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A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*

[autoinduction/gene activation/*las* genes/*N*-(tetrahydro-2-oxo-3-furanyl)butanamide/quorum sensing]

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ABSTRACT **Quorum sensing systems are used by a number of Gram-negative bacterial species to regulate specific sets of genes in a cell density-dependent manner. Quorum sensing involves synthesis and detection of extracellular signals termed autoinducers. As shown in recombinant *Escherichia coli*, the *Pseudomonas aeruginosa* autoinducer (PAI) *N*-(3-oxododecanoyl)homoserine lactone, together with the *lasR* gene product, activate the *P. aeruginosa lasB* gene. In this study, PAI was shown to activate *lasB–lacZ* expression in a *P. aeruginosa lasR* mutant containing a plasmid with *lasR* under the control of the *lac* promoter. The concentration of PAI necessary for half-maximal activation of the *lasB–lacZ* fusion was ≈1 μM, which is within the range of PAI levels found in *P. aeruginosa* culture fluids. The effect of PAI on a *P. aeruginosa lasR* mutant containing a plasmid with *lasR* under the control of its own promoter and containing the *lasB–lacZ* fusion was also tested. Although extracts of culture fluid activated the *lasB* promoter in this construct, concentrations of PAI as high as 10 μM did not. This indicates the presence of a second extracellular factor (factor 2) that is required for *lasB* activation in *P. aeruginosa* when *lasR* is controlled by its own promoter but not when *lasR* is controlled by a strong foreign promoter. Factor 2 was shown to be *N*-butyrylhomoserine lactone. Although recombinant *E. coli* cells containing the PAI synthase gene, *lasI*, produce PAI, these cells do not produce factor 2. Furthermore, a *P. aeruginosa* mutant that produced about 0.1% of the wild-type level of PAI made about 5% of the wild-type level of factor 2. This indicates that factor 2 synthesis results from the activity of a gene product other than PAI synthase. The role of factor 2 in virulence gene regulation remains to be determined, but this compound may affect the expression of *lasR*, which in turn activates transcription of numerous virulence genes in the presence of sufficient PAI. Apparently, multiple quorum sensing systems can occur and interact with each other in a single bacterial species.**

Pseudomonas aeruginosa lives in soil and freshwater environments and is a pathogen infecting immunocompromised individuals such as burn victims and people with cystic fibrosis (1). Survival and growth of *P. aeruginosa* in humans may be dependent in part on production of virulence factors such as the extracellular protease elastase (2). Regulation of the elastase gene, *lasB* (3), involves a phenomenon called quorum sensing, in which bacterial cells activate specific genes only at high population densities in response to chemical signals released by the bacterial cells into the growth medium (reviewed in ref. 4). At least two gene products comprise a quorum sensing system: a signal generator, encoded by a *luxI* homolog, and a cell-density dependent transcriptional activator, encoded by a *luxR* homolog (4). *luxI* and *luxR* regulate the luminescence genes of the marine bacterium *Vibrio fischeri* (5, 6). The chemical signal in each known example of quorum sensing is a specific *N*-acylhomoserine lactone (4, 7). For *V. fischeri*, it is *N*-(3-oxohexanoyl)homoserine lactone (8).

