Influence of OprM Expression on Multiple Antibiotic Resistance in Pseudomonas aeruginosa

KENDY K. Y. WONG,1 KEITH POOLE,2 NAOMASAH GOTOH,3 AND ROBERT E. W. HANCOCK1*

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z3; Department of Microbiology and Immunology, Queen’s University, Kingston, Ontario, Canada K7L 3N6; and Department of Microbiology, Kyoto Pharmaceutical University, Yamashina, Kyoto 607, Japan

Received 13 March 1997/Returned for modification 25 April 1997/Accepted 10 June 1997

Pseudomonas aeruginosa is well known for its intrinsic resistance to various structurally unrelated antimicrobial agents (29). This broad-spectrum resistance is largely due to the possession of an outer membrane with relatively low permeability (10, 22, 23, 32) coupled with secondary resistance mechanisms, such as efflux (14–16, 23, 25, 29). The efflux operon mexA-mexB-oprM has been identified in P. aeruginosa, and its products have been demonstrated to contribute to the high intrinsic antibiotic resistance of this organism as well as lead to multiple antibiotic resistance after overexpression in nalB mutants (7, 8, 18, 26–28). It has been suggested that the relatively hydrophilic and often negatively charged β-lactams, which have targets in the periplasm, can also be extruded directly from the periplasm or from the surface of cytoplasmic membrane through this system (16). Therefore, it was of interest whether the outer membrane component OprM could function independently. In this study, we overproduced OprM in various P. aeruginosa strains to investigate the role of OprM in efflux.

Two synthetic oligonucleotides were used to amplify oprM from plasmid pPV20 (27) and to incorporate NdeI and HindIII restriction sites at the 5′ and 3′ ends, respectively. The approximately 1.5-kb fragment was first cloned into plasmid pT7-7 (30), and the gene, together with the ribosome binding site on pT7-7, was then excised by XbaI and HindIII and ligated to plasmid pVL31 (17) to create pKPM-2. DNA sequencing confirmed the published (27) sequence of the subcloned oprM gene. The control vector pVL31 and the construct pKPM-2 were transformed into Escherichia coli DH5α, the P. aeruginosa wild-type strain H103 (laboratory collection), and two P. aeruginosa OprM-deficient ΔoprHΔ interposon mutants, K613 (27) and OCR63T (9). Expression of oprM from pKPM-2 was induced by isopropylthio- β- d- galactoside (IPTG) and confirmed by Western immunoblotting (20, 24, 31) with a murine monoclonal antibody against OprM. Surface expression of OprM was also confirmed in all clones expressing oprM by indirect immunofluorescence by the method of Hofstra et al. (13). The fluorescence signal from cells carrying pKPM-2 and induced by IPTG was the strongest. The wild-type P. aeruginosa strain H103 and the vector control strain H103/pVL31 gave weak signals due to OprM expressed from the chromosomal gene. However, excessive production of OprM from pKPM-2 seemed to be harmful to cells, as revealed by growth studies. Cell densities of strains carrying pKPM-2 started to decline after 2 h of induction with 0.1 or more mM IPTG (the results for strain K613/ pKPM-2 are shown in Fig. 1), at which point OprM was already substantially overproduced, as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of outer membrane isolated as described previously (12). It is possible that excess OprM perturbed the outer membrane and led to cell lysis, as observed in the case of overexpression of a mutant OmpA precursor protein in E. coli (5). A concentration of 0.05 mM IPTG led to no change in growth rate for at least 3 h and a normal yield of cells after overnight growth at 37°C.

OprM was overproduced from cells carrying pKPM-2 and induced with IPTG (Fig. 2A, lane 5; Fig. 2B, lane 6). Strain H103/pKPM-2 produced significantly larger amounts of OprM (Fig. 2B, lane 6) than the wild-type strain, H103; the vector control strain H103/pVL31 (Fig. 2B, lanes 1 through 4); and the nalB mutant OCR1 (Fig. 2B, lane 7). OprM was previously shown to be heat modifiable (6, 19). However, in this study we observed that the protein samples from strains carrying pKPM-2 in sample buffer alone did not give any noticeable change in the intensities of the 100-kDa oligomer band of OprM. Both the monomeric 50-kDa and the native oligomeric 100-kDa forms were associated with the outer membrane under such conditions (Fig. 2A, lane 5; Fig. 3, lanes 1 to 3). Only when β-mercaptoethanol was included did the 100-kDa band shift to the 50-kDa monomeric form (as confirmed by twodimensional, unheated versus heated SDS-PAGE [data not shown]), and this occurred even when solubilization was performed at room temperature or 37°C (Fig. 3, lanes 4 and 5). Many pores exist as oligomers in the outer membrane (2, 3, 11, 21). OprM might also exist as an oligomer in its native form. Overproduction of OprM could have overwhelmed the ability of the cell to correctly form the oligomer, or most oligomers formed may have been less SDS stable. We presume that those oligomers which formed were stabilized by disulphide bridges. In this regard, it should be noted that there are three cysteine residues in the predicted amino acid sequence of OprM.

