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Penicillin-binding proteins of *Bacteroides fragilis* and their role in the resistance to imipenem of clinical isolates

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In this study penicillin-binding proteins (PBPs) of *Bacteroides fragilis* and the resistance mechanisms of this micro-organism to 11 β-lactam antibiotics were analysed. The study focused on the role of PBP2Bfr and metallo-β-lactamase in the mechanism of resistance to imipenem. The mechanism of β-lactam resistance in *B. fragilis* was strain dependent. The gene encoding the orthologue of *Escherichia coli* PBP3 gene (*pbpBBfr*, which encodes the protein PBP2Bfr) was sequenced in five of the eight strains studied, along with the *ccrA* (*cfiA*) gene in strain 119, and their implications for resistance were examined. Differences were found in the amino-acid sequence of PBP2Bfr in strains AK-2 and 119, and the production of β-lactamases indicated that these differences may be involved in the mechanism of resistance to imipenem. *In vitro* binding competition assays with membrane extracts using imipenem indicated that the PBP that bound imipenem with the highest affinity was PBP2Bfr, and that increased affinity in strain 7160 may be responsible for the moderate susceptibility of this strain to imipenem. In the same way, the importance of the chromosomal class A β-lactamase CepA in the resistance mechanism of the *B. fragilis* strains NCTC 9344, 7160, 2013E, AK-4, 0423 and R-212 was studied. In these strains this is the principal resistance mechanism to antimicrobial agents studied other than imipenem.

INTRODUCTION

Bacteroides fragilis is a strict anaerobe that inhabits the intestines of humans and is the most common anaerobe recovered from various infections, such as intra-abdominal infection, foot ulcers and sepsis. Resistance to β-lactam antibiotics in Gram-negative anaerobic bacteria is an important problem in the treatment of the infectious diseases caused by these micro-organisms (Arpin *et al.*, 2002).

The existence of imipenem-resistant *B. fragilis* strains was reported nearly two decades ago (Cuchural *et al.*, 1986), and these strains were found to produce metallo-β-lactamase, which hydrolyses not only carbapenems but also other classes of β-lactams. This metallo-β-lactamase, encoded by the gene *cfiA* (or *ccrA*), is classified as class B (subclass B1) based on its primary genetic structure and activity, or group 3 (subclass 3a) based on its functional characteristics and inhibitor profile. Elements resembling insertion sequences were found adjacent to *cfiA* in metallo-β-lactamase-producing and imipenem-resistant strains but not in *cfiA*-carrying strains with no detectable metallo-β-lactamase activity. This enzyme is inhibited by EDTA but not by clinically used β-lactamase inhibitors (Yamazoe *et al.*, 1999).

B. fragilis shows inherently poor susceptibility to most β-lactam antibiotics, including monobactams and temocillin, because of the poor affinity of its penicillin-binding proteins (PBPs) for these compounds (Edwards, 1997). However, it is not easy to relate β-lactam activity and PBP affinity, because there are conflicting results from investigations into the numbering and molecular mass of PBPs from this micro-organism. In *B. fragilis*, three PBPs of 91 kDa, 80 kDa and 69 kDa have been described as ubiquitous, while two others of 63 kDa and 47 kDa are detected occasionally (Edwards & Greenwood, 1996). Several workers have reported an association between the reduced affinity of

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Abbreviations: COG, cluster of orthologous groups of proteins; IC50, concentration of imipenem that reduced the level of binding of Bocillin-FL to 50 %; PBP, penicillin-binding protein.

The GenBank/EMBL/DDBJ accession numbers for the *Bacteroides fragilis pbpBBfr* gene sequences for penicillin-binding protein 3 of strains NCTC 9344, 119, 7160, AK-2 and R212 are AJ831484–AJ831488, respectively, and for the *cfiA* gene of strain 119 is AJ831823.

An amino acid sequence alignment of PBP2Bfr from several *B. fragilis* strains is available as supplementary material in JMM Online.

β-lactam compounds for the PBPs of *Bacteroides* species and resistance to these agents. Georgopapadakou *et al.* (1983) observed reduced affinity of an 80 kDa PBP in the case of imipenem, piperacillin, cefoperazone, cefotaxime and ceftazidime in a resistant strain of *B. fragilis*, although no precise IC50 calculations were performed.

Preliminary analysis of the incomplete genome sequence for *B. fragilis* against the amino-acid sequences for *Escherichia coli* PBPs identified the gene sequences with the closest homologues in the *B. fragilis* genome. These were *pbp1abBfr*, *pbp1cBfr*, *pbpABfr*, *pbpBBfr*, *pbp4Bfr* and *pbp7Bfr* as the orthologues for *ponBEco* (PBP1bEco), *pbpCEco* (PBP1cEco), *pbpAEco* (PBP2Eco), *pbpBEco* (PBP3Eco), *dacBEco* (PBP4Eco) and *pbpGEco* (PBP7Eco) *E. coli* genes, respectively (Píriz *et al.*, 2004). However, no correlation was found between the deduced molecular mass of PBPs from patterns described in the literature and those obtained from the translated gene sequences.

Analysis of the whole sequenced bacterial genomes defined a database of orthologous genes at the NCBI (Clusters of Orthologous Groups of Proteins, COGs) (http://www.ncbi.nlm.nih.gov/COG/). All cell-wall-surrounded micro-organisms contain a set of PBPs that belong to some of the following COGs: COG0744, COG0768, COG1680, COG1686 and COG2027. The first two COGs correspond to class A and B high-molecular-mass PBPs, and the last three correspond to low-molecular-mass PBPs (COG1686 and COG2027) and class C β-lactamases (COG1680). Other COGs corresponding to metallo-carboxypeptidases (m-CPase) (COG1619), metallo-β-lactamases (m-BLase) (COG0595, COG1234, COG1235 and COG1237), class A β-lactamases (COG2367), class D β-lactamases (COG2602), VanY-type DD-peptidases (COG1876), VanX-type DD-peptidases (COG2173) and MepA (COG3770) were also identified. They represent proteins involved in one way or another in the modification of peptidoglycan and in conferring resistance to β-lactams. Only the proteins in COG0744 (transglycosylases/transpeptidases) and COG0768 (transpeptidase) are essential for growth and survival. Proteins in COG1680 (β-lactamases/DD-peptidases), COG1686 (DD-carboxypeptidases) and COG2027 (DD-endopeptidases) are usually dispensable for growth under laboratory conditions.

