Antisense PNA effects in *Escherichia coli* are limited by the outer-membrane LPS layer

Liam Good,¹ Rickard Sandberg,¹ Ola Larsson,¹ Peter E. Nielsen² and Claes Wahlestedt¹

Author for correspondence: Liam Good. Tel: +46 8 728 6697. Fax: +46 8 323950.
e-mail: liam.good@cgr.ki.se

¹ Center for Genomics Research, Karolinska Institute, Berzelius väg 37, 171 77, Stockholm, Sweden

² Center for Biomolecular Recognition, Panum Institute, Blegdamsvej 3c, DK 2200 Copenhagen N., and Pantheco A/S, Fruebjergvej 3, DK 2100 Copenhagen Ø., Denmark

Antisense peptide nucleic acids (PNAs) can inhibit *Escherichia coli* gene expression and cell growth through sequence-specific RNA binding, and this opens possibilities for novel anti-infective agents and tools for microbial functional genomics. However, the cellular effects of PNAs are limited relative to effects in cell extracts, presumably because of cell barrier components such as the outer-membrane lipopolysaccharide (LPS) layer or drug efflux pumps, both of which function to exclude antibiotics and other foreign molecules. To evaluate the importance of such cellular factors on PNA effects, the authors developed a positive assay for antisense inhibition by targeting the *lac* operon repressor and compared PNA susceptibilities in mutant and wild-type *E. coli* by assessing *lacZ* induction. Strains with defective LPS (AS19 and D22) were more permeable to the antibiotic nitrocefin and more susceptible to PNA than the wild-type. Also, PNA potency was improved in wild-type cells grown in the presence of certain cell-wall-permeabilizing agents. In contrast, the activities of the Acr and Emr drug efflux pumps were not found to affect PNA susceptibility. The results show that the LPS layer is a major barrier against cell entry, but PNAs that can enter *E. coli* are likely to remain active inside cells.

Keywords: peptide nucleic acid, bacteria, lipopolysaccharide

INTRODUCTION

Bacteria possess endogenous antisense gene control mechanisms (Simons & Kleckner, 1988), and recent experiments show that antisense gene inhibition in bacteria is possible using synthetic antisense agents. Peptide nucleic acids (PNAs) targeted to 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 (Good & Nielsen, 1998a, b). Therefore, PNAs can provide tools for functional analyses of bacterial genes and there are attractive possibilities for novel sequence-designed anti-infective agents (Good & Nielsen, 1998a, b; Ecker & Freier, 1998). To improve the approach, the challenge is to learn more about cellular limits to PNA activity and design molecules to more efficiently enter cells and access RNA targets.

PNA is a DNA mimic with nucleobases attached to a

pseudopeptide backbone (Nielsen *et al.*, 1991) (Fig. 1a). PNA hybridizes to complementary DNA, RNA or PNA oligomer sequences through Watson and Crick base pairing and helix formation (Egholm *et al.*, 1993; Wittung *et al.*, 1994; Jensen *et al.*, 1997). The peptide backbone provides improved hybridization affinity and specificity properties (Egholm *et al.*, 1993; Jensen *et al.*, 1997), resistance to enzymic degradation (Demidov *et al.*, 1994) and access to a variety of chemical modifications (Nielsen & Haaima, 1997; Püschl *et al.*, 1998). For antisense applications, target-bound PNA can hinder the activities of DNA and RNA polymerases, reverse transcriptase, telomerase and the ribosome (Hanvey *et al.*, 1992; Knudsen & Nielsen, 1996; Good & Nielsen, 1998a). Also, *in vitro* assays for telomerase activity and mitochondrial genome replication show that PNAs are often effective in applications where other (modified) oligonucleotides perform poorly (Norton *et al.*, 1996; Taylor *et al.*, 1997).

