Target optimization for peptide nucleic acid (PNA)-mediated antisense inhibition of the CmeABC multidrug efflux pump in *Campylobacter jejuni*

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Objectives: CmeABC is a resistance-nodulation-cell division (RND)-type multidrug efflux pump conferring resistance to clinically important antibiotics in *Campylobacter*. This study aimed to identify the optimal target sites for the inhibition of CmeABC with antisense peptide nucleic acid (PNA).

Methods: Eighteen PNAs were designed to bind to the translational initiation regions of *cmeABC*, spanning the ribosome-binding site (RBS) and the start codon of the *cmeABC* genes. *Campylobacter jejuni* was treated with CmeABC-specific PNAs (CmeABC-PNAs) at various concentrations and subjected to western blotting to measure changes in the level of CmeABC expression. The MICs of ciprofloxacin and erythromycin were measured to evaluate the impact of CmeABC knockdown on antibiotic susceptibility.

Results: While antisense PNA significantly affected CmeA and CmeB expression, interestingly, CmeC expression was not altered by any of the CmeC-PNAs used in this study. A CmeA-PNA targeting the RBS of *cmeA* and its upstream region reduced CmeA expression most efficiently, and CmeB expression was most significantly decreased by PNA binding to the RBS of *cmeB* and its downstream region. CmeA- and CmeB-PNAs increased the susceptibility of *C. jejuni* to ciprofloxacin and erythromycin in proportion to the inhibition levels observed in western blotting.

Conclusions: The *cmeA* gene is the best target to knockdown CmeABC with antisense PNA. The RBS is the major target for the PNA-mediated antisense inhibition of CmeABC. However, regions in its vicinity also significantly influence the effectiveness of the PNA-based knockdown of CmeABC.

Keywords: efflux pump inhibitors, fluoroquinolones, macrolides

Introduction

Campylobacter jejuni is a leading foodborne pathogen resulting in ~400 million – 500 million cases of human infection around the world annually.[1] *C. jejuni* infection is usually self-limiting and does not require antibiotic treatment; however, antibiotics are prescribed for severe cases or in immunocompromised patients.[2] The drugs of choice for the treatment of human campylobacteriosis include fluoroquinolones and macrolides, but *C. jejuni* has developed increasing resistance to these clinically important antibiotics, particularly fluoroquinolones.[3]

Drug efflux pumps mediate the extrusion of structurally unrelated antibiotics out of the cell and are considered to be a major mechanism responsible for multidrug resistance.[4] To potentiate existing antibiotics and control antibiotic resistance associated with drug efflux pumps, numerous studies have been reported to develop efflux pump inhibitors.[5] However, inhibitors of prokaryotic efflux pumps can also interfere with eukaryotic efflux systems, resulting in toxicity problems, and this is considered to be a major barrier in the development of efflux pump inhibitors.[5]

In *Campylobacter*, CmeABC is a key resistance determinant that confers both intrinsic and acquired resistance to clinically important antibiotics.[6,7] In addition, CmeABC significantly affects the ability of *C. jejuni* to colonize chicken intestines due to its importance in resistance to bile salts.[8] CmeABC is a resistance-nodulation-cell division (RND)-type efflux pump consisting of three protein components located in the inner membrane (CmeB), periplasm (CmeA) and outer membrane (CmeC), which are encoded by the polycistronic *cmeABC* operon.[6] In our previous report, we demonstrated that an antisense peptide nucleic acid (PNA) targeting CmeA sensitized *C. jejuni* to ciprofloxacin and erythromycin.[9] However, the targets for antisense PNA have not been optimized to maximize the susceptibility of *C. jejuni* to antibiotics by CmeABC knockdown. In this study, we identified the

optimal target sites in *cmeABC* that are most susceptible to the inhibition of antisense CmeABC-specific PNAs (CmeABC-PNAs).

