Biological Characterization of an Enterobacter cloacae Outer Membrane Protein (OmpX)

JOKE STOORVOGEL, MARIO J. A. W. M. VAN BUSSEL, AND JOS A. M. VAN DE KLUNDERT*

Department of Medical Microbiology, University Hospital, P.O. Box 9600, 2300 RC Leiden, The Netherlands

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We have described a gene coding for an Enterobacter cloacae protein, provisionally called OmpX (J. Stoovogel, M. J. A. W. M. van Bussel, J. Tommassen, and J. A. M. van de Kluinert, J. Bacteriol. 173:156-160, 1991). In the work reported here, OmpX was localized in the cell envelope by means of sucrose gradient fractionation of membrane vesicles. Overproduction of OmpX in Escherichia coli from a multicopy plasmid resulted in a reduction in the amount of OmpF. No accumulation of OmpF, of its uncleaved precursor, or of its degradation products could be detected in various cell fractions by Western immunoblot analysis using monoclonal antibodies produced in response to OmpF. A decrease in the rate of synthesis of ompF mRNA was indicated by a β-galactosidase assay in an ompF-lacZ fusion strain containing the cloned ompX gene and by Northern (RNA) blot analysis. These results indicate that the inhibition is at the level of transcription. Colony hybridization, using an internal ompX fragment as a probe, showed a widespread distribution of the ompX gene among clinical isolates of members of the family Enterobacteriaceae. To study the function of the OmpX protein and its role in the regulation of porin protein synthesis, the ompX gene was deleted from the Enterobacter cloacae chromosome and replaced by the aphA gene. The absence of the ompX gene had no apparent effect on cell growth or on the regulation of the porin proteins.

In a previous report (27), we described the cloning of a chromosomal gene of Enterobacter cloacae that codes for a membrane protein, OmpX. When cloned in a multicopy plasmid, this gene caused a decrease in the level of OmpF and OmpC in Escherichia coli and of a comparable porin-like outer membrane protein in E. cloacae. In the accompanying paper (28), the DNA sequence of the ompX gene is presented. OmpX encodes a precursor of the OmpX protein consisting of 172 amino acid residues and containing an N-terminal signal sequence of 23 amino acid residues. Here we present data on the localization of OmpX in the cell, on the effects of OmpX on the regulation of expression of the ompF gene, and on the occurrence of the ompX gene among members of the family Enterobacteriaceae, including E. coli. Given the knowledge that the effect of overproduction of OmpX in E. coli resembles the effect in E. cloacae and that the regulation of the porin proteins is well described for this species, we used an E. coli model to study the interference of OmpX with the porin proteins. Furthermore, an ompX deletion mutant was constructed to study the functions of OmpX.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used are listed in Table 1. Clinical isolates that were screened for the presence of the ompX gene were obtained from our laboratory stock. They included five Acinetobacter calcoaceticus, one Citrobacter diversus, four Citrobacter freundii, five Enterobacter cloacae, five E. coli, five Hafnia alvei, four Klebsiella pneumoniae, one Klebsiella oxytoca, five Pseudomonas aeruginosa, five Salmonella spp., four Serratia marcescens, one Serratia liquefaciens, one Staphylococcus epidermidis, four Staphylococcus aureus, two Morganella morganii, one Providencia rettgeri, one Proteus mirabilis, and one Proteus vulgaris.

DNA techniques. Plasmid DNA was prepared as described by Birnboim and Doly (2). Restriction endonucleases, HindIII linker (dGAAAGCTT) (Boehringer Mannheim Biochemicals), Klenow enzyme (Bethesda Research Laboratories), and T4 DNA ligase (Promega Biotec) were used as specified by the manufacturers. DNA fragments were excised from 1 to 2% agarose gels in TAE (40 mM Tris-acetate, 2 mM EDTA, pH 8.0) electrophoresis buffer, and the DNA was recovered with Gene Clean (Bio 101, Inc.). Southern transfer was carried out as described by Maniatis et al. (22).

