form triplexes that inhibited gene expression (55). However, it soon became clear that the PNA backbone was an excellent substitute for the normal sugar phosphate backbone, forming stable complexes with complementary ONs following Watson-Crick base pairing rules (56).

1.3.6.1 Chemical properties of PNA

The unusual backbone structure of PNA maintains a normal '6 + 3' number of bonds between the bases and permits efficient pairing with complementary ONs (Figure 7). At

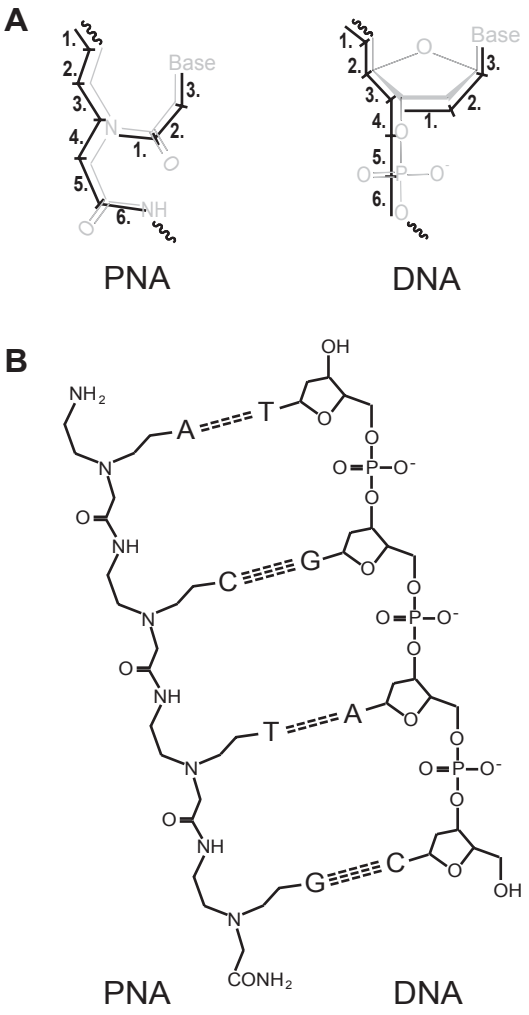


Figure 7. Base spacing in PNA and DNA. A. '6 + 3' arrangement between the bases in PNA and DNA. The bases in both PNA and DNA are separated by three bonds to the backbone structure and have an intra-backbone distance of six bonds. **B.** PNA oligomer hybridised to a complementary DNA. The similar spacing between the bases in PNA and DNA (or RNA) allows hybridisation through Watson-Crick pairing between the molecules.

the same time the backbone structure provides PNA with a number of unique chemical properties. First, PNA oligomers are uncharged. This gives an exceptionally high binding affinity to complementary nucleic acids (although not as high as LNA (45)) as electrostatic repulsion from the normally negatively charged backbones are diminished (56). Also, PNA hybridises more strongly with RNA than with DNA and hybridisation with either results in the formation of a helix like structure (57, 58). In addition, an important property for target specificity is that sequence discrimination is stronger for duplexes of PNA/DNA than for DNA/DNA duplexes (56). Depending on the specific substitution and surrounding bases, a one base pair mismatch in a PNA/DNA complex lowers the T_m by 8-20°C compared to a fully matching complex (59, 60). However, the uncharged nature of PNA makes the molecule less soluble than most other nucleic acids. Nevertheless, the peptide-like backbone of PNA is amenable to a number of different modifications (57), and the addition of a positively charged lysine ligand improves solubility (55). The effects from linking amino acids to PNAs will be further discussed in later sections.

1.3.6.2 Biological properties of PNA

As previously mentioned, the inherent susceptibility to nucleases of unmodified ONs was a major reason for developing DNA and RNA analogues and mimics. PNA has demonstrated high resistance to nucleases, proteases and peptidases (61). This high

stability is explained by the non-natural amide bonds between the glycine subunits in the backbone that can not be recognised by degrading enzymes.

A property that brings both advantages and disadvantages is that PNA, unlike PS, does not appear to bind to proteins and other cellular components (62), possibly due to the uncharged backbone. An obvious advantage is improved specificity, whereas a disadvantage is that PNA, unlike PS oligomers (41), displays relatively poor biodistribution and shows quite rapid body clearance (63-65). Nevertheless, PNA has demonstrated antisense and antigene *in vivo* effects (66-68) and, as will be discussed below, modifications can improve the efficiency. Finally, it appears that PNA/RNA duplexes are not substrates for RNase H (69). Instead, antisense PNA seems to inhibit RNA activity through tight target binding and steric hindrance.

1.3.6.3 Applications for PNA

The chemical and biological properties of PNA have generated numerous applications. With DNA as a target or interacting partner, applications include (i) duplex and triplex forming invasion of dsDNA (55, 70, 71) that can be used for gene expression and replication control (72, 73), (ii) PCR clamping that can be used for selective PCR amplification and single base pair mutation analysis (59, 74, 75), (iii) mutation screening (76) and (iv) PNA/DNA chimeras that can act as molecular decoys for nucleic acid binding proteins (77).

When targeting RNA, PNA has demonstrated efficient inhibition of (i) RNA processing by blocking splice sites (78, 79), (ii) translation by binding the rRNA component of ribosomes (80) and (iii) telomere elongation by blocking telomerase activity (81, 82). Also, PNA has been targeted to genomic regions of RNA viruses with repression of viral replication as a result (83-87). The most frequent target for PNA is mRNA and translational inhibition was early demonstrated both *in vitro* and *in vivo* (88). Since this study, mRNAs from a range of mainly eukaryotic genes have been targeted, mainly aiming at downregulation of oncogene expression. However, there are few reported applications in bacteria, and this will be further discussed in section 1.5.

1.4 DESIGN OF MRNA TARGETING ANTISENSE PNAS

Although simple in theory, the design of efficient antisense agents is a complex field when it comes to issues such as delivery and specificity. PNA and other antisense agents are generally poorly taken up by cells and, once inside, they may display low

efficiency and unspecific binding. Therefore, it is commonly necessary to design up to 20 different ONs to get one that is both efficient and specific (89).

1.4.1 Delivery and uptake

Unmodified PNA molecules display very slow diffusion through phospholipid membranes (90) and poor penetration through the lipopolysaccharide (LPS) layer of bacteria (91). Moreover, side by side comparisons of PNA and other ONs show that PNA exhibits lower cellular uptake and has a higher tendency to accumulate in endosomes and lysosomes than most other ONs (92, 93). Due to these weak internalisation properties, early *in vivo* studies used microinjection of PNA molecules into individual cells in culture (88). This delivery method is obviously both impractical and has narrow applicability and today other more practical methods have been developed.

The most commonly applied general method to internalise ONs uses cationic lipids as a delivery vehicle. However, this method is not adequate for delivery of non-ionic molecules like PNA (94). To improve delivery with cationic lipids, lipophilic molecules can be conjugated to PNA (95). Also, several studies have used partially complementary DNA molecules that hybridise to the PNA and thereby allow complex formation with the cationic lipids (81, 96, 97). This method requires that the hybridisation affinity between the PNA and the carrier DNA is carefully balanced. The binding must be strong enough to get the PNA into the delivery vehicle and sufficiently weak for the DNA to detach from the PNA inside the cell (96, 98).