In *P. aeruginosa*, cell-density dependent transcription of *lasB* occurs when the LasR protein becomes activated by PAI, which is released into the growth medium by bacteria with a functioning *lasI* (9, 10). PAI [*N*-(3-oxododecanoyl)homoserine lactone], together with the product of the *P. aeruginosa lasR* gene, under control of the *lac* promoter, activates the *lasB* promoter in *Escherichia coli* (11). In this report we show that with the same reporter plasmid as that used in the studies with *E. coli*, PAI and LasR activate the *lasB* promoter in *P. aeruginosa*. However, somewhat higher concentrations of PAI are required for *lasB* promoter activity in *P. aeruginosa*. We also show that in *P. aeruginosa* carrying a plasmid with *lasR* under the control of its own promoter, the *lasB* promoter is not activated by PAI. Furthermore, we describe a second factor produced by *P. aeruginosa*, *N*-(tetrahydro-2-oxo-3-furanyl)butanamide (or *N*-butyrylhomoserine lactone). The role of this second *N*-acylhomoserine lactone in *P. aeruginosa* virulence gene regulation remains unclear, but identification of this factor and our initial studies of its activity indicate that there may be a cascade of quorum sensing regulatory events involved in *P. aeruginosa* virulence gene expression.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. The *P. aeruginosa* strains used were PAO1 and PAO-R1. PAO-R1 is a *lasR* deletion mutant derived from PAO1 (9). The *E. coli* strains used were TB1 (12) and MG4 (13). The plasmids used were pTS400, pTS400-1.7, and pKDT17. pTS400 contains a *lasB–lacZ* translational fusion (10, 14), pTS400-1.7 is identical to pTS400 except that it also contains *lasR* under control of its own promoter (10), and pKDT17 contains the *lasB–lacZ* fusion and a *lac* promoter-controlled *lasR* (11). Unless otherwise indicated, *P. aeruginosa* cultures were grown at 37°C with shaking in the chemically defined medium of Jensen *et al.* (15) supplemented with 0.4% glycerol instead of 70 mM glucose. *E. coli* TB1 was grown in LB at 37°C with shaking. *E. coli* MG4 was grown as described (11). Carbenicillin (200 μg/ml) or ampicillin (100 μg/ml) was used to ensure plasmid maintenance in cultures of *P. aeruginosa* or *E. coli*, respectively.

Extraction of Factor 2 Activity from Culture Fluids. Ethyl acetate extracts of bacterial culture fluids were prepared as described (8, 11), except that culture supernatants were not filtered prior to extraction. Extracts were prepared from cultures in early stationary growth phase.

PAI Bioassays. We found that unlike *E. coli* MG4(pKDT17), without added PAI *P. aeruginosa* PAO-R1(pKDT17) showed

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Abbreviation: PAI, *Pseudomonas aeruginosa* autoinducer.

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a cell density-dependent induction of the *lasB–lacZ* fusion such that levels of β-galactosidase were high in fully grown cultures (data not shown). To study the effect of PAI on induction of the *lasB–lacZ* fusion, cells were grown at low culture densities so that β-galactosidase levels were low. To accomplish this, cells were grown in the following manner: First, PTSB medium (16) was inoculated at a low starting optical density (OD_{660} of $<10^{-4}$) and the cultures were grown to an OD_{660} of 0.1. These cultures were then used as the inocula for bioassays (1% inoculum). Bioassay cultures were in 10 ml of medium containing the test sample in a 125-ml flask incubated at 37°C with shaking. These cultures were grown to an OD_{660} of 0.05, and *lasB* promoter activity was determined by measuring β-galactosidase levels as described by Miller (17). Although we have not shown the data for experiments at 30°C, the dose–response to PAI at this temperature was comparable to that at 37°C.

***P. aeruginosa* Factor 2 Bioassays.** For factor 2 bioassays, cultures of *P. aeruginosa* PAO-R1 (pTS400-1.7) were diluted in PTSB to an OD_{660} of 0.1. Inocula were cells from late-logarithmic-phase cultures that had been washed and resuspended in PTSB. Bioassays were in 1 ml of medium plus the test sample. After an incubation period of 12 hr with shaking at 30°C, cells were washed and resuspended in A medium (18), and β-galactosidase activity was measured. The 12-hr incubation time was chosen because this was when the response to factor 2 was maximal.

Purification of *P. aeruginosa* Factor 2. The purification was based on methods for purification of the *Vibrio fischeri* autoinducer described by Eberhard *et al.* (8). Midlogarithmic-phase cultures of *P. aeruginosa* PAO1 were used to inoculate larger volumes (initial OD_{660} of 0.005). When the cultures reached early stationary phase, the medium was extracted and the extracts were concentrated and dried (8). The residue from the ethyl acetate extracts was extracted with ethanol and the ethanol solution was centrifuged at 12,000 × *g* for 10 min. The supernatant was evaporated and the remaining residue was extracted in ethyl acetate. The ethyl acetate was evaporated under N_2 gas, the resulting residue was extracted in methanol, and the methanol solution was applied to a C_{18} reverse-phase HPLC column and eluted with a linear gradient of methanol in water (11). Active fractions were pooled, solvents were removed by rotary evaporation, and the residue was extracted in water. The water solution was separated in a step gradient from 100% water to 5% methanol in water on a C_{18} reverse-phase HPLC column. Factor 2 activity was recovered as a single peak in 5% methanol. The purified factor 2 was dried and stored at −20°C.