Cell fractionation and preparation of membrane vesicles. Exponentially growing cultures in 500 ml of minimal M9 medium (22) were harvested at an A665 of 1.3 to 1.5 and converted to spheroplasts by treatment with 12.5 μg of lysozyme in 40 ml of 0.25 mM EDTA–250 mM sucrose–100 mM Tris hydrochloride (pH 8.0) at 0°C (31). Spheroplasts were disrupted by sonication as described by De Ley and Witholt (10). Inner membrane vesicles were separated from outer membrane vesicles by equilibrium centrifugation in a discontinuous sucrose gradient (30 to 70%) in a Spinc6 SW65 rotor (115,000 × g; 4°C for 30 h). Fractions were collected and analyzed on 14% polyacrylamide gels containing 0.2% sodium dodecyl sulfate (SDS) as described by Lugtenberg et al. (20). Gels were stained with fast green FCF.

Membrane isolation (small scale). Bacteria were grown in brain heart infusion broth (Oxoid Ltd.) supplemented when required with chloramphenicol (34 μg/ml). Cell fractions were obtained as described by Matsuyama et al. (23). Cells were harvested at an A665 of 1.2 and broken by sonication. After centrifugation at 1,500 × g for 10 min to remove intact cells, the membrane proteins were separated from the cytoplasmic proteins by centrifugation at 160,000 × g for 30 min. The cell envelope pellet was suspended in 2% Triton X-100–10 mM sodium phosphate buffer (pH 7.2) and incubated at 37°C for 15 min. The insoluble fraction, containing most of the outer membrane proteins, was separated from the soluble fraction, containing most of the inner membrane proteins, by centrifugation at 160,000 × g for 30 min.
Western immunoblot analysis. The protein concentration of each sample was estimated by measuring A280 and A260, assuming that 1.46 × A280 – 0.76 × A260 corresponds to the concentration of protein in milligrams per milliliter. Then 15 μg of cytoplasmic proteins, 180 μg of inner membrane proteins, and 30 μg of outer membrane proteins were loaded onto 14% polyacrylamide gels containing 0.2% SDS as described by Lugtenberg et al. (20) except that the gels were supplemented with 4 M urea. After electrophoresis, the proteins were electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Inc.) in transfer buffer (25 mM Tris, 150 mM glycine, 20% methanol, pH 8.3) for 16 h at 4°C and 20 V. Western blots were blocked with saturant (5% fetal calf serum in phosphate-buffered saline [10 mM sodium phosphate, 0.9% NaCl, pH 7.4]) supplemented with 0.05% Tween 20) and treated with saturant containing monoclonal OmpF antibodies raised against heat-inactivated (1 h, 100°C) E. coli cells (a gift of R. Toresmna). Blots were washed with phosphate-buffered saline supplemented with 0.05% Tween 20 and incubated with peroxidase-conjugated rabbit anti-mouse antibodies (Dakopatts, Copenhagen, Denmark) diluted in saturant.

β-Galactosidase assay. Activity of the ompF and ompC promoters was measured by assaying the β-galactosidase activity of ompF-lacZ and ompC-lacZ operon fusion strains as described by Miller (25). After growth in minimal A medium, β-galactosidase activity was determined at 28°C, using o-nitrophenyl-β-D-galactoside (ONPG) as the substrate. At least 12 assays were performed for each strain. The high and low values were discarded, and the averages and standard deviations of the remaining values were expressed in units according to Miller (25).

