Relationship between penicillin-binding protein patterns and β-lactamases in clinical isolates of *Bacteroides fragilis* with different susceptibility to β-lactam antibiotics

Segundo Píriz, Santiago Vadillo, Alberto Quesada, Jerónimo Criado, Rosario Cerrato and Juan Ayala

1,2 Medicine and Animal Health Department and Biochemistry, Molecular Biology and Genetics Department, University of Extremadura, 10071 Cáceres, Spain

3 ‘Severo Ochoa’ Molecular Biology Centre, CSIC-UAM, 28049 Cantoblanco (Madrid), Spain

This study examines the role of the penicillin-binding proteins (PBPs) of *Bacteroides fragilis* in the mechanism of resistance to different β-lactam antibiotics. Six of the eight strains used were β-lactamase-positive by the nitrocefin assay. These strains displayed reduced susceptibility to imipenem (MIC, 2–16 mg l⁻¹) and some of them were resistant to the actions of ampicillin, cefuroxime, cephalaxin, cefoxitin and piperacillin. When studying specific enzymic activity, the capacity to degrade cefuroxime was only detected in strains AK-4, R212 and 0423 and the capacity to degrade cephalaxin was only detected in strains R212 and 2013E; no specific activity was detected on imipenem. Metallo-β-lactamase activity was only detected in strains AK-2 and 119, despite the fact that the *cfaA* gene was identified in four strains (AK-2, 2013E, 119 and 7160). The *cepA* gene was detected in six of the eight strains studied. Three high-molecular-mass PBPs were detected in all strains; however, in some cases, PBP2Bfr and/or PBP3Bfr appeared as a faint band. PBP4Bfr and PBP5Bfr were detected in six strains. PBP6Bfr only was detected in *B. fragilis* strains AK-2, 0423, 119 and 7160. By analysis of the sequence of *B. fragilis* chromosomal DNA and comparison with genes that are known to encode PBPs in *Escherichia coli*, six genes that encode PBP-like proteins were detected in the former organism. The gene that encodes the PBP2 orthologue of *E. coli* (pbpABfr, PBP3Bfr) was sequenced in six of the eight strains and its implications for resistance were examined. Differences in the PBP3Bfr amino acid sequences of strains AK-2 and 119 and their production of β-lactamases indicate that these differences are not involved in the mechanism of resistance to imipenem and/or cephalaxin.

**INTRODUCTION**

Antimicrobial resistance has continued to increase significantly among various anaerobes, particularly members of the *Bacteroides fragilis* group. Resistance to many active agents, such as β-lactam/β-lactamase inhibitor combinations, clindamycin, metronidazole and carbapenems, has emerged, often associated with antimicrobial use. *B. fragilis* is often responsible for intra-abdominal sepsis and, as such, is an important cause of nosocomial infection (Arpin *et al.*, 2002). Resistance to β-lactam antibiotics in anaerobic bacteria could be due to the production of β-lactamases, alterations in penicillin-binding proteins (PBPs) or reduced penetration of antibiotics through the bacterial outer membrane (Nord & Hedberg, 1990).

Chromosomal class A β-lactamases are the most common among micro-organisms of the genus *Bacteroides*. Cephalosporinase activity is the most predominant of this group of enzymes. Rogers *et al.* (1993) cloned and sequenced the chromosomal cephalosporinase gene (*cepA*) from a high-level β-lactamase-producing clinical isolate, *B. fragilis* CS30. CepA is a β-lactamase that contains at least four amino acid motifs that are characteristic of class A, active-site-serine β-lactamases. Class B, carbapenem-hydrolysing metallo-
β-lactamases are less common. These enzymes are inhibited by EDTA, but not by clinically used β-lactamase inhibitors. These enzymes are detected in B. fragilis at varying frequencies, ranging from 1-6% of isolated strains in France to 6-9% in the UK. The gene that encodes this enzyme is cfiA (also known as ccaA). However, this gene may be ‘silent’ or expressed at various degrees, resulting in a wide range of levels of carbapenem resistance. Strains of B. fragilis that possess the silent gene cfiA may be converted spontaneously into strains with a high level of resistance to β-lactamases, including carbapenem. Expression of this gene (production of metallo-β-lactamase) requires the existence of an insertion element (IS) upstream of cfiA. This occurs spontaneously at a frequency of 10⁻⁷ (Söki et al., 2000).