Materials and methods

Bacterial strains and CmeABC-PNAs

C. jejuni NCTC 11168 was the first *Campylobacter* to have its genome sequenced,[10] and was used throughout this study. *C. jejuni* was grown at 42°C with Mueller–Hinton (MH) medium (Oxoid) in microaerobic conditions (5% O_2, 85% N_2 and 10% CO_2). CmeABC-PNAs were designed based on the genome sequence of *C. jejuni* NCTC 11168,[10] and were commercially synthesized by Panagene (Daejeon, South Korea; Table 1). To improve antisense potency, CmeABC-PNAs were conjugated with the cell-permeabilizing peptide KFFKFFKFFF, as described elsewhere.[11]

PNA treatment and western blot analysis

C. jejuni NCTC 11168 was grown overnight on MH agar plates and used to prepare bacterial suspensions in MH broth to an optical density at 600 nm of 0.05–0.07. Broth cultures of *C. jejuni* were grown in the presence of various concentrations of CmeABC-PNAs for 7 h with shaking at 200 rpm. SDS-PAGE was carried out using a 10% polyacrylamide gel for CmeA in Tris-Tricine buffer and CmeC in Tris-Glycine buffer, and a 6% polyacrylamide gel for CmeB in Tris-Glycine buffer. Western blot analysis was performed using polyclonal antibodies against CmeA, CmeB or CmeC as described previously.[6]

Determination of C. jejuni susceptibility to ciprofloxacin

The MICs of ciprofloxacin (Enzo Life Sciences, USA) and erythromycin (Sigma, USA) were measured with a microtitre broth dilution method in the presence or absence of CmeABC-PNAs as previously described.[9]

Results

DNA sequence analysis of translation initiation regions of CmeABC in C. jejuni and Campylobacter coli

PNA-mediated gene knockdown is based on blocking translation initiation in both eukaryotes and prokaryotes.[12] The start codon

Table 1. CmeABC-PNAs used in this study

PNA	Sequence (N→C)
CmeA-PNA1	KFFKFFKFFK–TGCCTTGAAAAA
CmeA-PNA2	KFFKFFKFFK–TTTTGCCTTGAA
CmeA-PNA3	KFFKFFKFFK–TGGTTTTGCCTT
CmeA-PNA4	KFFKFFKFFK–TCATGGTTTTGC
CmeA-PNA5	KFFKFFKFFK–ATTTCATGGTTT
CmeA-PNA6	KFFKFFKFFK–AATAATTTCATG
CmeB-PNA1	KFFKFFKFFK–CTCCAATTTCTT
CmeB-PNA2	KFFKFFKFFK–GTGCTCCAATTT
CmeB-PNA3	KFFKFFKFFK–ATTGTGCTCCAA
CmeB-PNA4	KFFKFFKFFK–ATTATTGTGCTC
CmeB-PNA5	KFFKFFKFFK–AAAACATTATTG
CmeB-PNA6	KFFKFFKFFK–TTAGAAAACATT
CmeC-PNA1	KFFKFFKFFK–ACCTCTTTTTTT
CmeC-PNA2	KFFKFFKFFK–TTACCTCTTTTT
CmeC-PNA3	KFFKFFKFFK–ACCTTACCTCTT
CmeC-PNA4	KFFKFFKFFK–ATGAACCTTACC
CmeC-PNA5	KFFKFFKFFK–TATTCATGAACC
CmeC-PNA6	KFFKFFKFFK–ATTTTATTCATG

regions, including the ribosome-binding sites (RBSs), are known to be most susceptible to antisense inhibition by PNA in *Escherichia coli*.[13] To design PNAs targeting *cmeABC*, we first performed a DNA sequence analysis of the translational initiation regions of *cmeABC*. Since *C. jejuni* and *C. coli* are responsible for >95% of human campylobacteriosis, *C. jejuni* in particular accounting for >92% of cases,[14,15] the sequence analysis focused on the two *Campylobacter* species whose genome sequences are available in the GenBank sequence database. Overall, the translation initiation regions of *cmeABC* are well conserved in both species (Figure 1). *C. jejuni* doylei has one nucleotide change from C to T at a point 2 bp upstream of the *cmeA* start codon (Figure 1a), and *C. jejuni* ICDCCJ07001 showed a nucleotide change from C to T at a point 7 bp upstream of the *cmeB* start codon (Figure 1b). However, all other *C. jejuni* strains showed identical sequences in the translation initiation regions of *cmeABC* (Figure 1). Relatively high sequence variation was observed between *C. jejuni* and *C. coli* in the translation initiation region of *cmeA* (Figure 1a), suggesting that species-specific PNAs may be required for the effective inhibition of CmeABC.

