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Molecular analysis of the effector mechanisms of cefoxitin resistance among *Bacteroides* strains

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Objectives: The characterization of *Bacteroides* strains with regard to the *cfxA* gene, the MTn*4555* mobilizable transposon, the role of penicillin-binding proteins (PBPs) and heterogeneous cefoxitin resistance.

Methods: Eighty-four randomly selected and 11 heterogeneously or highly cefoxitin-resistant *Bacteroides* isolates were included. Agar dilution and Etest methods were used for the determination of cefoxitin MICs. PCR experiments and nucleotide sequencing were used to detect the *cfxA* gene and the molecular features of MTn*4555*. Cefoxitin-binding experiments to determine its affinity (IC_{50}) for PBPs and cefoxitinase assays were also applied. Southern blotting was used to determine the copy number of the *cfxA* genes.

Results: Sixteen strains from the random collection proved to be positive for *cfxA*, and the MIC distribution for the *cfxA*-negative and -positive strains did not display a clear separation. The majority of the *cfxA*-positive strains in this collection harboured a 1.2 kb common region at the 3′ end of MTn*4555*. This region encoded an open reading frame that exhibited homology to abortive phage infection proteins (AbiD). The *cfxA* genes were transferable only at low frequencies in conjugation experiments. In PBP affinity studies, the PBP-A and PBP3 species were largely insensitive to cefoxitin, whereas the other PBP species were affected at very low concentrations. Seven of the heterogeneously resistant strains were positive for *cfxA* and most of them had mutations in the regulatory regions of *cfxA*.

Conclusions: Major and minor roles for *Bacteroides fragilis* PBPs and the CfxA cefoxitinase, respectively, were inferred. The role of the newly recognized *abiD* may be to control the copy number of *cfxA*.

Keywords: cephamycinases, *cfxA*, MTn*4555*, penicillin-binding protein(s)

Introduction

Bacteroides species are the most important anaerobic pathogens with regard to the frequency of their isolation, their antibiotic resistance rates and their various mechanisms of resistance. They cause a wide variety of soft tissue infections (some of which are life-threatening), such as abdominal, pelvic and brain abscesses and sepsis, where *Bacteroides fragilis* is the most frequently encountered and the most virulent species.[1]

The cephamycins are a good option for the treatment of these infections. These drugs (cefoxitin, cefotetan and cefmetazole) are cephalosporins whose usefulness is based on the specific 7α-methoxy modification of their β-lactam ring, which makes them fairly resistant to the action of most β-lactamases and furnishes them with a lower binding capacity for penicillin-binding proteins (PBPs).[2] Only the C and B molecular type β-lactamases are capable of readily hydrolysing them.[3]

While low resistance rates were reported among the *B. fragilis* group strains in the 1980s (2%), moderate levels emerged in the 1990s both in Europe and in the USA (3%–7.9%).[4–6] This trend was still observed in Europe in the late 2000s (17.2%),[7] but the level of resistance seems to be decreasing in the USA in the 2000s (12% versus 9%), presumably due to the removal of cefoxitin from clinical use.[8,9]

In the 1980s, the lack of known cefoxitin-degrading *Bacteroides* β-lactamases directed attention to PBPs as a possible cause of resistance. However, strains with transferable cefoxitin resistance were also found.[10] The role of PBPs was determined in cephalosporin resistance, including cefoxitin, in clinical *Bacteroides* strains, and was also determined in cefoxitin and piperacillin resistance using cefoxitin-resistant mutants.[11] These initial experiments, carried out with [^{14}C]benzylpenicillin and fluorography, revealed four to seven PBP species.[12,13] A more accurate detection of the PBP species of *B. fragilis* was achieved

by exploiting the simple use of the fluorescent penicillin analogue, bocillin-FL. This was facilitated by the assignments of the genes of PBP species in clusters of orthologous groups in the genome of *B. fragilis* NCTC 9343. Thus, in a recent study, nine PBP species (A, 1ab, 1c, D, 2, 2x, 3, 4 and 7) were identified in *B. fragilis*.[14]

The transferable cefoxitin resistance phenotype was identified as being due to a class A β-lactamase in the 1990s. Its gene, *cfxA*, has been cloned and sequenced, and demonstrated to be localized on a mobilizable transposon, MTn*4555*.[15,16] The nucleotide sequence and functions participating in the excision, integration (*int*, *xis* and *tnpC*) and mobility (*mobA*) of MTn*4555* were soon characterized.[17–19] Our own investigations on the imipenem resistance of *Bacteroides* demonstrated an insertion sequence (IS) element, IS*943*, which had homology to the *orf-9* of MTn*4555*, and this part was later proved to be an IS element, IS*Bf8*.[20,21] A recent study identified a correlation between the β-lactamase activity levels and the upstream regions, which mainly differed in IS content (IS*Bf8* of MTn*4555* or IS*614*B), of the *cfxA* genes of some *Bacteroides* isolates, and also showed that *cfxA* genes may have multiple copies in the harbouring strains and that these copies may sometimes be variants.[22]

The roles of the two above-mentioned resistance mechanisms that participate in the cephamycin resistance of *Bacteroides* have been investigated only separately; thus, an estimation of their overall contributions was timely. In order to gain information on this and to acquire more data on these resistance mechanisms, we examined CfxA and PBP-mediated cefoxitin resistance of *Bacteroides* strains. Additionally, we recognized a subgroup of *Bacteroides* strains that displayed heterogeneous resistance to cefoxitin and, in light of the available data, we attempted to elucidate how this peculiar phenotype might develop.

