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A MATE Family Multidrug Efflux Transporter Pumps out Fluoroquinolones in *Bacteroides thetaiotaomicron*

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We cloned a gene, *bexA*, that codes for a multidrug efflux transporter from the chromosomal DNA of *Bacteroides thetaiotaomicron* ATCC 29741 by using an *Escherichia coli* Δ*acrAB* Δ*acrEF* mutant as a host. Although the initial recombinant construct contained other open reading frames, the presence of *bexA* alone was sufficient to confer to the *E. coli* host elevated levels of resistance to norfloxacin, ciprofloxacin, and ethidium bromide. Disruption of *bexA* in *B. thetaiotaomicron* made the strain more susceptible to norfloxacin, ciprofloxacin, and ethidium bromide, showing that this gene is expressed in this organism and functions as a multidrug efflux pump. The deduced BexA protein sequence was homologous to the protein sequence of *Vibrio parahaemolyticus* NorM, a multidrug efflux transporter, and thus, BexA belongs to the multidrug and toxic compound extrusion (MATE) family.

Members of the *Bacteroides fragilis* group are anaerobic bacteria of the highest clinical relevance, and *B. fragilis* and *Bacteroides thetaiotaomicron* are often isolated from patients with suppurative anaerobic infections. They are gram-negative, obligately anaerobic organisms with a broad spectrum of recognized resistance to antimicrobial agents (23), including aminoglycosides, most of the penicillins and cephalosporins, and fluoroquinolones (except for a few recently developed compounds such as trovafloxacin and clinafloxacin) (2, 3).

Active multidrug efflux processes, usually involving secondary transporters belonging to the major facilitator superfamily, small multidrug resistance family, and resistance-nodulation-division (RND) superfamily, are now known to be important, especially in the baseline or intrinsic resistance of many bacteria to antimicrobial agents (16, 21). More recently, a new family, the multidrug and toxic compound extrusion (MATE) family, has been discovered (4), but its contribution to drug resistance has been known only for a few isolated cases (12, 13).

We found previously that at least a portion of the remarkable norfloxacin resistance (MICs, 16 to 32 μg/ml) of *B. fragilis* was attributed to active efflux of this agent (12). To elucidate the efflux mechanism, we attempted to clone the genes responsible for norfloxacin efflux from the chromosomal DNAs of *B. fragilis* and *B. thetaiotaomicron* using as the host an *Escherichia coli* mutant strain with defects in multidrug efflux pumps. Here we report on the cloning and sequencing of a gene, *bexA*, that is involved in the efflux of norfloxacin, ciprofloxacin, and ethidium bromide from *B. thetaiotaomicron*. Interestingly, the BexA transporter is a member of the MATE family.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741 5482 and were grown in general anaerobic GAM broth (Nissui, Tokyo, Japan) and supplemented Trypticase soy broth (sTSB) (12) in an anaerobic chamber.

For cloning, two host strains were used. They were *E. coli* DH5α [K-12 *supE44* Δ*lacU169* (φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] (26) and AG102AX [K-12 *argE3 thi-1 rpsL xyl mtl* Δ(*gal-uvrB*) *marR1* Δ*acrAB*::*kan* Δ*acrEF*::*spc*]. The latter strain was constructed by transducing into AG102A (18) an Δ*acrEF*::*spc* allele (made in strain JC7623 [9]) by deleting a 1,133-bp fragment between the *Eco*47III site about 43% of the way into *acrE* gene and the *Eco*RV site about 15% of the way into the *acrF* gene and by introducing the Ω interposon containing the spectinomycin and streptomycin resistance marker (22) in its place. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth or LB agar supplemented with ampicillin (20 μg/ml) or norfloxacin (50 ng/ml) when necessary.

Susceptibility testing. The MICs of drugs for *E. coli* and *Bacteroides* strains were determined by a broth microdilution assay in LB broth and sTSB, respectively, as described previously (12). For *Bacteroides* strains, gradient plate assays for MICs were also used when necessary (6). Cultures were incubated at 37°C overnight or for 2 days before the growth was assessed.

