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Active Efflux and Diffusion Are Involved in Transport of *Pseudomonas aeruginosa* Cell-to-Cell Signals

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Many gram-negative bacteria communicate by *N*-acyl homoserine lactone signals called autoinducers (AIs). In *Pseudomonas aeruginosa*, cell-to-cell signaling controls expression of extracellular virulence factors, the type II secretion apparatus, a stationary-phase sigma factor (σ^s), and biofilm differentiation. The fact that a similar signal, *N*-(3-oxohexanoyl) homoserine lactone, freely diffuses through *Vibrio fischeri* and *Escherichia coli* cells has led to the assumption that all AIs are freely diffusible. In this work, transport of the two *P. aeruginosa* AIs, *N*-(3-oxododecanoyl) homoserine lactone ($3OC_{12}$-HSL) (formerly called PAI-1) and *N*-butyryl homoserine lactone (C_4-HSL) (formerly called PAI-2), was studied by using tritium-labeled signals. When [^{3}H]C_4-HSL was added to cell suspensions of *P. aeruginosa*, the cellular concentration reached a steady state in less than 30 s and was nearly equal to the external concentration, as expected for a freely diffusible compound. In contrast, [^{3}H]$3OC_{12}$-HSL required about 5 min to reach a steady state, and the cellular concentration was 3 times higher than the external level. Addition of inhibitors of the cytoplasmic membrane proton gradient, such as azide, led to a strong increase in cellular accumulation of [^{3}H]$3OC_{12}$-HSL, suggesting the involvement of active efflux. A defined mutant lacking the *mexA-mexB-oprM*-encoded active-efflux pump accumulated [^{3}H]$3OC_{12}$-HSL to levels similar to those in the azide-treated wild-type cells. Efflux experiments confirmed these observations. Our results show that in contrast to the case for C_4-HSL, *P. aeruginosa* cells are not freely permeable to $3OC_{12}$-HSL. Instead, the *mexA-mexB-oprM*-encoded efflux pump is involved in active efflux of $3OC_{12}$-HSL. Apparently the length and/or degree of substitution of the *N*-acyl side chain determines whether an AI is freely diffusible or is subject to active efflux by *P. aeruginosa*.

Pseudomonas aeruginosa remains a leading cause of both nosocomial infections in immunocompromised patients and chronic infections in cystic fibrosis patients (reviewed in references 9 and 57). *P. aeruginosa* virulence depends on cell-associated factors, including alginate and pili (5, 9), and secreted factors, including toxins, exotoxin A, and exoenzyme S (14, 28); proteases, elastase, alkaline protease, and LasA protease (13, 27, 47); and hemolysins, rhamnolipid, and phospholipase (25). Cell-to-cell signaling (quorum sensing) is required for expression of many *P. aeruginosa* virulence factors (see below) (6, 43).

Intrinsic resistance of *P. aeruginosa* to many antibiotics and disinfectants also causes clinical problems (49). The intrinsic resistance is due to low outer membrane permeability and to multidrug efflux pumps that reduce the cellular level of antibiotics (reviewed in references 11 and 30). Three known *P. aeruginosa* multidrug efflux pumps are encoded by the *mexAB-oprM*, *mexCD-oprJ*, and *mexEF-oprN* operons, respectively (18, 45, 46). These pumps consist of a cytoplasmic membrane component of the resistance-nodulation-cell division (RND) family (39) thought to function as a proton antiport exporter (i.e., MexB), an outer membrane component thought to form channels (i.e., OprM), and a membrane fusion protein thought to link MexB and OprM (reviewed in reference 29). Bacterial RND pumps have also been shown to cause the efflux of many other organic compounds, including solvents and inhibitors (24, 29, 50). However, no natural products of *P. aeruginosa* have yet been shown to be subject to efflux via RND pumps.

Quorum sensing (or autoinduction) is the controlled expression of specific genes in response to extracellular chemical signals produced by the bacteria themselves (6). Typically cells emit an *N*-acyl homoserine lactone signal called an autoinducer (AI), which is usually synthesized by a LuxI-type AI synthase, into the environment (6). At high cell densities, the AI reaches a threshold concentration and binds to a LuxR-type protein which is then able to activate target genes (6). To date, *luxR-luxI*-type quorum-sensing systems and their *N*-acyl homoserine lactone AIs have been found in many different gram-negative bacteria (6). In *P. aeruginosa* the *las* (*lasR-lasI*) (7, 37) and *rhl* (*rhlR-rhlI*) (33, 34) quorum-sensing systems direct the synthesis of two distinct AIs, *N*-(3-oxododecanoyl) homoserine lactone ($3OC_{12}$-HSL) (formerly called PAI-1) (40) and *N*-butyryl homoserine lactone (C_4-HSL) (formerly called PAI-2) (41, 59), respectively. LasR and $3OC_{12}$-HSL activate expression of several genes, including *lasI* itself (54), *lasB* (encoding elastase) (37), *lasA* (encoding LasA protease) (8), both *xcp* operons (*xcpPQ* and *xcpR-Z*, encoding the type II secretion apparatus [3]), and *rhlR* (20, 44). Recently, the *las* quorum-sensing system was shown to be involved in *P. aeruginosa* biofilm differentiation (4). C_4-HSL and RhlR also activate expression of numerous genes, including *rhlI* itself (20), the rhamnolipid biosynthesis operon *rhlAB* (32, 42), *lasB* (1, 41, 42), and *rpoS* (encoding the stationary-phase sigma factor σ^s) (20).

