Mechanisms responsible for reduced susceptibility to imipenem in *Bacteroides fragilis*

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The mechanisms responsible for reduced susceptibility to imipenem (MIC 2–16 mg/L) were investigated in eight strains of *Bacteroides fragilis*. All the strains produced elevated levels of β-lactamase. Four *B. fragilis* strains produced metallo-β-lactamase capable of marked imipenem hydrolysis, and these enzymes were shown to be responsible for resistance. There was evidence, from testing susceptibility to imipenem in the presence of clavulanic acid, that increased resistance in two strains was associated with susceptibility to β-lactamases other than metallo-enzymes. With the remaining two strains there was no evidence of enzymic breakdown of imipenem. Neither of these strains showed evidence of decreased permeability to nitrocefin as judged by a method which takes into account the substrate concentration in the periplasmic space. A low molecular weight PBP, that was not seen in fully sensitive strains of *B. fragilis*, was detected in four strains in which reduced susceptibility to imipenem was not associated with metallo-β-lactamase activity.

**Introduction**

Imipenem exhibits high activity against *Bacteroides fragilis* (MICs typically ≤0.12 mg/L) and is effective in serious infections in which *B. fragilis* is involved (Edwards *et al.*, 1989; Sanders & Aldridge, 1992). This activity is associated with β-lactamase stability, ability to penetrate the cell, and high affinity for penicillin binding proteins (PBPs) (Quinn, 1994).

High level resistance to imipenem in *B. fragilis* (MIC > 100 mg/L) is presently rare and is associated with the production of metallo-β-lactamases (Podglajen *et al.*, 1992; Payne, 1993). However, *B. fragilis* strains for which the MIC of imipenem is in the range 2–4 mg/L, concentrations which are up to 50 times greater than those of ‘normal’ sensitive strains, are more common, representing 7% of *B. fragilis* isolated from clinical specimens (Edwards & Greenwood, 1992). Some of these isolates produce carbapenemases (Eley & Greenwood, 1986; Edwards & Greenwood, 1992), although the extent of their contribution to decreased susceptibility is unknown. Reduced affinity for the PBPs or the presence of a permeability barrier are the most likely reasons for increased resistance in strains which do not show carbapenemase activity.
Several studies have described the PBPs of *B. fragilis*, but the findings are conflicting (Georgopapadakou, Smith & Sykes, 1983; Botta, Privitera & Menozzi, 1983; Piddock & Wise, 1986; Yotsuji et al., 1988; Wexler & Halebian, 1990). Reduced affinity for the PBPs of *B. fragilis* has been implicated in resistance of cephalosporins and cephemycins (Georgopapadakou et al., 1983; Piddock & Wise, 1987; Yotsuji et al., 1988), but altered PBPs have not been identified as a factor in imipenem resistance in *B. fragilis*. In highly resistant strains, a permeability barrier associated with metallo-β-lactamase production has been described (Cuchural, Malamy & Tally, 1986).

We have investigated the role of β-lactamases, reduced permeability and affinity for PBPs in eight strains of *B. fragilis* which displayed reduced susceptibility to imipenem. We also examined the PBPs of fully sensitive *B. fragilis* strains to provide a yardstick for comparison with the more resistant isolates.

**Materials and methods**

**Bacterial strains**

*B. fragilis* R186, R208, R212, R240, R251 and 119 were clinical isolates, and *B. fragilis* 2013E and 0423 were laboratory culture collection strains originally obtained from Glaxo Laboratories, Greenford, UK and Wadsworth Veterans Administration Center, Los Angeles, CA, USA, respectively. *B. fragilis* NCTC 9344 was included as a sensitive control strain. Three fully sensitive *B. fragilis* clinical isolates were also used in investigations of PBPs.

**Antibiotic titrations**

Appropriate concentrations of imipenem (Merck, Sharp & Dohme) and clavulanic acid (SmithKline Beecham) were dissolved in 0.01 M phosphate buffer (pH 7.0) and in sterile distilled water respectively.

MICs of imipenem, with and without clavulanic acid 4 mg/L, and clavulanic acid alone were determined by the broth dilution method in Brain Heart Infusion broth (BHIS) supplemented with yeast extract 5 g/L, haemin 5 mg/L and menadione 1 mg/L. An inoculum of c. 10⁶ organisms/mL was used. The MICs were read as the lowest antibiotic concentration required to suppress visible growth after incubation at 37°C for 48 h in an atmosphere of 80% nitrogen, 10% hydrogen and 10% carbon dioxide.

