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# Role of *Pseudomonas aeruginosa* Outer Membrane Protein OprH in Polymyxin and Gentamicin Resistance: Isolation of an OprH-Deficient Mutant by Gene Replacement Techniques

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**The cloned *oprH* gene of *Pseudomonas aeruginosa* was mutated by inserting a 1.4-kbp fragment that encodes tetracycline resistance. This mutated gene was then incorporated into the *P. aeruginosa* chromosome by homologous recombination. Growth of the resultant *oprH::tet* mutant in $Mg^{2+}$-deficient medium had little effect on susceptibility to polymyxin B, gentamicin, or EDTA unless the mutant was complemented by the cloned *oprH* gene in plasmid pGB25. In contrast, growth of the parent strain on $Mg^{2+}$-deficient medium resulted in resistance to all three agents. These data support the hypothesis that overexpression of OprH under $Mg^{2+}$-deficient growth conditions is conditionally required for resistance to these three agents.**

In recent years, there has been a significant increase in our understanding of the mechanisms of uptake of antibiotics across the outer membranes of *Pseudomonas aeruginosa*. It is now well established that low outer membrane permeability to hydrophilic compounds contributes substantially to the high intrinsic resistance of this bacterium to antibiotics such as β-lactams (3). Conversely, polycationic antibiotics, such as polymyxin B and aminoglycosides, access the self-promoted uptake pathway (3, 4, 8). This pathway involves the competitive displacement by these bulky polycations of the divalent cations which noncovalently cross bridge adjacent lipopolysaccharide (LPS) molecules. Consequently, the outer membrane becomes distorted and more permeable, permitting increased (self-promoted) uptake of the permeabilizing compounds. The isolation of outer membrane mutants with altered susceptibilities to these agents argues powerfully that self-promoted uptake is relevant to eventual cell killing (3). The classical outer membrane permeabilizer EDTA, a divalent cation chelator, functions by accessing the same divalent cation-binding sites on LPS as do polycationic antibiotics (8).

Nicas and Hancock (8) proposed that the *P. aeruginosa* outer membrane protein OprH (or H1) blocked the self-promoted uptake pathway. Thus, overexpression of OprH by 20-fold or more, as a result of either adaptation to $Mg^{2+}$-deficient medium in parent strain H103 or mutation in the polymyxin-resistant derivative strain H181, was associated with resistance to polymyxin B, gentamicin, and EDTA (1, 3, 8). In contrast, there was no change in the susceptibility of these cells to β-lactams or tetracycline. It was hypothesized that OprH acts by binding to LPS sites which are normally occupied by divalent cations, thereby preventing access of polymyxin, gentamicin, and EDTA to these sites (3). Consistent with this theory, sequence analysis of the *oprH* gene indicated that OprH was a basic protein (theoretical pI, 9.6), permitting potential association with negatively charged LPS molecules, whereas the OprH protein was shown to copurify with LPS (2).

However, overexpression of OprH from the cloned gene in the wild-type strain H103/pGB25 resulted in only partial resistance to EDTA, ambiguous results for aminoglycosides, and no resistance to polymyxin, compared to the OprH-overproducing mutant H181, which was resistant to all three agents (1). This led to the hypothesis that strain H181 (and strain H103 adapted to $Mg^{2+}$-deficient medium, which had an identical phenotype) contained a second alteration. On the basis of pseudorevertant studies, in which it was shown that rough, LPS-altered mutants of strain H181 had reverted to wild-type polymyxin susceptibility despite overexpressing OprH, it was proposed that this second alteration was in LPS (1). However, these data led us to reexamine whether OprH had any role in polymyxin resistance. Therefore, we utilized gene replacement techniques to replace the wild-type *oprH* gene in strain H103 with an *oprH::tet* interposon-mutated gene. The resultant OprH-deficient strain permitted us to demonstrate that OprH had an obligate role in resistance to polymyxin, gentamicin, and EDTA because of adaptation to $Mg^{2+}$-deficient growth conditions.