An alternative delivery strategy is to conjugate cell penetrating peptides (CPPs) directly to the PNA backbone. Synthesis of peptide-PNA uses standard methods for protein synthesis (99) and, to date, a range of different CPPs have been evaluated with substantial improvements in PNA uptake (100-106). An important observation is that the peptide part in a peptide-PNA does not seem to interfere with target specificity (99). Interestingly, a comparative study using either DNA/lipid-mediated delivery or conjugation of the PNA to a nuclear localisation signal peptide showed higher efficiency with the former strategy although toxicity was lower with the latter, when applied to cells in culture (97). However, such comparisons can be difficult to interpret as a number of variables such as target site, peptide sequence, DNA hybridisation properties, and type of target cells may influence the outcome.

1.4.2 Target site selection

A first important criterion for selecting specific antisense ONs is that target site uniqueness is assured. Rapid advances in genome sequencing projects greatly facilitate the selection of unique target sites within mRNA sequences; however a number of difficulties remain. Due to the highly structured nature of most RNA molecules it can be difficult to find accessible antisense target sites. As mentioned previously, RNase H competence of the antisense ON is also an important determinant for target site selection. Efficient antisense target sites may also vary between prokaryotes and eukaryotes, especially when sterically hindering antisense agents like PNA are applied.

In eukaryotic cells, the predominant target for antisense PNA and other sterically blocking antisense agents has been the 5' UTR or sites near the translation start where they are thought to inhibit either start codon scanning or ribosome initiation (52, 96). A few studies also show antisense effects from PNAs targeted to the coding region (37, 93, 98). However, the antisense PNAs used in these studies were all relatively long or GC rich, and this suggests that only high affinity binding antisense molecules are able to stop an elongating ribosome (37, 98).

There is less information about efficient target sites in prokaryotes, however antisense effects have been obtained when the translation start codon region has been targeted (107). Observations that natural antisense RNAs commonly target the start codon region (10) and that this part of mRNAs is generally less structured (89) rationalise selection of a target site within this area. Also, avoiding target sites within the downstream coding region eliminates the risk of having truncated proteins produced (108).

1.4.3 Length and base composition

Smaller molecules are generally more readily taken up by a cell, which argues for shorter antisense ONs. On the other hand, a certain length is required for the antisense molecule to be unique within the transcriptome. Therefore, longer sequences are generally needed when organisms with larger transcriptomes are studied. Binding affinity is another important determinant for antisense efficiency and specificity and it is largely dependent on ON length. Naturally, sequences with T_ms much lower than the required experimental conditions are useless. Also, very high T_ms can create problems as unspecific binding to sequences with one or more mismatching bases becomes more likely. Therefore, in theory, the high binding affinity (56) and sequence discrimination (59, 60) of PNA should allow relatively short sequences to act specifically.

Base composition considerations are similar for most antisense ONs. First, hairpin forming and self-complementary sequences should be avoided. Second, antisense sequences that contain stretches of four or more guanines can impair antisense efficiency as they may aggregate as tetramers (109). A final consideration is to avoid G-rich sequences as they can be difficult to synthesise.

1.4.4 Specificity controls

From a specificity perspective there are two major motivations to attempt to improve the sequence selection properties of antisense agents. First, a lower concentration of the antisense molecule leads to a decreased binding to unintended RNA targets. Second, the antisense molecule has to be considered as a foreign substance that may alter cellular processes in more general ways. Therefore, as has been pointed out by Stein and co-workers, it is of central importance to have a critical eye when working with antisense and that a number of specificity controls are always included (37, 46, 110-112). Some commonly applied specificity controls are listed below:

- Comparisons with a mismatching antisense sequence
- Comparisons with a scrambled antisense sequence
- Evaluation with dose response curves
- Alterations in the target sequence
- Overexpression of the target sequence
- Repression of the target sequence
- Comparison of two or more antisense molecules targeted to different sites at the same mRNA
- Comparison of two or more antisense chemistries with the same base sequence
- Protein quantification with western blotting or enzymatic assays
- mRNA quantification with northern blotting or quantitative PCR

1.5 ANTISENSE INHIBITION OF BACTERIAL GENES

Today, antisense is one of the dominating techniques used to regulate gene expression and study protein function. Compared to other commonly applied methods like gene knockout, protein overexpression, small molecule inhibition and monoclonal

antibodies, an antisense approach displays generally broad applicability, high specificity, high cost efficiency and high probability of success (33, 113, 114).

Despite relative technical ease and many successful examples in eukaryotic cells, there has been surprisingly little interest in regulating bacterial gene expression with antisense ONs. One reason might be difficulties with delivery across stringent bacterial cell barriers. This problem becomes larger when considering antisense as a possible antibacterial therapeutic, as many bacterial pathogens are intracellular. Another reason for the few bacterial studies using antisense ONs can be that eukaryotic systems have been considered more relevant to investigate. However, improved delivery methods, the availability of a large number of complete bacterial genome sequences and increased problems with antibiotic resistance (as will be discussed below) argue for more investigation of antisense ONs in bacteria.

1.5.1 Rational for selecting a bacterial system for gene function studies

If thinking of a cell as an enormous jigsaw puzzle, gene functions would be the pieces and molecular interactions would determine the placing of the pieces. Bacterial genomes are generally much smaller than eukaryotic and with the puzzle analogy they would be considered both smaller and less complex. Furthermore, a vast majority of the already sequenced genomes are bacterial and, in general, these are much better annotated than the eukaryotic genomes. This background information provides the bacterial puzzles with a larger number of characterised pieces that are already properly placed. An important characteristic for most bacteria is a short generation time and simple handling. This implies that strokes and colours on the individual pieces may be identified faster and therefore may be more quickly and correctly placed. Also, bacteria are useful models for more complex organisms. With the puzzle analogy this would mean that motifs of different puzzles show resemblance and that having parts of smaller puzzles completed can help doing the larger picture. Therefore, considering that cellular puzzles are not two-dimensional but at least four-dimensional it seems surprising that so few choose to play with the smaller versions.

1.5.2 Antisense ONs as possible antimicrobials

The rapid development and spread of antibiotic resistance provides a strong motivation for intensified functional studies on bacterial genes. Currently used antibiotics target the products of only a few percent of several hundred estimated essential genes (115, 116). Therefore, the identification of essential genes as new appropriate drug targets

constitutes an important first step in the development of new antimicrobials. There are numerous methods available for such identification and some commonly applied involve gene deletions, insertion of thermosensitive lethal mutants, insertion of upstream inducible promoters and random transposon-insertion tests (117). Another approach involve expression of antisense RNA in mutant microorganisms, and this strategy has been applied to identify growth essential genes in both bacteria (116, 118) and yeast (119). An alternative strategy that does not rely on genomic modification is to use externally added antisense ONs. The advantage is the possibility to bring growth inhibitory antisense ONs into further development as antimicrobial agents themselves.

In theory, externally added antisense ONs could provide excellent antimicrobial agents. In practise, antimicrobial properties of antisense ONs have been demonstrated in several bacterial species using MP (120), PS (121-124), PMO (125, 126) and PNA (80, 107, 127). Less studied is the efficiency of such antimicrobial antisense ONs *in vivo*, although one examination using PMO in a mouse peritonitis infection model shows partial bacterial clearance (128). Still, it remains to see how antimicrobial antisense agents are best delivered and how well they penetrate infecting bacteria.

Another poorly studied area concerns the ability of bacteria to develop resistance against antisense agents. Bacteria possess several defence mechanisms that may neutralise an antimicrobial attack. These include enzymatic inactivation of the drug, increased drug efflux, drug target alteration, drug target overexpression and limitations in drug penetration (129). It is likely that the unnatural chemical structure of many antisense ONs can help them escape enzymatic inactivation. Also, target alterations are easily dealt with by simple changes in the antisense base composition. However, it is not unlikely that future antisense antimicrobials would encounter efficiency problems due to poor uptake, increased efflux and target overexpression (129). Nevertheless, antimicrobial antisense agents can benefit from the diversity in chemical structures and the huge amount of possible targets. This makes antisense a promising strategy for the development of new target specific antimicrobials.