Knockdown efficiency of CmeABC-PNAs

Eighteen PNAs were designed to bind to various regions involved in the translation initiation of *cmeA*, *cmeB* and *cmeC*, primarily targeting either the RBS or the start codon (Figure 2). Since it has been reported that, due to cell permeability, a 12-mer PNA exhibits better knockdown efficiency than longer PNAs,[11] we also designed 12-mer PNAs in this study. CmeA-PNAs 1, 4 and 5 significantly reduced CmeA expression at 1 μM, and CmeA-PNA1 further inhibited CmeA expression substantially even at 0.5 μM (Figure 3a). Among the three CmeA-PNAs (1, 2 and 3) binding to the RBS of *cmeA*, CmeA-PNA1 notably inhibited CmeA expression, while the effect of CmeA-PNAs 2 and 3 in CmeA knockdown was not significant (Figure 3a). Three CmeA-PNAs (4, 5 and 6) bind to the start codon of *cmeA* (Figure 2a). Treatment with 1 μM CmeA-PNAs 4 and 5 resulted in significant reductions in the level of CmeA expression (Figure 3a); however, CmeA-PNA6 had little effect on CmeA expression (Figure 3a). Among the six CmeA-PNAs, CmeA-PNA1 showed the best efficacy in CmeA knockdown (Figure 3a). CmeB-PNAs 1, 2 and 3 bind to the RBS of *cmeB* (Figure 2b). Compared with CmeB-PNAs 1 and 2, CmeB-PNA3, which binds to the RBS and its downstream region, was most effective in inhibiting CmeB expression (Figure 3b). Interestingly, CmeC expression was not affected by any of the six CmeC-PNAs even at high PNA concentrations, such as 4 μM (Figure 3c). Since CmeA-PNA1 and CmeB-PNA3 were most effective in inhibiting CmeA and CmeB, respectively, their knockdown efficiency was further tested by exposing *C. jejuni* to various concentrations of PNA. CmeA-PNA1 and CmeB-PNA3 demonstrated obvious knockdown efficacy in a concentration-dependent manner (Figure 4).

C. jejuni susceptibility to ciprofloxacin and erythromycin following treatment with CmeABC-PNAs

To evaluate the effect of CmeABC knockdown on antibiotic susceptibility, we measured the MICs of ciprofloxacin and erythromycin after PNA treatment because they are clinically important and known substrates of the CmeABC efflux pump.[3,6] The changes in MIC were proportional to the levels of CmeABC inhibition observed

