

## Molecular Characterization of an *Enterobacter cloacae* Outer Membrane Protein (OmpX)

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**A chromosomal gene of *Enterobacter cloacae* encoding an outer membrane protein (OmpX) has been cloned. Overproduction of the OmpX protein decreased the quantity of porins in the outer membrane of the parental strain and of *Escherichia coli* HB101. The *ompX* gene was located by insertions of the  $\gamma\delta$  sequence into the recombinant plasmid. The polarity of the gene was determined by in vitro transcription and translation of the  $\gamma\delta$ -containing plasmids. The nucleotide sequence of the *ompX* gene was elucidated by using both inverted terminal repeats of the  $\gamma\delta$  sequence as starting points for M13 dideoxy sequencing. The gene was found to encode a precursor of the OmpX protein consisting of 172 amino acid residues with a molecular mass of 18.6 kDa. The protein contains an N-terminal signal sequence of 23 amino acid residues. The exact cleavage point was established by sequencing the N-terminal part of the mature protein. The OmpX protein has several characteristics in common with outer membrane proteins of gram-negative bacteria. The protein is rather hydrophilic and is devoid of long hydrophobic stretches. On the basis of these results, we present a model for the OmpX protein folding in an outer membrane.**

The outer membranes of gram-negative bacteria obtain their nonspecific permeability for hydrophilic solutes from the presence of porins, a class of proteins that form water-filled diffusion channels (22). Lack of these proteins leads to resistance to most beta-lactam antibiotics and quinolones (1, 21). In a previous report (28), we described the cloning of a restriction fragment of *Enterobacter cloacae* chromosomal DNA that was associated with resistance to beta-lactam antibiotics in transformants both of *Escherichia coli* and of the parental *E. cloacae* strain. In these transformants, the amount of the porin proteins OmpF and OmpC present decreased, and an outer membrane protein of approximately 18 kDa was overproduced. In vitro transcription and translation of the recombinant plasmid showed that this protein was encoded by a gene on the cloned fragment. The function of the protein is still unknown, but its overproduction clearly interferes with the presence of OmpF and OmpC in the outer membrane. We propose the name OmpX for this protein, as long as its proper function is obscure. In the accompanying paper (29), the biological characterization of OmpX is presented. In this report, we present the nucleotide sequence of the *ompX* gene and the deduced amino acid sequence. From these data, some physical properties of OmpX are derived.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains, plasmids, and bacteriophages used are listed in Table 1.

**Growth conditions.** Bacteria were grown at 37°C in brain heart infusion broth (Oxoid Ltd.) supplemented when required with chloramphenicol (34  $\mu\text{g/ml}$ ).

**DNA techniques.** Plasmid DNA was prepared as described by Birnboim and Doly (3). Digestion of DNA with restriction endonucleases (Boehringer Mannheim Biochemicals) and ligation (T4 DNA ligase from Promega Biotec) of DNA fragments were performed as specified by the manufacturer.

Transformation of competent bacterial cells with plasmid DNA was carried out as described by Maniatis et al. (18).

**Preparation and characterization of outer membrane proteins.** Outer membrane proteins were isolated as described by Matsuyama et al. (20). The proteins were analyzed on 14% polyacrylamide gels containing 0.2% sodium dodecyl sulfate (SDS) as described by Lugtenberg et al. (17) except that the gels were supplemented with 4 M urea. Gels were stained with fast green FCF.

**Isolation of plasmids carrying an inserted  $\gamma\delta$  sequence.** Insertions of the transposable element  $\gamma\delta$  (11) in plasmid pJS04 were obtained as follows. DNA of plasmid pJS04 was introduced into the F' *lac*<sup>+</sup>-containing *E. coli* CE1304 by transformation. Transformants were selected on Iso-Sensitest agar (Oxoid CM471) containing chloramphenicol (34  $\mu\text{g/ml}$ ). Several transformants were subsequently mated with *E. coli* W3110 by filter mating on agar plates for 18 h at 37°C. Cells were collected from the filter, and the mixture was plated on Iso-Sensitest agar plates containing chloramphenicol and nalidixic acid (80  $\mu\text{g/ml}$ ). Transconjugants were screened subsequently for growth on Iso-Sensitest agar plates containing cephalothin (10  $\mu\text{g/ml}$ ). The positions of the  $\gamma\delta$  sequence insertions were localized by restriction enzyme digestions.