B. fragilis is naturally resistant to some β-lactam antibiotics, including monobactams and temocillin, because of the poor affinity shared by its PBPs for these compounds (Edwards, 1997). The PBPs of Bacteroides spp. differ from those of E. coli in terms of their affinity for β-lactam antibiotics and in the morphological consequences of inhibition of these proteins. The numbering and molecular mass of PBPs from these micro-organisms are conflicting. In B. fragilis, three PBPs of 91, 80 and 69 kDa are ubiquitous, whereas two others (of 63 and 47 kDa) are detected only occasionally (Edwards & Greenwood, 1996). The primary target in Bacteroides spp. for most β-lactam antibiotics is PBP2Bfr (78 kDa), which is involved in septation and corresponds to PBP3 of E. coli (PBP3Eco) (Georgopapadakou et al., 1983; Piddock & Wise, 1986).

Several workers have reported an association between the reduced affinity of β-lactam compounds for the PBPs of Bacteroides spp. and resistance. Georgopapadakou et al. (1983) observed reduced affinity of PBP2Bfr for imipenem, piperacillin, ceferozapen, cefotaxime and ceftazidime in a resistant strain of B. fragilis, although no precise IC₅₀ calculations were performed. Following that approach, the aim of this study was to test antimicrobial susceptibilities to various β-lactam antibiotics and β-lactamase activity in eight strains of B. fragilis and to correlate this behaviour with differences in the PBPs of these strains.

**METHODS**

**Bacterial strains.** B. fragilis strains 119 and R212 are human clinical isolates that were supplied by Professor Edwards from the University Hospital, Queen’s Medical Centre, Nottingham, UK, and B. fragilis strains 2013E and O423, also provided by Professor Edwards, are laboratory culture collection strains from Glaxo Laboratories, Greenford, UK, and Wadsworth Veterans Administration Center, Los Angeles, CA, USA, respectively. B. fragilis strains 7160, AK-2 and AK-4 were isolated from human intra-abdominal infections by Professor Garcia-Sánchez at the Clinical Hospital of the University of Salamanca, Spain. B. fragilis strain NCTC 9344 was included as a control strain. Isolates were identified by means of biochemical tests or kits that rely largely on sugar fermentation probes (Rapid ID 32A system; bioMérieux). These were further supplemented with volatile and non-volatile fatty acid analysis. These acids, which were end products of glucose metabolism, were extracted by means of GLC (Summanen et al., 1993).

**Antimicrobial titrations.** Standard compounds were obtained from the following sources: imipenem (Merck Sharp & Dohme), ampicillin (SmithKline Beecham), cefuroxime (Glaxo Wellcome), clavulanic acid (SmithKline Beecham). MICs of different β-lactam antibiotics, with or without clavulanic acid at 4 μg ml⁻¹, were determined by the agar dilution method in Wilkins-Chalgren agar (Oxoid) by following the proposed standard for antimicrobial susceptibility testing of anaerobic bacteria (National Committee for Clinical Laboratory Standards, 2000). 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 (ATCC, Manassas, VA, USA) were included in all MIC determinations: B. fragilis ATCC 25285T and Clostridium perfringens ATCC 13124T. The MIC was interpreted as the lowest concentration of each antimicrobial agent that permitted no growth, one discrete colony or a barely visible haze.

**Determination of β-lactamase activity.** β-Lactamase activities were determined quantitatively by using the nitrocefin test (Oxoid) with 0-10 mM nitrocefin in 50 mM sodium phosphate buffer (pH 7.0, 22 °C) (PiNa) 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. Development of a red colour in the test-tube after 5 min was considered +++ (high), but appearance of slight or no colour after 60 min was considered − (low). Absorbance was measured at 486 nm (A₄₈₆). Eppendorf tubes were centrifuged at 12 000 r.p.m. for 3 min in an Eppendorf centrifuge before being measured. Specific activity was calculated by using an extinction coefficient for nitrocefin of 20 500 at 486 nm.

**Iodosometric assay.** An aliquot (10 μl) of each extract was loaded onto a plate of agar/starch (1-5-0:5%) that contained I₂/I (0.032/0.88%) and the corresponding antibiotic. A final concentration of 100 μg ml⁻¹ was used for each of ampicillin, cefuroxime, cephalexin, piperacillin and imipenem. Appearance of a clear halo of activity was observed after 30 min (++), 60 min (+++) or 120 min (+) incubation at 30 °C, or was not observed after 24 h (−) incubation at room temperature.

**Biological assay.** Total cell extracts were prepared as described by Edwards & Greenwood (1992). Total cell extract (30 μl), imipenem, piperacillin, cefuroxime and cephalexin at 500 μg ml⁻¹ (10 μl, final concentration 100 μg ml⁻¹) and 10 μl PiNa were dispensed into wells in the agar. The assay was read in accordance with the criteria described by Edwards et al. (1999).

