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Peptide Nucleic Acid (PNA) Antisense Effects in *Escherichia coli*

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Antisense peptide nucleic acid (PNA) can be used to control cell growth, gene expression and growth phenotypes in the bacteria *Escherichia coli*. PNAs targeted to the RNA components of the ribosome can inhibit translation and cell growth, and PNAs targeted to mRNA can limit gene expression with gene and sequence specificity. In an *E. coli* cell extract, efficient inhibition is observed when using PNA concentrations in the nanomolar range, whereas micromolar concentrations are required for inhibition in growing cells. A mutant strain of *E. coli* that is more permeable to antibiotics also is more susceptible to antisense PNAs than the wild type. This chapter details methods for testing the antisense activities of PNA in *E. coli*. As an example of the specific antisense inhibition possible, we show the effects of an anti-ß-galactosidase PNA in comparison to control PNAs. With improvements in cell uptake, antisense PNAs may find applications as antimicrobial agents and as tools for microbial functional genomics.

Introduction

Bacteria possess endogenous antisense mechanisms for gene control and there would be many attractive applications for effective antisense agents. Standard antisense oligodeoxynucleotides have been used in bacterial cell free systems to study ribonucleoprotein complexes and there have been attempts to use oligodeoxynucleotides to control gene expression in bacterial cells (1-3). The results for *E. coli* when using oligodeoxynucleotides have been variable (4,5), but certain bacterial species with less stringent outer barriers can take-up oligodeoxynucleotides (6,7). Therefore, results from several studies indicate that antisense agents have some potential in bacterial systems. The challenge is to design molecules that can cross bacterial membranes and inactivate RNA targets.

Peptide nucleic acid (PNA) is a DNA mimic with nucleobases attached to a pseudo-peptide backbone (8). PNA hybridises with complementary sequences through Watson and Crick base pairing and helix formation (9). The peptide backbone provides superior hybridisation (10), resistance to enzymatic degradation (11) and access to a variety of chemical modifications (12). For antisense applications, target bound PNA can cause steric hindrance of DNA and RNA polymerases, reverse transcriptase, telomerase and the ribosome (13, 14, 15 16). For an introduction to PNA and its properties see the review in this issue by Nielsen and Egholm (17).

Antisense PNAs were applied to bacterial systems with the rational that improved hybridisation and stability properties should provide more potent inhibitory effects. By using standard *in vitro* transcription and translation reactions and *in vivo* growth

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and reporter gene expression assays we have demonstrated reproducible antisense inhibitory effects that are dependent on base-pairing at the target site (15, 16). In retrospect, one can think of additional reasons for using PNA as an antisense agent in *E. coli*. For example, the positively charged lysine residues included within typical PNA constructions could help localise PNA to the negatively charged surface of gram negative bacteria, whereas standard nucleic acids would be repelled. Therefore, the pseudo-peptide backbone of PNA may provide several advantages for the development of bacteria targeted antisense agents.

Protocols

Target Site Selection

Anti-Ribosomal PNAs
PNAs have been designed to target accessible sequences within ribosomal RNA. Target sites were chosen according to evidence of functional importance and accessibility to translation factors, antibiotics, probing agents or oligonucleotides (15). Ribosomal RNA is well characterised in this respect, but the RNA components of other ribonucleoprotein complexes are also potential targets. For example, PNA can act as a potent inhibitor of telomerase *in vitro* (18). In our experience with bacteria, those PNAs that inhibited translation and cell growth are triplex forming bis-PNAs, which are expected to form very stable complexes. Duplex forming PNAs were not found to be inhibitory (15). Therefore, in some cases, efficient inhibition may depend on the higher stability of triplex structures (19).

Anti-Sense PNAs
There are only loose theoretical rules for antisense target site selection. Nevertheless, some helpful guidelines are available from eukaryotic studies. For example, the start codon region is considered to be a susceptible target for antisense inhibition, and *in vitro* experiments have shown that eukaryotic translation can be inhibited by PNA binding to this region (13,14). Accordingly, the antisense PNAs that have been designed for *E. coli* have been targeted against the start codon region, however, there may be more susceptible sites within the long mRNA sequences. Also, the antisense PNAs that have been used in the bacterial studies are 15 mer molecules (16). This is a reasonable size for stability and sequence selection, but shorter PNAs may provide sufficient specificity and better uptake. Experiments are underway to evaluate the antisense activities of PNAs that vary in size and PNAs that are targeted to different gene regions. Finally, as a control, gene sequence mutations at the target site can be introduced in *E. coli* and used as a stringent test of the antisense mechanism of inhibition.

In Vitro Assays
Efficient *in vitro* translation in prokaryotic cell extracts requires simultaneous or coupled transcription and translation from added template DNA. *E. coli* systems for coupled transcription and translation are based on cell extracts that have been cleared by centrifugation and depleted of endogenous templates and small molecular weight components. Synthesis is started with the addition of plasmid-borne template DNA and the substrates needed for transcription and translation. Kits for coupled transcription and translation are available from several suppliers, however, we have found that large quantities of extract can be produced at a low cost using the protocols described in detail by Thorson et al. (20).