Wild-type strain H103 and its polymyxin B-resistant, OprH-overproducing derivative strain H181 have been described previously (8) and were maintained routinely on LB agar (0.8% Bacto Tryptone, 0.5% yeast extract, 1.5% Bacto Agar) with the inclusion of 8 μg of polymyxin B per ml for maintenance of the H181 phenotype. Construction of the OprH-deficient mutant H703 proceeded as follows. Plasmid pUC18T2 (5), containing the *tet* gene from pBR322 that had been adapted to contain flanking *Pst*I restriction sites, was kindly provided by S. Lory, Department of Microbiology, University of Washington, Seattle. This *tet* gene was excised and inserted between the tandem *Pst*I sites placed 15 bp apart in the *oprH* gene of plasmid pGB32 (2). All DNA manipulations utilized the methods outlined by Maniatis et al. (7). Plasmid pGB32 was linearized by using *Eco*RI and cloned into the *Eco*RI site of the gene replacement vector pRZ102 (6), which contains a *mob* site for transfer to *P. aeruginosa* but lacks an origin of replication that works in *P. aeruginosa*. The resultant plasmid pGB32B (Fig. 1) was then transformed into the mobilizing *Escherichia coli* strain S17-1 [*pro endA*::RP4(Tc::Mu Kn::Tn*7*)] (9) and transferred by conjugation into strain H103. Since pGB32B could not replicate in *P. aeruginosa*, isolation of colonies resistant to

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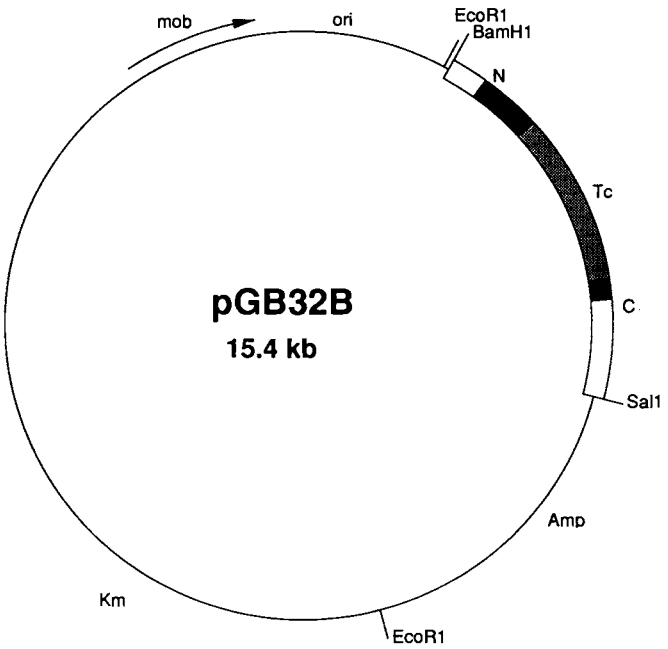


FIG. 1. Diagram of plasmid pGB32B utilized for gene replacement mutagenesis. The thick bar between the *Bam*HI and *Sal*I restriction sites represents *Pseudomonas* DNA flanking (white bars) or encoding (black bars) the *oprH* gene, whereas the stippled bar represents the *Pst*I fragment containing the tetracycline (Tc) resistance gene that was used to interrupt *oprH*. The orientation of the *oprH* gene is given by N and C. Km, kanamycin resistance gene (part of Tn*5*); Amp, ampicillin resistance gene; ori, *E. coli* specific origin of replication; mob, mobilization sequence.