Materials and methods

Bacterial isolates and cultivation

Ninety-five isolates belonging to the *Bacteroides* genus (81 *B. fragilis*, 6 *Bacteroides thetaiotaomicron*, 4 *Bacteroides ovatus*, 3 *Bacteroides uniformis* and 1 *Bacteroides vulgatus*) were analysed. They were collected in Hungary and were selected either randomly from a larger collection to be examined for the above-mentioned mechanisms of cefoxitin resistance ($n=84$), or were those exhibiting high or heterogeneous resistance to cefoxitin from an antibiotic susceptibility survey focusing on the efficacy of tigecycline ($n=11$).[23] Species identification was performed by routine methods.[24] The strains were stored at −70°C in CryoBank vials (Mast Diagnostica, Rheinfeld, Germany) and were cultivated at 37°C anaerobically on Columbia agar supplemented with 5% (v/v) blood, 5 g/L haemin and 1 g/L vitamin K_1, in Wilkins–Chalgren (WC) broth or in BHIS broth [i.e. brain heart infusion broth supplemented with 0.5% (w/v) yeast extract, 5 g/L haemin and 1 g/L vitamin K_1], either in an anaerobic cabinet under a gas composition of 85% N_2/10% H_2/5% CO_2 or in jars, using gas-generating sacs (Oxoid), for 24 h (for BHIS and WC broth) or 48 h.

The susceptibilities of the strains to cefoxitin were determined either by agar dilution or by Etest methods, as recommended by the CLSI or the supplier, respectively, with *B. fragilis* ATCC 25285 and *B. vulgatus* CLA341 as control strains. For resistance categorization, the CLSI breakpoints were used.[25] *B. vulgatus* CLA341 and *B. fragilis* 638R were used in PCR experiments as positive and negative controls, respectively. *B. fragilis* 638R was the host strain in conjugation experiments, which were carried out as described previously.[26] The conjugative transfer of MTn*4555* was induced by the addition of 1 mg/L tetracycline to cultures incubated overnight.

Population analysis profiles (PAPs) were recorded on WC agar plates containing the appropriate dilution of cefoxitin by the plating of parallel 10-fold dilutions of a BHIS culture of *Bacteroides* strains grown for 16 h.

Cefoxitin binding competition and cefoxitinase assays

Selected *B. fragilis* strains in 85 mL of WC broth were grown until the stationary phase. Cells were collected by centrifugation (JS25.5 rotors, 8000 rpm, 4°C, 10 min) and resuspended in 7 mL of ice-cold PBS buffer. The suspensions were passed twice through a French press (11 000 lb/inch^2) in a cold room and the remaining debris was removed by centrifugation (JS25.5 rotors, 8000 rpm, 4°C, 5 min). The membrane vesicles (2×3 mL) were collected by ultracentrifugation (Beckman TL100, TLA-100.3 rotor, 70 000 rpm, 4°C, 15 min) and resuspended in either 250 or 500 μL of ice-cold PBS. The protein concentrations of the preparations were determined spectrophotometrically (DC Protein Assay, Bio-Rad).

For the inhibition of interfering β-lactamase activities, we used clavulanic acid and EDTA at concentrations that inhibited the action of β-lactamases on nitrocefin for ≥30 min.

Membrane preparations with a protein content of 60 μg were incubated with the titrated amount of clavulanic acid or EDTA in 30 μL volumes at 37°C for 30 min. Cefoxitin dilutions in 10 μL were added, giving final concentrations of 0, 0.25, 1, 4, 16, 64 and 256 mg/L cefoxitin, and the mixtures were incubated at 37°C for 30 min. Unaffected PBPs were labelled with bocillin-FL (5 μM; Invitrogen) at 37°C for 30 min. Aliquots of these samples, which contained 30 μg of membrane proteins, were subjected to 8% NuPAGE electrophoresis, using 1× MOPS or 1× MES buffer and a constant voltage of 90 V. Bocillin-FL fluorescence was detected with a Typhoon 9410 fluorescence scanner (excitation at 488 nm, detection at 532 nm, high sensitivity, 200 μm resolution) The amounts of labelled PBPs were quantified by using TIF images with Bio-Rad Quantity One 4.6.3 software. IC_{50} values were calculated by the BioData Fit program (available at http://www.changbioscience.com/stat/ec50.html).

Specific cefoxitinase assays were carried out as described by Malouin *et al.*[27]

Chromosomal DNA preparation, restriction endonuclease treatment, PCR and Southern hybridization

Regular restriction endonuclease digestions were performed in a volume of 20 μL with 0.2–1 μg of DNA under the conditions recommended by the suppliers. PCR reaction mixtures (usually either 20 or 50 μL) contained half volumes of 2× ReadyMix PCR reaction mix (Sigma), 0.7 μM primers, 1/10 volume of template DNA and sterile water supplemented to the indicated final reaction volume. The PCR primer sequences and the cycling conditions applied are listed in Table S1 (available as Supplementary data at *JAC* Online). All the PCR experiments started with a 2.5 min starting denaturation at 94°C and had a 10 min final elongation at 72°C. Figure 1 outlines the PCR strategy used to detect the *cfxA* gene and the structure of MTn*4555*. The *cepA* and *cfiA* genes were detected as described by García *et al.*[22] PCR products or the fragments of the restriction digestion were analysed by 0.7% or 1.2% agarose gel electrophoresis, using 0.5 mg/L ethidium bromide and TBE (45 mM Tris-borate/1 mM EDTA) buffer.

