An Early Response to Environmental Stress Involves Regulation of OmpX and OmpF, Two Enterobacterial Outer Membrane Pore-Forming Proteins

Myrielle Dupont,† Chloë E. James,† Jacqueline Chevalier, and Jean-Marie Pagès*
UMR-MD1, IFR48, Faculté de Médecine et de Pharmacie, Université de la Méditerranée, Marseille, France

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Bacterial adaptation to external stresses and toxic compounds is a key step in the emergence of multidrug-resistant strains that are a serious threat to human health. Although some of the proteins and regulators involved in antibiotic resistance mechanisms have been described, no information is available to date concerning the early bacterial response to external stresses. Here we report that the expression of ompX, encoding an outer membrane protein, is increased during early exposure to drugs or environmental stresses. At the same time, the level of ompF porin expression is noticeably affected. Because of the role of these proteins in membrane permeability, these data suggest that OmpF and OmpX are involved in the control of the penetration of antibiotics such as β-lactams and fluoroquinolones through the enterobacterial outer membrane. Consequently, the early control of ompX and ompF induced by external stresses may represent a preliminary response to antibiotics, thus triggering the initial bacterial line of defense against antibiotic resistance.

The emergence of bacterial resistance to several structurally unrelated drugs has substantially impaired the efficacy of antibiotic therapy. In Enterobacteriaceae, resistance to β-lactam antibiotics is frequently associated with the production of hydrolytic enzymes and the alteration of membrane permeability, resulting in reduced intracellular drug accumulation (23, 25, 27). Among emerging resistant bacteria, Enterobacter aerogenes is now one of the most frequently described gram-negative pathogens involved in nosocomial respiratory tract infections in France, Belgium, Spain, and the United States (5, 6, 8, 15, 27). OmpX is a small outer membrane protein (OMP; 18 kDa) described in various enterobacterial species, including Escherichia coli, E. aerogenes, E. cloacae, etc., forming an eight-stranded antiparallel β-barrel (1, 20, 34). Overexpression of OmpX has been associated with a decrease in the expression of Omp36, a major porin in E. aerogenes cells, and a decreased susceptibility to β-lactams (9). This observation was reported in clinical isolates of E. aerogenes exhibiting a multidrug resistance (MDR) phenotype (10, 36). Recent studies indicated that OmpX expression is regulated by some environmental factors (9, 28, 32); however, its role and its significance in outer membrane permeability are unknown.

Among the global regulators of OMP expression, H-NS (histone-like structuring nucleoid protein), initially described as a transcription factor, plays a role in the structure and functioning of chromosomal DNA. H-NS controls ca. 5% of E. coli genes, most of which are involved in bacterial virulence or in bacterial adaptability to stressful environmental conditions (2, 4, 12). This protein regulates the expression of porins and several efflux pumps in E. coli and E. aerogenes (12, 19, 24), and it is possible that this regulator also controls expression of OmpX.

With the increasing number of drug resistant isolates of Enterobacteriaceae, it is important to decipher the regulation mechanisms involved in the control of omps including porins and OmpX, in response to various environmental stresses, and to understand how this might contribute to the development of a drug-resistant phenotype.

We report here the use of ompX-lacZ and ompF-lacZ reporter fusions to investigate their possible regulation in response to selected compounds and conditions. We report that, after exposure to many external stresses (salicylate, quinolones, novobiocin, high ionic strength, and dipiridyl) ompX expression was noticeably increased within minutes, and similar conditions lead to decreased porin synthesis. Further analysis examined the effect of such conditions on both transcriptional and translational levels of ompF expression.