(a)
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                                          RBS          cmeA start codon
C. jejuni 11168       TTTTAATTTAATTTTTCAAGGCAAAA-CCATGAAATTATTTC
C. jejuni IA3902      TTTTAATTTAATTTTTCAAGGCAAAA-CCATGAAATTATTTC
C. jejuni 81116       TTTTAATTTAATTTTTCAAGGCAAAA-CCATGAAATTATTTC
C. jejuni 81_176      TTTTAATTTAATTTTTCAAGGCAAAA-CCATGAAATTATTTC
C. jejuni RM1221      TTTTAATTTAATTTTTCAAGGCAAAA-CCATGAAATTATTTC
C. jejuni S3          TTTTAATTTAATTTTTCAAGGCAAAA-CCATGAAATTATTTC
C. jejuni S3B         TTTTAATTTAATTTTTCAAGGCAAAA-CCATGAAATTATTTC
C. jejuni M1          TTTTAATTTAATTTTTCAAGGCAAAA-CCATGAAATTATTTC
C. jejuni ICDCCJ07001 TTTTAATTTAATTTTTCAAGGCAAAA-CCATGAAATTATTTC
C. jejuni doylei      TTTTAATTTAATTTTTCAAGGCAAAA-TCATGAAATTATTTC
C. jejuni 154KU       TTTTAATTTAATTTTTCAAGGCAAAA-CCATGAAATTATTTC
C. coli CIT382        TTTTAATTTAATTTTTCAAGGCAAAA-CCATGAAATTATTTC
C. coli 993868        TTTAAATTAAAAATTTTAAGGCAAAAATCATGAATTTGTTTC
C. coli TF1_13        TTTAAATTAAAAATTTTAAGGCAAAAATCATGAATTTGTTTC
                      *** **** **   *** ********* ****** ** ****
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(b)
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                                         RBS        cmeB start codon
C. jejuni 11168       TTAAAGAAATTGGAGCACAATAATGTTTTCTAAATTTTTTAT
C. jejuni IA3902      TTAAAGAAATTGGAGCACAATAATGTTTTCTAAATTTTTTAT
C. jejuni 81116       TTAAAGAAATTGGAGCACAATAATGTTTTCTAAATTTTTTAT
C. jejuni 81_176      TTAAAGAAATTGGAGCACAATAATGTTTTCTAAATTTTTTAT
C. jejuni RM1221      TTAAAGAAATTGGAGCACAATAATGTTTTCTAAATTTTTTAT
C. jejuni S3          TTAAAGAAATTGGAGCACAATAATGTTTTCTAAATTTTTTAT
C. jejuni S3B         TTAAAGAAATTGGAGCACAATAATGTTTTCTAAATTTTTTAT
C. jejuni M1          TTAAAGAAATTGGAGCACAATAATGTTTTCTAAATTTTTTAT
C. jejuni ICDCCJ07001 TTAAAGAAATTGGAGTACAATAATGTTTTCTAAATTTTTTAT
C. jejuni doylei      TTAAAGAAATTGGAGCACAATAATGTTTTCTAAATTTTTTAT
C. jejuni 154KU       TTAAAGAAATTGGAGCACAATAATGTTTTCTAAATTTTTTAT
C. coli CIT382        TTAAAGAAATTGGAGCACAATAATGTTTTCTAAATTTTTTAT
C. coli 993868        TTAAAGAAATTGGAGCACAATAATGTTTTCTAAATTTTTTAT