Synthesis of *N*-Butyryl-L-homoserine Lactone. The synthesis was conducted as described by Eberhard *et al.* (19). The resulting product was extracted in ethyl acetate, purified by HPLC, and stored as described above.

Spectra. Proton NMR was performed on a Varian model Unity 500-MHz instrument. Gas chromatography/electrical ionization mass spectrometry (GC/MS) was performed on a Hewlett–Packard 5890 equipped with a Hewlett–Packard Ultra-1 capillary column (25 m × 0.2 mm with 0.33-μm film thickness) using helium as the carrier gas. The temperature gradient was from 70°C to 240°C at 10°C/min. Temperatures of the injection port and detector were 220°C and 280°C, respectively. High-resolution fast atom bombardment (FAB) was performed on a ZAB-HF mass spectrometer (VG Analytical, Manchester, U.K.).

RESULTS

N-(3-Oxododecanoyl)homoserine lactone is the PAI responsible for LasR-mediated activation of *lasB* transcription. This was discovered by using a sensitive PAI bioassay that employed *E. coli* MG4 containing a plasmid, pKDT17, which carries *lasR* under the control of the *lac* promoter and a *lasB–lacZ* gene fusion (11). To show a PAI response in *P. aeruginosa* and to determine whether the response was quantitatively similar to that seen in *E. coli*, we examined the influence of PAI on expression of the *lasB–lacZ* fusion on pKDT17 in *P. aeruginosa* PAO-R1, a *lasR* mutant. Not only does the *lasR* mutation results in a loss of LasR, but because *lasI* expression depends on LasR (31), this strain contains only little LasI. Thus PAO-R1 produces very low levels of PAI (7 nM in stationary-phase cultures, whereas the level in PAO1 cultures is on the order of 5 μM; data not shown). We have used the *lasR* mutant because our efforts to construct a *lasI* null mutation by allelic exchange with insertionally inactivated *lasI* genes in *P. aeruginosa* have been unsuccessful. We have used these techniques with success to construct null mutations in other *P. aeruginosa* genes (20). *P. aeruginosa* PAO-R1(pKDT17) responded to ethyl acetate extracts of *P. aeruginosa* PAO1 culture fluid and to PAI in a fashion similar to that previously reported for *E. coli* (Fig. 1*A* and ref. 11). However, the concentration of PAI that resulted in half-maximal activation of the reporter in *P. aeruginosa* was about 1 μM, whereas it was about 5 nM in *E. coli* (11). Addition of PAI to cultures of *P. aeruginosa* PAO-R1(pKDT17) also caused a small but reproducible stimulation of growth (without PAI the doubling time was 45 min; with PAI it was 33 min).

We also studied the effect of culture fluid extracts and PAI on *lasB* activation in *P. aeruginosa* PAO-R1(pTS400-1.7), and the results were surprising. The difference between pKDT17 and pTS400-1.7 is that in the latter, *lasR* is under the control of its own promoter. As expected, addition of an ethyl acetate extract of wild-type *P. aeruginosa* (PAO1) culture fluid resulted in elevated levels of β-galactosidase (Fig. 1*B*). However, synthetic PAI had no significant effect on β-galactosidase activity, suggesting that another factor in PAO1 culture fluid is required for activation of the *lasB* reporter in PAO-R1(pTS400-1.7). With this reporter the maximum level of β-galactosidase activity achieved in the presence of this factor, factor 2 found in PAO1 culture fluid, was about 20% of that observed when PAI was added to *P. aeruginosa* PAO-R1(pKDT17) (Fig. 1). This could reflect a limitation in the LasR level, PAI concentration, or both.

To address whether the PAI synthase gene, *lasI*, is required for production of the *P. aeruginosa* factor 2 activity, culture fluids of *E. coli* TB1 containing *lasI*, which has previously been shown to produce PAI (10, 11), were extracted and tested in the factor 2 bioassay. The *E. coli* extracts showed no activation