Limited cellular uptake is a general difficulty with antisense agents. Furthermore, restricted uptake is expected with *Escherichia coli* because of stringent

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PNA, peptide nucleic acid.

cellular barriers against foreign molecules (Nikaido, 1994). The outer barrier of Gram-negative bacteria consists of a peptidoglycan layer, a lipid bilayer and an external lipopolysaccharide (LPS) coating. Furthermore, foreign compounds that do enter cells can be exported by various translocases, which are believed to 'pump out' a wide range of foreign compounds including certain peptides (Nikaido, 1994; Lewis, 1994). Therefore, typical bacterial cells are well equipped to resist foreign molecules, such as PNA. Indeed, results from cell extract and cell culture experiments suggest that only a small proportion of PNAs can enter and act within cells (Good & Nielsen, 1998a, b).

To study factors that limit PNA antisense effects apart from nonspecific inhibition, we designed a PNA to target the *lac* repressor mRNA. Limited repressor expression was seen as induced β -galactosidase (LacZ) activity in *E. coli* cells and taken as an indicator of antisense effect. The anti-*lac* repressor PNA was used together with mutant *E. coli* strains and chemicals known to disrupt cell wall components. The results show that the outer-membrane LPS layer is a major limit to PNA cell entry and antisense effects in *E. coli*.

METHODS

Bacteria and plasmids. The *E. coli* strains used in this study are listed in Tables 1 and 3. Plasmid pBX1, which carries a wild-type copy of the *acr* operon, was obtained from Kevin Bertrand (Washington State University), and plasmid pEMR2.1, which carries a wild-type copy of the *emrB* drug translocase, was obtained from Kim Lewis (Tufts University).

PNAs and other chemicals. The PNAs used in this study were synthesized as described by Christensen *et al.* (1995), and their structures are shown in Fig. 1. The cell-wall-active chemicals were from Sigma and the nitrocefin was from Oxoid.

Bacterial growth conditions. Growth on solid media was initiated with an inoculum of 10^4 cells ml^{-1} in an overlay of agar media, and growth in liquid media was initiated with an inoculum of 10^4 cells ml^{-1} in 200 μl cultures in siliconized polypropylene microdilution tubes. The cultures were grown in 10% Luria-Bertani medium at 37 °C for 18 h. The chemicals listed in Table 2 were included in liquid cultures over the nanomolar concentration range. PNAs and the chemicals listed in Table 2 were added to cultures prior to overnight growth.

β -Galactosidase activity assays. To indicate LacZ activity in solid cultures, the chromogenic substrate X-Gal was included in the agar plates at 250 $\mu\text{g ml}^{-1}$ and 4 μl PNA aliquots of PNA were applied by pipette directly onto the agar surface before growth. β -Galactosidase activity in liquid cultures was measured by using the chromogenic LacZ substrate ONPG, essentially as described by Miller (1972).

Nitrocefin outer cell barrier permeability assay. Outer-membrane permeability was determined essentially as described by Angus (1982). *E. coli* cells transformed with pBR322 were cultured to mid-exponential phase, recovered by centrifugation at 3000 g and rinsed with 5 mM HEPES (7.4). Reactions were in 96-well microtitre plates containing 0.1 OD₅₅₀ units of cells in 5 mM HEPES (7.4), 5 mM carbonyl

Fig. 1. PNA-mediated induction of *lacZ*. (a) Structure of PNA; 'b' represents a purine or pyrimidine base. (b) Sequence of the *lac* repressor mRNA start codon region (start codon GUG underlined) and the antisense and control PNAs. (c) Structure of the *lac* operon and the PNA-mediated induction strategy. (d) PNA-mediated up-regulation of the *lac* operon *E. coli* strain AS19 grown on agar medium. Antisense and control PNAs were applied by direct pipetting onto overlay cultures before overnight growth. The chromogenic LacZ substrate X-Gal was included at 250 $\mu\text{g ml}^{-1}$ and PNA solutions were applied by pipette directly onto the agar surface before growth by using 4 μl applications. β -Galactosidase induction by the anti-*lacI* PNA is visible as blue zones against the lawn of cells with repressed *lacZ* expression (top row).

cyanide *m*-chlorophenylhydrazine (CCCP), and 20 μg nitrocefin ml^{-1} . Nitrocefin cleavage was monitored by measuring A_{500} .

