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Biochemistry

Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes

[density-dependent transcription/gene activation/*las* genes/*N*-acyl homoserine lactone/3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)dodecanamide]

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ABSTRACT **In *Pseudomonas aeruginosa* the LasR protein is required for activation of *lasB* and several other virulence genes. A diffusible signal molecule, the *P. aeruginosa* autoinducer (PAI), produced by the bacterial cell and released into the growth medium, is required for activity of LasR. By cloning a *lasB*::*lacZ* fusion and a *lasR* gene under control of the *lac* promoter in *Escherichia coli*, we have developed a quantitative bioassay for PAI. We have used this assay to follow the purification of PAI from cell-free culture supernatant fluids in which *P. aeruginosa* or *E. coli* containing the *P. aeruginosa* gene required for autoinducer synthesis, *lasI*, had been grown. Chemical analyses indicated the purified material was 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)dodecanamide. To confirm this assignment, the compound was synthesized and the synthetic compound was shown to have chemical and biological properties identical to those of PAI purified from culture supernatant fluids. The elucidation of the PAI structure suggests therapeutic approaches toward control of *P. aeruginosa* infections.**

The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic human pathogen that causes infections in immunocompromised hosts and colonizes the lungs of individuals with cystic fibrosis (1, 2). This bacterium produces a number of extracellular virulence factors including exotoxin A, encoded by the *toxA* gene (3, 4), two elastolytic proteases, encoded by the *lasA* and *lasB* genes, and an alkaline protease, encoded by the *aprA* gene (5–7).

The *P. aeruginosa lasR* gene encodes a transcriptional activator, the LasR protein (8). Because the LasR protein activates transcription of *aprA*, *lasA*, and *lasB* and enhances *toxA* expression (8–10), this transcription factor is considered to be a global regulator of *P. aeruginosa* virulence genes. Activity of LasR is dependent on a diffusible factor produced by *P. aeruginosa* containing a functional *lasI* gene or by *Escherichia coli* containing a *lasI* gene cloned from *P. aeruginosa* (11). LasR and LasI, the *lasI* gene product, show significant sequence identity with the LuxR and LuxI proteins of the luminescent marine bacterium, *Vibrio fischeri* (8, 11). LuxR is a transcriptional activator of the luminescence genes, and activity of LuxR is dependent on a diffusible substance, termed the autoinducer, that is synthesized by cells containing a functional *luxI* gene (12, 13). The *V. fischeri* autoinducer (VAI) is 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)-hexanamide, commonly termed *N*-(3-oxohexanoyl)homoserine lactone (14). Because cells of *V. fischeri* are freely permeable to it, VAI accumulates in the medium during growth. Thus the response of LuxR to autoinducer defines a cell-density-dependent system for gene regulation. At high cell densities, autoinducer concentrations will increase above a threshold level and luminescence gene transcription will be activated. At low cell densities, autoinducer will diffuse out of cells and be diluted into the surrounding medium, and the luminescence genes will not be activated (ref. 15; for a recent review, see ref. 16).

It has been reported that *P. aeruginosa* can synthesize VAI (17) and that the *P. aeruginosa* autoinducer (PAI) might in fact be VAI (18). In this report we show that the diffusible compound produced by bacteria containing the *lasI* gene and required for activity of the LasR protein is not identical to VAI, *N*-(3-oxohexanoyl)homoserine lactone. Rather it is the related *N*-acyl-homoserine lactone, 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)dodecanamide [or *N*-(3-oxododecanoyl)homoserine lactone].