By comparing the expression levels of the two omp genes in response to environmental stresses in different genetic backgrounds (marA and ompX mutations), we determined that OmpX and OmpF are able to respond to external stresses via different regulation cascades, and we hypothesize that OmpX is involved in a complex regulatory network to modulate outer membrane permeability and adaptability.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used are listed in Table 1. Bacteria were grown routinely in Luria-Bertani (LB) broth supplemented with kanamycin (50 μg ml⁻¹), chloramphenicol (30 μg ml⁻¹), and streptomycin (50 μg ml⁻¹) when required. Various compounds, including antibiotics (nimipenic, cephaline, cefazidime, nalidixic acid, norfloroxacin, ciprofloxacin, novobiocin, tetraxyline, and clavulanic acid), biocides and disinfectants (trichlosan, pararquat, and phenoxynethanol), and chemicals (deoxyccholate, salicylate, 2'-2' dipiridy, ethanol, and phenethyl alcohol [PEA]), were used at subinhibitory concentrations (determined by performing standard MIC assays). Sodium chloride (300 mM NaCl) was used to induce osmotic shock.

* Corresponding author. Mailing address: UMR-MD1, Faculté de Médecine, 27 Blvd. Jean Moulin, 13385 Marseille Cedex 05, France. Phone: 33(0)4 91 32 45 87, Fax: 33(0)4 91 32 46 06. E-mail: Jean-Marie.Pages@medecine.univ-mrs.fr.
† M.D. and C.E.J. contributed equally to this study.
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TABLE 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<tr>
<td>Strains</td>
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<td>pcJafl2         with the ompF-lacZ translation fusion</td>
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a. Te', tetracycline resistance; Amp', ampicillin resistance; Sm', streptomycin resistance; Km', kanamycin resistance. WT, wild type.

Plasmid construction. In order to explore the regulation of ompX and ompF, a number of reporter gene constructs were made in which the lacZ gene was placed under the direct control of the promoter of each gene. The promoter region of ompX was amplified from pMD05 (9) harboring the complete ompX gene from E. aerogenes, using the primer pair X7 (5'-AATGGATCCTTAGGA-3') and 16S (16S1 [5'-GTGTTGCCGAAATGTTGTG-3'] and X8 [5'-TAAAGGTATCCACCACTCCAATGTG-3'], both containing the restriction site for BamHI. The 220-bp PCR fragment obtained was cloned into the cloning vector pDrive (pMD07). After BamHI digestion of pMD07, the 220-bp fragment was cloned into the pFus2K vector to obtain a transcriptional fusion of the ompX promoter region to the promoter-less lacZ gene, generating pMD08. The construction was confirmed by sequencing.

Two different ompX-lacZ fusion plasmids were constructed by amplifying ompF promoter regions from chromosomal extracts of E. coli strain MC1061. A transcriptional fusion was constructed by using primer pair F1 (5'-GTGTTMGCGGATCCATGTTTGATGCTGCTGCAAG-3') and F3 (5'-GTGTTMGCGGATCCATGTTTGATGCTGCTGCAAG-3') to amplify the region between nucleotides (nt) 685 to -11, where '1' is the transcriptional start site. The translational fusion required the use of an alternative 3' primer F2 (5'-ACCTCTCTGCCATGCTGCAAGATTATTGCTGCTGCAAG-3') and 16S (16S1 [5'-GTGTTGCCGAAATGTTGTG-3'] and X8 [5'-TAAAGGTATCCACCACTCCAATGTG-3'], both containing the restriction site for BamHI. The 220-bp PCR fragment obtained was cloned into the cloning vector pDrive (pMD07). After BamHI digestion of pMD07, the 220-bp fragment was cloned into the pFus2K vector to obtain a transcriptional fusion of the ompX promoter region to the promoter-less lacZ gene, generating pMD08. The construction was confirmed by sequencing.