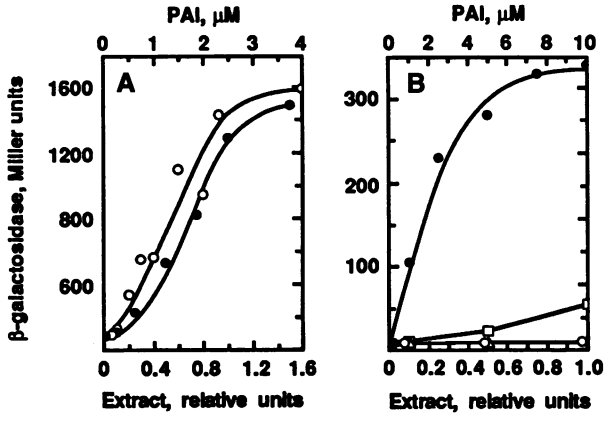

FIG. 1. Bioassays for extracellular factors in *P. aeruginosa*. (*A*) PAI bioassay in *P. aeruginosa* PAO-R1(pKDT17). Culture fluid extract of *P. aeruginosa* PAO1 (●) or synthetic PAI (○) was added in the amounts shown. Relative units correspond to the amount of culture fluid extracted. (*B*) Responses of *P. aeruginosa* PAO-R1(pTS400-1.7) to ethyl acetate extracts of bacterial culture fluids of *P. aeruginosa* PAO1 (●), PAO-R1 (□), and *E. coli* TB1(pLasI-1) (△) and to PAI (○).

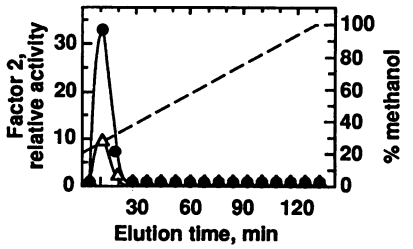

FIG. 2. HPLC analysis of factor 2 extracted from *P. aeruginosa* PAO1 (●) or PAO-R1 (△) culture fluids. HPLC conditions are described in *Materials and Methods*. The dashed line indicates the percentage of methanol in water (vol/vol).

of the *lasB* reporter in *P. aeruginosa* when *lasR* was under control of its own promoter (Fig. 1*B*). This suggests that a gene other than *lasI* directs the synthesis of factor 2. Culture fluids of the *P. aeruginosa lasR* mutant (PAO-R1) were also extracted and showed a somewhat reduced level of factor 2 activity, about 5% that of the parent PAO1 (Fig. 1*B*). This suggests that LasR may regulate factor 2 production.

To learn more about the active components within the extracts, methanol-gradient HPLC was used as described (11), and the fractions collected were bioassayed for factor 2 and PAI. Factor 2 activity from both wild-type *P. aeruginosa* (PAO1) and from the *lasR* mutant (PAO-R1) was eluted in the void volume from the HPLC column (Fig. 2). PAI bioassays [in *E. coli* MG4(pKDT17)] of the fractions showed that a single peak of PAI was eluted at the expected position [73–78% methanol in water (vol/vol)] (data not shown; ref. 11).

