Outer Membrane Permeation of \textit{Bacteroides fragilis} by Cephalosporins

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Outer membrane permeation of \textit{Bacteroides fragilis} by cephalosporins was examined by a previously described method. The permeation parameters of cephalosporins in \textit{B. fragilis} were close to $10^{-8}$ cm$^2$/min per $\mu$g of cell dry weight. These values were about an order of magnitude lower than those in \textit{Escherichia coli}. In \textit{B. fragilis}, the permeation was not directly proportional to the hydrophilicity of cephalosporins, and the ion selectivity was weak.

The mechanism of action of $\beta$-lactam antibiotics has been studied extensively in aerobic (4) and anaerobic (9) bacteria. Gram-negative bacteria, including \textit{Bacteroides fragilis}, are covered by an outer membrane which acts as a permeability barrier for various toxic materials such as antibiotics and detergents (6). \textit{B. fragilis} strains have been isolated from clinical specimens with an increasing frequency and are known to be moderately or highly resistant to penicillins and cephalosporins (2).

In this study, we performed a detailed analysis of the outer membrane permeation of \textit{B. fragilis} by cephalosporins.

**Bacterial strains.** \textit{B. fragilis} G-210, G-237, and G-242 were used in this study. These strains were isolated from clinical specimens and were highly resistant to $\beta$-lactam antibiotics owing to $\beta$-lactamase. \textit{B. fragilis} G-210 and G-242 constitutively produce typical species-specific cephalosporinases of \textit{B. fragilis} (14, 15). \textit{B. fragilis} G-237 constitutively produces a novel cephalosporinase which shows a unique substrate profile in hydrolyzing cephalosporins, cephams, penicillins, and carbenapens (14). The $\beta$-lactamase produced in the periplasmic space was used for the measurement of outer membrane permeation by cephalosporins. \textit{Escherichia coli} W3110 RGN823, which produces a TEM-type 3-lactamase, was also used in this study. \textit{B. fragilis} and \textit{E. coli} strains were stored in skim milk (10%) at $\sim70^\circ$C.

**Antibiotics.** The antibiotics used in this study were commercially available: cefamandole and cephaloridine, Shionogi Chemical Co., Ltd.; cefazolin and ceftezole, Fujisawa Pharmaceutical Co., Ltd.; cefoperazone, Toyama Chemical Co., Ltd.; cefusulodin, Takeda Chemical Industries, Ltd.; ceftalothon; Torii Pharmaceutical Co., Ltd.

**Media.** GAM broth and GAM agar (Nissui Pharmaceutical Co., Ltd.) were used to culture \textit{B. fragilis}. Antibiotic medium 3 (Difco Laboratories, Detroit, Mich.) was used to culture \textit{E. coli}.

**Reverse-phase thin-layer chromatography.** The hydrophobic character of the cephalosporins was expressed as the $R_f$ value, which was measured by reverse-phase thin-layer chromatography (TLC). The polar mobile phase was acetate-Veronal buffer (pH 7.0; Winthrop Laboratories, Div. Sterling Drug Co., New York, N.Y.)-methanol (4:1; vol:vol). TLC silica gel 60 F$_{254}$, siliconized precoated plates (Merck & Co., Inc., Rahway, N.J.) were used as the nonpolar stationary phase. A sample was dissolved in the acetate-Veronal buffer to give about 3 mg/ml, and 1 to 2 $\mu$l of the solution was loaded onto the TLC plate.

**$\beta$-Lactamase assay.** $\beta$-Lactamase activity was assayed by a modification of the microiodometric method of Novick (8).

**Assay of outer membrane permeation by cephalosporins.** The assay of outer membrane permeation by cephalosporins was carried out as described previously (11, 16), except that the bacterial cells were grown in GAM broth for \textit{B. fragilis} and on antibiotic medium 3 for \textit{E. coli}. Cultures of 200 ml in the mid-logarithmic growth phase were harvested by centrifugation at 5,000 $\times$ g for 15 min at 20°C. Cells were washed once with 0.1 M phosphate buffer (pH 7.0) containing 1 mM magnesium sulfate and were suspended in 30 ml of the same buffer. A portion of cell suspension was sonicated for 2 min at 4°C with an ultrasonic disruper. This suspension was used to measure the velocity of hydrolysis by disrupted cells ($v_{\text{disrupt}}$). The rest of the cell suspension was used directly for the measurement of the velocity of hydrolysis by intact cells ($v_{\text{intact}}$). At the same time that $v_{\text{intact}}$ was measured, the intact cell suspension was centrifuged quickly for 2 min and $\beta$-lactamase activity of the supernatant was measured ($v_{\text{sup}}$).

The permeation parameter $C$ (12) was calculated as follows: $C = (S_0 - S_f) [V_{\text{max}}S_f/(K_m + S_f)], S_f = (v_f/v_{\text{intact}}) \cdot [K_mS_f/(K_m + S_f)],$ and $V_{\text{max}} = [1 + (K_m/S_f)]v_{\text{sup}}$, where $v_f = v_{\text{disrupt}}$ $- v_{\text{sup}}$ and $v_f = v_{\text{intact}}$ $- v_{\text{sup}}$. $V_{\text{max}}$ and $K_m$ are the maximum velocity of hydrolysis of a test $\beta$-lactam antibiotic and Michaelis constant, respectively, $S_f$ and $S_i$ are concentrations of $\beta$-lactam antibiotics in the medium and in

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Hydrophilicity*</th>
<th>Permeation parameter (cm$^2$/min per $\mu$g of cell dry wt [10$^{-8}$])</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>\textit{G-210}</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>0.35</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>Cefalothin</td>
<td>0.40</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>Cefamandole</td>
<td>0.48</td>
<td>0.8 ± 0.5</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>0.71</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>Ceftezole</td>
<td>0.80</td>
<td>0.9 ± 0.3</td>
</tr>
</tbody>
</table>

* A negative electrical charge was used for all permeation determinations.

