Involvement of a Novel Efflux System in Biofilm-specific Resistance to Antibiotics

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ABSTRACT

Bacteria growing in biofilms are more resistant to antibiotics when compared to their planktonic counterparts. How this transition occurs is unclear, but it is likely there are multiple mechanisms of resistance that act together in order to provide an increased overall level of resistance to the biofilm. We have identified a novel efflux pump in *Pseudomonas aeruginosa* that is important for biofilm-specific resistance to a subset of antibiotics. Complete deletion of the genes encoding this pump, PA1874-1877, in a PA14 background results in an increase in sensitivity to tobramycin, gentamicin and ciprofloxacin, specifically when this mutant strain is growing in a biofilm. This efflux pump is more highly expressed in biofilm cells than planktonic cells, providing an explanation for why these genes are important for biofilm but not planktonic resistance to antibiotics. Furthermore, expression of these genes in planktonic cells increases their resistance to antibiotics. We have previously shown that *ndvB* is important for biofilm-specific resistance (Mah, T. F., B. Pitts, B. Pellock, G. C. Walker, P. S. Stewart, and G. A. O’Toole, Nature 426:306-310, 2003). Our discovery that combining the *ndvB* mutation with the *PA1874-1877* deletion results in a mutant strain that is more sensitive to antibiotics than either single mutant strain suggests that *ndvB* and *PA1874-1877* contribute to two different mechanisms of biofilm-specific resistance to antibiotics.
Bacteria growing in biofilms are more resistant to antimicrobial agents than their planktonic counterparts (16). Various hypotheses have been put forward to explain biofilm resistance, but to date there are no data that entirely explain this phenomenon (24). For instance, it has been suggested that the exopolysaccharide matrix that surrounds the cells in the biofilm prevents diffusion of the antimicrobial agents through the biofilm, thus preventing access of the antimicrobial agent to the cells. While this may be the case for some antimicrobial agents, for others it has been shown that they can penetrate the matrix but still cannot kill the cells in the biofilm (2, 39). It has also been suggested that cells within the biofilm grow slowly in response to oxygen, nutrient deprivation, or environmental stress. While a number of studies support the idea that a slowed growth rate can explain some level of biofilm-specific resistance, other studies have suggested that the full extent of resistance cannot be accounted for by this mechanism (7, 12, 39). Furthermore, it has been suggested that high cell-density signalling or quorum sensing play a role in resistance to antimicrobial agents, but again, their exact role is not clear (6).

Conversely, resistance mechanisms utilized by planktonic cells have been well studied. These mechanisms, such as expression of antibiotic-degrading enzymes, decrease in antibiotic influx or increase in antibiotic efflux, are often encoded by transmissible elements and can spread rapidly through different species of bacteria (20). In particular, ATP-binding cassette (ABC) transporters and resistance-nodulation-division (RND) efflux pumps contribute to planktonic resistance to a variety of antibiotics and biocides (21, 35). While no ABC transporters have been identified in Pseudomonas aeruginosa that specifically contribute to drug resistance (34), 9 RND efflux pumps have been identified in P. aeruginosa that account for this organism’s high intrinsic resistance to antibiotics (22, 28, 35). Like ABC transporters, RND
transporters in *P. aeruginosa* also have a wide range of substrates (10). For instance, MexAB-OprM, MexCD-OprJ and MexXY extrude quinolones, macrolides, tetracyclines and β-lactams and more, while aminoglycosides are more specific to MexXY-OprM (26).

Since RND multidrug efflux pumps contribute to antibiotic resistance in planktonic cells, it has been suggested that expression of efflux pumps in biofilms might lead to antibiotic resistance in these surface-attached communities (11). It was found that four of the best-studied RND efflux pumps in *Pseudomonas aeruginosa*, MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY had no impact on biofilm-specific resistance when mature biofilms were tested (11). However, a recent study suggested that MexAB-OprM and MexCD-OprJ are involved in biofilm resistance to the macrolide azithromycin (14). Furthermore, a putative multidrug efflux pump, YhcQ, has been implicated in *Escherichia coli* biofilm antibiotic resistance (23).

