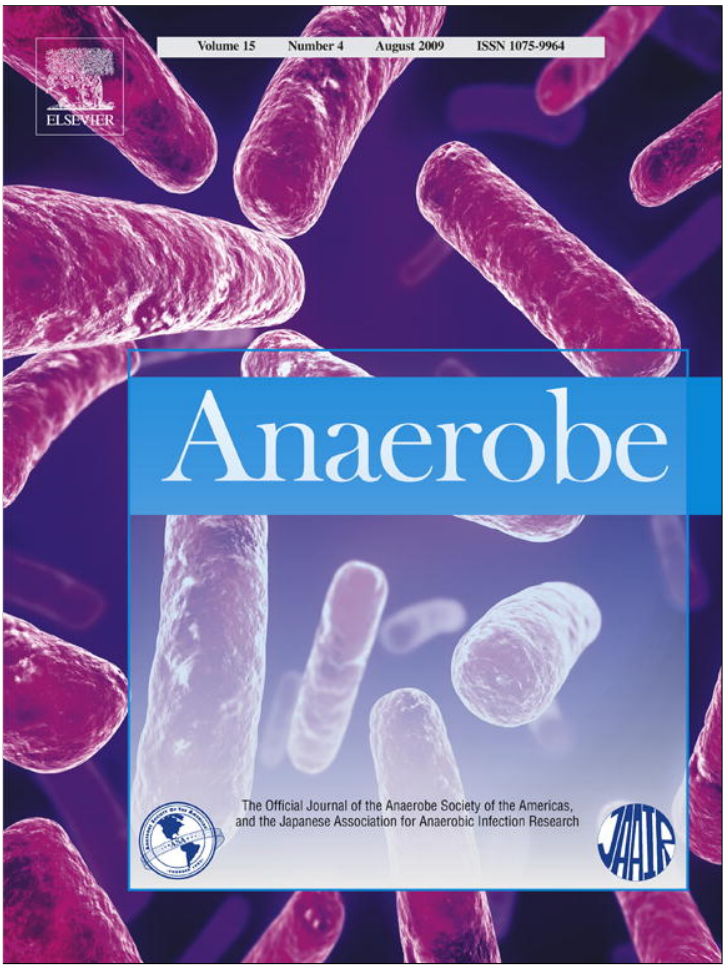

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Antimicrobial susceptibility

Characterization of an ATP-binding cassette from *Clostridium perfringens* with homology to an ABC transporter from *Clostridium hathewayi*

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ABSTRACT

A ciprofloxacin-resistant mutant of *Clostridium perfringens*, strain VPI-C, which had stable mutations in the topoisomerase genes, accumulated less norfloxacin and ethidium bromide than the wild type, strain VPI. Efflux pump inhibitors both increased the accumulation of ethidium bromide by cells of the mutant and enhanced their sensitivity to this toxic dye. Cloning a gene, which codes for a putative ABC transporter protein (NP_562422) of 527 amino acids, from the mutant strain VPI-C into the wild-type strain VPI not only reduced the accumulation of ethidium bromide by the recombinant strain but also reduced its sensitivity to norfloxacin and ciprofloxacin. Efflux pump inhibitors decreased the rate at which ethidium bromide was removed from the cells of the recombinant strain. It appears that the putative ABC transporter protein (NP_562422) may contribute to extrusion of drugs from *C. perfringens*.

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1. Introduction

Multidrug-resistance efflux pumps of ABC (ATP-binding cassette) transporters and protein/sodium-motive force transporters use the energy of ATP and ion gradients, respectively, to transport a variety of compounds from bacterial cells [1,2]. Their activities may be responsible for antimicrobial resistance in different bacterial strains [3–11]; their overexpression in bacteria can be detected by decreased ethidium bromide accumulation compared to the wild type [7,12].