**Analysis of plasmid-encoded proteins.** Plasmid-encoded proteins were synthesized in vitro in a procaryotic DNA-directed transcription-translation system (Amersham, U.K.). L-[<sup>35</sup>S]methionine [NEN Research Products, Du Pont]-labeled proteins were detected by autoradiography after SDS-polyacrylamide gel electrophoresis as described by Laemmli (16).

**DNA sequence analysis.** DNA restriction fragments were subcloned into the multiple cloning site of bacteriophage vectors M13mp18 and M13mp19 and sequenced by the dideoxy-chain termination method (26) with an M13 sequencing kit (Boehringer Mannheim Biochemicals) as prescribed by the manufacturer. [ $\alpha$ -<sup>32</sup>P]dCTP was from NEN

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TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid, or phage	Relevant characteristics <sup>a</sup>	Reference or origin
<b>Bacterial strains</b>		
<i>E. coli</i> K-12		
HB101		4
W3110	F <sup>-</sup> Nd <sup>r</sup>	5
CE1304(F' <i>lac</i> <sup>+</sup> $\gamma\delta$ )		7
<i>E. cloacae</i> 2249-1	Clinical isolate	28
<b>Plasmids</b>		
pJS04	Cm <sup>r</sup> <i>ompX</i>	28
pJS04:: $\gamma\delta$	pJS04 carrying a $\gamma\delta$ insertion	This study
pJS05	Cm <sup>r</sup> ; <i>ompX</i> deletion derivative of pJS04	28
<b>Phages</b>		
M13mp18	M13 sequencing vector	32
M13mp19	M13 sequencing vector	32

<sup>a</sup> Abbreviations: Nd<sup>r</sup>, resistance to nalidixic acid; Cm<sup>r</sup>, resistance to chloramphenicol.

Research Products, Du Pont. Fragments obtained from restriction digestion of plasmid pJS04 were sequenced by using the universal M13 primer. The fragments obtained from restriction digestion of plasmid pJS04 carrying  $\gamma\delta$  sequences were sequenced by using a 20-mer primer (TTC CATTGGCCCTCAAACCC) homologous to the inverted terminal repeats of the  $\gamma\delta$  sequence (10) according to Van de Klundert (30a). The labeled fragments were analyzed on 0.4-mm-thick sequencing gels containing 6% polyacrylamide and 8 M urea (6). The gels were exposed to X-ray films (Fuji RX) for autoradiography at  $-80^{\circ}\text{C}$ .

**Analysis of the sequence data.** Nucleotide sequences were analyzed using the computer programs of the University of Wisconsin Genetics Computer Group (8).

**Amino acid sequence analysis of the N-terminal part of the mature OmpX protein.** A membrane fraction containing the mature OmpX protein was loaded on a 12% polyacrylamide gel and electrophoresed as described by Laemmli (16). The gel was electroblotted on a polyvinylidene difluoride membrane (19). This membrane was stained with 0.1% Coomassie brilliant blue R-250. The protein band was cut out and processed in a Protein Sequenator (Applied Biosystems model 470A) equipped with on-line PTH (phenylthiohydantoin) analysis, using a model 120A PTH analyzer.

**Nucleotide sequence accession number.** The nucleotide sequence of the *ompX* gene has been deposited in the EMBL data library under accession number M33878.