2 AIMS

When this thesis project started it had already been demonstrated that antisense PNA could provide a specific inhibitor of gene expression in cultured bacteria (107) and that PNA was capable of inhibiting bacterial growth through an antisense mechanism (80). However, antisense PNA molecules had also shown to be efficiently excluded by the bacterial lipopolysaccharide layer of the outer membrane (91) and therefore diluted growth media and hyperpermeable bacterial strains were required for potent antisense inhibition (80, 91, 107). Our aims with this work have been to improve antisense PNA efficiency and assess practical applications for antisense PNA as a specific gene expression inhibitor in bacteria. The following specific aims were treated in the separate papers:

I. To improve antisense efficiency by conjugating cell penetrating peptides to the PNA molecules and to investigate the relationship between antisense length and efficiency. To examine bactericidal properties of antisense PNA directed to an essential bacterial gene and further study these properties in a non-invasively infected culture of eukaryotic cells.

II. To evaluate the possibility to use antisense PNA targeted to an essential bacterial gene as an alternative to antibiotics for bacterial strain selection.

III. To determine sites on bacterial mRNA that are susceptible to antisense PNA inhibition.

IV. To examine the combined effects of protein targeting antibacterial drugs and mRNA target specific antimicrobial antisense PNAs.

V. To examine the effects on cotranscribed bacterial genes after inhibition with antisense PNA.

3 OVERVIEW OF THESIS OUTCOME

3.1 PAPER I

Antisense PNA can specifically inhibit *Escherichia coli* gene expression and growth and holds promise as anti-infective agents and as tools for microbial functional genomics (80, 107). However, antisense PNAs are larger than most drugs and PNA size or length is likely to be an important parameter for efficiency. To evaluate the size/activity relationship for antisense PNAs in *E. coli* cultures, PNAs targeted to the start codon region of the chromosomal β-galactosidase gene (*lacZ*) were synthesised over a 7 to 15 mer size range. Inhibition of *lacZ* expression was determined using an *E. coli* liquid culture assay with a wild type *E. coli* strain (K12) and a permeable mutant (AS19) (130). In both strains, PNAs in the 9 - 12 mer range were most active. The 7 and 8 mer PNAs tested were the least active. One would indeed expect an optimum PNA length because binding affinity to the RNA target increases with length, whereas uptake efficiency is expected to decrease dramatically with length. Also, shorter oligomers naturally offer higher sequence specificity at the expense of gene target specificity. Finally, as an initial test of selectivity, a four bp mismatched 12 mer PNA was tested and found to be significantly less active than the fully matched version.

Further, we considered that chemical modifications of antisense PNA could improve potency for wider applications. The *E. coli* outer cell wall is a major barrier to PNA (91). However, bacteria are permeabilised by cationic antimicrobial peptides and such compounds can act synergistically with antimicrobials that enter cells poorly (131). As was previously shown, *E. coli* cells permeabilised by the polymyxin nonapeptide are more susceptible to PNAs (91). We therefore considered that covalent attachment of such a permeabilising agent could further improve cell entry, as has been demonstrated in related applications with eukaryotic cells (102, 105, 106, 132). Instead of the polymyxin nonapeptide, we decided to search for a peptide that did not exhibit the pronounced antibacterial activity. Indeed a quite simple synthetic peptide (KFFKFFKFFK) was shown by Vaara and Porro (133) to posses the desired properties. We tested whether this cell wall permeating peptide, when conjugated to PNA oligomers, could enhance the uptake and thus efficacy of antisense PNAs. For this, 12 and 15 mer anti-*lacZ* PNAs were synthesised with the KFFKFFKFFK peptide attached via a flexible ethylene glycol-lysine linker. Both the 12 and 15 mer conjugates were 15-20 fold more potent than the corresponding PNAs without attached peptides. Also,

consistent with the length comparison experiment, the 12-mer peptide-PNA conjugate was significantly more active than the 15-mer version. Furthermore, the free peptide did not inhibit *lacZ* production (*e.g.* via general toxicity), and there was no additive inhibitory effect from a mixture of free peptide and naked PNA. Thus, covalent attachment of PNA to the carrier peptide was needed for efficient antisense effects.

To evaluate the specificity of the more potent anti-*lacZ* peptide-PNAs, one 15 mer and two 12-mer mismatched peptide-PNA conjugates were included in the study. These control constructs showed poor *lacZ* inhibition when compared to the fully matched constructs of similar size and composition. Also the anti-*lacZ* constructs showed very low inhibition of a control reporter gene (luciferase) and low general toxicity. Therefore, the peptide-PNAs retain good overall specificity, although a couple of the peptide-PNAs showed general toxicity at low micromolar concentrations. This must be appreciated when considering applications for the approach – particularly for gene functional studies. We further examined the effects of conjugating a few other peptides to the most efficient 12 mer PNA sequence either with the ethylene glycol-lysine linker or with a maleimide SMCC coupling. The PNA construct assembled via maleimide SMCC coupling was more than 10 fold less potent than the ethylene glycol linked PNA. The other peptides were also able to carry PNA oligomers into bacteria, however not as efficiently as the KFFKFFKFFK motif.

It was previously shown that a triplex-forming PNA oligomer targeted to the α-sarcin loop of the 23S ribosomal RNA inhibits the growth of *E. coli* (80). To test whether attached peptides can also improve the potency of such anti-microbial PNAs, the KFFKFFKFFK peptide was conjugated to this PNA. The "naked" version of this anti-ribosomal PNA was only capable of inhibiting cell growth of the permeable *E. coli* strain AS19 in diluted growth media. In contrast, the new anti-ribosomal peptide-PNA conjugate exhibited an MIC of 3 μM against wild type *E. coli* K12 grown in Mueller Hinton broth and an MIC of 700 nM when grown in dilute broth. This shows that conjugation of the peptide with PNA is needed for potent growth inhibitory effects.

To test the potential for PNAs targeted against mRNA to provide antibacterial effects, we evaluated a PNA complementary to the start codon region of the essential *acpP* gene encoding the acyl carrier protein ACP, which is central to fatty acid biosynthesis in *E. coli* (134). This anti-*acpP* peptide-PNA conjugate showed an MIC against *E. coli* K12 of 1 μM in rich broth and an even lower MIC in dilute broth. Antisense specificity of the anti-*acpP* peptide-PNA was demonstrated in two ways.