RNA analysis. Exponentially growing cultures in Luria broth (22) without NaCl were harvested at an A660 of 0.8 to 1.0. Total RNA was isolated by the method of Cohen et al. (9). A 20-μg sample of total RNA was denatured with formaldehyde and formamide and subjected to electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde (22). Transfer of RNA to nylon membranes (Biotrans; ICN Biomedicals, Inc.) and hybridization of Northern (RNA) blots were as described for Southern blots (22). The 1.5-kb PstI fragment from pJP33, which contains the ompF gene, was used as a probe. The fragments were labeled with [α-32P]dCTP (NEN Research Products, Du Pont) by using a random-primed DNA-labeling kit (Promega Biotec).

 Colony blot hybridization. Clinical isolates were grown overnight in brain heart infusion broth (Oxoid Ltd.) by using an automatic multipoint inoculator (MIC 2000; Dynatech Laboratories, Inc.). 1.5 μg of culture was spotted on brain heart infusion agar plates covered with a nitrocellulose membrane (Schleicher & Schuell). Plates were incubated for about 8 h at 37°C. Cell lysis, DNA immobilization, and hybridization were performed as described by Maniatis et al. (22). A 370-bp KpnI-TaqI fragment of pJS04, an internal fragment of the ompX gene, was used as a probe. This fragment was labeled with [α-32P]dCTP (NEN Research Products, Du Pont), using a nick translation DNA-labeling kit (Bethesda Research Laboratories). After hybridization at 65°C for 16 h, filters were washed for 15 min in 2x SSPE (0.36 M NaCl, 20 mM NaH2PO4, 2 mM EDTA, pH 7.4)–0.1% SDS and for 15 min in 1x SSPE–0.1% SDS at 65°C. The filter was exposed to X-ray film (Fuji RX) for autoradiography at −80°C for 16 h.

Construction of an ompX deletion mutant. A deletion mutant of E. cloacae lacking the ompX gene was constructed by the method of Matsuyama and Mizushima (24) (see Results).

**RESULTS**

Localisation of OmpX. In a previous paper (27), we described the separation of inner membrane proteins from outer membrane proteins of both E. cloacae and E. coli, using a small-scale preparation as described by Matsuyama et al. (23). In such a separation (which is based on the different solubilities of these proteins in 2% Triton X-100), part of the total OmpX present was found in the outer membrane fraction. In combination with the physical properties of OmpX, derived from the nucleotide sequence of the ompX gene presented in the accompanying paper (28), this finding strongly suggests that OmpX is an integral outer membrane protein. To verify this hypothesis, membrane vesicles were prepared from E. coli HB101 transformed with pJS04, in which OmpX is overproduced. E. coli HB101 transformed with the vector pACYC184 served as a control. Outer membrane vesicles were separated from those derived from the inner membrane in a sucrose density gradient. The A260 profile (Fig. 1) showed that the proteins were concentrated in two main bands. Analysis of the proteins on SDS-polyacrylamide gels showed that the upper band contained inner membrane proteins, whereas the lower band contained outer membrane proteins (Fig. 2). Although OmpX was found in both bands (Fig. 2A), the bulk of the OmpX was in the lower band, thus proving that OmpX is an outer membrane protein.

Presence of OmpF in transformed cells. In E. coli cells transformed with plasmid pJS04 carrying the ompX gene, the amount of OmpF and OmpC in the outer membrane fraction was shown to be reduced (27). To determine whether the decrease in these porin proteins was due to a block in export of these proteins or to inhibition of transcription or translation, we investigated the effect of overproduction of OmpX.
on the synthesis of the OmpF protein. The presence of OmpF in different cell fractions was studied by Western blot analysis, using monoclonal antibodies produced in response to OmpF. No OmpF was found in the soluble protein (Fig. 3A) or inner membrane (Fig. 3B) fractions. In the outer membrane fraction, the amount of OmpF was decreased in the presence of pJS04 (Fig. 3C, lane 2). These findings suggest that the decrease in the OmpF protein in the outer membrane was due to an inhibition of synthesis rather than to a block in the export process.