The aim of this work was to study the role of the PBPs, the class B metallo-β-lactamase CfiA and the chromosomal class A β-lactamase CepA in the resistance of eight *B. fragilis* strains to different β-lactam antimicrobial agents.

METHODS

Bacterial strains. The strains used in this study, *B. fragilis* 119 and R212 (human clinical isolates), 2013E and 0423 (laboratory culture collection strains), 7160, AK-2 and AK-4 (isolated from human intra-abdominal infections) and NCTC 9344 (control strain), have been described previously (Píriz *et al.*, 2004). Isolates were identified by means of biochemical tests or kits that rely largely on sugar fermentation probes (Rapid ID 32A system, BioMérieux). These tests were further supplemented with the analysis of volatile and non-volatile fatty acids extracted by means of gas–liquid chromatography (Summanen *et al.*, 1993).

Antimicrobial susceptibility. Standard compounds were obtained from the following sources: imipenem, Merck, Sharp & Dohme; ampicillin, amoxycillin and clavulanic acid, GlaxoSmithKline; cefoxitin, cefotaxime and cefpirome, Hoeschst Marion Roussel; piperacillin and methicillin, Sigma; cefoperazone, Pfizer; ceftriaxone, Roche Farma; penicillin, Antibioticos SA; cefadroxil, Bristol-Myers Squibb; and cefotetan, AstraZeneca.

MICs of different β-lactam antibiotics, with or without 4 μg clavulanic acid ml^{-1}, were determined by the agar dilution method in Wilkins-Chalgren agar (Oxoid) following the proposed standard for antimicrobial susceptibility testing of anaerobic bacteria (NCCLS, 2000). The plates were incubated at 37 °C in Gas-Pak jars (Oxoid) for 24 and 48 h. Two control strains from the American Type Culture Collection were included in all MIC determinations: *B. fragilis* ATCC 25285 and *Clostridium perfringens* ATCC 13124. The MIC was interpreted as the lowest concentration of each antimicrobial agent producing no growth, one discrete colony or a barely visible haze.

Determination of β-lactamase activity. The β-lactamase activities were determined using the nitrocefin test (Oxoid) and quantified with 0·10 mM nitrocefin in 50 mM sodium phosphate buffer (PiNa) (pH 7·0, 22 °C) by a spectrophotometric method. Nitrocefin (50 μg ml^{-1}) and 10 μl extract were incubated for 1 h in a final volume of 500 μl at room temperature in 50 mM PiNa pH 7·0 (22 °C). Eppendorf tubes were centrifuged at 12 000 r.p.m. for 3 min in an Eppendorf centrifuge. Absorbance was measured at 486 nm. The appearance of a red colour in the test tube after 5 min was considered high (+++), but the appearance of little or no colour after 60 min was considered low (−). Specific activity was calculated by using a molar absorption coefficient for nitrocefin of 20 500 mol^{-1} cm^{-1} at 486 nm.

Spectrophotometry. Metallo-β-lactamase and β-lactamase activities against different antibiotics were detected by a change in optical density at the wavelength associated with maximum absorption for each compound in 1 mm silica cells (Píriz *et al.*, 2004). The absorption of a mixture of total cell extract (20 μl) or membrane extract (20 μl), and 0·2 ml imipenem (299 nm, 250 μg ml^{-1}), or 0·2 ml piperacillin (257 nm, 250 μg ml^{-1}), or 0·2 ml cefuroxime (274 nm, 250 μg ml^{-1}), or 0·2 ml cefoperazone (275 nm, 50 μg ml^{-1}), or 0·2 ml ceftriaxone (270 nm, 25 μg ml^{-1}), or 0·2 ml cefotaxime (260 nm, 50 μg ml^{-1}), or 0·2 ml amoxycillin (273 nm, 300 μg ml^{-1}) or 0·2 ml methicillin (281 nm, 50 μg ml^{-1}) and phosphate buffer, 50 mM, pH 7·0 (0·8 ml) was measured for 1 h at 37 °C. Numbers in parentheses indicate maximum absorption wavelength and final concentration for each antibiotic, respectively. The final concentrations were chosen as the highest possible for each antibiotic that would still allow detection of the decay of the absorption at the selected absorption wavelength.

In those cases in which β-lactamase activity was detected by this technique, the capacity of the extracts for degrading the antimicrobial was tested again in the presence of clavulanic acid, at a fixed concentration of 4 μg ml^{-1}, or EDTA at 2 mM added 10 min before the spectrophotometric assay.

Amplification of the *cfiA* and *cepA* genes by PCR. The PCR amplification reaction conditions were as described previously (Píriz *et al.*, 2004). For the detection and amplification of the cephalosporinase gene *cepA*, two previously described primers (Píriz *et al.*, 2004) based on the nucleotide sequence with the GenBank accession number L13472 (*B. fragilis* CS30) were used. For amplification and sequencing of the class B β-lactamase gene *cfiA* (*ccrA*) from *B. fragilis*, a pair of primers (P1, 5′-AAAGAATAAAATGAAAACAGT-3′, positions 81–101, and P4, 5′-GATAAAAGTTTTCGCCTCTTC-3′, complementary to nucleotides 877–897 of this gene) based on the GenBank accession number M63556 sequence (*B. fragilis* TAL 3636) was used.

Preparation of bacterial envelopes and assay of PBPs. Membrane extracts were prepared from overnight anaerobic cultures grown at 37 °C in a Wilkins-Chalgren broth medium (Oxoid) by differential centrifugation as described elsewhere (Spratt, 1977). Thirty micrograms of membrane protein in 15 µl phosphate buffer (50 mM pH 7·0) was labelled at 37 °C for 30 min with a final concentration of 5 µM of Bocillin-FL (Molecular Probes) and separated on a 7 % acrylamide, 3·3 % cross-linkage gel SDS-PAGE. When appropriate, samples were incubated at 37 °C with clavulanic acid at a final concentration of 10 µg ml^{-1} or EDTA at a final concentration of 10 mM for 30 min before labelling, so as to avoid degradation of the fluorescent penicillin by β-lactamases. The PBPs were visualized directly on the gel by fluorescence using Typhon9410 (Amersham Biosciences) with an excitation wavelength of 588 nm and emission filter 520BP40. These assays were repeated three times.

