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Sensitization of *Campylobacter jejuni* to fluoroquinolone and macrolide antibiotics by antisense inhibition of the CmeABC multidrug efflux transporter

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Objectives: **The aim of this study was to investigate the feasibility and efficacy of antisense-mediated gene silencing by peptide nucleic acid (PNA) for specific inactivation of the CmeABC multidrug efflux transporter in *Campylobacter jejuni*.**

Methods: **PNA was designed to bind to the *cmeA* transcript and to inhibit the translation of CmeA, the periplasmic component of the RND-type CmeABC efflux transporter of *C. jejuni*. Inhibition of CmeA production was determined by western blotting. MICs of clinically important antibiotics, including ciprofloxacin and erythromycin, were measured in the presence of the CmeA-specific PNA (CmeA-PNA).**

Results: **CmeA-PNA greatly reduced the expression level of CmeA. Consistent with the reduced CmeA production, CmeA-PNA rendered *C. jejuni* strains more susceptible to ciprofloxacin and erythromycin. At a concentration of 2 μM, CmeA-PNA resulted in 8- and 4-fold reductions in the MICs of ciprofloxacin and erythromycin, respectively, in *C. jejuni* NCTC 11168. CmeA-PNA also increased the susceptibility to the antibiotics in *C. jejuni* strains that were resistant to ciprofloxacin or erythromycin.**

Conclusions: **Antisense technology is a feasible method to suppress the function of the CmeABC multidrug efflux transporter, which may be further exploited to control antibiotic-resistant *Campylobacter*.**

Keywords: gene silencing, antibiotic resistance, peptide nucleic acid

Introduction

Campylobacter jejuni is a leading bacterial cause of human gastroenteritis worldwide and is increasingly resistant to various antibiotics.[1] Among the multiple mechanisms utilized by *C. jejuni* for antibiotic resistance, the multidrug efflux pump CmeABC plays an important role and confers resistance to a broad range of antimicrobials, including clinically important antibiotics (e.g. fluoroquinolones and macrolides), toxic chemicals and bile acids.[2] As an RND-type efflux transporter, CmeABC consists of the periplasmic fusion protein (CmeA), inner membrane transporter (CmeB) and outer membrane channel protein (CmeC). The three components are encoded by a single polycistronic operon (*cmeABC*).[2] Because of the significant role of drug efflux transporters in antibiotic resistance, there have been many studies attempting to control drug resistance in pathogenic bacteria including *C. jejuni* using efflux pump inhibitors.[3] These efflux pump inhibitors block the function of drug efflux transporters by either disrupting the driving forces of the transporters or competitive binding to efflux transporters. However, these inhibitors are usually of low specificity and are potentially toxic to animal hosts,[3] underlining the need for exploring novel approaches for efflux transporter inhibition.

Peptide nucleic acid (PNA) antisense agents are DNA-mimic synthetic polymers, carrying a pseudo-peptide backbone with nucleic acid bases.[4] Because of its specific recognition of nucleotide sequences, strong affinity to nucleic acids and resistance to nuclease degradations, PNA is often used in antisense technology to inhibit gene expression with high specificity. PNA designed to bind *acpP*, an essential gene encoding the acyl carrier protein in *Escherichia coli*, effectively suppressed the translation of *acpP* by sterically hindering the attachment of translational machineries to the *acpP* transcript.[5] Despite its demonstrated usefulness and specificity in gene silencing in

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several bacteria, this antisense technology has not been utilized in the inhibition of multidrug efflux transporters in bacteria and has not been used in *C. jejuni* for specific silencing of gene functions. In this study, we applied this novel technology to inhibit the CmeABC multidrug transporter, which is an important antibiotic resistance determinant in *C. jejuni*. We demonstrated that a CmeA-specific PNA significantly inhibited CmeA production and sensitized *C. jejuni* strains to ciprofloxacin and erythromycin.

Materials and methods

Bacterial strains and PNA

C. jejuni NCTC 11168,[6] its isogenic CmeB mutant,[7] three fluoroquinolone-resistant *C. jejuni* isolates (S3BR1, CT2-2 and CB2-1) and three erythromycin-resistant *C. jejuni* isolates (S3BE2-2, S3BE4-2 and CT8-19) were used in this study. *C. jejuni* strains were cultured at 42°C in Mueller–Hinton (MH) medium (Difco) microaerobically (85% N_2, 5% O_2 and 10% CO_2). The PNA (TCATGGTTTTGC) complementary to the leader sequence (spanning the start codon and part of the ribosomal binding site) of *cmeA* was designed from the genome sequence of *C. jejuni* NCTC 11168.[6] The CmeA-specific PNA was conjugated to the oligopeptide KFFKFFKFFK, which was first used by Good *et al.*[5] to improve PNA entry into bacterial cells. The peptide-conjugated PNA was synthesized by PANAGENE (Taejeon, South Korea) and designated as CmeA-PNA in this study.