For Southern blotting, PCR products or chromosomal DNA preparations were resolved on 0.7% agarose gels after restriction endonuclease treatment of the common 1.2 kb amplified region of MTn*4555* (DdeI) or the chromosomal DNA of *cfxA*-positive strains (Bsu15I), and were transferred to nylon membranes (Amersham, UK) by capillary

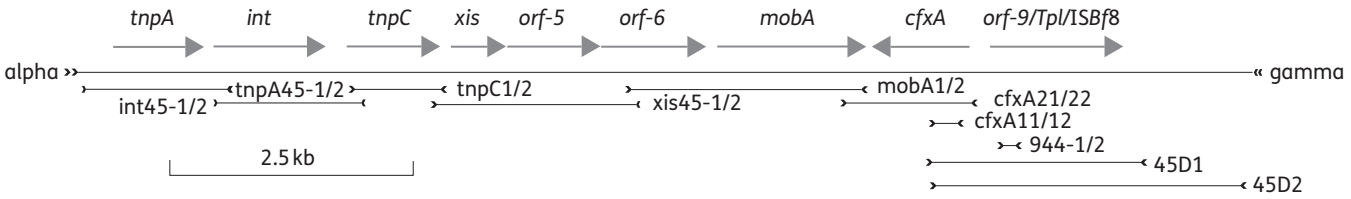

Figure 1. Structure of MTn*4555* and the PCR strategy applied. The genes and the ends for MTn*4555* are displayed at the top of the figure. At the bottom of the figure, the PCR fragments amplified from MTn*4555* are displayed, with the primer pairs indicated to the left. In the case of the amplifications with the 45D1 and 45D2 primers, cfxA11 was used as a forward primer.

action. Labelling, hybridization and detection were carried out with the GenImages AlkPhos System (Amersham, UK). The 1.2 kb PCR fragment of *B. fragilis* 540/1 amplified with primers cfxA11 and 45D2 was used for labelled probe preparation.

Inverse PCR and nucleotide sequencing

For inverse PCR, the method of García *et al.*[22] was essentially followed. Chromosomal DNA (0.5 μg) was restricted with BsuRI (HaeIII isoschizomer) enzyme and precipitated with ethanol, and 20 ng of digested DNA was circularized by T4 ligase [1× T4 ligation buffer, 1.5 mM $Co(NH_3)_6Cl_3$, 50 mM KCl and 2 U of T4 ligase in a final volume of 50 μL]. Subsequent PCR amplification was performed as described previously.[22]

Nucleotide sequencing was carried out as described previously.[26] The sequence data obtained were deposited into GenBank (www.ncbi.nlm.nih.gov) under accession numbers FJ875532 (*B. fragilis* 540, ~1.2 kb 'common fragment') and HQ378596 (upstream region of the *cfxA* gene in *B. ovatus* 32456/2).

Statistical analysis

The distributions of heterogeneous resistance and numbers of *cfxA*-specific chromosomal DNA fragment copies in the different *Bacteroides* populations were compared by means of χ^2 analysis or the Mann–Whitney test, using the SPSS program package (SPSS, Inc., Chicago, IL, USA). The tests were considered significant if the *P* values were <0.05.

Results

Analysis of random clinical Bacteroides isolates for the cfxA gene, cefoxitin MIC, cefoxitinase activity and conjugative transfer of cefoxitin resistance

Eighty-four randomly selected *Bacteroides* isolates were tested for their cefoxitin MICs by agar dilution and for the presence of the *cfxA* gene by PCR. The rate of resistance (MIC ≥64 mg/L) was 8.3%. Sixteen *cfxA*-positive strains (14 *B. fragilis*, 1 *B. ovatus* and 1 *B. uniformis*; 19.0% positivity) were detected (Table 1). The MIC distribution with respect to the presence of *cfxA* is displayed in Figure 2. The MIC range was 1–128 mg/L, with a shift of the modes of the *cfxA*-negative and -positive strains from 8 to 32 mg/L (2-fold dilution), respectively. The prevalences of *cfxA*-positive and -negative strains in the susceptible category (MICs ≤16 mg/L) were 6.3% and 93.7%, respectively; those in the resistant category (MICs ≥64 mg/L) were 6.0% and 2.4%, respectively. Out of the seven resistant strains, five were *cfxA*-positive (71.4%). Thus, the *cfxA* gene had an effect on the cefoxitin resistance of the strains, but it was only modest.