RESULTS

Prevalence of the modification of OmpX levels in MDR clinical isolates. Recent studies have shown that a number of E. aerogenes clinical isolates exhibit increased levels of OmpX expression (10, 36). It is interesting to evaluate the level of OmpX expression in MDR clinical isolates with a lack of porins. Immunodetection experiments probed total cell extracts of 47 randomly chosen clinical isolates collected over a 2-year period for relative levels of porin and OmpX expression. We observed that 21 strains exhibited altered porin profiles (noticeable decrease or absence of porin content). Among these porin-altered isolates, 15 strains presented an increase of OmpX expression compared to the nonmodified porin strains for which no modification of OmpX content was observed (data not shown). These results suggest that, in 71% of resistant clinical strains, the decrease in porin expression is associated with an increase in OmpX expression.

The expression of ompX is mediated by the presence of antibiotics and stress compounds in the environment. Modulation of envelope permeability plays a role in the development of resistance to antimicrobial agents by gram-negative bacteria. Since a decrease in porin expression is associated with an increased level of OmpX, it is hypothesized that this small pore-forming OMP may be involved in the control of the outer membrane permeability and consequently in the MDRO phenotype. We used an ompX-lacZ fusion construct to investigate the effect of subinhibitory concentrations of various chemical stresses on the modulation of ompX expression using β-galac-
tosidase assays. The effect of antibiotic stress was investigated with a wide range of compounds, including imipenem, cefepime, ceftazidime, nalidixic acid, norfloxacin, ciprofloxacin, novobiocin, tetracycline, and clavulanic acid. Also under investigation were the effects of various biocides and disinfectants (trichlosan and phenoxethanol) and chemicals, including deoxycholate, salicylate (activator of the \textit{mar} operon), paraquat (inducer of the \textit{sotRS} regulon), 2′-2′ dipiridyl (reported as a \textit{Rob} activator), ethanol, and PEA and temperatures (32 and 42°C compared to 37°C) (28, 29, 30).

Salicylate, dipiridyl, and novobiocin were found to be the most potent chemical inducers of \textit{ompX} upregulation, causing a two- to threefold increase within 60 min. Norfloxacin, nalidixic acid, paraquat, ethanol, and PEA also noticeably increased the β-galactosidase activity compared to the control (Fig. 1). Similar results were obtained when the assays were performed in \textit{E. coli} JM109 and ATCC 15038 \textit{E. aerogenes} reference strains (data not shown).

High osmolarity and temperature are well-known inducers of porin regulation. Since OmpX is also located in the outer membrane and associated with altered porin profiles, it was hypothesized that its expression would be affected by varying these conditions of the growth media. Figures 1 and 3 show that increased osmotic strength and temperature greatly increased \textit{ompX} expression at the transcriptional level. Osmolarity and salicylate play a major role in the control of OMP expression, and we searched for an additive action or synergy of these two factors. We observed that the activity was only

![Graph showing the activation of the \textit{ompX-lacZ} fusion in response to several compounds and external factors in \textit{E. coli} \textit{DompX}](http://aac.asm.org/)
1.4-fold higher in conditions of high osmotic strength supplemented with salicylate compared to the effect of salicylate or osmotic stress alone, suggesting that the two stress conditions at least partially induced a common regulation step (Fig. 1).

Previously, microarray studies have shown that *ompX* transcription was under the control of MarA in *E. coli* (3). MarA controls the expression of various genes involved in multiple antibiotic resistance (3, 7, 11). In the present study the reporter plasmid encoding the *ompX-lacZ* fusion was introduced into the isogenic *E. coli* mutant CH164 and its parent AG100, and the activity measured clearly showed that MarA positively controls *ompX* expression (Fig. 2). Salicylate failed to significantly upregulate the expression of *ompX* beyond levels observed under control conditions in strain CH164 (Fig. 2).

