Effect of putative efflux pump inhibitors and inducers on the antimicrobial susceptibility of *Campylobacter jejuni* and *Campylobacter coli*

Minna Hannula and Marja-Liisa Hänninen

Correspondence

Minna Hannula
minna.hannula@helsinki.fi

Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki, Helsinki, Finland

Received 13 December 2007

Accepted 29 February 2008

The CmeABC efflux pump plays an important role in the antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli*. The aim of this investigation was to study the effect of putative efflux pump inhibitors, phenyl-arginine- β -naphthylamide (PA β N) and 1-(1-naphthylmethyl)-piperazine (NMP), as well as the effect of putative efflux pump inducers, sodium salicylate and sodium deoxycholate, on the MIC levels of erythromycin, ciprofloxacin, kanamycin, tetracycline and rifampicin for *C. jejuni* and *C. coli*. Our results indicated that susceptibility to erythromycin and rifampicin increased, respectively, 8- to 32- and 8- to 64-fold in the presence of PA β N and to a lesser extent in the presence of NMP. Salicylate produced a 2- to 4-fold increase in ciprofloxacin MIC values, whereas little effect was observed in the presence of deoxycholate.

INTRODUCTION

Campylobacteriosis is the leading cause of bacterial gastroenteritis in industrialized countries (Friedman *et al.*, 2000). Although in most cases the illness is self-limiting, in severe cases and in immunocompromised patients, the use of antimicrobial agents is warranted. However, the rapid increase in resistant strains and the emergence of strains with multiple resistances are of growing concern (Ruiz *et al.*, 2007).

It has been established that efflux pumps, particularly the resistance–nodulation–cell division (RND) type CmeABC pump, have an important role in the antimicrobial resistance of *Campylobacter jejuni* (Lin *et al.*, 2002). The same efflux pump has recently been characterized in *Campylobacter coli* (Corcoran *et al.*, 2005). This pump is able to significantly decrease the susceptibility of *Campylobacter* to a number of antimicrobials (Lin *et al.*, 2002).

Efforts have been made to find substances capable of reversing the action of the efflux pumps and which therefore have the potential to prevent antimicrobial resistance *in vivo*. A broad-spectrum efflux pump inhibitor (EPI), phenyl-arginine- β -naphthylamide (PA β N), was first characterized in *Pseudomonas aeruginosa* (Lomovskaya *et al.*, 2001) and has since been studied in several bacteria, including *Campylobacter* (Cagliero *et al.*, 2005; Gibreel *et al.*, 2007; Payot *et al.*, 2004). Another putative EPI is 1-

(1-naphthylmethyl)-piperazine (NMP), characterized in *Escherichia coli* (Bohnert & Kern, 2005). It is able to partially reverse multidrug resistance in some members of the *Enterobacteriaceae* and in *Acinetobacter baumannii* (Bohnert & Kern, 2005; Pannek *et al.*, 2006; Schumacher *et al.*, 2006) but its effects on *Campylobacter* have not been studied previously.

In *E. coli*, salicylic acid binds to a repressor protein, MarR, which results in the overexpression of the global regulatory gene *marA*. This causes the down-regulation of the outer-membrane protein OmpF and an increase in the synthesis of the RND multidrug efflux pump AcrAB-TolC, which leads to decreased antimicrobial accumulation (Price *et al.*, 2000). Salicylate has also been reported to increase the effect of antimicrobial aminoglycosides in *E. coli* (Aumercier *et al.*, 1990). Finally, Lin *et al.* (2005) have shown that bile salts, such as deoxycholate, significantly induce the expression of *cmeABC* in *C. jejuni* by preventing the binding of the transcriptional repressor of *cmeABC* (CmeR). This can lead to increased resistance to several antibiotics (Lin *et al.*, 2005).

The aim of this study was to analyse the effect of these two EPIs, as well as the two putative efflux pump inducers, on the susceptibility of *C. jejuni* and *C. coli* to erythromycin, ciprofloxacin, kanamycin, tetracycline and rifampicin.

METHODS

Bacterial strains. Five *C. jejuni* and four *C. coli* strains, isolated from poultry, human patients or pigs, were included in this study. The *C. jejuni* strain ATCC 33560 was used as a reference strain. Strain 49/

Abbreviations: EPI, efflux pump inhibitor; NMP, 1-(1-naphthylmethyl)-piperazine; PA β N, phenyl-arginine- β -naphthylamide; RND, resistance–nodulation–cell division.