**Presence of ompF mRNA in transformed cells.** Since no accumulation of the OmpF protein could be detected, we examined whether the inhibition of expression of OmpF by OmpX was due to inhibition of transcription. The concentration of ompF mRNA was determined by Northern blot analysis, and ompF expression was determined by measuring the β-galactosidase production of an ompF-lacZ gene fusion strain.

The presence of ompF mRNA was determined in *E. coli* HB101 transformed with plasmid pJS04 carrying the ompX gene and in the same strain transformed with plasmid pJS05 (pJS04 from which the ompX gene has been deleted). *ompF* mRNA could be detected in *E. coli* HB101 transformed with

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**FIG. 1.** Optical density at 280 nm (OD_{280}) profile of membrane fractions separated in a discontinuous sucrose gradient. Symbols: △, *E. coli* HB101(pJS04); +, *E. coli* HB101(pACYC184).

**FIG. 2.** SDS-polyacrylamide gel electrophoresis patterns of fractions of the sucrose gradients shown in Fig. 1. (A) *E. coli* HB101(pJS04); (B) *E. coli* HB101(pACYC184). Molecular mass standards are indicated at the left (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).

**FIG. 3.** Western blot analysis of OmpF. Proteins derived from *E. coli* HB101(pACYC184) (lanes 1) and *E. coli* HB101(pJS04) (lanes 2) were run in SDS-polyacrylamide gels, and OmpF was detected as described in Materials and Methods. (A) Soluble proteins; (B) inner membrane fractions; (C) outer membrane fractions. Positions of the molecular mass standards are indicated at the left (see legend to Fig. 2).
Similarly, mRNA and DNA data, with functional control of the expression of OmpX which was indicated in kilobases as the RNA (Fig. 4). Northern blot analysis of total RNA. Samples (20 μg) of total RNA from E. coli HB101(pJS05) (lane 1) and E. coli HB101(pJS04) (lane 2) were electrophoretically separated, and HindIII fragments of phage lambda DNA, treated in the same way as the RNA samples, were used as fragment length standards, indicated in kilobases at the left.

pJS05 but not in E. coli HB101 transformed with pJS04, in which OmpX is overproduced (Fig. 4). These results indicate that the inhibition of expression of ompF by overexpression of the ompX gene occurs at the level of transcription.

To determine the influence of OmpX overproduction on the expression of ompF, an E. coli ompF-lacZ fusion strain, MH513, in which the expression of lacZ is under transcriptional control of the ompF promoter, was transformed with pJS04. Similarly, inhibition of expression of the ompC gene was measured in the E. coli ompC-lacZ fusion strain, MH221. The β-galactosidase activities of the two strains transformed with pJS04 (95 ± 40 and 257 ± 32 U/ml for MH513 and MH221, respectively) were considerably lower than the activities of the strains transformed with vector plasmid pACYC184 (424 ± 167 and 632 ± 171 U/ml). From these data, it can be concluded that overproduction of OmpX results in a drastic decrease in transcription of the ompF gene.

Distribution of the ompX gene among the bacterial flora. To determine whether OmpX is a common outer membrane protein of gram-negative bacteria, we examined the distribution of the ompX gene among several clinical isolates. The DNA of these isolates was hybridized with an internal KpnI-TaqI fragment of the ompX gene in a colony hybridization (Fig. 5). E. coli HB101(pJS04) and E. cloacae 2249-1 and JS101 (an ompX deletion mutant; see below) were used as positive and negative controls, respectively. Hybridization was observed with the DNA of the positive controls (Fig. 5, dots A6 and B6) and with the DNA of strains of closely related species (5): E. cloacae (dots C1 to C5), Citrobacter spp. (dots D1 to D5), Klebsiella spp. (dots F1 to F5), Serratia spp. (dots I1 to I5), and three of five Salmonella spp. (dots H1 to H3). No hybridization was seen with the DNA of E. cloacae JS101 (dot C6), the more distantly related species H. alvei (dots E1 to E5), M. morganii (dots L1 and L4), Proteus spp. (dots L2 and L5), and Providencia rettgeri (dot L3), or the nonrelated species A. calcoaceticus (dots A1 to A5), P. aeruginosa (dots G1 to G5), and Staphylococcus spp. (dots K1 to K5). These data indicate that the ompX gene is rather well conserved in members of the family Enterobacteriaceae.