Although many different *N*-acyl homoserine lactone AIs have been isolated from various gram-negative bacteria, to date, all differences are in the *N*-acyl side chain length (from

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
P. aeruginosa strains		
PAO1	Wild type	This laboratory
PAO-JP2	Δ*lasI* Δ*rhlI* Tc^r $HgCl_2^r$	42
PAO-JP3	Δ*lasR rhlR*::Tn*501* Tc^r $HgCl_2^r$	42
PAO200	Δ(*mexA-mexB-oprM*) unmarked	53
E. coli MG4λ$I_1$4	Δ(*argF-lac*)U169 *zah-735*::Tn*10* *recA56 srl*::Tn*10* λ::*lasIp-lacZ*	54
Plasmids		
pPCS1	ColE1 *lasR*$^+$ *bla*	54
pECP61.5	ColE1 *ori*$_{P.\ aeruginosa}$ *rhlAp-lacZ* *tacp-rhlR*$^+$ *bla*	42
pUCP21T	*ori*$_{P.\ aeruginosa}$ *bla*	53
pPS952	pUCP21T derivative; *mexA*$^+$ *mexB*$^+$ *oprM*$^+$	53

C_4 to C_{14}) or degree of substitution (either 3-oxo, 3-hydroxy, saturated, or unsaturated) (6, 26, 48, 55). All AIs have been assumed to be freely diffusible in bacterial cells. This assumption is based on the fact that a radiolabeled *Vibrio fischeri* AI, *N*-3-oxo-hexanoyl homoserine lactone ([^{3}H]3OC_6-HSL), was shown to freely diffuse into and out of *V. fischeri* and *Escherichia coli* cells (15, 42). Here, we studied the uptake and efflux of 3OC_{12}-HSL and C_4-HSL by *P. aeruginosa* cells. Our results indicate that [^{3}H]C_4-HSL freely diffuses into and out of *P. aeruginosa* cells. In contrast, cellular concentrations of [^{3}H]3OC_{12}-HSL are higher than external levels. Our results show that the increased cellular level of [^{3}H]3OC_{12}-HSL is not due to association with LasR or RhlR and suggest that it is not due to active (inward) transport. We propose that the high cellular level of 3OC_{12}-HSL is probably due to partitioning into the cell membranes. By use of *P. aeruginosa* Δ(*mexAB-oprM*) mutant cells or poisoned wild-type cells, we also show that 3OC_{12}-HSL is subject to active efflux by the MexAB-OprM pump.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The strains and plasmids used are shown in Table 1. Bacteria were grown at 37°C. When needed for plasmid maintenance, ampicillin (100 μg/ml) was included in cultures of *E. coli* and carbenicillin (200 μg/ml) was included in *P. aeruginosa* cultures. For β-galactosidase determinations, *P. aeruginosa* and *E. coli* were grown with shaking in PTSB medium (35) and supplemented A medium (40), respectively. Otherwise, *P. aeruginosa* cultures were grown with shaking in M9 medium (52) containing 0.2% glucose and 1 mM $MgSO_4$. Overnight cultures were centrifuged (10,000 × *g* for 10 min at 20°C), and cell pellets were washed in fresh M9 medium and resuspended in M9 medium to an optical density at 660 nm of 0.075. These cultures were then grown to mid-logarithmic growth phase (optical density at 660 nm of 0.7). This corresponded to 1.3×10^9 CFU/ml.

Preparation of cell suspensions. Cells were washed in KG buffer (10 mM potassium phosphate [pH 7.0], 0.03% glycerol) and resuspended in KG buffer to a cell density of 8×10^{10} CFU/ml.

Chemicals. The syntheses of unlabeled 3OC_{12}-HSL, [^{3}H]3OC_{12}-HSL (specific activity, 229 Ci/mmol), unlabeled C_4-HSL, and [^{3}H]C_4-HSL (specific activity, 29.4 Ci/mmol) have been described previously (38, 41, 42). [^{3}H]3OC_{12}-HSL and [^{3}H]C_4-HSL are labeled with tritium on the respective acyl side chains (38, 42). Antibiotics and all other chemicals, including the three radioactive compounds [^{14}C]ethylene glycol (specific activity, 1.5 mCi/mmol), [^{14}C]dextran (specific activity, 1.25 mCi/g; average molecular weight of 70,000), and [^{14}C]leucine (specific activity, 0.27 Ci/mmol), were purchased from Sigma Chemical Co., St. Louis, Mo.

Determination of cellular AI concentrations. The measurement of AI concentration was based on previously described techniques (15) with the following modifications. A 250-μl volume of cell suspension (2×10^{10} cells) was mixed with 50 μl of KG buffer containing the compound of interest (unless otherwise specified, the concentrations were as follows: [^{3}H]3OC_{12}-HSL or [^{3}H]C_4-HSL, 60 nM; [^{14}C]leucine, 2.0 μM; [^{14}C]ethylene glycol, 720 μM; or [^{14}C]dextran, 1.7 μg/μl). Assay mixtures were incubated for 5 min (20 to 22°C) unless otherwise specified, and then duplicate 140-μl aliquots were centrifuged through 75 μl of Nyosil M25 silicone fluid (Nye Lubricants, New Bedford, Mass.) into 25 μl of aqueous 2% trichloroacetic acid–10% glycerol and radioactivity was counted as described previously (15). One percent of the input [^{3}H]3OC_{12}-HSL radioactivity entered the silicone fluid regardless of whether cells were present, and this was corrected for in subsequent calculations. No radioactivity was detected in the silicone fluid for the other radioactive compounds.

Cell volumes were calculated by a modification of a previously described method (51). We substituted *P. aeruginosa* PAO1 and its derivatives for *E. coli*. We measured accumulation of the freely permeative [^{14}C]ethylene glycol in place of the [^{3}H]-labeled AIs. Trapped extracellular fluid was measured by using the impermeative [^{14}C]dextran.

[^{14}C]leucine accumulation was also assayed by using the above-described technique. Prior to addition of the radiolabeled compounds, cells were pretreated for 30 min with chloramphenicol (final concentration of 5 mM) to inhibit protein synthesis. Accumulation of both ^{3}H-AIs was unaffected by pretreating the cells with chloramphenicol. Thus, chloramphenicol was not included in further experiments with ^{3}H-AIs.