First, a control peptide-PNA with a scrambled PNA sequence showed 15 fold higher MIC than the fully matched version. Second, the *E. coli acpP* gene was cloned and selected bases altered to eliminate the potential for PNA binding while encoding of native ACP was retained. The target-site-altered version of *acpP* was introduced into *E. coli* on a plasmid vector and the transformed strain was rescued from PNA inhibition with restored growth (Figure 8). Complementation by the altered *acpP* allele gives

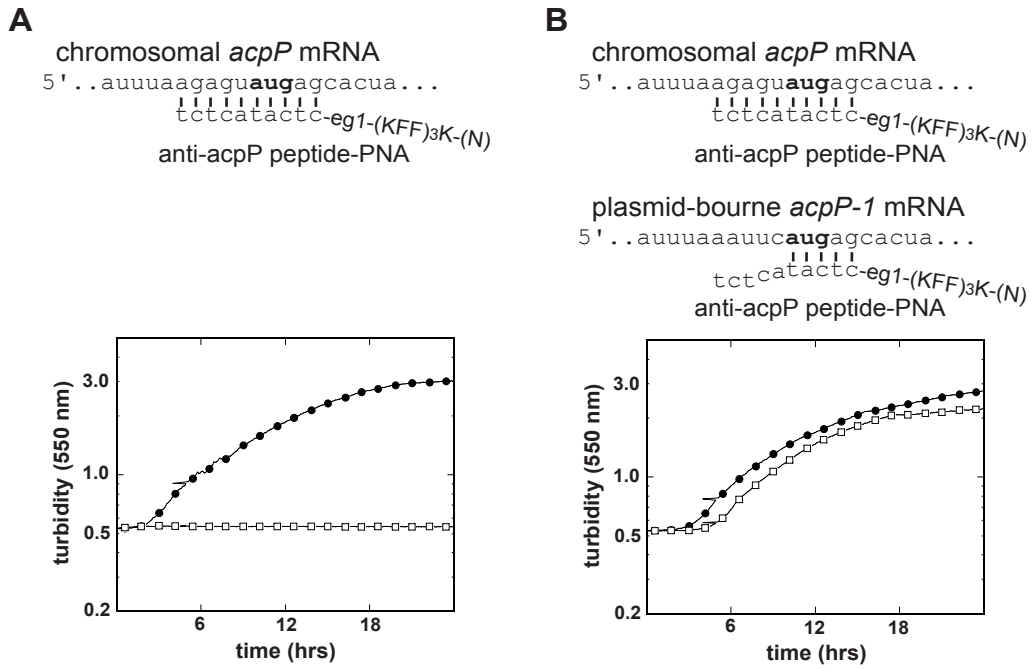


Figure 8. *E. coli* **K12 growth and inhibition by an anti-*acpP* peptide-PNA conjugate.** Cell growth is indicated by increased turbidity using OD550 measurements. **A.** *E. coli* K12 cells. **B.** *E. coli* K12 cells with plasmid pKK223-3/*acpP-1*. Cultures were grown in the absence (closed circles) or presence (open squares) of added PNA (2 μM). The structure of the wild type chromosomal and mutant plasmid-borne copies of the *acpP* gene present in the two strains are shown, along with the antisense PNA, which is complementary only to the native *acpP* mRNA start codon region.

strong genetic evidence for specificity and confirms an antisense mechanism of action for the anti-*acpP* peptide-PNA. As a tool for genetics, the experimental approach can be extended to a wide variety of experimental questions.

The effects of antibacterial peptide-PNA conjugates on *E. coli* cells were further examined by assessing cell viability. Within one hour of exposure to 2 μM anti-*acpP* PNA, the number of colony forming units decreased by three orders of magnitude from an initial 10^5 cells/ml and no viable cells were detected after three hours, showing that the PNA treatment was bactericidal. The results suggest that antisense peptide-PNAs can be used to evaluate microbial genes for suitability as drug targets.

To evaluate any possible anti-*acpP* PNA toxicity to eukaryotic cells as well as to examine the anti-bacterial potential of antisense inhibition in the presence of eukaryotic cells, the anti-*acpP* PNA was tested against *E. coli* grown in cell culture medium. In this medium, which should be more relevant for any *in vivo* application, the PNA was ten fold more potent (MIC = 200 nM) than in MH broth. Further, HeLa cell cultures non-invasively infected with 10^4 CFU *E. coli* K12 were treated with PNA. The PNA did not visibly affect HeLa cell growth at the highest concentration tested (20 μM), and PNA at 2 μM or higher appeared to fully cure the HeLa culture of the infection. No live bacteria were detected from these samples in an agar colony forming unit test, confirming that the anti-*acpP* PNA is bactericidal under these conditions. Again, the sequence specificity of the effect was evaluated in cultures infected with *E. coli* carrying the binding-site-altered *acpP* allele (K12-*acpP-1*) and this infection was resistant to PNA up to at least 20 μM.

To summarise, we have shown that 9-12 mer antisense PNAs conjugated to cell permeating peptides provide potent and specific inhibitors of gene expression. Furthermore, antisense inhibition of an essential bacterial gene provides bactericidal effects that can cure a cell culture non-invasively infected by *E. coli*. Therefore peptide-PNA conjugates are promising agents for antimicrobial drug development and microbial functional genomics studies.

3.2 PAPER II

Antibiotics are widely useful in medicine, agriculture and industrial fermentations. However, increasing problems with resistant strains call for restrained use and alternative strategies. Paper I demonstrated that antisense PNAs can provide potent bactericidal effects when targeted against the essential *E. coli acpP* gene ((135), see Figure 8). Aside from attractive antimicrobial therapeutic possibilities for such antisense PNAs, we considered that they could be used as a substitute for antibiotics in bacterial strain selection and construction. To test whether antisense PNA treatment could select a genetically modified strain in a mixed culture, wild type *E. coli* cells were grown together with transformants containing a plasmid that provides both PNA resistance and an ampicillin resistance (Ap^r) selection marker (pKK223-3/*acpP-1*). The plasmid contains a copy of the *acpP* gene in which the PNA binding site has been altered, and this change can rescue *E. coli* growth in the presence of anti-*acpP* PNA (135). Therefore, given that the antisense agent can kill wild type bacteria without harming the modified strain, it should be possible to select only the modified strain in a

mixed culture. In a first experiment, selection was carried out using 1 μM of anti-*acpP* PNA, and a strong selection effect was seen after just one hour (83.3 % Ap^r clones as compared to 50 % in the start culture) and 100 % selection efficiency was obtained within only two hours. Also, a strong bactericidal effect of PNA on wild type K12 cells was seen when grown as a monoculture. It can therefore be concluded that the PNA inhibit growth in a strain specific manner, and antisense PNAs can be used for strain selection from a mixed culture.

The strain selection capacity observed when using a mixed bacterial culture suggested that antisense PNA selection of transformants should be possible. To obtain high transformation efficiency competent *E. coli* DH5α cells were electrotransformed with purified plasmid. Within 6 hours after PNA treatment the selection efficiency was almost complete (96.6 % Ap^r), and after 18 hours the culture contained only Ap^r bacteria. Analysis of plasmid content in 11 PNA resistant clones revealed that all contained plasmids of the same size as the original pKK223-3/*acpP-1*. Therefore, the clones were true transformants selected without using Ap. To demonstrate that the procedure was not restricted to a particular strain, the same selection procedure was performed with electrocompetent K12 cells and a similar result was obtained. Therefore, antisense PNAs can be used for antibiotic-free selection of transformants, a procedure that requires the selection of relatively few cells from a large background of non-transformed bacteria.

PNA oligomers will naturally be more expensive to use than conventional antibiotics, but for laboratory use this is not prohibitive. Relatively small quantities are used and both PNA monomers and oligomers (by custom synthesis) are commercially available (at a cost comparable to peptides). Furthermore, PNA oligomers are readily attainable by solid phase peptide synthesis (136, 137). However, cost will of course be an issue if this technique is to be used for larger scale fermentation production.

The present results open possibilities to use PNA as an efficient and flexible alternative for antibiotic resistance markers in strain selection procedures and maintenance. Previously described methods for antibiotic-free selection strategies require special strains or selective conditions that reduce growth rate and thereby limit the flexibility of the approach (138-140). In contrast, the method described here is not limited to such special conditions, and may even be suitable for a range of other strains and growth conditions. This flexibility is important if antibiotic-free selection methods are to be practical alternatives to antibiotics. Therefore, in addition to their promise as

new antimicrobials for medicine, antisense PNAs offer advantages as selective agents for antibiotic-free strain construction and maintenance.