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions. The *E. coli* strains used were TB1 (19), MG4 (20), and VJS533 (21). The *P. aeruginosa* strains used were PAO1, which contains functional *lasR* and *lasI* genes, and PAO-RI, which is a *lasR*$^-$*lasI*$^-$ mutant derived from PAO1 (8). The plasmids used were pLasI-1, a *lasI* expression vector (11), pKDT17, which contains a *lasB*::*lacZ* reporter of *lasB* promoter activity and *lasR* under control of the *lac* promoter, and pHV200I$^-$, which contains the *V. fischeri* luminescence gene cluster with an inactive *luxI*. These plasmids are all ColE1 replicons containing an ampicillin-resistance marker. Construction of pKDT17 involved cloning into *Sma* I-digested pUCP18 (22), an 800-bp *lasR* fragment from pMJG1.7 (8) that extended from the *Eco*RV site 59 bp upstream of the *lasR* transcriptional start to the *Alu* I site 22 bp beyond the *lasR* translational stop codon to construct pKDT11. In this plasmid, *lasR* is under control of the *lac* promoter. An intermediate construct containing only two *Pvu* II sites was made by subcloning the 800-bp fragment from pKDT11 into pUC18 to form pKDT13. This intermediate construct was digested with *Pvu* II and the p*lac*–*lasR* fragment was cloned in *Tth* III-digested pTS400. The plasmid pTS400 contains a *lasB*::*lacZ* translational fusion (11, 23). The resulting plasmid was called pKDT17. The plasmid pHV200I$^-$ was derived from the *lux* regulon-containing pHV200 (24) by introducing a frameshift mutation in *luxI*. This was accomplished by digesting pHV200 with *Bgl* II, filling-in the single-stranded overhangs with *Taq* polymerase, and treating the product with T4 DNA ligase.

For production of PAI, cultures of *P. aeruginosa* PA01 or *E. coli* TB1 containing pLasI-1 were grown to late-

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Abbreviations: PAI, *Pseudomonas aeruginosa* autoinducer; VAI, *Vibrio fischeri* autoinducer; AAI, *Agrobacterium tumefaciens* autoinducer.

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exponential phase in A medium (25) supplemented with 0.4% glucose, 0.05% yeast extract, and 1 mM $MgSO_4$ with shaking at 37°C. For use in autoinducer bioassays, *E. coli* MG4 containing pKDT17 was grown in supplemented A medium at 30°C with shaking, and *E. coli* VJS533 containing pHV200I$^-$ was grown in L broth (26) at 30°C with shaking. For plasmid screening and maintenance, ampicillin (100 μg/ml) was included in *E. coli* cultures.

Plasmid Purification and Transformation Procedure. Plasmids were purified and manipulated as described (25). The transformation procedure used was described by Hanahan (27).

Autoinducer Bioassays. When grown in a medium conditioned by the growth of a recombinant *E. coli* containing *lasI*, *E. coli* MG4 containing a plasmid with *lasR* and a *lasB*::*lacZ* transcriptional fusion shows a 25-fold induction of β-galactosidase (11). This formed the basis of our quantitative PAI assay. We found that PAI activity could be extracted in ethyl acetate as described for VAI (14, 28). For our bioassays, overnight cultures of *E. coli* containing pKDT17 were diluted in supplemented A medium to an OD_{660} of 0.1 and stored on ice. Each bioassay consisted of 2 ml of the cell suspension plus the test sample. After 5.5 h at 30°C, β-galactosidase activity was measured as described by Miller (29). Without addition of autoinducer, β-galactosidase activities were 25 ± 10 Miller units and, with saturating amounts of autoinducer, β-galactosidase activities were 1500 ± 200 Miller units. A unit of PAI activity is defined as that amount required to achieve 50% saturation in the bioassay.

The assay for VAI was based on those described elsewhere (15, 17, 28). Because luminescence of *E. coli* containing pHV200I$^-$ requires the addition of VAI, this recombinant strain was used to test for VAI activity. Overnight cultures of *E. coli* containing pHV200I$^-$ were diluted to an OD_{660} of 0.01 in 0.05% tryptone/0.03% glycerol/100 mM NaCl/50 mM $MgSO_4$/10 mM potassium phosphate, pH 7. Each bioassay consisted of 1 ml of the cell suspension plus the test sample. After 3 h at room temperature, luminescence was measured in a photometer. Synthetic VAI (30) was used to construct a standard dose–response curve. A unit of activity is defined as that amount required to achieve a half-maximal response.