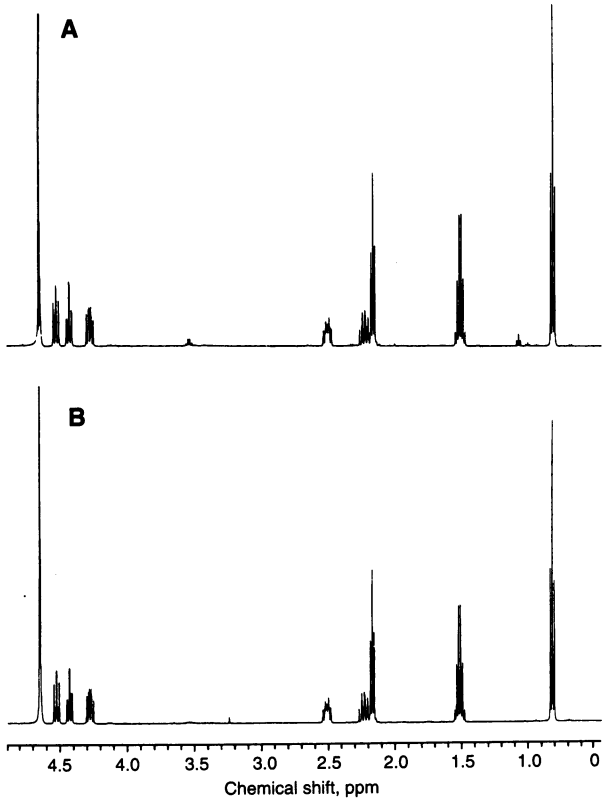

FIG. 3. High-resolution proton NMR spectra of natural and synthetic *P. aeruginosa* factor 2 in ^2H_2O. (*A*) Natural *P. aeruginosa* factor 2 purified from strain PA01 culture medium (1.6 mg in 0.7 ml): δ_H 0.81 (3H, t, CH_3), 1.52 (2H, sextet, CH_2CH_2CO), 2.18 (2H, t, CH_2CO), 2.24 (1H, m, β-CH_2), 2.52 (1H, m, β-CH_2), 4.29 (1H, m, γ-CH_2), 4.45 (1H, m, γ-CH_2), 4.55 (1H, m, α-CH_2). (*B*) Synthetic *P. aeruginosa* factor 2 (0.7 mg in 0.7 ml). The chemical shifts and integrations were indistinguishable from those in *A*. The resonance at 4.67 ppm in both *A* and *B* is from water. No resonances were observed downfield of the water resonance in either *A* or *B*.

Structure of Factor 2. Factor 2 was purified from culture fluid extracts of *P. aeruginosa* PAO1 as described in *Materials and Methods*. The proton NMR spectrum of the purified material was similar to the proton NMR spectra of other *N*-acylhomoserine lactones (8, 11, 20, 21) (Fig. 3*A*). The spectrum of purified factor 2 contained a triplet at 2.18 ppm and a sextet at 1.52 ppm, which correspond, respectively, to methylene protons in positions 2 and 3 on an *N*-acyl side chain (Fig. 3*A*). This indicated that purified factor 2 was *N*-(tetrahydro-2-oxo-3-furanyl)butanamide (or *N*-butyrylhomoserine lactone). GC/MS of the purified factor 2 yielded a single GC peak (retention time, 13.26 min). This compound had a molecular ion with an *m/z* of 171 (Fig. 4*A*). High-resolution FAB MS showed that the *m/z* of the $(M+H)^+$ of factor 2 was 172.0972, which corresponds to a chemical composition of $C_8H_{13}NO_3$. This is consistent with the composition of *N*-butyrylhomoserine lactone.

Analysis of Synthetic *N*-Butyryl-L-Homoserine Lactone. As a confirmation that *P. aeruginosa* factor 2 is *N*-butyrylhomoserine lactone, this compound was synthesized and shown to have biological activity. GC/MS analysis of the synthetic product showed a single GC peak (retention time, 13.29 min), the molecular ion had an *m/z* of 171, and the mass spectrum was indistinguishable from that of the purified factor 2 (Fig. 4). High-resolution FAB MS of the synthetic product gave an *m/z* of the $(M+H)^+$ at 172.0973, which is consistent with that observed for the purified factor 2 and with the expected value of 172.0974 for *N*-butyrylhomoserine lactone. The proton NMR spectra for the synthetic *N*-butyryl-L-homoserine lactone and the natural *P. aeruginosa* factor 2 were indistinguishable (Fig. 3).

The biological activities of the natural *P. aeruginosa* factor 2 and the synthetic *N*-butyryl-L-homoserine lactone in *P. aeruginosa* [PAO-R1(pTS400-1.7)] were similar (Fig. 5). The half-maximal response to either was at ≈1 μM. To determine whether factor 2 was interacting with the *lasB* promoter, we tested its affect on β-galactosidase activity in PAO-R1(pTS400), which contains the *lasB–lacZ* fusion but does not contain a functional *lasR*. In this strain, β-galactosidase levels were low (6–10 Miller units) with or without factor 2 added at concentrations as high as 5 μM. When factor 2 (up to 500 nM) was tested in *E. coli* containing pTS400-1.7, no *lasB* activation occurred. However, unlike *P. aeruginosa* PAO-R1(pTS400-1.7), *E. coli*(pTS400-1.7) did respond to exogenous addition of