Materials

S-30 cell extract	8.5 µl
Transcription/translation buffer	7.5 µl
50 mM magnesium acetate	4 µl
Plasmid template DNA	1 µg
Water to	30 µl

Method

1. Thaw reaction components and keep all reagent and reaction tubes on ice.
2. Aliquot all reagents, excepting the S-30 extract, into siliconised microfuge tubes, vortex to mix and ensure that all of the liquid is at the bottom of the tube. PNA or other inhibitors can be added at this point. For *in vitro* reactions, PNA is typically effective when present in nanomolar concentrations.
3. Pipet the S-30 extract onto the inside wall of the microfuge tube and start the reactions by vortexing the mixture. Again, ensure that all of the liquid is at the bottom of the tube. Incubate tubes at 37°C for 30-60 minutes. Stop the reactions by placing the tubes on ice.

Assay for Translation Inhibition

To assay the overall level of protein synthesis, the buffers for coupled transcription and translation must lack at least one amino acid, which is provided separately as a radioactive isotope (typically methionine S^{35}). Incorporation of the isotope is measured as the radioactivity that can be precipitated in cold 25% TCA (containing 1% casein hydrolysate) and retained on filter paper (e.g. Watman GFA) after vacuum filtration and repeated washing with cold 5% TCA.

Assay for ß-Galactosidase and ß-Lactamase Reporter Gene Expression

ß-galactosidase and ß-lactamase are convenient reporter genes and widely available on plasmids. Enzyme production from these genes can be measured using the chromogenic ß-galactosidase substrate, *o*-nitrophenyl-ß-galactoside (ONPG), and a ß-lactamase substrate, pyridinium-2-azo-p-dimethylaniline chromophore (PADAC). ONPG can be stored at -20°C at 4 mg/ml in water, used at a final concentration of 0.4 mg/ml and assayed by absorbance measurements at 420 nm (21). PADAC can be stored at -20°C in methanol/1% acetic acid at 1 mg/ml, used at a final concentration of 10 ug/ml and assayed by absorbance measurements at 570 nm (22).

Liquid Culture Assays

E. coli can be grown conveniently as 100-200 µl cultures in siliconised microfuge tubes or microtitre plate wells. To limit PNA binding to the surface, polypropylene plates are recommended (eg, Costar # 3790). Also, improved antisense effects have been obtained using the permeable *E. coli* strain AS19 and Luria-Bertani (LB) media diluted to 10% of normal strength. Aliquote PNAs from stock solutions into diluted LB media to final concentrations in the high nanomolar to micromolar range. Inoculate the cultures with a 1% volume of an overnight *E. coli* culture and incubate overnight at 37°C. The next day, the cultures can be scored for growth by optical density measurements taken at 550 nm. *Beta*-galactosidase and ß-lactamase activities can be assayed as described above.

Solid Culture Assays

The solid culture assay described here is a modification of the "zone-of-inhibition" assay that is often used to test antibiotic potency. *E. coli* and many other species of bacteria can be grown as a "lawn" of cells on the surface of agar media in a petri plate. For antibiotic testing, it is standard practice to soak a small piece of filter

paper with a known concentration of an antimicrobial agent and then place the paper on the surface of agar medium. After bacterial growth, the size of the zone of inhibition is taken as an indication of the antimicrobial potency and susceptibility of the cells. PNA can be tested in a similar way, however, PNA diffuses only slowly within agar media. Therefore, it is appropriate to prepare a thin lawn of cells and apply the PNA solution by direct pipetting onto the surface of the agar.

Materials

Petri plates containing solid 10% LB/1.5% agar media.
Molten 10% LB/1% agar media, stored at 55°C.
Overnight culture of *E. coli* adjusted to approximately 1.0 $OD_{550\ nm}$.

Method

1. Pre-warm petri plates to 37°C.
2. Add a 1% inoculum of overnight culture to the molten agar. Quickly pour approximately 5 ml of the inoculated agar media onto the surface of an agar plate. Tilt the plate to spread the molten media across the entire surface and pour-off the excess.
3. PNA solutions with concentrations in the low to mid micromolar range are pipetted directly onto the surface, 3-5 µl per application.
4. Incubate the plates overnight at 37°C.

