Pseudomonas aeruginosa Outer Membrane Protein OprH: Expression from the Cloned Gene and Function in EDTA and Gentamicin Resistance

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Overexpression of major outer membrane protein OprH of Pseudomonas aeruginosa as a result of mutation (in strain H181) or adaptation to low Mg2+ concentrations (in parent strain H103) is accompanied by increased resistance to polymyxin B, gentamicin, and EDTA. A 2.8-kb EcoRI fragment containing the oprH gene was subcloned into several different expression plasmids in Escherichia coli. These experiments showed that significant levels of OprH could be produced from a promoter on the EcoRI fragment; that the cloned oprH gene was not regulated by Mg2+ deficiency; that there were no differences in the expression of OprH in any construction, regardless of whether the gene from strain H103 or its OprH-overexpressing, polymyxin B-resistant derivative, strain H181, was used; and that overexpression of OprH in E. coli to the level observed in P. aeruginosa H181 did not result in a resistance phenotype. These results favored the conclusion that the mutation in strain H181 was a regulatory rather than a promoter mutation. The oprH gene was cloned behind the benzoate-inducible pEX promoter in plasmid pGB25 and transferred to P. aeruginosa H103. Overexpression of OprH from the cloned gene in H103/pGB25 resulted in EDTA resistance but not polymyxin B resistance. This result suggested that another factor, possibly lipopolysaccharide, was affected by the mutation in strain H181. Consistent with this suggestion was the demonstration that mutants of strain H181 with alterations in lipopolysaccharide had reverted to wild-type polymyxin B susceptibility but had unaltered gentamicin and EDTA resistance. These data were consistent with the hypothesis that OprH replaces outer membrane-stabilizing divalent cations.

The outer membrane of gram-negative bacteria constitutes a semipermeable barrier which restricts the uptake of antibiotic molecules to specific uptake pathways and thus influences intrinsic resistance to antibiotics. One such pathway is the self-promoted uptake pathway (6, 8), which affects the action on cells of polycations, such as polymyxins and aminoglycosides, and chelators, such as EDTA. This pathway involves sites at the outer membrane surface, at which divalent cations cross-bridge adjacent lipopolysaccharide (LPS) molecules (15, 21). Thus, the above-mentioned cationic molecules competitively displace these divalent cations to increase the uptake of (i.e., increase the permeability of outer membranes to) various probe molecules (8, 19) and, by inference, to self-promote their own uptake. Conversely, EDTA removes these divalent cations by chelation and consequently increases the permeability of outer membranes. Outer membrane mutants of Pseudomonas aeruginosa with apparent alterations in this pathway are resistant to suppersusceptible to polycations and/or EDTA (17, 22), indicating that the self-promoted uptake pathway is relevant to cell killing.

One example involves chemically induced mutants of P. aeruginosa that are cross-resistant to EDTA, polymyxin B, and aminoglycosides (17). Such mutants were found to overproduce constitutively an outer membrane protein, H1 (= OprH), by up to 24-fold. Wild-type cells grown in media deficient in certain divalent cations (Mg2+, Ca2+, Mn2+, Sr2+) had similar resistance properties and were induced for OprH expression (17, 19). Cells with mutational overproduction of OprH displayed altered kinetics of streptomyacin uptake (8) and had reduced Mg2+ levels in their envelopes (17). There were no changes, however, in susceptibility to other antibiotics, such as beta-lactams and tetracyclines (17), or in outer membrane permeability to the beta-lactam nitrocefin (18). It was concluded that OprH probably blocks a common uptake pathway that is essential to the bacteriocidal action of polycationic antibiotics and EDTA. Since these compounds are known to disrupt LPS-divalent cation interactions and increase the permeability of the outer membrane (see above), OprH was hypothesized to inhibit self-promoted uptake by replacing divalent cations at negatively charged sites on LPS. The protein, being stably anchored in the membrane, would not be displaced by the permeabilizers and thus could prevent membrane disruption and consequent uptake of the disrupting polycation. In agreement with this idea, purified OprH was shown to be strongly LPS associated, whereas the sequence of the cloned gene predicted a basic protein (putative pI = 8.6) which might interact with anionic LPS (1a).