Imipenem-binding competition assay. A 30 µg sample of total membrane proteins was preincubated in phosphate buffer (50 mM, pH 7·0) with 10 µg clavulanic acid ml^{-1} or 10 mM EDTA plus imipenem over a range of concentrations from 1·25 to 60 µg ml^{-1} in a 20 µl reaction for 30 min at 37 °C. Then, Bocillin-FL was added at a final concentration of 5 µM and incubated for another 30 min at 37 °C. Loading buffer was added to each of the samples, which were then boiled for 5 min to stop the reaction. The samples were then centrifuged at 12 000 r.p.m. and supernatant fractionated on a 7 % acrylamide, 3·3 % cross-linkage gel SDS-PAGE. Immediately after running, the gel was washed in distilled water and PBPs were visualized directly on the gel by fluorescence using Typhon9410 with an excitation wavelength of 588 nm and emission filter 520BP40. The profiles of each line were quantified by means of the ImageQuant programme (Amersham Biosciences) and the IC50 was measured as the concentration of imipenem that reduced the binding of Bocillin-FL to half the value when no imipenem was added to the reaction.

PCR and DNA sequencing of identified PBP sequences of *B. fragilis*. For the analysis of the *pbpBBfr* gene, the specific primers used in this study were 83U21, 5′-ACTTCTTCGTCATCCTGTTGA, and 2268L21, 5′-TACCTGTCTGGAGTCGATGTC, where the numbers at the start of the primers refer to the position in the sequence related to the A in the ATG start codon. Both oligonucleotides were designed with the Oligo Primer Analysis Software v6.57 (PREMIER Biosoft International) and synthesized by Isogen Life Sciences. PCR reactions were performed by using a cocktail of *Taq* (BioTools) and *Pfu* (Stratagene) polymerases designed for high fidelity and long distance amplifications (Barnes, 1994). Reactions included 25 mU *Taq* µl^{-1} and 1·5 mU *Pfu* µl^{-1}, 1 ng genomic DNA µl^{-1}, 0·5 pmol primer 83U21 µl^{-1}, 0·5 pmol primer 2268L21 µl^{-1}, 200 µM dNTPs and 1× standard concentration of the *Pfu* buffer, as supplied by the manufacturer (including Mg^{2+} at 2 mM). Conditions for PCR were: an initial step of 3 min at 94 °C; 30 cycles of three steps, 1 min at 94 °C, 30 s at 52 °C and 5 min at 72 °C; and a final extension of 10 min at 72 °C. The 2124 bp PCR fragment was separated by electrophoresis in 1 % agarose and 1× TAE (40 mM Tris/acetate, 2 mM EDTA, pH 8·5) gels (Sambrook & Russell, 2000), purified by using the Gene Clean Turbo purification kit (Bio 101), following the manufacturer's specifications, and cloned in pBluescriptIISK− or used directly for sequencing or digestions.

The DNA fragments corresponding to the *pbpBBfr* gene were amplified by PCR using chromosomal DNA of the *B. fragilis* strains NCTC 9344, AK-2, R212, 119 and 7160 under the conditions described above. DNA fragments obtained by PCR were sequenced with the oligonucleotides 83U21, 2268L21, 3BF-D (5′-TTGCAGACATCAACCCATCCA) and 3BF-INT (5′-ATGCAGGATATCTGCGAGAAG) as primers and using the ABI PRISM 377 DNA sequencer. Sequences were compared with the genome of *B. fragilis* strains NCTC 9343 and 638R (http://www.sanger.ac.uk/Projects/B_fragilis/) by means of the CLUSTAL V and BLAST programmes (http://www.ncbi.nlm.nih.gov/blast/blast.cgi).

RESULTS AND DISCUSSION

Antimicrobial susceptibility and β-lactamase activity

Table 1 shows the susceptibilities of eight strains of *B. fragilis* to 11 β-lactam antimicrobial agents. As discussed in a previous study (Píriz *et al.*, 2004), the most resistant strains to imipenem are AK-4 and 119 (MICs, 16 µg ml^{-1}) and AK-2 (MIC, 8 µg ml^{-1}). On studying the behaviour of these microorganisms in the presence of clavulanic acid to a concentra-

Table 1. MICs of 11 β-lactam antibiotics for *B. fragilis* isolates

Antibiotics: I, imipenem; C, clavulanic acid; P, penicillin G; A, amoxycillin; Ch, cefadroxil; Co, cefoperazone; Cp, cefpirome; Ct, ceftriaxone; Cx, cefotaxime; Cn, cefotetan; M, methicillin.

B. fragilis strain	MIC (µg ml^{-1})										
	I*	I+C*†	C*	P	A	Ch	Co	Cp	Ct	Cx	Cn	M
NCTC 9344	0·12	0·06	⩾256	8	32	16	8	16	2	4	4	64
119	16	8	⩾256	16	4	16	16	32	8	8	8	64
AK-2	8	4	⩾256	8	1	8	16	8	4	4	4	16
7160	4	2	⩾256	16	16	16	16	8	4	4	2	32
2013E	2	0·25	⩾256	256	⩾256	256	128	256	64	256	128	⩾256
AK-4	16	0·5	128	256	⩾256	32	16	256	32	16	128	256
0423	2	1	64	⩾256	128	8	16	256	32	32	256	256
R212	4	2	128	⩾256	128	128	128	⩾256	256	256	256	⩾256

*Previously reported data (Píriz *et al.*, 2004).

†Clavulanic acid at a fixed concentration of 4 µg ml^{-1}.

tion of 4 μg ml^{-1} as well as imipenem, we found that the susceptibility of strain AK-4 was considerably increased (MIC, 0·5 μg ml^{-1}). However, the change in susceptibility of strains 119 and AK-2 was very slight (MICs 8 and 4 μg ml^{-1}, respectively).

Table 2 shows that the gene *cfiA*, which encodes a metallo-β-lactamase, was found in strains 119, AK-2, 7160 and 2013E; however, metallo-β-lactamase activity has only been detected in strains 119 and AK-2. The activity of this enzyme is inhibited by the presence of EDTA, but not by clavulanic acid. The expression of the *cfiA* gene requires the presence of an insertion sequence immediately upstream (Arpin *et al.*, 2002). Strains of *B. fragilis* that possess the silent *cfiA* gene may be converted spontaneously into strains with a high level of resistance to β-lactam antimicrobial agents. This occurs at a frequency of 10^{-7} per cell per generation (Sóki *et al.*, 2000).

