ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1998, p. 399–403 Vol. 42, No. 2
0066-4804/98/$04.00+0


# β-Lactamase Inhibitors Are Substrates for the Multidrug Efflux Pumps of *Pseudomonas aeruginosa*

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Received 10 June 1997/Returned for modification 9 October 1997/Accepted 1 December 1997

**The MexAB-OprM multidrug efflux system exports a number of antimicrobial compounds, including β-lactams. In an attempt to define more fully the range of antimicrobial compounds exported by this system, and, in particular, to determine whether β-lactamase inhibitors were also accommodated by the MexAB-OprM pump, the influence of pump status (its presence or absence) on the intrinsic antibacterial activities of these compounds and on their abilities to enhance β-lactam susceptibility in intact cells was assessed. MIC determinations clearly demonstrated that all three compounds tested, clavulanate, cloxacillin, and BRL42715, were accommodated by the pump. Moreover, by using β-lactams which were readily hydrolyzed by the *Pseudomonas aeruginosa* class C chromosomal β-lactamase, it was demonstrated that elimination of the *mexAB-oprM*-encoded efflux system greatly enhanced the abilities of cloxacillin and BRL42715 (but not clavulanate) to increase β-lactam susceptibility. With β-lactams which were poorly hydrolyzed, however, the inhibitors failed to enhance β-lactam susceptibility in MexAB-OprM$^+$ strains, although BRL42715 did enhance β-lactam susceptibility in MexAB-OprM$^-$ strains, suggesting that even with poorly hydrolyzed β-lactams this inhibitor was effective when it was not subjected to efflux. MexEF-OprN-overexpressing strains, but not MexCD-OprJ-overexpressing strains, also facilitated resistance to β-lactamase inhibitors, indicating that these compounds are also substrates for the MexEF-OprN pump. These data indicate that an ability to inactivate MexAB-OprM (and like efflux systems in other bacteria) will markedly enhance the efficacies of β-lactam–β-lactamase inhibitor combinations in treating bacterial infections.**

*Pseudomonas aeruginosa* is an opportunistic human pathogen characterized by an innate resistance to a variety of antimicrobial agents. Previously attributed to a highly impermeable outer membrane (22), this resistance is now recognized to result from the synergy between broadly specific drug efflux pumps and low outer membrane permeability (16). One such efflux system, encoded by the *mexAB-oprM* operon (8, 28, 29), effluxes a range of antibiotics, including tetracycline, chloramphenicol, quinolones, β-lactams, novobiocin, macrolides, and trimethoprim (8, 9, 12, 29). Expressed constitutively in wild-type cells, where it contributes to intrinsic drug resistance (5, 12, 29), the operon is hyperexpressed in *nalB* mutants (30), producing elevated levels of resistance to substrate antibiotics (8, 9, 12, 29). Homologous efflux systems encoded by the *mexC-mexD-oprJ* (27) and *mexE-mexF-oprN* (10) operons have also been described. Apparently not expressed during growth under normal laboratory conditions, these systems are expressed in *nfxB* (27) and *nfxC* (10) multidrug-resistant mutants, respectively. *nfxB* strains are resistant to chloramphenicol, tetracycline, quinolones, macrolides, novobiocin, and newer cephalosporins such as cefepime and cefpirome but display hypersusceptibility to most β-lactam antibiotics (18). *nfxC* strains exhibit resistance to chloramphenicol, trimethoprim, quinolones, and carbapenems, including imipenem, although the resistance to imipenem results from the loss of the porin protein OprD in these mutants and not from the overexpression of MexEF-OprN (6, 10).

The tripartite efflux pumps consist of an inner membrane component (MexB, MexD, and MexF) which functions as a resistance-nodulation-division family $H^+$ antiport exporter (21, 31), an outer membrane, a presumed channel-forming component (OprM, OprJ, and OprN) (16, 23), and a so-called membrane fusion protein predicted to link the membrane-associated efflux components (MexA, MexC, and MexE) (16, 23). Recent data suggest that the operation of MexAB-OprM (and by analogy the remaining efflux systems) is at least partially dependent upon the TonB energy-coupling protein implicated in the opening of outer membrane gated channels responsible for iron-siderophore uptake across the *P. aeruginosa* outer membrane (36). Thus, the outer membrane components of these efflux pumps may be gated channels.