These conclusions were disputed by Gilleland and Conrad (5), who reported other changes in outer membrane proteins and lipids in the above-mentioned mutants or adapted strains and demonstrated decreased levels of OprH in these strains grown in subinhibitory concentrations of polymyxin B. They proposed that the mechanism of resistance in these strains was similar to that found in P. aeruginosa strains that had been made adaptively resistant by growth in polymyxin B, involving changes in phospholipid content caused by reduced divalent cation concentrations in the cell envelope (5).
However, Moore et al. (16) drew a distinction between mutational alterations in the initial susceptibility of a strain to polymyxin B (mimicked by growth in divalent cation-deficient medium) and adaptive alterations occurring in cultures grown in the presence of the drug. This detailed study failed to demonstrate changes in these strains, other than the overproduction of OprH, that could account for the resistance phenotype. Moreover, Nicas and Hancock (19) showed that OprH levels corresponded well with initial susceptibility to polymyxin B and EDTA when cells were shifted from low to high concentrations of Mg$^{2+}$.

Recently, Said et al. (23) failed to find a relationship between susceptibility to polymyxin B and EDTA and levels of OprH in 12 clinical isolates of *P. aeruginosa*. These data confirm the idea that factors other than OprH are involved in susceptibility or resistance to those agents and underscore the need for study of the effects of OprH overproduction independent of those of other variables. For example, it might be predicted that alterations in LPS would influence self-promoted uptake. Indeed, in the family *Enterobacteriaceae*, polymyxin B-resistant mutants with alterations in LPS have been isolated (20, 24), whereas both gentamicin-supersusceptible and -resistant mutants with alterations in LPS have been demonstrated in *P. aeruginosa* (2, 22) and *E. coli* (5a).

In this study, we used the cloned oprH gene in conjunction with expression vectors to overproduce OprH to investigate whether it has a direct role in polymyxin B, aminoglycoside, and EDTA resistance and whether the original OprH-overproducing mutants were regulatory mutants.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The *E. coli* strain used was DH5αF' endA1 recA1 hsdR17 (rK- mK-) supE44 thi-1 gyrA relA1 ΔlacZΔM15 (lac-proAB-u169)/ F'φ80lacZ-ΔM15. *P. aeruginosa* PAO strains were the wild-type prototroph H103 and its polymyxin-resistant derivative, strain H181 (17). LPS-altered variants of strain H181, H222 and H223, were selected as resistant to LPS-specific (17) phages A7 and A16, respectively. They were confirmed as O-antigenic (B-band) LPS altered by typing with a variety of phages with different outer membrane receptors. The vector plasmids used were the high-copy-number vectors pUC18 (25) and pT7Z18 and pT7Z18U (US Biochemical Corp., Cleveland, Ohio), the related low-copy-number shuttle vectors pRK404 and pRK767 (3), and the shuttle expression vector pNM185 (14). All strains were kept as stocks at −70°C in 8% dimethyl sulfoxide. For short-term maintenance, *P. aeruginosa* H103, H222, and H223 were maintained on 1% proteose peptone no. 2 (PP2)-2% Bacto-Agar (Difco Laboratories, Detroit, Mich.), and H181 was maintained on PP2 agar with 8 μg of polymyxin B sulfate per ml. *E. coli* DH5αF' was maintained on LB agar (13). All antibiotic susceptibility tests were performed after growth of strains in M9 (13) or BM2 (17) minimal medium with glucose as a carbon source, and OprH expression studies were done with these minimal media as well as with BM2-succinate minimal medium (17). Plasmids were maintained in bacteria with the following antibiotic concentrations added to growth media: for *E. coli*, 25 μg (in LB medium) or 15 μg (in M9-glucose medium) of ampicillin sodium per ml, 12.5 μg of tetracycline hydrochloride per ml, 25 μg of kanamycin sulfate per ml, and 25 μg of streptomycin sulfate per ml; for *P. aeruginosa*, 150 μg of tetracycline hydrochloride per ml and 300 μg (in LB medium with a low salt concentration, i.e., 1 mg of NaCl per ml) or 500 μg (in BM2-succinate or M9-glucose medium) of kanamycin sulfate per ml. The expression of streptomycin resistance or inserted genes in pNM185 was coinduced by the addition of sodium benzoate to 5 mM (for *E. coli*) or 2 mM (for *P. aeruginosa*). Antibiotics were obtained from Sigma Chemical Co., St. Louis, Mo.