From Table 1 it can be seen that the *B. fragilis* strains 2013E, AK-4, 0423 and R212 were highly resistant (MIC $\geqslant$ 256 μg ml^{-1} in many cases) to most of the antimicrobials used in our study (penicillin G, amoxycillin, cefadroxil, cefoperazone, cefpirome, ceftriaxone, cefotaxime, cefotetan and methicillin). These four strains possess the *cepA* gene, which encodes the β-lactamase CepA (an enzyme containing at least four amino-acid motifs that are characteristic of class A active-site-serine β-lactamases), and have significant activity in the nitrocefin assay (Table 2). This β-lactamase activity was completely inhibited, in all cases, by clavulanic acid at a fixed concentration of 4 μg ml^{-1}; however, it did not change in the presence of EDTA at 2 mM added 10 min before the nitrocefin assay, indicating the expression of *cepA*, which plays a determinant role in the resistance of these bacteria to the antimicrobial agents studied.

In the same way, on studying the enzymic activity of the eight strains of *B. fragilis* by spectrophotometric assay, it was found that the extracts from *B. fragilis* R212 were very active in degrading cefoperazone, cefpirome, ceftriaxone and cefotaxime (Table 2). This activity was inhibited in the presence of clavulanic acid. Moreover, we found cephalosporinase activity against cefoperazone and ceftriaxone in strains AK-4 and 0423 and against ceftriaxone in strains NCTC 9344, 7160 and 2013E. These enzymic activities were inhibited in the presence of clavulanic acid and did not change when EDTA was added, which is characteristic of class A β-lactamases. It is noteworthy that low enzymic activity by strain NCTC 9344 against ceftriaxone was maintained in the presence of clavulanic acid and EDTA. Although no β-lactamase activity was detected by the nitrocefin assay in this strain, this behaviour could be caused by a silent *cepA* gene that it carries, or otherwise by other known class A β-lactamases not tested in our study (CfxA or CblA) that confer resistance to cefoxitin (Parker & Smith, 1993; Smith *et al.*, 1994). Finally, we would like to stress that the extract from strain 119 (*cfiA*+, *cepA*−) showed weak activity against cefoperazone only. This activity was inhibited in the presence of EDTA, but not clavulanic acid, which leads us to conclude that the metallo-β-lactamase produced by this micro-organism may also degrade cefoperazone.

Even though β-lactamase activity has been detected by the nitrocefin assay in strains 2013E, AK-4, 0423 and R212, and they had high MICs for cephalosporins, the spectrophotometric assay did not reveal much activity except in strain R212. So, the simplest explanation for why amoxycillin was not affected by the cephalosporinase is that the method, under our assay conditions, was not accurate enough to identify activity on amoxycillin. But, it could also be possible that the cephalosporinase activity of CepA is greater than the penicillinase activity.

On analysing the susceptibility data of the eight strains of

Table 2. β-Lactamase activity of total extract of *B. fragilis* isolates

***B. fragilis* strain**	**Presence of gene***		**Nitrocefin assay result*†**			**Spectrophotometric assay result‡**			
	cfiA	***cepA***	**Total**	**Total+C**	**Total+E**	**Co**	**Cp**	**Ct**	**Cx**
119	+	–	++	++	–	+ (E)	–	–	–
AK-2	+	–	+	++	–	–	–	–	–
7160	+	+	–	–	–	–	–	++ (C)	–
2013E	+	+	+++	–	+++	–	–	++ (C)	–
NCTC 9344	–	+	–	–	–	–	–	+	–
AK-4	–	+	+++	–	+++	++ (C)	–	++ (C)	–
0423	–	+	+++	–	+++	++ (C)	–	+ (C)	–
R212	–	+	+++	–	+++	+++ (C)	+++ (C)	+++ (C)	+++ (C)

*Previously reported data (Píriz *et al.*, 2004).

†Activity is indicated as an inverse function of the time required for colour detection: –, 60 min; +, 30 min; ++, 10 min; +++, 5 min. C, indicates addition of potassium clavulanate (10 μg ml^{-1}) 10 min before nitrocefin assay; E, indicates addition of EDTA (2 mM) 10 min before the assay.

‡Results are expressed as related to the maximal activity: +++, 100 % detected in the assay; ++, 10–99 %; +, 0·1–9·9 %; –, no detectable activity. Total inhibition of activity was produced by either 4 μg clavulanic acid ml^{-1} (C) or 2 mM EDTA (E) treatments. Co, cefoperazone; Cp, cefpirome; Ct, ceftriaxone; Cx, cefotaxime. Imipenem, amoxycillin and methicillin were not degraded by any of the strains studied.

B. fragilis for the 11 β-lactam antimicrobials and relating them to the production of β-lactamases, we consider that the production of these class A and B enzymes would explain the resistance or the low susceptibility of these bacteria to some of the antimicrobials used, although in some cases the resistance mechanism may be due to other reasons. For example, with strains 7160 and NCTC 9344, moderate resistance to ceftriaxone (MICs of 4 and 2 μg ml^{-1}, respectively) is most probably due to the occurrence of β-lactamase activity with low affinity for nitrocefin under our assay conditions but active against ceftriaxone. As these two strains carry the silent *cepA* gene, this activity may also be due to low level expression of this enzyme, and also may influence the reduced susceptibility to imipenem in strain 7160, in addition to the low IC50 of PBP2Bfr (0·4 μg ml^{-1}) for imipenem in this strain.

Correlation between binding patterns and sequence data of PBPs of *B. fragilis*

Provisional DNA sequences of the whole genome of *B. fragilis* NCTC 9343 (http://www.sanger.ac.uk/Projects/B_fragilis/) with the highest matches to the *E. coli* proteins were identified by BLAST, and then the gene sequences for the closest homologues in the *B. fragilis* genome were deduced and compared with the annotated sequence of *Bacteroides thetaiotaomicron* VPI-5482 (Xu *et al.*, 2003). Orthologous PBPs for these micro-organisms are shown in Table 3.