**Hydrolysis of imipenem**

Hydrolysis of imipenem was measured in the presence and absence of the chelating agent EDTA, or EDTA and zinc sulphate. Crude β-lactamase extracts prepared as described by Edwards & Greenwood (1992) were concentrated five-fold by freeze-drying; 100 μL of the extract were mixed with 200 μL of EDTA (5 mM) or phosphate buffer (0.02 M; pH 7.0). After incubation at 37°C for 30 min, 200 μL of zinc sulphate (5 mM) or phosphate buffer were added with imipenem to give an antibiotic concentration of 50 mg/L. The absorbance of the imipenem solutions was measured at 299 nm after incubation over a period of 2 h at 37°C.
Reduced susceptibility to imipenem in *B. fragilis*

Specific imipenemase activity (nmol of imipenem hydrolysed/min/mg of protein) was determined from the rate of hydrolysis measured over the initial linear part of the hydrolysis curve (Edwards & Greenwood, 1992).

**Turbidimetric experiments**

Continuous opacity records of anaerobic bacterial growth were obtained with a 12-channel turbidimeter operated under anaerobic conditions (Electrotek, Keighley, UK).

For low inoculum experiments, an appropriate dilution of imipenem (or sterile distilled water control) was added to the tubes containing BHIS broth and a bacterial inoculum of $c. 10^5$ organisms/mL. To test high inocula, imipenem was added to cultures when growth had raised the opacity to 30% of that of a fully grown culture, equivalent to $c. 10^8$ organisms/mL. Antibiotic was added through two-way syringe needles located in the lid of the turbidimeter, without loss of anaerobiosis.

From the growth response curves, MICs and minimum antibacterial concentrations (MACs) were determined and compared. The MIC was defined as the lowest antibiotic concentration required to suppress growth for the 30 h period of observation, and the MAC as the lowest antibiotic concentration causing deviation from normal growth.

**Permeability measurements**

Permeability was assessed by the method of Cuchural et al. (1988) in which the rate of hydrolysis (μmols/min) of β-lactam substrate by intact cells per μg of protein is divided by the difference in substrate concentration across the outer membrane. Nitrocefin (50 μM) was used in the assay medium and protein estimations were carried out with a Sigma Protein Assay kit. The concentration of nitrocefin in the periplasmic space was derived from crypticity values defined as the ratio of hydrolytic activity observed in preparations of sonicated cells to that of intact cells, and the Michaelis-Menton constant estimated with the Hanes plot using measurements of initial rates of hydrolysis at various concentrations of nitrocefin.

**Penicillin binding proteins (PBPs)**

Bacteria from a 4 h BHIS broth culture (200 mL) were harvested by centrifugation, suspended in 10 mL of phosphate buffer, sonicated and centrifuged to remove the unbroken cells. The supernate was centrifuged (40,000 rpm at 4°C for 30 min) to sediment the cell membranes, which were then washed five times and the protein content adjusted to 10 mg/mL. The sample was tested for penicillinase (Escamilla, 1976) and stored at -70°C.

Twenty μL of cell membrane suspension and 5 μL of distilled water were pre-warmed to 30°C; 5 μL of βH-benzylpenicillin (Amersham International, Aylesbury, UK), with a radioactive concentration of 1.0 mCi/mL and a benzylpenicillin concentration of 18.8 mg/L (final penicillin concentration 3.1 mg/L and a radioactive content of 5 μCi), were added. After 10 min at 30°C, excess non-radioactive penicillin (5 μL of a solution containing 120 mg benzylpenicillin/L) was added. After addition of 35 μL of double strength Laemmli sample buffer (Laemmli, 1970), the mixture was boiled for 5 min and stored at -70°C.
In some experiments, the amount of $^3$H-benzylpenicillin used was increased to 15 $\mu$L. Other experiments were carried out in which the membrane suspension was exposed to 10 $\mu$L $^3$H-benzylpenicillin and clavulanic acid at a final concentration of 5 mg/L. For competition assays, 5 $\mu$L volumes of doubling dilutions of imipenem were used with 5 $\mu$L of $^3$H-benzylpenicillin.

The cell membrane proteins and protein molecular weight standards (Biorad, Hemel Hemstead, UK) were separated on SDS-PAGE gels (Laemmli, 1970) and stained with 0.1% Page Blue.