Purification of PAI. The procedure for purification of PAI was based on that described previously for purification of VAI (14). Cells and culture fluid were separated by centrifugation (10,000 × *g* for 10 min at 4°C). The culture fluid was passed through a 0.2-μm pore-size filter and then extracted twice with equal volumes of ethyl acetate plus glacial acetic acid at 0.1 ml/liter (glacial acetic acid at 0.1 ml/liter was included with ethyl acetate throughout). Water was removed with magnesium sulfate, and the ethyl acetate was removed by rotary evaporation at 40–45°C. The residue was extracted in 5 ml of ethyl acetate, followed by sequential extractions with 5 ml of ethanol, 5 ml of ethyl acetate, 5 ml of ethanol, and 5 ml of ethyl acetate. The solvents were removed by rotary evaporation after each extraction. Finally, the sample was dissolved in 0.2 ml of methanol and further purified by HPLC with a C_{18} reverse-phase column (0.46 × 25 cm). The PAI activity was recovered as a sharp peak at 73–78% methanol in a linear 20–100% (vol/vol) gradient of methanol and water. Fractions constituting this peak were pooled and dried by rotary evaporation, and the residue was dissolved in ethyl acetate. The ethyl acetate was removed, the residue was dissolved in 0.1 ml of methanol, and this solution was subjected to further purification by HPLC using a step gradient from 60 to 65% methanol in water. PAI was recovered as a single peak in 65% methanol. The active fractions were dried, dissolved in ethyl acetate, and stored at −20°C.

Chemical Synthesis of PAI. Synthesis of PAI, *N*-(3-oxododecanoyl)-L-homoserine lactone, was similar to that described by Eberhard *et al.* (14) for synthesis of the VAI, *N*-(3-oxohexanoyl)homoserine lactone. The major difference was that ethyl 3-oxododecanoate was used instead of ethyl 3-oxohexanoate. The ethyl 3-oxododecanoate was prepared from decanoyl chloride and the dilithio dianion of monoethyl hydrogen malonate (31). The ethylene glycol ketal of ethyl 3-oxododecanoate was prepared as described for ethyl 3-oxohexanoate (14) except that Dowex-50 sulfonic acid cation-exchange resin was used in place of *p*-toluenesulfonic acid as described by Goswami *et al.* (32). The sodium salt was prepared (14) and incubated with equimolar amounts of L-homoserine lactone hydrochloride (Sigma) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (Aldrich, Milwaukee, Wisconsin). The resulting ethylene glycol ketal of *N*-(3-oxododecanoyl)-L-homoserine lactone was deprotected by acid treatment (14) to yield *N*-(3-oxododecanoyl)-L-homoserine lactone. This compound was purified by HPLC and stored as described above.

Spectra. Proton NMR was performed on a Varian Unity 500-MHz instrument. Infrared spectroscopy of intermediates in the synthesis of PAI was performed on a Nicolet 205 Fourier transform infrared spectrometer. Chemical ionization mass spectrometry was performed on a Nermag R10-10C instrument with a desorption chemical ionization probe. The reagent gas was ammonia. High-resolution fast atom bombardment was performed at the University of Nebraska, Midwest Center for Mass Spectrometry.

RESULTS

Purification of PAI from Culture Media. Ethyl acetate extracts of media from cultures of *E. coli* (pLasI-1) or *P. aeruginosa* PAO1 contained PAI activity in amounts equivalent to those in the culture media prior to extraction. Bioassays on extracts of media from cultures grown to an OD_{660} of 0.3 indicated that *P. aeruginosa* and *E. coli* (pLasI-1) produced equivalent amounts of PAI (Fig. 1).

Because it has been reported that *P. aeruginosa* produces VAI, *N*-(3-oxohexanoyl)homoserine lactone (17), we tested this compound for activity as an inducer for the *lasB* promoter. We also tested a homolog of VAI, *N*-(3-oxooctanoyl)-homoserine lactone, that serves as the autoinducer in conjugal transfer gene activation in *Agrobacterium tumefaciens*, a plant pathogen (33). VAI had no detectable activity when