7RAT was a laboratory-derived variant of 49/7R, obtained by selection on media containing increasing concentrations of ampicillin and tetracycline. Also included were ciprofloxacin-resistant derivatives of ATCC 33560, Brt1, B25, B42, B67, 49/7R and 49/7RAT, obtained *in vitro* by growing the parent strains in media containing 1 mg ciprofloxacin l⁻¹ (Hänninen & Hannula, 2007).

Determination of MICs. MICs of ciprofloxacin, erythromycin, rifampicin and tetracycline (all Sigma-Aldrich) in the presence and absence of NMP (100 mg l⁻¹) (Chess), PA β N (50 mg l⁻¹) (Sigma-Aldrich), sodium salicylate (100 mg l⁻¹) or sodium deoxycholate (1 mg ml⁻¹) (both Merck) were determined by using the agar dilution method following the guidelines set by the CLSI (2006). All experiments were repeated at least three times. The growth of each strain was verified on control plates containing the indicated amounts of the studied efflux pump inhibitors and inducers.

RESULTS AND DISCUSSION

MIC values for the *C. jejuni* and *C. coli* strains and their variants

The MIC levels were determined for each strain studied and are shown in Table 1. Ciprofloxacin-resistant variants were obtained by selection on media containing 1 mg ciprofloxacin l⁻¹ (Hänninen & Hannula, 2007). The resistant variants had 16- to 128-fold greater MIC values than the respective parent strains, which corresponded to MIC values from 2 to 64 mg l⁻¹.

Effect of PA β N

In congruence with previous studies (Cagliero *et al.*, 2005; Gibreel *et al.*, 2007; Payot *et al.*, 2004), PA β N at a concentration of 50 mg l⁻¹ resulted in an 8- to 32-fold decrease in the erythromycin MIC levels, corresponding to MIC values from 0.032 to 1 mg l⁻¹. Our study included only erythromycin-susceptible and low-level resistant strains that most probably lacked the A2075G mutation in the 23S rRNA gene associated with high-level macrolide resistance. A previous study, involving high-level resistant strains, showed that when all copies of the target gene carry an A2074C mutation, PA β N is unable to decrease the resistance level, indicating that efflux has little significance in the high-level resistance to erythromycin in these instances (Gibreel *et al.*, 2007).

PA β N also decreased rifampicin resistance 8- to 64-fold in the studied strains. *C. jejuni* and *C. coli* are intrinsically resistant to rifampicin, and PA β N's ability to reduce this resistance suggests that rifampicin resistance in *Campylobacter* is at least partly associated with efflux. A recently published work on tetracycline reported 1.3- to 2.6-fold decrease in tetracycline resistance in highly resistant *C. jejuni* strains in the presence of 30 μ g PA β N ml⁻¹ (Gibreel *et al.*, 2007). Congruently, the two tetracycline-resistant strains (6590 and 49/7RAT CIP) included in our study also had a 2-fold reduction in the tetracycline MIC values, but the same phenomenon was not observed in tetracycline-susceptible strains. With

regards to kanamycin and ciprofloxacin, there was no evidence of PA β N affecting the MICs of these antimicrobials for the bacteria.

The inactivation by insertional mutagenesis of the *cmeB* gene of *C. jejuni* has led to an 8-fold reduction in ciprofloxacin MIC levels, 4- to 16-fold reduction in erythromycin MIC levels, 64- to 128-fold decrease in rifampicin MIC levels and an 8-fold decrease in tetracycline MIC levels (Lin *et al.*, 2002). The effects on the MIC levels obtained when the PA β N pump inhibitor was added to the growth media were less pronounced or absent, suggesting that the action of PA β N does not result in the complete inactivation of CmeABC and that this substance may have other modes of action. However, the strong decreasing effect of PA β N on the MIC levels of erythromycin and rifampicin warrants the use of PA β N in further studies involving these antimicrobials.

Effect of NMP

Studies conducted with NMP showed a 2- to 8-fold decrease in erythromycin and rifampicin MIC levels for *C. jejuni* and *C. coli*, but did not show an increase in the susceptibility of *Campylobacter* to tetracycline, kanamycin or ciprofloxacin. According to our results, NMP confers an increase in susceptibility to the same antimicrobials as PA β N but with less effectiveness, which is in agreement with the studies on members of the *Enterobacteriaceae* and *Acinetobacter baumannii* (Bohnert & Kern, 2005; Pannek *et al.*, 2006; Schumacher *et al.*, 2006), indicating that NMP confers effects on MIC levels that differ from those seen with PA β N, suggesting a different mode of action for these pump inhibitors.