For fluorography the gel was covered with En($^3$H)ance (NEN, Boston, MA, USA), and after 1 h this was replaced with deionised water and left for a further hour. The gel was dried at 80°C under vacuum on a model 443 slab drier (Bio-Rad). In a dark room, the gel was laid on the pre-fogged side of a Kodak X-OMAT XAR 5 film. After 3 weeks storage at $-70^\circ$C, the film was developed and the position of the bands measured.

For competition assays, gels were divided into two segments representing the molecular weight ranges c. 100-60 and 60-20 kDa. Each segment was placed in a glass vial with 3 mL of scintillation fluid (Koch-Light, Hatfield, UK) and after 10 days, the level of radioactivity was counted in a scintillation counter. Segments of gel away from the sample tracks were similarly processed to give a count of the background radioactivity.

Results

Susceptibility to imipenem

The MICs of imipenem determined by broth dilution, with and without clavulanic acid, are shown in Table I. The MICs of imipenem alone for all the test strains were 2 or 4 mg/L, except for B. fragilis 119 for which the MIC was 16 mg/L.

Clavulanic acid 4 mg/L had little effect on the MIC of imipenem, except in the case of B. fragilis R208 and 2013E for which the respective MICs of imipenem were reduced by four- and eight-fold. The MIC of clavulanic acid alone was $>8$ mg/L for all strains.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>MIC (mg/L)</th>
<th>imipenem</th>
<th>imipenem + clavulanic acid$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. fragilis R186</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>B. fragilis R240</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>B. fragilis R251</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>B. fragilis 119</td>
<td>16</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>B. fragilis R208</td>
<td>2</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>B. fragilis R212</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>B. fragilis 2013E</td>
<td>2</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>B. fragilis 0423</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>B. fragilis NCTC 9344</td>
<td>0.12</td>
<td>0.06</td>
<td></td>
</tr>
</tbody>
</table>

$^*$Clavulanic acid at a fixed concentration of 4 mg/L.

Table I. MICs of imipenem in the presence and absence of clavulanic acid
Reduced susceptibility to imipenem in *B. fragilis*

Table II. Hydrolysis of imipenem by the test strains

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>% imipenem hydrolysed* alone</th>
<th>Inhibition by EDTA with ZnSO₄</th>
<th>Specific imipenemase activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. fragilis</em> R186</td>
<td>90</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td><em>B. fragilis</em> R240</td>
<td>100</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td><em>B. fragilis</em> R251</td>
<td>100</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td><em>B. fragilis</em> 119</td>
<td>100</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td><em>B. fragilis</em> R208</td>
<td>&lt;10</td>
<td>no</td>
<td>NA</td>
</tr>
<tr>
<td><em>B. fragilis</em> R212</td>
<td>&lt;10</td>
<td>no</td>
<td>NA</td>
</tr>
<tr>
<td><em>B. fragilis</em> 2013E</td>
<td>&lt;10</td>
<td>no</td>
<td>NA</td>
</tr>
<tr>
<td><em>B. fragilis</em> 0423</td>
<td>&lt;10</td>
<td>no</td>
<td>NA</td>
</tr>
<tr>
<td><em>B. fragilis</em> NCTC 9344</td>
<td>&lt;10</td>
<td>no</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Percentage of antibiotic control after 2 h incubation at 37°C.
*nmoles imipenem hydrolysed/min/mg protein.
NA, Not applicable.

Imipenemase activities

Concentrated crude β-lactamase extracts of *B. fragilis* R240, R251 and 119 rapidly hydrolysed imipenem; less rapid degradation was observed with *B. fragilis* R186 (Table II). EDTA inhibited hydrolysis of imipenem by all these strains. Hydrolysis was restored when zinc sulphate was added. Enzymes from the remaining strains produced only a small rate of change in absorbance in the presence and absence of EDTA.

*B. fragilis* 119 exhibited specific activity two to ten times greater than that of the other metallo-β-lactamase producing strains (Table II).

Inoculum effect

The effect of imipenem on inocula of c. 10⁵ and 10⁸ organisms/mL was examined turbidimetrically to investigate the influence of β-lactamase on the response to the antibiotic of whole cells.

An inoculum effect that was reflected most markedly in a change in the MAC of imipenem, but also to a lesser extent in the turbidimetric MIC, was observed with *B. fragilis* R186, R240, R251 and 119. The effect was particularly marked with *B. fragilis* 119 (Table III). For these strains, MACs of imipenem in the high inoculum experiments were at least eight-fold higher than those obtained with the low inoculum, and the MICs for the high inoculum were at least twice the low inoculum MICs. No inoculum effect was observed with *B. fragilis* R212 and 0423.