In conjugation experiments, the *cfxA* gene of 9 out of 10 strains could be detected, but only with low frequency (5.5×10^{-9}–3.35×10^{-6}/input donor cell) and was not significantly affected by tetracycline. The MIC values of cefoxitin for the transconjugant colonies were determined by the Etest method (Table 1). These values were 2–3-fold higher, comparable to the MIC distribution shift between the *cfxA*-positive and -negative strains (2-fold) and the parent strain *B. fragilis* 638R (8 mg/L).[15]

B. vulgatus CLA341 displays a high cefoxitin MIC and a high cefoxitinase activity (0.359 nmol cefoxitin hydrolysed/mg of protein/min). For the other examined strains, the hydrolysis of cefoxitin under the assay conditions was usually within the range of cefoxitin self-decay.

Cefoxitin affinities of PBP species of selected strains

In order to analyse the roles of PBPs in general and in the overlapping cefoxitin MIC distribution for *cfxA*-negative and -positive *B. fragilis* strains, cefoxitin competition assays were carried out, using both *cfxA*-negative ($n=11$) and *cfxA*-positive ($n=8$) strains. Strains covering the obtained cefoxitin MIC ranges were included in order to observe the effect of the PBP affinities on the MICs; five of the seven resistant strains were also included. A representative fluorogram is depicted in Figure S1 (available as Supplementary data at *JAC* Online) and results are presented in Table S2 (available as Supplementary data at *JAC* Online). For some PBP species (PBP-A and PBP3), the IC_{50} values were high (>256 mg/L) and not calculable, because the binding of cefoxitin was very low and approximately constant in the concentration range applied (insensitive/low-affinity PBPs). Some PBPs (PBP-D and PBP4) could not be detected in the majority of strains. The other PBP species were high-affinity binders. For PBP1ab and PBP1c, the IC_{50} values were usually very low and 0.25 mg/L cefoxitin generally caused full binding. The case for PBP2 was similar among *cfxA*-negative strains, but IC_{50} values could be calculated; for the *cfxA*-positive strains, this PBP was often lacking.

Analysis of the structural variety of MTn4555

The structure of MTn*4555* in the 16 *cfxA*-positive strains from our random collection was analysed. We identified (i) whether the *cfxA* genes are on complete MTn*4555* transposons and (ii) the structures of the regulatory regions of the *cfxA* genes (the 3′ regions of MTn*4555*).

Table 1. Characteristics of the *cfxA*-positive *Bacteroides* strains from the randomly selected collection

Strain	Cefoxitin MIC (mg/L)										Conjugation[d]		
	agar dilution	Etest	*cepA*	*cfxA*	*cfiA*	IS*Bf8*	MITE*Bf2*	*cfxA* up[a]	MTn*4555*[b]	No. of *cfxA* fragments[c]	Tc−	Tc+	MIC (mg/L)
B. vulgatus CLA341	256	256	NT	+	NT	+	+	3.2	C	NT	ND	4.9×10^{-6}	NT
B. fragilis 7979	64	16–(256)[e]	−	+	+	+	+	3.2	C	3	5.5×10^{-9}	ND	NT
B. uniformis 32456/1	32	16	−	+	−	+	+	3.2	C	2	2.0×10^{-8}	ND	NT
B. fragilis 6269	32	16	+	+	−	+	−	2.9	C	2	3.3×10^{-6}	ND	32
B. fragilis 540/1	64	32–(256)	+	+	−	−	−	1.2	C	6	2.3×10^{-9}	3.4×10^{-8}	NT
B. fragilis 8887	64	4–(64)	+	+	−	−	−	1.2	C	7	2.1×10^{-7}	ND	64
B. fragilis 66653	64	32	+	+	−	−	−	1.2	C	4	1.9×10^{-8}	1.0×10^{-7}	32
B. fragilis 38470/2	64	32	+	+	−	−	−	1.2	C	1	NT	NT	NA
B. fragilis 724/4	32	32	−	+	−	−	−	1.2	C	5	ND	ND	NA
B. fragilis 59481	32	16	+	+	−	−	−	1.2	C	10	NT	NT	NA
B. fragilis 13202	32	16	+	+	−	−	−	1.2	C	4	NT	NT	NA
B. fragilis 427/31	32	16	−	+	−	−	−	1.2	C	1	ND	1.9×10^{-9}	64
B. fragilis 84219/4	16	16	+	+	−	−	−	1.2	C	4	4.6×10^{-9}	ND	64
B. fragilis 59483	16	16	−	+	−	−	−	1.2	C	5	NT	NT	NA
B. fragilis 84219/2	16	8	+	+	−	−	−	1.2	C	3	NT	NT	NA
B. fragilis 71928/4	32	16–(64)	+	+	−	−	−	0.7	C	3	1.4×10^{-8}	1.8×10^{-8}	64
B. ovatus 32456/2	4	2	−	+	−	−	−	NA	cfxA	2	NT	NT	NA

NT, not tested; ND, not detected; NA, not applicable.
[a]The sizes of PCR fragments obtained by the cfxA11 and 45D2 primers.
[b]C denotes that complete copies of MTn*4555* were found, while cfxA denotes that, using PCR, products could only be obtained using the cfxA21/22 primer pair.
[c]The numbers of the detected *cfxA*-hybridizing chromosomal fragments are shown.
[d]The frequencies of cefoxitin resistance transfer are displayed, expressed as the number of transconjugants per input donor cell. The MICs for the transconjugant isolates recorded by Etests are displayed. Tc+, tetracycline induction; Tc−, no tetracycline induction.
[e]In the heterogeneous resistance readings, the first number denotes the value for even growth, while the number in parentheses denotes the value where the separate colonies disappeared.