Most fluoroquinolones are not effective against anaerobes [13]; several fluoroquinolone-resistant bacterial strains that efflux the drugs or have mutations in topoisomerase genes have been found [3,4,14]. We previously generated fluoroquinolone-resistant mutants from several strains of *Clostridium perfringens* with stable mutations in the fluoroquinolone target genes, DNA gyrase and topoisomerase IV [15].

Some of the mutants, including those derived from strains NCTR and VPI by growth at different fluoroquinolone concentrations, had identical mutations in the target genes but different levels of sensitivity to fluoroquinolones. Both of the mutants derived from strain VPI with 5 μg/ml and 50 μg/ml of ciprofloxacin had mutations resulting in the substitution of Asp87Tyr in GyrA and Asp93Tyr in ParC. However, the MICs of different fluoroquinolones for the mutants derived from strain VPI with 5 μg/ml ciprofloxacin had increased from 2 to 96-fold and the MICs for mutants developed with 50 μg/ml ciprofloxacin had increased from 8 to >128-fold [15]. Similarly, the mutants derived from strain NCTR with 5 μg/ml and 50 μg/ml of ciprofloxacin had had only the mutation resulting in the substitution of Asp87Tyr in GyrA. The MICs of different fluoroquinolones for the mutants derived from strain NCTR with 5 μg/ml ciprofloxacin had increased from 2 to 8-fold and the MICs for those mutants developed with 50 μg/ml ciprofloxacin had increased from 8 to >128-fold [15]. This indicated that other factors, in addition to the mutations in topoisomerase genes [16], contributed to fluoroquinolone resistance in these strains.

Multidrug-resistance pumps have been shown to be involved in the transport of drugs out of cells [5,7,11,12]. A GenBank search shows 118 genes in *C. perfringens* that are annotated as putative ABC transporters as well as other types of transporters. A 527 amino acid long protein (NP_562422) which is annotated as an ABC transporter in *C. perfringens* had homology and conserved functional domains with an ABC transporter of *Clostridium hathewayi*, CmpA [17]. CmpA is homologous to the multidrug-resistance

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transporters of several other bacteria and appears to decrease the sensitivity of *C. hathewayi* to fluoroquinolones [17]. In the present study, we have evaluated the role of transporters and the contribution of protein (NP_562422) to drug transport in *C. perfringens*.

2. Materials and methods

2.1. Bacterial cultures

The parental strains, *C. perfringens* VPI and NCTR, and a ciprofloxacin-resistant mutant, VPI-C (Cip-VPI-50 in Ref. [15]), were grown on Brain Heart Infusion (BHI) agar under anaerobic conditions in a glove box (85% nitrogen, 10% CO_2 and 5% hydrogen) [15]. The MIC of ciprofloxacin for the wild type strains VPI and NCTR was 0.25 μg/ml. The double mutant VPI-C, which had substitutions of Asp87Tyr in GyrA and Asp93Tyr in the ParC quinolone resistance determining region, was also grown on BHI agar in the presence of 50 μg/ml of ciprofloxacin. An E-test determined that the MIC of ciprofloxacin for this strain was >32 μg/ml.

2.2. Accumulation of ethidium bromide

The sensitivity of *C. perfringens* strains VPI and VPI-C to ethidium bromide was measured by applying disks containing 10 μg of ethidium bromide to lawns of strains VPI and VPI-C on BHI agar plates with and without 20 μg/ml of an efflux pump inhibitor, reserpine [10]. The zones of inhibition around the ethidium bromide disks were measured after incubation at 37 °C under anaerobic conditions.