Construction of an ompX deletion mutant of E. cloacae. To gain more insight into the functions of the OmpX protein and its role in the regulation of porin protein synthesis, we decided to construct an ompX deletion mutant. The strategy chosen was that of Matsuyama and Mizushima (24): constructing a plasmid containing a temperature-sensitive replicon and a chromosomal DNA fragment of E. cloacae, carrying both flanking regions of the ompX gene but not the ompX gene itself. An antibiotic resistance gene was introduced instead of ompX. The procedure is schematically shown in Fig. 6. Plasmid pJS05, containing the flanking regions of the ompX gene, was linearized. The ClaI site of this linear DNA was modified into a HindIII site with a HindIII linker, resulting in plasmid pJS06. The 1.9-kb HindIII fragment of pJS06 was ligated into the HindIII site of pMAN031, yielding pJS07. A 4.2-kb KpnI fragment of pCR1 containing the apha gene (a kanamycin resistance determinant) was inserted in the KpnI site of pJS07, resulting in pJS08.

An ompX deletion mutant of E. cloacae 2249-1 was constructed by replacing the ompX gene with the apha gene of pJS08 via homologous recombination (Fig. 7). E. cloacae 2249-1, the strain from which the ompX gene was isolated, was transformed with pJS08 (Fig. 7a). Since pJS08 includes the temperature-sensitive replicon of pMAN031, transfor-
Transformants grow on brain heart infusion agar plates containing kanamycin (50 μg/ml) at 42°C only if pJS08 has been integrated into the host chromosome (15). Most of the isolated colonies were ampicillin (100 μg/ml) and kanamycin (50 μg/ml) resistant, indicating that pJS08 had been integrated by a single crossover at one of the E. cloacae chromosomal borders of the aphA gene (Fig. 7b). About 0.1% of the colonies were ampicillin sensitive and kanamycin resistant, indicating that a second crossover event had occurred at the other chromosomal border region of pJS08. This event resulted in the loss of the ompX gene and most of pJS08 from the chromosome, leaving the aphA gene behind (Fig. 7c).

The chromosomal DNA of one of the presumed ompX deletion mutants, E. cloacae JS101, was analyzed by Southern hybridization to confirm the deletion of the ompX gene (Fig. 8). When the 0.85-kb KpnI fragment of pJS04 carrying the ompX gene was used as a probe, the chromosomal DNA of E. cloacae 2249-1 hybridized with 0.8-kb KpnI, 3.8-kb EcoRI, and 7.5-kb BamHI fragments. This probe did not hybridize with chromosomal DNA of E. coli. E. cloacae JS101. On the other hand, when the 4.2-kb KpnI fragment of pCR1 carrying the aphA gene was used as a probe, hybridization occurred only with 4.2-kb KpnI, 6.8-kb EcoRI, and 9.0-kb BamHI fragments of the chromosomal DNA of E. cloacae JS101. These results demonstrated that the ompX gene was deleted and replaced by the aphA gene in E. cloacae JS101. It also showed that there is only one copy of the ompX gene in the wild-type E. cloacae chromosome.

**Characteristics of the ompX deletion mutant.** The ompX deletion mutant was analyzed to determine the influence of ompX on cell metabolism and porin expression. The colony morphology of the ompX deletion mutant E. cloacae JS101 is the same as that of the parental strain E. cloacae 2249-1, and the cells also have the same appearance when observed under light microscopy. Growth rate and carbohydrate metabolism of parental and mutant strains, tested with an API 50 CHE strip (API System S.A.), were indistinguishable. Analysis of the outer membrane proteins on polyacrylamide gels showed that production of porin proteins in the ompX deletion mutant was normal and changed in response to variations in osmolarity or temperature in the same way as in the parental strain. Susceptibility to beta-lactam antibiotics was the same as for the wild-type strain (results not shown).