In order to identify antibiotic resistance mechanisms utilized by biofilm bacteria, we developed a high-throughput system to search for Tn5-insertion mutants of *P. aeruginosa* that do not develop the characteristic increase in resistance to antimicrobial agents when grown in a biofilm (25). This method allowed us to previously identify *ndvB* as a gene important for biofilm-specific resistance and describe a novel mechanism of resistance whereby *ndvB*-derived glucans interact with the antibiotic tobramycin and prevent it from accessing its cellular target (25). Here we present the characterization of another mutant from this screen, which has allowed us to identify a new, putative efflux pump with a role in biofilm antibiotic resistance.
MATERIALS AND METHODS

Bacterial strains, plasmids, media and chemicals. *P. aeruginosa* PA01 (17) and PA14 (36) and *E. coli* DH5α (3) were grown in rich medium (Luria Bertani, LB) or minimal medium at 37 ºC. The minimal medium was minimal M63 salts (33) supplemented with arginine (0.4%) and MgSO₄ (1 mM). The *P. aeruginosa* PA01 transposon-insertion mutants were obtained from the University of Washington Genome Center (17). The *P. aeruginosa* PA14 deletion strains ∆PA1874, ∆PA1875, ∆PA1876, ∆PA1877 and ∆PA1874-1877 were constructed as reported (25) using the following primers: P504: 5’–GGCGGGATCCGACCACGTCGTTGACCACTG-3’ and P455: 5’-GGCGGCGCATATGGATAGGGGAATCTTCGCCTCCTG-3’ for the 5’ PA1874 fragment, P456: 5’-GGCGGCGCATATGGCCTTCGGTGTTTTCAGCGGAACC-3’ and P505: 5’-GGCGGGAATCTCCTGCAGTTGATCAGTGAGC-3’ for the 3’ PA1874 fragment, P506: 5’-GGCGGGATCCGACATCACCACGACATCCTCAAC-3’ and P459: 5’-GGCGGCGCATATGCCGACTTCCCTGCGCGTACTG-3’ for the 5’ PA1875 fragment, P460: 5’-GGCGGCGGATATGCAGCCGAGGCCAAGGCGTCGCTG-3’ and P507: 5’-GGCGGGAATTCGTCGTCGAGCAGCAGCAG-3’ for the 3’ PA1875 fragment, P462: 5’-GGCGGCGCATATGCCGACTTCCCTGCGCGTACTG-3’ and P463: 5’-GGCGGGAATCTCCTGCAGTTGATCAGTGAGC-3’ for the 3’ PA1876 fragment, P464: 5’-GGCGGCGGATATGCCGACTTCCCTGCGCGTACTG-3’ and P465: 5’-GGCGGCGGATATGCAGCCGAGGCCAAGGCGTCGCTG-3’ and P466: 5’-GGCGGCGGATATGCAGCCGAGGCCAAGGCGTCGCTG-3’ and P467: 5’-GGCGGCGGATATGCAGCCGAGGCCAAGGCGTCGCTG-3’ and P468: 5’-GGCGGCGGATATGCAGCCGAGGCCAAGGCGTCGCTG-3’ and P469: 5’-
GGCGGAATTCCGTCGCCGCGGATATTTTATC-3’ for the 3’ PA1877 fragment, PA1874F2: 5’-GATCAAGCTTACATGGTGCGGCCGTTCG-3’ and PA1874R2: 5’-GGAATCTAGGCGTTGCTGGCATTGACCT-3’ for the 5’ PA1874-1877 fragment, PA1877F2: 5’-GGAGTCTAGATCCAGCGGCAGATCCACCT-3’ and PA1877R2: 5’-CACAGAATTCAAGCGATCCACGCGACAT-3’ for the 3’ PA1874-1877 fragment.

Tobramycin, gentamicin, ciprofloxacin, nalidixic acid and rifampicin were purchased from Sigma-Aldrich Co. (St. Louis, MO).

Biofilm formation assay. Biofilm formation was measured by using the microtitre dish assay system, as described previously (31, 32). Biofilms were grown for 24 hours before they were stained with 0.1% crystal violet.

MBC-P and MBC-B assays. Minimal bactericidal concentration for biofilms (MBC-B) (25): Bacterial strains were inoculated into the wells of the 96-well microtitre dish and biofilms were allowed to form over 24 hours. Serial dilutions of the antibiotic of choice were added into the wells and the biofilms were exposed to the drug for 24 hours. The media containing the antibiotic was replaced with fresh media so that after 24 additional hours, bacteria from the biofilm that survived antibiotic treatment would detach from the biofilm and repopulate the planktonic phase. These bacteria were transferred using a multipronged device (Dan-Kar Corp., Woburn, MA) onto an agar growth plate in order to determine the concentration of the drug that killed the biofilm cells. Minimal bactericidal concentration for planktonic cells (MBC-P) (25): Bacterial strains were inoculated into the wells of the 96-well microtitre dish at the same time as serial dilutions of the antibiotic of choice. The planktonic bacteria were exposed to drug for 24
hours and viability was assessed by transferring a small amount of the culture onto an agar growth plate using a multipronged device.