Gene cloning and sequencing. Chromosomal DNAs were prepared from cells of *B. fragilis*, *B. thetaiotaomicron*, and their mutants by the method of Smith et al. (30) and later with a MasterPure Genomic DNA purification kit (Epicentre Technologies, Madison, Wis.). The DNA was partially digested with *Sau*3AI, and digested fragments with sizes of 2 to 10 kb were separated by agarose gel electrophoresis. The DNA fragments extracted from the gels were ligated into vector pBR322 that was cut with *Bam*HI and were then dephosphorylated with calf alkaline phosphatase. Cells of *E. coli* AG102AX were transformed with the ligated recombinant plasmids by electroporation and were spread on LB agar plates containing 50 ng of norfloxacin and 20 μg of ampicillin per ml. This led to the isolation of pBRBT20 containing 5.3 kb of *B. thetaiotaomicron* DNA. Other DNA manipulations were carried out by standard procedures as described elsewhere (26).

Subcloning of the insert in pBRBT20 were first performed by removing the 5′ end of the insert by double digestion with *Eco*RV and *Bam*HI and then inserting the shortened fragments into pBR322 digested with both *Eco*RV and *Bam*HI, generating pBRBT201 (Fig. 1). The same fragment was also inserted into the multiple cloning site of pUC18, generating pUC18BT201. Digestion of pBRBT201 with *Mlu*I and *Eco*RV, followed by conversion of the *Mlu*I-digested terminus into a blunt end by filling in with T4 DNA polymerase (Takara DNA Blunting kit; Takara Biotechnology, Kyoto, Japan) and finally religation, generated pBRBT201ME (Fig. 1). Similarly, digestion of pBRBT201 with *Kpn*I and *Bam*HI, followed by conversion of the ends into blunt ends by digestion with

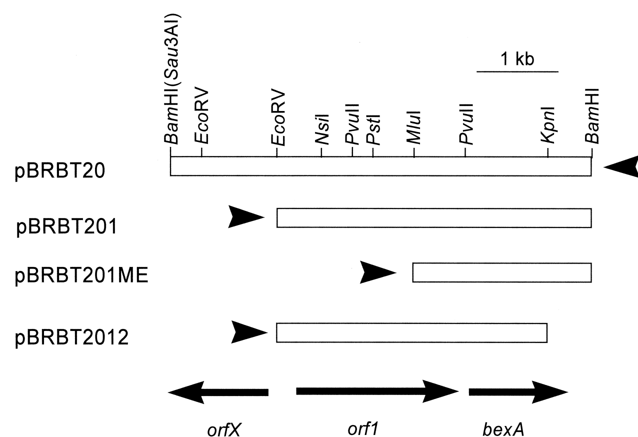

FIG. 1. Physical map of a cloned fragment containing the *orfX*, *orf1*, and *bexA* (*orf2*) genes from *B. thetaiotaomicron*. Although a 5.3-kb DNA fragment partially digested with *Sau*3A was cloned into the *Bam*HI site of pBR322, the right side of the insert-vector junction remained cleavable with *Bam*HI. The arrows at the bottom indicate putative open reading frames and their directions of transcription. Arrowheads indicate the approximate positions and the directions of transcription of the *tet* promoter in the vector pBR322.

mung bean nuclease and religation, gave pBR2012 (Fig. 1). In addition, subcloning of *orf1* and *bexA* (Fig. 1) was performed by PCR amplification of these genes. Primers contained 5′ extensions corresponding to *Eco*RV and *Bam*HI target sequences for directed insertion into the pBR322 vector. The forward primers for *orf1* and *bexA* started at residues 1,536 and 3,417 of the insert sequence in pBRBT20, respectively (or 85 and 147 bp upstream from the putative translation initiation codons of *orf1* and *bexA*, respectively). The backward primers started at nucleotides 3,511 and 4,998, respectively, in the insert DNA of pBRBT20. The amplified fragments were inserted into pBR322 digested with *Eco*RV and *Bam*HI, to yield plasmids pYEB (containing orf1) and pNEB (containing *bexA*); in these plasmids the cloned genes are inserted in the orientation in which the *tet* promoter of the vector could initiate their transcription.