Accumulation of ethidium bromide in *C. perfringens* strains VPI and VPI-C was measured in cells adjusted to an A_{600} of 0.2 (10^8 cfu/ml) by measuring the increase in fluorescence intensity as it accumulated in the cells [12]. Ethidium bromide (2 μg/ml) was added to washed cells suspended in MOPS/glucose buffer immediately before measurement of the fluorescence with a spectrofluorometer (Molecular Devices, Sunnyvale, CA). The ethidium bromide incorporated in the cells was measured every 40 s at excitation and emission wavelengths of 530 and 600 nm, respectively. After 10 min, the efflux pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 100 μM) was added to the cells and its effect on the accumulation of ethidium bromide was measured for another 10 min. CCCP, an ionophore which disperses the transmembrane proton gradient [18], also inhibits ATP synthesis and decreases the intracellular ATP concentration in *Escherichia coli* [19].

2.3. Accumulation of norfloxacin

Accumulation of norfloxacin was measured over time in the lysates of washed, norfloxacin-treated cells of strains VPI and VPI-C [7,10]. The cultures of *C. perfringens* strains VPI and VPI-C were centrifuged at 4 °C. The cells were washed in sodium phosphate buffer (pH 7) and resuspended in the same buffer to an A_{600} of 12. Norfloxacin (final concentration 10 μg/ml) was added to the cells. Five min after the addition of norfloxacin, 100 mM of the efflux pump inhibitor CCCP was added. Samples (0.5 ml) were removed before the addition of norfloxacin and CCCP and at 1-min intervals thereafter, diluted with 1 ml of ice-cold buffer, and placed on ice. The cells were centrifuged; pellets were washed once with ice-cold sodium phosphate buffer (50 mM, pH 7) and lysed overnight at room temperature in 1 ml of 0.1 M glycine–HCl buffer (pH 3). Cell debris was removed by centrifugation at 12,000 × g. The norfloxacin released from the cells into the supernatants was determined at an excitation wavelength of 281 nm and an emission wavelength of 440 nm. The norfloxacin concentrations were calculated by using norfloxacin standard curves and the kinetics of accumulation for each sample were determined by measuring the amount of norfloxacin accumulated per mg of cells dry weight [7,10].

2.4. Cloning of the gene coding for NP_562422

A gene coding for a protein of 527 amino acids (NP_562422) http://www.ncbi.nlm.nih.gov/entrez, which is annotated as an ABC transporter of *C. perfringens* in GenBank (Gene ID: 18310488), was amplified, cloned and its role in drug transport in *C. perfringens* was analyzed. The sequence of this gene was used to design the following primers, to amplify the entire fragment coding for 527 amino acids, as follows: One μg each of DNA, Taq polymerase, forward 21-mer primer 5′ TTC ATG TGC ATA AAA ATA GTT 3′, and reverse 24-mer primer 5′ GGA AAA GAA AAG ACA GTA AAA GTA 3′ were used in a reaction mixture with amplification conditions described previously [16]. Using these primers a fragment of 1801 base pairs was amplified that included the coding region in addition to 71 base pairs upstream of this region. Using the TOPO TA cloning system (Invitrogen), this fragment was ligated to the Topo vector and cloned in *E. coli*. The presence of the gene was confirmed by sequencing after plasmid extraction. For introduction into *C. perfringens*, the fragment was excised from the Topo vector with *Bam*HI and *Xbo*I and ligated to the plasmid pECU001. pECU001 [16] is a shuttle vector constructed from the pJIR750 plasmid of *C. perfringens* and pUC18. It has the origins of replication of *E. coli* and *C. perfringens*, the *lacZ* (β-galactosidase) gene, and the *cat* gene. The pECU001 plasmids, with and without the inserted fragment coding for 527 amino acids, were transferred to the wild-type *C. perfringens* strains VPI and NCTR. The *C. perfringens* transformants were selected using chloramphenicol (20 μg/ml) and the presence of the insert was verified by PCR in pECU527 (pECU001 with the fragment coding for 527 amino acids). The recombinant strains were designated VPI (pECU001), VPI (pECU527), NCTR (pECU001), and NCTR (pECU527).