**Tobramycin accumulation assay.** Biofilms were formed over 24 hours and then exposed to tobramycin for 8 hours. After exposure to tobramycin, the wells containing the biofilms were rinsed with phosphate buffered saline to remove the external tobramycin and the media was replaced with a glycine lysis buffer (0.1 M glycine, pH 3.0). The biofilms were left overnight at 37 °C to allow for complete lysis of the biofilm cells. After ~16 hrs, the biofilm/glycine mixture was evaporated to dryness and resuspended in water. To measure the amount of tobramycin that had accumulated in the biofilms, the resuspended biofilm/glycine mix was spotted onto a lawn of sensitive *E. coli*. The presence of tobramycin was noted as a zone of clearing on the lawn of *E. coli* (25).

**RT-PCR.** Total bacterial RNA was isolated from the planktonic or biofilm cells. Briefly, the planktonic cells were prepared from the log-phase cells subcultured at 37°C in LB broth (OD$_{600}$ of ca. 0.7); while the biofilm cells were prepared from colony biofilms grown on agar plates overnight at 37°C followed by another night at room temperature as described previously (8). The RNA samples were prepared by the Trizol method using Trizol Reagent (Invitrogen). After treatment of the RNA samples with RNase-free DNase I (Sigma), the DNase I was inactivated according to the manufacturer’s instruction. The RNA samples were further purified with the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen) and then quantitated using the SmartSpec Plus Spectrophotometer (Bio-Rad) with the confirmation on agarose gel. Equal amounts of DNase-treated RNA samples were used as templates for reverse transcription (RT) with the iScript cDNA Synthesis Kit (Bio-Rad), followed by PCR amplification of the cDNA
samples with Taq DNA polymerase (Invitrogen). A pair of primers specific for and internal to PA1874 (i.e., PA14_40260 in PA14 strain) (5'-GCGTCGGCATCGATACCAAT-3' [forward] and 5'-ACGATCACCCTCACCCTC-3' [reverse]) was used to amplify mRNA as a measure of PA1874 expression. As a control, the mRNA of the constitutively expressed rpoD gene was amplified and quantitated by the two-step RT-PCR with the primers (5'-
GATCCGGAACAGGTGAAGAC-3' [forward] and 5'-TCAGCAGTTCCACGGAAC-3' [reverse]). RT was performed in a volume of 50 µl with the incubation of 5 min at 25°C, 40 min at 42°C and 5 min at 85°C. PCR was subsequently carried out in a 50 µl reaction containing the primers and the cDNA samples with the incubation of 1 min at 94°C followed by 35 cycles of 1 min at 94°C, 30 s at 56°C, and 20 s at 72°C; and a final step at 10 min at 70°C. The RT-PCR products were analyzed by agarose gel electrophoresis for the expected products (PA1874, 382 bp; rpoD, 226 bp). To control for DNA contamination of RNA samples, non-RT reactions (i.e., PCR) were carried out. In no instance was a product obtained in the absence of a RT reaction.

We estimate the difference in expression between the planktonic and biofilm cells to be 10-fold, based on the fact that dilution of the reaction by 1/10 reduced the planktonic PA1874 signal to zero yet reduced the biofilm PA1874 signal to a level equal to the undiluted planktonic PA1874 signal.