In an effort to further define the range of antibiotic compounds which are accommodated by the known *P. aeruginosa* efflux systems, we examined β-lactamase inhibitors as possible pump substrates by assessing the influence of pump status (its presence or absence) on the intrinsic antibacterial activities of these compounds and on their abilities to enhance the efficacies of β-lactam compounds.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacterial strains used in this study are listed in Table 1. K1115 was derived from K1114 via the introduction of a *mexAB-oprM* deletion by a previously described approach (34). K1117 and K1118 were selected by plating 100 μl of a 10-fold-concentrated overnight culture of K1115 and K1116, respectively, onto L-agar plates supplemented with ciprofloxacin (0.2 μg/ml) and chloramphenicol (150 μg/ml). Colonies arising after 48 h of growth were screened for additional antibiotic resistances, and two such multidrug-resistant isolates, K1117 and K1118, were selected for further study. Luria-Bertani broth (1% [wt/vol] Difco tryptone, 0.5% [wt/vol] Difco yeast extract, 0.5% [wt/vol] NaCl) was the growth medium used throughout the study. Bacteria were cultivated at 37°C with shaking (200 rpm) except during susceptibility testing, during which cultures were not shaken.

**Antibiotics.** Ampicillin, carbenicillin, cephaloridine, piperacillin, and cloxacillin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Clavulanate (lithium salt) and BRL42715 were kindly provided by SmithKline Beecham Pharma Inc. (Oakville, Ontario, Canada). Imipenem (Merck Sharp Dohme Can-

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TABLE 1. *P. aeruginosa* strains used in this study

| Strain | Description | Reference or source |
|---|---|---|
| ML5087 | *aphA ilv-220 leu-9001 met-9011 pur-67 thr-9001* | 25 |
| K1110 | ML5087 Δ*oprM* | This study |
| K1121 | ML5087 Δ*mexAB-oprM* | 34 |
| K1131 | K1121 *nfxB* | 34 |
| K1112 | ML5087 *nalB* | 33 |
| K1113 | K1112 Δ*oprM* | This study |
| K1114 | ML5087 Δ*mexCD-oprJ* | 34 |
| K1111 | ML5087 *nfxB* | 33 |
| K1115 | ML5087 Δ*mexCD-oprJ* Δ*mexAB-oprM* | This study |
| K1117 | Multidrug-resistant derivative of K1115; elevated expression of OprN | This study |
| K1116 | ML5087 Δ*mexCD-oprJ* Δ*oprM* | This study |
| K1118 | Multidrug-resistant mutant of K1116; elevated expression of OprN | This study |
| K767 | PAO1 prototroph | 17 |
| K1119 | PAO1 Δ*mexAB-oprM* | This study |
| OCR1 | PAO1 *nalB* | 17 |

ada) was purchased from the pharmacy at the Kingston General Hospital. The concentrations reported take into account the fact that this source of the antibiotic is a mixture. Its use, however, was restricted to induction of β-lactamase and not susceptibility testing. Nitrocefin (Glaxo) was purchased from Becton Dickinson and Company (Cockeysville, Md.).

**Drug susceptibility testing.** Susceptibility testing was carried out by the two-fold serial broth dilution method with an inoculum of $5 \times 10^5$ cells/ml (12). Data were reported as MICs, which reflected the lowest concentration of antibiotic inhibiting visible growth after 18 h of incubation. In some experiments β-lactamase inhibitors were included to ascertain their effects on β-lactam MICs.