**Spectrophotometry.** Metallo-β-lactamase activity and β-lactamase activities against imipenem, piperacillin, cefuroxime and cephalexin were detected by a change in A₃₉₅, A₄₅₇, A₅₃₂ and A₆₅₅, respectively, in a mixture of total cell extract (0-02 ml) or membrane extract (0-02 ml), imipenem, piperacillin, cefuroxime or cephalexin (0-2 ml; 250 μg ml⁻¹) and 50 mM PiNa (0-8 ml) over 1 h at 37 °C.

**Detection of the cfiA gene by PCR.** Two pairs of primers were designed, based on the published nucleotide sequence of the class B β-lactamase cfiA (ccaA) gene from B. fragilis TAL 3636. The sequences of primers P1 (5’-AAAGAAATTTAGAAAAACGT-3’) and P2 (5’-GCTGGATCGTGTAATCC-3’) were identical to the nucleotide sequence at positions 81–101 and complementary to positions 506–522 of the B. fragilis TAL 3636 cfiA gene, respectively. A product with a length of 442 bp was expected when primed by oligonucleotides P1 and P2. In order to amplify another fragment of the same gene, another pair of primers was used: P3 (5’-TGAAGAAAGGTTTGGTGCC-3’) and P4 (5’-GGATATAAGTTGCGGTTCCT-3’), complementary to nt 877–897 of this gene) amplified a 423 bp PCR product. Primers were synthesized by Invitrogen. B. fragilis TAL 3636 was included as a control PCR. PCR amplifications were performed...
by following the conditions described by Fang et al. (1999). Southern blotting and hybridization with a cepA-specific probe were used to confirm the PCR results; the protocol described by Ausubel et al. (1994) was used.

Detection of the cepA gene by PCR. For detection of the cephalosporinase gene cepA from B. fragilis, one pair of primers was used, based on the nucleotide sequence deposited in GenBank with accession number L13472 (Rogers et al., 1993). The sequence of the first primer (5’-TACCTTTTGAGGCGGATTAC-3’) was identical to the sequence of nt 398–419 of the cepA gene in B. fragilis NCTC 9343, whilst the sequence of the second primer (5’-ATTGTAACCCGAGGTATTTCG-3’) was complementary to nt 1333–1354. A fragment with a length of 956 bp was expected. Primers were provided by Invitrogen. PCR amplification conditions were: an initial step of 3 min at 94°C; 30 cycles of three steps: 1 min at 94°C, 30 s at 50°C and 6 min at 68°C; followed by a final extension of 10 min at 72°C. B. fragilis NCTC 9343 was used as a positive control. Southern blotting and hybridization with a cepA-specific probe were used to confirm the PCR results, following the protocol described by Ausubel et al. (1994).

Preparation of bacterial envelopes and PBP assay. Membrane extracts were prepared from overnight anaerobic cultures grown at 37°C in Wilkins–Chalgren broth (Oxoid) by differential centrifugation, as described by Spratt (1977). Protein (30 ng in 10 μl PiNa) was labelled at 30°C for 10 min with a final concentration of 10 mg [3H]-benzylpenicillin l-1 (ca. 670 GBq mmol-1; Amersham Biosciences) and separated by 10% SDS-PAGE. Before labelling, samples were incubated for 10 min at 30°C with clavulanic acid at a final concentration of 10 mg l-1, to avoid degradation of the labelled penicillin by β-lactamases. PBDs were visualized by fluorography after 3 weeks storage at –70°C. The film was developed and positions of the bands were measured. These assays were repeated three times and the results were absolutely reproducible.

Southern blot hybridization with the pbbABf gene. Chromosomal DNA of eight B. fragilis strains was isolated as described previously (Fang et al., 2002), digested with the restriction enzyme KpnI (Roche) and resolved on 9.8% agarose gel in TAE (Tris/acetate/EDTA) buffer. Digested DNA was then blotted onto positively charged nylon membranes (Roche). A DNA fragment that contained the pbbABf gene, amplified from B. fragilis NCTC 9344, was labelled by PCR using the PCR DIG labelling mix (Roche). The membrane was then hybridized with the digoxigenin-pbbABf gene probe at 42°C overnight. The procedure for immunological and luminescent detection with CSPD (Roche) was performed by following the manufacturer’s instructions.