β-Galactosidase assays showed that a number of other stress conditions induced the upregulation of *ompX*. The absence of a functional mar operon blocked the ability of salicylate to upregulate *ompX* expression. However, Fig. 2 shows that norfloxacin and osmotic shock induced a noticeable increase (1.5- to 2-fold) in *ompX* expression beyond the level of the control in the marA-null mutant strain CH164. This indicates that an alternative pathway of *ompX* upregulation may exist that acts independently of MarA. This phenomenon was also observed in response to treatment with novobiocin and dipyridyl. These data indicated that salicylate (a well-known inducer of the mar regulon) had a positive effect on *ompX* expression, which was tightly MarA dependent, whereas the response to other compounds, such as norfloxacin, may involve two independent regulatory mechanisms, only one of which is dependent on marA.

RT-PCR experiments were performed to determine the point of control (transcription and/or translation) of the *ompX* regulation pathways triggered by various stresses. Total RNA was extracted from bacterial cultures that had been treated with subinhibitory concentrations of salicylate and novobiocin. Serial dilutions were carried out, and RT-PCR was performed with specific primers to amplify *ompX* cDNA. 16S rRNA was used as an internal control, since expression levels of this gene were not affected by the different conditions used (Fig. 3).

![Fig. 2](http://aac.asm.org/)

**FIG. 2.** Effect of several compounds and the mar background on the *ompX-lacZ* fusion in *E. coli* after 60 min of incubation. Values are means from five independent determinations, and standard deviation are represented.

![Fig. 3](http://aac.asm.org/)

**FIG. 3.** Semiquantitative analysis of *ompX* expression using RT-PCR. (A and B) RT-PCR of 16S rRNA (A) or *ompX* (B) on total RNA extracted from ATCC strain 15038 with no treatment (lane 1), novobiocin added (lane 2); osmotic shock (in the presence of NaCl) (lane 3), or salicylate added (lane 4). The concentrations of total RNA were evaluated as described in Materials and Methods. Serial dilutions of total RNA template were performed; lanes 1a and 1b show cDNA products amplified from dilutions 1/10^4 and 1/10^5, respectively. Only the relevant parts of the gel are shown.
When bacterial cells were grown in the absence of toxic compounds, ompX mRNA transcript levels were low, since they could only be detected in the least-diluted samples (1/10^4) (Fig. 3). In contrast, expression was detected in extracts from cultures treated with salicylate and novobiocin that were diluted 1/10^5 (Fig. 3). These results show that both salicylate and novobiocin induced the upregulation of ompX at the level of transcription.

Furthermore, it has been shown previously that overexpression of the E. coli regulator baeR, a two-component regulator system, induced a novobiocin resistance phenotype by upregulating the RND type drug exporter MdtABC, which required TolC (22). A recent study of a ceftriaxone-resistant S. enterica isolate pointed out that the inactivation of baeR gene abolished the expression of an OmpX homologue and restored ceftriaxone susceptibility (13). Moreover, baeR is involved in a novobiocin resistance phenotype via the induction of drug transporter expression and also via the reduction of membrane permeability associated with a decrease in porin production due to ompX expression.

OmpX is under the control of the global regulatory gene hns. To investigate the role of H-NS in OmpX expression, an E. coli hns mutant (PS2652) and its parental strain (PS2209) were transformed with pMD08, encoding the ompX-lacZ fusion, and β-galactosidase assays were performed. The hns mutant strain exhibited a twofold increase in β-galactosidase activity compared to the parental strain (Fig. 4A).

H-NS controls the expression of OmpF and OmpC synthesis in response to osmotic stress in E. coli (12, 35). In addition, a number of E. coli strains were transformed with a high-copy-number plasmid into which the ompX gene had been cloned (pMD05) to mimic the overproduction of OmpX by external factors. Strains with or without constitutive OmpX overproduction were analyzed by Western blotting for modified levels of porin expression. Figure 4B clearly indicates an association between overproduction of OmpX and a drastic downregulation of OmpC and OmpF synthesis. The hns-null strain (PS2652) exhibited a complete switch in the balance of OmpF and OmpC production compared to the parental strain PS2209. When both strains were transformed with pMD05, the
overall level of porin expression was decreased. These data suggest that OmpX may play a major role in the downregulation of porins in response to environmental stresses that induce its overproduction. This could constitute an essential early step in the reduction of porin-dependent permeability of the outer membrane, leading to a decreased uptake of compounds using the porin-dependent pathway.