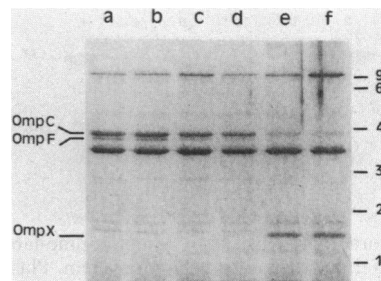


FIG. 2. SDS-polyacrylamide gel electrophoresis pattern of outer membrane proteins of *E. coli* W3110 transformed with pJS05 (lane a), pJS04:: $\gamma\delta$ -3 (lane b), pJS04:: $\gamma\delta$ -5 (lane c), pJS04:: $\gamma\delta$ -6 (lane d), pJS04:: $\gamma\delta$ -7 (lane e), and pJS04 (lane f). Positions of molecular mass standards are indicated at the right (rabbit muscle phosphorylase *b*, 97.4 kDa; bovine serum albumin, 66.2 kDa; hen egg white ovalbumin, 42.7 kDa; bovine carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; hen egg white lysozyme, 14.4 kDa). Positions of OmpF, OmpC, and OmpX are indicated at the left.

## RESULTS

**Location of *ompX* on plasmid pJS04.** The gene encoding OmpX was located by  $\gamma\delta$  sequence insertion mapping. pJS04 plasmids carrying a  $\gamma\delta$  sequence were isolated. Since the physical map of the  $\gamma\delta$  sequence is known (10, 11), the sites of the insertions in or near *ompX* could be determined by using *Bam*HI-, *Eco*RI-, and *Hind*III-generated restriction profiles (Fig. 1).

Susceptibility data for transconjugants showed that about 92% of them were able to grow on agar plates containing 10  $\mu\text{g}$  of cephalothin per ml, indicating that the insertion was located outside the gene. On the other hand, transconjugants containing a plasmid with a  $\gamma\delta$  sequence in a region of 500 bp between the two *Kpn*I sites of pJS04 had lost their cephalothin resistance. Analysis of the outer membrane proteins of the transconjugants by SDS-polyacrylamide gel electrophoresis demonstrated that the 18-kDa protein band could be detected only in resistant strains (Fig. 2).

**Polarity of the gene.** To determine the polarity of the *ompX* gene, the synthesis of proteins encoded by pJS04:: $\gamma\delta$ -3, -5, -6, and -7 was studied in an in vitro transcription-translation system, followed by SDS-polyacrylamide gel electrophoresis (Fig. 3). Plasmid pJS04 (lane f) and plasmid pJS04:: $\gamma\delta$ -7 (lane e) produced a protein of approximately 19 kDa. Since in such an in vitro system no processing occurs, this result implies that OmpX is synthesized as a precursor protein, proOmpX. Plasmid pJS04:: $\gamma\delta$ -3 yielded no OmpX protein

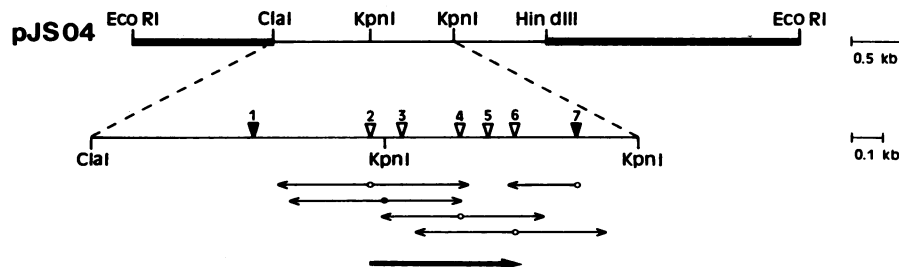


FIG. 1. Schematic representation of the restriction map of plasmid pJS04 and the  $\gamma\delta$  sequence insertion sites. pJS04 is linearized by *Eco*RI. Vector pACYC184 DNA is indicated by the solid bar. The  $\gamma\delta$  insertion sites are numbered 1 to 7 and marked to indicate beta-lactam-sensitive ( $\nabla$ ) or -resistant ( $\blacktriangledown$ ) phenotypes. Light arrows indicate the direction and extent of each DNA sequencing run.  $\circ$ , DNA sequences that begin at a  $\gamma\delta$  sequence insertion;  $\bullet$ , DNA sequences that begin at the *Kpn*I restriction site. The location of *ompX* and the direction of transcription are indicated by the heavy arrow.