**DNA techniques.** All DNA techniques were carried out as described by Maniatis et al. (13). Transfer of plasmids from *E. coli* to *P. aeruginosa* was done by triparental mating with plasmid pRK2013 as a helper plasmid (4). Successful transfer was monitored by antibiotic resistance and with quick-lysis plasmid preparations (9).

**Gel electrophoretic analyses.** Whole-cell preparations and cellular subfractions were isolated as described previously (17) with overnight cultures of *E. coli* and midlogarithmic-phase cultures (A_{600} = 0.4 to 0.6) of *P. aeruginosa* (since OprH is overproduced in *P. aeruginosa* when cells reach the stationary phase in most media [17]). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Hancock and Carey (7) with the inclusion of 70 mM NaCl in the running gel to permit the separation of OprH (protein H1) and OprL (protein H2). Quantitation of OprH relative to OprL was performed by densitometry analyses of Coomassie blue-stained gels of identically loaded whole-cell or outer membrane preparations (17). Western immunoblotting with specific anti-OprH serum was performed as described previously (1a).

**Antibiotic susceptibility tests.** MIC determinations were performed by the agar dilution procedure as previously described (17). Killing assays were performed as described by Nicas and Hancock (17) for polymyxin B and EDTA, whereas for gentamicin killing a modification was used in which 0.5% (wt/vol) glucose was added to the 30 mM sodium phosphate (pH 7.0) buffer. In each case, survival was determined at a single time point, 5 min after antibiotic addition. Killing assays were performed on midlogarithmic-phase cultures.

**RESULTS**

Expression of the cloned oprH gene in *E. coli*. Previous results localized the wild-type strain H103 oprH gene to a stretch of 603 nucleotides within an internal 0.8-kb *Smal-KpnI* fragment from a larger 2.8-kb *EcoRI* restriction fragment (13). In this study, two equivalents 2.8-kb fragments from OprH-overproducing mutant strain H181 was also cloned. In all expression studies performed with the 2.8-kb *EcoRI* fragments from strains H103 and H181, in two different vectors in *E. coli* (Table 1) and in one in *P. aeruginosa* (Table 2), very similar levels of OprH were expressed from both fragments. Although the oprH gene was flanked by more than 1 kb upstream and 1 kb downstream of the coding region, it would seem probable that the factor resulting in the overexpression of OprH in strain H181 was not present on this 2.8-kb fragment.

Cloning of the 2.8-kb *EcoRI* fragment into pUC18 resulted in the production of OprH in *E. coli* cells containing plasmids pGB22 and pGB122 (Table 1). In all of the cloning and subcloning experiments, no clones in which the lac promoter and the oprH gene were in the same orientation in a high-copy-number vector were obtained, unless the gene was truncated. This result suggested that expression might be driven from the oprH promoter in pGB22 and pGB122 and that overexpression of OprH was lethal. Subcloning into pUC or pTZ vectors of any of the 0.8-kb *Smal-KpnI*
VOL. 173, 1991

UNIQUE OUTER MEMBRANE PROTEIN FUNCTION 6659

TABLE 1. Levels of expression in E. coli DH5α F' of cloned oprH DNA from wild-type P. aeruginosa H103 and its OprH-overproducing derivative, H181

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Vector</th>
<th>Promoter</th>
<th>oprH gene source</th>
<th>Orientation of insert relative to vector promoter</th>
<th>Level of oprH gene expression in:</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGBK2</td>
<td>pRK767</td>
<td>lac</td>
<td>H103</td>
<td>-</td>
<td>LB: +, M9-glucose: +</td>
</tr>
<tr>
<td>pGBK124</td>
<td>pRK404</td>
<td>lac</td>
<td>H103</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>pGBK23</td>
<td>pRK767</td>
<td>lac</td>
<td>H103</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pGBK124</td>
<td>pRK404</td>
<td>lac</td>
<td>H103</td>
<td>+</td>
<td>+ +</td>
</tr>
<tr>
<td>pGBK2</td>
<td>pUC181</td>
<td>lac</td>
<td>H103</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>pGBK122</td>
<td>pUC181</td>
<td>lac</td>
<td>H103</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pGBK25</td>
<td>pNM185</td>
<td>P_m</td>
<td>H103</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>pGBK25</td>
<td>pNM185</td>
<td>P_m + benzoate</td>
<td>H103</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

a pRK767 and pRK404 are related low-copy-number vectors (3); pUC181 is a high-copy-number vector (25), and pNM185 is an intermediate-copy-number vector with a P_m promoter activated by benzoate (14). All plasmids contained as inserts the 2.8-kb EcoRI fragment indicated by the heavy line in Fig. 1.