The turbidimetric MICs were lower than those obtained by conventional titration (Table I) for some strains, possibly due to the shorter incubation period in the turbidimetric system.

Permeability measurements

The lowest permeability value (log₁₀ −5.4) was displayed by *B. fragilis* 2013E and the greatest (log₁₀ −4.7) by *B. fragilis* 119. Values for *B. fragilis* R208, 0423 and R212 were of log₁₀ −5.2, log₁₀ −5.0 and log₁₀ −4.9 respectively. *B. fragilis* R186, R240 and R251 were not tested due to their low affinity for nitrocefin.
Table III. Minimum antibacterial concentration (MAC) and MIC of imipenem for the high and low inocula of each isolate assessed by continuous turbidimetric monitoring

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Inoculum</th>
<th>MAC (mg/L)</th>
<th>MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. fragilis</em> R186</td>
<td>low*</td>
<td>≤0.25</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>high*</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td><em>B. fragilis</em> R240</td>
<td>low</td>
<td>0.12</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td><em>B. fragilis</em> R251</td>
<td>low</td>
<td>≤0.25</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td><em>B. fragilis</em> 119</td>
<td>low</td>
<td>≤1</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>16</td>
<td>64</td>
</tr>
<tr>
<td><em>B. fragilis</em> R208</td>
<td>low</td>
<td>0.12</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>0.25</td>
<td>1</td>
</tr>
<tr>
<td><em>B. fragilis</em> R212</td>
<td>low</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>B. fragilis</em> 2013E</td>
<td>low</td>
<td>0.06</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>0.12</td>
<td>0.25</td>
</tr>
<tr>
<td><em>B. fragilis</em> 0423</td>
<td>low</td>
<td>0.12</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>0.12</td>
<td>0.5</td>
</tr>
<tr>
<td><em>B. fragilis</em> NCTC 9344</td>
<td>low</td>
<td>0.06</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>high</td>
<td>0.12</td>
<td>0.25</td>
</tr>
</tbody>
</table>

*c. 10^5 organisms/mL.

Penicillin binding proteins

The PBPs of the fully sensitive *B. fragilis* strains were resolved by fluorography in experiments in which 5 μL 3^H-benzylpenicillin was used. Three proteins with mean molecular weights of 91, 80 and 69 kDa, designated PBPs 1, 2 and 3 respectively, were found in all four strains in four duplicate experiments. In one experiment, a fainter band representing an additional PBP with a molecular weight of 63 kDa (designated PBP 4) was detected in two strains, one of which also yielded an additional diffuse band of c. 47 kDa (designated PBP 5).

Table IV. Penicillin binding proteins (PBPs) in *B. fragilis* strains that showed reduced susceptibility to imipenem that was not attributable to metallo-β-lactamase

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Presence of PBP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td><em>B. fragilis</em> R208</td>
<td>(+)</td>
</tr>
<tr>
<td><em>B. fragilis</em> R212</td>
<td>(+)</td>
</tr>
<tr>
<td><em>B. fragilis</em> 0423</td>
<td>(+)</td>
</tr>
<tr>
<td><em>B. fragilis</em> 2013E</td>
<td>+</td>
</tr>
<tr>
<td><em>B. fragilis</em> NCTC 9344</td>
<td>+</td>
</tr>
</tbody>
</table>

Round brackets indicate that the band was detected only in experiments in which clavulanic acid (5 mg/L) and 10 μL 3^H-benzylpenicillin were present in the PBP assay. Square brackets indicate that the band was detected only in the absence of clavulanic acid and with 15 μL 3^H-benzylpenicillin.

*Diffuse or faint band.
Reduced susceptibility to imipenem in *B. fragilis*

1500

947

1000 - t

500 - l

V77?? ^7777

1500

1500

ic)

(d)

c

M

1000

500

1000

l

\[
\begin{array}{c}
0.015 \\
0.03 \\
0.06 \\
0.12 \\
0.25 \\
0.015 \\
0.03 \\
0.06 \\
0.12 \\
0.25
\end{array}
\]

1500

1500

1000

1000

\[
\begin{array}{c}
0.015 \\
0.03 \\
0.06 \\
0.12 \\
0.25 \\
0.015 \\
0.03 \\
0.06 \\
0.12 \\
0.25
\end{array}
\]