2.5. Assay for activity of the putative transporter gene

The effect of the putative transporter gene on the accumulation of ethidium bromide was measured by comparing the kinetics of dye accumulation in strains VPI (pECU001) and VPI (pECU527) before and after addition of CCCP. The effect of the putative transporter gene on the efflux of ethidium bromide was measured as described previously [20]. *C. perfringens* strains VPI (pECU001) and VPI (pECU527) were grown in BHI overnight, washed, and suspended in 50 mM sodium phosphate buffer (pH 7.0) to 10^8 cfu/ml. 10 μg/ml of ethidium bromide and, in some tubes, CCCP (final concentration 100 μM) were added to the cells and shaken for 30 min. The cells were washed with 50 mM sodium phosphate buffer (pH 7.0) containing 10 μg/ml of ethidium bromide and resuspended in the same buffer. Ethidium bromide efflux was initiated by adding 0.2% glucose and measured with time using excitation of 500 nm and emission of 590 nm. Fluorescence was monitored continuously by using a fluorescence spectrophotometer and expressed as a percentage of the original fluorescence [20].

For detecting the effect of the putative transporter gene on protecting the cells from fluoroquinolones, the growth of *C. perfringens* strains VPI (pECU001), VPI (pECU527), NCTR (pECU001), and NCTR (pECU527) was compared in BHI containing 1% Oxyrase with or without ciprofloxacin and norfloxacin in a microtiter plate. The wells containing 10^6 cells were overlaid with mineral oil and placed inside a spectrophotometer [17]. The E-test was used for measuring the MIC of norfloxacin for the strains.

3. Results

3.1. Effect of efflux pump inhibitors on drug accumulation

The wild type *C. perfringens* strain VPI was more sensitive to ethidium bromide than the fluoroquinolone-resistant mutant strain VPI-C. Reserpine, an efflux pump inhibitor, increased the zone of ethidium bromide inhibition from 8 to 10 mm for the wild type and from 7 to 12 mm for the mutant, indicating that the mutant had a more active efflux pump.

The kinetics of ethidium bromide accumulation showed less of the dye in the mutant VPI-C than in the wild type VPI, both at the beginning and after 10 min, in the absence of an efflux pump inhibitor. Addition of the inhibitor CCCP resulted in an increased rate of accumulation of ethidium bromide in both strains, with a higher rate of increase in the mutant than in the wild type. When CCCP was added at the beginning of the experiment, the rate of ethidium bromide accumulation was 7.46 relative fluorescence units (RFU)/min for the wild type and 12.18 RFU/min for the mutant (Fig. 1). When CCCP was added to cells after incubation for 10 min with ethidium bromide, the increased rate of ethidium bromide accumulation was 3.88 RFU/min for the wild type but 6.79 RFU/min for the mutant (Fig. 1).

Less norfloxacin accumulated in the mutant strain VPI-C than in the wild type VPI in the absence of CCCP, but addition of CCCP increased the rate of norfloxacin accumulation per unit of cells dry weight more in the mutant strain than in the wild type (Fig. 2).

3.2. Effects of a putative ABC transporter gene on ethidium bromide accumulation and efflux

A GenBank search for proteins with sequence homology to an ABC transporter of *C. hathewayi*, CMPA (AY273188), which has a duplicated ATPase domain, detected a protein of 527 amino acids (NP_562422) which was annotated as an ABC transporter for *C. perfringens*. The gene coding for this protein was amplified from