3.3 PAPER III

Antisense PNA can inhibit bacterial gene expression with gene and sequence specificity. Using attached carrier peptides that aid cell permeation, the antisense effects when targeting essential genes are sufficient to prevent growth and even kill bacteria. However, many design uncertainties remain, including the difficult question of target sequence selection. In this study we synthesised 90 ten mer antisense peptide-PNAs with the cell penetrating peptide KFFKFFKFFK attached to target sequences in a head to tail manner across the entire length of the mRNA encoding β-lactamase. This gene was selected because it is the shortest of the convenient reporter systems available for *E. coli*. Also, β-lactamase is often used in molecular biology and closely related versions pose a significant problem for medicine by conferring penicillin resistance to pathogens.

Antisense activities were assayed using a cell culture assay for β-lactamase production. Of the 90 antisense peptide-PNAs tested only one inhibited β-lactamase activity to around 50% of the control level when present at 500 nM, a concentration that was not growth inhibitory for any of the PNA-peptide conjugates. Interestingly, the inhibitory peptide-PNA was complementary to a site in the translation start codon region. Therefore, a higher resolution scan around this region was performed in which ten additional peptide-PNAs were tested. This resulted in the detection of an additional strongly inhibitory peptide-PNA that was targeting the AUG start codon.

Susceptibility within the start codon region provides very useful design information, but use of such guidelines can not guarantee predictable antisense effects. Indeed, two peptide-PNAs complementary to the start codon region were much less inhibitory. This could be due to poor uptake properties or subtle differences in local accessibility or affinity within the target region (108). Also, several peptide-PNAs complementary to downstream sequences significantly elevated gene expression. The reason for this is unclear, but it is possible that the antisense agent stabilised the mRNA against degradation by nucleases (141).

In order to address the difference in target affinity due to sequence variations, we calculated the melting temperature, or T_m (for a sequence-identical DNA target) (142), for all the PNA oligomers. A scatter plot of T_m vs effect on relative β-lactamase

expression suggested little or no correlation between T_m and antisense efficacy, possibly reflecting the fact that antisense efficiency is dependent on many factors. Also, we found no relationship between antisense efficacy and codon usage near the target site.

To further investigate the start codon region as a susceptible target on a naturally occurring essential gene, a scan was performed for the essential fatty acid biosynthesis *acpP* gene. Thirteen peptide-PNAs twelve residues in length where synthesised and minimal inhibitory concentrations (MICs) were determined by the lowest peptide-PNA concentration required for complete growth inhibition through 24 h of incubation. Again, the results showed that the antisense peptide-PNAs were most efficient when targeted to the Shine-Dalgarno/start codon region. A susceptibility region was apparent where the MIC values drop to the low micromolar level for sites spanning from the start codon to 24 bases upstream. T_m values for the individual sequences showed no correlation with efficacy. However, in a previous study we targeted the *acpP* start codon with a ten mer PNA conjugated to the same peptide as we have used here (135). Interestingly, this peptide-PNA (targeting -5 to +5 relative to the translation start site) resulted in a two-fold decrease in MIC compared to the twelve mer peptide-PNA used in this study (targeting -5 to +7). It is interesting to note that the shorter PNA has a lower T_m (41.5 compared to 54.8) and likely gains in potency by having improved uptake properties, as indicated in our previous comparison of PNA targeted against the β-galactosidase gene (135).

The results obtained from scanning the β-lactamase and *acpP* genes for susceptible sites point to the translation start codon region as being most susceptible to translation inhibition by antisense peptide-PNAs. The results are consistent with previous work with the non-essential β-galactosidase gene (107). Also, the results are consistent with results for antisense PNA and morpholino antisense agents targeted against eukaryotic genes (52); although in this case intron/exon junctions, which are not prevalent in bacterial genes are also exquisitely sensitive (78). In bacterial systems, it is not surprising that the mRNA start codon region is susceptible to antisense inhibition. First, bases within this region are recognised by ribosomal components during recruitment and assembly of an active translation apparatus (143, 144). Indeed, data from footprinting studies show that in bacteria the start codon region is particularly active in ribosomal assembly for translation initiation, with ribosomal RNA sequences recognizing bases at or around the start codon (145). Second, in natural examples of

antisense inhibition in bacteria, the start codon region is the most frequent target (10). Third, there are several examples of attenuated mRNAs where the start codon region is sequestered within a double stranded region (146). Therefore, knowledge of translation initiation in bacteria and the results described here point to the start codon region as being most susceptible to antisense PNAs.

A comparison between the region that is sensitive to antisense PNAs and the region that is protected by binding of the ribosomal 30S subunit during translation initiation (145) showed that the zone of sensitivity observed in this study is very similar to the 30S subunit footprint. This similarity supports the notion that the antisense PNAs sterically block the initial step in ribosome binding when the 30S ribosomal subunit recognises and binds to mRNA. The PNAs complementary to sequences downstream of the start codon were not inhibitory at the concentrations tested, presumably because they are not blocking the ribosome binding site (RBS) and are displaced during elongation, as has been observed *in* vitro (69). Although we have analyzed only two genes here, the β-galactosidase gene is also sensitive at the start codon region (107, 135), and on-going experiments indicate that other *E. coli* genes examined also are sensitive to antisense PNAs directed to the translation start site (unpublished data).

Localised sensitivity at the start codon region should improve the probability of achieving gene specific effects. Most sites within mRNA, even if similar in sequence, are likely to be less sensitive to inhibition. Nevertheless, it is important to check that new antisense PNAs are not complementary to other genes, particularly within start codon regions as sequences upstream the AUG are strongly conserved amongst many bacterial genes. Thus we emphasise that promiscuous binding is of particular concern within the start codon region of other genes. New PNAs can be checked for non-target-gene complementarity using on-line sequence analyses tools, which can indicate potential binding sites and the binding position relative to the start of the open reading frame. We recommend sequence analyses tools available at the Institut Pasteur. Finally, to design effective antisense PNAs, previous work indicates 10-12 residues as an effective length, and there are some general sequence composition guidelines to be observed while designing PNAs for any purpose. Also, an estimate of relative target binding affinity may be obtained from calculated T_m values (142).

In summary, for bacterial applications, we conclude that the translation start codon region is generally the most susceptible to inhibition by antisense PNAs. While designing antisense PNAs to target the start codon region does not guarantee specific

effects, this region is clearly most sensitive, and by focusing on this region the sequence screen may be limited to a dozen compounds.

3.4 PAPER IV

The lack of many distinct classes of antimicrobials limits the scope for single and combined drug treatment of resistant infections. Here we wanted to evaluate antimicrobial effects from combinations of protein specific drugs and mRNA specific antisense inhibitors.

To establish a set of antisense inhibitors, we designed PNAs complementary to unique sequences within the start codon region of mRNAs encoded by essential genes including drug targets in *E. coli* and *S. aureus* (147). The carrier peptide KFFKFFKFFK was attached to each PNA to improve cell uptake (125, 135). Inhibitory drug and PNA concentrations were determined in *E. coli* wild type (K12) and permeable (AS19) strains and *S. aureus* (RN4220). As expected, the permeable strain was most susceptible to both drugs and PNAs and the thick-walled *S. aureus* was generally less susceptible (148). Inhibitory concentrations also varied between target genes, possibly due to differences in uptake, binding kinetics and gene product requirement. As a control, the cell penetrating peptide KFFKFFKFFK was tested at a 20 μM concentration in *S. aureus* without showing any effect upon bacterial growth.