Proteins encoded by the *pbp1abBfr* and *pbp1cBfr* genes must be located in COG0744, and correspond with the PBP1Bfr complex (88 kDa) previously identified (Píriz *et al.*, 2004). The molecular mass of the protein coded by *pbpABfr* (69 kDa) must correspond to PBP3Bfr (69 kDa), identified by the binding of labelled penicillin in *B. fragilis* membrane extracts, while the protein coded by *pbpBBfr* (78 kDa) is PBP2Bfr (80 kDa) based on the binding pattern; these proteins could be placed in COG0768. The protein encoded by *pbp4Bfr* (52 kDa) is not identified in the binding pattern, because it binds the clavulanic acid that is added in the assay to inhibit β-lactamases. However, a band of 52 kDa was seen in strains 119 and AK-2 when the binding assay was performed in the presence of 10 mM EDTA (Fig. 2a).

In an attempt to characterize the three different patterns of mutant strains identified in our previous study (Píriz *et al.*, 2004), non-β-lactamase-producers (NCTC 9344 and 7160), CepA producers (*cepA*+, *cfiA*−; AK-4, 0423 and R212) and CfiA producers (*cepA*−, *cfiA*+; strains AK-2 and 119), we sequenced the *pbpBBfr* gene of five representative strains and compared our results with the sequences of strains NCTC 9343 and 638R. DNA sequences of the *pbpBBfr* genes and the deduced PBP2Bfr proteins of strains NCTC 9344, AK-2, R212, 119 and 7160 were compared with the unpublished sequences of strains NCTC 9343 and 638R. The results obtained by multiple sequence alignment (Supplementary Figure) indicated that in the strains NCTC 9344, 7160 and R212 there were no amino-acid changes present in PBP2Bfr compared with strain NCTC 9343, and only six, four and 10 silent nucleotide changes, respectively. However, the number of amino-acid changes in PBP2Bfr proteins from strains AK-2 and 119 was very large (19 changes) compared with the sequences from the other strains (Supplementary Figure). Differences were mainly silent nucleotide changes that correlated precisely within the two patterns, i.e. a large number in strains AK-2 (245 nucleotide changes) and 119 (246 nucleotide changes), and low numbers for NCTC 9344, 7160 and R212. As previously described for PBP3Bfr (Píriz *et al.*, 2004), major changes in nucleotide and amino-acid sequences for PBP2Bfr occurred in CfiA-positive strains (119 and AK-2). As described below, these amino-acid changes in PBP2Bfr do not alter significantly the IC50 for imipenem binding. However, a great difference in the imipenem IC50 of PBP2Bfr was observed between strains NCTC 9344 and R212, although no amino-acid change was seen for these two proteins. So, factors other than PBP2Bfr structure must be responsible for the low imipenem affinity of this protein in isolated membranes of strain R212.

PBP profiles and imipenem-binding competition assay

Membrane extracts from the different strains were used in a binding assay with 5 μM Bocillin-FL in the presence of 10 μg clavulanic acid ml^{-1} and the results for strain NCTC 9344 are shown in Fig. 1. The four major PBPs (PBP1abBfr, PBP1cBfr, PBP2Bfr and PBP3Bfr), previously identified by sequence comparison (Table 3), were clearly seen on the gel. The band corresponding to PBP4Bfr was only seen when there was no treatment with 10 μg clavulanic acid ml^{-1}, indicating that this PBP binds to that antibiotic. PBP7Bfr does not have the three conserved domains of DD-serine peptidases and was not seen on the gel. Sequence comparison suggests that this protein may correspond to metallo-LD-carboxypeptidase (*lcdA* gene in *E. coli*). Other proteins encoded by *cepA*, a fragment of PBP2, a PBPx gene, a putative DD-dipeptidase, a putative metallo-endopeptidase and several putative metallo-β-lactamase genes were also identified in the complete sequenced genomes of *B. thetaiotaomicron* VPI-5482 and *B. fragilis* NCTC 9343. However, no *cfiA* gene was identified by the BLAST search in these strains (Table 3), nor in NCTC 9344, AK-4, 0423 or R212 as previously described (Píriz *et al.*, 2004).

A band of 95 kDa (band A) was observed on the binding assay for all the membrane extracts, but does not correspond to any previously identified PBP. This band did not compete with imipenem at 60 μg ml^{-1}, but it disappeared in the assay previously treated with 100 μg ampicillin ml^{-1} or 30 μg aztreonam ml^{-1}. We termed this band PBP1ABfr (Fig. 1). Other bands with low intensity may correspond to degradation products of the main PBPs and are labelled by a white dot in Fig. 1. To our knowledge this is the first description of a real pattern of PBPs that correlates with that expected from the whole sequence analysis of *B. fragilis*.

Membrane extracts of all representative strains, 119, AK-2, 7160, 2013E, NCTC 9344, AK-4, 0423 and R212, were used in

Table 3. Homologies between *B. fragilis* and *B. thetaiotaomicron* genes involved in the susceptibilities to *β*-lactam antimicrobial agents