In addition, another set of primers starting at nucleotides 3,418 and 4,992, respectively, were used to amplify *bexA* for insertion in the reverse direction. The fragments were amplified by using pBRBT20 as the template, purified, and cut with *Eco*RV and *Bam*HI. These fragments were inserted into the pBR322 vector that had been cut with *Eco*RV and *Bam*HI to yield plasmid pNBE, in which the *bexA* gene was inserted in a reverse orientation in relation to the *tet* promoter of the vector. The recombinant plasmids were confirmed to contain the correct genes in the expected direction by PCR amplification as well as digestion with several restriction enzymes.

Preparation of a gene disruption mutant in *B. thetaiotaomicron*. pGERM, a suicide vector for *Bacteroides* spp. (24), was used for preparation of a gene disruption mutant in *B. thetaiotaomicron*. This vector contains markers selectable in *E. coli* (*bla*) as well as in *Bacteroides* (*ermG*), replicates in *E. coli*, and can be mobilized for transfer into other bacteria because of the presence of a transfer origin (*oriT*) from plasmid RK2. It also carries the multiple cloning sites from pUC19. An approximately 1-kb internal fragment of *bexA* was cut out from pBRBT201 by digestion with *Pvu*II and *Kpn*I (Fig. 1) and was inserted into pGERM cut with *Sma*I and *Kpn*I. *E. coli* S17-1 was transformed by this recombinant construct of pGERM containing an internal fragment of *bexA*, and a transformant was used as the donor in a mating with *B. thetaiotaomicron* ATCC 29741. In *Bacteroides*, pGERM derivatives cannot replicate, so a single homologous recombination with the chromosome within *bexA*, resulting in the insertion of pGERM sequence into this gene, is the most likely way to make the recipient cell erythromycin resistant (25, 29). Several erythromycin-resistant colonies recovered on plates with erythromycin (10 μg/ml) and gentamicin (200 μg/ml) (added in order to counterselect the donor strain, as *Bacteroides* spp. are intrinsically resistant to aminoglycosides [23]) were picked up for further analysis. These possible disruption mutants were tested for drug susceptibility and were then tested by genomic Southern analysis to confirm the occurrence of a disruption within *bexA*. A disruption mutant of *bexA* in *B. thetaiotaomicron*, OUT4, was isolated in this manner.

Genomic Southern blot analysis. Whole-plasmid DNAs of pBRBT20 (carrying the full insert DNA) and of pGERM recombinants pFY13A (containing the 0.65-kbp *Pst*I-*Nsi*I fragment, corresponding to an internal fragment of *orf1*) and pFY28 (containing the 1-kb *Kpn*I-*Pvu*II fragment, corresponding to an internal fragment of *bexA*) were biotinylated by nick translation with the BioNick Labeling System (Life Technologies, Frederick, Md.) and were then used as probes. Chromosomal DNAs were extracted as described above, digested with various restriction enzymes, and separated by gel electrophoresis. The Southern hybridization was detected by using the PhotoGene nucleic acid detection system (version 2.0; Life Technologies) according to the manufacturer's instructions.

Norfloxacin efflux assay. Norfloxacin accumulation in the cells was assayed by filtration of cells incubated with [^{14}C]norfloxacin (12). Energy-dependent efflux was assessed by measurement of an increase in the level of accumulation upon the addition of a proton conductor, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), at 100 μM.

Nucleotide sequence accession number. The nucleotide sequence data have been deposited in the DDBJ database under accession no. AB067769.