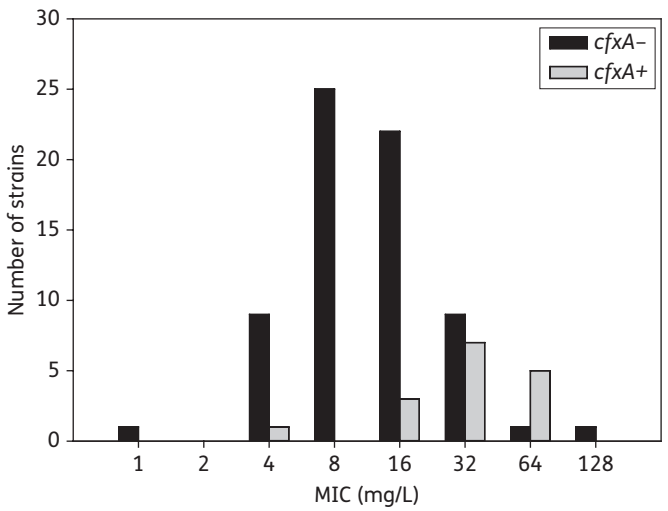

Figure 2. Cefoxitin MIC distribution for the *cfxA*-negative and -positive strains.

All but one of the test strains harboured a complete copy of MTn*4555*; with a few exceptions, the 3′ regions of the MTn*4555*s displayed a common structure. IS*Bf8* was detected and mapped adjacent to the *cfxA* genes in three strains. For the analysis of the 3′ regions of MTn*4555* transposons, two upstream primer sequences (45D1 and 45D2; Table S1) were selected to amplify the region. Primers cfxA11 and 45D2 yielded an ∼1.2 kb common sequence for the majority (11 out of 16) of *cfxA*-positive strains. Upstream fragments with other sizes (3.0 or 0.7 kb) were also found. The nucleotide sequence of the 1.2 kb cfxA11-45D2 fragment of *B. fragilis* 540/1 was determined. Its structure and sequence are depicted in Figure 3. Comparison of the corresponding sequence of *B. vulgatus* CLA341 with that of *B. fragilis* 540/1 demonstrated that there are two insertions in the sequence of *B. vulgatus* CLA341; one is IS*Bf8*, and the other is a 360 bp sequence with terminal direct and inverted repeats. This structure resembles an IS element, but does not harbour an open reading frame (ORF). It may therefore belong to the novel and growing group of miniature transposable elements (MITEs), and was termed MITE*Bf2* (Figure S2, available as Supplementary data at *JAC* Online). Additionally, the 3′ end of the common 1.2 kb sequence coded an ORF that has not been documented previously. On BLAST searches it exhibited close homology (with scores between 33.5 and 154, and E-values between 2e-36 and 3e-4) to a number of cytoplasmic abortive phage infection proteins (Abis), including species of Bacteroidetes, and it could be aligned with the lactococcal AbiD proteins (22% identity, 32% homology). The 1.2 kb cfxA11-45D2 fragments of all strains that yielded them displayed a common structure and homology after restriction

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10         20         30         40         50         60         70         80
AACTACGATTTGTTTTTTTCTGTTTTTTTCCATGTTTATATTATTTATATTTGTTTGACGAGAATATCTTTATTTTGCCGA
CAAAGGTACATAACTTTACGGAAAAATATGTTTCTAAAATATATAAATAGACAGCTGTGGGGAAAATGTGGGGAAAAATT
                                                  ->         ->
AGATAATTAAAAAGGCTAAATGACTGGAAATAATCACTTAGCCTTTTAATCCGTACCCAGACCCGTACTTCGTAATTGCT
                                                        M  M  P  M  R  M  P  N  T  W  I
GCGCCCATCATTCAAGACTGTCAAATCGGGTCTTTCCTGTCAAGCCAATGATGCCTATGCGTATGCCAAACACATGGATT
T  D  F  S  F  R  E  Q  T  L  Y  P  Q  L  C  Y  V  V  Y  W  L  N  S  I  S  M  G
ACGGATTTCTCGTTCCGTGAACAAACACTTTATCCGCAACTCTGCTATGTGGTGTATTGGCTTAACTCCATTTCTATGGG
 N  T  F  V  A  D  F  K  Q  L  L  S  K  Y  P  S  V  R  T  R  L  L  G  F  P  H  N
CAACACTTTTGTTGCAGATTTCAAGCAGCTTTTATCGAAATAcCCATCAGTAAGAACTCGTTTATTAGGCTTTCCTCATA
  W  E  Q  E  P  L  W  R  *
ATTGGGAACAAGAGCCTTTGTGGAGATAAAATAATTGCCCTGTTTCTTAAAAAGCACTGGGGCAAACCAGCTCCGTCTTT
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Figure 3. Nucleotide sequence of the characteristic part of the common 1.2 kb regulatory region of the *cfxA* gene with the Abi protein ORF. The amino acid sequence of the Abi protein is displayed above the first nucleotides of its codons. The start codon of the *cfxA* gene is in italic+bold and the proposed promoter sequence for *abiD* is underlined+bold. The detected mutations of the region, the insertions of IS*Bf8* and MITE*Bf2*, and the deletions are marked with underlining and stems of arrows, respectively.