Protein*	*Bacteroides thetaiotaomicron*† VPI-5482	*Bacteroides fragilis* NCTC 9343‡ ATCC 25285	Homology§	COG/MEROP no. and prototype enzyme‖
PBP1ab	BT0743, 779 aa, STIK, SDN, KTG	PBP1abBfr, 777 aa, STIK, SNN, KTG	Score, 3212; identities, 599/756 (79 %); similarities, 660/756 (87 %)	COG0744, HMM PBP class A Eco1a
PBP1c	BT0162, 772 aa, SILK, SLN, KTG	PBP1cBfr, 779 aa, SILK, SLN, KTG	Score, 3205; identities, 593/750 (79 %); similarities, 661/750 (88 %)	COG0744, HMM PBP class A Eco1c
PBP2	BT3453, 708 aa, STFK, SSN, KTG	PBP2Bfr, 707 aa, STFK, SSN, KTG	Score, 2864; identities, 554/709 (78 %); similarities, 620/709 (87 %)	COG0768, HMM PBP class B Eco3
PBP2x	BT2501, 649 aa, SLPK, YEN	PBP2xBfr, 640 aa, SLPK, YEN	Score, 2215; identities, 417/609 (68 %); similarities, 500/609 (82 %)	COG0768, HMM PBP class B
PBP3	BT3816, 620 aa, STFK, SCN, KTG	PBP3Bfr, 618 aa, STFK, SCN, KTG	Score, 2851; identities, 530/620 (85 %); similarities, 576/620 (92 %)	COG0768, HMM PBP class B Eco2
PBP4	BT3187, 468 aa, SAGK, SDN, KTG	PBP4Bfr, 469 aa, SAGK, SDN, KTG	Score, 1709; identities, 317/450 (70 %); similarities, 381/450 (84 %)	COG2027, MEROPS-S13, LMM PBP class B Eco4
PBP7	BT2549, 302 aa, Put. M-CPase, ldcA	PBP7Bfr, 304 aa	Score, 1158; P = e−128; identities, 219/298 (73 %); similarities, 246/298 (82 %)	COG1619, MEROPS-U61, metallo-CPase Eco-ldcA
PBP2′	BT0943, 300 aa	PBP2′Bfr fragment, 311 aa	Score, 601; P = 2e−63; identities, 130/315 (41 %); similarities, 182/315 (57 %); gaps 20/315 (6 %)	
DD-DPasa	BT3007, 292 aa, VanX-type	Putative Dala-Dala dipeptidase Bfr, 263 aa	Score, 713; P = 1·7e−70; identities, 144/205 (70 %); similarities, 158/205 (77 %)	COG2173, MEROPS-M15D, DD-DPase VanX-type
CepA	BT4507, 293 aa, SVFK, SDN, KTG	Putative class A-betalactamase Bfr, 300 aa, SVFK, SDN, KTG	Score, 1163; P = 9·0e−116; identities, 215/277 (77 %); similarities, 245/277 (88 %)	COG2367, DD-BLase class A
CfiA	ND	ND		COG1404, MEROPS-S8A, metallo-BLase class B
M-BL-1	BT1410, 273 aa	Putative metallo-betalactamase Bfr, 268 aa	Score, 904; P = 2e−98, identities, 169/264 (64 %); similarities, 207/264 (78 %)	COG1237, metallo-BLase
M-BL-2	BT0822, 267 aa	Putative metallo-betalactamase Bfr, 267 aa	Score, 1258; P = 5·2e−126; identities, 234/267 (87 %); similarities, 251/267 (94 %)	COG1235, metallo-BLase
M-BL-3	BT1146, 203 aa	Putative metallo-betalactamase Bfr, 203 aa	Score, 1010; P = 5·9e−102; identities, 180/203 (88 %); similarities, 194/203 (95 %)	COG0491, metallo-BLase
M-BL-4	BT4346, 316 aa	Putative metallo-betalactamase Bfr, 304 aa	Score, 1326; P = 1·0e−133; identities, 242/304 (79 %); similarities, 274/304 (90 %)	COG1234, metallo-BLase
M-EPase	BT4391, 212 aa	Putative metallo-endopeptidase Bfr, 243 aa	Score, 722; P = 8·8e−70; identities, 133/180 (73 %); similarities, 156/180 (86 %)	COG1619, metallo-EPase

*Identified penicillin-binding protein in the whole sequence analysis.

†File name, number of amino acids of the protein and conserved motifs of the protein in *B. thetaiotaomicron*.

‡Protein name, number of amino acids of the protein and conserved motifs of the protein in *B. fragilis*.

§Percentage identity and similarity between the *B. thetaiotaomicron* and *B. fragilis* proteins. Score and P are BLAST values used to compare the quality of the homology search. Parameters obtained with the BLAST program at NCBI.

‖Identified cluster of orthologous groups of proteins (http://www.ncbi.nlm.nih.gov/COG/), the MEROPS database (http://merops.sanger.ac.uk/) and most representative protein of the cluster. HMM, high molecular mass; LMM, low molecular mass.

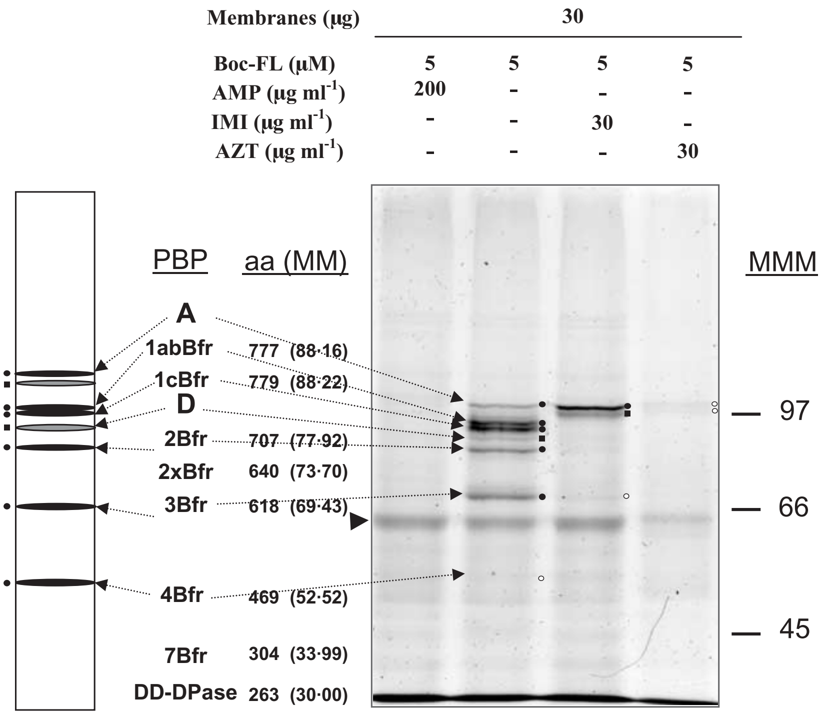

Fig. 1. Bocillin-FL binding profile of membranes from *B. fragilis* strain NCTC 9344. Concentrations of Bocillin-FL (Boc-FL), ampicillin (AMP), imipenen (IMI) and aztreonam (AZT), and the amounts of membrane used in the binding assays are indicated for each line. A profile is shown on the left. Black circles and black squares indicate the major and minor bands, respectively, and white circles mark the remaining bands after different treatments. For each identified penicillin-binding protein (PBP) the number of amino acids (aa) and the molecular mass (MM) are displayed. The positions of the molecular mass markers (MMM) are shown on the right. A black arrowhead shows an unspecific band. Putative PBPs that could not be detected in the assay (see the text) are not shown. Non-identified PBPs by whole genome sequence analysis are shown as letters (A and D).

binding competition assays at different imipenem concentrations to calculate the IC50 of each individual PBP. The results are shown in Table 4. All PBPs showed low affinities for imipenem as described elsewhere (Edwards, 1997), but PBP2Bfr had the highest affinity, except in strains 0423 and R212. The affinity of PBP2Bfr from strain AK-4 could be considered as intermediate. Only one of the IC50 values correlated with the observed MIC for imipenem. We do not know how much higher a PBP IC50 needs to be to cause resistance. Actually we cannot correlate directly the IC50 for a defined PBP and the MIC for an antibiotic, except if this was the only reason for resistance, because other factors influence resistance. However, if a particular MIC for an antibiotic correlates precisely with the IC50 for a PBP, and no other mechanism could affect resistance (for example no change in the MIC with clavulanic acid or no β-lactamase is produced),

Table 4. IC50 for imipenem-binding competition assay of membrane extracts of *B. fragilis* strains

NA, Not applicable, PBP does not compete for imipenem; ND, not detected as a separate entity in this strain.