RESULTS AND DISCUSSION

Cloning of norfloxacin resistance gene from *B. thetaiotaomicron*. Since norfloxacin is actively pumped out from *B. fragilis* (12) and *B. thetaiotaomicron* (unpublished data) and since the efflux is catalyzed by a multidrug efflux pump (12), we tried to clone a gene(s) responsible for the efflux activity from these two species. We successfully obtained from *B. thetaiotaomicron* but not from *B. fragilis* several recombinant plasmids that decreased the norfloxacin susceptibility of the hypersusceptible *E. coli* host strain lacking major multidrug efflux pumps (strain AG102AX). One of the recombinants carrying a 5.3-kb insert, pBRBT20, was used for further experiments (Fig. 1). As shown in Table 1, the norfloxacin and ethidium bromide MICs were eight times higher and the ciprofloxacin MIC was four times higher for AG102AX carrying pBRBT20 in comparison with the MICs for the host strain or the strain containing the vector (pBR322) alone. pBRBT20 did not increase the MIC of sparfloxacin, a lipophilic fluoroquinolone, or the MICs of chloramphenicol and puromycin (Table 1).

Sequence and characteristics of the putative gene products. Sequencing of the whole insert of pBRBT20 revealed three open reading frames shown as *orfX*, *orf1*, and *orf2* (*bexA*) in Fig. 1. The *orf2* product is predicted to contain 443 amino acids. BLASTP analysis of the sequence showed that it had strong similarity to the *Vibrio parahaemolyticus* NorM sequence (13) and that of its *E. coli* homolog, VdhE (14), as well as to those of many other proteins discussed below (see "Homology among members of the MATE family" below). Because of its homology with NorM, a known multidrug efflux transporter (13, 14), we propose that *orf2* be named *bexA* (bacteroides exporter A). The family containing NorM was earlier defined as the MATE family (5).

The *orf1* sequence is predicted to code for a protein of 618 amino acid residues. The BLASTP program suggested that this protein was a homolog of YidC, the *E. coli* protein recently shown to be involved in the insertion of membrane proteins (27, 28). Although the similarity was 39%, this involved the creation of many gaps corresponding to 19% of the aligned sequence, and it is not clear if this protein performs a similar function in *Bacteroides*. In any case, partial deletion of the insert sequence (see below) showed that this gene was unlikely to be involved in drug efflux.

The *orfX* sequence, transcribed in the opposite direction, was predicted to code for a 403-residue protein, although its C

TABLE 1. Antibiotic susceptibilities of various strains[a]

Strain	MIC (μg/ml)					
	Norfloxacin	Ciprofloxacin	Sparfloxacin	Ethidium bromide	Chloramphenicol	Puromycin
E. coli						
AG102AX[b]	0.03	0.004	0.001	8	1	4
AG102AX/pBRBT20	0.25	0.016	0.001	64	1	4
AG102AX/pBRBT201	0.25	0.016	0.001	64	1	4
AG102AX/pBRBT201ME	0.25	0.016	0.001	64	1	4
AG102AX/pBRBT2012	0.03	0.004	0.001	8	1	4
AG102AX/pYEB	0.03	0.004	0.001	8	1	4
AG102AX/pNEB	0.25	0.016	0.001	64	1	4
AG102AX/pNBE	0.03	0.004	0.001	8	1	4
DH5α[b]	0.25	0.06	0.03	64	4	64
DH5α/pUC18BT201	1.0	0.25	0.03	512	4	64
B. thetaiotaomicron						
ATCC 29741	128	16	2	128	14	4
OUT4 (*bexA* mutant)	32	8	2	32	14	4

[a] MIC determination by the broth microdilution assay was repeated at least three times in each case, and the consistencies of the MICs were confirmed.
[b] No change in the MIC occurred for strain AG102AX or DH5α when the host was transformed with the vector plasmid alone.

terminus appears to be truncated in pBRBT20. This polypeptide showed strong similarity to the C-terminal halves of dozens of bacterial helicases, with the strongest similarity to those of *Staphylococcus aureus* (M63176_2) and *Neisseria meningitidis* (AJ391262-1). The function of this open reading frame is not clear, and its deletion did not affect the drug efflux phenotype, as described below.