Figure Scintillation counts/min (above the background count) of bound \(^3\)H-benzylpenicillin in gel segments containing cell membrane proteins from *B. fragilis* strains showing sensitivity ((a), (b)) or increased resistance ((c), (d)) to imipenem, in competition assay with imipenem. (a) 100–60 kDa gel segment; (b) 60–20 kDa gel segment, (c) 100–60 kDa gel segment; (d) 60–20 kDa gel segment

Analysis of PBPs of strains showing increased resistance to imipenem was restricted to those isolates which did not produce metallo-\(\beta\)-lactamase: *B. fragilis* R208, R212, 0423 and 2013E. These strains were originally selected on the basis of elevated \(\beta\)-lactamase production and low penicillinase levels were found to persist in their cell membrane preparations, despite repeated washing. No PBPs were detected in experiments in which 5 \(\mu\)L \(^3\)H-benzylpenicillin was used. However, with 15 \(\mu\)L of labelled benzylpenicillin or 10 \(\mu\)L of penicillin tested in the presence of clavulanic acid 5 mg/L, PBPs were visualised as listed in Table IV. An additional PBP of 40 kDa, designated PBP 6, was detected in all four resistant isolates, but was not seen when *B. fragilis* 2013E was tested in the presence of clavulanic acid. PBP 6 was not seen in *B. fragilis* NCTC 9344 on any occasion. PBPs 1, 2 and 3 were detected in all four strains tested in the presence of clavulanic acid (5 mg/L), although PBPs 2 and 3 appeared faintly in *B. fragilis* R212. *B. fragilis* 2013E was the only resistant strain tested in which PBPs 1, 2 and 3 were detected in the absence of clavulanic acid. PBPs 4 and 5 were detected in *B. fragilis* 0423 and 2013E.

In a competition assay performed with a fully sensitive clinical isolate, imipenem was able to displace benzylpenicillin from proteins of molecular mass 100 to 60 kDa (Figure). Counts of radioactivity were reduced to 75% in the presence of imipenem 0.015 mg/L and declined further to 19% as the imipenem concentration was raised to 0.25 mg/L. In keeping with the fluorography results, only 4% of labelled
benzylpenicillin bound to proteins of molecular weights 60 to 20 kDa and this was minimally affected by the presence of imipenem.

The results of competition assays with the resistant *B. fragilis* R212 were markedly different from those obtained with the sensitive strain. In the absence of imipenem, binding to the 100–60 kDa segment was reduced ten-fold, although scintillation counts in the 60–20 kDa segment doubled. The effect of imipenem was more apparent in the gel segment containing proteins of molecular weights <60 kDa. Bound penicillin associated with these low molecular weight proteins was reduced by 43% in the presence of imipenem 2 mg/L.

**Discussion**

None of the *B. fragilis* strains examined in the present study was highly resistant to imipenem. However, isolates for which the MIC of imipenem is 2–4 mg/L are considerably more resistant than fully sensitive strains, although they are inhibited at the breakpoint of 8 mg/L that is usually cited as indicating clinically significant resistance (Phillips *et al.*, 1992). Only one of the strains examined, *B. fragilis* 119, exhibited resistance to imipenem according to this criterion.

Concentrated crude enzyme extracts of four of the eight *B. fragilis* isolates (R186, R240, R251, and 119) markedly hydrolysed imipenem. Inhibition of hydrolysis by EDTA and its restoration by excess ZnSO₄ indicate that these strains produce a zinc dependent metallo-β-lactamase. As expected, clavulanic acid failed to restore the activity of imipenem against these strains. Each of these strains exhibited an inoculum effect which was most marked according to the criterion of a change in the turbidimetric MAC. The largest inoculum effect was seen with *B. fragilis* 119, and this strain also displayed the largest specific imipenemase activity. In these four strains, the enzymic activity appeared to be the sole or predominant resistance mechanism.

Reduced susceptibility to imipenem in the other four *B. fragilis* strains was not associated with metallo-β-lactamase activity. A possible role for other types of β-lactamase in *B. fragilis* R208 and 2013E was indicated by an increase in imipenem sensitivity in the presence of a subinhibitory concentration of clavulanic acid and a slight inoculum effect. Thus, elevated production of the β-lactamases of these strains may play a part in the increased resistance, although the hydrolysis of imipenem demonstrated by crude cell extracts after incubation for 2 h was minimal. However, other resistance factors are suggested by the inability of clavulanic acid to fully restore sensitivity to imipenem.

