

## Influence of the MexAB-OprM Multidrug Efflux System on Quorum Sensing in *Pseudomonas aeruginosa*

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***Pseudomonas aeruginosa* nalB mutants which hyperexpress the MexAB-OprM multidrug efflux system produce reduced levels of several extracellular virulence factors known to be regulated by quorum sensing. Such mutants also produce less acylated homoserine lactone autoinducer PAI-1, consistent with an observed reduction in lasI expression. These data suggest that PAI-1 is a substrate for MexAB-OprM, and its resulting exclusion from cells hyperexpressing MexAB-OprM limits PAI-1-dependent activation of lasI and the virulence genes.**

*Pseudomonas aeruginosa* is an opportunistic human pathogen characterized by an innate resistance to a wide array of antimicrobial agents. Once attributed to a highly impermeable outer membrane (24), this property is now recognized to result from the operation of broadly specific drug efflux pumps which act synergistically with low outer membrane permeability to elicit multidrug resistance (20). One such efflux system, encoded by the *mexAB-oprM* operon (11, 34, 35), expels a range of antibiotics, including tetracycline, chloramphenicol, quinolones,  $\beta$ -lactams, novobiocin, macrolides, and trimethoprim (11, 14, 17, 18). Although expressed in wild-type cells (7), the operon is hyperexpressed in *nalB* mutants (36), which display markedly elevated levels of resistance to substrate antibiotics.

Homologues of this system have been reported for *P. aeruginosa* (*mexCD-oprJ* [33]; *mexEF-oprN* [15]), *Escherichia coli* (*acrAB-tolC*) (9, 20), *Neisseria gonorrhoeae* (*mtrCDE*) (12), and *Burkholderia cepacia* (*ceoA-ceoB-opcM*) (4, 5). An *oprM* gene probe was used to demonstrate the presence of *oprM* homologues in *Burkholderia pseudomallei* and *Pseudomonas putida* (2), suggesting the presence of such systems in these organisms as well. Although the aforementioned systems all play a role in resistance to clinically relevant antibiotics, the likely natural function has been addressed only with respect to the *E. coli* AcrAB and *N. gonorrhoeae* MtrCDE systems, which appear to play a role in the export of toxic environmental lipids or hydrophobic agents (e.g., bile salts) (12, 19, 44).

During studies intended to elucidate the natural function of the MexAB-OprM efflux system, including the identification of natural, cell-associated substrates, we noted an inverse correlation between the presence of *mexAB-oprM* in *P. aeruginosa* and the production of the blue-green pigment pyocyanin, a virulence factor in this organism (6). (Intriguingly, a similar observation was made regarding the *mexEF-oprN* operon: strains expressing this system were demonstrably pyocyanin deficient compared to strains lacking this system [15]). A more detailed study subsequently revealed that this effect on pyocyanin was due to the apparent influence of MexAB-OprM on

autoinducer (AI) levels, pyocyanin production being AI dependent (16).

AIs are a family of acylated homoserine lactones found in a number of gram-negative bacteria whose accumulation in the growth medium mirrors cell density, triggering the expression of certain target genes upon reaching a critical AI (i.e., cell concentration) (10). Quorum sensing, as this process is now known, involves an AI synthase, which produces AI destined for release into the growth medium, and a transcriptional activator, which acts in concert with the AI upon its reentry into cells to activate target genes in response to increases in bacterial cell density (10). Two homoserine lactone AIs have been characterized in detail for *P. aeruginosa*, *N*-(3-oxo)-dodecanoyl-L-homoserine lactone (29) (also called PAI-1 [31]) and *N*-butanoyl-L-homoserine lactone (30) (also called PAI-2 [10]), synthesized by the products of the *lasI* (28) and *rhII* (*vsmI*) (16, 25) genes, respectively. Together with their cognate quorum-sensing regulators, LasR (31) and RhIR (26) (also called VsmR [16]), these act to stimulate production of a number of extracellular virulence factors in *P. aeruginosa* (16, 31). We report here that hyperexpression of MexAB-OprM compromises production of PAI-1 and, thus, expression of LasR-LasI-dependent virulence factors. Apparently, reentry of PAI-1 is prevented by the efflux activity of MexAB-OprM, leading to a reduction in intracellular PAI-1 and, thus, reduced expression of PAI-1-dependent genes.