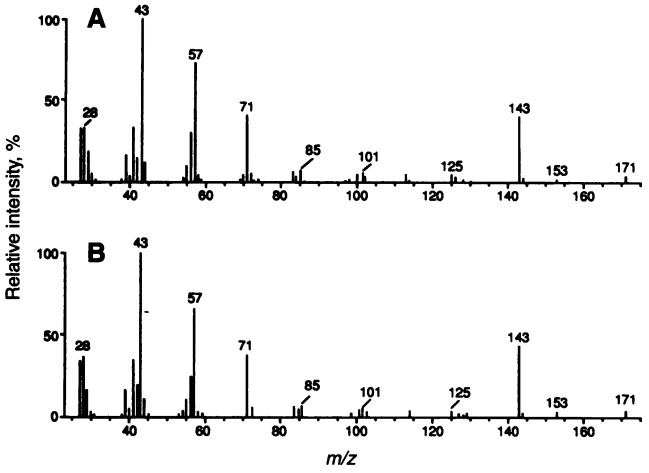

FIG. 4. Electrical ionization mass spectra of natural factor 2 purified from *P. aeruginosa* PAO1 culture medium (*A*), and synthetic factor 2 is shown (*B*). The *m/z* of the molecular ion was 171. This is the expected value for *N*-butyrylhomoserine lactone.

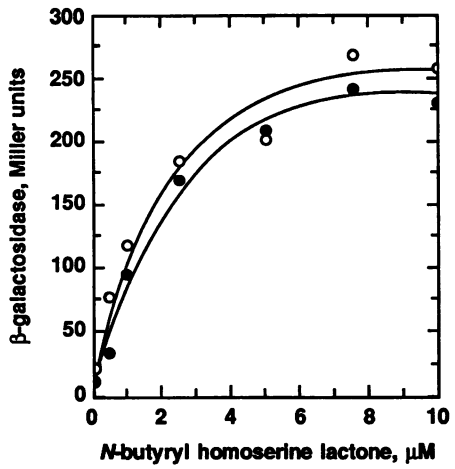

FIG. 5. Dose–response curves for activity of natural *P. aeruginosa* factor 2 (○) and synthetic *N*-butyryl-L-homoserine lactone (●). Concentrations of either natural or synthetic factor 2 at ≈1 μM gave a half-maximal response.

PAI, showing a dose response that was similar to that of *E. coli*(pKDT17). These results indicate that factor 2 cannot interact directly with LasR. They also suggest that factor 2 serves to derepress or activate something in *P. aeruginosa* so that LasR and PAI can then activate the *lasB* promoter. It appears that the target with which factor 2 interacts is not present in *E. coli*(pTS400-1.7). Finally, in *E. coli*(pTS400-1.7), *lasB* activation by PAI (10 nM) was not affected by addition of factor 2 at concentrations of 100 nM or 500 nM (β-galactosidase activity was 1700–1800 Miller units with or without factor 2). This finding supports the conclusion that factor 2 does not directly interact with LasR.

DISCUSSION

We have shown that in *P. aeruginosa*, exogenously added PAI can activate the *lasB* promoter when *lasR* is present and under the control of a strong foreign promoter. The concentration of PAI needed for *lasB* activation in *P. aeruginosa* was ≈200-fold that needed in *E. coli* (Fig. 1 and ref. 11) and about one-fifth that found in stationary-phase culture fluids of PAO1 (Fig. 1*A*). Previous reports have shown that in *V. fischeri* the autoinducer concentration needed for half-maximal luminescence gene activation is ≈50 nM (23), whereas *E. coli* containing *V. fischeri* luminescence genes requires 2.5 nM (K. M. Gray and E.P.G., unpublished work). Recently, it has been shown that expression of the *traM* gene in *Agrobacterium tumefaciens* results in this organism requiring higher concentrations of the *A. tumefaciens* autoinducer for the function of its LuxR homolog, TraR (24). It remains to be determined why higher concentrations of PAI are required for *lasR* activation in *P. aeruginosa* as compared to *E. coli*, but a function analogous to that of the *A. tumefaciens traM* gene could account for this.

When *lasR* is under control of its own promoter, extracts of PAO1 culture fluid but not PAI activate the *lasB* promoter in *P. aeruginosa*. This is due to an additional extracellular factor, factor 2, which was purified from PAO1 culture fluid extracts and identified as *N*-butyrylhomoserine lactone (Fig. 6). The unsubstituted *N*acyl side chain of factor 2 is unique in comparison with the side chains of other known bacterial *N*-acylhomoserine lactone autoinducers, which contain either a 3-oxo (8, 11, 21, 25) or a 3-hydroxy (22) moiety. Based on the data in Figs. 1*B* and 5, we calculate the level of *N*-butyrylhomoserine lactone (factor 2) in early-stationary-phase cultures of PAO1 (OD_{660} of 5) to be about 10 μM.