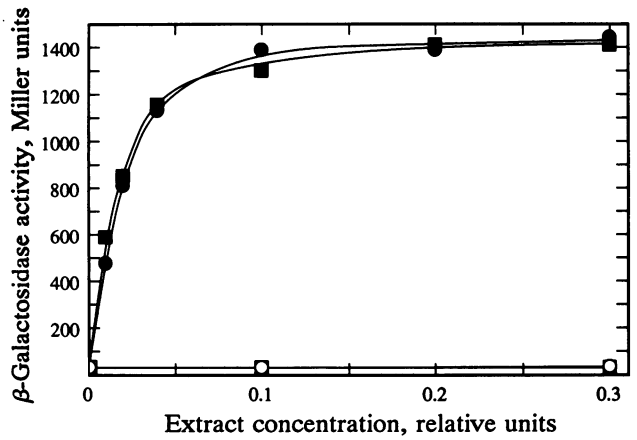

FIG. 1. Dose–response curves for PAI in ethyl acetate extracts of bacterial culture fluid. Culture fluid extracts of *P. aeruginosa* PA01 (●), *P. aeruginosa* PAO1-RI (○), *E. coli* (pLasI-1) (■), and *E. coli* without pLasI-1 (□) were added in the relative amounts shown on the horizontal axis. Relative amounts of extract correspond to the amount of culture fluid extracted. One milliliter of *P. aeruginosa* culture fluid contained ≈50 units of PAI activity. A unit of autoinducer activity is defined as that amount required for a half-maximal activation of the *lasB* promoter in *E. coli* (pKDT17) in the bioassay.

tested in the PAI bioassay at concentrations as high as 500 nM (Table 1). This compound gives a half-maximal response at ≈25 nM in the VAI bioassay. The *A. tumefaciens* autoinducer (AAI), however, showed considerable activity (Table 1). This suggested that PAI is *N*-(3-oxooctanoyl)homoserine lactone or a related compound.

When PAI extracted from supernatant fluids of *E. coli* (pLasI-1) or *P. aeruginosa* PAO1 cultures was subjected to HPLC, one major peak of activity was observed (Fig. 2). For PAI produced by *E. coli* (pLasI-1), there was a small peak of activity that was eluted just after the major peak. Synthetic AAI, *N*-(3-oxooctanoyl)homoserine lactone (34), was eluted well ahead of the major peak of PAI (Fig. 2). This shows that PAI is not *N*-(3-oxooctanoyl)homoserine lactone. Synthetic VAI (30) was eluted ahead of both AAI and PAI. The chromatographic behavior of PAI and the finding that AAI shows activity in the bioassay for PAI suggest that PAI is an *N*-acyl-homoserine lactone with a hydrophobic side chain longer than that of AAI.

Because it has been reported that *P. aeruginosa* produces VAI (17), we tested HPLC fractions of *P. aeruginosa* PAO1 culture fluid in the VAI bioassay. In fact, there was a peak of VAI activity that was eluted at the same location as synthetic *N*-(3-oxohexanoyl)homoserine lactone (data not shown). There also was a peak that was eluted at the same location as synthetic *N*-(3-oxooctanoyl)homoserine lactone, which shows some activity with *V. fischeri* (34), and as we have shown, is an inducer of the *P. aeruginosa lasB* gene (Table 1). However, only low levels of these compounds were present in extracts of either *P. aeruginosa* or *E. coli* (pLasI-1) culture medium.

Analysis of Purified PAI. The proton NMR spectrum of purified PAI (Fig. 3*A*) was remarkably similar to that of VAI (14, 35) except for the presence of a large methylene peak at 1.22 ppm in the PAI spectrum. The integration of this methylene peak indicated the purified PAI was *N*-(3-oxododecanoyl)homoserine lactone. Chemical ionization mass spectrometry showed a strong quasimolecular $(M+H)^+$ ion with an m/z of 298 (Fig. 4*A*). This is consistent with the conclusion that PAI is *N*-(3-oxododecanoyl)homoserine lactone. The chemical composition was confirmed by high-resolution fast atom bombardment, which showed the m/z of the $(M+H)^+$ was 298.2018. This corresponded to a chemical composition of $C_{16}H_{27}NO_4$. This is the composition of *N*-(3-oxododecanoyl)homoserine lactone.

Analysis of Synthetic *N*-(3-Oxododecanoyl)homoserine Lactone. To confirm that PAI is *N*-(3-oxododecanoyl)homoserine lactone, we synthesized this compound and showed that the synthetic compound has biological activity. The mass spectrum was comparable to that of natural PAI (Fig. 4). High-resolution fast atom bombardment showed the m/z of the $(M+H)^+$ was 298.2026. This is in agreement with the value for natural PAI. The NMR spectra of synthetic *N*-(3-oxododecanoyl)-L-homoserine lactone and natural PAI were essentially identical (Fig. 3). In addition, when coinjected, the natural and synthetic compounds were recovered as a single peak in methanol-gradient HPLC.

