Outer-membrane Permeability to $\beta$-Lactam Antibiotics in 
*Yersinia enterocolitica*

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Two outer-membrane (OM) proteins of *Yersinia enterocolitica* YOMP-C and YOMP-F appear to function as porins. Mutants that were YOMP-C- and YOMP-F- exhibited changes in cephaloridine and [$^3$H]glucose uptake and increased resistance to $\beta$-lactam antibiotics (especially cephalosporins) and tetracycline. Alterations in OM permeability may contribute to antibiotic resistance in *Yersinia*.

INTRODUCTION

*Yersinia enterocolitica* is a human enteropathogen. Most strains isolated from patients are resistant to $\beta$-lactam antibiotics (Cornelis & Abraham, 1975; Cornelis et al., 1987). The main mechanisms of resistance of pathogenic bacteria to $\beta$-lactams are: (i) changes in the target sites of the drug (penicillin-binding proteins), (ii) the activity of hydrolysing enzymes ($\beta$-lactamases), and (iii) the reduced accessibility of the target sites to the drug (permeability barrier). The latter barrier in Gram-negative bacteria consists of the outer membrane (OM) (Spratt, 1983; Nakazawa & Ogawara, 1982; Nakaido & Varra, 1985).

Mutations associated with lowered OM permeability of *Escherichia coli* and *Serratia marcescens* have been isolated in the course of antibiotic therapy (Benson & Decloux, 1985; Goldstein et al., 1983). One *E. coli* strain was deficient in the synthesis of OmpC protein. This indicates that enteric bacteria can increase their $\beta$-lactam resistance by mutational loss of porins – a loss of possible clinical revelance.

There is little information about the contribution of permeability mutations to *Y. enterocolitica* antibiotic resistance in clinical isolates. The composition of the OM of *Y. enterocolitica* is only partly known. Two major proteins (37 and 36 kDa) have been identified and correspond to a porin and OmpA in *E. coli* (Zaleska et al., 1985; Ogasawara et al., 1985). In this study we demonstrate that the 37 kDa porin consists of two protein species, designated here as YOMP-C and YOMP-F. We suggest that YOMP-C and YOMP-F are separate porins and they can play a role in permeability of the OM and in $\beta$-lactam resistance.

METHODS

Organisms. The following were used: *Yersinia enterocolitica* 5 (YE 5) O:5 biotype 3 from the collection of the State Institute of Hygiene, Warsaw; *Escherichia coli* K12 AB1157 Thr-Leu-proATsx-Gal-His-Mtl-Xyl-argED-Thi-Str+ (Howard-Flanders et al., 1966), and *E. coli* 15T+/RP-4 Thy- (Ap'Tc'Km') both from the Institute of Microbiology, Warsaw University. Plasmid RP-4 was transferred to *E. coli* AB1157 and thence to 15T- from the original strain *Pseudomonas aeruginosa* by the method of sexduction (Datta et al., 1971).

Isolation of cefoxitin-resistant mutants. The procedure of Jaffe et al. (1982) was applied. Spontaneous mutants resistant to 10 $\mu$g cefoxitin ml$^{-1}$ (Cfx') were isolated from YE 5 grown at 24 °C for 76 h with a frequency of $10^{-8}$. Single Cfx' colonies were purified by successive platings on media with antibiotic. MICs were determined according to Inouye et al. (1978) for the following antibiotics: penicillin (Serva), cephaloridine, cephalaxin,

Abbreviation: OM, outer membrane.
ampicillin, carbenicillin, cephalosporin C, tetracycline (Sigma), cefoxitin (MSD, Pharma), cefazolin (Allar), nitrocefin (Glaxo).

**Isolation of OM.** YE 5 and Cfx' mutants were grown at 24 °C in LB or BHI medium overnight. The OM proteins were isolated according to Hantke (1981). Protein concentrations were determined by the Lowry method. Trypsin digestion of OM proteins was done according to Zaleska et al. (1985).

**SDS-PAGE.** Gels for protein electrophoresis were prepared according to Laemmli (1970). The final concentration of acrylamide in gels was 12.5% (w/v). Protein samples for electrophoresis were prepared according to Lugtenberg et al. (1975). The gels were stained for 15 min in aqueous solutions of Coomassie blue (Serva) with acetic acid (9% w/v) and ethanol (4.5% w/v).