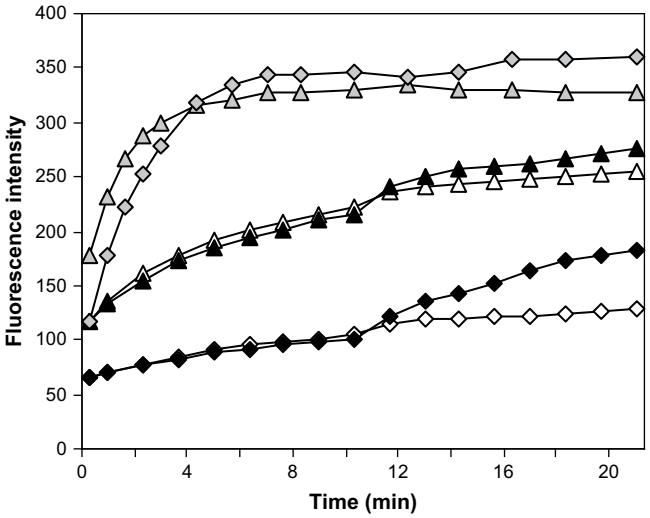

Fig. 1. Ethidium bromide accumulation (Relative fluorescence units) over time in the wild type *C. perfringens* strain VPI and a ciprofloxacin-resistant mutant, strain VPI-C, in the presence and absence of CCCP. The open symbols (△ and ◇) show ethidium bromide accumulation in the absence of CCCP in VPI (△) and VPI-C (◇). The shaded symbols (△ and ◇) show ethidium bromide accumulation when CCCP was added at zero time to VPI (△) and VPI-C (◇). The solid symbols (▲ and ◆) show ethidium bromide accumulation when CCCP was added after 10 min of incubation to VPI (▲) and VPI-C (◆). Data represent triplicate experiments in duplicate wells.

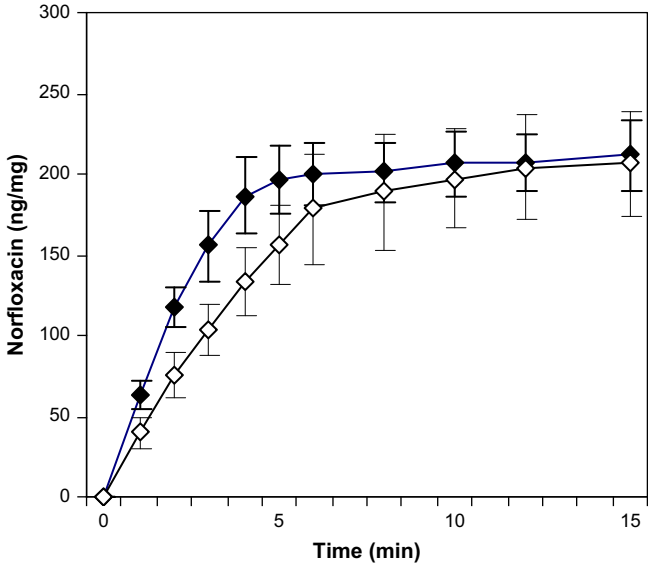

Fig. 2. Norfloxacin accumulation (nanograms per milligram dry weight of cells) with time in the wild type *C. perfringens* strain VPI (◆) and the ciprofloxacin-resistant mutant VPI-C (◇) with CCCP added after 5 min. Data represent the mean of the results of five experiments.

C. perfringens VPI-C and cloned into *C. perfringens* strains NCTR and VPI.

The accumulation and efflux of ethidium bromide were compared in *C. perfringens* strains VPI (pECU001) and VPI (pECU527) (Fig. 3A). There was more accumulation of ethidium bromide in *C. perfringens* strain VPI (pECU001) than in strain VPI (pECU527), both at the beginning and after 10 min incubation. As expected, the addition of inhibitors resulted in a higher rate of accumulation of ethidium bromide (5.3 RFU/min) in strain VPI (pECU527), which carried the plasmid with the putative transporter gene, than the rate of 3.95 RFU/min in strain VPI (pECU001), which had the plasmid only.

The effect of the putative transporter gene on the efflux of ethidium bromide was also measured (Fig. 3B). There was a higher rate of efflux of ethidium bromide in the strain containing the putative transporter gene than in the one with the plasmid only. After addition of CCCP, the rate of ethidium bromide efflux decreased in both strains, with a more pronounced effect in strain VPI (pECU527), containing the putative transporter gene.