Table 1. Influence of PAI, VAI, and AAI on *lasB* promoter activity in *E. coli* MG4 (pKDT17)

Autoinducer added	β-Galactosidase activity, Miller units
None	26 ± 2
VAI (50 nM)	33 ± 3
VAI (500 nM)	34 ± 1
AAI (50 nM)	56 ± 5
AAI (500 nM)	733 ± 50
PAI (1.0 unit)	735 ± 110
PAI (3.0 units)	1470 ± 90

β-Galactosidase activity is a measure of *lasB*::*lacZ* promoter activity. Numbers are the average (± the range) of four experiments. PAI was an ethyl acetate extract of the culture medium in which *E. coli* (pLasI-1) was grown. Synthetic VAI (30) and synthetic AAI (34) were used.

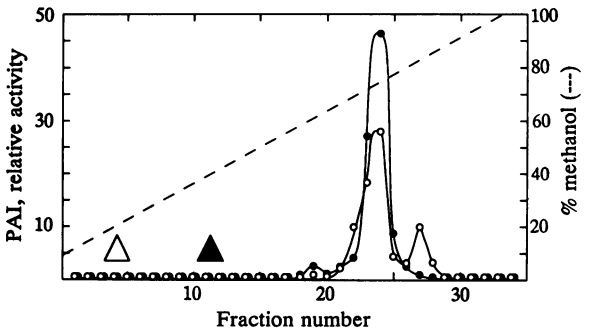

FIG. 2. HPLC analysis of PAI extracted from culture medium. Autoinducer was extracted from medium in which a culture of *E. coli* (pLasI-1) had been grown (○) and from medium in which a culture of *P. aeruginosa* PAO1 had been grown (●). Each fraction was 2 ml. The dashed line indicates the methanol concentration. The percent of activity recovered in the major peak for either bacterium was >75%. The open triangle indicates where *N*-(3-oxohexanoyl)homoserine lactone was eluted, and the solid triangle indicates where *N*-(3-oxooctanoyl)homoserine lactone was eluted.

The biological responses to natural PAI and synthetic *N*-(3-oxododecanoyl)-L-homoserine lactone were similar (Fig. 5). For either, the bioassays indicate that the half-maximal response occurs at 3–5 nM. This is in the range found for the VAI (15) and AAI (32) systems, where half saturations occur at ≈25 nM and 5 nM, respectively.

DISCUSSION

We conclude that the autoinducer, which serves in conjunction with the LasR protein to activate a number of *P. aeruginosa* virulence genes, is *N*-(3-oxododecanoyl)homoserine lactone. This autoinducer has a longer acyl side chain than related autoinducers from other bacteria (Fig. 6). It was