Identification of PBP sequences from the B. fragilis genome. The preliminary genome sequence for B. fragilis (http://www.sanger.ac.uk/Projects/B_fragilis/) was screened against the amino acid sequences of E. coli PBPs by using the tblastn program (Altschul et al., 1997). Provisional DNA contingencies that had the highest match with E. coli proteins were identified and the gene sequences for the closest homologues in the B. fragilis genome were deduced: pbbABf, pbbABf, pbbABf, pbbABf, pbbABf, pbbABf, and pbbABf, as the orthologues for the E. coli genes pbp1E (PBP1E), pbp1E (PBP1E), pbp1E (PBP1E), pbp1E (PBP1E), pbp1E (PBP1E), and pbp1E (PBP1E), respectively. It should be noted that the molecular mass of the protein encoded by pbbABf (69 kDa) must correspond with PBP3Bfr (69 kDa), identified by the binding of labelled penicillin in B. fragilis membrane extracts. For analysis of the pbbABf gene, the specific primers used in this study were: –26up, 5’-GGCTTAGAAGGAGGTAGAGGAGA-3’, and +31down, 5’-GATGTGACACCATACCATGTG-3’; where numbering refers to ATG (start) and TAG (stop) codons of the protein and lower-case letters indicate mismatches that generate restriction sites. Both oligonucleotides were devised with Oligo primer analysis software version 6.57 and synthesized by Invitrogen. PCRs were performed by using a cocktail of Taq (BioTools) and Pfu (Stratagene) polymerases that was designed for high-fidelity and long-distance amplifications (Barnes, 1994). Reactions contained Taq and Pfu polymerases at 25 and 1.5 mM Mg2+ l-1, respectively; genomic DNA at 1 ng μl-1; –26up and +31down primers at 0.5 pmol μl-1 each; dNTPs at 200 μM; and 1x the standard concentration of Pfu buffer, as supplied by the manufacturer (including Mg2+ 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; followed by a final extension of 10 min at 72°C. The 1914 bp PCR product was separated by electrophoresis in 1% agarose/1% TAE gels (Sambrook & Russell, 2000), purified by using the Gene Clean Turbo purification kit (Bio101), following the manufacturer’s specifications, and cloned into the vector pBlueScriptHSK(–) (Stratagene) or used directly for sequencing or digestions.

DNA fingerprinting analysis of the pbbABf gene of B. fragilis. The pbbABf gene was amplified by PCR from the eight B. fragilis strains used in this study and digested with TruI1 and HmoI (MBI Fermentas). Digestion profiles for pbbABf were visualized directly by staining with ethidium bromide and separation in 1.8% agarose/TAE gel.

PCR and DNA sequencing. DNA fragments that corresponded to the pbbABf gene were amplified by PCR using chromosomal DNA of B. fragilis strains NCTC 9344, AK-2, AK-4, R212, 119 and 7160, under the conditions described above. DNA fragments obtained by PCR were sequenced with oligonucleotides –26up, +31down and 2BF-2 (5’-GTCCCATGTTGTGTTGAGAT-3’) as primers and by using an ABI PRISM 377 DNA sequencer. Sequences were compared with the unpublished genomes of B. fragilis strains NCTC 4393 and 683R (http://www.sanger.ac.uk/Projects/B_fragilis/) by means of the CLUSTAL V and BLAST programs (http://www.ncbi.nlm.nih.gov/blast/blast.cgi).

RESULTS AND DISCUSSION

Antimicrobial susceptibility and β-lactamase activity

Table 1 shows the susceptibilities of the eight B. fragilis strains analysed in this study to six β-lactam antibiotics. B. fragilis strains AK-4 and 119 (MIC, 16 mg l-1) and AK-2 (MIC, 8 mg l-1) were the most resistant strains to imipenem. However, when we tested the activity of imipenem in the presence of 4 mg clavulanic acid l-1, it was observed that the sensitivity of B. fragilis AK-4 increased substantially (MIC, 0.5 mg l-1), but the change in strains 119 and AK-2 was relatively small (MICs, 8 and 4 mg l-1, respectively). All of these strains were resistant to clavulanic acid alone at a concentration higher than 128 mg l-1. As shown in Table 2, six of the eight strains produced β-lactamases and the cfa gene was present in strains 119, AK-2, 2013E and 7160. However, we were only able to detect metallo-β-lactamase activity for strains 119 and AK-2. Expression of the group 3a, class B metallo-β-lactamase encoded by the silent cfa chromosomal gene required the location of an IS immediately upstream (Arpin et al., 2002). Edwards (1997) reported that various strains of B. fragilis that produced β-lactamases were resistant to the action of benzylpenicillin and cefoxitin and displayed moderate resistance to imipenem. However, the β-lactamases of these micro-organisms were unable to hydrolyse either cefoxitin or imipenem in vitro. Similar results were obtained in this study, as in strains 119, AK-2...
Table 1. MICs of seven β-lactam antibiotics

Abbreviations: Imi, imipenem; Cla, clavulanic acid; Amp, ampicillin; Cefu, cefuroxime; Cepha, cephalexin; Cefo, cefoxitin; Pip, piperacillin.