RESULTS AND DISCUSSION

PNA-mediated up-regulation of the *lac* operon

To establish a positive assay for PNA antisense effects, we designed a new PNA (#1709) to target the start codon region of the *E. coli lac* repressor gene (*lacI*). The *lacI* gene normally represses the *lac* operon and limits expression of the *lacZ* gene (Miller, 1972). To test the potential of the anti-*lacI* antisense strategy, PNA #1709 was applied to a lawn of *E. coli* AS19 cells grown on an LB agar plate containing the LacZ substrate X-Gal, but not the *lac* operon inducer IPTG (Fig. 1). Increased *lacZ* expression was apparent as blue zones at sites of antisense PNA application (Fig. 1). Control PNAs did not up-regulate *lacZ*. Therefore, induction of *lacZ* by PNA #1709 provides a positive assay for PNA antisense effects. The assay can be used to assess factors that limit

Table 1. PNA-mediated *lacZ* induction and outer-membrane permeability of various *E. coli* strains

The table includes data for wild-type *E. coli* and strains with LPS defects. Bacteria were cultured with 2 μ M PNA #1709. Values for *lacZ* induction show the level of enzyme activity in cultures treated with PNA over the level for untreated cultures. Cell permeation by nitrocefin is indicated as the time in minutes to observe a 50% change from the initial to final A_{500} ($t_{1/2}$). The values are means $\pm$ SD for three replicates. The F42 episome was introduced into strain D22 by mating to confer an active *lac* operon (Miller, 1972). Outer-membrane permeability to nitrocefin (Angus *et al.*, 1982) was determined using cells carrying the β -lactamase gene within pBR322. CGSC indicates the Coli Genetic Stock Center, Yale University.

Strain	Relevant phenotype or genotype	<i>lacZ</i> induction (PNA treated/untreated)	Cell permeation by nitrocefin ($t_{1/2}$, min)	Source
K-12		1.3 $\pm$ 0.3	30 $\pm$ 6	CGSC 5743
B		0.9 $\pm$ 0.2	29 $\pm$ 5	CGSC 6739
ML35		1.2 $\pm$ 0.6	34 $\pm$ 4	R. Lehrer, UCLA
AS19	LPS defect	9.0 $\pm$ 0.9	4.0 $\pm$ 1	S. Pedersen, University of Copenhagen
D22	LPS defect (<i>envA</i>)	3.0 $\pm$ 0.8	10 $\pm$ 2	H. Boman, Karolinska Institute

or improve the antisense approach, apart from non-specific inhibitory effects.

LPS layer and limited antisense effects

The outer cell membrane of Gram-negative bacteria is coated with an LPS layer that stringently limits the influx of foreign molecules (Nikaido, 1994; Hancock, 1997), and this barrier also may restrict PNA. In our previous experiments, efficient inhibition *in vitro* was observed with PNA concentrations in the nanomolar range, whereas growth inhibition in cells required micromolar concentrations, dilute growth medium and the cell-wall-deficient strain AS19 (Good & Nielsen, 1998a, b). These results suggest that cell uptake, rather than limited intracellular activities, is the major limit against the use of antisense PNAs in bacteria. Therefore, we aimed to determine whether PNA susceptibility correlates with outer-barrier permeability and whether PNA antisense effects can be improved by compromising the integrity of the LPS. It was hoped that the results would clarify the need for improved uptake properties in the next generation of antisense PNAs.

Wild-type and permeable mutant *E. coli* strains were tested for susceptibility to antisense PNA #1709. Among the strains listed in Table 1, AS19 and to a lesser extent D22 showed increased induction of *lacZ* by PNA #1709; both of these susceptible strains are LPS defective. Strain AS19 has a severely depleted LPS layer (Zorzopulos *et al.*, 1989) and is more susceptible to antibiotics (Sekiguchi & Iida, 1967). Also, this strain is unusually susceptible to PNAs targeted to the rRNA and reporter gene mRNAs (Good & Nielsen, 1998b). Strain D22 is defective in the lipid A component of its LPS and

Fig. 2. Antisense PNA induction of the *lac* operon and effect of polymyxin B nonapeptide. The cell wall permeability increasing agent polymyxin B nonapeptide was included in cultures of wild-type *E. coli* K-12 containing 2 μ M anti-*lacI* PNA #1709. Induction of the *lac* operon was measured by assaying LacZ activity after growth in the presence and absence of PNA (2 μ M) and a range of polymyxin B nonapeptide concentrations (see also Table 1).

more susceptible to antibiotics (Normark *et al.*, 1969). Therefore, LPS defects in *E. coli* appear to confer unusual PNA susceptibility.