**Conjugation and β-lactamase activity.** E. coli 15T'/RP-4 (AprTcrKmr) was crossed with the recipient YE 5 and Cfx' mutants. Both partners were mixed 1:1 (v/v) and incubated for 72 h at 24 °C. Kanamycin-resistant clones (Km') of YE 5 and Cfx' mutants were isolated on Davies medium with kanamycin added (10 μg ml⁻¹). The frequency of plasmid transfer counted per donor cells was 1 x 10⁻³ for YE 5. The presence of β-lactamase TEM in Km' clones of YE 5 and Cfx' was checked by the reaction of nitrocefin hydrolysis according to O'Callaghan et al. (1972). The method described by Zimmerman & Rosselet (1977) was used. β-Lactamase activity was expressed as nmol cephaloridine hydrolysed during 1 min in 1 g dry weight mass calculated according to Gerhardt (1981).

**[3H]Glucose uptake.** The method of Sawai et al. (1982) was used. The amount of [3H]glucose (specific activity 3-3 mCi mmol⁻¹, 122-1 MBq mmol⁻¹; Amersham) taken up by the bacteria was standardized for a constant number of cells in the sample.

**RESULTS**

**Characterization of Cfx' mutants**

A total of 20 Cfx' strains were isolated; 12 could grow in the presence of 10 μg cefoxitin ml⁻¹ and eight in the presence of 20 μg cefoxitin ml⁻¹ on LB medium. Growth of all mutants on LB medium with the drug added was observed at 20-24 °C, but not at 37 °C. Four mutants were subjected to closer analysis: two were more resistant to cefoxitin (Cfx'-1, Cfx'-11) and two were less resistant (Cfx'-2, Cfx'-7). MICs of several β-lactam antibiotics are given in Table 1. When compared to the parent YE 5, increased resistance to β-lactam antibiotics, particularly to cephalosporins, was observed in all Cfx' strains.

**SDS-PAGE of OM proteins of YE 5 and Cfx' mutants is shown in Fig. 1.** The amount of YOMP-A (corresponding to OmpA in E. coli, Fig. 1a, lane 11) was similar in all samples tested. In the wild-type YE 5, a broad band (Fig. 1a, lanes 5 and 10) migrated to near the position of porins OmpF, C in E. coli (Fig. 1a, lane 11). This pattern did not change with temperature (Fig. 1a). In contrast, mutants Cfx'-1 and Cfx'-11 (Fig. 1a, bands 1, 2, 6 and 7) synthesized both of those major proteins in only trace amounts (denoted in Fig. 1 as F and C or YOMP-F and YOMP-C; *Yersinia* OM proteins).

When a smaller amount (10 μg) of the *Y. enterocolitica* preparation was placed on the gel, the YOMP-C and YOMP-F of YE 5 appeared as separate bands (Fig. 1b, lane 2); in the OM of the

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>YE 5 (control)</th>
<th>MIC (μg ml⁻¹)*</th>
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<tbody>
<tr>
<td></td>
<td>Cfx'-1</td>
<td>Cfx'-2</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>Penicillin</td>
<td>55</td>
<td>65</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>Cephalozin</td>
<td>2</td>
<td>80</td>
</tr>
<tr>
<td>Cephahoridine</td>
<td>2</td>
<td>40</td>
</tr>
<tr>
<td>Cephalosporin C</td>
<td>100</td>
<td>300</td>
</tr>
<tr>
<td>Carbencillin</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>5</td>
<td>15</td>
</tr>
</tbody>
</table>