<table>
<thead>
<tr>
<th>B. fragilis strain</th>
<th>MIC (mg l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Imi</td>
</tr>
<tr>
<td>NCTC 9344</td>
<td>0.12</td>
</tr>
<tr>
<td>119</td>
<td>16</td>
</tr>
<tr>
<td>AK-2</td>
<td>8</td>
</tr>
<tr>
<td>7160</td>
<td>4</td>
</tr>
<tr>
<td>2013E</td>
<td>2</td>
</tr>
<tr>
<td>AK-4</td>
<td>16</td>
</tr>
<tr>
<td>0423</td>
<td>2</td>
</tr>
<tr>
<td>R212</td>
<td>4</td>
</tr>
</tbody>
</table>

*Clavulanic acid at a fixed concentration of 4 mg l⁻¹.

and 7160 (three of the four strains in which the cfIA gene was detected), no specific β-lactamase activity was observed against imipenem, piperacillin or cefoxitin. This may be due to the fact that the use of nitrocefin enables the detection of a basal level of β-lactamase production, whereas specific determination of the activity against an antimicrobial requires the presence of increased levels of this enzyme. Interestingly, specific β-lactamase activity could be observed in the iodometric assay for piperacillin in the presence of 10 mg clavulanic acid l⁻¹ for these three strains. B. fragilis NCTC 9344 showed the highest sensitivity to imipenem.

As shown in Table 1, the strains under study were resistant to some cephalosporins, such as cefuroxime, cephalexin and cefoxitin. As indicated previously, no specific enzymic activity was detected in strains AK-2, 119 or 7160. However, strain AK-2 showed moderate resistance to cephalexin and cefoxitin, strain 119 was moderately resistant to the action of cephalexin and strain 7160 was highly resistant to the action of cefuroxime and cephalexin. Edwards & Greenwood (1992) reported the isolation of five strains of B. fragilis with moderate resistance to cefoxitin, latomoxef and imipenem, which was not associated with β-lactamase activity. Several reports have established an association between antimicrobial resistance and the reduced affinity for β-lactam compounds of Bacteroides spp. PBPs. Georgopapadakou et al. (1983) observed reduced affinity of piperacillin, cefoperazone, cefotaxime, cefazidine and imipenem for PBP2 in a resistant strain of B. fragilis. Changes in the affinity of PBP1 or PBP2 in laboratory-derived mutants have also been correlated with a decrease in susceptibility to cefoxitin (Piddock & Wise, 1987). Yotsuji et al. (1988) reported that five cephalosporins used in their study displayed a high affinity for PBP3 (72 kDa), as these cephalosporins bound strongly to PBPs at 0.1–0.2 μg ml⁻¹. The aforementioned authors concluded that the affinity of cephalosporins for high-molecular-mass (HMM) PBPs may play an important role in the antibacterial activity of cephalosporins for the susceptible B. fragilis strain G-253, although no IC₅₀ calculation was carried out. In our study, only the cephalexin resistance of strain 119 could be attributed to β-lactamase activity, but no differences were found in the PBP profiles of these three strains.

The cepA gene was detected in six of the eight strains studied. Only B. fragilis strains 119 and AK-2 were cepA-negative. This fact explains the differential behaviour of these two isolates in respect to their resistance to ampicillin, cefuroxime, cephalexin and piperacillin. As can be seen in Table 1, higher MIC values were observed for these antibiotics in strains where the cepA gene was detected than in strains 119 and AK-2 (cepA⁻). Gutacker et al. (2002) concluded that the β-lactamase-encoding genes cepA and cfIA were never found together in the same isolate. This apparent mutual exclusion may be explained by the acquisition of these genes in separate and unique events. In our study, six of the eight strains [119 and AK-2 (cfIA⁺ and cepA⁻) and NCTC 9344, AK-4, 0423 and R212 (cfIA⁻ and cepA⁺)] are in accordance with the observations of the cited authors. However, we detected the presence of both genes in strains 7160 and 2013E, which are both cfIA- and cepA-positive, but it seems that either no or very low CfaA carbapenemase activity is expressed in strains that contain both genes (Table 2).

PBP profiles in B. fragilis

Table 3 shows the PBP profiles for eight strains of B. fragilis. Three HMM-PBPs were detected in all strains analysed. We observed that PBP1Bfr (91 kDa) appeared clearly in all strains, but that PBP2Bfr (80 kDa) was only seen clearly in strain 0423, as a faint band appeared for the rest of the strains; this may indicate differences in affinity for the labelled benzylpenicillin. PBP3Bfr (69 kDa), the protein encoded by the pbp4Bfr gene, was detected in all strains, but appeared as a diffuse band in B. fragilis strains NCTC 9344 and 2013E.
These two strains displayed the greatest sensitivity to the combination of imipenem and clavulanic acid, as they presented MICs of 0.06 and 0.25 mg l\(^{-1}\), respectively. This observation could be related to the behaviour displayed by the PBP3Bfr proteins of these micro-organisms against \[^{3}H\]-benzylpenicillin, whereby both proteins may present a greater affinity for imipenem than the PBP3Bfr proteins of the other six \textit{B. fragilis} strains that were used in this study. However, no differences were found at the amino acid level between the PBP3Bfr sequences of strains NCTC 9344 and 2013E, which could explain their differing affinity for imipenem.