Where indicated, either sodium azide (30 mM) or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (250 μM) was added to de-energize cells as described previously (16, 22).

Efflux assay. Efflux of AI from cells was measured by comparing the cellular AI level with that remaining associated with the cells after removal of the external AI and then suspending the cells in a large excess of AI-free buffer as described previously (15). Incubation times were 20 and 5 min for cells with [^{3}H]3OC_{12}-HSL or [^{3}H]C_4-HSL, respectively, to reach steady-state levels. Duplicate 140-μl samples were transferred to 1.5-ml tubes and centrifuged for 1 min at 13,000 × *g* (20 to 22°C). Cells from one of the samples were suspended in 1.4 ml of KG buffer and centrifuged for 2 min as before. AI levels remaining with the cells after washing was compared with those remaining with cells that were not washed. Where indicated, sodium azide or CCCP was included in the wash buffer at the same concentrations as described above. Trapped extracellular fluid was corrected for by using [^{14}C]dextran in place of the ^{3}H-labeled AIs.

AI bioassays. *E. coli* MG4λ$I_1$4(pPCS1) was used to measure 3OC_{12}-HSL as described previously (54). *P. aeruginosa* PAO-JP2(pECP61.5) was used to measure C_4-HSL as described previously (42).

HPLC analysis of cellular AIs. Analysis of cellular AIs was based on a previously described technique (15). Briefly, cells were incubated with [^{3}H]3OC_{12}-HSL or [^{3}H]C_4-HSL as described above and centrifuged (13,000 × *g* for 1 min at 20 to 22°C). Cell pellets were suspended in 20 μl of KG buffer and extracted twice in 0.2 ml of ethyl acetate, and then 20 nmol of unlabeled 3OC_{12}-HSL or 24 nmol of unlabeled C_4-HSL was added as a carrier to 0.15 ml of the respective [^{3}H]3OC_{12}-HSL or [^{3}H]C_4-HSL extract. Each mixture was then evaporated under N_2 gas, and the material was dissolved in 10% (vol/vol) acetonitrile in water and subjected to reverse-phase high-performance liquid chromatography (HPLC) (C_{18} column, 0.46 by 25 cm). The HPLC flow rate was 1 ml/min, and elution conditions are indicated in Fig. 1.

RESULTS

Cellular concentrations of *P. aeruginosa* AIs. In order to study AI uptake in *P. aeruginosa*, [^{3}H]3OC_{12}-HSL or [^{3}H]C_4-HSL was incubated with suspensions of wild-type cells (strain PAO1). After separation of the cells from the extracellular fluid by centrifugation through a layer of silicone fluid, radiolabeled AI was found to be associated with the cells. In order to calculate the internal concentration of each AI and compare it with external concentrations, cellular AI is assumed to be unmodified. A structurally similar AI molecule, [^{3}H]3OC_6-HSL, was reported to be unmodified in *V. fischeri* cells (15). To verify this assumption, *P. aeruginosa* PAO1 cells that had been loaded with either [^{3}H]3OC_{12}-HSL or [^{3}H]C_4-HSL were extracted with ethyl acetate as described in Materials and Methods. For [^{3}H]C_4-HSL-loaded cells, 100% of the radioactivity was recovered from the cells that had been loaded with this AI. In the case of [^{3}H]3OC_{12}-HSL, 75.2 ± 2.3% (average ± standard deviation [SD]; $n = 3$ independent experiments) of the radioactivity was recovered from cells. In both cases, when the extracted radioactivity was analyzed by HPLC, >90% of the radioactivity applied to the HPLC column eluted at the same point as the [^{3}H]3OC_{12}-HSL and [^{3}H]C_4-HSL standards, respectively (Fig. 1). For C_4-HSL these results support the assumption that cellular radiolabel was in the form of unmodified [^{3}H]C_4-HSL. For [^{3}H]3OC_{12}-HSL, nearly all of the radiolabel extracted from cells was unmodified [^{3}H]3OC_{12}-HSL (Fig. 1), but 25% of the [^{3}H]3OC_{12}-HSL remained associated with the cells. It is possible that this fraction of the total

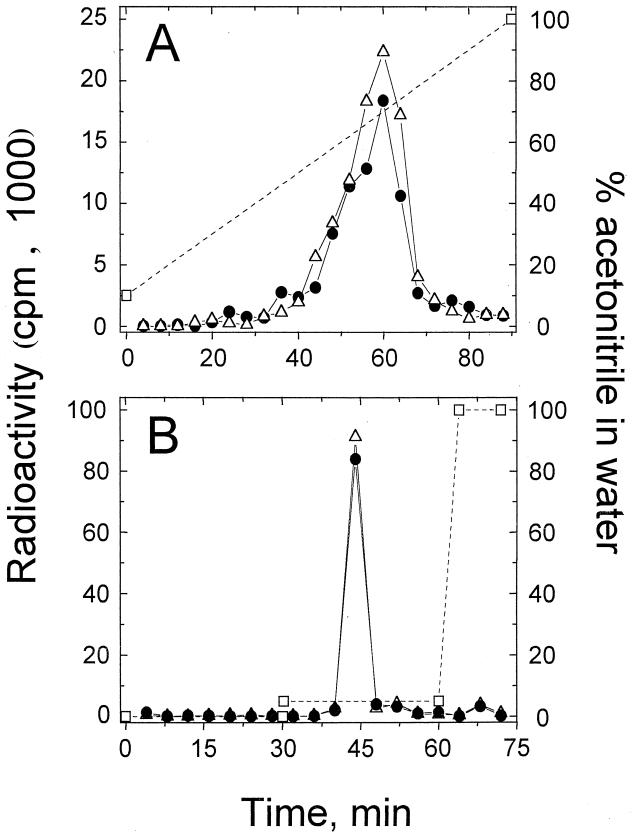

FIG. 1. HPLC analysis of tritium-labeled material extracted from *P. aeruginosa* PAO1 that had been loaded with [^{3}H]3OC$_{12}$-HSL (A) or with [^{3}H]C$_4$-HSL (B) compared with [^{3}H]3OC$_{12}$-HSL and [^{3}H]C$_4$-HSL, respectively. Symbols: ●, material extracted from cells; △, ^{3}H-AI; □, percent (volume/volume) acetonitrile in water.