To analyse the level of interaction between mRNA and protein level inhibitors, PNAs and drugs were used in pairwise combinations in *E. coli* and *S. aureus* cultures. Interactions were assessed using the chequerboard assay ((149), Figure 9), and fractional inhibitory concentrations (FICs) were calculated:

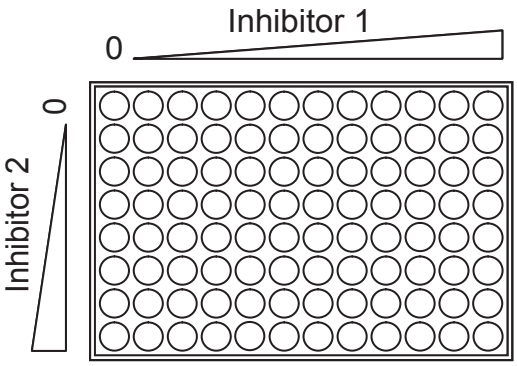


Figure 9. Experimental setup for the checkerboard assay. Combinatory effects are assessed by titrating separate inhibitors into bacterial cultures in perpendicular dimensions. Growth inhibitory combinations help calculate a fractional inhibitory concentration for each inhibitor.

$$FIC_A = \frac{MIC_A \text{ in combination}}{MIC_A}$$

and

$$FIC_B = \frac{MIC_B \text{ in combination}}{MIC_B}$$

FIC indices (FICIs) were determined by adding the FIC values for the separate inhibitors:

$$FIC_A + FIC_B = FICI$$

Antimicrobial synergy was defined by FICIs ≤ 0.5 (150).

A total of 14 PNA/drug combinations were tested in *E. coli*; six with unrelated targets, four with targets in the same biosynthetic pathway and four sharing genetic targets. A pattern was observed where inhibitor combinations with unrelated targets resulted in the highest FICIs, and combinations with targets in the same pathway generally showed a lower FICI. The four combinations with shared genetic targets gave the lowest FICIs, and three of these could be defined as synergistic. In *S. aureus*, nineteen antisense PNA/drug combinations were tested; fifteen with unrelated targets, one with functionally related targets and three sharing genetic targets. Again, combinations with unrelated targets displayed no interaction with relatively high FICIs, whereas combinations with shared genetic targets gave much lower values, and in one case displayed antimicrobial synergy. Therefore, the synergistic or more than additive interactions for antisense/drug combinations sharing genetic targets suggest a new strategy to improve antimicrobial efficiency in practice. Moreover, as protein- and mRNA-level inhibitors are chemically distinct and inhibit sequential steps in gene expression, combined treatment could help limit drug resistance. Although gene selective mRNA inhibitors for antimicrobial applications are still far from the clinic, there are possibilities to develop mRNA sequence (125, 127, 135) and structure (151) targeting antimicrobials, which could follow the progress of antisense agents in viral and cancer treatment (152).

In one noteworthy case where the targets were functionally related, rather than shared, a more than additive PNA/drug interaction was observed. *S. aureus* treated with anti-*fmhB* PNA and ampicillin showed an FICI of 0.62, whereas combinations including only one of these inhibitors displayed much higher FICIs. Interestingly, the target of anti-*fmhB* PNA is involved in the synthesis of peptidoglycans (153), and ampicillin inhibits the formation of peptidoglycan cross-links (154). The result suggests that the low FICI is due to mutual inhibition of peptidoglycan synthesis, although different steps are affected by the two inhibitors. Therefore, in some cases, antisense/drug inhibitors with functionally related targets can display positive interactions. The result also shows that effective *fmhB* inhibitors could potentiate penicillins used in the clinic.

When assessing antisense inhibition, sequence specificity of the PNA is an important issue. In bacteria, sequence alterations in both the PNA and the target sequence lower PNA efficiency (127, 135). Here, target specificity is indicated by the low FICI observed for each PNA/drug combination with shared genetic target relative to other combinations. Moreover, antisense/drug combinations maintained similar FICIs when the antisense target was shifted within the translation start region of the mRNA. Although this likely reflects mRNA specific effects, we aimed to evaluate specificity more thoroughly.

To test whether the positive inhibitor interactions hold true at the biochemical level, the activity of the essential EACPR enzyme (*fabI* gene product) was determined in *E. coli* K12 cultures pre-treated with anti-*fabI* PNA and a control PNA (anti-*folA*) in the presence or absence of the EACPR specific inhibitor triclosan. The results demonstrated a dose-dependent inhibition of EACPR with anti-*fabI* PNA, whereas high doses of the unrelated anti-*folA* PNA did not affect the level of enzyme activity. Moreover, combined anti-*fabI* PNA and triclosan treatment showed more potent inhibition than either inhibitor used alone. Also, enzyme activity in samples treated with both anti-*folA* PNA and triclosan did not differ from samples treated with triclosan alone. Therefore, the result demonstrates anti-*fabI* PNA inhibition of EACPR production and mutual PNA/drug inhibitory effects on FabI activity.

Antimicrobial synergy has been attributed to the hyperbolic or logarithmic nature of dose-responses (149, 155). In a similar way, dose response effects could explain positive interactions between mRNA and protein level inhibitors. To test this, we examined pairwise inhibition of the nonessential β-galactosidase gene, a reporter system that enables inhibition over a large range without altering growth. As expected, inhibition kinetics for treatment with anti-*lacZ* PNA or the competitive β-galactosidase inhibitor D-galactal displayed hyperbolic dose-response curves. When cultures were pre-treated with low doses of anti-*lacZ* PNA, the response curve for D-galactal shifted towards more complete inhibition at every dose tested. By setting arbitrary threshold inhibition levels it is possible to calculate FICIs for combinations of inhibitors. Calculations using several threshold levels and two different PNA concentrations all resulted in low FICIs, suggesting that inhibition kinetics is a major and consistent contributor to synergy. For each gene target, the dose response profiles as well as factors such as threshold level for growth inhibition and feedback regulation could influence FICIs. Therefore, while low FICIs are consistently observed for combinations of mRNA- and protein-level inhibitors with shared genetic target, the level is expected

to vary between target genes. Previous studies report positive interactions and synergy between antisense and non-specific chemotherapeutics in cancer therapy (156), however, the present results are unique in being the first to report synergy when inhibiting the same genetic target at both the mRNA- and protein-level, and we speculate that the mechanism is likely to hold true in a range of bacterial and eukaryotic systems.

Knowledge of protein-small molecule interactions is based on genetic and *in vitro* studies which often lack physiological relevance or fail to uncover significant interactions (157). For example, sulfa antimicrobials are the oldest synthetic anti-infectives used in the clinic, yet their mechanism(s) of action and the mechanism(s) underlying their synergy with trimethoprim remain controversial (149). The main target of sulfa drugs is dihydropteroate synthase (FolP), which provides an early step in folate biosynthesis. A later step in the same pathway is provided by dihydrofolate reductase (FolA), the target of trimethoprim. Synergy between sulfa and trimethoprim is typically explained as a case of sequential inhibition (158, 159). However, several studies suggest that sulfonamides target both FolP and FolA. Therefore, mutual inhibition of FolA could explain the observed synergy (160-162). Interestingly, our results for combinations of PNAs and drugs against folate biosynthesis targets showed more than additive effects when anti-*folA* PNA was combined with sulfamethoxazole, but not when anti-*folP* PNA was used in combination with trimethoprim. Poor specificity for the anti-*folP* PNA can not explain the result as this PNA gave more than additive effects with sulfamethoxazole. Therefore, our results are consistent with sulfa inhibition of FolA (161). This suggestion requires further investigation, but the analysis shows how combinations of mRNA and protein level inhibitors can be applied to help decipher drug mechanism of action.