There is a certain disorder with respect to ascertaining the

## Table 2. \(\beta\)-Lactamase activity

<table>
<thead>
<tr>
<th>B. fragilis strain</th>
<th>NCTC 9344</th>
<th>119</th>
<th>AK-2</th>
<th>7160</th>
<th>2013E</th>
<th>AK-4</th>
<th>0423</th>
<th>R212</th>
</tr>
</thead>
<tbody>
<tr>
<td>cfA*</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>cepA</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Nitrocefin assay†‡</td>
<td>–1.36(+), 28.78 (+), 13.58 (-), 0.82 (+), 36.40 (+), 44.55 (+), 47.05 (+), 37.78 (+)</td>
<td>–0.62 (+), 9.28 (+), 0.38 (-), 0.44 (+), 17.22 (+), 20.76 (+), 22.21 (+), 16.69 (+)</td>
<td>–0.24 (+), 29.08 (+), 20.81 (-), 0.13 (-), 0.26 (+), 0.52 (+), 0.93 (+), 2.20 (+)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Clavu§</td>
<td>–0.22 (+), 12.04 (+), 0.51 (-), 0.24 (-), 0.34 (-), 1.11 (-), 1.65 (-), 1.38 (-)</td>
<td>–0.30 (-), 0.13 (-), 0.09 (-), 0.80 (+), 37.78 (+), 42.19 (+), 49.35 (+), 39.10 (+)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membrane ED Ta</td>
<td>–0.18 (+), 0.22 (+), 0.19 (-), 0.23 (+), 16.83 (+), 22.24 (+), 22.87 (+), 17.61 (+)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The same result was obtained by using oligonucleotides P1/P2 or P3/P4, which produced DNA fragments of 442 or 423 bp, respectively.

†Appearance of a red colour under the conditions described in Methods was evaluated after 5 min (+++), 10 min (++) or 30 min (+) incubation or no colour after 60 min incubation (–).

‡Specific activity (nmol mg\(^{-1}\) min\(^{-1}\)) was calculated by using an extinction coefficient for nitrocefin of 20 500 at 486 nm.

§Total cell extract.

∥Membrane fraction.

¶Potassium clavulanate at 10 mg l\(^{-1}\) was added to the assay 10 min before the nitrocefin.

#EDTA at 2 mM was added 10 min before the nitrocefin.

**Ampicillin; I, imipenem; P, piperacillin; Cf, cefuroxime; Cp, cephalexin; C, clavulanic acid. Production of a clear halo of activity was observed after 30 min (+++), 60 min (++) or 120 min (+) or was not observed after 24 h (–) incubation at 30 °C.
number, size and nomenclature of PBPs from \textit{B. fragilis}. However, most authors agree on the presence of three HMM-PBPs, with molecular masses of 88–94 (PBP1Bfr), 80–83 (PBP2Bfr) and 69–72 (PBP3Bfr) kDa (Wexler & Halebian, 1990). In the present study, these three proteins were detected in all strains studied, although, in some cases, PBP2Bfr and/or PBP3Bfr appeared as a faint band. Imipenem and meropenem bind initially to PBP3Bfr to produce round cells and then interact with PBP2Bfr. Imipenem also binds to PBP1Bfr at concentrations that correlate with the MIC (Piddock & Wise, 1986). In a competition assay, imipenem can displace benzylpenicillin from \textit{B. fragilis} proteins that have a molecular mass of 60–100 kDa (Edwards & Greenwood, 1996). These observations suggest that changes in the pattern of one or several HMM-PBPs may play a role in the resistance of \textit{B. fragilis} to imipenem, but that does not seem to be the case for strains NCTC 9344 and 2013E.