Subcloning of insert sequence. Several subclones were made from pBRRT20 in order to identify the gene essential for the norfloxacin resistance (Fig. 1). pBRBT201, in which an approximately 1.3-kb *Bam*HI-*Eco*RV fragment (containing *orfX*) was deleted from the *orfX*-proximal end of the insert in pBRBT20, still exhibited the same level of resistance as the initial clone (Table 1). When the insert in pBRBT201 was further shortened on the 5′ side by removing the *Eco*RV-*Mlu*I fragment that contains most of *orf1*, the resultant construct, pBRBT201ME, still maintained the high level of resistance (Table 1), showing that *orf1* is not required for resistance. In contrast, pBRBT2012, with a deletion of the 0.6-kb *Kpn*I-*Bam*HI fragment at the *bexA*-proximal end of the insert, produced no increase in the level of resistance (Table 1), suggesting that the *bexA*-containing region is essential for resistance.

A promoter-like sequence (TATAAT and TGACAA) in front of *orf1* shows strong similarity to the *E. coli* consensus −10 and −35 sequences, and no rho-independent terminator is found downstream of *orf1*. Thus, in pBRBT201 and pBRBT2012, which contain *orf1* and its upstream sequence, *bexA* could have been transcribed at least in part by "readthrough" from *orf1*. The same explanation also applies to pBRBT20, in which the tetracycline promoter of the vector is located at the 3′ end (the right end in Fig. 1) of the insert. In pBRBT201ME, which lacks this putative *orf1* promoter, however, the tetracycline promoter of the vector pBR322, located on the 5′ side of the insert (the left side in Fig. 1), was likely to have been responsible for transcription of *bexA* in *E. coli*, as the sequence between *orf1* and *bexA* does not seem to contain a promoter sequence typical for *E. coli*. This hypothesis was supported by the observation that the level of norfloxacin resistance was drastically reduced when the tetracycline promoter was removed by cutting pBRBT201 with *Aat*II and *Eco*RV and then the upstream region and the 5′-terminal portion of *orf1* were removed by exonuclease III digestion (data not shown).

In order to confirm the conclusion that only *bexA* is needed for antibiotic resistance, we amplified the *orf1* and *bexA* genes (with their upstream sequences) separately through PCR and inserted these genes into pBR322 digested with *Eco*RV and *Bam*HI so that the tetracycline promoter was located upstream from these genes. The results with these constructs, pYEB and pNEB (Table 1), showed clearly that only *bexA* is necessary for resistance. However, when the *bexA* gene was inserted in the reverse orientation in plasmid pNBE, there was no increase in the level of resistance, suggesting that the immediate upstream sequence of *bexA* indeed did not function as an efficient promoter, at least in *E. coli*, and that the successful expression of *bexA* in pNEB was due to the presence of the tetracycline promoter of the vector.

The insert sequence in pBRBT201 was recloned in expression vector pUC18 in a direction that allowed the P*lac* promoter of the vector to drive the transcription of both *orf1* and *bexA*. The resultant plasmid, pUC18BT201 increased the norfloxacin MIC for strain DH5α, in which the Δ*lacU169* deletion covers *lacI* and allows the constitutive expression of the P*lac* promoter (Table 1). Since, in contrast to strain AG100AX, DH5α has no known defects in its multidrug efflux systems and its OmpF porin level is not reduced by the *marR* mutation (15), these features of AG100AX are not needed to see the effect of the BexA pump in the increased level of resistance. Since NorM also pumps out aminoglycosides (14), we tested the MICs of kanamycin and streptomycin using DH5α/pUC18BT201. No detectable increase in the MICs of these compounds for DH5α/pUC18BT201 in comparison with those for the parent strain, DH5α, was found (data not shown).