**β-Lactamase activity.** The induction and assay of the *P. aeruginosa* chromosomal β-lactamase were based on a previously published protocol (14). Briefly, stationary-phase cells were diluted 1:59 into 30 ml of prewarmed (37°C) Luria-Bertani broth and incubated (with shaking) for 2 h at 37°C. Following the addition of imipenem (0.25 μg/ml) (to induce the chromosomal β-lactamase), the cultures were incubated with shaking for an additional 3 h, at which time they were harvested by centrifugation (5,000 × *g* for 10 min). Cell pellets were washed once with 50 mM sodium phosphate buffer (pH 7.2) and were resuspended in a final volume of 2 ml of the same buffer. Following disruption of the cells on ice with sonication (three 30-s pulses at 50% maximum power with a Vibra Cell sonicator [Sonics and Materials Inc., Danbury, Conn.]), the cell lysate was centrifuged at 150,000 × *g* for 30 min at 4°C and the β-lactamase-containing supernatant was retained. Two different substrates, cephaloridine and nitrocefin, were used to assess β-lactamase activity. In the first instance, 3 μl of supernatant was incubated at room temperature with cephaloridine (final concentration, 100 μM) in a final volume of 1 ml of assay buffer (50 mM sodium phosphate buffer [pH 7.2]), and hydrolysis of cephaloridine was monitored spectrophotometrically at a λ value of 260 nm. In the latter instance, 2 to 4 μl of a 1:49 dilution of the β-lactamase-containing supernatant was added to nitrocefin (final concentration, 100 μM) at room temperature in a final volume of 1 ml of assay buffer, and nitrocefin hydrolysis was measured spectrophotometrically at a λ value of 482 nm. To assess β-lactamase inhibition by the inhibitors, the aforementioned assays were repeated by the method of Dixon (4) by using cephaloridine as a substrate at concentrations of 50 and 100 μM. In some experiments, the β-lactamase inhibitors cloxacillin (100 μg/ml) and BRL42715 (20 μg/ml) replaced imipenem as inducers of β-lactamase.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblotting.** Cell envelopes of *P. aeruginosa* were prepared as described previously (20) and were electrophoresed on 11% (wt/vol) acrylamide gels (15) prior to being electroblotted onto Immobilon-P membranes (Millipore, Mississauga, Ontario, Canada) at 25 mA (constant current) overnight at 4°C by a previously published protocol (35). Membranes were processed as described previously (3), with the exception that 10% (wt/vol) skim milk powder (Difco) replaced bovine serum albumin in the initial blocking step and an anti-OprN monoclonal antibody (7) and a horseradish peroxidase-coupled donkey anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) (diluted 1/5,000) were used as the primary and secondary antibodies, respectively. Blots were developed with the Enhanced Chemiluminescence system (Amersham) according to the manufacturer's protocol.

## RESULTS

**β-Lactamase inhibitors as substrates for the MexAB-OprM efflux system.** MexAB-OprM is the sole known drug efflux system expressed constitutively in *P. aeruginosa*, in which it contributes to intrinsic antibiotic resistance. To determine whether this system could accommodate β-lactam inhibitors and, thus, thwart their activities, we took advantage of the intrinsic antibacterial activities of these compounds and examined the sensitivities of *P. aeruginosa* strains expressing or deficient in the *mexAB-oprM* operon. Strains expressing wild-type levels of the efflux system (e.g., ML5087 and K767) were quite resistant to killing by the three agents tested, cloxacillin, clavulanate, and BRL42715 (Table 2), consistent with the generally poor antibacterial activity of each of these agents when used alone. Still, mutants deficient in *mexAB-oprM*-encoded components exhibited increased susceptibilities to all three agents in the case of the ML5087 derivatives K1110, K1115, and K1116 and to clavulanate and cloxacillin in the case of the K767 derivative K1119 (Table 2). The high level of resistance of K767 to BRL42715 precluded any determination of differences in susceptibility between this strain and K1119. *nalB* strains overexpressing *mexAB-oprM* also showed measurable increases in resistance to cloxacillin and clavulanate (K1112) and to cloxacillin (OCR1) (Table 2). These increases were, however, abrogated when components of *mexAB-oprM* were deleted from these strains (e.g., K1113) (Table 2). Thus, susceptibility to β-lactamase inhibitors inversely correlated with the presence of MexAB-OprM, indicating that this efflux pump affords resistance to β-lactamase inhibitors, which are thus substrates for the pump.

**β-Lactamase inhibitors as substrates for additional efflux systems in *P. aeruginosa*.** *mexCD-oprJ* is not expressed in wild-type cells grown under normal laboratory conditions, so it was not surprising that a *mexCD-oprJ* deletion of ML5087 (K1114) showed no alteration in susceptibility to the β-lactamase inhibitors (Table 2). Still, a *nfxB* derivative of ML5087 (K1111) also showed no change in susceptibility (Table 2), despite the fact that this strain demonstrates decreased susceptibility to a variety of other agents. Moreover, an *nfxB* derivative of the ML5087 Δ*mexAB-oprM* strain K1121 (designated K1131) also