The expression of OmpF is modulated by the presence of antibiotics and other environmental stress conditions. In order to test the hypothesis that OmpX plays a direct role in the regulation of membrane permeability in response to various environmental stresses, regulation of the porin OmpF was investigated. Two genetic probes were constructed: a transcriptional ompF:lacZ gene fusion and a translational ompF-lacZ protein fusion. Similar constructs have been used previously to study ompF expression levels in response to unbalanced membrane phospholipid compositions (14). β-Galactosidase assays were performed to observe changes in ompF expression in response to salicylate, dipiridyl, and novobiocin, which were all shown to be potent inducers of OmpX upregulation (the present study). The response to norfloxacin and cefepime, as representatives of different antibiotic classes, was also observed.

The involvement of both marA and ompX in regulation of ompF was investigated by performing such β-galactosidase assays in ompX- and marA-null backgrounds. It is important to note that porin expression is controlled by a complex network of regulatory mechanisms that are particularly active during the stationary phase of growth. The ompF response to various environmental stresses was assayed during the exponential phase of cell growth to decrease the involvement of additional factors, such as the alternative sigma factor σE synthesized in the late exponential or stationary phase of cell culture that causes a rapid downregulation of various OMPs (26).

**ompF regulation at the level of transcription.** After treatment of bacterial cultures with salicylate for 60 to 120 min, a clear decrease in ompF transcription was detected (Table 2). In addition, this effect was observed to be independent of OmpX since a similar decrease in ompF transcription was observed in the ompX-null mutants. In contrast, this effect was partially dependent of MarA. Dipiridyl had no significant effect on ompF transcription regardless of the strain background (wild type, marA mutant, or ompX mutant). Novobiocin induced a noticeable decrease in ompF transcription, and this effect was partially dependent on the MarA control, but OmpX was not involved in the regulation mechanism since a decrease in ompF expression of up to 36% was observed in response to novobiocin in the ompX-null mutant. Norfloxacin caused a downregulation of ompF expression. This effect was not dependent on OmpX, but MarA seemed to be involved in this downregulation. No significant effect on ompF transcription was detected after treatment with cefepime during the same period of incubation.

**ompF regulation at the level of translation.** Salicylate treatment induced a major decrease in ompF translation in both parent and ompX-null mutant strains (Table 3). However, this effect was not observed in the marA-null strain (CH164) compared to the parent (AG100). This indicates that salicylate induces an ompF regulatory pathway that is strongly associated with the mar regulon at the level of translation. In contrast, no
involvement of OmpX was observed (Table 3). Dipyridyl induced a decrease in \textit{ompF} translation, with a strong effect in AG100 strain, and this effect was eliminated in a \textit{marA}-null background. Novobiocin treatment was observed to induce a strong decrease in \textit{ompF} translation that was quite similar to that induced by salicylate. This effect of novobiocin was partially preserved in the \textit{marA}-null background. This suggests that an alternative \textit{mar}-independent pathway, involved in the downregulation of \textit{ompF}, may be recruited by novobiocin. However, there is no evidence to indicate that \textit{ompX} plays a role during this regulation pathway. Norfloxacin induced a decrease in \textit{ompF} translation. Again, this reduction was not linked to any OmpX regulatory effect. Cefepime did not induce any decrease in \textit{ompF} translation regardless of the genetic background.