Homology among members of MATE family. The MATE family was reported to contain three branches: the NorM branch, a branch containing several plant proteins (such as T51035 of *Arabidopsis thaliana*; see below), and a branch containing *E. coli* DinF (5). Among these three clusters, BexA had

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B. thetaiotaomicron BexA         156 TKTLTLNSIVMVLSNVVFNYILIFGKFGFPQLGIAGAAIGS
T. maritima T0815                168 TRTPMIVTGLTNFLNIFLDYAMIFGKFGFPEMGVRGAAVAT
P. abyssi PAB0243                154 TKTPMKLNILMNVINAVLDYLLVFGKFGFPRLGPVGAAWAS
P. horikoshii PH1807             154 TKTPMKLNILMNVINGILDYLLVFGKLGFPKLGPVGAAWAS
B. halodurans AP001517           157 TKDVMYVTIGMNILNVILDYLLVFGKLGFPRMGPVGAAWAS
Pyrococcus sp. X91006            155 TKTPMLLNILMNVVNAVGNYLFIFGPFGIPVLGVTGVALST
B. thuringiensis AF305387        168 TKQAMFISLGMNIIHIAGNYVLIFGKFGFPELGVQGAAISS
B. subtilis YoeA Z99114          169 SKTPFYTLIVSTVINIALLPVLILGMFGFPKLGIYGSAYAT
Synechocystis Slr0896            172 ARPIMLIVIAATLFNILGNYGLGFGKWGFPALGITGLAIAS
V. parahaemolyticus NorM         156 TKPAMVIGFIGLLLNIPLNWIFVYGKFGAPELGGVGCGVAT
V. cholerae NorM                 160 TKPAMVIGFIGLLLNIPLNWIFVYGKFGAPELGGVGCGVAT
E. coli YdhE (NorM)              157 TKPGMVMGFIGLLVNIPVNYIFIYGHFGMPELGGVGCGVAT
H. influenzae HI1612             161 TKPAMVITFLGLLINIPLNYIFIYGKFGMPAFGAVGCGIAT
B. subtilis YojI                 160 TRVTMMITLSSLPINFVLNYVFIFGKFGMPALGGVGAGLAS

A. thaliana T05135               183 TLPLTYSAFFAVLLHIPINYLLVSSL----GLGLKGVALGA
A. thaliana Orf4 CAA66809        195 IMVMAVISAVALVIHVPLTWFVIVKL----QWGMPGLAVVL

E. coli DinF P28303              176 ARAPVILLVVGNILNIVLDVWLVMGL----HMNVQGAALAT
S. pneumoniae DinF CAA84073      165 SLAALGFLIFSALVNVVLDLYFITQL----HLGVQSAGLAT
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FIG. 2. Multiple alignment of the new signature region of MATE family members.

a high degree of similarity to members of the NorM branch. Thus, the Expect (*E*) value in BLASTP was 9×10^{-17} with *V. parahaemolyticus* NorM and 4×10^{-20} with the *Haemophilus influenzae* homolog of NorM (HI1612). The closest homologs of BexA in terms of *E* values, however, were MATE family members that have not been tested for their efflux functions and that have often been described as DinF homologs in databases, such as *Thermotoga maritima* T0815 (AAD35897.1) and *Pyrococcus abysii* PAB0243 (CAB49288.1), with *E* values of 4×10^{-30} and 2×10^{-29}, respectively. Nevertheless, BexA does not appear to belong to the DinF branch or the plant protein branch, because a two-sequence BLAST search between BexA and representatives of these clusters (DinF of *E. coli* and T05135 of *A. thaliana* [F7H19.220, listed as H19.22 in Fig. 3 of reference 5]) produced rather high *E* values (4×10^{-4} and 0.047, respectively).