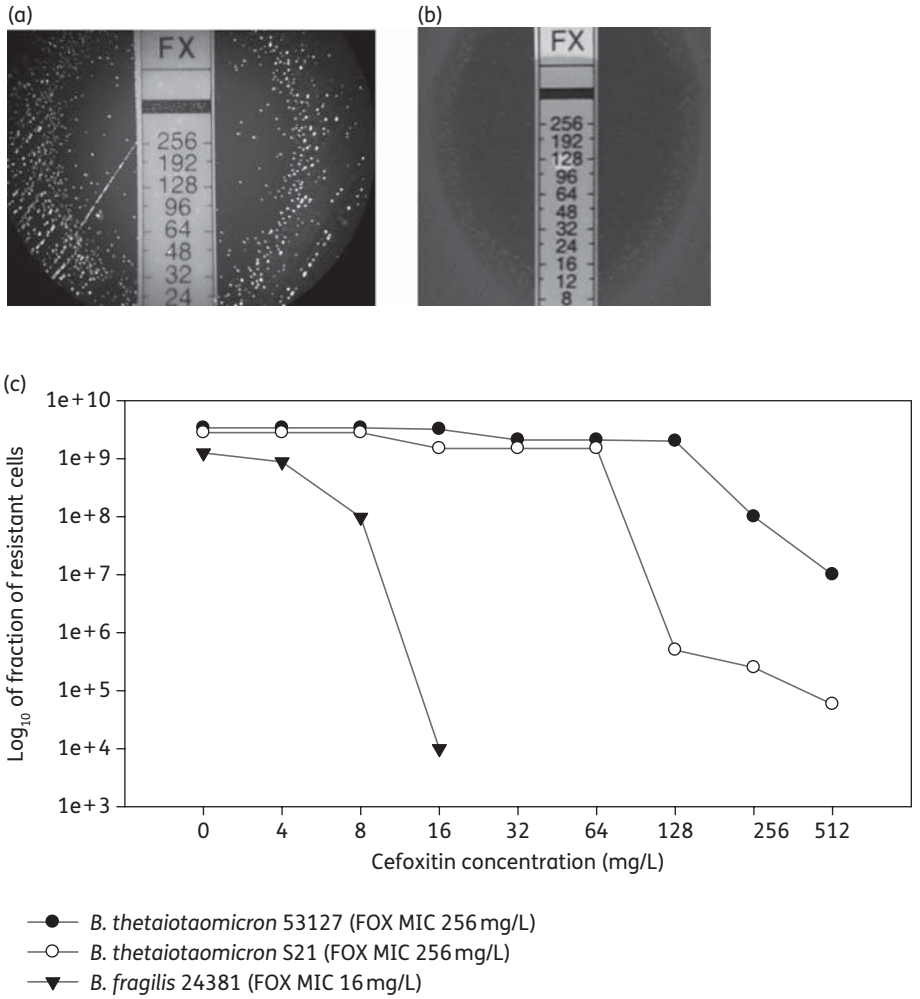

Figure 4. Characteristics of heterogeneously cefoxitin-resistant strains. Appearance of the resistance patterns using cefoxitin Etests for a *cfxA*-negative strain (*B. thetaiotaomicron* S21) (a) and for a *cfxA*-positive strain (*B. fragilis* 53148) (b). Population analysis profiles for cefoxitin (c). FOX, cefoxitin.

digestion (DdeI) and Southern blotting (data not shown). Primers cfxA11 and 45D1 amplified 2.8 kb fragments in two test strains. Since primer 45D1 fell into the sequence of MITE*Bf2*, it was possible to detect this element independently. The exception to the strains carrying full copies of MTn*4555* was *B. ovatus* 32456/2, which had only the *cfxA* region. For this strain, the upstream sequence of the

Table 2. Characteristics of the heterogeneously cefoxitin-resistant *Bacteroides* strains

Strain	Cefoxitin MIC (mg/L)		*cepA*	*cfxA*	*cfiA*	IS[a]	*cfxA* (kbp)[b]	No. of *cfxA* fragments[c]
	agar dilution	Etest						
B. fragilis 53148	256	8–(256)[d]	+	−	−			
B. fragilis 5269	256	256	+	+	−	−	0.5	2
B. fragilis 34581/2	64	64–(256)	−	+	−	IS*Bf8*, MITE*Bf2*	3.2	2
B. fragilis 13405	32	32–(256)	+	−	−			
B. fragilis D46	32	16–(256)	+	+	−	−	ND	1
B. fragilis D34	16	8–(256)	+	−	−			
B. fragilis 76240	NT	128–(256)	+	+	−	IS*614*B	NT	3
B. thetaiotaomicron 53127	256	32–(128)	−	+	−	IS*Bf8*, MITE*Bf2*	3.2	2
B. thetaiotaomicron S21	256	32–(256)	−	−	−			
B. thetaiotaomicron 85395	256	16–(64)	−	+	−	IS*Bf6*	2.9	2
B. fragilis D21	NT	>256	−	+	−	−	1.2	3

ND, not detected; NT, not tested.
[a]For the *cfxA*-positive strains, the absence (−) or presence of specific insertion elements is displayed.
[b]The sizes of PCR fragments obtained with the cfxA11 and 45D2 primers.
[c]The numbers of the detected *cfxA*-hybridizing chromosomal fragments are shown.
[d]In the heterogeneous resistance readings the first number denotes the value for even growth, while the number in parentheses denotes the value where the separate colonies disappeared.

cfxA gene was determined after an inverse PCR (Figure S3, available as Supplementary data at *JAC* Online).