**DISCUSSION**

The early response of bacterial pathogens to antibiotic therapy is a key step to consider in the treatment of infectious diseases. We describe here, for the first time, the early regulation of two OMPs which form channels, OmpX and OmpF, by environmental stresses, including antibiotic molecules. The effects of subinhibitory concentrations of antibiotics on the regulation of bacterial membrane permeability were investigated by analyzing the expression of OmpX and the effect on porin transcription and translation in various genetic backgrounds. Interestingly, the effect on OmpX expression takes place during the first 60 to 90 min after the addition of chemicals to the medium, suggesting the efficiency of the cascade and the rapid impact on the OMP equilibrium. The same timing was observed for OmpF. The present study addresses the early regulation (up to 120 min) during the exponential phase of cell growth in order to rule out additional effects induced by stationary-phase regulators previously mentioned (26).

This is the first evidence of such a rapid response to antibiotic stress by regulation of \textit{omp} expression and, in turn membrane permeability. Only preliminary microarray results have previously been reported that \textit{ompX} transcription is activated by salicylate and MarA (1.5- to 1.8-fold) and by paraquat and SoxS (1.2- to 1.7-fold) (3). In addition, other studies mention the role of \textit{F}-controlled small RNAs, RybB and MicA, which act during the envelope stress response (26, 37). Recently, a posttranslational hypothesis, involving the role of Deg protease, has been proposed to explain the strong decay of porin content in a resistant selected strain that conjointly overproduced efflux pumps and OmpX (38).

In our study, toxic compounds were reproducibly shown to upregulate OmpX expression, and the central role of MarA in the activation induced by salicylate is clearly demonstrated. Interestingly, our results indicate that, for specific cases of external stimuli or stresses, a different mechanism of activation may exist via a MarA-independent pathway. Exposure to norfloxacin, novobiocin, and dipyridyl induces strong upregulation of OmpX in a \textit{marA}-null strain; similar results were observed with osmotic shock. These results provide evidence that these compounds play a key role in the early bacterial antibiotic response by altering the level of an outer membrane component without the involvement of the Mar regulon.
In the case of porin regulation, MarA plays a central role during brief exposure to salicylate, dipryridyl, and norfloxacin and a partial role in response to novobiocin treatment. In addition, during the exposure time, cefepime has no significant effect. Several reports have previously indicated the selection of resistant strains devoid of porins after clinical antibiotic therapy or during in vitro selection (25). However, these strains are collected from long-term exposure (several days for clinical isolates) and may result from complex and multistep cascades of regulation. This is the first report analyzing the rapid effect of subinhibitory concentrations of toxic compounds and antibiotics on the balance of membrane permeability in marA- and ompX-null backgrounds. Taking into account recent results addressing the question of OMP balance in a resistant strain selected after long-term drug exposure (38) or in strains undergoing envelope stress response in the stationary phase (26) via small RNAs that control OMP expression (37), it is interesting that at least two key regulatory levels are evidenced—transcriptional and translational control—and their respective involvement depends on the chemical used and on the presence or absence of marA regulator.

Bacteria have developed various signaling systems for eliciting selective responses to their environmental stresses. These adaptive responses are usually mediated by two-component regulatory systems involving a histidine kinase sensor located in the inner membrane and a cognate response regulator in the cytoplasm or small RNAs controlling the posttranscriptional expressions of various OMPs (33, 37). Similar systems control the expression of genes for nutrient acquisition, virulence, antibacterial resistance, and numerous other pathways in diverse bacteria (31).

However, despite extensive investigations, at this time the dialogue between bacterial pathogens and antibiotics remains unclear, especially with respect to the role played by membrane proteins in the signaling process. Our data identified OmpX and OmpF as first targets in the early stages of response to toxic conditions. Membrane permeability is a bacterial function associated with the regulation of chromosomal multiple antibiotic resistance inEscherichia coli (32). The H-NS protein is involved in the biogenesis of flagella inEscherichia coli (33). The H-NS protein is involved in the biogenesis of flagella inEscherichia coli (34). The H-NS protein is involved in the biogenesis of flagella inEscherichia coli (35). The H-NS protein is involved in the biogenesis of flagella inEscherichia coli (36).

**REFERENCES**