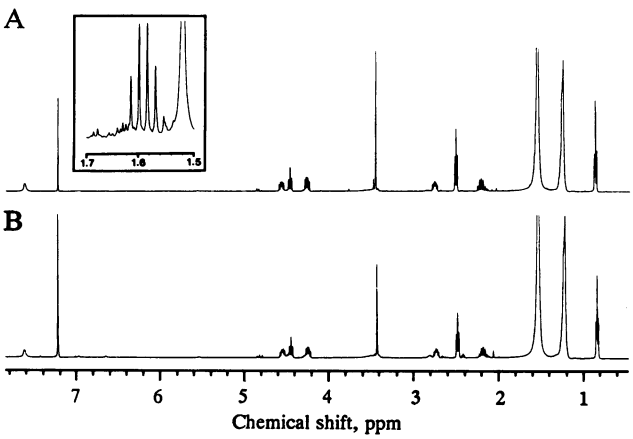

FIG. 3. High-resolution proton NMR spectra of natural and synthetic PAI in C^2HCl_3. (*A*) Natural PAI purified from spent *P. aeruginosa* PAO1 culture medium (500 μg in 0.8 ml): δ_H 0.85 (3H, t, CH_3), 1.22 [12H, broad s, $CH_3(CH_2)_6$], 2.18 (1H, m, β-CH_2), 2.48 (2H, t, CH_2CO), 2.73 (1H, m, β-CH_2), 3.43 (2H, s, $COCH_2CO$), 4.24 (1H, m, γ-CH_2), 4.44 (1H, m, γ-CH_2), 4.54 (1H, m, α-CH_2), 7.62 (1H, broad d, NH). The signal at 1.5 ppm was reduced by addition of ^2H_2O. This allowed resolution of a CH_2CH_2CO quintet at 1.60 and indicated that the shift at 1.5 ppm was due to H_2O. (*Inset*) Expanded scale with the quintet at 1.60 revealed by addition of ^2H_2O. (*B*) Synthetic PAI (400 μg in 0.8 ml). The chemical shifts and integrations were indistinguishable from those in *A*.

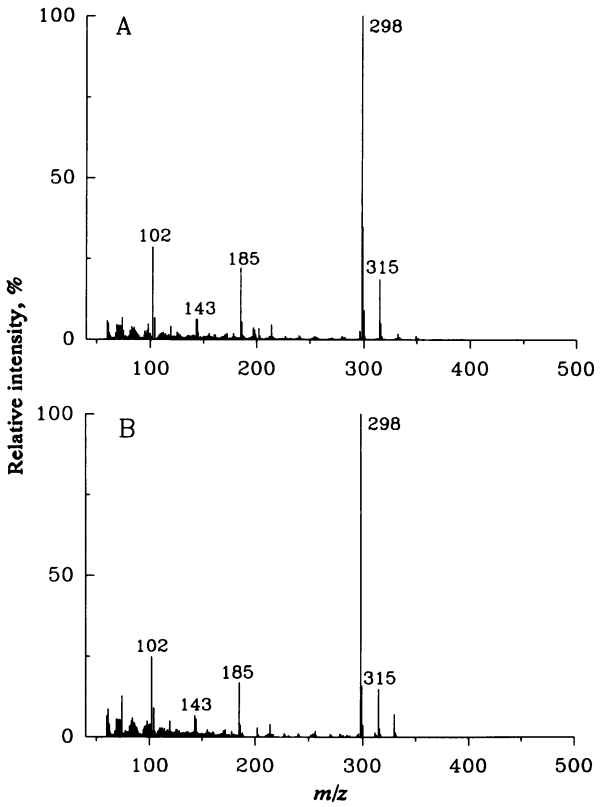

FIG. 4. Chemical ionization mass spectra of natural PAI purified from a spent *P. aeruginosa* PAO1 culture medium (*A*) and synthetic PAI (*B*). The m/z of the $(M+1)^+$ was 298. This is consistent with that expected for *N*-(3-oxododecanoyl)homoserine lactone.

reported elsewhere that *P. aeruginosa* produces the VAI, *N*-(3-oxohexanoyl)homoserine lactone (17), and it was suggested that this VAI was the PAI (18). Our analysis confirmed that a compound that serves to induce the *V. fischeri lux* genes is produced by the *lasI* gene product and that this compound behaves like VAI in HPLC. However, relatively low levels of this compound were synthesized by *lasI*-containing *P. aeruginosa* or *E. coli* (0.5% of the PAI concentration in extracts of *P. aeruginosa* PAO1, data not shown). Neither this compound nor synthetic VAI showed PAI activity. Based on the data in Fig. 1, which show that 1 unit of PAI activity can be extracted from ≈0.02 ml of culture