To establish a more direct link between LPS integrity and PNA susceptibility for strains AS19 and D22, we tested outer-barrier permeability to nitrocefin, a chromogenic cephalosporin antibiotic. Nitrocefin is normally excluded from cells, but nitrocefin molecules that do permeate the outer membrane are cleaved by periplasmic β -lactamase and undergo a change in absorbance maxi-

Table 2. PNA-mediated *lacZ* induction in the presence of cell-wall-active agents

The relative level of *lacZ* induction in *E. coli* K-12 cells grown in the presence of subinhibitory concentrations of cell-active agents is shown. Bacteria were cultured with 2 µM PNA #1709. Relative values indicate the increase in LacZ activity for cultures containing added chemical agents relative to control cultures lacking added chemical agents. The values are means $\pm$ SD for three replicates. The chemical agents were tested over a range of concentrations; the most effective are shown in parentheses. As an example, *lacZ* induction in the presence of polymyxin B nonapeptide is shown in Fig. 2.

Chemical	Relative <i>lacZ</i> induction	Reference for permeabilizing activity
Ampicillin*	1 $\pm$ 0.1	Sieradzki & Tomasz (1997)
Cefotaxime*	1 $\pm$ 0.1	Sieradzki & Tomasz (1997)
EDTA	1 $\pm$ 0.1	Gerberick & Castric (1980)
Polymyxin B nonapeptide	6 $\pm$ 1.0 (50 nM)	Chunhong <i>et al.</i> (1998)
Polymyxin B sulfate	2 $\pm$ 0.9 (20 nM)	Chunhong <i>et al.</i> (1998)
Polyethyleneimine	1 $\pm$ 0.1	Helander <i>et al.</i> (1997)
Prochlorperazine	2 $\pm$ 0.4 (50 nM)	Motohashi <i>et al.</i> (1997)
Promethazine	1 $\pm$ 0.1	Motohashi <i>et al.</i> (1997)
Sodium hexametaphosphate	1 $\pm$ 0.3	Vaara & Jaakkola (1989)

* Asterisks indicate chemicals that compromise formation of the peptidoglycan layer; all other agents listed permeabilize the outer cell wall.

mum from 385 nm to 490 (Angus *et al.*, 1982). *E. coli* strains were transformed with pBR322, selected for ampicillin resistance and subjected to the nitrocefin permeability assay (Table 1). To block respiration and eliminate the potential for active efflux of nitrocefin, CCCP was included at 5 µM. The results showed high outer cell barrier permeability for strain AS19 and to a lesser extent for strain D22 (Table 1, Fig. 2). These strains also showed high PNA susceptibilities (Table 1). The link between PNA susceptibility and LPS permeability suggests that the integrity of the LPS layer is a major determinant of PNA entry into *E. coli*.

To further investigate the importance of the LPS layer in the susceptibility of *E. coli* to PNA, we asked whether PNA antisense effects in wild-type *E. coli* strain K-12 can be improved by using chemicals that compromise LPS integrity. The anti-*lac* repressor PNA #1709 was used in combination with a range of compounds that permeabilize *E. coli* and can increase the uptake or potency of standard antibiotics (Table 2). Several of these agents enhanced PNA potency; the most effective was polymyxin B nonapeptide, which enhanced *lacZ* induction by approximately sixfold when present at low nanomolar concentrations (Fig. 2). An important factor in the effectiveness of polymyxin B nonapeptide in this application may be its ability to permeabilize the outer membrane with relatively low toxicity to *E. coli* (Chunhong *et al.*, 1998). Antibiotics that block peptidoglycan formation did not enhance PNA effects (Table 2). Synergism between polymyxin B nonapeptide and antisense PNAs is unlikely to be useful in practical applications, but it is important as further evidence that the LPS is a major barrier to PNA uptake.