[^{3}H]3OC$_{12}$-HSL accumulated by strain PAO1 may be chemically modified (such as by lactone ring hydrolysis or deacylation of the side chain) by the cells. Chemical modification of [^{3}H]3OC$_{12}$-HSL by the cells seems unlikely, because other AIs with structurally similar lactone rings and shorter acyl side chains, i.e., [^{3}H]C$_4$-HSL and [^{3}H]3OC$_6$-HSL, are not modified by *P. aeruginosa* (Fig. 1) or *V. fischeri* (15), respectively. It is, however, more likely that the 25% of [^{3}H]3OC$_{12}$-HSL remaining with cells may be unmodified yet is strongly associated with the cells (i.e., partitioned into the membranes) and could not be liberated by our method of extraction.

P. aeruginosa PAO1 incubated with [^{3}H]C$_4$-HSL yielded a cellular/extracellular concentration ratio of 0.92, which is close to the ratio of 0.96 obtained with the freely permeative [^{14}C]ethylene glycol (Table 2). In contrast, the cellular [^{3}H]3OC$_{12}$-HSL concentration was 3.35-fold higher than the external concentration (Table 2). The fact that 3OC$_{12}$-HSL appeared to be concentrated by cells opened the possibility that active (inward) transport could be involved.

To address whether the silicone fluid centrifugation technique would be able to distinguish between the active and passive transport, the time course of *P. aeruginosa* uptake of [^{14}C]leucine, which is known to be actively transported inward by *P. aeruginosa* (12), was measured (Fig. 2A). After 5 min of incubation in the presence of [^{14}C]leucine, the cellular [^{14}C]leucine concentration was greater than 20-fold higher than the external concentration (Fig. 2A), as previously reported (12). To demonstrate inhibition of active (inward) transport, cells were preincubated with sodium azide, which has been shown to block the active transport of leucine in *P. aeruginosa* (12, 16). As expected, the [^{14}C]leucine level in cells pretreated with sodium azide reached only approximately twice the external level (Fig. 2A). These experiments demonstrated that the silicone fluid centrifugation technique would be able to detect active (inward) transport systems.

We then measured the time courses of [^{3}H]3OC$_{12}$-HSL and [^{3}H]C$_4$-HSL accumulation in strain PAO1 under the same conditions (Fig. 2B). In less than 30 s, the cellular concentration of [^{3}H]C$_4$-HSL reached a steady-state level that was approximately equal to the external concentration (Fig. 2B). The same results were obtained with the freely diffusible compound [^{14}C]ethylene glycol (data not shown). *P. aeruginosa* cells seem to be freely permeable to C$_4$-HSL, as *V. fischeri* cells are to 3OC$_6$-HSL (15). In contrast, [^{3}H]3OC$_{12}$-HSL required about 5 min to reach a steady-state level, with a cellular-to-extracellular ratio of nearly 3 (Fig. 2B), suggesting that 3OC$_{12}$-HSL transport was more complex than simple diffusion.

Because active (inward) transport of [^{14}C]leucine was strongly inhibited when cells were pretreated with sodium azide, the effect of this poison on ^{3}H-AI accumulation was also studied, and the results were surprising. Cellular [^{3}H]3OC$_{12}$-HSL concentrations rose to 10 times the extracellular concentrations (Fig. 2B), but azide treatment had no effect on accumulation of [^{3}H]C$_4$-HSL (Fig. 2B). These results suggest that 3OC$_{12}$-HSL is not actively transported into *P. aeruginosa* as is leucine.

Azide and other agents, such as potassium cyanide and CCCP, that de-energize the bacterial cytoplasmic membrane potential (i.e., proton motive force [PMF]) are known to cause increases in cellular accumulation of various substances, including certain antibiotics (i.e., tetracyclines, fluoroquinolones, and some β-lactams) (21, 29). The increased accumulation occurs because PMF-dependent multidrug efflux pumps become inactivated in de-energized cells (29). Thus, these amphipathic antibiotics are no longer subject to active efflux out of the cells, resulting in the higher cellular antibiotic levels (29). In *P. aeruginosa* the *mexAB-oprM* operon constitutively expresses a PMF-dependent multidrug efflux pump (23, 46). To address whether the increased cellular [^{3}H]3OC$_{12}$-HSL concentrations observed in azide-treated cells were due to inhibition of this efflux pump, the [^{3}H]3OC$_{12}$-HSL concentration in a defined *P. aeruginosa* Δ(*mexAB-oprM*) mutant, strain PAO200 (53), was measured. This strain is derived from wild-type strain PAO1 and carries an unmarked deletion of the entire *mexAB-oprM* operon (53). The time course of accumulation of [^{3}H]3OC$_{12}$-HSL by strain PAO200 cells (Fig. 2C)

TABLE 2. *P. aeruginosa* PAO1 cellular and external AI concentrations

Compound	External concn[a]	Cellular concn[b]	Ratio of cellular to external concn[c]
[^{3}H]3OC$_{12}$-HSL	69.1 ± 6.03 nM	231 ± 16.8 nM	3.35
[^{3}H]C$_4$-HSL	56.4 ± 6.93 nM	51.9 ± 11.4 nM	0.92
[^{14}C]ethylene glycol	2.10 ± 0.07 mM	2.00 ± 0.06 mM	0.96

[a] Calculated from the amount of radioactivity remaining above the silicone fluid after centrifugation. Values are the averages ± SDs from three or four experiments.