C. coli TF1_13        TTAAAGAAGTTGGAGCACAATAATGTTTTCTAAATTTTTTAT
                      ******** ****** *************************
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(c)
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                                       RBS        cmeC start codon
C. jejuni 11168       AAAAAAAGAGGTAAGGTTCATGAATAAAATAATTTCAATTAG
C. jejuni IA3902      AAAAAAAGAGGTAAGGTTCATGAATAAAATAATTTCAATTAG
C. jejuni 81116       AAAAAAAGAGGTAAGGTTCATGAATAAAATAATTTCAATTAG
C. jejuni 81_176      AAAAAAAGAGGTAAGGTTCATGAATAAAATAATTTCAATTAG
C. jejuni RM1221      AAAAAAAGAGGTAAGGTTCATGAATAAAATAATTTCAATTAG
C. jejuni S3          AAAAAAAGAGGTAAGGTTCATGAATAAAATAATTTCAATTAG
C. jejuni S3B         AAAAAAAGAGGTAAGGTTCATGAATAAAATAATTTCAATTAG
C. jejuni M1          AAAAAAAGAGGTAAGGTTCATGAATAAAATAATTTCAATTAG
C. jejuni ICDCCJ07001 AAAAAAAGAGGTAAGGTTCATGAATAAAATAATTTCAATTAG
C. jejuni doylei      AAAAAAAGAGGTAAGGTTCATGAATAAAATAATTTCAATTAG
C. jejuni 154KU       AAAAAAAGAGGTAAGGTTCATGAATAAAATAATTTCAATTAG
C. coli CIT382        AAAAAAAGAGGTAAGGTTCATGAATAAAATAATTTCAATTAG
C. coli 993868        AAGAAAAGGGGTAAAGTTCATGAATAAAATAATTTCAATAAG
C. coli TF1-13        AAGAAAAGGGGTAAAGTTCATGAATAAAATAATTTCAATAAG
                      ** ***** ***** ********************** **
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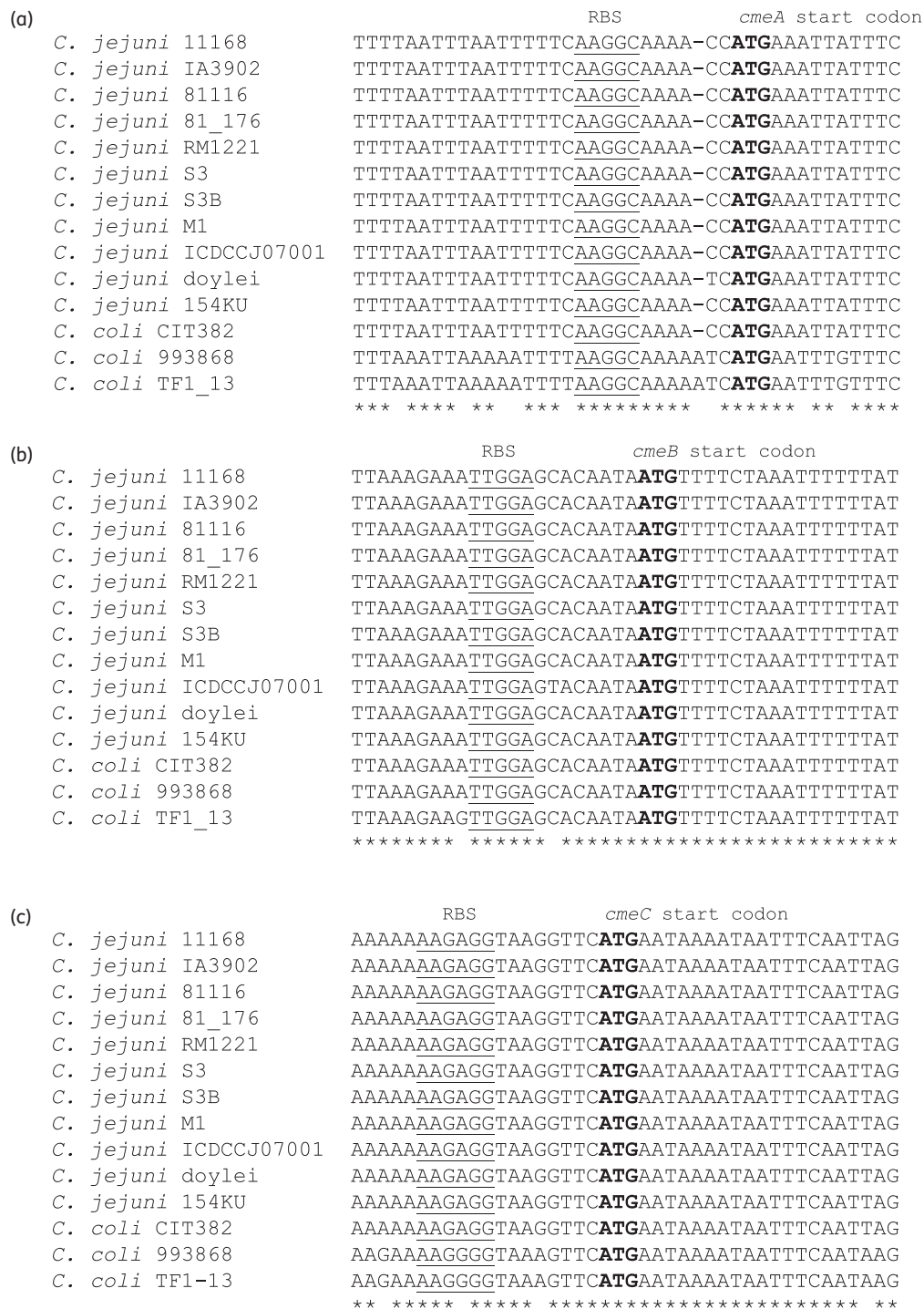
Figure 1. Multiple alignment of the translation initiation regions of *cmeA* (a), *cmeB* (b) and *cmeC* (c). The *C. jejuni* and *C. coli* DNA sequences available in the GenBank database were used for the analysis. Start codons are shown in bold and RBSs are underlined. GenBank accession numbers are AL111168 (*C. jejuni* NCTC 11168), CP001876 (*C. jejuni* IA3902), CP000814 (*C. jejuni* 81116), CP000538 (*C. jejuni* 81_176), NC_003912 (*C. jejuni* RM1221), CP001960 (*C. jejuni* S3), FJ797669 (*C. jejuni* S3B), CP001900 (*C. jejuni* M1), CP002029 (*C. jejuni* ICDCCJ07001), CP000768 (*C. jejuni* doylei 269.97), DQ333454 (*C. jejuni* 154KU), AY598796 (*C. coli* CIT382), FJ797671 (*C. coli* 993868) and FJ797673 (*C. coli* TF1_13). The alignment was performed using ClustalW2.