Multiple alignment (with the Clustal W program [31]) also confirmed this conclusion. A previous report (5) concluded that a region corresponding to putative transmembrane helices 5 and 6, as well as the segment containing helix 8 and the following loop, are most characteristic in the proteins of MATE family. However, many more MATE family members have been sequenced since then, and in particular, inclusion of proteins from extremophiles produces a somewhat different picture. In the regions noted earlier (5), BexA and its most closely related homologs, from *Thermotoga* and *Pyrococcus*, show only low degrees of similarity to "classical" MATE family members such as NorM. However, other regions of the protein turn out to be more instructive. In Fig. 2 we show the alignment of the segment corresponding to the putative transmembrane helix 6 and the segment that follows it. All members of the putative NorM branch show a remarkable conservation of the sequence GKFGXP. In contrast, this sequence was not conserved at all in the plant protein branch (*A. thaliana* proteins) or the DinF branch. This confirms that BexA indeed belongs to the NorM cluster, although the group that includes BexA and the *Thermotoga* and *Pyrococcus* proteins seems to be somewhat more distantly related to the close relatives of NorM from the facultatively anaerobic gram-negative bacteria. Thus, the sequences that are remarkably conserved in the latter group (TKPXMVIGFIGLLXNIPLN or LGGVGCGVAT) are not well conserved in the former group. Apparently, this distance was responsible for the misclassification of some of the extremophile proteins, such as the proteins from the two *Pyrococcus* species, as DNA damage-induced protein or homologs of DinF in the databases.

Norfloxacin efflux activity. As shown in Fig. 3, the addition of CCCP to *E. coli* AG102AX carrying pBRBT201 produced a large increase in the level of norfloxacin accumulation. In addition, in the absence of CCCP the steady-state level of norfloxacin accumulation in AG102AX/pBRBT201 was lower than that in the same host carrying the pBR322 vector alone. AG102AX with no plasmid showed a similar, higher level of accumulation of norfoxacin (data not shown). These results suggest that this lower steady-state level of accumulation in AG102AX/pBRBT201 was achieved by an active efflux driven by the gene product expressed from the recombinant plasmid in *E. coli*.

Effects of inhibitors. We examined the effects of various inhibitors of bacterial multidrug efflux pumps. As shown in Table 2, several compounds appeared to inhibit the increase in the norfloxacin MIC, presumably by inhibiting drug efflux. These compounds included reserpine and verapamil, both of which are known to be inhibitors of major facilitator superfamily pumps (1, 15), and MC-207,110, which is known to inhibit RND pumps in *Pseudomonas aeruginosa* (10). We note that reserpine and verapamil were earlier shown to decrease the norfloxacin MICs for *B. fragilis* strains (12).

Disruption mutant of *bexA*. A disruption mutant of *B. thetaiotaomicron bexA*, OUT4, was isolated by using a suicide

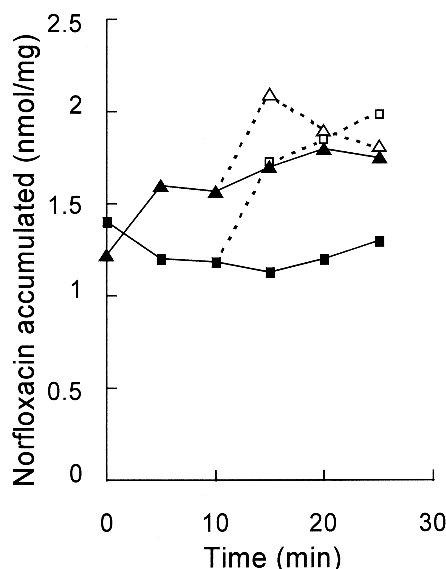

FIG. 3. Accumulation of [^{14}C]norfloxacin by *E. coli* AG102AX cells with either the recombinant plasmid, pBRBT201 (squares), or the vector alone (triangles). At 10 min a proton conductor, CCCP, was added to one-half of the bacterial cell suspension. Closed and open symbols, norfloxacin accumulation in cells in the absence and presence of CCCP, respectively.

vector, pGERM (see Materials and Methods). Genomic Southern analysis confirmed that the *bexA* gene was interrupted in the mutant (data not shown). It was more susceptible than the parent strain, *B. thetaiotaomicron* ATCC 29741, to norfloxacin, ciprofloxacin, and ethidium bromide (Table 1), confirming that the gene is expressed in *Bacteroides* and contributes to its intrinsic resistance to fluoroquinolones.