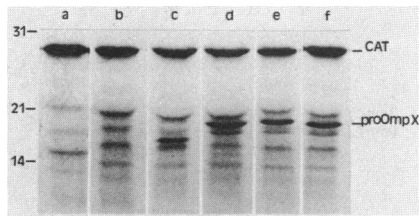


FIG. 3. Autoradiogram of L-[<sup>35</sup>S]methionine-labeled proteins from an in vitro transcription-translation system. Plasmids added to the incubation mixtures: lane a, pJS05; lane b, pJS04:: $\gamma\delta$ -3; lane c, pJS04:: $\gamma\delta$ -5; lane d, pJS04:: $\gamma\delta$ -6; lane e, pJS04:: $\gamma\delta$ -7; lane f, pJS04. Positions of molecular mass standards are indicated at the left (see legend to Fig. 2). Positions of the precursor of OmpX (proOmpX) and chloramphenicolacetyltransferase (CAT) are indicated at the right.

(lane b), whereas plasmid pJS04:: $\gamma\delta$ -5 produced a truncated form of this protein (lane c). pJS04:: $\gamma\delta$ -6 directed the synthesis of an almost full-length protein in vitro (lane d). Remarkably, the transconjugant containing this plasmid was sensitive to beta-lactam antibiotics in vivo, and no OmpX protein band could be detected among the outer membrane proteins of this strain (Fig. 2, lane d). From these data we conclude that insertion -6 mapped at the end of the gene and that the direction of transcription of the gene is as indicated in Fig. 1.

**Nucleotide sequence of the *ompX* gene.** The data obtained from the  $\gamma\delta$  sequence insertion mutations were used to design the sequencing strategy shown in Fig. 1. The *KpnI* restriction site inside the gene and restriction endonuclease fragments from several  $\gamma\delta$  sequence insertion plasmids were used to construct M13 sequencing templates. The nucleotide sequence of the *ompX* gene is shown in Fig. 4. The open reading frame appeared to code for a protein of 172 amino acid residues with a calculated molecular mass of 18.6 kDa. The *ompX* RNA is composed of codons corresponding to abundant tRNAs (13).

Our sequencing strategy, using both  $\gamma\delta$  termini as priming regions, made it possible to establish that the  $\gamma\delta$  sequence of pJS04:: $\gamma\delta$ -6 was inserted about 9 bp before the stop codon. The nucleotide sequence preceding the ATG start codon of the *ompX* gene contains a putative promoter sequence (12) and a ribosome binding site (27) (Fig. 4). The proposed stop codon TAA (nucleotides 517 to 519) is followed by a stable hairpin structure (nucleotides 538 to 567;  $\Delta G_{37}^{\circ} = -14.6$  kcal [ca.  $-61.1$  kJ/mol (9)] ending with a T track. Such a hairpin structure is characteristic of rho-independent terminators of transcription in prokaryotes (25).

Comparison of the *ompX* sequence with those stored in GenBank and the EMBL data library did not reveal homology with other known genes.

**Signal sequence of OmpX.** The derived amino acid sequence of the OmpX protein suggested that the protein is synthesized as a precursor with an N-terminal signal peptide, characteristic of exported proteins (24, 31). It starts with two basic lysine residues (positions -22 and -21), followed by a stretch of hydrophobic amino acid residues. The exact cleavage point was determined by sequencing the N-terminal part of the purified mature protein (underlined in Fig. 4). We conclude that the precursor protein is cleaved between the alanine -1 and alanine +1 residues. The resulting mature OmpX protein consists of 149 amino acid residues with a calculated molecular mass of 16.5 kDa,