[b] Calculated from the amount of radioactivity centrifuged through silicone fluid and the cell volume, with a correction for the small amount of extracellular material carried through the silicone fluid (measured with [^{14}C]dextran). Values are the averages ± SDs from four independent experiments.

[c] An unpaired *t* test determined that the ratio of 4.4 for 3OC$_{12}$-HSL was significantly higher than the ratio of 0.92 for C$_4$-HSL ($P < 0.001$) and the ratio of 0.96 for ethylene glycol ($P < 0.001$).

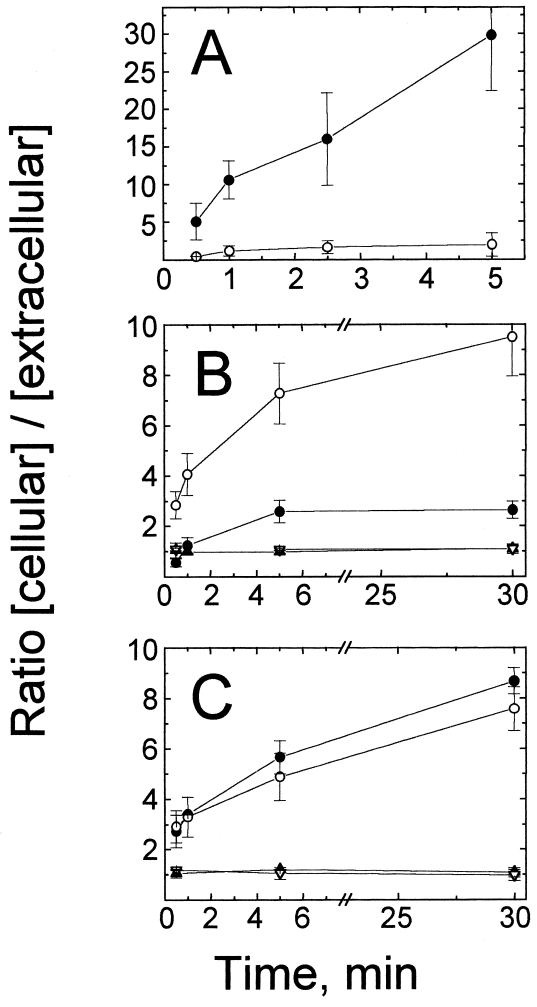

FIG. 2. Accumulation of radiolabeled compounds by *P. aeruginosa*. (A) [^{14}C] leucine accumulation in strain PAO1 treated with chloramphenicol (5 mM); (B) accumulation of [^{3}H]3OC$_{12}$-HSL (circles) and [^{3}H]C$_4$-HSL (triangles) in strain PAO1; (C) accumulation of [^{3}H]3OC$_{12}$-HSL (circles) and [^{3}H]C$_4$-HSL (triangles) in *mexAB-orpM* mutant strain PAO200. Closed symbols, non-azide-treated cells; open symbols, cells treated with sodium azide (30 mM). Accumulation of each compound was determined as described in Materials and Methods. Data are the averages (± SDs) of results from two to five independent experiments.

resembled that by azide-treated strain PAO1 cells (Fig. 2B). The [^{3}H]3OC$_{12}$-HSL concentration observed in strain PAO200 cells was about eightfold higher than the external concentration, as we had observed for azide-treated PAO1 cells (Fig. 2B and C). Azide treatment of strain PAO200 resulted in almost no change in the cellular concentration of [^{3}H]3OC$_{12}$-HSL (Fig. 2C). These results suggested that the cellular level of [^{3}H]3OC$_{12}$-HSL is influenced by the presence of the *mexAB-oprM*-encoded efflux pump and further suggested that the high level of [^{3}H]3OC$_{12}$-HSL accumulated in azide-treated wild-type cells is due to inactivation of this PMF-dependent efflux pump. In contrast, when [^{3}H]C$_4$-HSL was added to strain PAO200 cells, the cellular-to-external concentration ratios were not significantly increased compared to those in strain PAO1 cells, and these levels were unaffected by poison (Fig. 2C). Therefore, C$_4$-HSL accumulation in *P. aeruginosa* appears to be independent of MexAB-OprM.

Efflux of AIs. To further investigate the role of the *mexAB-oprM*-encoded PMF-dependent efflux pump on [^{3}H]3OC$_{12}$-HSL accumulation in *P. aeruginosa*, and to confirm that cells are freely permeable to [^{3}H]C$_4$-HSL, AI efflux was studied (Fig. 3). In theory, a freely diffusible compound would be expected to completely escape when cells loaded with the compound are transferred into a large volume of medium lacking the compound. Moreover, this free diffusion process would occur independently of the presence or absence of an active-efflux system. When strains PAO1 and PAO200 (Δ*mexAB-oprM*) were loaded with [^{3}H]C$_4$-HSL and transferred to AI-free buffer, cellular levels of this AI decreased 100 and 95%, respectively (Fig. 3A). When CCCP-treated cells (strains PAO1 and PAO200, respectively) were loaded with [^{3}H]C$_4$-HSL and then transferred to AI-free buffer, 100% of the radiolabel escaped from the cells (Fig. 3A). These results confirmed that *P. aeruginosa* cells are freely permeable to [^{3}H]C$_4$-HSL and that the *mexAB-oprM*-encoded efflux pump has no effect on efflux of this AI.