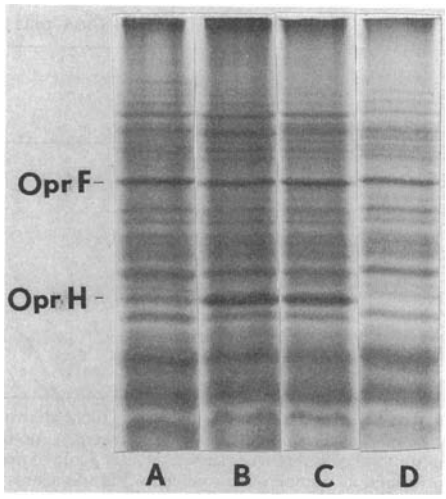


FIG. 2. SDS-PAGE of cell envelope preparations (methods are described in reference 8) demonstrating expression of OprH on $Mg^{2+}$-sufficient medium. Lanes: A, strain H103; B, H181; C, H703/pGB25 induced with 5 mM *m*-toluate; D, H703 *oprH*::*tet*. The running positions of OprH and the major porin OprF are indicated. The small amount of protein observed just above the running position of OprH in strain H703 was not heat modifiable and was not inducible by growth in $Mg^{2+}$-deficient medium, unlike OprH, and thus was concluded to be another protein.

150 μg of tetracycline per ml resulted in selection for mutants in which the chromosomal *oprH* gene was replaced by homologous recombination with the interposon-mutated *oprH*::*tet* gene (data not shown). For one strain, H703, Southern hybridizations of chromosomal DNA with a $^{32}P$-labelled *oprH* gene probe confirmed that the *oprH* chromosomal gene was interrupted by a 1.4-kbp *Pst*I fragment containing the *tet* gene. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cell envelopes confirmed the lack of OprH protein under both $Mg^{2+}$-sufficient and -deficient conditions (Fig. 2), while the gene probe also confirmed the lack of a second cross-hybridizing gene.

It was previously demonstrated that the overexpression of OprH in the parent strain H103 grown on $Mg^{2+}$-deficient (0.02 mM $Mg^{2+}$) BM2 glucose minimal medium (1, 8) was accompanied by resistance to killing by polymyxin B, gentamicin, and EDTA compared to strain H103 grown on $Mg^{2+}$-sufficient (0.5 mM $Mg^{2+}$) BM2 glucose minimal medium (1, 8). In contrast, strain H181 overexpressed OprH and was resistant to all three agents, regardless of the $Mg^{2+}$ concentration in the medium (8). This observation was confirmed here (Table 1) by growing cells to an $OD_{600}$ of 0.5 on $Mg^{2+}$-sufficient or -deficient medium, centrifuging them down and resuspending them in buffer (for polymyxin B and EDTA) or buffer plus glucose (for gentamicin) (1, 8) prior to addition of 4 μg of polymyxin B per ml, 80 μg of gentamicin per ml, or 10 mM EDTA and plating for survivors after 5 min at 37°C, in accordance with the methods of Nicas and Hancock (8) as modified by Bell et al. (1). In contrast to the situation with parent strain H103, the OprH-deficient, *oprH*::*tet* mutant strain H703 remained susceptible to all three agents after growth in either $Mg^{2+}$-sufficient or $Mg^{2+}$-deficient medium (Table 1). These killing data were confirmed by MIC experiments which demonstrated that susceptible cells (2 to 12% survivors) were affected by MICs of gentamicin and polymyxin of 2 μg/ml, whereas resistant cells (78 to 95% of survivors) were affected by MICs of 8 to 16 μg/ml. These data supported the hypothesis that overproduction of OprH during growth on $Mg^{2+}$-deficient medium was required to permit expression of the polymyxin, gentamicin, and EDTA resistance phenotype.

To confirm that this result reflected an inability to produce and induce OprH, two control experiments were performed. First, it was demonstrated that H703 was affected by the same MIC (1 μg/ml) of the β-lactam cefpirome as the parent strain H103 in both $Mg^{2+}$-sufficient and -deficient medium, arguing against a nonspecific change in permeability. Second, plasmid pGB25, which contains the *oprH* gene behind an *m*-toluate-inducible promoter, was introduced into strain H703. The resultant strain, H703/pGB25, when grown on $Mg^{2+}$-deficient medium supplemented with *m*-toluate to induce OprH production, was resistant to both polymyxin B and EDTA compared to the vector plasmid control strain H703/pNM185 and the parent strain H703 (Table 1) (note that gentamicin resistance could not be tested in the presence of these plasmids [1]).