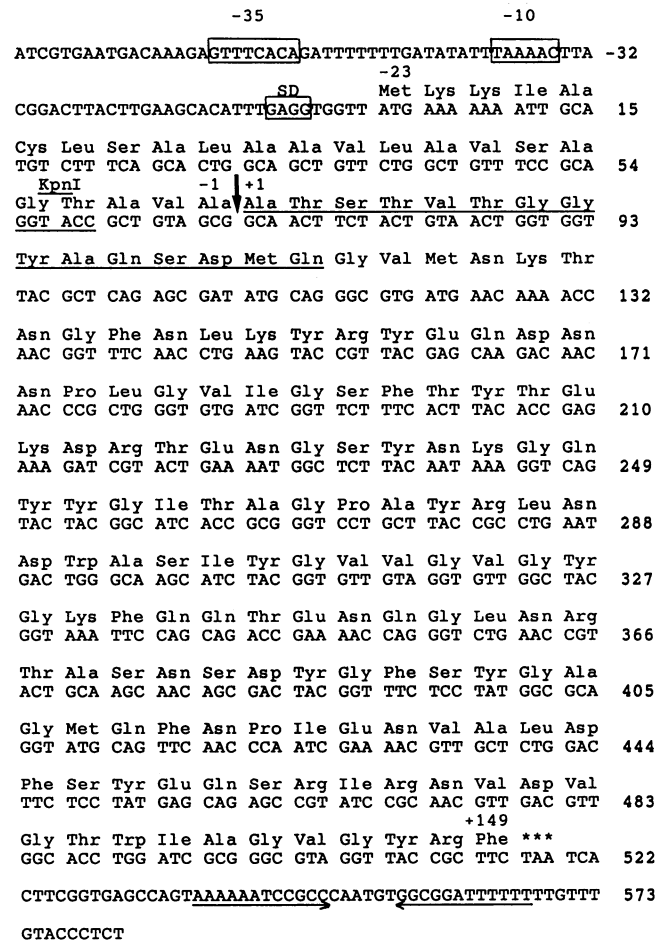


FIG. 4. Nucleotide sequence of *ompX*. Putative -35, -10, and Shine-Dalgarno (SD) sequences are boxed. The transcription terminator is indicated by horizontal arrows. Nucleotide numbering is relative to the ATG start codon. The sequence matching the amino acid sequence analysis of the N-terminal region of mature OmpX is underlined. The cleavage site between the signal peptide and mature OmpX is indicated by the vertical arrow. Numbering of the amino acid residues of the mature OmpX starts with +1, whereas the residues of the signal sequence have negative numbers. The asterisks mark the stop codon.

slightly less than the value estimated from SDS-polyacrylamide gel electrophoresis (18 kDa).

**Physical properties of OmpX.** The OmpX protein contains 16.8% charged amino acid residues, which are scattered along with protein. Hydrophobic regions of the OmpX protein were traced by using a computerized version of the method of Kyte and Doolittle (15). A hydropathy plot of the OmpX protein is shown in Fig. 5. Apart from the signal sequence, no stretches of more than 10 hydrophobic amino acids were found. The derived amino acid sequence and hydropathy plot of the OmpX protein showed some striking features, also occurring in known outer membrane proteins (30).

Characteristic are the amino acid residues of the C-terminal part of the protein, Thr-Trp-Ile-Ala-Gly-Val-Gly-Tyr-Arg-Phe, following the consensus sequence found in many outer membrane proteins of X-Z-X-Z-X-Z-X-Tyr-X-Phe (X is any amino acid residue; Z is a hydrophobic amino acid



protein. In the accompanying paper (29), we present more data on the localization of OmpX and on its role in the loss of the porin proteins.

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#### ADDENDUM IN PROOF

After submission of the manuscript, the sequences of two outer membrane proteins were published: the Ail protein of *Yersinia enterocolitica* (V. L. Miller, J. B. Bliska, and S. Falkow, *J. Bacteriol.* **172**:1062–1069, 1990) and the Lom protein of *Escherichia coli* ( $\lambda$ ) (J. J. Barondess and J. Beckwith, *Nature (London)* **346**:871–874, 1990). Both proteins show striking homologies to OmpX. Variations are found mainly in regions exposed at the cell surface according to the model of Fig. 6. These findings firmly support our model for the folding of OmpX in an outer membrane.

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