A compound utilizing an efflux pump would escape more readily from cells containing an active-efflux system than from mutants lacking the system. As shown above, in both azide-poisoned strain PAO1 cells and mutant cells lacking the *mexAB-oprM*-encoded efflux pump (strain PAO200), the cellular accumulation of [^{3}H]3OC$_{12}$-HSL was higher than that in nonpoisoned strain PAO1 cells (Fig. 2B and C). In efflux experiments, the level of [^{3}H]3OC$_{12}$-HSL initially loaded in strain PAO200 [Δ(*mexAB-*

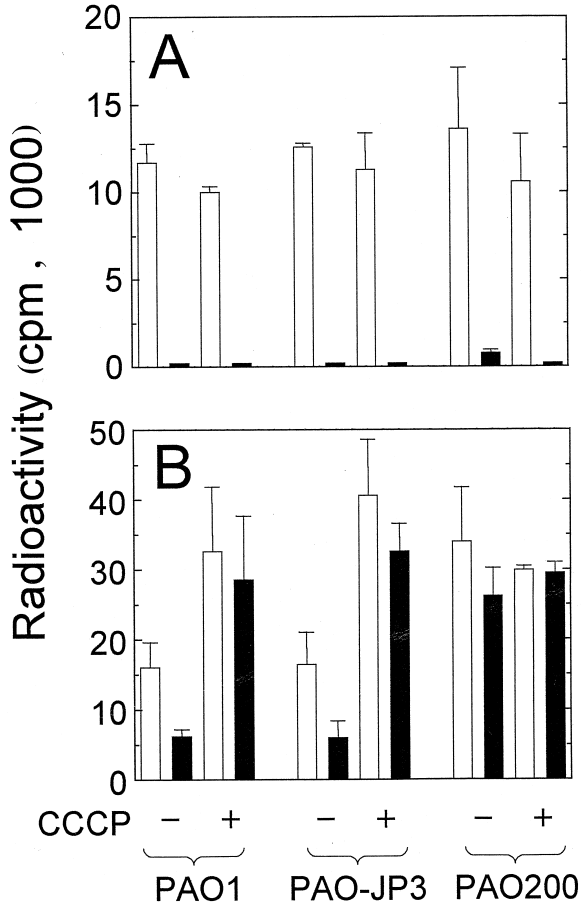

FIG. 3. Efflux of [^{3}H]C$_4$-HSL (A) and [^{3}H]3OC$_{12}$-HSL (B) from *P. aeruginosa* PAO1 (wild type), PAO-JP3 (*lasR rhlR*), or PAO200 (*mexABA-oprM*). Open bars, radioactivity loaded in cells; closed bars, radioactivity remaining in cells after suspension in AI-free buffer. Cells were de-energized with CCCP (250 μM) as indicated. Data are the averages (± SDs) of results from two to four independent experiments.

oprM)] cells and in poisoned PAO1 cells was therefore higher than the level of [^{3}H]3OC$_{12}$-HSL initially loaded in unwashed strain PAO1 cells (Fig. 3B). When the external [^{3}H]3OC$_{12}$-HSL was removed and cells were suspended in AI-free buffer, 60% of the cellular radioactivity escaped from the wild-type cells and 30% escaped from mutant cells (strain PAO200) that lacked the efflux pump (Fig. 3B). This strongly suggested that *P. aeruginosa* cells are not freely permeable to [^{3}H]3OC$_{12}$-HSL and that the *mexAB-oprM*-encoded efflux pump facilitates efflux of this AI. Furthermore, when de-energized (CCCP-treated) strain PAO1 or PAO200 cells were loaded with [^{3}H]3OC$_{12}$-HSL and then transferred to AI-free buffer, very little [^{3}H]3OC$_{12}$-HSL escaped from either strain (Fig. 3B).

Therefore, unlike the freely permeative [^{3}H]C$_4$-HSL, which completely escaped from cells, 40% of cellular [^{3}H]3OC$_{12}$-HSL remained after transfer of strain PAO1 cells to AI-free buffer. To address the possibility that the remaining [^{3}H]3OC$_{12}$-HSL was bound to the AI-dependent transcriptional activator proteins encoded by *lasR* and *rhlR*, strain PAO-JP3, a defined *lasR rhlR* double mutant, was tested for [^{3}H]3OC$_{12}$-HSL efflux (and for [^{3}H]C$_4$-HSL efflux as a control). The results were nearly identical to those for efflux in strain PAO1 cells (Fig. 3). Therefore, the remaining cellular [^{3}H]3OC$_{12}$-HSL in strain PAO1 was not irreversibly bound to the LasR or RhlR protein. In a time course efflux experiment in which both CCCP-treated and nontreated wild-type cells that had been loaded with [^{3}H]3OC$_{12}$-HSL were suspended in AI-free buffer, 40% of the [^{3}H]3OC$_{12}$-HSL remained after 1 min with the nontreated cells and 80% remained with the CCCP-treated cells, as before. By 60 min (the duration of this experiment), 25% of the [^{3}H]3OC$_{12}$-HSL remained with the nontreated cells whereas 50% remained with the CCCP-treated cells (data not shown). The fact that some of the [^{3}H]3OC$_{12}$-HSL was released by CCCP-treated cells may be due to diffusion through the cell membranes in an efflux pump-independent fashion. This explanation would also account for the minor release of [^{3}H]3OC$_{12}$-HSL by strain PAO200 shown in Fig. 3.

To confirm that the effects seen in strain PAO200 were due to the absence of *mexAB-oprM*, autoinducer efflux was measured in PAO200 cells containing either pUCP21T, a vector control plasmid, or pPS952, a pUCP21T derivative carrying a wild-type *mexAB-oprM* operon. When strain PAO200 contained pPS952, [^{3}H]3OC$_{12}$-HSL accumulated in these cells to levels similar to those observed in wild-type PAO1 cells (Fig. 4B and 3B), about fourfold less than for PAO200 containing the vector control. This indicated that efflux of [^{3}H]3OC$_{12}$-HSL was restored when strain PAO200 contained a functional *mexAB-oprM*-encoded pump. This efflux was strongly inhibited when strain PAO200(pPS952) cells were de-energized (Fig. 4B). As a control, efflux of [^{3}H]C$_4$-HSL from the *mexAB-oprM* mutant containing the *mexA*$^+$ *mexB*$^+$ *oprM*$^+$ plasmid was also assayed. As expected, 98 to 100% of the [^{3}H]C$_4$-HSL was transported from the cells regardless of whether cells were de-energized or contained a functional *mexAB-oprM* operon (Fig. 4A).