b +, same orientation; -, inverse orientation.

c Levels of production of OprH in E. coli DH5α F' were judged visually and by densitometry from SDS-PAGE gels of cell lysates (Fig. 2) and/or from Western immunoblots of these gels, developed with OprH-specific antiserum (10). -, no observable expression; +, trace expression; +, expression equivalent to that of OprH in P. aeruginosa H103 grown in either LB or M9-glucose (Table 2); + +, intermediate expression; + ++, expression equivalent to that of OprH in OprH-overproducing P. aeruginosa H181 grown in either LB or M9-glucose (Table 2).

(pGBK52), 1.4-kb BamHI-KpnI (pGBK142), 1.8-kb BamHI-SalI (pGBK32), 1.9-kb EcoRI-KpnI (pGBK62 and pGBK162), and 2.3-kb EcoRI-SalI (pGBK172) fragments (all of which contained the full coding sequence in inverse orientation to the lac promoter) abolished the production of OprH by E. coli. This surprising observation suggested that the 0.5-kb SalI-EcoRI region downstream of oprH (Fig. 1) was influencing the expression of oprH. Although this suggestion might reflect control by processing of the 3' end of the message, no experiments were performed to test this possibility.

Cloning of the 2.8-kb EcoRI fragment into the related low-copy-number vectors pRK404 and pRK767 revealed that the expression of oprH could also be driven by the lac promoter (Fig. 2, lane 3; Table 1). When the lac and oprH promoters were in the same orientation (pGBK3 and pGBK123), the levels of protein OprH produced were higher than those obtained from the oprH promoter alone in a high-copy-number vector (Table 1). When the lac and oprH promoters were in opposite orientations, the OprH protein levels were a small fraction of those obtained with pGBK3 and pGBK123 (Fig. 2, lanes 3 and 4). This result indicated that in E. coli, the lac promoter was capable of driving much higher production of OprH than was the oprH promoter. Presumably, if expression from the lac promoter occurred in a high-copy-number vector, the resulting overproduction of OprH would be lethal. The expression of oprH could also originate from the twin P_m promoters of vector pNM185 (in pGBK25, Fig. 1) when coinduced by added benzoate. The production of OprH in all of the above-mentioned constructions was higher in M9-glucose medium than in LB medium but was not increased by growth in Mg2+- and/or Ca2+-deficient medium. Fractionation studies with sucrose density gradient centrifugation separations of outer and cytoplasmic membranes and coisolation of soluble fractions indicated that the bulk of the OprH produced in E. coli was fractionated with the outer membrane.

Overproduction of OprH from the cloned oprH gene in P. aeruginosa. OprH is produced at a low level from the chromosomal oprH gene when P. aeruginosa PAO1 is grown in Mg2+-sufficient (500 μM) minimal medium and at a high level when this strain is grown in Mg2+-deficient (20 μM) medium (16). To boost the production of OprH, independently of either growth in Mg2+-deficient medium or the mutation in H181, we transferred plasmids pGBK23 and pGBK123, containing the oprH gene in the same orientation as the lac promoter in the low-copy-number broad-host-range plasmids pRK404 and pRK767, respectively, by triparental conjugation from E. coli to P. aeruginosa PAO1 strain H103. Strain H103 with either pGBK23 or pGBK123 did not produce OprH at levels significantly higher than PAO1 without a plasmid when cells were grown in PP2 broth or BM2-succinate medium (Table 2). Slightly higher levels were produced in M9-glucose medium, but these were still substantially lower than those produced by strain H181. These results may reflect the observation that the lac promoter is relatively weak in P. aeruginosa, especially in minimal medium (12).