Analysis of cefoxitin-heteroresistant strains; copy number determination of the cfxA genes in both sets of strains

Our studies to detect the efficacy of common anti-*Bacteroides* agents revealed strains with very high or heterogeneous resistance in cefoxitin Etests (11 of 280; 3.9%) (Figure 4a and b). These strains were subjected to the same molecular analysis as the 17 *cfxA*-positive *Bacteroides*; the molecular data are listed in Table 2. Out of the 11 strains, 7 were positive for *cfxA*. The PAPs were determined for three representative strains: one susceptible strain with homogeneous phenotype, *B. thetaiotaomicron* 53127; one heterogeneously resistant without a *cfxA* gene, *B. thetaiotaomicron* S21; and one heteroresistant with a *cfxA* gene, *B. fragilis* 24381 (Figure 4c). This experiment confirmed that strains with heterogeneous resistance in Etests also demonstrated heterogeneous resistance in PAP (compare two strains in Table 2 and Figure 4c). After this observation, we also conducted Etests on the strains with *cfxA* genes from the random collection to see whether there were heteroresistant strains among them; three such strains were found. The most prominent molecular feature of the *cfxA*-positive cefoxitin-heteroresistant strains was the accumulation of mutations in the 3′ region of MTn*4555* (Figure 3). The difference between the proportion of heteroresistant strains having the 'basic', 1.2 kb region (3/12, 25.0%) and those with mutation (8/11, 72.7%) was statistically significant ($P=0.03$) among the strains with *cfxA* genes. The mutations might affect the expression of the AbiD encoded in this region by inhibiting its transcription (see the promoter sequence upstream; Figure 3). To relate the resistance levels and the molecular characteristics of the MTn*4555* elements, copy numbers in individual *cfxA*-positive strains were determined by Southern blotting [Figure S4 (available as Supplementary data at *JAC* Online) and Tables 1 and 2]. Besides the heteroresistant strains having more mutations in the regulatory region, they exhibited a tendency to have fewer copies of the *cfxA* gene.

Discussion

In this study, we investigated the roles of PBPs and the CfxA cephamycinase in the cefoxitin resistance of *Bacteroides* strains. The genetic element MTn*4555*, harbouring the *cfxA* gene, was also characterized. The prevalence of the *cfxA* gene among Hungarian clinical isolates was found to be 19%, a value in the range determined previously.[22,28–30] The prevalences of the *cfxA* gene in Brazil among all *Bacteroides* and *B. fragilis* strains, and all *Bacteroides* in Spain were 15.1%, 12.0% and 27.3%, respectively. Our results demonstrated that, with a nomenclature proposed previously, the PBP-A and PBP3 species are weak binders of cefoxitin (the IC_{50} values were usually >256 mg/L), whereas the others (PBP1ab, PBP1c and PBP2) can be inhibited at very low concentrations (<1 mg/L). This phenomenon indicates that insensitive PBPs are good candidates in the development of high cefoxitin MICs experienced frequently in this and in other studies and the PBP affinity results indicate a balanced action of PBPs under antibiotic stress—the insensitive ones can undertake the role of peptidoglycan synthesis of the sensitive ones ensuring high MIC values, but the inhibition of the sensitive ones decreases the possibility of attaining high MICs. In previous studies of the effects of cephalosporins on the antibiotic resistance levels of different *Bacteroides* species, similar molecular weight PBPs were detected, but the effect of cefoxitin was not well elucidated. Yotsuji *et al.*[12] detected PBPs with molecular weights of 94 (1a), 90 (1b), 88 (1c), 82 (2) and 72 (3) kDa, and correlated the benzylpenicillin competition of