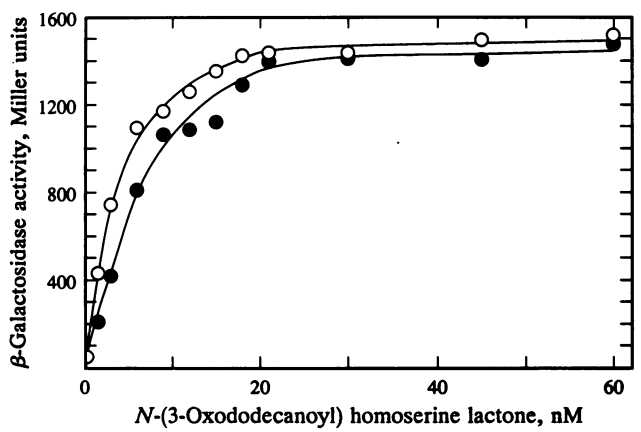

FIG. 5. Dose–response curves for activity of natural PAI purified from *P. aeruginosa* culture medium (●) and activity of synthetic *N*-(3-oxododecanoyl)-L-homoserine lactone (○). Concentrations of either natural or synthetic PAI in the range of 3–5 nM gave a response equivalent to 1 unit of PAI activity.

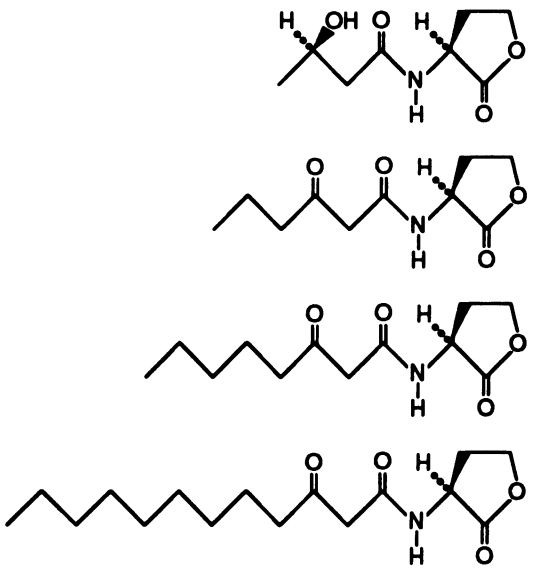

FIG. 6. Known autoinducer structures. Structures, from the top, are as follows: *N*-(3-hydroxybutanoyl)homoserine lactone, the inducer of luminescence in *Vibrio harveyi* (36, 37); *N*-(3-oxohexanoyl)-homoserine lactone, the inducer (VAI) of *V. fischeri* luminescence (14); *N*-(3-oxooctanoyl)homoserine lactone, the inducer (AAI) of conjugal transfer genes in *A. tumefaciens* (33); *N*-(3-oxododecanoyl)-homoserine lactone, the PAI.

fluid, and the data in Fig. 5, which show that 1 unit of activity corresponds to ≈4 nM PAI, we calculated that ≈200 nM PAI was present in a mid-logarithmic-growth-phase culture of *P. aeruginosa* grown in supplemented A medium (in a stationary-phase culture, the PAI concentration was 1–2 μM, data not shown). This is in considerable excess of the concentrations required to fully activate the *lasB* promoter.

It has recently become apparent that regulatory circuits homologous to LuxR–LuxI in *V. fischeri* and LasR–LasI in *P. aeruginosa* are common to several diverse Gram-negative bacteria. With this report four autoinducer structures are known (Fig. 6). The luminescence genes in *Vibrio harveyi* are controlled by *N*-(3-hydroxybutanoyl)homoserine lactone (36, 37), but homologs of *luxI* and *luxR* in this organism have not been identified. A number of bacteria have been reported to produce *N*-(3-oxohexanoyl)homoserine lactone, VAI (17). In one of these bacteria, *Erwinia carotovora*, the *luxI* and *luxR* homologs *expI* and *expR*, respectively, have been identified (38). The *expI* gene directs the synthesis of an autoinducer that is required with the *expR* product for induction of extracellular protease in a fashion reminiscent of PAI control of extracellular protease induction in *P. aeruginosa*. Conjugal transfer genes in *A. tumefaciens* are controlled by AAI (33) and a transcriptional activator encoded by *traR* (39). The gene or genes required for AAI synthesis have not yet been described.

The notion that PAI and LasR serve as a global regulatory system for virulence factors in *P. aeruginosa* suggests they might be targets for therapeutic approaches to control *P. aeruginosa* infections. Two points in particular bear on this possibility: Certain analogs of VAI with substitutions in the homoserine lactone ring or with altered alkyl side chains serve as inhibitors of VAI (34). Also, for the plant pathogen *Erwinia carotovora*, *expI* mutants are avirulent (38).

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