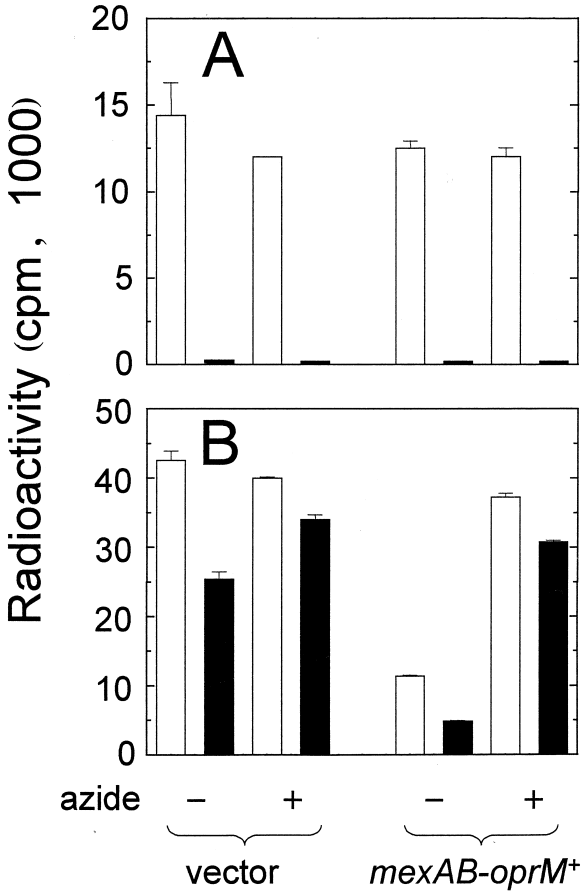

FIG. 4. Efflux of [^{3}H]C$_4$-HSL (A) and [^{3}H]3OC$_{12}$-HSL (B) from *P. aeruginosa* PAO200 in the presence (labeled *mexAB-oprM*$^+$ [pPS952]) or absence (labeled vector [pUCP21T]]) of *mexAB-oprM*. Open bars, radioactivity loaded in cells; closed bars, radioactivity remaining in cells after suspension in AI-free buffer. Cells were de-energized with sodium azide (30 mM) as indicated. Data are the averages (± SDs) of results from two independent experiments.

DISCUSSION

This study shows that both C$_4$-HSL and 3OC$_{12}$-HSL diffuse into and out of *P. aeruginosa*. We found that the cellular concentration of [^{3}H]C$_4$-HSL quickly reached a steady state in wild-type *P. aeruginosa* cells at a level that was nearly equal to the external level, suggesting a passive diffusion process. Thus, we have shown that *P. aeruginosa* is freely permeable to C$_4$-HSL as both *V. fischeri* and *E. coli* are freely permeable to 3OC$_6$-HSL (15). In contrast, [^{3}H]3OC$_{12}$-HSL required about 5 min to reach a steady-state cellular concentration of nearly threefold the external level (Fig. 2B), indicating a more complex transport process. HPLC analysis indicated that *P. aeruginosa* did not modify the cellular [^{3}H]C$_4$-HSL and did not modify 75% of the [^{3}H]3OC$_{12}$-HSL that was loaded into the cells. The remaining 25% of the cellular [^{3}H]3OC$_{12}$-HSL could have been chemically modified; however, it is more likely that it is unmodified and partitioned into the cell membranes. Most importantly, this 25% does not significantly influence our conclusions about active efflux of [^{3}H]3OC$_{12}$-HSL from *P. aeruginosa* cells.

Thus, while 3OC$_{12}$-HSL freely diffuses into and out of *P. aeruginosa*, the cells accumulate more of this compound because of passive partitioning into membranes. Superimposed on these processes, 3OC$_{12}$-HSL is subject to active efflux from *P. aeruginosa* via the MexAB-OprM pump.

[^{3}H]3OC$_{12}$-HSL does not seem to be actively transported into *P. aeruginosa*. Indeed, accumulation of this AI was not inhibited in de-energized cells but actually increased (Fig. 2B). De-energization of the cytoplasmic membrane potential (i.e., PMF) by azide and other poisons such as CCCP results in a strong increase in the accumulation of certain antibiotics by some gram-negative bacteria (21, 29). In *P. aeruginosa* these effects have been associated with inhibition of the PMF-dependent *mexAB-oprM*-encoded efflux pump (23, 46).

We have observed that a *P. aeruginosa* Δ(*mexAB-oprM*) mutant accumulated about threefold more [^{3}H]3OC$_{12}$-HSL than

untreated wild-type cells. Because de-energization of strain PAO200 cells had essentially no effect on the time course of accumulation of [^{3}H]3OC$_{12}$-HSL (Fig. 2C), these results strongly suggest that the MexAB-OprM efflux is the only PMF-dependent efflux pump involved in regulation of cellular levels of 3OC$_{12}$-HSL under these conditions. However the *P. aeruginosa mexCD-oprJ*- and *mexEF-oprN*-encoded multidrug pumps may also be involved in efflux of 3OC$_{12}$-HSL under other conditions where those operons would be expressed.