by western blotting. Compared with PNAs targeting CmeB, most CmeA-PNAs rendered *C. jejuni* highly susceptible to ciprofloxacin and erythromycin (Table 2). In particular, CmeA-PNA1 decreased the MICs of ciprofloxacin and erythromycin 4-fold even at 0.5 μM (Table 2). CmeB-PNAs required higher doses than CmeA-PNAs to achieve similar MIC changes (Table 2). Consistent with the results

(a)

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                       RBS          cmeA start codon
              attttttcaaggcaaaaccatgaaattatttca

    CmeA-PNA1:  aaaaagttccgt
    CmeA-PNA2:    aagttccgtttt
    CmeA-PNA3:      ttccgttttggt
    CmeA-PNA4:        cgttttggtact
    CmeA-PNA5:          tttggtactttta
    CmeA-PNA6:            gtactttaataa
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(b)

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                       RBS          cmeB start codon
              ttaaagaaattggagcacaataatgttttctaaa

    CmeB-PNA1:    ttcttttaacctc
    CmeB-PNA2:      tttaacctcgtg
    CmeB-PNA3:        aacctcgtgtta
    CmeB-PNA4:          ctcgtgttatta
    CmeB-PNA5:            gttattacaaaa
    CmeB-PNA6:              ttacaaaagatt
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(c)

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                       RBS          cmeC start codon
              ataaaaaaagaggtaaggttcatgaataaaata

    CmeC-PNA1:    ttttttttctcca
    CmeC-PNA2:      tttttctccatt
    CmeC-PNA3:        ttctccattcca
    CmeC-PNA4:          ccattccaagta
    CmeC-PNA5:            ccaagtacttat
    CmeC-PNA6:              gtacttatttta
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Figure 2. Target sequences of PNAs against *cmeA* (a), *cmeB* (b) and *cmeC* (c). The RBSs are underlined, and the start codon is in bold and marked with a rectangle.
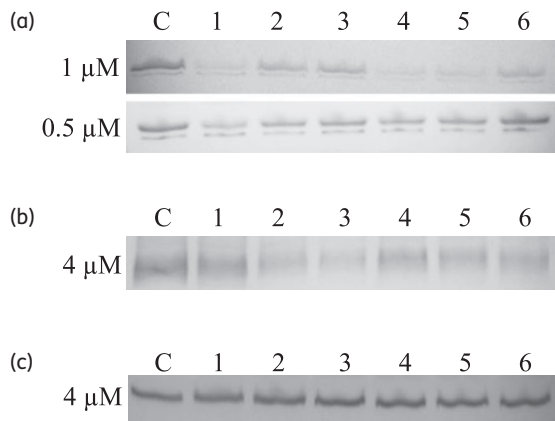
Figure 3. Inhibition efficiency of CmeABC-PNAs. (a) CmeA expression level visualized by western blotting following treatment with six CmeA-PNAs at two different concentrations (1 μM and 0.5 μM), as indicated on the left. CmeA is a glycosylated protein and appears as a doublet band in western blotting. C, *C. jejuni* NCTC 11168 without PNA; lanes 1–6, CmeA-PNA1–CmeA-PNA6, respectively. (b) Changes in the level of CmeB expression caused by 4 μM CmeB-PNAs. C, *C. jejuni* NCTC 11168 without PNA; lanes 1–6, CmeB-PNA1–CmeB-PNA6, respectively. (c) Protein levels of CmeC expression following treatment with 4 μM CmeC-PNAs. C, *C. jejuni* NCTC 11168 without PNA; lanes 1–6, CmeC-PNA1–CmeC-PNA6, respectively. An equal amount of the protein sample was used for each lane, and total proteins were visualized by SDS-PAGE and Coomassie blue staining as a control in all experiments by using the same protein amount used for western blotting (data not shown).
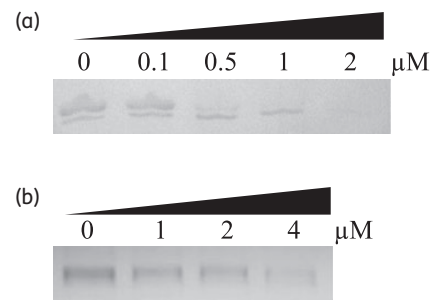

Figure 4. Concentration-dependent knockdown by CmeA-PNA1 (a) and CmeB-PNA3 (b). PNA concentrations are indicated at the top.

of western blotting, CmeC-PNAs did not affect the antimicrobial susceptibility of *C. jejuni* (data not shown).

Discussion

In this study, we optimized the antisense inhibition of CmeABC by using 18 PNAs targeting the translation initiation regions of *cmeABC*. The *cmeA* gene is the first gene in the *cmeABC* operon and was the best target for the PNA-mediated knockdown of CmeABC (Figure 3 and Table 2). Interestingly, CmeC expression was not altered by antisense PNA even at high doses (Figure 3c), although CmeC-PNAs were extensively designed to span various regions associated with translational initiation (Figure 2c). Treatment with antisense PNAs targeting CmeA and CmeB substantially increased the susceptibility of *C. jejuni* to ciprofloxacin and erythromycin (Table 2). Consistent with the observations from the western blot analysis, most CmeA-PNAs significantly reduced the MIC levels (Table 2).