**Cloning of PA1875-1877 genes.** A ca. 4.7-kb fragment containing the putative operon of the PA1875-1877 genes (beginning immediately after the ATG start codon of PA14_40250) was amplified from the genomic DNA of *P. aeruginosa* PA14 using Platinum Taq HF DNA polymerase (Invitrogen) with primers (forward: 5’-
ACAAGAGCTCATCGCAGGCGCAGCAGTCGCAGCGG-3’, *SacI* site underlined; and reverse:
5’-GACCGAATTCTTGCTCCTGACGCTTC-3’, *EcoRI* site underlined). PCR reaction
mixtures were subjected to 35 cycles of 1 min at 94°C, 1 min at 60°C, and 6 min at 70°C before finishing with a 10 min incubation at 72°C. Following digestion of the two purified PCR products with *Sac*I and *Eco*RI enzymes, the fragment was cloned into the *Sac*I/*Eco*RI-restricted pJB866 vector (5) to yield pLZ866A. Subsequently, the plasmid (pLZ866A) obtained in *E. coli* DH5α was used to transform S17-1, from which pLZ866A and pJB866 (empty vector) were mobilized into *P. aeruginosa* PA14 via conjugation (43) by selection of the transconjugants on LB agar containing tetracycline (100 µg/ml) and ciprofloxacin (0.1 µg/ml, for counter selection). The presence of the appropriate plasmids in either *E. coli* or *P. aeruginosa* was confirmed by plasmid isolation, PCR amplification and/or restriction enzyme digestion. The role of the plasmidborne PA14_40250/40240/40230 operon in antibiotic resistance was assessed in LB broth by MIC determination, in which the cells carrying pJB866 or pLZ866A were pre-grown in the presence or absence of 2 mM m-toluic acid.

RESULTS

Identification of genes important for biofilm-specific resistance to antibiotics. We carried out a genetic screen to identify mutants of *P. aeruginosa* that are more sensitive to the antibiotic tobramycin, specifically when this microbe is growing in a biofilm (25). One of the mutants from the screen had a Tn5-insertion in the first gene of a predicted four-gene operon PA14_40250-40240/40230 locus in PA14, which corresponds to PA1874-1877 in PA01 (Fig. 1). The probability that these genes are co-transcribed is high: between 0.94 and 0.99 (40, 42). Based on the published annotation, PA1874 is predicted to encode a large outer membrane protein with sequence similarity to LapA of *P. putida* and Bap of *Staphylococcus aureus* (42). LapA is
important for biofilm formation in *P. fluorescens* and Bap was identified in a screen for mutants of *S. aureus* unable to make a biofilm (9, 15).

The other genes in this operon are predicted to encode an outer membrane protein with homology to an RND efflux pump outer membrane protein (PA1875), an ABC transporter protein (PA1876) and a membrane fusion protein predicted to be part of an ABC transporter (PA1877) (Fig. 1) (42). Given the predicted functions of the gene products of the PA1874-1877 operon and their phenotype in the initial genetic screen, we initiated this study in order to determine if these gene products are important for biofilm-specific efflux of antibiotics.

**Confirmation of biofilm-specific resistance defect.** To confirm the defect in biofilm-specific resistance to tobramycin that we observed in the original transposon insertion mutant from our screen, we obtained individual transposon-insertion mutants in *PA1874, PA1875, PA1876* and *PA1877* from the PA01 transposon-insertion library at the University of Washington Genome Center (17). Using these mutants, we performed biofilm assays, growth assays and determined the MBC-P for tobramycin for each strain and found that the mutant strains all behaved like the wild type strain in these assays (Table 1). However, the MBC-B values for tobramycin for these mutant strains were lower (Table 1) than the MBC-B value for the wild type strain, suggesting that these genes are important for biofilm-specific resistance to tobramycin and confirming our original observation with the original transposon-insertion mutant described above.

Because we did not know if the phenotypes of the transposon-insertion mutants (Table 1) were due to the transposon insertion in *PA1874*, or the polar effect of this transposon insertion on the downstream genes in the operon, we constructed in-frame deletion mutations in of each individual gene in the operon in a PA14 background and tested each knockout mutant for its
biofilm-specific sensitivity-phenotype (Table 2). Interestingly, while knockouts of the three genes predicted to encode components of an efflux pump, PA1875, PA1876 and PA1877, were more sensitive to tobramycin in the MBC-B assay, the PA1874 mutation showed a sensitivity profile identical to the wild type strain, suggesting that the large, outer membrane protein is not important for biofilm-specific resistance to tobramycin (Table 2).

To confirm that the knockout mutant phenotypes were specific to biofilm-resistance, we tested the growth, biofilm formation (using a static 96-well microtitre dish assay) and planktonic sensitivity phenotypes of each mutant. For all three assays, there was no defect in any of the mutants when compared to the wild type; the knockout mutants were able to grow as well as wild type (data not shown), form a biofilm and had a planktonic resistance level (MBC-P) equivalent to the wild type strain (Table 2).