Factor 2 cannot substitute for PAI, as evidenced by the fact that no significant activity was exhibited in the *E. coli*

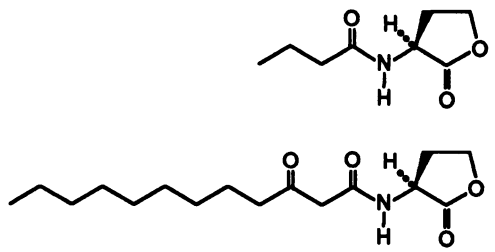

FIG. 6. Structure of *P. aeruginosa* factor 2, *N*-butyrylhomoserine lactone (*Upper*), and the structure of PAI, shown for reference (*Lower*). Factor 2 and PAI showed no crossreactivity in our experiments.

MG4(pKDT17) bioassay, even with concentrations of factor 2 as high as 10 μM. Thus factor 2 does not appear to interact with the LasR protein directly to activate *lasB* gene expression. The *lasB* promoter itself is also unlikely to be regulated by factor 2 directly, because this signal has no effect on *lasB* activation in *P. aeruginosa* lacking *lasR* [PAO-R1(pTS400)]. In addition, factor 2 is unlikely to activate *lasB* through induction of *lasI*, because PAI, whose synthesis is directed by the LasI protein, does not substitute for factor 2. The observation that factor 2 activity in *P. aeruginosa* occurs specifically when *lasR* is under control of its own promoter suggests that factor 2 may be somehow involved in *lasR* control.

The autoinducer-like structure of factor 2 and its high concentration in culture fluids of *P. aeruginosa* suggest that this molecule is likely to be part of a quorum sensing system in this bacterium. Because factor 2 has no activity in *E. coli* carrying the *lasB–lacZ* system [MG4(pTS400-1.7)], it seems probable that a LuxR-type protein not found in *E. coli* but present in *P. aeruginosa* may sense factor 2. RhlR, a transcriptional activator showing significant sequence similarity with the LuxR family of regulators, has been found in *P. aeruginosa* (26). Therefore it is not surprising that another autoinducer-like molecule should exist. It is enticing to speculate that factor 2 activity is dependent upon RhlR and that this interaction is part of a series of events. A complex method of regulation involving more than one autoinducer has already been proposed for the quorum sensing system of *V. harveyi* (27–29). However, there is no information on the structure of the hypothetical second *V. harveyi* autoinducer.

The number of known quorum sensing systems is increasing as more bacterial species are examined. With this report, *P. aeruginosa* is shown to produce and use two *N*-acylhomoserine lactones, *N*-(3-oxododecanoyl)homoserine lactone (PAI) and *N*-butyrylhomoserine lactone (factor 2). From our data it appears that the *lasI* product is not responsible for factor 2 synthesis and that factor(s) in *P. aeruginosa* other than LasR may interact with factor 2. Answers to how factor 2 is involved in virulence gene regulation and what is required for its synthesis await further study. The discovery that *P. aeruginosa* makes a second *N*-acylhomoserine lactone, factor 2, involved in activation of *lasB* suggests that a quorum sensing circuit with multiple signals and receptors exists and that PAI–LasR defines only one of the important aspects of this circuit.

Note. During revision of this manuscript we learned that an article by Kuo *et al.* (30) was accepted for publication. This report shows that *V. fischeri* synthesizes a second *N*-acylhomoserine lactone. In addition to *N*-(3-oxohexanoyl)homoserine lactone, *V. fischeri* produces *N*-octanoylhomoserine lactone. Synthesis of the second *N*-acylhomoserine lactone is independent of *luxI* and requires a second locus, *ain* (30). Furthermore, we have learned that the DNA immediately downstream of *rhlR* in *P. aeruginosa* codes for a LuxI homolog required for production of a signal, which together with RhlR activates *rhlAB* (U. A. Ochsner and J. Reiser, personal communication). In a different strain of *P. aeruginosa*, genes termed *vsmR* and *vsmI* have been found. The *vsmR* and *vsmI* genes may be equivalent to *rhlR* and *rhlI*. The *vsmI* gene directs the synthesis of *N*-butyrylhomoserine lactone (factor 2)

and *N*-hexanoylhomoserine lactone in *E. coli* or *P. aeruginosa* (P. Williams, G. Stewart, B. Bycroft, and A. Lazdunski, personal communication).

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