Effect of sodium salicylate

Salicylate and acetylsalicylate are commonly used non-steroidal anti-inflammatory agents that are often used in combination with antimicrobials for the treatment of microbial diseases. However, salicylate has been shown to decrease the susceptibility of bacteria, such as *E. coli*, to antimicrobials by altering membrane proteins and inducing efflux pumps (Price *et al.*, 2000). Since *C. jejuni* possesses an outer-membrane protein (MOMP) belonging to the same porin family as *E. coli* OmpF (Bolla *et al.*, 1995) and a multidrug efflux pump (CmeABC) belonging to the same RND family as AcrAB-TolC (Lin *et al.*, 2002), it has been speculated that salicylate might also decrease antimicrobial accumulation in *C. jejuni*. A previous study by Randall *et al.* (2003) showed a small but significant effect of salicylate on the ciprofloxacin resistance of *Campylobacter*, and our results confirm a 2- to 4-fold increase in MIC values for ciprofloxacin in almost all strains, with ciprofloxacin-susceptible strains and their respective variants reacting alike. In the study by Randall *et al.* (2003), no effect was observed on kanamycin MIC levels, which was confirmed by our study, but unlike the

Table 1. MICs (mg l⁻¹) of antimicrobial agents in the presence and absence of PAβN (50 mg l⁻¹), NMP (100 mg l⁻¹), sodium salicylate (100 mg l⁻¹) or sodium deoxycholate (1 mg ml⁻¹)

At least 4-fold changes are indicated in bold.

Strain	Ciprofloxacin	Erythromycin	Kanamycin	Rifampicin	Tetracycline
ATCC 33560 <i>C. jejuni</i>	0.25	2	4	512	2
+ PAβN	0.25	0.25	4	64	4
+ NMP	0.25	0.5	4	256	1
+ Salicylate	0.25	2	4	512	2
+ Deoxycholate	0.25	2	4	512	2
ATCC 33560 CIP <i>C. jejuni</i>	16	4	4	512	4
+ PAβN	8	0.25	4	64	4
+ NMP	8	0.5	4	256	1
+ Salicylate	8	2	4	512	2
+ Deoxycholate	8	4	4	512	4
Br1 <i>C. jejuni</i>	0.125	1	4	512	1
+ PAβN	0.125	0.125	4	8	1
+ NMP	0.125	0.25	4	128	0.5
+ Salicylate	0.25	2	4	1024	1
+ Deoxycholate	0.125	1	4	512	1
Br1 CIP <i>C. jejuni</i>	4	1	4	1024	1
+ PAβN	8	0.125	4	16	1
+ NMP	4	0.25	4	256	0.5
+ Salicylate	16	2	4	1024	2
+ Deoxycholate	8	1	4	1024	2
B42 <i>C. jejuni</i>	0.063	0.5	4	256	0.5
+ PAβN	0.063	0.063	4	8	0.5
+ NMP	0.063	0.25	4	64	0.25
+ Salicylate	0.25	1	4	512	1
+ Deoxycholate	0.125	1	4	512	1
B42 CIP <i>C. jejuni</i>	4	0.5	4	256	0.5
+ PAβN	4	0.063	4	8	0.5
+ NMP	4	0.25	4	64	0.25
+ Salicylate	16	1	4	512	1
+ Deoxycholate	8	1	4	512	0.5
B67 <i>C. jejuni</i>	0.125	1	8	512	0.5
+ PAβN	0.125	0.125	8	32	0.5
+ NMP	0.125	0.5	8	256	0.25
+ Salicylate	0.5	1	4	1024	1
+ Deoxycholate	0.125	1	8	1024	0.5
B67 CIP <i>C. jejuni</i>	16	1	8	512	0.5
+ PAβN	16	0.125	4	32	0.5
+ NMP	16	1	8	256	0.25
+ Salicylate	64	1	2	1024	1
+ Deoxycholate	16	1	8	1024	0.5
49/7R <i>C. jejuni</i>	0.063	0.5	4	1024	0.5
+ PAβN	0.063	0.063	4	64	0.5
+ NMP	0.063	0.25	4	512	0.25
+ Salicylate	0.25	1	4	2048	1
+ Deoxycholate	0.063	0.5	4	2048	0.5
49/7R CIP <i>C. jejuni</i>	2	4	4	1024	4
+ PAβN	1	0.5	4	128	4
+ NMP	1	2	4	512	2
+ Salicylate	2	4	2	1024	4
+ Deoxycholate	4	4	4	2048	4
49/7RAT <i>C. jejuni</i>	1	16	4	512	8
+ PAβN	1	1	4	32	8
+ NMP	0.5	4	4	256	4
+ Salicylate	1	8	4	512	4

Table 1. cont.