TABLE 2. Influence of efflux gene expression on susceptibility of *P. aeruginosa* to β-lactamase inhibitors

| Strain | Efflux system expressed[a] | MIC (μg/ml) | | |
|---|---|---|---|---|
| | | Cloxacillin | Clavulanate | BRL42715 |
| ML5087 | MexAB-OprM | 5,120[b] | 256 | >1,024 |
| K1110 | | 1,024 | 128 | 256 |
| K1121 | | 1,024 | 64 | 512 |
| K1112 | MexAB-OprM$^{++}$[c] | >10,240[b] | 512–1,024 | >1,024 |
| K1113 | | 64 | 64 | 64 |
| K1114 | MexAB-OprM | >1,024 | 256 | >1,024 |
| K1111 | MexCD-OprJ/ MexAB-OprM | >1,024 | 256 | >1,024 |
| K1131 | MexCD-OprJ | 1,024 | 64 | 512 |
| K1115 | | 1,024 | 64 | 256 |
| K1117 | MexEF-OprN | >1,024 | 256 | >1,024 |
| K1116 | | 1,024 | 64 | 512 |
| K1118 | MexEF-OprN | >1,024 | 128–256 | >1,024 |
| K767 | MexAB-OprM | 5,120[b] | 512 | >1,024 |
| K1119 | | 2,560[b] | 128 | >1,024 |
| OCR1 | MexAB-OprM$^{++}$ | >10,240[b] | 512 | >1,024 |

[a] Those efflux systems that are known to be expressed and, thus, that contribute to drug resistance in the indicated strains are indicated.

[b] These values were all >1,024 μg/ml in the initial assays. In order to assess differences between the *nalB* strains OCR1 and K1112 and their parent strains K767 and ML5087, respectively, a second assay was performed with higher concentrations of cloxacillin.

[c] The two plus signs denote high-level expression of MexAB-OprM.

TABLE 3. Influence of MexAB-OprM on β-lactamase inhibitor enhancement of β-lactam activity[a]

| β-Lactam | β-Lactamase inhibitor | MIC (μg/ml) for the following strain: | | |
|---|---|---|---|---|
| | | K767 (wild type) | K1119 (Δ*mexAB-oprM*) | OCR1 (*nalB*) |
| Ampicillin | None | 1,024 | 256–512 | ND[b] |
| | Cloxacillin | 256 | 4 | ND |
| | BRL42715 | 64 | <2 | ND |
| | Clavulanate | 1,024 | 512 | ND |
| Cephaloridine | None | >10,240[c] | 2,560[c] | ND |
| | Cloxacillin | >1,024 | 258 | ND |
| | BRL42715 | 32 | 4–8 | ND |
| | Clavulanate | >1,024 | >1,024 | ND |
| Carbenicillin | None | 64 | 1–2 | 256 |
| | Cloxacillin | 64 | 1 | 256 |
| | BRL42715 | 64 | 0.125 | 256 |
| | Clavulanate | 64 | 2 | 256 |
| Piperacillin | None | 4 | 0.25 | 16 |
| | Cloxacillin | 4 | 0.25 | 16 |
| | BRL42715 | 8 | 0.125 | 16 |
| | Clavulanate | 4 | 0.25 | 16 |

[a] Determinations of the MICs of the various β-lactams were carried out in the presence of constant amounts (cloxacillin, 100 μg/ml; BRL42715, 20 μg/ml; and clavulanate, 10 μg/ml) of the indicated β-lactamase inhibitors.

[b] ND, not determined.

[c] These values were >1,024 μg/ml in the initial assays. In order to assess differences between these strains, a second assay was performed with higher concentrations of cephaloridine.

failed to demonstrate any lessening of β-lactamase inhibitor susceptibility, indicating that MexCD-OprJ does not accommodate β-lactamase inhibitors. To determine if the MexEF-OprN system afforded resistance to these compounds, attempts were made to select MexEF-OprN-overexpressing derivatives of Δ*mexAB-oprM* Δ*mexCD-oprJ* (K1115) and Δ*oprM* Δ*mexCD-oprJ* (K1116) double-knockout strains. Several multidrug-resistant isolates were obtained from K1115 and K1116, and two, K1117 and K1118, were screened for OprN production with an available OprN-specific antiserum. Both strains showed elevated levels of OprN (data not shown), consistent with the overexpression of *mexEF-oprN* in this strain. Assessment of the antibacterial activities of the β-lactamase inhibitors subsequently revealed that K1117 and K1118 were more resistant to all three inhibitors than the parent strain (Table 2), indicating that the MexEF-OprN efflux system, like MexAB-OprM, accommodates β-lactamase inhibitors.