Deletion of the PA1874-1877 operon affects biofilm-specific resistance to several antibiotics. Tobramycin, a key aminoglycoside antibiotic for therapy of P. aeruginosa infection, was used in the screen and the initial experiments. In order to assess the specificity of the biofilm-specific sensitivity phenotype, we determined the MBC-B values of the whole operon deletion (PA1874-1877) for the following antibiotics: gentamicin (aminoglycoside), ciprofloxacin (fluoroquinolone) (Table 3), nalidixic acid (quinolone) and rifampicin. None of the MBC-P values for the various antibiotics differed from that of the wild type strain but MBC-B values for each individual PA1874-1877 deletion mutant were two- to three-fold lower compared to the MBC-B values for the wild type for gentamicin and ciprofloxacin. In the MBC-B assay, there was no loss of viability observed for either the wild type or mutant strains for the maximum deliverable concentration of nalixidic acid and rifampicin (data not shown). Furthermore, using
a Kadouri drip-fed biofilm reactor (27), we confirmed the tobramycin biofilm-sensitivity phenotype of the PA1874-1877 deletion mutant (data not shown). These data suggest that PA1874-1877 is acting like a multidrug efflux pump rather than a specific transporter for one antibiotic or one class of antibiotics.

Loss of the PA1874-1877 operon results in the accumulation of tobramycin in the mutant biofilm. Because the gene products encoded by PA1874-1877 are predicted to function as a transporter, we wanted to determine if the loss of the PA1874-1877 operon had an effect on the ability of the mutant to pump tobramycin out of the cells of the biofilm. Because the PA1874-1877 operon deletion showed no impairment in planktonic resistance, we devised a biofilm-specific accumulation bioassay that was adapted from other efflux/accumulation assays used for planktonic cells (4, 30). Biofilms of the wild type and PA1874-1877 knockout mutant were formed over 24 hours and then exposed to tobramycin for 8 hours. After exposure to tobramycin, the wells containing the biofilms were rinsed with phosphate buffered saline to remove the external tobramycin and the media was replaced with a glycine lysis buffer. The biofilms were left overnight at 37 °C to allow for complete lysis of the biofilm cells. After ~16 hrs, the biofilm/glycine mixture was evaporated to dryness and resuspended in water.

To measure the amount of tobramycin that had accumulated in the biofilms, the resuspended biofilm/glycine mix was spotted onto a lawn of sensitive E. coli (Fig. 2). The presence of tobramycin was noted as a zone of clearing on the lawn of E. coli (25). As predicted, the PA1874-1877 knockout mutant biofilm accumulated more tobramycin than the wild type biofilm, implying that the loss of this predicted efflux system results in an increase in the accumulation of an antibiotic that is normally an efflux substrate.
**PA1874 is more highly expressed in biofilm bacteria.** Deleting the entire **PA1874-1877 operon** or each individual gene in the operon had no effect of the planktonic resistance phenotypes of these mutants but in all cases except for the individual **PA1874** knockout, the biofilm resistance of the mutants was reduced compared to wild type (Table 2). In an effort to understand this biofilm-specific phenotype, we studied the expression profile of the **PA1874** in planktonic and biofilm cells, by semi-quantitative reverse-transcriptase PCR (Fig. 3). We observed that the **PA1874** gene was ~10-fold more highly expressed in biofilm cells compared to planktonic cells (Fig. 3). The control for the experiment, **rpoD**, displayed equal expression levels in planktonic and biofilm cells, as expected. These data suggest that the **PA1874-1877** mutants are specifically impaired in biofilm-specific resistance because the **PA1874-1877** operon is preferentially expressed in the biofilm.