SDS–PAGE and western blotting

Overnight culture of *C. jejuni* NCTC 11168 grown on MH agar plates was resuspended in MH broth to an optical density of 0.05 at 600 nm and was microaerobically cultured in the presence of various concentrations of CmeA-PNA for 7 h with shaking at 200 rpm. SDS–PAGE and western blotting analysis were performed with anti-CmeA and anti-MOMP (where MOMP stands for major outer membrane protein) antibodies as described previously using a 10% polyacrylamide gel in Tris-Tricine buffer.[2]

Bacterial susceptibility to ciprofloxacin and erythromycin

The MICs of ciprofloxacin and erythromycin were determined with a microtitre broth dilution method as described previously with or without CmeA-PNA.[2] Ciprofloxacin and erythromycin were purchased from MP Biomedicals (Irvine, CA, USA) and from Sigma Chemical Co. (St Louis, MO, USA), respectively.

Results and discussion

CmeA-PNA reduced the translation of CmeA, but did not affect the production of other proteins as shown by SDS–PAGE analysis of whole cell fractions (Figure 1a). The specific inhibition was further demonstrated by western blotting using CmeA-specific antibodies (Figure 1b). As a negative control, the production of MOMP was not altered by CmeA-PNA (Figure 1b). CmeA is a known *N*-glycosylated protein. Thus, both the glycosylated and non-glycosylated forms of CmeA are shown by western blotting analysis, which was also observed in this study (Figure 1b). Interestingly, the glycosylated form was reduced more than the non-glycosylated form by the CmeA-PNA treatment (Figure 1b). In an antibiotic-free medium, CmeA-PNA did not affect the growth rate of *C. jejuni* at a concentration of 2 μM, which is the highest concentration tested in the study (Figure 1c). We chose CmeA for antisense inhibition because it is the first gene in the *cmeABC* operon and it has been previously shown that antisense inhibition of genes in a polycistronic operon, such as the *lac* operon of *E. coli*, also reduces the expression of downstream genes in the operon by interfering

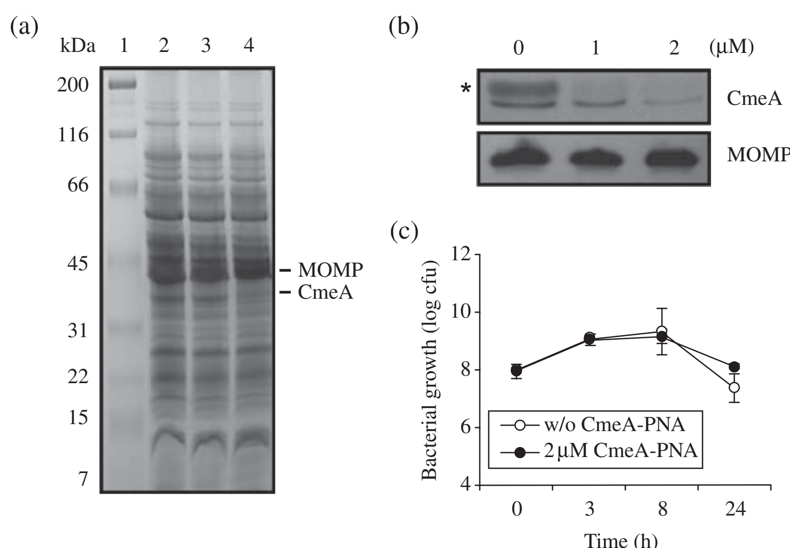

Figure 1. Effect of CmeA-PNA on CmeA production and bacterial growth of *C. jejuni* NCTC 11168. (a) SDS–PAGE analysis and Coomassie Blue staining of whole cells grown with different concentrations of CmeA-PNA. Lane 1, protein size markers; lanes 2–4, cells cultured with 0, 1 and 2 μM CmeA-PNA, respectively. The locations of CmeA (38 kDa) and MOMP (43 kDa) are indicated on the right-hand side. (b) Antisense inhibition of CmeA production demonstrated by western blotting. The CmeA-PNA concentration is marked above the top panel. The glycosylated form of CmeA is indicated with an asterisk on the left-hand side. The bottom panel shows the levels of MOMP in different preparations as a control. (c) *C. jejuni* growth in MH broth without (w/o) or with CmeA-PNA (2 μM). Results show the average ± SD of triplicate cultures.