Therefore, the 2.8-kb EcoRI fragment was cloned into a second type of broad-host-range vector, pNM185, to yield pGBK25 (Fig. 1). In this plasmid, oprH expression could now be driven from the twin P_m promoters derived from the TOL plasmid. These promoters are under the control of the XylS protein and its coinducer benzoate and are highly active in Pseudomonas species (14). When pGBK25 was transferred to P. aeruginosa H103, it resulted in substantially elevated production of OprH, even without added benzoate (Fig. 3). Indeed, the level was similar to that found with P. aeruginosa H181 (Fig. 3, lanes 2 and 3). The ratios of levels of

TABLE 2. Levels of expression of OprH in P. aeruginosa H103 with or without specified plasmids and in P. aeruginosa H181

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid*</th>
<th>Level of expression of OprH in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LB</td>
</tr>
<tr>
<td>H103</td>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>H103</td>
<td>pGBK23</td>
<td>+</td>
</tr>
<tr>
<td>H103</td>
<td>pGBK123</td>
<td>+</td>
</tr>
<tr>
<td>H103</td>
<td>pGBK25</td>
<td>+</td>
</tr>
<tr>
<td>H103 + 2 mM benzoate</td>
<td>pGBK25</td>
<td>+</td>
</tr>
<tr>
<td>H181</td>
<td>None</td>
<td>+++</td>
</tr>
</tbody>
</table>

* See Table 1 for details about plasmids. All plasmids contained as inserts the 2.8-kb EcoRI fragment indicated by the heavy line in Fig. 1.

* As in Table 1, footnote c; densitometer traces revealed that ratios of OprH to OprL were as follows: +, ±1; ++, 1 to 6; ++++, 7 to 9. ND, not determined.
OprH to levels of OprL in cell envelopes were determined by densitometric scanning of gel lanes. OprL levels remain relatively constant under different growth conditions and thus were used as a standard (17). OprH/OprL ratios were 0.2 for strain H103, 7.4 for strain H181, 7.1 for H103/pGB25 without added benzoate, and 9.0 for H103/pGB25 with added benzoate. This lesser effect of induction of the $p_m$ promoter in *P. aeruginosa* (Table 1) than in *E. coli* (Table 2) could have several explanations, including the putative relatively

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**FIG. 1.** Schematic representation of plasmid pGB25. The *oprH*-containing insert is represented as a heavy line with the relevant restriction sites shown. The vector sequence (thin line) is pNM185 (14). Km, kanamycin resistance gene; xylS, activator gene; $P_m$, promoter at which xylS and benzoate act; Sm, streptomycin resistance gene; ori, origin of vegetative replication; mob, genes for mobilizing functions; rep, genes for vegetative replication functions.

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**FIG. 2.** SDS-PAGE (A) and Western immunoblotting with OprH-specific antiserum (B) of cell lysates of *E. coli* carrying plasmids with *oprH* DNA inserts. Lanes: 1, *P. aeruginosa* H181 outer membrane (positive control); 2, *E. coli* DH5αF'/pUC18 (negative control); 3, *E. coli* DH5αF'/pGB23 (+++ expression in Table 1); 4, *E. coli* DH5αF'/pGB24 (± expression in Table 1). Running positions of heat-modified OprH (labelled OprH*) and unmodified OprH (labelled OprH) bands are shown to the right of each panel. Both bands were usually observed in OprH-containing outer membranes (1a).

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**FIG. 3.** SDS-PAGE of *P. aeruginosa* cell lysates from wild-type strain H103 (lane 1), OprH-overproducing mutant H181 (lane 2), and strain H103/pGB25 (lane 3). Forty micrograms of protein was loaded in each lane. The running position of heat-modified OprH is shown to the right. Cells were grown in M9-glucose with 500 μM Mg²⁺ and 2 mM benzoate to an $A_{600}$ of 0.4 to 0.6. Strain H103/pNM185 (the vector control for H103/pGB25) showed a profile identical to that seen in lane 1.
greater efficiency of the oprH promoter than of the p_m promoter in P. aeruginosa, the fact that benzoate is metabolized by P. aeruginosa (14), or the leakiness of the p_m promoter. As with E. coli, growth in M9-glucose medium was required to obtain the highest level of OprH expression. However, when individual components of M9-glucose medium that were different from those of BM2-succinate medium (carbon source, added Ca^{2+}, added NaCl, lack of added Fe^{2+}) were tested, none affected oprH expression.