As well as the three HMM-PBPs, we also observed up to three low-molecular-mass (LMM) PBPs in some strains: PBP4Bfr (63 kDa), PBP5Bfr (47 kDa) and PBP6Bfr (40 kDa). All LMM-PBPs, including PBP6Bfr, were absent from strains NCTC 9344 and R212, whereas only PBP6Bfr was absent from isolates AK-4 and 2013E, in which imipenem-hydrolysing activity may play a major role in resistance. It has been reported that PBP6Bfr may be the putative \(\beta\)-lactamase of these strains or an LMM cell-membrane protein that is able to bind a degradation product of radioactive benzylpenicillin (Edwards & Greenwood, 1996). Our observations are similar to those reported by Wexler & Halebian (1990), who detected PBP6Bfr (40 kDa) in strains of \textit{B. fragilis} that were both susceptible and resistant to imipenem; however, our results differed from those published by Edwards & Greenwood (1996), who only detected this protein in resistant strains. This behaviour, even with the higher affinity of imipenem and cephalosporins for HMM-PBPs of \textit{Bacteroides} spp., prompts us to consider that, in the strains under study, these proteins (PBP1Bfr, PBP2Bfr and PBP3Bfr) are less relevant than PBP6Bfr in the resistance mechanism of this microorganism to these \(\beta\)-lactam antibiotics.

### DNA fingerprinting analysis of the \textit{pbpABfr} gene

Fig. 1 shows the results of the hybridization of chromosomal DNA from the eight strains of \textit{B. fragilis} that were studied, using the digoxigenin–\textit{pbpABfr} gene fragment as the probe. We detected two types of behaviour during analysis of these strains. An initial pattern was exemplified by strains AK-2 and 119: in both strains, there were two bands of about 10 kbp after digestion with the enzyme \textit{KpnI}. In strain 119, the molecular size of one of the bands was slightly greater, but in strain AK-2, the bands were similar in size (about 10 kbp). The other six strains displayed a different pattern: they all contained a single band of >15 kbp.

By using the primers and conditions described in Methods, we were able to amplify a single band of 1914 bp from all strains analysed by using primers –26up and +31down. In order to study the sequence variability that may exist among

### Table 3. PBPs in \textit{B. fragilis} strains labelled with [3H]-benzylpenicillin

<table>
<thead>
<tr>
<th>\textit{B. fragilis} strain</th>
<th>Presence of PBP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBP1Bfr</td>
</tr>
<tr>
<td>NCTC 9344</td>
<td>+</td>
</tr>
<tr>
<td>119</td>
<td>+</td>
</tr>
<tr>
<td>AK-2</td>
<td>+</td>
</tr>
<tr>
<td>7160</td>
<td>+</td>
</tr>
<tr>
<td>2013E</td>
<td>+</td>
</tr>
<tr>
<td>AK-4</td>
<td>+</td>
</tr>
<tr>
<td>0423</td>
<td>+</td>
</tr>
<tr>
<td>R212</td>
<td>+</td>
</tr>
</tbody>
</table>

Parentheses indicate a diffuse or faint band.

**Fig. 1.** \textit{B. fragilis} chromosomal DNA hybridization with the \textit{pbpABfr} probe. Chromosomal DNA of strains NCTC 9344 (lane 1), 119 (2), 0423 (3), 7160 (4), AK-2 (5), R212 (6), 2013E (7) and AK-4 (8) was digested with \textit{KpnI} and resolved in 0·8% agarose/TAE gel. The \textit{pbpABfr} gene from \textit{B. fragilis} NCTC 9344 was labelled by PCR using the PCR DIG labelling mix and used as the probe. Strains AK-2 and 119 show a different pattern to the others, with a band of approximately 10 000 bp. The other six strains displayed similar patterns.
these DNA fragments from the different strains of *B. fragilis*, which are expected to encode PBP3Bfr (69 kDa), DNA fingerprinting analysis was performed by using the restriction enzymes *Tru*I and *Hin*4I. With both enzymes, as shown in Fig. 2, two patterns were observed, which corresponded precisely to those already established by means of Southern blot analysis. The size and number of bands from *B. fragilis* strains AK-2 and 119 (cepA⁺) were identical and differed from those of the other six strains (cepA⁻), which displayed a different pattern. These observations prompted us to select six strains in order to sequence the gene that encodes PBP3Bfr, identified by homology comparison as *pbpABfr* (see Fig. 3). The strains selected were NCTC 9344, AK-2, AK-4, R212, 119 and 7160. Strains AK-2 and 119 displayed one of the patterns identified by Southern blot analysis and DNA fingerprinting and the other four displayed the other pattern.

**PCR and DNA sequencing**

DNA sequences of the *pbpABfr* genes and deduced PBP3Bfr proteins of strains NCTC 9344, AK-2, AK-4, R212, 119 and 7160 were compared with the unpublished sequences of strains NCTC 9343T and 638R. The results obtained by multiple sequence alignment, shown in the Supplementary Figure (available in JMM Online), indicate that the number of amino acid changes is small, conservative and mainly restricted to the non-transpeptidase domain (N-terminal) of PBP3Bfr. However, the number of nucleotide changes in the PBP3Bfr proteins from strains AK-2 and 119 was very large, compared to the sequences of the other strains, but most of them were in the third codon position (see Supplementary Figure in JMM Online).