**Influence of efflux systems on the efficacies of β-lactamase inhibitors.** Having demonstrated that MexAB-OprM is able to accommodate β-lactamase inhibitors, we found it of interest to assess the influence that this might have on the efficacies of these inhibitors in enhancing β-lactam activity. Using defined, subinhibitory levels of each inhibitor, we assessed the abilities of inhibitors to enhance β-lactam susceptibility in wild-type PAO1 (K767), pump-deficient (K1119), and pump-hyperexpressing (OCR1) strains. As can be seen in Table 3, cloxacillin and BRL42715 increased the susceptibility of K767 to the β-lactams ampicillin and cephaloridine, drugs which are readily hydrolyzed by the *P. aeruginosa* chromosomal β-lactamase (2). The fourfold increase in susceptibility to ampicillin seen with cloxacillin in K767 became, however, a >64-fold increase in susceptibility in the *mexAB-oprM* deletion strain K1119, indicating that the inhibitor was having a markedly greater impact in the absence of the efflux pump. Similarly, the 16-fold increase in susceptibility of K767 to ampicillin seen in the presence of BRL42715 became a >256-fold increase in susceptibility in K1119. Thus, a strain which is intrinsically very resistant to ampicillin (MIC, 1,024 μg/ml) can be made very sensitive in the presence of an inhibitor such as BRL42715 when the MexAB-OprM efflux system is nonoperational (MIC, <2 μg/ml). Similar results were obtained for cloxacillin, while clavulanate had no effect on β-lactam susceptibility. These data were consistent with results demonstrating that cloxacillin and BRL42715 were effective inhibitors of the class C β-lactamase of *P. aeruginosa*, while clavulanate was not (data not shown).

The relative increase in susceptibility to cephaloridine in the presence of cloxacillin or BRL42715 could not be determined in strain K767, since this wild-type strain was incredibly resistant to this cephalosporin (Table 3). Thus, the relative abilities of these β-lactamase inhibitors to potentiate cephaloridine activity in K767 versus K1119 could not be accurately assessed. Nonetheless, *P. aeruginosa* was ultimately very susceptible to cephaloridine when an inhibitor was present and the efflux pump was not (Table 3). Intriguingly, BRL42715 appeared to have a greater impact on susceptibility (in K767) to cephaloridine than to ampicillin, reflecting, perhaps, the lower affinity of the *P. aeruginosa* class C β-lactamase for the cephalosporin (13).

With carbenicillin and piperacillin, the presence or absence of MexAB-OprM, while influencing susceptibility to the β-lactams, did not markedly affect the influence of the β-lactamase inhibitors on β-lactam susceptibility (the presence or absence of cloxacillin or clavulanate had no influence on susceptibility to these β-lactams in K767 or K1119). The one exception was BRL42715, which failed to affect carbenicillin resistance in K767 but which managed to increase susceptibility more than eightfold in the *mexAB-oprM* deletion strain (Table 3). These data likely reflect the fact that these agents either are poorly hydrolyzed by the *P. aeruginosa* chromosomal β-lactamase in the first place (14) or are poor inducers of the β-lactamase (14, 26), and thus, inhibition of this enzyme is unlikely to substantially affect susceptibility.

**Influence of MexAB-OprM on β-lactamase induction by β-lactams and β-lactamase inhibitors.** To be sure that the changes in β-lactam susceptibility seen in *nalB* and pump deletion strains did not result from any changes in β-lactamase levels or activities, β-lactamase was assayed in K767 (wild type), K1119 (Δ*mexAB-oprM*), and OCR1 (*nalB*). By using cephaloridine or nitrocefin as a substrate, the β-lactamase levels measured were uniformly low in the uninduced cells (<0.1 μmol of nitrocefin hydrolyzed/min/mg of protein for all three strains) and were comparable in cells induced with imipenem (190 to 535 μmol of nitrocefin hydrolyzed/min/mg of protein and 1.95 to 2.80 μmol of cephaloridine hydrolyzed/min/mg of protein), indicating that the changes in sensitivity seen were attributable to efflux and not changes in β-lactamase activity in these strains.