**DISCUSSION**

In this report, we present additional evidence that OmpX is an outer membrane protein. These data are based on an OmpX-overproducing E. coli strain. However, the resemblance of the phenomena observed in E. coli and in E. cloacae made it plausible that our data also hold true for E. cloacae. Previous work has shown that the presence of multiple copies of the ompX gene has a negative effect on the quantity of porin proteins present in the cell (27, 28).

In this study, we demonstrate that this effect is due to diminished transcription of the ompF gene. This inhibition may be due
to either a direct or indirect effect of OmpX on regulation of expression of the ompF gene.

Regulation of expression of the porin proteins has been extensively studied. The total cellular levels of OmpF and OmpC proteins are rather consistent, but the ratio varies according to environmental conditions (21, 30). The expression of these proteins is regulated by the products of the ompB locus, OmpR and EnvZ, in response to the osmolarity of the medium (17, 18). The present model proposes that the positive regulatory protein OmpR (26) binds to sequences approximately 90 bp upstream from the ompF and ompC promoters, activating the transcription of these genes. The inner membrane protein EnvZ (12) is the sensory protein. EnvZ influences the DNA-binding activity of OmpR, probably by phosphorylation, in response to the osmolarity of the medium (17, 19). A direct role for OmpX in this process is not very likely, since in the ompX deletion mutant JS101 the ompF and ompC genes are normally expressed.

A more plausible explanation is that overproduction of OmpX saturates the outer membrane and interferes with the production of outer membrane proteins because of the limited capacity of the outer membrane to accommodate these proteins. The protein concentration per unit surface area is known to remain nearly constant; it is not dependent on the growth rate or the induction of new surface proteins (3, 11). This suggests that the synthesis of these proteins must be strictly regulated. The phenomenon of a decrease in outer membrane proteins caused by high-level expression of one of these proteins has been observed by several investigators. Click et al. (7) have shown that overproduction of the OmpC protein results in the inhibition of synthesis of the outer membrane proteins OmpA and LamB. The inhibition of these proteins is at the level of translation (7, 8). Synthesis of the Lc protein encoded by lambdoid phages also results in a drastic reduction in the amounts of OmpF and OmpC (14), which is proportional to the increase in Lc. This down-regulation is due to a posttranscriptional effect (16). Induction of lamB also leads to a reduction in levels of OmpF and OmpC (11); at least part of the reduction is due to reduced transcription, as monitored by ompC-lacZ operon fusion experiments. Our data show clearly that inhibition of the synthesis of OmpF protein due to overproduction of OmpX is at the level of transcription. Our results cannot be explained by the hypothesis that overproduction of exported proteins is prevented by coupling posttranscriptional gene regulation to export capacity (3, 7). Boyd and Holland (3) have proposed that hypothetical membrane sites specifically involved in translation and translocation of outer membrane proteins limit the overall rate of synthesis of these proteins or that the growth rate of the peptidoglycan layer determines the assembly of outer membrane proteins. Click et al. (7) have proposed that the production of exported proteins is regulated by sensing the occupancy of some limiting component in the export machinery and using this information to regulate translation of these proteins. The exact role of OmpX in regulation of expression of the porin genes, as well as its functions, remains to be elucidated. The ompX deletion mutant of E. cloacae did not differ from the parental strain in appearance, metabolism, or viability. These data show that the presence of OmpX is not essential for cell growth under the conditions tested. However, the colony hybridization analysis clearly shows that the ompX gene is well conserved among related members of the family Enterobacteriaceae and thus may play an important role. Our present research is aimed at reconciling these seemingly conflicting results.

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