Strain	Ciprofloxacin	Erythromycin	Kanamycin	Rifampicin	Tetracycline
+ Deoxycholate	1	16	4	512	8
49/7RAT CIP <i>C. jejuni</i>	64	16	4	512	16
+ PA β N	32	1	4	64	8
+ NMP	32	4	4	256	4
+ Salicylate	32	8	2	512	8
+ Deoxycholate	32	16	4	512	16
B25 <i>C. coli</i>	0.063	1	2	128	1
+ PA β N	0.063	0.032	1	4	1
+ NMP	0.063	0.125	1	64	1
+ Salicylate	0.25	0.5	1	256	1
+ Deoxycholate	0.063	1	2	128	1
B25 CIP <i>C. coli</i>	8	1	2	128	0.5
+ PA β N	4	0.032	1	8	1
+ NMP	8	0.125	1	16	1
+ Salicylate	16	0.5	1	128	2
+ Deoxycholate	8	1	2	256	0.5
S140R <i>C. coli</i>	0.125	1	2	128	1
+ PA β N	0.125	0.063	2	8	0.5
+ NMP	0.125	0.5	4	32	0.5
+ Salicylate	0.25	1	2	128	1
+ Deoxycholate	0.125	0.5	2	256	1
72277 <i>C. coli</i>	0.25	1	8	1024	2
+ PA β N	0.125	0.063	8	64	2
+ NMP	0.125	0.25	8	256	1
+ Salicylate	0.5	1	8	1024	2
+ Deoxycholate	0.25	1	8	1024	2
6590 <i>C. coli</i>	0.25	2	8	512	64
+ PA β N	0.25	0.125	4	16	32
+ NMP	0.25	0.25	8	256	32
+ Salicylate	0.5	2	8	512	64
+ Deoxycholate	0.25	2	8	512	64

previous study, we were unable to observe changes in tetracycline or erythromycin MIC levels in the presence of sodium salicylate (Randall *et al.*, 2003). In 7 out of 17 strains, a 2-fold increase in rifampicin resistance was found when salicylate was included in the medium.

Effect of sodium deoxycholate

In the study by Lin *et al.* (2005), the expression of *cmeABC* was significantly induced by various bile salts, such as deoxycholate. Cholate (1 mg ml⁻¹) and taurocholate (25 mg ml⁻¹) included in the MH broth produced, respectively, a 0- to 2-fold and a 2- to 4-fold increase in the MIC levels of various antimicrobials, such as ciprofloxacin and erythromycin, in *C. jejuni*. In our study, sodium deoxycholate (1 mg ml⁻¹) used in the agar dilution experiments had little effect on the MIC values of the studied antimicrobials. Strains with 2-fold increases in rifampicin MIC values were variably found in each round of testing, suggesting that although deoxycholate may have had an increasing effect on rifampicin MIC values, this effect was too modest to be confirmed by these experiments.

In summary, PA β N induced an 8- to 32-fold and 8- to 64-fold reduction in the MIC values of erythromycin and rifampicin for the studied strains, respectively. NMP produced a smaller, 2- to 8-fold reduction, for the same antimicrobials. With regards to the putative efflux pump inducers, sodium deoxycholate had little or no effect on the MIC values of the studied antimicrobials, whereas sodium salicylate brought about a small (2- to 4-fold) but reproducible increase in the MIC values for ciprofloxacin, and in 7/17 of the studied strains, a 2-fold increase in the MIC values for rifampicin. These results further confirm the importance of efflux in *Campylobacter* resistance and highlight the value of agents that affect efflux pumps as tools for research aimed at the inhibition of CmeABC and the reversal of antimicrobial resistance in *Campylobacter*.

ACKNOWLEDGEMENTS

This work was supported by the Finnish Graduate School on Applied Bioscience: Bioengineering, Food & Nutrition, Environment (ABS), and the Academy of Finland. We are grateful to Urszula Hirvi for her technical assistance.

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