The inhibition efficiency of antisense PNA targeting the *cmeABC* operon was highest for *cmeA*, moderate for *cmeB*, and low for *cmeC*, suggesting that the gene organization in the *cmeABC* operon affects the knockdown efficiency. Similarly, Dryselius *et al.*[16] reported that the upstream *lacZ* was more strongly inhibited by PNA than were the downstream *lacY* and *lacA* proteins in the *lac* operon of *E. coli*. Based on our findings and the previous report, upstream genes appear to be most susceptible to PNA-mediated antisense inhibition in a polycistronic operon.

In addition, the unresponsiveness of CmeC to antisense PNA might possibly be related to the unique organization of *cmeABC* in the operon. The *cmeABC* genes constitute a polycistronic operon, in which *cmeB* and *cmeC* overlap by 8 bp and the start codon of *cmeC* is embedded in the coding region of *cmeB*.[6] The tight binding of PNA to mRNA disrupts the function of the ribosomes and interferes with translation, resulting in gene knockdown.[17] While translation initiation regions are susceptible to PNA inhibition, PNA binding to a coding region does not result in knockdown effects in *E. coli*.[13] The start codon and RBS of *cmeC* are located in the coding region of *cmeB* as the start codon of *cmeC* is 5 bp upstream of the stop codon of *cmeB*.[6] Although CmeC-PNAs bind to the translation initiation regions of *cmeC*, which is located within the coding region of *cmeB*, in this case ribosomes translating *cmeB* may dissociate CmeC-PNAs from mRNA. This possibility is high because Dryselius *et al.*[16] observed a fully dis-coordinated reduction in a *gfp-DsRed* operon that was artificially constructed placing RBS in an intergenic region between the stop

Table 2. Changes in the MICs of ciprofloxacin and erythromycin caused by CmeA- and CmeB-PNAs

PNAs	CmeA-PNA[a]			PNAs	CmeB-PNA[a]		
	0.5 μM	1 μM	2 μM		0.5 μM	1 μM	2 μM
Ciprofloxacin				Ciprofloxacin			
control[b]	0.5	0.5	0.5	control[b]	0.5	0.5	0.5
A1	0.125 (4)	0.0625 (8)	0.0625 (8)	B1	0.5	0.5	0.25 (2)
A2	0.5	0.25 (2)	0.25 (2)	B2	0.5	0.5	0.25 (2)
A3	0.5	0.25 (2)	0.125 (4)	B3	0.5	0.25 (2)	0.125 (4)
A4	0.25 (2)	0.125 (4)	0.125 (4)	B4	0.5	0.5	0.25 (2)
A5	0.5	0.25 (2)	0.25 (2)	B5	0.5	0.5	0.5
A6	0.5	0.5	0.25 (2)	B6	0.5	0.5	0.5
Erythromycin				Erythromycin			
control[b]	0.5	0.5	0.5	control[b]	0.5	0.5	0.5
A1	0.125 (4)	0.0313 (16)	0.0156 (32)	B1	0.5	0.5	0.25 (2)
A2	0.5	0.125 (4)	0.0625 (8)	B2	0.5	0.5	0.25 (2)
A3	0.5	0.25 (2)	0.125 (4)	B3	0.5	0.25 (2)	0.25 (2)
A4	0.25 (2)	0.125 (4)	0.0625 (8)	B4	0.5	0.25 (2)	0.125 (4)
A5	0.5	0.25 (2)	0.125 (4)	B5	0.5	0.5	0.25 (2)
A6	0.5	0.5	0.0625 (8)	B6	0.5	0.5	0.25 (2)

[a]MICs are expressed in mg/L. Fold changes are indicated in parentheses. The results are representative of three independent experiments that all presented similar results. All the tests were performed with *C. jejuni* NCTC 11168.
[b]A negative control tested without PNA.

codon of *gfp* and the start codon of *DsRed*. In their artificial operon construct, antisense PNAs directed at *gfp* or *DsRed* exhibited discoordinated inhibitory effects on both upstream and downstream genes.[16]