B. fragilis strain	IC50 of imipenem for PBP (μg ml^{-1})*						Presence of gene†		Nitrocefin assay result†‡			MIC (μg ml^{-1})†§	
	A	1abBfr	1cBfr	D	2Bfr	3Bfr	*cfiA*	*cepA*	Nitro	Nitro+C	Nitro+E	I	I+C
119	NA	>60	>60	ND	2·01	>60	+	–	+	+	–	16	8
AK-2	NA	43·16	43·16	ND	2·41	15·0	+	–	–	–	–	8	4
7160	NA	6	2·5	2·2	0·4	5	+	+	–	–	–	4	2
2013E	NA	13·03	11·67	ND	1·16	59·44	+	+	+++	–	+++	2	0·25
NCTC 9344	NA	4·53	4·53	6·0	1·05	15·6	–	+	–	–	–	0·12	0·06
AK-4	NA	4·5	4·5	2·85	8·1	57·4	–	+	+++	–	+++	16	0·5
0423	NA	>60	>60	ND	>60	>60	–	+	+++	–	+++	2	1
R212	NA	>60	>60	ND	57·78	>60	–	+	+++	–	+++	4	2

*Identified PBP on the PAGE profile of Bocillin-FL binding.

†Previously reported data (Píriz *et al.*, 2004).

‡Activity of membrane extracts is indicated as an inverse function of the time required for colour detection: –, 60 min; +, 30 min; ++, 10 min; +++, 5 min. The assay was also preformed with the present of potassium clavulanate (C; 10 μg ml^{-1}) and EDTA (E; 2 mM) 10 min before the nitrocefin assay.

§Imipenem (I) MIC was tested with and without clavulanic acid (C) at a fixed concentration of 4 μg ml^{-1}.

then blocking of that specific PBP could be considered as the cause of resistance. But still, we cannot say that only a specific block of a PBP is the resistance mechanism if the MIC for an antibiotic is lower than the IC50 for that PBP.

For those strains producing CepA β-lactamase (2013E, AK-4, 0423 and R212), the IC50 of imipenem for all PBPs was higher than the MIC of imipenem in the presence of 4 μg clavulanic acid ml^{-1}. In strains 2013 and AK-4 a high MIC of imipenem is reversed by clavulanic acid, and the lowest IC50 value for a PBP (1·16 μg ml^{-1} for PBP2Bfr in 2013E and 4·5 μg ml^{-1} for PBP1abBfr and PBP1cBfr in AK-4) is still higher than the MIC of imipenem in the presence of clavulanic acid (0·25 μg ml^{-1} for 2013E and 0·5 μg ml^{-1} for AK4). Therefore, resistance must be due to the CepA β-lactamase. Strains 0423 and R212 both have high PBP IC50s and strong CepA activity but the imipenem MICs are not as high as with other strains. Thus, an unknown factor in addition to CepA production or very low affinity for PBP may be responsible for the imipenem MIC (as no other β-lactamase seems to be produced, the most plausible explanation is that the permeability barrier could be raised).

The IC50 of imipenem was also higher than the MIC of imipenem when combined with 4 μg clavulanic acid ml^{-1} for strains producing metallo-β-lactamase CfiA (119 and AK-2), and in the non-β-lactamase-producer NCTC 9344. Strain 119 could be resistant to imipenem due to high IC50s for PBPs, but the imipenem MIC is lower than the IC50 for PBP1abBfr, PBP1cBfr and PBP3Bfr and higher than the IC50 for PBP2Bfr. However, the β-lactamase activity is not low (see nitrocefin assay). It appears to be difficult to measure imipenem IC50s in our assay conditions, and the higher imipenem MIC in this strain compared with strains 0423 and R212 must be due to a higher affinity of CfiA for imipenem compared with CepA.

The only cases of an IC50 lower than or similar to the MIC for imipenem were PBP2Bfr and PBP1cBfr in the non-β-lactamase-producer strain 7160, indicating that inhibition of these PBPs may account for the MIC observed in this strain. Surely a higher IC50 would cause an increase in imipenem MIC, but actually the MIC drops to 2 μg ml^{-1}, and under these conditions the IC50 for PBPs 1cBfr, D and 2Bfr correlate precisely with the imipenem MIC. So, any of these PBPs may be responsible for the moderate susceptibility to imipenem of this strain. Also, even if no β-lactamase was detected by using nitrocefin or imipenem in the *in vitro* assay, some activity could be observed with ceftriaxone (reversed by the addition of clavulanic acid), which may explain the imipenem MIC change for this strain in the presence of clavulanic acid.

Also, a 30 μg sample of membrane extract of all strains was used in a binding assay in the presence of 10 μg clavulanic acid ml^{-1} or 10 mM EDTA, and the patterns of the remaining bands are shown in Fig. 2. In the strains producing the class A β-lactamase CepA (2013E, AK-4, 0423 and R212), all bands corresponding to PBPs disappeared in the samples treated with 10 mM EDTA (Fig. 2a), and only traces of PBP2Bfr and PBP4Bfr remained in strains 2013E and AK-4, which produced a lower level of β-lactamase. This may indicate a higher affinity of these two PBPs for Bocillin-FL. For strains 7160 and NCTC 9344, the Bocillin-FL binding pattern was indistinguishable in the presence or absence of clavulanic acid and EDTA. Strains 119 and AK-2 only showed the whole set of PBP bands in the presence of 10 mM EDTA. However, it is interesting to note that the pattern does not disappear completely in the absence of EDTA, most probably due to the lower affinity of CfiA for Bocillin-FL.