Strains used in this study are listed in Table 1. Luria (L) broth (Difco), pyocyanin production broth (6), and peptone tryptic soy broth (27) have been described previously. Assays for the exoproducts pyocyanin (6), elastase (27), and casein protease (13) have been previously described. A *nalB* derivative of streptomycin-resistant PAO1 strain K1171 (designated K1168) was selected on L agar containing 0.4  $\mu$ g of ciprofloxacin and 100  $\mu$ g of carbenicillin per ml as described previously and was screened for *nalB*-type multidrug resistance (43) and OprM hyperexpression by a Western immunoblotting procedure with an OprM-specific antiserum (42). The use of a streptomycin-resistant strain was necessitated by the need to subsequently introduce a *mexAB-oprM* deletion (via conjugation; see below) into the *nalB* strain via a procedure involving streptomycin counterselection of the donor strain. For the construction of *mexAB-oprM* deletion strain K1169, vector pELCT04 was constructed. First, the mercury resistance  $\Omega$

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TABLE 1. Bacterial strains

Strain	Description <sup>a</sup>	Source or reference
<i>P. aeruginosa</i>		
PAO1 (K767) <sup>b</sup>	Prototroph	
OCR1	PAO1 (K767) <i>nalB</i>	21
K784	Spontaneous Sm <sup>r</sup> derivative of OCR1	36
K1170	K784 $\Delta$ <i>mexAB-oprM</i>	This study
PAO1 (K867) <sup>b,c</sup>	Prototroph; Cm <sup>r</sup>	B. H. Iglewski
K1171	Spontaneous Sm <sup>r</sup> derivative of K867	This study
K1168	K1171 <i>nalB</i>	This study
K1169	K1168:: $\Delta$ <i>mexAB-oprM</i>	This study
PDO100	PAO1 (K867) $\Delta$ <i>rhlI</i> ::Tn501	3
PAO-JP1	PAO1 (K867) $\Delta$ <i>lasI</i> :: <i>tet</i>	B. H. Iglewski
<i>E. coli</i>		
DH5 $\alpha$	<i>endA hsdR17 supE44 thi-1 recA1 gyrA relA1</i> $\Delta$ ( <i>lacZYA-argF</i> )U169 <i>deoR</i> [ $\phi$ 80 <i>dlac</i> $\Delta$ ( <i>lacZ</i> )M15]	1
S17-1	<i>thi pro hsdR recA</i> Tra <sup>+</sup>	41

<sup>a</sup> Cm<sup>r</sup>, chloramphenicol resistant; Tra<sup>+</sup>, mobilizes nonconjugative *mob*-carrying vectors.

<sup>b</sup> Two different PAO1 strains were used in this study and are distinguished by the laboratory designations K767 and K867.

<sup>c</sup> This PAO1 strain displays substantial resistance to chloramphenicol, in contrast to PAO1 strain K767.

Hg interposon from pHP45:: $\Omega$  Hg (8) was cloned into *Hind*III-restricted pK18*mobsacB* (38) on a 4.6-kb *Hind*III fragment, to yield pELCT02. The previously constructed *mexAB-oprM* deletion fragment was then cloned from vector pRSP14 (43) into pELCT02 on a 1.4-kb *Bam*HI fragment to yield pELCT04. All manipulations were carried out with *E. coli* DH5 $\alpha$ . Following transformation (37) of pELCT04 into *E. coli* S17-1, the vector was mobilized into *P. aeruginosa* *nalB* strain K1168 via conjugation as described previously (34) and pELCT04-containing *P. aeruginosa* was selected on L agar supplemented with 15  $\mu$ g of HgCl<sub>2</sub> per ml (to select the vector) and 10  $\mu$ g of tetracycline per ml (to counterselect *E. coli* S17-1). HgCl<sub>2</sub>-resistant colonies were recovered and streaked for single colonies on L agar containing 10% (wt/vol) sucrose. Sucrose-resistant colonies were screened for loss of HgCl<sub>2</sub> resistance (and kanamycin resistance), and those carrying the *mexAB-oprM* deletion were identified following PCR amplification of chromosomal DNA with *Taq* DNA polymerase as described previously (43). Where indicated, AIs PAI-1 and PAI-2, synthesized as described previously (29, 30), were included in the culture medium at a final concentration of 0.5 to 5  $\mu$ M. In cross-streaking experiments, bacteria were streaked onto the surfaces of L agar plates at right angles so that areas of bacterial growth approached but did not contact. Pyocyanin production was then assessed visually on plates, although control experiments confirmed that the pigment observed was pyocyanin. This involved the recovery of pigmented agar, following the removal of bacterial cells, and extraction and assay of pyocyanin as described above. AI levels were quantitated by previously described bioassays (29, 30) following the extraction of AIs from cell-free culture supernatant with ethyl acetate (29). Synthetic PAI-1 and PAI-2 were used to construct a standard dose-response curve, which permitted the quantification of the extracted AIs based on the results of the bioassay (29). Expression of *lasI* was assessed with a plasmid-borne *lasI-lacZ* fusion vector (40).  $\beta$ -Galactosidase assays were carried out as described previously (22) with cells cultured in pyocyanin production broth.