The results of western blotting demonstrated that the RBS is the major target for the PNA-mediated knockdown of CmeA and CmeB (Figure 3). However, it should be noted that the sequence in the vicinity around the RBS is also important in determining the knockdown efficiency of PNA. Although CmeA-PNA2, CmeA-PNA3 and CmeB-PNA1 harbour the whole sequence of the RBS (Figure 2a and b), their knockdown efficiency was very low (Figure 3a and b). These results suggest that targeting the RBS sequence alone is not sufficient for achieving the best knockdown efficiency and that both RBS and the sequence in its vicinity play a critical role in determining the knockdown efficacy of antisense PNA. The start codon of *cmeA*, but not of *cmeB* and *cmeC*, was susceptible to antisense PNA (Figure 3). Among the three CmeA-PNAs (4, 5 and 6) harbouring the *cmeA* start codon, only CmeA-PNAs 4 and 5 significantly reduced the level of CmeA expression, while the antisense effect of CmeA-PNA6 was small (Figure 3a). Similar to the case of targeting the RBS, the results suggest that both the start codon and its nearby sequence affect the knockdown efficiency.

In this study, we demonstrated that the target optimization of antisense PNA for CmeABC knockdown significantly affects the effectiveness of sensitizing *C. jejuni* to ciprofloxacin and erythromycin. Although the translation initiation regions are the known major target for antisense PNA, the knockdown efficiency of antisense PNA is still dependent on other unknown factors. Presumably, the secondary structure of mRNA may be associated with the inhibition mechanism of antisense PNA, which will be examined in future investigations.

Transparency declarations

None to declare.

References

1 Ruiz-Palacios GM. The health burden of *Campylobacter* infection and the impact of antimicrobial resistance: playing chicken. *Clin Infect Dis* 2007; **44**: 701–3.

2 Allos BM. *Campylobacter jejuni* infections: update on emerging issues and trends. *Clin Infect Dis* 2001; **32**: 1201–6.

3 Luangtongkum T, Jeon B, Han J *et al.* Antibiotic resistance in *Campylobacter*: emergence, transmission and persistence. *Future Microbiol* 2009; **4**: 189–200.

4 Poole K. Efflux-mediated antimicrobial resistance. *J Antimicrob Chemother* 2005; **56**: 20–51.

5 Mahamoud A, Chevalier J, Alibert-Franco S *et al.* Antibiotic efflux pumps in Gram-negative bacteria: the inhibitor response strategy. *J Antimicrob Chemother* 2007; **59**: 1223–9.

6 Lin J, Michel LO, Zhang Q. CmeABC functions as a multidrug efflux system in *Campylobacter jejuni*. *Antimicrob Agents Chemother* 2002; **46**: 2124–31.

7 Yan M, Sahin O, Lin J *et al.* Role of the CmeABC efflux pump in the emergence of fluoroquinolone-resistant *Campylobacter* under selection pressure. *J Antimicrob Chemother* 2006; **58**: 1154–9.

8 Lin J, Sahin O, Michel LO *et al.* Critical role of multidrug efflux pump CmeABC in bile resistance and *in vivo* colonization of *Campylobacter jejuni*. *Infect Immun* 2003; **71**: 4250–9.

9 Jeon B, Zhang Q. Sensitization of *Campylobacter jejuni* to fluoroquinolone and macrolide antibiotics by antisense inhibition of the CmeABC multidrug efflux transporter. *J Antimicrob Chemother* 2009; **63**: 946–8.

10 Parkhill J, Wren BW, Mungall K *et al.* The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* 2000; **403**: 665–8.

11 Good L, Awasthi SK, Dryselius R *et al.* Bactericidal antisense effects of peptide-PNA conjugates. *Nat Biotechnol* 2001; **19**: 360–4.

12 Good L. Translation repression by antisense sequences. *Cell Mol Life Sci* 2003; **60**: 854–61.

13 Dryselius R, Aswasti SK, Rajarao GK *et al.* The translation start codon region is sensitive to antisense PNA inhibition in *Escherichia coli*. *Oligonucleotides* 2003; **13**: 427–33.

14 Gillespie IA, O'Brien SJ, Penman C *et al.* Demographic determinants for *Campylobacter* infection in England and Wales: implications for future epidemiological studies. *Epidemiol Infect* 2008; **136**: 1717–25.

15 Jeon B, Muraoka WT, Zhang Q. Advances in *Campylobacter* biology and implications for biotechnological applications. *Microb Biotechnol* 2010; **3**: 242–58.

16 Dryselius R, Nikravesh A, Kulyte A *et al.* Variable coordination of cotranscribed genes in *Escherichia coli* following antisense repression. *BMC Microbiol* 2006; **6**: 97.

17 Good L, Nielsen PE. Antisense inhibition of gene expression in bacteria by PNA targeted to mRNA. *Nat Biotechnol* 1998; **16**: 355–8.