Two sets of results suggested that a second cellular alteration was required to give the full resistance phenotype observed in strain H103 grown in $Mg^{2+}$-deficient medium. First, as previously observed for strain H103/pGB25 (1), overexpression of OprH in strain H703/pGB25 was not sufficient to give the full resistance phenotype when strains were grown in $Mg^{2+}$-sufficient medium. Indeed, both H703/pGB25 (Table 1) and H103/pGB25 (1) demonstrated only partial resistance to EDTA and full susceptibility to polymyxin B when grown on $Mg^{2+}$-sufficient medium, despite

TABLE 1. Influence of the OprH phenotype on killing by polymyxin B, gentamicin, and EDTA

| Strain | $Mg^{2+}$ concn (mM) during growth | OprH level[a] | % Survivors (mean ± SD) after treatment with[b]: | | |
|---|---|---|---|---|---|
| | | | Polymyxin (4 μg/ml) | Gentamicin (80 μg/ml) | EDTA (10 mM) |
| H103 | 0.02 | +++ | 90 ± 3[c] | 78 ± 3[c] | 89 ± 3[d] |
| H103 | 0.5 | + | 4 ± 3 | 2 ± 2 | 2 ± 3 |
| H181 | 0.02 | +++ | 95 ± 3[c] | 85 ± 3[c] | 87 ± 7[c] |
| H181 | 0.5 | +++ | 81 ± 5[c] | 78 ± 5[c] | 85 ± 18[c] |
| H703 | 0.02 | − | 8 ± 4 | 12 ± 5 | 7 ± 1 |
| H703 | 0.5 | − | 2 ± 1 | 4 ± 3 | 5 ± 2 |
| H703/pGB25 | 0.02 | +++ | 79 ± 5[c] | —[e] | 77 ± 9[c] |
| H703/pGB25 | 0.5 | +++ | 3 ± 1 | — | 30 ± 1[c,d] |
| H703/pNM185 | 0.02 | − | 6 ± 5 | — | 8 ± 4 |
| H703/pNM185 | 0.5 | − | 3 ± 2 | — | 2 ± 2 |

[a] Determined by SDS-PAGE experiments such as those shown in Fig. 2. +++, overexpression of OprH such that this was the major cell envelope protein (Fig. 2, lanes B and C); +, small amount of OprH produced; −, no observable expression of OprH.

[b] Percentage of total cells that were survivors after 5 min of treatment with polymyxin B, gentamicin, or EDTA (1, 8). Results are means ± standard deviations of three experiments; all numbers are rounded off to the nearest integer.

[c] Significantly different ($P < 0.05$ by Student's $t$ test) from the result for H103 grown on $Mg^{2+}$-sufficient medium.

[d] Significantly different ($P < 0.05$) from the result for H703/pGB25 grown on $Mg^{2+}$-deficient medium.

[e] Because of the aminoglycoside-modifying enzyme genes on the plasmids pGB25 and pNM185, gentamicin susceptibility could not be tested.

overexpression of OprH. Second, growth of strain H703 (and H703/pNM185) on $Mg^{2+}$-deficient medium consistently resulted in a small increase in resistance to these agents, although the increase was far less than that observed for OprH-overexpressing strains. Thus, these data suggest that although OprH overexpression is required for the polymyxin, gentamicin, and EDTA resistance phenotype, it is not by itself sufficient to explain this phenotype. Since previous pseudorevertant studies suggested that LPS might also play a role (1), we are currently developing methods for examining the rather complex LPS of *P. aeruginosa*.

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