Cultures of *P. aeruginosa* PAO1 (strain K767) elicited a blue-green pigment, reminiscent of pyocyanin, during growth

on L agar (Table 2). Indeed, extraction of this pigment from agar plates and subsequent spectrophotometric examination confirmed it as pyocyanin (data not shown). Interestingly, the *nalB* derivative of this strain, OCR1, lacked this pigmentation (Table 2). When both strains were cultured on the same L-agar plate, however, OCR1 growth in the vicinity of PAO1 strain K767 growth was pigmented (Table 2). This suggested that the *nalB* strain was deficient in pyocyanin production and that PAO1 strain K767 produced something that restored pyocyanin production in OCR1. Similarly, a second PAO1 strain, K867, and its streptomycin-resistant derivative, K1171, were also pyocyanin proficient in L broth, while a *nalB* derivative of K1171, strain K1168, was pyocyanin deficient (Table 2). Moreover, elimination of *mexAB-oprM* in either OCR1 or K1168 (yielding K1170 and K1169, respectively) restored pyocyanin production (Table 2). Thus, there appeared to be an inverse correlation between levels of *mexAB-oprM* expression and pyocyanin production by *P. aeruginosa*.

Although a possible explanation for the above-mentioned influence of *mexAB-oprM* on pyocyanin production was that *nalB* strains expel a precursor necessary for pyocyanin production, the latter being a substrate for MexAB-OprM, the observation that PAO1 could, in effect, cross-feed OCR1 (Table 2), restoring pyocyanin production, argued against this. Intriguingly, cells overexpressing the *mexAB-oprM* operon (e.g., OCR1) failed to elicit this cross-feeding phenomenon, while those deficient in or with reduced (such as the wild type) *mexAB-oprM* expression were proficient at cross-feeding (Table 2). Given that pyocyanin production is regulated by quorum sensing (3, 16), however, it was likely that strains overexpressing MexAB-OprM (e.g., OCR1 and K1168) were somehow defective in the quorum-sensing process. Moreover, given their inability to cross-feed but their ability to be cross-fed, it was likely that they were defective in a diffusible component of quorum sensing, namely the AI. Consistent with this, pyocyanin produced by K1168 in liquid culture could be increased (by two-fold) upon the addition of 1 to 2  $\mu$ M PAI-1 (data not shown). Initially, the *nalB* strain K1168 was examined for the production of pyocyanin and additional AI-dependent components, including elastase and casein protease (3), to see if there was, indeed, a general deficiency in quorum sensing in this strain. An examination of pyocyanin (Fig. 1A), casein protease (Fig. 1B), and elastase (Fig. 1C) levels revealed that K1168 produced reduced levels of these compared with levels produced by the parent strain, K1171. Deletion of *mexAB-oprM* in K1168 restored the production of pyocyanin (Fig. 1A), casein pro-

TABLE 2. Pyocyanin production by *P. aeruginosa*

Strain	Efflux genotype	Pyocyanin production <sup>a</sup>	Cross-feeds OCR1 <sup>b</sup>
K767	Wild type	+	+
OCR1	<i>nalB</i>	-	-
K784	<i>nalB</i>	-	-
K1170	<i>nalB</i> $\Delta$ <i>mexAB-oprM</i>	+	ND
K1171	Wild type	+	ND
K1168	<i>nalB</i>	-	ND
K1169	<i>nalB</i> $\Delta$ <i>mexAB-oprM</i>	+	ND
PAO-JP1	Wild type	-	-
PDO100	Wild type	-	+

<sup>a</sup> Assessed visually following growth on L agar. +, produces pyocyanin; -, no pyocyanin produced.

<sup>b</sup> The indicated strains were streaked at right angles to OCR1 on L agar, and production of pyocyanin by OCR1 was assessed as described above in the vicinity of the cross-streaked strain. +, cross-feeds OCR1; -, does not cross-feed OCR1; ND, not determined.