Effect of OprH expression on antibiotic susceptibility. E. coli clones producing OprH, including those containing plasmids pGB23 and pGB123, which resulted in OprH levels equivalent to those in strain H181, had unaltered susceptibility to polymyxin B and gentamicin (data not shown). Overproduction of OprH from the cloned oprH gene in P. aeruginosa H103 did not affect the MICs of polymyxin B for the organism (Table 3). Unfortunately, plasmids pNM185 and pGB25 each encoded two separate aminoglycoside-modifying enzymes resulting in kanamycin and streptomycin resistance (14). Although cross-resistance to gentamicin due to these enzymes was minimal (Table 3; MICs of kanamycin and streptomycin were >500 μg/ml), it was sufficient to mask any potential influence of OprH. Similarly, although strain H103/pGB25 (grown without benzoate) was killed as poorly by gentamicin as was strain H181 (Fig. 4), we were unable to conclude with certainty that this result was due to OprH overexpression, since H103 containing control plasmid pNM185 was almost as resistant to gentamicin as was H103/pGB25. Susceptibility to the anionic beta-lactam ceftazidime was unaffected by OprH overproduction.

The minimal effect of OprH overexpression from the cloned gene on polymyxin B susceptibility was also confirmed in killing assays (Fig. 4). In contrast, cells were significantly protected from EDTA killing by OprH overexpression. Over a range of EDTA concentrations from 0.1 to 10 mM, strain H103/pGB25 had 12- to >40-fold more survivors than did strain H103 with or without the vector (Fig. 4). However, the survival rates of EDTA-treated strain H103/pGB25 were consistently below those of strain H181 treated in an identical manner (Fig. 4), with the difference in survival rates varying between 4- and 100-fold, depending on the EDTA concentration and the induction of OprH expression by benzoate.

Influence of LPS alterations on antibiotic resistance. The above-mentioned data indicated that polymyxin B resistance in strain H181 might be due to an alteration in a surface molecule other than OprH. One possibility was an LPS alteration, since polymyxin B has been shown to interact with LPS. Therefore, we examined the influence of rough mutations leading to the loss of O-antigenic (B-band) LPS on polymyxin B resistance by selecting phase-resistant rough mutants that continued to overproduce OprH. Strains H222 and H223, derived from strain H181, became polymyxin B susceptible, despite overproducing OprH, as judged by MIC determinations (data not shown) and killing assays (Fig. 5).

![FIG. 4. Effect of OprH overexpression on survival after 5 min in the presence of polymyxin B (PX) (2 μg/ml), gentamicin (GM) (80 μg/ml), or EDTA (10 mM). The strains used were parent strains H103 and H103/pNM185 and OprH-overexpressing strains H103/pGB25 and H181. Strains H103/pNM185 and H103/pGB25 were grown in the presence of benzoate to induce the p_m promoter. The results shown are the means of three to five assays after pregrowth of cells in BM2-glucose with 0.5 mM Mg^{2+}.](attachment:image)
In contrast, they remained as resistant as strain H181 to both EDTA and gentamicin (Fig. 5).

DISCUSSION

To determine the role of OprH overexpression in resistance to cationic antibiotics and EDTA in *P. aeruginosa* H181, we overexpressed OprH from the cloned gene reintroduced into *P. aeruginosa* on expression plasmid pGB25. The results suggested that, by itself, OprH overexpression accounted largely for EDTA resistance and not much, if at all, for polymyxin B resistance. Thus, these data are consistent with OprH overexpression being a codeterminant rather than the sole cause of resistance in strain H181. Since reversion studies implied that the OprH-overexpressing mutant had a single point mutation (17), it is difficult to explain these data unless strain H181 contains a regulatory rather than a promoter mutation resulting in the overexpression of OprH. A regulatory mutation could result in two changes which could differentially influence the resistance phenotype of strain H181.