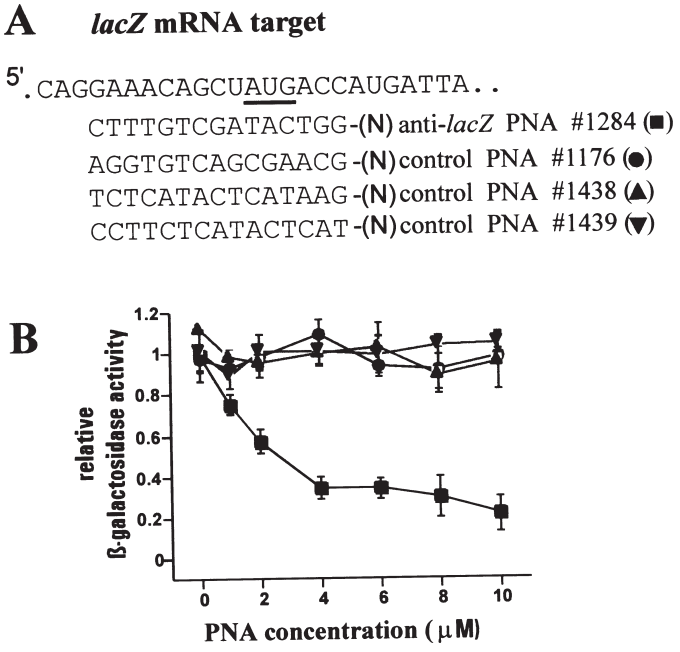


Figure 1. Inhibition of ß-galactosidase expression in *E. coli* AS19. Relative enzyme activities are shown for cultures containing anti-ß-galactosidase PNA and control PNAs. The values shown are the average of two replicates with relative values calculated by division with those obtained for cultures lacking PNA. The error bars indicate the standard deviation.

Example

PNA-Mediated Down-Regulation of ß-Galactosidase

Here we show the results obtained when using an antisense PNA targeted to the start codon region of the *E. coli* ß-galactosidase gene (*lacZ*). The anti-*lacZ* PNA was included in liquid cultures prepared as described above, using 50 µM of the *lac* operon inducer isopropyl ß-D-thiogalactopyranoside (IPTG). For comparison, control PNAs of similar size and composition were included in the experiment. After overnight growth, the level of ß-galactosidase activity was determined using the chromogenic substrate ONPG. The results are illustrated in Figure 1 (16). Dose dependent inhibition was observed when using antisense PNA concentrations ranging from 1 to 10 µM, while the control PNAs were not inhibitory. The results show that antisense PNAs can be used to specifically decrease gene expression in *E. coli*.

Applications and Limitations

There are many situations where it would be useful to specifically control bacterial gene expression with antisense agents. The methods described here provide a starting point for developing antisense PNAs for bacterial research and possible practical applications. The most attractive application is to develop sequence-designed antimicrobials that could be used to control infections. However, this application requires improved *in vivo* activities. Also, anti-bacterial PNAs may prove useful in studies of growth and infection. Antisense strategies can be helpful in identifying drug targets when used in animal models (23) and there are possibilities to use PNA to reveal susceptible gene targets for antibiotics (24).

A general problem with the development of antisense agents is poor cellular uptake. The outer membrane of gram negative bacteria can provide impressive resistance against a wide variety of compounds. However, there are examples of permeation by large molecular weight antibiotics (25). In our experience with PNA, i*n vivo* activities relative to *in vitro* results suggest that only a small proportion of added PNA is able to act within cells. Also, the increased susceptibility of the permeable *E. coli* strain AS19 indicates that the bacterial cell wall is a significant barrier against efficient antisense effects. Fortunately, there are opportunities to understand more about how PNAs can enter bacteria and there are possibilities to overcome cell barriers by introducing further modifications or conjugations.

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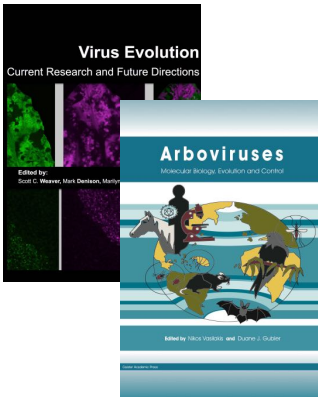
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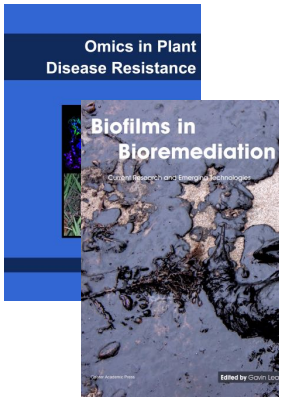
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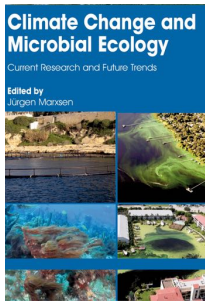
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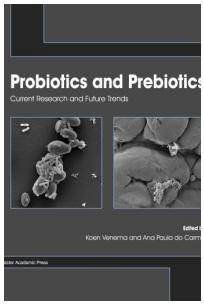
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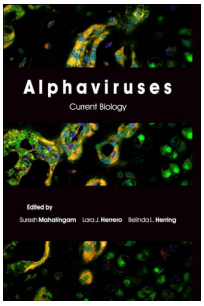
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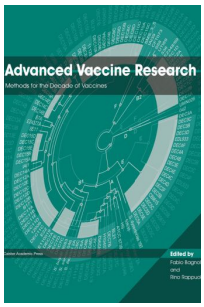


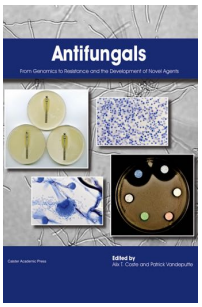












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