Antibiotic susceptibilities of the various clones were studied by broth microdilution assays in Mueller-Hinton broth by the method described by Amsterdam (1). MICs of different antimicrobial agents were determined after 20 to 22 h of incubation, and controls demonstrated that the growth of cells carrying pKPM-2 was not inhibited by 0.05 mM IPTG. As shown

* Corresponding author. Phone: 604-822-2082. Fax: 604-822-6041. E-mail: bob@cbdn.ca.
in Table 1, overproduction of OprM in the two OprM-deficient strains led to complementation of their mutations. Tetracycline resistance was the selective marker on pVLT31; thus, strains carrying pVLT31 or pKPM-2 were highly resistant to tetracycline. The MICs of some antibiotics for control vector strains K613/pVLT31 and OCR03T/pVLT31 were increased compared to the MICs of those antibiotics for the OprM-deficient parents of those strains. This is possibly due to the tet gene on pVLT31 or the requirement to include tetracycline to maintain the plasmids in growing bacteria to seed the MIC plates. Nevertheless, when comparing isogenic strains with the oprM-expressing plasmid or with the vector plasmid alone, complementation was observed. Most interestingly, overproducing OprM from the cloned gene in the wild-type P. aeruginosa PAO strain H103 did not alter the MICs of any of the antibiotics tested. Without IPTG induction, H103/pKPM-2 gave MIC results similar to those obtained by IPTG induction (data not shown). This indicated that OprM cannot function independently as an antibiotic efflux channel. In strains K613 and OCR03T, only the most distal gene, oprM, of the operon was interrupted and mexA and mexB could still be expressed. Thus, OprM produced from pKPM-2 could function with these MexA and MexB molecules to complement the OprM deficiency. The excess molecules of OprM produced in these pKPM-2-containing strains might not be able to function properly, since there would be too little MexA and MexB available to reconstruct additional complete efflux systems (assuming that the efflux systems involved stoichiometric amounts of the three components). Consistent with this view, there are small amounts of MexA, MexB, and OprM produced in the wild-type PAO strain H103 which assemble into an efflux apparatus and contribute to intrinsic antibiotic resistance (27). The lack of influence of OprM overexpression in strain H103 is consistent with the explanation that extra copies of OprM expressed from pKPM-2 would presumably not have any MexA and MexB molecules available to form additional efflux complexes. This would explain why, in this genetic background, there was no

FIG. 1. Growth of P. aeruginosa strain K613/pKPM-2 in Luria-Bertani medium induced with different concentrations of IPTG.

FIG. 2. SDS-PAGE of outer membrane proteins. (A) Samples from E. coli DH5α/pVLT31 (lanes 2 and 3) and DH5α/pKPM-2 (lanes 4 and 5) heated without β-mercaptoethanol in sample buffer. Molecular mass standards are shown in lane 1 and are as follows: phosphorylase B, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 30 kDa. (B) Samples from P. aeruginosa H103 (lanes 1 and 2), H103/pVLT31 (lanes 3 and 4), H103/pKPM-2 (lanes 5 and 6), OprM-overproducing strain OCR1 (lane 7), and OprM-deficient strain K613 (lane 8). β-Mercaptoethanol (10% [vol/vol]) was included in the sample buffer. Molecular masses are indicated on the left. –, samples from cultures without IPTG induction; +, samples from cultures with 0.05 mM IPTG induction. Position of OprM is shown by arrowheads on the right.

FIG. 3. SDS-PAGE of outer membrane proteins from strain H103/pKPM-2 induced with 0.05 mM IPTG. β-Mercaptoethanol (10% [vol/vol]) was included in the sample buffer in lanes 4, 5, and 6. After mixing with the sample buffer, the samples in lanes 1 and 4 were left at room temperature, the samples in lanes 2 and 5 were left at 37°C for 10 min, and the samples in lanes 3 and 6 were heated at 100°C for 10 min before being loaded onto the wells. Molecular masses are indicated on the left. The positions of the 50-kDa and 100-kDa OprM forms are shown by arrowheads on the right.
significant change in antibiotic susceptibility. OprM was also overproduced in an E. coli tolC mutant strain (4) and its parent strain AG100. There was no significant difference in their antibiotic susceptibilities (data not shown), indicating that OprM cannot replace TolC.

Our results do not provide concrete proof that OprM required MexA and MexB to function properly. However, these results indicated that OprM cannot function independently. Interestingly, a P. aeruginosa tonB homolog was recently cloned, and preliminary data indicated that drug resistance mediated by the mexAB-oprM operon might be TonB dependent (33). Perhaps the energy-dependent resistance to β-lactams mediated through this system is dependent on TonB as well. Alternatively, one of the other systems known to influence β-lactam susceptibility, including inducible β-lactamase and penicillin binding proteins, may be influential.

This research was financially supported by a grant from the Medical Research Council of Canada to R. E. W. Hancock and from the Canadian Cystic Fibrosis Foundation to K. Poole. K. Wong is supported by a studentship from the Canadian Cystic Fibrosis Foundation. R. Hancock is a recipient of the MRC Distinguished Scientist Award. K. Poole is a NSERC University Research Fellow. K. Poole’s contribution was carried out during a sabbatical leave and was partly funded by a travel award from the Canadian Cystic Fibrosis Foundation.

**REFERENCES**

33. Zhao, Q. Personal communication.