Multidrug resistance translocase activity and PNA susceptibility

Intracellular accumulation of PNAs and antisense effects also could be limited by multidrug resistance translocases, which can clear a diverse range of foreign agents from cells, including peptide antibiotics (Shafer *et al.*, 1998). Several drug translocases are associated with multidrug-resistance phenotypes in bacteria, and these 'efflux pumps' display surprising substrate flexibility. Therefore, we investigated whether the best-known *E. coli* drug pumps could limit PNA-mediated effects. The level of *lacZ* induction by PNA #1709 was determined in *E. coli* strains with altered drug efflux activities. Strains with defective copies of the *acrAB* or *emrA* transporter genes did not differ from the wild-type in susceptibility to PNAs (Table 3). Also, plasmids bearing cloned copies of the *acrA,B* and *emrB* transporter genes were introduced by transformation to complement the defective endogenous genes in strains N43 and SJ261, and active copies of these genes did not alter PNA susceptibilities (Table 3).

The *acr* and *emr* drug translocase genes are among the best-characterized *E. coli* drug pumps, but other translocases also could affect PNA susceptibility. A range of translocases are implicated in drug resistance and there may be as many as 18 ABC-type transporter homologues within the *E. coli* genome (Blattner *et al.*, 1997). Accordingly, drug translocase involvement in PNA susceptibility was tested in a more general way using the inhibitors verapamil and reserpine, which can reduce drug efflux in *E. coli* and other types of cells (Beja & Bibi, 1996; Ng *et al.*, 1994). Neither of these compounds altered the PNA susceptibility of *E. coli* K-12. Therefore,

Table 3. PNA-mediated *lacZ* induction in various *E. coli* strains with altered translocase activities

The table includes data for wild-type *E. coli* and strains with defects in translocase genes associated with drug efflux. The level of PNA-mediated *lacZ* induction shows the level of enzyme activity in cells treated with PNA relative to untreated cells. Bacteria were cultured with 2 μ M PNA #1709. The values are means $\pm$ SD for three replicates.

Strain	Relevant phenotype or genotype	<i>lacZ</i> induction (PNA treated/ untreated)	Source
K-12		1.3 $\pm$ 0.3	CGSC 5743
N43	Translocase defect (<i>acrA</i>)	1.4 $\pm$ 0.2	CGAS 5583
N2616	Translocase defect (<i>acrA</i>)	0.8 $\pm$ 0.2	CGSC 6907
SJ261	Translocase defect (<i>emrB</i>)	1.1 $\pm$ 0.3	Kim Lewis, Tufts Univ., Medford, MA
PM61R	Translocase defect (<i>acrE</i>)	1.0 $\pm$ 0.2	CGSC 6629
N43/pBX1	<i>acrA</i> overexpression	1.2 $\pm$ 0.4	This study
SJ261/pEMR2.1	<i>emrA,B</i> overexpression	1.1 $\pm$ 0.2	Kim Lewis

the activity of drug efflux translocases does not appear to alter the susceptibility of *E. coli* to PNAs.

Conclusions

Induction of *lacZ* by an anti-*lacI* PNA provides a positive assay for antisense effects. In this study the assay was used to learn which cell-barrier components limit PNA cell entry and antisense effects in *E. coli*. The results, together with our previous work, provide three lines of evidence indicating that the LPS layer is a major barrier against PNA uptake. First, the LPS mutant strains AS19 and D22 are more susceptible to PNA than wild-type. Second, mutant *E. coli* strains that are more susceptible to PNA also are more permeable to the antibiotic nitrocefin. Third, chemical agents that compromise LPS integrity can also increase cellular PNA susceptibility. Fortunately, for practical applications, there are opportunities to modify PNAs to better permeate the outer-membrane LPS layer. Also, the activity of multidrug resistance translocases did not significantly alter the susceptibility of *E. coli* to PNA. This resistance against active efflux, along with evidence for PNA stability in biological fluids, suggests that PNAs that are able to gain access into bacteria are likely to remain active within cells. Therefore, efforts to improve the technology can focus on achieving improved uptake. Finally, the observation that an antisense PNA can up-regulate, as well as down-regulate, gene expression supports the idea that antisense PNAs can provide sequence specific tools for the analysis of microbial gene expression and function.