To assess whether cloxacillin and BRL42715 were capable of inducing the chromosomal β-lactamase of *P. aeruginosa* and whether MexAB-OprM efflux activity influenced this inducibility, β-lactamase induction by these compounds was assessed in the MexAB-OprM$^+$ strain K767 and its *mexAB-oprM* deletion derivative K1119. At concentrations at which these β-lactamase inhibitors were previously shown to influence β-lactam activity (Table 3), no induction of the chromosomal β-lactamase was observed in K767 (<0.05 μmol of nitrocefin hydrolyzed/min/mg of protein for both inhibitors). Although BRL42715 also proved to be a weak inducer of β-lactamase in K1119 (0.14 μmol of nitrocefin hydrolyzed/min/mg of protein), cloxacillin markedly increased β-lactamase

levels in this strain (10.57 μmol of nitrocefin hydrolyzed/min/mg of protein), consistent with the increased level of accumulation of this inhibitor in the *mexAB-oprM* deletion strain.

## DISCUSSION

In the current study we have demonstrated that β-lactamase inhibitors are accommodated by the MexAB-OprM multidrug efflux system. As a result, their actions are enhanced in *mexAB-oprM* deletion strains, where they accumulate to a greater degree, and the activities of β-lactams such as ampicillin are potentiated to a greater degree by these inhibitors in MexAB-OprM$^-$ strains. This occurs despite the fact that cloxacillin, for example, induces markedly higher levels of β-lactamase in Δ*mexAB-oprM* strain K1119 than in MexAB-OprM$^+$ strain K767. Still, the β-lactams themselves, particularly ampicillin and cephaloridine, also induce expression of the *P. aeruginosa* β-lactamase and, thus, influence the net yield of β-lactamase in strains exposed to both inhibitor and β-lactam. At 0.1× the MIC of ampicillin (as determined in the presence of cloxacillin), for example, induction of β-lactamase by ampicillin is seen in K767 but not in K1119 (11), presumably because the exquisite ampicillin sensitivity of the latter strain in the presence of cloxacillin means that levels of ampicillin approaching the MIC are insufficient to induce the enzyme. The net result in this instance, then, is that comparable levels of β-lactamase are seen in K767 and K1119 in the presence of cloxacillin and ampicillin (at 0.1× the MIC) (11). In the case of BRL42715, which is a poor inducer of β-lactamase, it is likely that net β-lactamase levels will be reduced in K1119 compared to those in K767 (at 0.1× the β-lactam MIC and in the presence of BRL42715) due to the loss of or decreased induction of β-lactamase by β-lactams at concentrations (much lower in K1119 compared with those in K767) approaching the MIC for this strain. Differences in β-lactamase levels notwithstanding, inhibitor potentiation of β-lactam activity is best explained by inhibition of β-lactamase leading to increased β-lactam susceptibility, and any differences in β-lactamase levels seen will be a reflection of the overall concentration of inducer (inhibitor and β-lactam) entering the cell and not a determinant of β-lactam susceptibility per se. As such, greater potentiation in K1119 is likely due to increased accumulation of β-lactamase inhibitors in the absence of the MexAB-OprM efflux system, and these elevated levels will be more effective at inhibiting the available β-lactamase. The absence of inhibitor potentiation in the cases of carbenicillin and piperacillin has been noted previously (19) and strongly suggests that β-lactamase is not an important determinant of *P. aeruginosa* resistance to these β-lactams. Certainly, neither is a strong inducer of the *P. aeruginosa* chromosomal β-lactamase (1, 14, 26), and carbenicillin, at least, is poorly hydrolyzed by this enzyme (14).

Potentiation of β-lactam efficacy by the various β-lactamase inhibitors, particularly in wild-type strains, could conceivably have been due to interference with β-lactam export since, as substrates of the MexAB-OprM efflux system, these inhibitors might compete with β-lactams for export via MexAB-OprM. While such competition for export may, in fact, occur, it is unlikely to explain the potentiation attributed to the inhibitors in wild-type cells because even greater potentiation was seen in MexAB-OprM$^-$ strains. Thus, any increase in β-lactam accumulation in wild-type cells due to competition with inhibitors is not as important as the effect of the inhibitor on β-lactamase activity. Moreover, since β-lactamase seems relatively unimportant with regard to resistance to carbenicillin and piperacillin, the major determinant of resistance to these agents is likely to be efflux (certainly MexAB-OprM$^-$ strains are more sensitive than wild-type strains). The observation, then, that β-lactamase inhibitors had no effect on carbenicillin or piperacillin susceptibility with or without MexAB-OprM also indicates that the inhibitors do not significantly affect the β-lactam export component of β-lactam resistance in *P. aeruginosa.*