**Cloned PA1875-1877 genes increase the resistance of planktonic cells.** Intact **PA1875-1877** genes from PA14 were cloned into a board-host-range vector, pJB866, which carries **Pm** promoter by induction of **m**-toluic acid (5), resulting in the creation of pLZ866A. Compared with planktonic PA14 cells carrying the vector only, the pLZ866A-containing cells (pre-induced with **m**-toluic acid) showed decreased susceptibility to tobramycin (four-fold MIC change from 0.5 to 2 µg/ml), gentamicin (two-fold MIC change from 4 to 8 µg/ml) and ciprofloxacin (two-fold MIC change from 1 to 2 µg/ml). Consistent with these observations, when pre-induced with **m**-toluic acid, **E. coli** DH5α with pLZ866A also showed decreased susceptibility to tobramycin and gentamicin (2-4 fold MIC increase), but not to ciprofloxacin.
Multiple resistance mechanisms in biofilm-specific resistance. Previously, we showed that ndvB-derived glucans are important for biofilm-specific resistance to tobramycin through a proposed mechanism involving sequestration of the tobramycin by the glucans (25). The glucans are produced in the cytoplasm of the cell, yet function in the periplasm, and possibly the extracellular medium. Since we have not identified the glucan transporter, nor have we identified the whole range of substrates of the PA1875-1877 transporter, it is formally possible that the products of the PA1874-1877 operon transport the ndvB-derived glucans, in addition to antibiotics, thus contributing to a documented mechanism of biofilm-specific resistance.

To test whether ndvB and PA1875-1877 function in the same pathway, we constructed a mutant combining the ndvB mutation and the PA1874-1877 deletion, and tested the antibiotic resistance of this strain mutant in the 96-well microtitre dish assay (Table 3). The MBC-P values for tobramycin, gentamicin and ciprofloxacin for this mutant were two-fold lower than the wild type strain, indicating a small role for these genes in planktonic resistance. However, the MBC-B of the ndvB and PA1875-1877 mutant to tobramycin, gentamicin and ciprofloxacin was 8-fold lower than the wild type strain and 2-fold lower than each individual mutation. This cumulative effect of the combined ndvB and PA1875-1877 mutations over the phenotypes of the single mutants (Table 2) (25) implies that these two genes contribute to two different mechanisms of resistance in biofilms, supporting the hypothesis that there are multiple mechanisms of resistance in these surface-attached communities.

DISCUSSION

Biofilm cells are notably more resistant to antimicrobial agents than their planktonic counterparts. The protective mechanisms are not well understood. We present the identification
of a novel biofilm-specific efflux pump in P. aeruginosa. Our conclusions are based on the gene annotations of PA1874-1877 provided by the Pseudomonas genome database (42), confirmation of the importance of these genes in biofilm-specific resistance to antibiotics (Tables 1 and 2) and demonstration of the accumulation of tobramycin in the PA1874-1877 operon knockout mutant biofilm (Fig. 2). Furthermore, MIC results from the planktonic cells carrying the cloned genes have suggested that the PA1874-1877 operon contributes resistance to multiple antibiotics.

The increased accumulation of tobramycin in the PA1874-1877 operon knockout mutant could be due to either an increase in permeability or a decrease in efflux. We favor the idea that the increase in accumulation is due to a decrease in efflux because deletion of PA1874 has no effect on biofilm-resistance to tobramycin, while deletion of the individual components of the predicted efflux pump, PA1875, PA1876 and PA1877, reduces biofilm-resistance to two classes of antibiotic (Table 2). If the increase in accumulation of tobramycin in the mutant biofilm were due to altered permeability, we would expect that deletion of PA1874, a predicted large outer membrane protein, would have the greatest effect on biofilm-specific resistance to tobramycin.

Based on the annotations compiled by the Pseudomonas aeruginosa Community Annotation project, it is likely that the PA1875-1877 operon encodes an ABC transporter. PA1876 is predicted to be an ATP-binding protein due to the presence of several conserved motifs, including Walker boxes A and B, suggesting that the pump should be classified as an ABC transporter (10, 42). Interestingly, there has not been an antibiotic-specific ABC transporter identified in P. aeruginosa. PA1875 has homology to OprM, an outer membrane component of at least two different RND efflux systems in P. aeruginosa (1, 29). In fact, PA1875 has been named opmL (42), for outer membrane protein M family, protein L (18). Additionally, PA1875 is homologous to tolC, the E. coli oprM homolog. TolC is the outer
membrane component for several RND and ABC transporters in *E. coli* (19). Finally, the protein product of *PA1877* is a predicted membrane fusion protein of the ABC transporter family that is homologous to HlyD, the membrane fusion protein of the *E. coli* α–hemolysin secretion system (13).

We have shown that *PA1874* is not required for biofilm-specific resistance to tobramycin, gentamicin and ciprofloxacin (Tables 1 and 2). However, the protein product of *PA1874* may be one of the substrates of this efflux system as it is predicted to encode an outer membrane protein (42). Interestingly, *PA1875-1877* show strong sequence similarity to the genes that encode the ABC transporter system for LapA, another large outer membrane protein with homology to PA1874. LapB is an ATPase with 58% identity to PA1876, LapC is a membrane fusion protein with 57% identity to PA1877 and LapE is an outer membrane protein with 42% similarity to PA1875 (15).