The expression studies described here provided substantial evidence that strain H181 contained a regulatory rather than a promoter mutation. Results with *E. coli* containing plasmid pGB22 indicated that OprH could be expressed from a promoter within the 1 kb of DNA upstream of the oprH gene within the 2.8-kb insert in this plasmid. While we have no direct evidence that this is the oprH gene promoter in *P. aeruginosa*, the extremely high maximal expression of OprH under inducing conditions (approximately 10⁸ molecules per cell) suggests that oprH is the first gene in the operon and makes it seem unlikely that the oprH gene contains a very large leader sequence. Therefore, it seems almost certain that the oprH gene promoter is contained within the 2.8-kb EcoRI fragment.

The cloned 2.8-kb EcoRI fragment from strain H181 caused no higher expression in *E. coli* than did the cloned fragment from strain H103. Also, Mg²⁺ concentrations in the medium, which strongly influenced OprH levels in strain H103 but not in strain H181, had no influence on expression from the cloned oprH gene in *E. coli*. We conclude, therefore, that this 2.8-kb fragment does not contain the sequences which regulate OprH expression differentially in strains H103 and H181 and that strain H181 is therefore most probably a regulatory mutant.

It should be noted that expression from the cloned oprH gene was substantially stimulated by growth in M9-glucose minimal medium in both *E. coli* and *P. aeruginosa*. Consistent with this result, the resistance phenotypes associated with OprH overexpression were optimally expressed in M9-glucose minimal medium (1). Thus, this influence of growth medium on phenotype appears to be associated with the 2.8-kb EcoRI restriction fragment containing the oprH gene.

We propose here a model which accounts for these findings (Fig. 6). Previous data have shown that LPS mutations can alter susceptibility to polymyxins and aminoglycosides (2, 17, 22). Furthermore, phage selection of rough mutants with alterations in LPS in an OprH-overproducing background (strain H181 [Fig. 5] or strain H103 grown on Mg²⁺-deficient medium [1]) leads to partial reversion of the resistance phenotype without influencing OprH overexpression. Interestingly, polymyxin B resistance is affected substantially whereas EDTA resistance and gentamicin resistance are unaffected by such mutants. These data are consistent with a model in which strain H181 or strain H103 grown under divalent cation-deficient conditions contains two alterations regulated at the level of gene expression, namely, an alteration in LPS and an overproduction of OprH. Thus, OprH overproduction by itself in H103/pGB25 would result in the replacement of LPS-stabilizing divalent cations with (putative LPS-stabilizing) OprH (Fig. 6). The resultant substantial decrease in surface divalent cations (21) would render the outer membrane more resistant to attack by EDTA and aminoglycosides. Although no conclusions regarding the influence of OprH overexpression on gentamicin susceptibility could be made on the basis of data obtained with the cloned oprH gene, the lack of influence of LPS alterations on gentamicin resistance implied that OprH may be involved in such resistance in strain H181. However, we propose that polymyxin B, perhaps because of its higher net positive charge and hydrophobic tail, could still attack the sites of interaction between positive charges on OprH and negative charges on LPS (or, because of its higher affinity [15, 21] for residual LPS-divalent cation binding sites, could preferentially attack such sites). The enhanced resistance of
strain H181 could then be due to an additional alteration in LPS so as to make such sites resistant to attack by polymyxin B (Fig. 6). It should be noted that while previous evidence has revealed no apparent alterations in fatty acids (16), 2-keto-3-deoxyoctulosonic acid/phosphate ratios (19), or SDS-PAGE profiles of strain H181 LPS (16), given the apparent role of phosphates in polycation binding (15, 20, 21) and the complexity and large number of phosphate residues in Pseudomonas LPS (11, 21, 22), it seems possible that such changes might easily escape detection by the crude chemical analyses used to date.

Despite the implication that adaptive resistance and mutational resistance to polymyxin B, gentamicin, and EDTA are more complicated than previously suggested by us (8), these data provide clear evidence that OprH overproduction, by itself, can cause resistance to EDTA (Fig. 4) and also suggest (i.e., via a lack of effect of an LPS alteration) a role in gentamicin resistance (Fig. 5). We propose, therefore, that this protein has as its normal function the stabilization of the outer membrane under conditions in which environmental divalent cations become depleted. The consequent replacement of divalent cations by OprH thus results in the resistance phenotype. However, we have found no evidence that OprH is expressed in substantial quantities in animal model experiments (10) and propose that conditions resulting in OprH overexpression are more likely to occur during growth in soil, a primary habitat for P. aeruginosa.

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