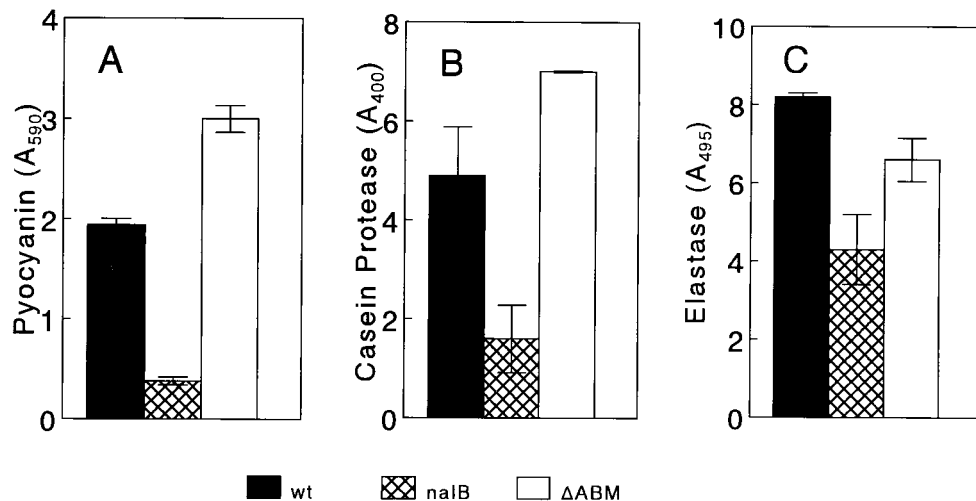


FIG. 1. Production of AI-dependent virulence factors as a function of *mexAB-oprM* expression. *P. aeruginosa* K1171 (wild type [wt] for *mexAB-oprM*), K1168 (*nalB*), and K1169 (*nalB*  $\Delta$ *mexAB-oprM*;  $\Delta$ ABM) were examined for the production of elastase (values reported have been multiplied by 100), casein protease (values reported have been multiplied by 10), and pyocyanin. Results reported are per milliliter of cells at an  $A_{600}$  of 1.0 and are the means of the results of three separate experiments  $\pm$  the standard deviations.

tease (Fig. 1B), and elastase (Fig. 1C) in the resultant strain, K1169, indicating that the quorum-sensing defect identified in *nalB* strain K1168 resulted from overexpression of the efflux pump and not some other manifestation of the *nalB* mutation.

These data strongly argued that *nalB* strains were AI deficient. To assess this directly, we measured the levels of PAI-1 and PAI-2 in spent culture supernatants of K1171, K1168, and K1169 by previously described bioassays (29, 30). As can be seen in Fig. 2A, *nalB* strain K1168 consistently produced ca. threefold less PAI-1 than its parent strain, K1171 (wild type with respect to MexAB-OprM). A similar result was observed for a number of independently isolated *nalB* derivatives of *P. aeruginosa* (data not shown). Interestingly, this decline in PAI-1 levels was abrogated upon the deletion of the *mexAB-oprM* efflux genes (see K1169; Fig. 2A), indicating that this reduction was a function solely of MexAB-OprM overproduction. In contrast, PAI-2 levels remained constant in all three strains (Fig. 2B), indicating that PAI-2 production is not influenced by the status of MexAB-OprM.

It is apparent from these data that MexAB-OprM hyperexpression yields a specific decline in PAI-1 levels which is correlated with a decline in the levels of several known AI-dependent products. That PAI-1 levels alone were impacted in the *nalB* strain was, in fact, consistent with the observation that an *rhlI* mutant, which produces PAI-1 but not PAI-2, was able to cross-feed *nalB* strain OCR1 (with respect to pyocyanin production) while a *lasI* mutant (produces no PAI-1) was not (Table 2). One explanation for these observations is that a precursor for PAI-1 synthesis is exported by MexAB-OprM, leading to a reduced synthesis of this AI in MexAB-OprM-overexpressing *nalB* strains such as K1168. Still, in light of evidence indicating that AIs are synthesized from *S*-adenosylmethionine and acylated-acyl carrier protein (23, 39), neither of which is a likely candidate for export via MexAB-OprM, this is improbable. Alternatively, PAI-1, but not PAI-2, may be a substrate for the MexAB-OprM efflux system. According to currently accepted models of quorum sensing, whereby AI released by cells in a population accumulates in the extracellular milieu and then diffuses back into the cell to stimulate the expression of cell density-dependent genes, the increased expression of MexAB-OprM in a *nalB* strain would serve to compromise this reentry of PAI-1. The resulting reduction in

PAI-1 accumulation within the cell would limit LasR-PAI-1 formation and subsequent activation of target genes (e.g., elastase and casein protease). Moreover, since *lasI* expression is also LasR-PAI-1 dependent (31), this would also lead to a reduction in PAI-1 synthesis in a *nalB* strain. In fact, we did