The demonstration of the biofilm-specific expression of *PA1874-1877* (Fig. 3) provides an explanation for the observation that mutations of this operon affect biofilm resistance to antibiotics but not planktonic resistance. This type of biofilm-specific expression was also observed for *ndvB*, another gene shown to be important for biofilm-specific resistance to tobramycin (25). None of these genes have been identified as biofilm-specific genes in genomic and proteomic studies (37, 38, 41), likely due to differences between *P. aeruginosa* strains and experimental conditions used in these studies, but they may represent a class of genes that are regulated by a common regulatory system that is turned on at different stages in the biofilm lifecycle.

Several factors, such as the extracellular polysaccharide matrix, high cell density and slow growth, have been implicated in the increase in resistance that biofilm cells exhibit over
their planktonic counterparts (24). Due to the success of a genetic screen that we carried out, some additional mechanisms are being proposed (25). Individually, these factors and mechanisms might only add a modest level of resistance, as evidenced by the 2- or 3-fold reduction in resistance that we have observed in our studies (Table 2) (25). However, when added together, they contribute to the overall level of increased resistance that allows biofilms to survive in conditions where their planktonic counterparts do not.

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504
TABLE 1: Phenotypes of PA01 and PA1874-1877 operon transposon (tn)–insertion mutants of 
P. aeruginosa

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<th>Tobramycin MBC-B (µg/ml)</th>
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TABLE 2: Phenotypes of PA14 and PA1874-1877 knockout mutants of *P. aeruginosa*

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TABLE 3: MBC-P and MBC-B values for tobramycin, gentamicin and ciprofloxacin for PA14, \( \Delta ndvB \), \( \Delta PA1874-1877 \) and a double \( ndvB/PA1874-1877 \) mutant

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<td>MBC-B</td>
<td>MBC-P</td>
</tr>
<tr>
<td>PA14</td>
<td>32*</td>
<td>100*</td>
<td>64**</td>
</tr>
<tr>
<td>( \Delta PA1874-1877 )</td>
<td>32*</td>
<td>25*</td>
<td>64</td>
</tr>
<tr>
<td>( \Delta ndvB )</td>
<td>32**</td>
<td>25**</td>
<td>64**</td>
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<td>( \Delta ndvB/PA1874-1877 )</td>
<td>16</td>
<td>12.5</td>
<td>32</td>
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* results presented in Table 2

** results presented in ref. 25
Figure 1: Genomic context of PA1874-1877 operon

PA01 gene designations are indicated in the figure. PA14 gene designations are as follows:
PA1874=PA14_40260, PA1875=PA14_40250, PA1876=PA14_40240 and PA1877=PA14_40230. Predicted location or function of the protein product is listed below (42).

Figure 2: Tobramycin accumulates in the PA1874-1877 deletion mutant biofilm but not the wild type biofilm.

Wild type and mutant (Δ1874-1877) biofilms were exposed to 200 µg/mL tobramycin. The control biofilms were not exposed to tobramycin. The amount of tobramycin that had accumulated in the biofilms was observed as a zone of clearing on the lawn of E. coli, using the white disks as focal points.

Figure 3: Analysis of PA1874 expression by RT-PCR

Expression of PA1874 gene in planktonic (lanes 6 and 7) and biofilm (lanes 8 and 9) cells of P. aeruginosa PA14 measured by RT-PCR amplification of RNA with primers specific to PA1874 gene (lanes 6 to 9) and rpoD gene (control, lanes 2 to 5). The RNA templates were isolated from planktonic and biofilm cells, respectively, and the different amounts of the templates were used (undiluted in lanes 2, 4, 6 and 8; 1:10 dilution in lanes 3, 5, 7 and 9; data with 1:100 dilution not shown). DNA mass markers (0.1 to 12 kb) (1 kb plus DNA ladder; Invitrogen) are shown in lane 1.
<table>
<thead>
<tr>
<th>Year</th>
<th>Protein/Transporter</th>
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<tr>
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<tr>
<td>1875</td>
<td>OpmL</td>
</tr>
<tr>
<td>1876</td>
<td>ABC transporter</td>
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