ACKNOWLEDGEMENTS

This research was supported by the Swedish Foundation for Strategic Research, Pharmacia Corp. and the Danish Biotechnology Programme. We thank Mary Berlyn, Kevin

Bertrand, Ole Micheson, Kim Lewis and Hans Boman for providing *E. coli* strains and plasmids.

REFERENCES

- Angus, B. L., Carey, A. M., Caron, D. A., Kropinski, A. M. & Hancock, R. E. (1982). Outer membrane permeability in *Pseudomonas aeruginosa*: comparison of a wild-type with an antibiotic-supersusceptible mutant. *Antimicrob Agents Chemother* **21**, 299–309.
- Beja, O. & Bibi, E. (1996). Functional expression of mouse Mdr1 in an outer membrane permeability mutant of *Escherichia coli*. *Proc Natl Acad Sci U S A* **93**, 5969–5974.
- Blattner, F. R., Plunkett, G., 3rd, Bloch, C. A. & 14 other authors (1997). The complete genome sequence of *Escherichia coli* K-12. *Science* **277**, 1453–1474.
- Chunhong, L., Peters, A. S., Meredith, E. L., Allman, G. W. & Savage, P. B. (1998). Design and syntheses of potent sensitizers of gram-negative bacteria based on cholic acid scaffolding. *J Am Chem Soc* **120**, 2961–2962.
- Christensen, L., Fitzpatrick, R., Gildea, B., Petersen, K. H., Hansen, H. F., Koch, T., Egholm, M., Buchardt, O. & Nielsen, P. E. (1995). Solid-phase synthesis of peptide nucleic acids. *J Peptide Sci* **3**, 175–183.
- Demidov, V., Potaman, V. N., Frank-Kamenetskii, M. D., Buchardt, O., Egholm, M. & Nielsen, P. E. (1994). Stability of peptide nucleic acids in human serum and cellular extracts. *Biochem Pharmacol* **48**, 1309–1313.
- Ecker, D. J. & Freier, S. M. (1998). PNA, antisense, and antimicrobials. *Nat Biotechnol* **6**, 332.
- Egholm, M., Buchardt, O., Christensen, L. & 7 other authors (1993). PNA hybridizes to complementary oligonucleotides obeying the Watson–Crick hydrogen bonding rules. *Nature* **365**, 566–568.
- Gerberick, G. F. & Castric, P. A. (1980). *In vitro* susceptibility of *Pseudomonas aeruginosa* to carbenicillin, glycine, and ethylenediaminetetraacetic acid combinations. *Antimicrob Agents Chemother* **17**, 732–735.