3.3. Effect of the putative ABC transporter gene on sensitivity to fluoroquinolones

Introduction of the putative transporter gene coding for NP_562422 into the wild type also decreased the sensitivity to fluoroquinolones. Using an E-test, the MIC of norfloxacin for strain VPI (pECU001) was 0.5 μg/ml and that for strain VPI (pECU527) was 0.75 μg/ml, indicating that NP_562422 contributed to protection against norfloxacin.

The presence of the putative transporter fragment also affected the kinetics of growth of recombinant strains with ciprofloxacin and norfloxacin in strains NCTR and VPI. Fig. 4 shows the growth of strains VPI (pECU001) and VPI (pECU527) with or without norfloxacin and ciprofloxacin. In the absence of any drug, strain VPI (pECU527) grew less than strain VPI (pECU001). In the presence of norfloxacin, however, a longer lag phase was observed in strain VPI (pECU001) than in strain VPI (pECU527). In the presence of ciprofloxacin, strain VPI (pECU001) did not grow but VPI (pECU527)

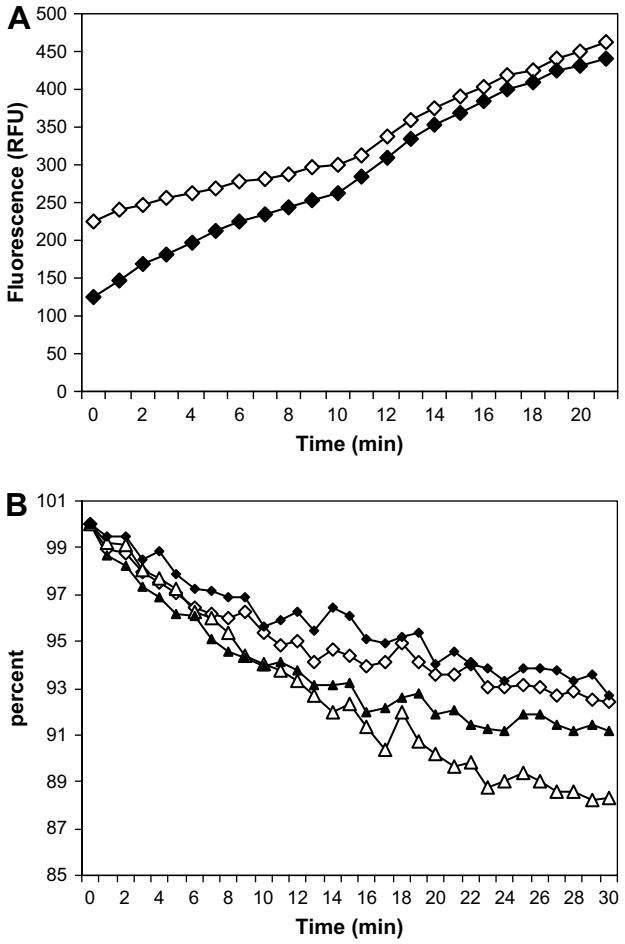

Fig. 3. Accumulation and efflux of ethidium bromide in *C. perfringens* strains in the presence and absence of CCCP. Strain VPI (pECU001) carries pECU001 without an insert and strain VPI (pECU527) carries pECU001 with the inserted gene from *C. perfringens* encoding for a fragment of 527 amino acids protein (NP_562422) which is annotated as putative ABC transporter (A) Accumulation of ethidium bromide in relative fluorescence units in strains VPI (pECU001) (◇) and VPI (pECU527) (◆), with CCCP added 10 min after ethidium bromide. (B) Efflux of ethidium bromide in percent from strain VPI (pECU001) without CCCP (◇), strain VPI (pECU527) without CCCP (△), strain VPI (pECU001) with CCCP (◆), and strain VPI (pECU527) with CCCP (▲).