* MIC for Cfx'-11 mutant was similar to Cfx'-1 and MIC for Cfx'-7 was similar to Cfx'-2.
Fig. 1. SDS-PAGE of major OM proteins of YE 5 (wild-type) and Cfx' mutants. (a) Bacteria grown at 24 and 37 °C. Protein samples (50 µg) were loaded onto the gel. Lanes 1 and 6, Cfx'-1; lanes 2 and 7, Cfx'-11; lanes 3 and 8, Cfx'-2; lanes 4 and 9, Cfx'-7; lanes 5 and 10, YE 5; lane 11, E. coli (control). (b) Bacteria grown at 24 °C. Protein samples (10 µg) were loaded onto the gel. Lane 1, Cfx'-1; lane 2, YE 5; lane 3, Cfx'-2. E. coli OM proteins: OmpA, 33 kDa (Hindennach & Henning, 1975); OmpF, 35 kDa; OmpC, 36 kDa; LamB, 50 kDa (Lugtenberg, 1981). LMW Calibration Kit Proteins (Pharmacia) were used. More details are given in the text.

Table 2. β-Lactamase activity of Y. enterocolitica Cfx' mutants and OM permeability of these strains to cephaloridine

<table>
<thead>
<tr>
<th>Strain</th>
<th>Intact cells</th>
<th>Spheroplasts (V_s)</th>
<th>V_i/V_s</th>
</tr>
</thead>
<tbody>
<tr>
<td>YE 5</td>
<td>3.24 ± 0.75</td>
<td>530 ± 53</td>
<td>111 ± 10</td>
</tr>
<tr>
<td>Cfx'-1</td>
<td>0.67 ± 0.14</td>
<td>628 ± 52</td>
<td>0.38 ± 0.04</td>
</tr>
<tr>
<td>Cfx'-2</td>
<td>0.75 ± 0.25</td>
<td>603 ± 25</td>
<td>0.43 ± 0.04</td>
</tr>
<tr>
<td>E. coli AB1157 (control)</td>
<td>3.00 ± 0.50</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* β-Lactamase activity before introduction of plasmid RP-4.
† Cells were converted to spheroplasts and complete liberation of β-lactamase was achieved by short ultrasonic treatment of the spheroplasts at 0 °C.

Y. enterocolitica outer-membrane permeability

As shown in Table 2, E. coli K12 AB1157/R− synthesized a small amount of β-lactamase, which is the product of the chromosomal gene ampC (Hrebenda & Heleszko, 1985). The
\[ \beta \text{-lactamase activity level in YE} \ 5 \text{ was comparable to that in } E. \ coli \ AB1157/R^- \text{. The enzyme activities in Cfx}^-1 \text{ and Cfx}^-2 \text{ mutants were about four times lower than in the wild-type strain. The differences in } \beta \text{-lactamase activity between wild-type and Cfx}^- \text{ mutants has not been further elucidated. The } Y. \ enterocolitica \text{ strain used in the studies contains only one plasmid, with a molecular mass of about 40 MDa (data not included), which, judging by the } \beta \text{-lactamase activity, does not carry determinants of resistance to ampicillin (Ap).}

\]

In order to determine the cryptic indices of YE 5 and of the mutants Cfx^-1, Cfx^-1 1, Cfx^-2 and Cfx^-7, plasmid RP-4 carrying genes Ap which code } \beta \text{-lactamase of the TEM type (Datta et al., 1971) was introduced into them. } 

\[ \beta \text{-Lactamase activity increased in all the tested strains. The cryptic indices } V_j/V_s \text{ (Table 2) calculated for Cfx}^-1 \text{ and Cfx}^-2 \text{ mutants indicated that, in contrast to YE 5, there was at least a 2-5-fold decrease in permeability of the OM for } \beta \text{-lactam antibiotics was observed.}

\[ [^3H] \text{Glucose uptake}

\]

For elucidation of the function of YOMP-C and YOMP-F in the OM, the kinetics of labelled glucose uptake by wild-type cells and Cfx^- mutants was studied (Fig. 2). A slight but significant inhibition of uptake of this sugar was observed in representative Cfx^- mutants as compared with that in the wild-type strain. The differences between Cfx^-1 and Cfx^-2 in [^3H]glucose uptake were not further studied.