Hydrophilicity is expressed as the $R_f$ value of reverse-phase TLC.

Values are means ± standard deviations; $n = 3$.  

† Corresponding author.
the periplasmic space of the cells, respectively, at steady state. The permeation parameter was normalized by the dry weight of cells in the assay mixture.

**Effect of hydrophilicity on penetration rates.** A large amount of leakage significantly alters the permeation parameter $C$. In *E. coli* W3110 RGN823, $v_{\text{intact}}$ with cephalothin was 0.0091 to 0.012 μmol/min per μg of cell dry weight, $v_{\text{sup}}$ was 0.0020 to 0.0069 μmol/min per μg of cell dry weight, and $v_{\text{disrupt}}$ was 0.15 to 0.21 μmol/min per μg of cell dry weight. In *B. fragilis* G-210, $v_{\text{intact}}$ with cephalothin was 0.0035 to 0.0054, $v_{\text{sup}}$ was 0.0026 to 0.0034, and $v_{\text{disrupt}}$ was 0.24 to 0.34 μmol/min per μg of cell dry weight, respectively. In our data, the overall $v_{\text{sup}}$ with cephalosporins was between 40 and 80% of the overall $v_{\text{intact}}$ in *B. fragilis* G-210, G-237, and G-242. On the contrary, the overall $v_{\text{intact}}$ was between 2 and 15% of the overall $v_{\text{disrupt}}$ of the same amount of cells. Although $v_{\text{intact}}$ was low, $v_{\text{sup}}$ was directly proportional to $v_{\text{intact}}$ and $v_{\text{sup}}$ was always lower than $v_{\text{intact}}$. So, the precision of the permeation parameter obtained might have been high. The permeation parameter $C$ of *E. coli* W3110 RGN823 outer membrane by cephalothin was $1.5 \times 10^{-4} \pm 0.5 \times 10^{-4}$ cm/min per μg of cell dry weight. When this value was compared with that of the *E. coli* YA21 outer membrane toward the same compound ($2 \times 10^{-4}$ cm/min per μg of cell dry weight) (12; A. Yamaguchi, N. Tomiyama, R. Hiruna, and T. Sawai, submitted for publication), the reliability of our result was found to be acceptable.

The relationship between outer membrane permeation by a series of monoanionic cephalosporins and the hydrophilicity of the cephalosporins in *B. fragilis* is shown in Table 1. The hydrophilicity of cepofepazone was low; this was followed by the hydrophilicities of cephalothin, cefamandole, cefazolin, and ceftezole. Permeation by cephalothin was nearly equal; i.e., the permeation parameter $C$ was $0.7 \times 10^{-5}$ to $1.4 \times 10^{-5}$ cm/min per μg of cell dry weight in *B. fragilis* G-210, 0.8 $\times 10^{-5}$ to $3.1 \times 10^{-5}$ cm/min per μg of cell dry weight in *B. fragilis* G-237, and 0.7 $\times 10^{-5}$ to $2.0 \times 10^{-5}$ cm/min per μg of cell dry weight in *B. fragilis* G-242. These values were about an order of magnitude lower than those of *E. coli* (12).

Although the permeation was directly proportional to the hydrophilicity of cephalosporins in *E. coli* (7, 12) and *Proteus* sp. (5), it was not in *B. fragilis*. It is also known that the additional positive charge of the solute molecule accelerates the diffusion process through the porin pores in *E. coli* and *Proteus* sp. but that an increase in negative charge markedly decreases the permeation by solutes (1, 5, 7, 13). In *B. fragilis*, the additional positive charge did not accelerate the diffusion process and the additional negative charge slightly decelerated the diffusion process. The ion selectivity was weak.

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**LITERATURE CITED**


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**TABLE 2. Effect of additional positive or negative charges on outer membrane permeations of *B. fragilis* G-210, G-237, and G-242**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Hydrophilicity&lt;sup&gt;#&lt;/sup&gt;</th>
<th>Electrical charge</th>
<th>Permeation parameter (cm&lt;sup&gt;3&lt;/sup&gt;/min per μg of cell dry wt [10&lt;sup&gt;-5&lt;/sup&gt;]&lt;sup&gt;α&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>G-210</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>0.41</td>
<td>−, +</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>0.40</td>
<td>−</td>
<td>1.5 ± 0.7</td>
</tr>
<tr>
<td>Ratio A/B</td>
<td></td>
<td></td>
<td>0.93</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>0.41</td>
<td>−, +</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>Cefsulodin</td>
<td>0.90</td>
<td>−, −, +</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Ratio A/B</td>
<td></td>
<td></td>
<td>4.67</td>
</tr>
</tbody>
</table>

<sup>#</sup> Hydrophilicity is expressed as the $R_f$ value of reverse-phase TLC.

<sup>α</sup> Values are mean ± standard deviations; $n = 3$. 
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