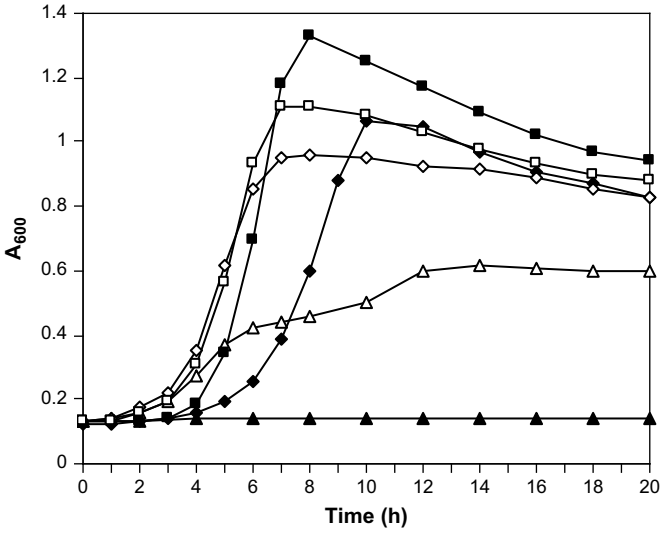

Fig. 4. Growth of *C. perfringens* strains in BHI broth (A_{600}) with time in the presence and absence of 0.125 μg/ml of norfloxacin or ciprofloxacin: strain VPI (pECU001) without any drug (■), with norfloxacin (◆), and with ciprofloxacin (▲); strain VPI (pECU527) without any drug (□), with norfloxacin (◇), and with ciprofloxacin (△).

grew (Fig. 4). Similar growth patterns were also observed in strains NCTR (pECU001) and NCTR (pECU527) data not shown.

3.4. Similarity of putative transporter NP_562422 to an ABC transporter from C. hathewayi

Sequencing of the cloned fragment showed homology of this fragment with the ATP-hydrolyzing fragment of the *C. hathewayi* ABC transporter. In addition to having identical conserved signature motifs with the ABC transporter of *C. hathewayi*, many of the amino acids throughout the fragments were conserved (Fig. 5).

4. Discussion

Since *C. perfringens* strain VPI-C contained resistance mutations in *gyrA* and *parC*, it could grow in the presence of different fluoroquinolones. The contribution of the efflux pump to fluoroquinolone resistance was measured by comparing the accumulation of ethidium bromide and norfloxacin in *C. perfringens* strains VPI (wild type) and VPI-C (mutant). Because the efflux pump extruded more ethidium bromide from the cells of strain VPI-C than from the wild type, there was less ethidium bromide accumulated in this strain in the absence of CCCP. By inhibiting ATP synthesis, CCCP decreases the concentration of ATP, which is needed to provide energy for ABC transporters [17–19]. CCCP inhibited transport activity in both strains but had a more pronounced inhibitory effect on the mutant strain with its more active pump, as shown by the 75% higher accumulation of ethidium bromide in the mutant than in the wild type when CCCP was added after 10 min. The accumulation of norfloxacin was also lower in the mutant than in the wild type. Addition of CCCP allowed more accumulation of norfloxacin in the mutant than in the wild type, indicating higher normal activity of the pump in the mutant strain. Reserpine, another efflux pump inhibitor, also increased ethidium bromide sensitivity more in the mutant than in the wild type. All these data confirm the involvement of an active efflux pump in the mutant.