Analysis of the whole sequenced bacterial genome defined a database of orthologous genes (clusters of orthologous groups of proteins; COGs) at the NCBI web site (http://www.ncbi.nlm.nih.gov/COG/). All cell-wall-surrounded microorganisms contain a set of PBPs that fall into some of the following COGs: COG0744, COG0768, COG1680, COG1686 and COG2027. The two first correspond to HMM-PBPs of classes A and B and the last three correspond to LMM-PBPs. Only the proteins in COG0744 (transglycosylases/transpeptidases) and COG0768 (transpeptidases) 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. Proteins that are encoded by the *pbp1abBfr* and *pbp1cBfr* genes can be identified in COG0744 and correspond to the PBP1Bfr complex, whereas proteins that are encoded by the *pbpABfr* and *pbpBBfr* genes correspond to PBP3Bfr and PBP2Bfr and could be placed in COG0768. The molecular mass of the protein encoded by *pbpABfr* (69 kDa) must correspond to that of PBP3Bfr (69 kDa), which was identified by the binding of labelled penicillin to *B. fragilis* membrane extracts, whilst the protein encoded by *pbpBBfr* (78 kDa) can be deduced to be PBP2Bfr (80 kDa) in the binding pattern, due to a long C-terminal extension of 193 aa (Fig. 3).

In an attempt to characterize the two different patterns of mutant strains identified in this study and exemplified by strains NCTC 9344, 7160, AK-4 and R212 (cepA⁺) and AK-2 and 119 (cepA⁻), respectively, we sequenced the *pbpABfr* gene of these six strains and compared our results with the sequences of strains NCTC 9343T and 638R (see Supplementary Figure in JMM Online). Compared with strain NCTC 9343T, sequences from strains NCTC 9344, 7160, AK-4 and R212 showed no differences in the amino acid composition of PBP3Bfr and only 11 (strains NCTC 9344, 7160 and AK-4) and three (strain R212) silent nucleotide changes. Differences were mainly silent nucleotide changes that correlated precisely within each pattern, i.e. a large number in strains AK-2 (229 nucleotide changes) and 119 (230 nucleotide changes) (see Supplementary Figure in JMM Online) and a low number in strains NCTC 9344, 7160, AK-4 and R212. All the strains that were sequenced, including strain 638R from the Sanger Centre, have the mutation H147R when compared to strain NCTC 9343T, with the exception of strain R212, which probably indicates accuracy in the sequence of strain NCTC 9343T. Strain 683R contains the
single amino acid mutation L444V. However, strains AK-2 and 119, despite the H147R change, have the following five conservative amino acid changes: L27V, K105R, Y203F, I235V and L493I, four of which occur in the non-penicillin-binding domain and only one of which (L493I) occurs in the transpeptidase domain. It is difficult to ascribe imipenem-resistance patterns to these changes in PBP3Bfr, although it is worthy of note that this larger number of nucleotide changes could be concluded that the molecular target of imipenem in strain 119 is due to high-level production of CepA cephalosporinase. Moreover, re-

![Fig. 3. Homologies between E. coli and B. fragilis PBPs 2 and 3. Numbers under conserved boxes indicate amino acid position and numbers between them show the distance in amino acids. Nt, N-terminal end; Ct, C-terminal end. *Probability PN and score (in brackets) as obtained by the tblastn program on the Sanger web server, using the protein sequences of PBP2 (GenBank accession no. P08150) and PBP3 (accession no. P04286) of E. coli as query.](image)

ACKNOWLEDGEMENTS

We would like to thank Professor Edwards of Queen’s Medical Centre, Nottingham, UK, and Professor García-Sánchez of the Clinical Hospital of the University of Salamanca, Spain, for providing the bacterial strains used in this study. We would also like to thank Ade Morales for her excellent technical assistance. This study was sponsored by grants AGL2001-1104, BMC2001-2346 and BMC2002-04126 (Ministerio de Ciencia y Tecnología, Madrid, Spain) and grant no. 2PR01A014 (Consejería de Educación, Ciencia y Tecnología, Mérida, Spain). We would also like to express our gratitude to the Fundación Ramón Areces for its institutional support to the Molecular Biology Centre. Sequence data of strains NCTC 9343 and 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/.

REFERENCES


<table>
<thead>
<tr>
<th>Homologies*</th>
<th>(pbpBfr)</th>
<th>(pbpBfr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBP2Eco (pba)</td>
<td>1.2e^-4 (139)</td>
<td>3.5e^-54 (567)</td>
</tr>
<tr>
<td>PBP3Eco (pbaB)</td>
<td>3.6e^-17 (252)</td>
<td>7.6e^-11 (194)</td>
</tr>
</tbody>
</table>