Although *B. fragilis* R212 and 0423 also possess β-lactamases that are moderately susceptible to inhibition by clavulanic acid (Edwards & Greenwood, 1992), the inhibitor had little effect on imipenem susceptibility and no inoculum effect was shown in turbidimetric experiments. Hydrolytic activity did not, therefore, appear to play any part in the reduced susceptibility of these strains to imipenem. Also, reduced permeability was not demonstrated with these strains. Moreover, we have been unable to find evidence of unusual outer membrane protein or lipopolysaccharide composition in strains with increased resistance to imipenem which do not produce metallo-β-lactamase (Edwards & Greenwood, 1995).

Since previous accounts of the number and molecular weights of PBPs of *B. fragilis* have provided conflicting data (Georgopapadakou *et al.*, 1983; Botta *et al.*, 1983;
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Piddock & Wise, 1986; Yotsuji et al., 1988; Wexler & Halebian, 1990), we undertook an analysis of fully sensitive strains of *B. fragilis* before studying the PBPs of our less susceptible variants. The results broadly support the findings of Yotsuji et al. (1988) and Wexler & Halebian (1990) who described three PBPs with molecular weights in the range 88–94 (PBP 1), 82–83 (PBP 2) and 69–72 kDa (PBP 3). Wexler & Halebian (1990) also found additional PBPs of molecular weights 64 and 40 kDa. The three main PBPs that we found in all four sensitive isolates of *B. fragilis* correspond closely to these values for PBPs 1–3. Also, the PBP of molecular weight 63 kDa, that was occasionally detected in two of the four strains, appears similar to PBP 4 described by Wexler & Halebian (1990).

The strains used for PBP analysis produced elevated levels of non-metallo-β-lactamases which were clavulanic acid sensitive. The presence of these enzymes considerably complicates investigations of PBPs, since the radioactive benzylpenicillin is hydrolysed during the course of the experiment. Attempts to overcome this by several methods, including repeated washing of the membrane preparations, were unsuccessful. PBPs, however, could be detected by increasing the amount of ²H-benzylpenicillin and by the simultaneous use of the β-lactamase inhibitor, clavulanic acid. In these experiments, a band corresponding to a 40 kDa protein (PBP 6) was shown in all four resistant strains tested, but not in the sensitive control. It is tempting to explain this finding as a new PBP associated with imipenem resistance. It is also possible that the putative PBP 6 was the β-lactamase of these strains, or a low molecular weight cell membrane protein that was able to bind a degradation product of the radioactive benzylpenicillin. Interestingly, Georgopapadakou et al. (1983) reported a low molecular weight PBP with weak penicillinase activity in *B. fragilis*. A 40 kDa PBP has previously been reported in *B. fragilis* by Wexler & Halebian (1990), although, in contrast to our findings, this PBP was observed in a sensitive strain.

Since PBPs 1, 2 and 3 were seen in all four resistant strains, resistance was not associated with the loss of one or more of the high molecular weight PBPs. However, in competition experiments with *B. fragilis* R212, benzylpenicillin appeared to bind poorly to the higher molecular weight PBPs, and the degree of imipenem binding was reduced. Thus, reduced affinity of imipenem for PBPs of high molecular weight may play a part in resistance. However, the situation is complicated by the presence of β-lactamases in the PBP competition assay of resistant strains which governed the amount of ²H-benzylpenicillin available for binding and may themselves be inhibited by imipenem.

This study has revealed several different mechanisms that may be implicated in the reduced susceptibility to imipenem of clinical isolates of *B. fragilis*: low level production of metallo-β-lactamase that does not, in some cases, confer resistance detectable by conventional breakpoint criteria (*B. fragilis* R186, R240, R251 and 119); raised amounts of β-lactamase, which is not a metallo-enzyme, but may slowly hydrolyse imipenem (*B. fragilis* R208 and 2013E); and reduced affinity for high molecular weight PBPs associated with the presence of a PBP of low molecular weight (*B. fragilis* R208, R212, 2103E and 0423).

Strains of *B. fragilis* that exhibit reduced susceptibility to imipenem are relatively common among clinical isolates. We have encountered one case in which therapeutic failure of imipenem was associated with a metallo-β-lactamase-producing strain of *B. fragilis* in which the MIC of imipenem rose from 4 to 32 mg/L during treatment with
the antibiotic (Turner et al., 1995). The therapeutic implications of the present findings require further investigation.

References


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