Efflux of drugs from cells by the activity of transporter genes has been shown to be the reason for decreased sensitivity of bacteria to different drugs, including fluoroquinolones [3,7,9–11]. Plasmid pECU001, which was previously shown to allow expression of the dominant wild type *gyrA* in a strain with a *gyrA* mutation [16], was used to clone the putative ABC transporter gene coding for NP_562422 from *C. perfringens* VPI-C. This gene increased extrusion of ethidium bromide, as was shown by comparing the efflux and accumulation of the dye, in the presence and absence of CCCP, in isogenic *C. perfringens* VPI strains with or without this gene. As the result of expression of the gene coding for 527 amino acids of NP_562422 protein less ethidium bromide accumulated in strain VPI (pECU527) than in strain VPI (pECU001). However addition of CCCP resulted in 34% increase in rate of ethidium bromide accumulation in strain containing this gene. CCCP also had a more pronounced effect on ethidium bromide efflux in strain VPI (pECU527), which had the putative ABC transporter insert, than in strain VPI (pECU001), confirming the involvement of the gene in drug transport.

The presence of the gene coding for a protein of 527 amino acids (NP_562422) also affected the sensitivity of strains VPI and NCTR to ciprofloxacin and norfloxacin.

Perhaps because of the burden of the transporter gene coding for NP_562422 protein on the bacteria, there was less growth in the strain with the putative transporter gene than in the strain without this gene (Fig. 4). In the presence of fluoroquinolones, there is

Fig. 5. Alignment of amino acids of NP_562422 protein a fragment (used for this study) annotated as an ABC transporter of *C. perfringens* (*Cp*) with those of the ABC transporter from *C. hathewayi* (*Ch*). Bold letters show identical amino acids.

additional burden because of antibiotic stress. This was manifested both in the extension of the lag phase and in final cell densities when cells were grown with norfloxacin and ciprofloxacin. Ciprofloxacin, which is more potent than norfloxacin [13], exerted more stress on the bacteria, resulting in even less growth. However, in the presence of antibiotics, expression of the putative transporter gene resulted in better growth in the strain containing the transporter than in the strain with the plasmid only. This was seen when the putative transporter gene was cloned into two different strains of *C. perfringens*: VPI (Fig. 4) and NCTR (data not shown).

We previously detected an ABC transporter in *C. hathewayi* [16]. Its duplicated ATPase domain, CmpA, has conserved regions with several ABC drug transporters in other bacteria [1,8]. This ATP-binding protein, CmpA, in combination with a transmembrane protein, CmpB (AY273189), confers fluoroquinolone resistance on heterologous hosts [17]. A 527-amino-acid fragment from *C. perfringens* had conserved ABC signature motifs, the Walker A and Walker B motifs, which are characteristic of all ATP-hydrolyzing segments of ABC transporters [1,21]. This fragment also had amino acid sequences throughout the protein that were conserved with those of CmpA from *C. hathewayi*. Involvement of this fragment in drug transport was experimentally established by cloning it into *C. perfringens* strains. The cloned fragment, coding for 527 amino acids, probably uses a transmembrane protein from the host to extrude drugs. Not all of the transmembrane proteins of ATP transporters have been identified [1,8]. A gene in the GenBank database listed as YP_694872.1 from *C. perfringens*, designated as a hypothetical protein, had similarity to the CmpB transmembrane protein of the *C. hathewayi* ABC transporter (34% identity and 61% positive conserved amino acids). The TMPRED program from http://www.ch.embnet.org identified it as a transmembrane protein with 5 helices. However, since the 527-amino-acid ATP-binding fragment was cloned into a homologous host, it had access to the necessary transmembrane protein. The role of this transmembrane protein in decreasing fluoroquinolone sensitivity and accumulation of ethidium bromide is not known. It appears that, as in other bacteria [4], exposure of *C. perfringens* to fluoroquinolones, in addition to inducing mutations in topoisomerases, may result in overexpression of efflux pumps in some strains. The transporter inhibitor affected accumulation of ethidium bromide more in the mutant strain VPI-C than in the wild type VPI (pECU527) indicating involvement of other transporter besides NP_56422 in drug transport. Like *Bacteroides fragilis* [4], *C. perfringens* has many transporter genes that may contribute to extrusion of drugs. We have identified one of the genes that may use the energy of ATP hydrolysis to do so.

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