Homologs in *B. fragilis*. Southern blot analysis with pGERM recombinants (pFY28) carrying an internal fragment of *bexA* as a probe did not reveal any homologous DNA fragments from *B. fragilis* ATCC 25285. Two clinical isolates of *B. fragilis* used previously (12) had no positive bands either, but *B. thetaiotaomicron* ATCC 29741 and 5482 contained similar positive bands carrying the genes (data not shown).

Since the genome sequencing of *B. fragilis* ATCC 25285 is in progress at the Sanger Centre and the raw sequences of the fragments are available at its website (www.sanger.ac.uk), we looked for homologs of the BexA protein by using the translated products of these sequences with the program TBLASTN. Indeed, the product of three sequences spliced together (Bf221 h02.q1c + Bf236a12 [reverse complement] + Bf142c11.qlc) had the strongest similarity (84% identity at the protein level), and at least three other sequences had strong similarity. However, this degree of similarity would not have been detected by Southern blotting under high-stringency conditions. We are trying to disrupt this and other homologs in *B. fragilis* in an attempt to evaluate their contribution to the drug resistance of this organism.

Other possible mechanisms contributing to intrinsic fluoroquinolone resistance of *Bacteroides*. When we inactivated the *bexA* gene in *B. thetaiotaomicron*, the norfloxacin MIC decreased from 128 to 32 μg/ml (Table 1), yet the MIC for the mutant was still much higher than those for the wild-type strains of *E. coli*, for example. The presence of at least four homologs of *bexA* in *B. fragilis* suggests the possibility that there may also be other homologs of *bexA* in *B. thetaiotaomicron* which could contribute to the fluoroquinolone resistance. In addition, the target genes in various *Bacteroides* species might already contain "mutations" that make these targets resistant to fluoroquinolones, as has been demonstrated in several species of *Mycobacterium* (7). In fact, a partial sequence of *B. thetaiotaomicron* GyrA has recently been reported to contain an Asp86-to-Tyr change (17), corresponding to a frequent mutation (Asp87Tyr) found in fluoroquinolone-resistant mutants of *E. coli* (8, 32). The *gyrA* and *gyrB* genes of *B. fragilis* have been sequenced (19), and interestingly, Asp86 of the GyrA enzyme is again changed into phenylalanine. As the mutation of this residue to various neutral amino acids increases the level of fluoroquinolone resistance (8, 20, 32), this change may also make the target less susceptible in *B. fragilis*. Mutations of the target enzymes, however, usually produce only moderate levels of resistance, and the additional increase in efflux activity is needed for the production of very high levels of resistance, at least in *E. coli* (see reference 11 and references cited therein). These considerations suggest that active efflux plays a major role in the intrinsic fluoroquinolone resistance in *Bacteroides*. (However, in *B. fragilis* it has been reported that mutations in *gyrA* are usually introduced at later stages in the creation of strains with high levels of fluoroquinolone resistance [4].)

TABLE 2. Susceptibility of BexA multidrug resistance pump to potential inhibitors

Inhibitor[a]	Norfloxacin MIC (μg/ml)		
	AG102AX/ pBR322	AG102AX/ pBRBT201	AG102AX/ pBRBT201ME
None	0.03	0.25	0.25
Reserpine (20 μg/ml)	0.015	0.06	0.06
Reserpine (100 μg/ml)	0.015	0.06	0.06
Verapamil (20 μg/ml)	0.015	0.06	0.06
Verapamil (100 μg/ml)	0.015	0.015	0.015
MC 207,110 (10 μg/ml)	0.015	0.125	0.125
MC 207,110 (15 μg/ml)	0.015	0.015	0.06

[a] These inhibitors inhibited the growth of the *E. coli* strains at high concentrations; and the MICs of reserpine, verapamil, and MC 207, 110 for AG102AX/pBR322 were >512, 512, and 64 μg/ml, respectively.

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