five cephalosporins with their MICs. Although that study detected mainly the same PBPs as the present study, cefoxitin was not included; however, the PBPs designated 1b, 1c, 2 and 3 (1ab, 1c, 2 and 3 according to our nomenclature) were also very susceptible to cefoperazone. A similar PBP pattern was detected by Piddock and Wise,[13] except that the ~94 kDa PBP-A (or PBP1a according to Yotsuji *et al.*[12]) was not investigated in great detail. However, this latter protein was barely visible in competition assays with benzylpenicillin. Fang *et al.*[11] focused on competition assays of piperacillin and cefoxitin with biotinylated ampicillin in mutants selected for cefoxitin resistance. They detected increased IC_{50} values of PBPs with molecular weights of 86 and 72 kDa for these antibiotics as compared with the parent *B. thetaiotaomicron* 238 strain.[11] However, we used only *B. fragilis* strains, since accurate PBP detection was available both by antibiotic labelling and genomic data. No appreciable difference was observed between the IC_{50} values for the *cfxA*-positive and -negative strains, except for the relative lack of PBP2 among the *cfxA*-positive strains. However, it would be better to examine the IC_{50} values of the insensitive PBPs above 256 mg/L and use not only membrane preparations but undertake the laborious task of measuring the cefoxitin affinities of purified PBPs (such studies have been initiated in our laboratories for PBP-A). No examination was carried out on differences in the outer membrane permeability among our tested strains. However, the outer membrane protein loss linked to the cefoxitin resistance of *Bacteroides* has only been detected in some selected highly resistant isolates,[31] whereas the role of efflux pumps has been demonstrated for fluoroquinolones and for other β-lactams, but not for cefoxitin.[32] Interestingly, the presence of the *cfxA* gene did not greatly elevate the cefoxitin MICs for the carrying strains as compared with the *cfxA*-negative strains; the increase was ~2-fold after the conjugation and between the *cfxA*-positive and -negative strains. These data invoke the previously mentioned high basal level resistance and inefficient hydrolysis of cefoxitin by CfxA. In accordance with this, the cefoxitin decay by CfxA has been given as <0.01% relative to that of cefaloridine.[15] The high cefoxitinase production by *B. vulgatus* CLA341 can be explained by the increase in the copy number of MTn*4555* (our unpublished preliminary results). This control strain differed from the examined strains in other aspects too: the conjugal transfer of its *cfxA* gene was very efficient and was inducible by a low level of tetracycline. The phenomenon that tetracycline conjugative transposons, such as CTnDOT and CTnERL, are inducible by tetracycline as regards the tetracycline resistance levels and their conjugative transfer efficiencies, has long been known, and the regulatory mechanism behind it was examined extensively. As helper elements, they can also mobilize co-resident plasmids and mobilizable transposons, e.g. MTn*4555*.[33] However, the conjugation frequency is not always high and is not always inducible by tetracycline. A specific region is responsible for this in the above-mentioned conjugative transposons, as was demonstrated recently.[34] We presume that there was no conjugative helper element in some of our strains or that the above-mentioned regulatory region was lacking from the CTns of these strains. These data imply a lesser spread than expected of the *cfxA*-mediated resistance mechanism among *Bacteroides* strains.

We described heterogeneously cefoxitin-resistant strains. Heterogeneous antibiotic resistance is best known for methicillin-resistant *Staphylococcus aureus* (MRSA),[35] penicillin-resistant *Streptococcus pneumoniae*[36] and carbapenem-resistant *Pseudomonas aeruginosa*.[37] In the case of MRSA strains, a low-affinity PBP (PBP2a or PBP2′) is the primary resistance determinant, which is encoded on a specific chromosomal resistance island, SCC*mec*,[38] and some auxiliary chromosomal genes (*fem* or *aux*) are also necessary for this resistance phenotype to develop. The role of these latter genes is to produce altered substrates during cell wall synthesis.[35] In the case of cefoxitin heteroresistance among *Bacteroides*, the role of the insensitive or low-affinity PBPs can also be expected, as for MRSA.

The 3′ region of the MTn*4555* elements, which is a regulatory region for the *cfxA* gene, displayed diversity. The strains from our random collection had moderate cefoxitin MICs, and most of them had a common 1.2 kb region at the 3′ end and harboured an ORF for AbiD. The modification of this region was characterized by insertional (IS*Bf8*, IS*614*B and MITE*Bf2*) or deletion mutations. The role of AbiD is to inhibit the intracellular replication of invading phages and mobile elements by cleavage of their replicative forms.[39] It is interesting, as compared with *B. vulgatus* CLA341, for which we expect a copy number increase behind the high level of cefoxitinase production and resistance, that the heteroresistant strains more frequently had lower copy numbers of *cfxA* genes and mutations at the 3′ end of the MTn*4555* elements (Mann–Whitney *U*-test, $P=0.005$). From these data we hypothesize that MTn*4555* increases its copy number in the case of highly resistant colonies of the heteroresistant strains, because of the lack of AbiD. Such copy number elevations are not rare in the development of antibiotic resistance and may be a cause of emerging antibiotic resistance.[40,41] García *et al.*[22] also found a high level of variability in the 3′ regulatory region of MTn*4555* among *Bacteroides* strains from Spain, where the prevalence of *cfxA* is quite high (see above). They could correlate the β-lactamase production and the structure of this 3′ regulatory region, where the presence of IS*614*B made the strains high β-lactamase producers.[22] In one of our strains, we also found an insertion of IS*614*B into the upstream region of *cfxA* (*B. fragilis* 76240 with a very high level of cefoxitin resistance; Table 2). Thus, further studies are needed to clarify the contributions of *cfxA* copy number and different promoter regions to the overall cefoxitinase production.

We can conclude that the molecular examination of MTn*4555* and the PBP affinity measurements was a start to better understand the cefoxitin resistance of *Bacteroides* species caused by multiple factors. According to our results, we hypothesize that PBPs play a major role and that *cfxA* expression is only a minor factor, noting (i) the IC_{50} values of >256 mg/L for PBP-A and PBPBf4, and (ii) the ~2-fold increase in the MICs for the transconjugants and *cfxA*-positive strains and the low cefoxitinase activity of CfxA. The interaction of these two resistance mechanisms is obvious but the exact nature of this interaction remains to be determined. This interaction may be simply additive but may be regulated in more complex ways giving rise to bi- or multistable states accounting for the heteroresistance experienced.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 and S2 and Figures S1 to S4 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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