Analysis and sequence of *cfiA* gene

The 750 bp *cfiA* gene in the imipenem-resistant *B. fragilis* strain 119 was analysed by PCR and DNA sequencing. The nucleotide sequence of the *cfiA* gene varied from that of the standard *cfiA* gene from *B. fragilis* TAL2480 and was identical to the *cfiA2* of an imipenem-resistant strain isolated in Japan (Kato *et al.*, 2003). The *cfiA* variant tested in this study, *cfiA-119*, encodes a protein with all the amino-acid residues that are involved in the binding of the two Zn^{2+} ions (His99, His101, Asp103, His162, Cys181 and His223). These results indicate that the metallo-β-lactamase generated from the *cfiA-119* variant is as active as that produced by standard *cfiA*, and it was shown to be inhibited by 10 mM EDTA (Píriz *et al.*, 2004). Resistance to imipenem in strain 119 is entirely due to the high expression of an active carbapenemase and no significant change in affinity for this β-lactam was observed in a competition assay (IC50 of 2·01) with this antibiotic, despite the fact that 19 amino-acid changes were found in PBP2Bfr of this strain.

The strains of *B. fragilis* 119 and AK-2 are therefore resistant to imipenem due to the production of a metallo-β-lactamase, because the changes in the affinity of the PBPs to the reference antimicrobial are less important or perhaps insignificant. On the other hand, the high affinity of PBP2Bfr of the 7160 *B. fragilis* strain to imipenem shows that this protein may be responsible for moderate susceptibility to this antibiotic. In short, depending on the strain of *B. fragilis*, two mechanisms of resistance to imipenem (i.e. the production of metallo-β-lactamases and the variation in the affinity to PBP2Bfr) would be involved in the resistance of these bacteria to the reference antimicrobial. Finally, the production of CepA (chromosomal class A β-lactamase) is the principal mechanism elicited by the *B. fragilis* strains NCTC 9344, 7160, 2013E, AK-4, 0423 and R212 against most of the β-lactam antibiotics analysed other than imipenem.

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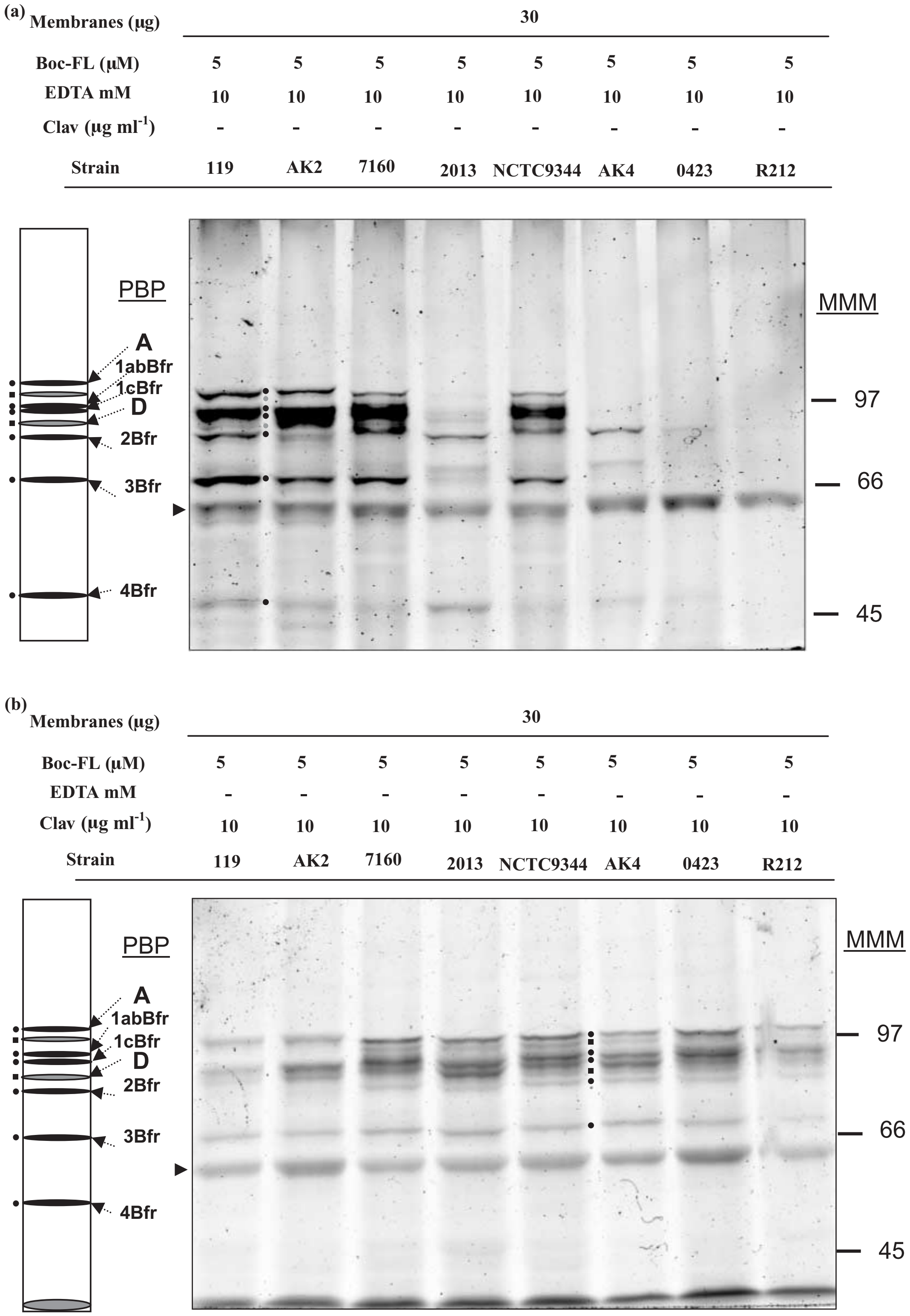

Fig. 2. Bocillin-FL binding of membrane extract of *B. fragilis* strains in the presence of (a) EDTA and (b) clavulanic acid. Concentrations of Bocillin-FL (Boc-FL), EDTA and clavulanic acid (Clav), and the amounts of membrane used in the binding assays are indicated for each lane. A profile is shown on the left of each panel; major and minor bands are labelled with circles and squares, respectively. A black arrowhead shows an unspecific band. Every identified penicillin-binding protein (PBP) of Fig. 1 is displayed. The positions of the molecular mass markers (MMM) are shown on the right.

638R were produced by the Microbial Genomes Sequencing Group at the Sanger Institute and can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/bf/.

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