The observation that certain combinations of mRNA- and protein-level inhibitors display antimicrobial synergy suggests that mRNA inhibition could provide a new strategy to improve drug efficiency. Another area of application for antisense agents is to reveal drug mechanism of action. Mutant bacteria and yeast expressing antisense transcripts have been used to examine drug interactions (116, 119, 163), and an inhibitor combination strategy can strengthen the general approach. Most importantly, the mRNA inhibitors are easily titratable, they can be added to growing cells, and there is no need for genome modifications or antibiotic selection. Furthermore, there are possibilities to access a wider range of microbial species. Finally, the results provide a basis to establish a simple and accurate cell based assay

for target specific inhibitor screens (164) where the use of sub-growth inhibitory concentrations of antisense PNA, other antisense chemistries (125) or small molecule RNA inhibitors (165) could help identify new inhibitors.

In conclusion, the results demonstrate that interactions between mRNA- and protein-level inhibitors having the same genetic target can be synergistic. Therefore, combined antisense/drug treatment provides a strategy to improve antimicrobial efficacy, facilitate drug mechanism of action studies and aid the search for new antimicrobials.

3.5 PAPER V

Most bacterial genes belong to tight clusters and operons, which complicates gene functional studies using conventional knock-out methods. Antisense agents, however, can down-regulate the expression of genes without disrupting the genome as they bind mRNA and block expression. However, it is unclear how antisense inhibition affects expression from genes that are cotranscribed with the target. To address this issue we constructed a plasmid expressing the two reporter genes *gfp* and *luc* as one transcriptional unit (Figure 10). Antisense PNA directed to the mRNA start codon region of the upstream *gfp* gene resulted in a coordinated reduction of the expression from both genes.

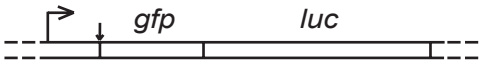


Figure 10. Structure of the artificial *gfp*/*luc* operon. The *gfp* and *luc* genes are expressed as one transcriptional unit under the control of a *lac* promoter. The small arrow indicates the anti-*gfp* PNA target site.

The same approach was applied to the three cotranscribed genes in the endogenously expressed *lac*-operon (*lacZ*, *Y* and *A*, Figure 11). Targeting of the 5' *lacZ* gene resulted in a stronger inhibition of LacZ than the downstream LacY and LacA proteins, demonstrating a partial discoordination in protein expression, a phenomena previously observed by Pestka and co-workers when "anti-mRNA" directed to *lacZ* mRNA was expressed from a plasmid (166). Moreover, when *lacY* was targeted, downstream LacA expression was reduced as much as LacY, whereas LacZ was unaffected. The general differences in expression response between the *gfp-luc* transcript and the *lac*-operon transcript are likely assigned to differences in expression

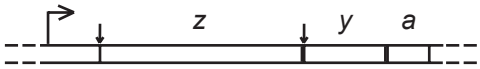


Figure 11. Structure of the *lac*-operon. The *lac*-operon contains the three co-transcribed structural genes *lacZ*, *lacY* and *lacA*. Anti-*lacZ* and anti-*lacY* PNA target sites are indicated by small arrows.

regulation: For the naturally occurring *lac*-transcript, and also for many other endogenously expressed polycistronic transcripts, a wealth of regulatory mechanisms have evolved to balance gene expression after the cells needs. The *gfp*-*luc* transcript, however, is not a natural component in bacteria and therefore not likely to be affected by specific regulatory mechanisms. Therefore, we conclude that antisense PNAs targeted to genes belonging to polycistronic transcripts are very likely to affect the cotranscribed genes, thereby rendering gene functional studies on such genes more difficult.

Antisense PNA inhibits gene expression by steric hindrance of ribosome binding to mRNA (69, 147). Also, it is known that efficient ribosomal binding is necessary for mRNA stability (167-169), and that actively translating ribosomes protect mRNA from RNase E processing (170). Moreover, it has been shown that enhanced transcription rates of *lacZ* mRNA leads to a decreased production of LacZ protein and lower transcript stability, probably due to prolonged exposure of unprotected mRNA behind the RNA polymerase (171). Therefore, we investigated the impact of antisense inhibition of *lacZ* and *lacY* expression at the mRNA level. We observed that the pattern of mRNA levels were similar to that of protein levels within the *lac*-operon, suggesting that antisense PNA inhibition of translation has a destabilising effect on mRNA. Destabilisation of target mRNA is in accordance with that observed by Forsyth and co-workers when they used expressed antisense RNA to downregulate gene expression (116). Therefore, in contrast to previous statements (37, 52), it appears that the activity of sterically blocking antisense agents can be assessed by examining mRNA levels. However, it must be considered that mRNA degradation pathways differ in prokaryotes and eukaryotes and also differ between individual mRNAs.

The altered expression pattern of the *lac* genes as a response to antisense treatment raises at least two new questions: (i) “Why is expression from the downstream *lacY* and *lacA* less inhibited than *lacZ* with anti-*lacZ* PNA?” and (ii) “How can *lacZ* expression remain unaffected when cells are treated with anti-*lacY* PNA?” The first question can be explained by direct protein synthesis inhibition of LacZ together with Rho-dependent premature transcription termination. Translational cessation induces Rho-dependent termination, probably due to increased exposure of Rho protein binding sites behind the transcribing RNA polymerase (172). As it has previously been shown that the *lacZ* coding region contains several Rho binding sites (173) antisense inhibition of ribosome binding at the *lacZ* mRNA should lead to increased Rho binding and therefore increased premature transcription termination.

However, as translation inhibition of, in this case, LacZ is the primary event; premature transcription termination is expected to be slightly delayed. Indeed, such a delay was also observed at both the protein and the mRNA expression levels after antisense inhibition.

Why, then, is *lacZ* not affected by downstream targeting anti-*lacY* PNA? Even though the *lac*-operon is thought to be transcribed to its entirety before being processed (174), it has been observed that *lacZ* transcripts are several fold more abundant than *ZYA* transcripts (174, 175). This imbalance can be explained by an RNase E cleavage in the *lacZY* intergenic region that stabilises the *lacZ* part of the transcript (176), an event that decouples the coding regions of *lacZ* and *lacYA* (177). According to this model, most of the LacZ product is likely to be translated from processed monocistronic transcripts that can not be affected by antisense binding to *lacY* mRNA.

In summary, our experiments show that antisense inhibition of genes within polycistronic operons can strongly affect cotranscribed genes. We further show that inhibition is followed by a decreased stability of the transcript that is coordinated with translational reduction. As a consequence, simple and specific methods for functional studies on the majority of bacterial genes are still lacking. Therefore, we suggest that antisense inhibition and other strategies for gene expression inhibition as a means for gene functional studies (118, 178, 179) and drug screening (116, 163) should be performed with extra caution placed on cotranscribed genes.

4 PERSPECTIVES

Antisense inhibition is a simple technology in theory, but has encountered a number of unexpected challenges on its way to become an efficient research tool (180). Nevertheless, antisense provides a valuable and widely applied technique for controlling gene expression, especially since the discovery of RNAi in eukaryotes. The present investigation has focused on the development of antisense PNA as a silencer of bacterial gene expression. This final section will concentrate on the (in)significance of our results and suggest further studies with the aim to improve efficiency and broaden the field of application for antisense PNA.