The demonstration that the presence or absence of MexAB-OprM had no effect on imipenem induction of β-lactamase (imipenem is apparently not a substrate for MexAB-OprM [18]) was significant in that it indicated that efflux systems do not influence the β-lactam resistance of *P. aeruginosa* via an effect on β-lactamase. Thus, export of β-lactams by MexAB-OprM is the most likely explanation for the role of this efflux system in β-lactam resistance. It is, perhaps, not surprising, then, that MexAB-OprM also accommodates β-lactamase inhibitors, because these are also β-lactams. Moreover, the demonstration here that β-lactamase inhibitors are exported via MexAB-OprM suggests that a previous report highlighting outer membrane permeability differences as factors influencing β-lactamase inhibitor accumulation in *P. aeruginosa* (32) needs reinterpreting, since it is likely that the differences in inhibitor entry seen were due to efflux effects, in particular, to differences in the relative ability of MexAB-OprM to accommodate each of the inhibitors examined, and not to differences in inhibitor permeation across the outer membrane.

The observation that an OprN-hyperexpressing strain (and, thus, a MexEF-OprN-hyperexpressing strain) elicited increased resistance to β-lactamase inhibitors was curious, in light of earlier descriptions of MexEF-OprN-hyperexpressing *nfxC* strains which do not display resistance to the structurally related β-lactams (except carbapenems) (6, 10). Examination of the resistance profiles of the OprN-hyperexpressing strains K1117 and K1118 revealed, however, that these strains were generally β-lactam resistant (fourfold increases in MICs of carbenicillin, cefoperazone, and cefotaxime were seen for K1117 and K1118 compared to those for the parent strains), suggesting that MexEF-OprN can, indeed, accommodate β-lactams. Since *nfxC* strains are typically selected from wild-type strains expressing MexAB-OprM, it is likely that the modest contribution of MexEF-OprN to β-lactam resistance is only observable in strains lacking MexAB-OprM. Perhaps this contribution is masked by the more efficient (as far as β-lactams are concerned) MexAB-OprM pump, or perhaps an increase in MexEF-OprN levels in *nfxC* strains is coupled to a decrease in MexAB-OprM levels, with no net change in β-lactam resistance resulting. MexAB-OprM expression is known, for example, to decline in MexCD-OprJ-overexpressing *nfxB* strains (7).

The accommodation of β-lactams, including β-lactamase inhibitors, by MexAB-OprM highlights an important feature of this efflux system, namely, that it appears to export agents active both within the periplasm and within the cytoplasm. Although it is not clear that β-lactams are unable to access the cytoplasm, it is unlikely that export of β-lactams from this compartment would affect β-lactam resistance since the targets of these agents do not exist in this compartment. Thus, MexAB-OprM must be able to accommodate agents present on either side of the cytoplasmic membrane. The recent observation that the outer membrane OprM does not facilitate β-lactam recognition and that the cytoplasmic membrane-associated components of this efflux system are responsible for recognition of this class of antibiotic (34) lends support to a model of MexAB-OprM activity which invokes drug partitioning into the cytoplasmic membrane, from which the drug is accessed by the MexAB-OprM system (16, 24). If this model is accurate, β-lactams would be expected to be accessed from the outer leaflet of the cytoplasmic membrane, while other agents would be accessed from the inner leaflet of this membrane

(24). Mechanistically, this model is appealing since it provides an explanation for a common mode of export of agents active in different cellular compartments. While this model implicates the integral cytoplasmic membrane protein MexB as the component of the MexAB-OprM system that recognizes the substrate, this has yet to be demonstrated, and such demonstration, particularly in the case of the β-lactams, will go a long way in supporting the proposed model.

## ACKNOWLEDGMENTS

This work was supported by an operating grant from the Canadian Cystic Fibrosis Foundation (to K.P.). X.-Z.L. acknowledges the support of the Canadian Cystic Fibrosis Foundation in the form of a studentship. R.S. is a Natural Sciences and Engineering Research Council (NSERC) postdoctoral fellow. K.P. is an NSERC University Research fellow.

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