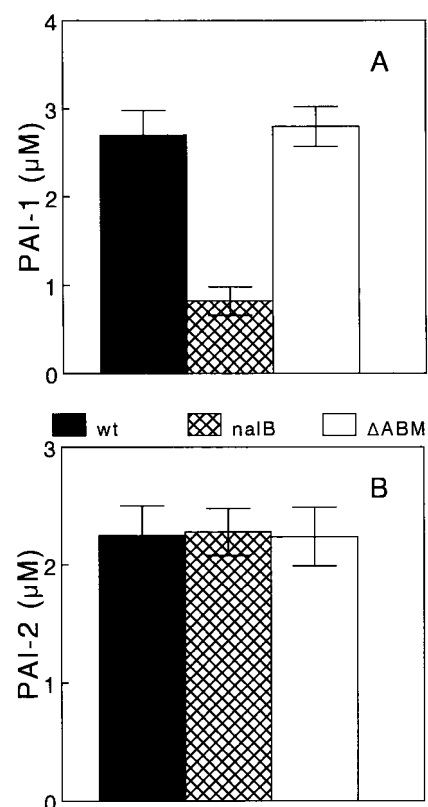


FIG. 2. AI production as a function of *mexAB-oprM* expression. PAI-1 (A) and PAI-2 (B) levels were measured in cell-free supernatants of 18-h cultures of *P. aeruginosa* K1171 (wild type for *mexAB-oprM*; wt), K1168 (*nalB*), and K1169 (*nalB*  $\Delta$ *mexAB-oprM*;  $\Delta$ ABM) as described in the text. Values reported are the means of results from five separate experiments  $\pm$  the standard deviations.



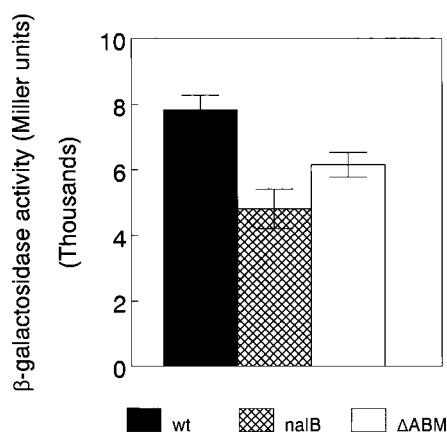


FIG. 3.  $\beta$ -Galactosidase activities of 18-h cultures of *P. aeruginosa* K1171 (wild type for *mexAB-oprM*; wt), K1168 (*nalB*), and K1169 (*nalB*  $\Delta$ *mexAB-oprM*;  $\Delta$ ABM) harboring *lasI-lacZ* fusions. Values reported are the means of results from three separate experiments  $\pm$  the standard deviations.

observe a ca. twofold decrease in *lasI* expression in *nalB* strain K1168 relative to expression in its parent strain (Fig. 3), consistent with this decline in PAI-1 levels in K1168. Although LasR-PAI-1 does not directly regulate pyocyanin biosynthesis, which appears to be controlled by the RhlRI system (16), *rhlRI* gene expression is positively regulated by LasR-PAI-1 (32) and *lasI* mutants are compromised as regards pyocyanin production (7). Thus, any reduction in PAI-1 formation would be expected to yield a decrease in pyocyanin levels.

What is less clear, given the involvement of LasR-PAI-1 in *rhlRI* gene expression, is why PAI-2 levels were not altered in a *nalB* strain. Certainly, in light of the effect on pyocyanin production, a *nalB* strain is compromised as regards the operation of some component of the *rhl* quorum-sensing system. Perhaps *rhlI* expression is less sensitive to changes in LasR-PAI-1 than is that of *rhlR*. Similarly, since RhlR is required for the expression of *rhlI* and the production of pyocyanin, the latter may be more affected by any decline in RhlR levels than is *rhlI*. Moreover, given suggestions that PAI-1 antagonizes PAI-2 association with RhlR (32), a reduction in PAI-1 might lessen this effect, enhancing PAI-2 interaction with available RhlR molecules. Thus, while there might be less RhlR in a *nalB* strain, what is present might be more active as a result of increased association with PAI-2.

Given that efflux gene hyperexpression compromises PAI-1 and AI-dependent virulence gene expression, it will be of interest to determine whether *nalB* strains are less virulent, despite their increased multidrug resistance. Moreover, the impact of MexAB-OprM on quorum sensing, possibly by compromising the reentry of PAI-1 into cells, represents the first *P. aeruginosa*-associated process or substrate which is influenced by this efflux system. Still, given the broad substrate specificity of MexAB-OprM, it is highly unlikely that the export of a quorum-sensing-related molecule such as PAI-1 would be a specific function of MexAB-OprM, and therefore any modulation of quorum sensing in response to MexAB-OprM is probably a secondary effect of its primary and hitherto unidentified primary role in the cell.

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