- Good, L. & Nielsen, P. E. (1998a). Inhibition of translation and bacterial growth by peptide nucleic acid targeted to ribosomal RNA. *Proc Natl Acad Sci U S A* **95**, 2073–2076.
- Good, L. & Nielsen, P. E. (1998b). Antisense inhibition of gene expression in bacteria by PNA targeted to mRNA. *Nat Biotechnol* **16**, 355–358.
- Hancock, R. E. W. (1997). The bacterial outer membrane as a drug barrier. *Trends Microbiol* **5**, 37–42.
- Hanvey, J. C., Peffer, N. J., Bisi, J. E. & 12 other authors (1992). Antisense and antigene properties of peptide nucleic acids. *Science* **258**, 1481–1485.
- Helander, I. M., Alakomi, H. L., Latva-Kala, K. & Koski, P. (1997). Polyethyleneimine is an effective permeabilizer of gram-negative bacteria. *Microbiology* **143**, 3193–3199.
- Jensen, K. K., Ørum, H., Nielsen, P. E. & Nordén, B. (1997). Kinetics for hybridization of peptide nucleic acids (PNA) with DNA and RNA studied with the BIAcore technique. *Biochemistry* **36**, 5072–5077.
- Knudsen, H. & Nielsen, P. E. (1996). Antisense properties of duplex- and triplex-forming PNAs. *Nucleic Acids Res* **24**, 494–500.
- Lewis, K. (1994). Multidrug resistance pumps in bacteria: variations on a theme. *Trends Biochem Sci* **19**, 119–123.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Motohashi, N., Kurihara, T., Kawasw, M. & 7 other authors (1997). Drug resistance reversal, anti-mutagenicity and anti-retroviral effect of phthalimido- and chloroethyl-phenothiazines. *Anticancer Res* **17**, 3537–3544.
- Ng, E. Y., Trucksis, M. & Hooper, D. C. Quinolone resistance mediated by *norA*: physiologic characterization and relationship to *flqB*, a quinolone resistance locus on the *Staphylococcus aureus* chromosome. *Antimicrob Agents Chemother* **38**, 1345–1355.
- Nielsen, P. E. & Haaime, G. (1997). Peptide nucleic acid (PNA). A DNA mimic with a pseudopeptide backbone. *Chem Soc Rev* **96**, 73–78.
- Nielsen, P. E., Egholm, M., Berg, R. H. & Buchardt, O. (1991). Sequence selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* **254**, 1497–1500.
- Nikaido, H. (1994). Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* **264**, 382–388.
- Normark, S., Boman, H. G. & Matsson, E. (1969). Mutant of *Escherichia coli* with anomalous cell division and ability to decrease episomally and chromosomally mediated resistance to ampicillin and several other antibiotics. *J Bacteriol* **97**, 1334–1342.
- Norton, J. C., Piatyczek, J. A., Wright, W. E., Shay, J. W. & Corey, D. R. (1996). Inhibition of human telomerase activity by peptide nucleic acid. *Nat Biotechnol* **14**, 615–619.
- Püschl, A., Sforza, S., Haaime, G., Dahl, O. & Nielsen, P. E. (1998). Peptide nucleic acids (PNAs) with a functional backbone. *Tetrahedron Lett* **39**, 4707–4710.
- Sekiguchi, M. & Iida, S. (1967). Mutants of *Escherichia coli* permeable to actinomycin. *Proc Natl Acad Sci U S A* **58**, 2315–2320.
- Shafer, W. M., Qu, X., Waring, A. J. & Lehrer, R. I. (1998). Modulation of *Neisseria gonorrhoeae* susceptibility to vertebrate antibacterial peptides due to a member of the resistance/nodulation/division efflux pump family. *Proc Natl Acad Sci U S A* **95**, 1829–1833.
- Simons, R. W. & Kleckner, N. (1988). Biological regulation by antisense RNA in prokaryotes. *Annu Rev Genet* **22**, 567–600.
- Sieradzki, K. & Tomasz, A. (1997). Suppression of beta-lactam antibiotic resistance in a methicillin-resistant *Staphylococcus aureus* through synergic action of early cell wall inhibitors and some other antibiotics. *J Antimicrob Chemother* **39**, suppl. A, 47–51.
- Taylor, R. W., Chinnery, P. F., Turnbull, D. M. & Lightowlers, R. N. (1997). Selective inhibition of mutant human mitochondrial DNA replication *in vitro* by peptide nucleic acids. *Nat Genet* **15**, 212–215.
- Vaara, M. & Jaakkola, J. (1989). Sodium hexametaphosphate sensitizes *Pseudomonas aeruginosa*, several other species of *Pseudomonas*, and *Escherichia coli* to hydrophobic drugs. *Antimicrob Agents Chemother* **33**, 1741–1747.
- Wittung, P., Nielsen, P. E., Buchardt, O., Egholm, M. & Nordén, B. (1994). DNA-like double helix formed by peptide nucleic acid. *Nature* **368**, 561–563.
- Zorzopulos, J., de Long, S., Chapman, V. & Kozloff, L. M. (1989). Evidence for a net-like organization of lipopolysaccharide particles in the *Escherichia coli* outer membrane. *FEMS Microbiol Lett* **52**, 23–26.

Received 2 May 2000; revised 21 June 2000; accepted 4 July 2000.