Paper I examine the relationship between antisense length and efficacy and show that PNA oligomers 9-12 bases in length are most efficient. It is further demonstrated that conjugation of the cell penetrating peptide KFFKFFKFFK to PNA can largely improve antisense effects with retained specificity. Although useful for several of our antisense studies, there is room for much improvement. Indeed, a study from our lab demonstrate potent antisense effects in *S. aureus* for PNAs linked to a number of other carrier peptides (127). Therefore, it is highly likely that extended examination in this area will reveal other more potent peptides. It is also possible that peptides with organism and cell type specific penetration properties can be developed as efficient future antisense carriers.

The first paper also describes that an antisense PNA directed to the mRNA of the essential *acpP* gene has gene specific bactericidal effects and that this PNA is able to cure eukaryotic cells from a non-invasive bacterial infection. A natural extension of this study would be to investigate bactericidal properties of PNA in an animal model. Interestingly, a recent publication on antisense PMO shows partial bacterial clearance of *E. coli* infected mice after a KFFKFFKFFK peptide conjugated antisense directed to *acpP* mRNA was injected intraperitonealy (128). Similar examinations would be interesting to perform with antisense PNA, especially considering that rather little is known about *in vivo* properties of PNA (94).

Paper I and II use a plasmid containing an extra copy of the essential *acpP* gene which has base alterations in the anti-*acpP* PNA target site. Therefore, anti-*acpP* PNA treatment of cells carrying this plasmid results in repression of the chromosomal copy of the *acpP* gene, whereas the plasmid copy can continuously produce ACP. Interestingly, this system allows insertion of additional mutations in the coding region

of the plasmid copy of the gene by standard mutagenesis procedures, and treatment with antisense PNA would make the cells produce mainly mutant protein. Such strains could be used to study amino acids within essential proteins and reveal their structural and functional roles. Indeed, we have evaluated the idea by mutating several evolutionary conserved amino acids in ACP and they were all detected as essential as inhibition of the chromosomal copy resulted in arrested bacterial growth. This strategy should be applicable to many bacterial genes, and could be particularly helpful for studying amino acids in essential proteins where other methods typically fail.

Paper III shows that antisense PNAs targeted to the start codon region of mRNA are most efficient as translation inhibitors. Moreover, the susceptibility area correlates well with the area where the ribosomal 30S subunit binds to initiate translation. From a design point of view this is helpful information as it limits the number of rational target sites for sterically blocking antisense agents like PNA. Also, the number of sites for unwanted binding to other mRNAs is considerably reduced. As a consequence, this strongly increases the likelihood of finding unique target sites for shorter and more efficient antisense sequences. However the use of both RNase H activating and high affinity steric blocking antisense agents requires that much of the transcriptome is considered as a target: RNase H activating agents because they cleave the RNA target and high affinity sterical blockers because they may arrest ribosome elongation (37, 93, 98). A concern that involves all kinds of antisense agents is possible interference of RNAs other than mRNA. Such interference can largely affect specificity and it is therefore problematic that knowledge about many of these other RNAs still is far from complete.

Paper IV demonstrate antimicrobial synergy between antisense PNAs and protein specific inhibitors when directed to the same genetic targets, and show that antisense PNA can help define drug mechanism of action. Furthermore, it is suggested that antisense agents may find future use in improving antimicrobial efficacy, and that strains treated with subinhibitory concentrations of target specific antisense could be screened against chemical libraries for detection of new target specific antimicrobials. This idea is not new (163, 181-184), but an approach using antisense agents to titrate down the expression of a specific gene is practically simple as no genome modifications are needed. This, in turn, enables screening in strains that are not compatible to cloning, of course provided that the antisense agent may penetrate into the cells.

While a screen using target specific antisense would enable detection of target specific antimicrobial compounds, an antisense agent aiming to silence the expression of a specific transcription factor could render screens for pathway specific compounds possible (Figure 12). It is likely that such screens could show more relevance as many

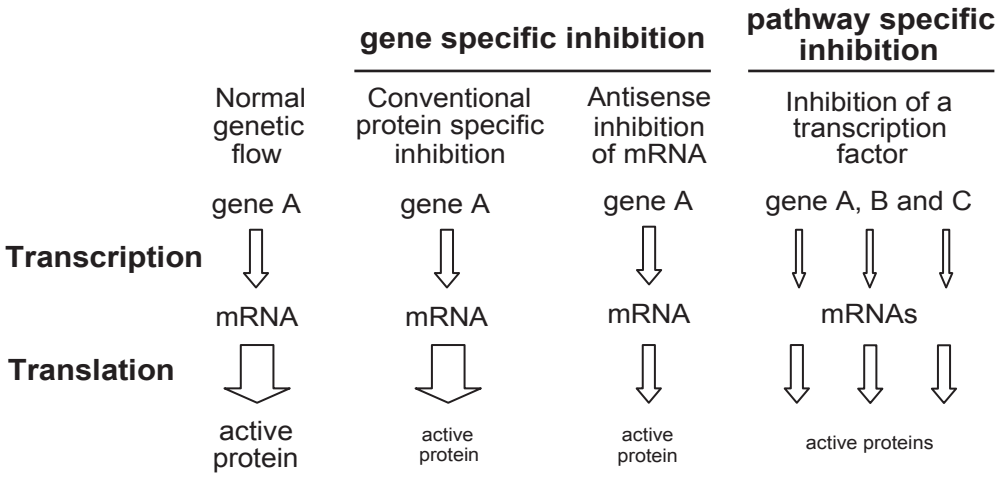


Figure 12. Different strategies for gene expression inhibition. The figure shows some key steps in the genetic flow and the target level for some different gene inhibition strategies with font size as an indicator of the amount of active protein(s). Gene specific inhibition can either occur at the protein level as is the case with some conventional inhibitors or at the mRNA level where antisense agents can inhibit translation. Pathway specific inhibition using antisense against a transcription factor is also suggested where the products of several genes are simultaneously reduced.

antimicrobial compounds display broader inhibitory activity than just against one gene product. However, it must initially be asked whether transcription factors constitute efficient targets for antisense and also if such inhibition can sensitise cells to inhibition of the genes whose expression the transcription factor regulates.

Finally, paper V shows that antisense PNA directed to genes within operons may largely affect the expression of cotranscribed genes and that the effects are not only detectable at the protein level, but also as increased transcript instability. Therefore, the specificity of sterically hindering antisense agents, at least in some cases, might be determined by quantifications of mRNA levels. This implies that relatively simple RNA quantification methods can be applied to determine specificity of sterically hindering antisense agents.

Several of the studies presented in this thesis investigate how to obtain specific and potent antisense effects in bacteria using PNA. Studies on the impact from conjugated peptides, antisense length, mRNA target site and the genetic environment of the targeted gene has generated a number of guidelines that may help improve antisense design. Also important, some outside resources have proven invaluable for designing

specific antisense PNAs, namely; the T_m-calculator developed by Giesen and co-workers (142), the Institute Pasteur web server that allows scanning for unique target sequences within desired parts of mRNA regions in a number of microorganisms (www.pasteur.fr/externe) and, finally, the RegulonDB where frequently updated information about *E. coli* transcription units and operons is available ((185), www.cifn.unam.mx/Computational_Genomics/regulondb/). Taken together, these tools allow the design of reasonably efficient antisense oligomers against one gene within half a day. This is relatively slow, especially considering that the design of antisense PNAs directed to the over 4400 genes of *E. coli* would require more than six years of work. In order to speed up this process, we are presently developing an automated design program that has the above mentioned factors integrated. Our longer-term aim is to provide a web-based tool that select antisense agents of different chemistries that target genes from a broad range of bacterial species.

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