Table 1. Effect of CmeA-PNA on the susceptibility of *C. jejuni* NCTC 11168 to ciprofloxacin and erythromycin

		MIC (mg/L)[a]	
C. jejuni strains	CmeA-PNA (μM)	ciprofloxacin	erythromycin
NCTC 11168	0	0.5	1
NCTC 11168	1	0.25 (2)	0.5 (2)
NCTC 11168	2	0.063 (8)	0.25 (4)
CmeB mutant	0	0.125 (4)	0.125 (8)

[a]The numbers in parentheses indicate the fold changes in MIC compared with that of NCTC 11168 without CmeA-PNA.

with mRNA stability.[8] Similarly, CmeA-PNA also decreased the protein levels of CmeB and CmeC when analysed by western blotting (data not shown), suggesting that antisense inhibition of CmeA also partially affected the production of CmeB and CmeC. Thus, CmeA-PNA impaired the production of the entire efflux pump.

CmeABC is known to confer resistance to ciprofloxacin and erythromycin in *C. jejuni*.[2] In order to investigate whether CmeA-PNA increased the susceptibility of *C. jejuni* to these antibiotics, the MICs of ciprofloxacin and erythromycin were determined in the presence of different concentrations of CmeA-PNA. While the MICs of both antibiotics decreased 2-fold at 1 μM CmeA-PNA, an increased concentration (2 μM) of CmeA-PNA reduced the MICs of ciprofloxacin and erythromycin 8- and 4-fold in *C. jejuni* NCTC 11168, respectively, compared with the control with no CmeA-PNA (Table 1). This level of MIC reduction was comparable to the observed MIC change with the CmeB mutant (Table 1), suggesting that the function of CmeABC in *C. jejuni* NCTC 11168 was almost completely inhibited by CmeA-PNA at a concentration of 2 μM. We further investigated the effect of CmeA-PNA on antimicrobial susceptibility using *C. jejuni* strains that are resistant to fluoroquinolone or erythromycin. The CmeA-PNA treatment at a 2 μM concentration resulted in a 2- to 4-fold reduction in the MIC of ciprofloxacin of the fluoroquinolone-resistant strains (S3BR1, CT2-2 and CB2-1) and a 4- to >64-fold reduction in the MIC of erythromycin of the erythromycin-resistant strains (S3BE2-2, S3BE4-2 and CT8-19). These results suggest that the effect of CmeA-PNA on MIC reduction varies with different strains. The exact reason for this variation is unknown, but there are a couple of possible explanations. First, bacterial lipopolysaccharide is a permeability barrier to PNA uptake.[9] In the *C. jejuni* genome, the locus responsible for lipooligosaccharide biosynthesis is known to be hypervariable,[6] which may cause variations in cell permeability to PNA and consequently varied efficiencies of CmeA-PNA in different strains. Secondly, CmeA-PNA only partially reduced the protein levels of CmeB and CmeC as measured by western blotting. Thus, the residual CmeB (inner membrane transporter) and CmeC (outer membrane channel protein) might continue to be functional, contributing to the varied efficiencies of CmeA-PNA. In *E. coli* and *Pseudomonas aeruginosa*, TolC and OprM, which are the outer membrane components of the efflux pumps AcrAB-TolC and MexAB-OprM, respectively, work with multiple drug transporters.[10] It is unknown whether CmeC in *C. jejuni* also partners with multiple efflux transporters. If this is the case, it may also explain the strain-dependent effect of CmeA-PNA.

Results from this study clearly demonstrated the feasibility of antisense technology in inhibiting gene expression and sensitizing *C. jejuni* to clinically important antibiotics. To our knowledge, this is the first study demonstrating the feasibility of using antisense technology to inhibit the function of multidrug efflux transporters in bacteria. This technology can be further optimized for enhanced specificity and efficiency and has a potential for use in treating antibiotic-resistant bacterial pathogens in which multidrug efflux transporters can be specifically targeted by PNA to enhance drug susceptibility.

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Transparency declarations

None to declare.

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