**DISCUSSION**

Two major OM proteins of } Y. \ enterocolitica \text{ were studied: YOMP-C and YOMP-F. The positions of YOMP-C and YOMP-F after PAGE approximately corresponded to the respective porins OmpC and OmpF of } E. \ coli \text{ (35 and 36 kDa, Lugtenberg, 1981) and to the } Y. \ enterocolitica \text{ protein of 37 kDa tentatively identified as a porin (Zaleska et al., 1985). OmpC and OmpF are the site of diffusion of } \beta \text{-lactam antibiotics and tetracycline (Van Alphen et al., 1978; Chopra & Eccles, 1978). We suggest that the 37 kDa protein of } Y. \ enterocolitica \text{ described by Zaleska et al. (1985) is a mixture of YOMP-C and YOMP-F, since: (i) we could separate the 37 kDa protein into two bands, (ii) the mutants Cfx^-2 and Cfx^-7 only synthesize protein F (YOMP-C is greatly reduced or lacking in the OM of these strains), (iii) under standard conditions of electrophoresis, OmpC and OmpF of } E. \ coli \text{ also appear in the form of one band. The 37 kDa protein and}
YOMP-C and YOMP-F were resistant to trypsin, suggesting that they may be associated with murein. As compared with *Yersinia pestis*, the amount of protein resistant to this enzyme in the OM of *Y. enterocolitica* was twice as much. The function of the great majority of these proteins is not known. Among the three proteins resistant to trypsin in *Y. pestis* (proteins E, F and J; 30, 32 and 15 kDa, respectively), protein E fulfills the function of a porin (Benz *et al.*, 1985).

In investigations on the permeability to β-lactam antibiotics, Cfx* mutants of *Y. enterocolitica* were used. Cfx* mutants of *E. coli*, *Proteus mirabilis* and *Enterobacter cloacae* were described by Jaffe *et al.* (1982) and Sawai *et al.* (1982). All these strains exhibit: (i) a lack of some major protein in the OM, (ii) disturbances in glucose uptake and (iii) an increase in resistance to β-lactam antibiotics. The inhibition of [3H]glucose uptake was observed in *E. coli* OmpF− and OmpC− mutants. We demonstrate that in Cfx* mutants of *Y. enterocolitica* the absence of either YOMP-C alone or reduction in amount of both YOMP-C and YOMP-F proteins was associated with a reduced cephaloridine penetration into the cells (permeability index, $V_p/V_o$, less than unity). In the YOMP-C− and YOMP-C′, YOMP-F− mutants, a relatively slight but distinct inhibition of uptake of [3H]glucose was also noted. We suggested on this basis that, like *E. coli* OmpF and OmpC, the proteins YOMP-C and YOMP-F may play the role of general porins in *Y. enterocolitica*.

SDS-PAGE of lipopolysaccharide (LPS) isolated from *Y. enterocolitica* and Cfx* mutants showed that these strains synthesize LPS of smooth type (S) and that there was no difference in LPS structure between the wild-type and Cfx* mutants (data not shown).

*Y. enterocolitica* synthesizes two β-lactamases (A and B) which differ in substrate specificity. The resistance of the wild-type to β-lactams, particularly of the penicillin type and cephalosporin C, may be due to the activity of these enzymes (Cornelis *et al.*, 1973; Cornelis & Abraham, 1975). As regards Cfx* mutants in which β-lactamase activity was somewhat lower than in the wild-type strain, a marked increase was observed in resistance to β-lactam antibiotics of the cephaloridine group and tetracycline. The increase resistance may be mainly due to the limited diffusion of both of these groups of drugs into mutant cells. An important role is played in this process by YOMP-C proteins since YOMP-C− mutants displayed moderate resistance to cefoxitin and moderately decreased sugar and cephaloridine uptake. Reduction of YOMP-C synthesis in Cfx*−2 and Cfx*−7 was associated with a distinct hyperproduction of YOMP-F. It would seem, therefore, that in some *Y. enterocolitica* a compensation mechanism of porin synthesis may function. The existence of regulation of this type has been described earlier in *E. coli* (Mallick & Herrlich, 1979; Kawai *et al.*, 1979; Ozawa & Mizushima, 1979; Koga-Ban *et al.*, 1983). Mutants with reduced production of both YOMP-C and YOMP-F were more resistant to cefoxitin and had a greater decrease in sugar uptake and cephaloridine penetration than mutants deficient in YOMP-C alone.

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**REFERENCES**