From our efflux experiments we conclude that 3OC$_{12}$-HSL efflux depends on the presence of an active MexAB-OprM pump. While this paper was being prepared, others also suggested that 3OC$_{12}$-HSL may be exported by the *mexAB-oprM*-encoded pump (5a). Here, we found that the percentage of [^{3}H]3OC$_{12}$-HSL retained after resuspension in AI-free buffer was higher in de-energized wild-type cells and in *mexAB-oprM* mutant cells than in nontreated wild-type cells (Fig. 3B). The observation that the remaining cellular 3OC$_{12}$-HSL slowly escaped even when the cells were de-energized suggests that a gradual release of this AI likely occurs in addition to active efflux of the majority of cellular 3OC$_{12}$-HSL via the MexAB-OprM pump. One explanation for the slow efflux of 3OC$_{12}$-HSL from the cells may be that 3OC$_{12}$-HSL partitions into the cell membranes. The respective lengths of the acyl side chains of the two AIs are probably responsible for the differences in accumulation observed between the relatively hydrophobic 3OC$_{12}$-HSL and the more hydrophilic C$_4$-HSL. We propose that 3OC$_{12}$-HSL transport by *P. aeruginosa* occurs by a mechanism similar to that of amphipathic antibiotics such as tetracycline, fluoroquinolones, and β-lactams. Nikaido has presented a model in which those antibiotics diffuse and partition in gram-negative bacterial cell membranes and are subject to active efflux from the cells by PMF-dependent RND pumps (29). Recent results with an RND pump (AcrAB) of *Salmonella typhimurium* have extended this model and suggested that penicillins and cephalosporins containing more-lipophilic side chains are more likely to partition into the lipid bilayer of the cytoplasmic membrane (31). The model suggests that once these β-lactam antibiotics are partitioned into the cytoplasmic membrane, they can then become substrates of the RND pump (31). Future studies will be required to elucidate the likely mechanism(s) (i.e., membrane partitioning) apparently involved in 3OC$_{12}$-HSL accumulation, besides the role of the MexAB-OprM pump described here.

A natural substrate of MexAB-OprM. PMF-dependent RND pumps in *P. aeruginosa* are known to cause the efflux of various toxic substances, such as antibiotics (18, 23, 45), fatty acid inhibitors (53), and organic solvents (24), out of cells. 3OC$_{12}$-HSL is the first example of a natural product of *P. aeruginosa* that is subject to efflux by a PMF-dependent RND pump. Our results show that the other *P. aeruginosa* AI, C$_4$-HSL, is not a substrate of MexAB-OprM, which indicates specificity for long-chain AIs. Based on the broad spectrum of compounds that RND pumps are known to transport, other small amphipathic molecules produced by *P. aeruginosa* are likely to be subject to efflux by these pumps as well.

Cell-to-cell signaling and RND pumps. The discovery that 3OC$_{12}$-HSL is subject to efflux by an RND pump suggests that regulation of genes controlled by 3OC$_{12}$-HSL and LasR, such as those encoding elastase (*lasB*) or the type II secretion apparatus (*xcp* operons), is likely to be affected by the presence of a MexAB-OprM pump and PMF. In theory, as PMF decreases, the efflux pump activity would also decrease, causing the cellular concentration of 3OC$_{12}$-HSL to rise. The expected result would be increased activation of *las* quorum-sensing target genes such as those mentioned above. Interestingly, in the early 1980s others observed that in *P. aeruginosa*, extracellular protease production increased as the PMF decreased (58). Those findings, together with the results presented here which demonstrate that 3OC$_{12}$-HSL concentrations in *P. aeruginosa* cells are higher in de-energized cells or those lacking the MexAB-OprM efflux pump, suggest that the timing of induction of genes controlled by LasR and 3OC$_{12}$-HSL may be affected by PMF-dependent AI efflux. A higher cellular 3OC$_{12}$-HSL concentration would be expected sooner during the growth of *P. aeruginosa* lacking a functional MexAB-OprM efflux pump, resulting in earlier expression of target genes. Indeed, *P. aeruginosa* quorum-sensing target genes are controlled by a hierarchy of induction based on the AI concentration (43, 54). Because of the roles that the *las* quorum-sensing system play in virulence and biofilm differentiation (4, 56), experiments with the *mexAB-oprM* mutant will need to examine if the timing of biofilm differentiation and virulence are affected.

Because 3OC$_{12}$-HSL may be partitioned into the *P. aeruginosa* membranes by the same mechanism as other amphipathic compounds, future studies will need to examine the role of *P. aeruginosa* membranes in quorum sensing. Although others have shown that in *V. fischeri* the 3OC$_6$-HSL-dependent transcriptional activator of the *lux* genes, LuxR, is associated with the inner membrane (19), it is not known whether LasR associates with the *P. aeruginosa* inner membrane.

Components of quorum-sensing systems (a *luxR-luxI* homologue and/or an *N*-acyl homoserine lactone AI) and RND-type efflux systems (a *mexA*, *mexB*, or *oprM* homologue or all three) have been identified in numerous gram-negative bacteria. *Agrobacterium tumefacians* synthesizes 3OC$_8$-HSL (60), *Rhizobium leguminosarum* is known to produce 7,8-*cis*-3-hydroxy-C$_{14}$-HSL (10), *Rhizobium meliloti* produces an AI of unknown structure (10) which comigrates in HPLC with 7,8-*cis*-C$_{14}$-HSL of *Rhodobacter sphaeroides* (48), and *Pseudomonas putida* and *Burkholderia cepacia* also produce AI activity detected in a 3OC$_8$-HSL assay (39a). Homologues of at least one component of the *P. aeruginosa* MexAB-OprM system have been identified in all of these species except *R. sphaeroides*. In the plant symbionts *R. meliloti* and *R. leguminosarum*, the respective *mexAB-oprM* homologues may be involved in efflux of nodulation signals (29, 39). In *P. putida*, the *srpABC* operon and *ttgB*, respectively, were shown to cause efflux of organic solvents (17, 50). In the human pathogen *B. cepacia*, a *mexAB-oprM* homologue is involved in multiple antibiotic resistance (2), and in the plant pathogen *A. tumefacians*, *ifeAB* encode an isoflavanoid-inducible efflux pump that is involved in colonization of plant roots (36). Thus, it is likely that cell-to-cell communication systems in species other than *P. aeruginosa* that rely on *N*-acyl homoserine lactones containing long-chain AIs will also involve RND pumps.

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