

Functional Analysis of the *Pseudomonas aeruginosa* Autoinducer PAI

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A series of structural analogs of the *Pseudomonas aeruginosa* autoinducer [PAI, *N*-3-oxo-dodecanoyl homoserine lactone] were obtained and tested for their ability to act as autoinducers in stimulating the expression of the gene for elastase (*lasB*) by measuring β -galactosidase production from a *lasB-lacZ* gene fusion in the presence of the transcriptional activator LasR. The data suggest that the length of the acyl side chain of the autoinducer molecule is the most critical factor for activity. Replacement of the ring O by S in the homoserine lactone moiety can be tolerated. Tritium-labelled PAI (³H]PAI) was synthesized and used to demonstrate the association of [³H]PAI with cells overexpressing LasR. The PAI analogs were also tested for their ability to compete with [³H]PAI for binding of LasR. Results from the competition assays suggest that once again the length of the acyl side chain appears to be crucial for antagonist activity. The presence of the 3-oxo moiety also plays a significant role in binding since analogs which lacked this moiety were much less effective in blocking binding of [³H]PAI. All analogs demonstrating competition with PAI in binding to LasR also exhibited the ability to activate *lasB* expression, suggesting that they are functional analogs of PAI.

An increasing number of gram-negative organisms have been shown to utilize quorum-sensing as a method of gene regulation (2, 4, 28). Quorum-sensing systems make use of the interaction of two components: a transcriptional activator protein (R protein) and an *N*-acyl homoserine lactone molecule termed the autoinducer (AI) (4). The AI is proposed to bind to the R protein, and the R protein-AI complex stimulates the expression of a target gene. The most extensively studied quorum-sensing system is that which regulates bioluminescence in the marine bacterium *Vibrio fischeri*. In that system, the transcriptional activator, LuxR, interacts with the *Vibrio* AI [VAI; *N*-3-(oxohexanoyl) homoserine lactone] to activate the genes required for bioluminescence (4). VAI is produced in bacteria expressing the *luxI* gene, which encodes an AI synthase (4).

The subsequent cloning and characterization of the *lasR* and *lasI* genes (6, 20) indicated the presence of a quorum-sensing system in *Pseudomonas aeruginosa*, a gram-negative, opportunistic pathogen of immunocompromised individuals. LasR was shown to activate the transcription of several genes, including the genes for PAI synthase (*lasI*), two elastases (*lasA* and *lasB*), and alkaline protease (*aprA*), and to enhance expression of the gene for exotoxin A (*toxA*) (5, 6, 27, 29). The *lasI* gene encodes a synthase required for the synthesis of an AI molecule named PAI (*Pseudomonas* autoinducer), which was subsequently characterized as *N*-(3-oxododecanoyl) homoserine lactone (22). LasR and PAI are both necessary and sufficient for efficient expression of *lasB* and *lasI* (20, 22, 27). A second AI molecule (factor 2) has been characterized in *P. aeruginosa* and shown to be *N*-butyrylhomoserine lactone (24). Subsequent studies (30) suggest that the *rhlI* gene product, previously reported by Ochsner and Reiser (18), is required for the synthe-

sis of the *P. aeruginosa* factor 2 AI. RhlR, a LasR-homolog, and RhlI were required for rhamnolipid biosynthesis through control of the *rhlAB* genes (17, 18). In addition maximal protease production also required RhlR and RhlI (17, 18). Interestingly, Kuo et al. (13) recently reported that *V. fischeri* also produces a second AI, *N*-octanoyl homoserine lactone. Synthesis of this AI is independent of *luxI* and requires the *ainS* gene (7, 13). Both of these AIs are involved in regulation of bioluminescence (7, 13).

In all systems thus far characterized, the AI molecules are similar in structure but each AI also contains unique features (e.g., length of acyl side chain). Early studies of *V. fischeri* (3) and *Erwinia carotovora* (2) investigated the ability of analogs of the respective AI to activate the target R protein. In the case of *V. fischeri* (3) the ability of these analogs to act as antagonists was also studied. Findings from these studies indicated that AI molecules contain various features critical for interaction with their target protein. In addition, association of radiolabelled VAI with cells overproducing *luxR* has been shown (10). A recent report (26) describes studies which have begun to probe the interactions between VAI and LuxR by examining VAI analogs for their ability to act as AIs to stimulate luminescence gene expression and for their ability to compete with radiolabelled VAI for binding to LuxR.

In the present study we report the ability of radiolabelled PAI to associate with cells overproducing LasR. In addition, we demonstrate the ability of various structural homologs of PAI to act as both agonists and antagonists.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* MG4 [Δ (*argF-lac*)U169 *zah-735::Tn10 recA56sr1::Tn10*] (25) was used as the host strain for all β -galactosidase assays. The pKDT33 control vector for these studies was constructed in a series of steps. The intact *lacZ* gene was isolated as a *NruI-SmaI* fragment from pTL61A (14) and ligated to *SmaI*-digested expression vector pEX1 (21) to place *lacZ* expression under the control of the *tac* promoter on plasmid pTSZ. The first one-third of *lacZ* was then removed by digestion with *EcoRI* and *ClaI* and replaced with an *EcoRI-ClaI* fragment from pSW205 (20). The resulting plasmid (pTLZ) allows for the construction of translational fusions with *lacZ*. A previously constructed fusion of *lasB* to *lacZ* on pTS400 (20) was digested with *EcoO109I* to isolate the *lasB-lacZ* fusion region on a 700-bp fragment, which was

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then ligated with *Eco*O109I-digested pTLZ to form pKDT33. This plasmid carries a *lasB-lacZ* translational fusion and a *tac* promoter but no *lasR*.

To construct pKDT37, a 759-bp *MscI-AluI* fragment carrying the entire *lasR* structural gene as well as 18 bp upstream of the *lasR* ATG was ligated into *EcoRV*-digested pBluescript KS⁻ to serve as an intermediate (pKDT*lasR*). pKDT*lasR* was then digested with *EcoRI* and *HindIII*, and the fragment carrying *lasR* was ligated into *EcoRI-HindIII*-digested pKDT33 to form pKDT34. pKDT34 was digested with *EcoRI* and the resulting ends made flush with the Klenow fragment. The majority of *lasR* was then removed by digestion with *SfiI*. The *lasR* gene was reconstructed by ligation of an *Eco47III-SfiI* fragment (carrying the *lasR* region from -3 to +741) into the prepared pKDT34 to form pKDT37. This plasmid carries the *lasR* gene under the control of the *tac* promoter as well as a *lasB-lacZ* translational gene fusion. The pGroESL plasmid contains the respective chaperonin genes under control of the *tac* promoter. This plasmid (10) was a generous gift from B. Hanzelka and P. Greenberg.

Chemical synthesis of PAI analogs. The acyl derivatives of L-homoserine lactone (and of its thiolactone and lactam analogs) were typically prepared by the coupling of the sodium or lithium salts of the appropriate fatty acids with the aminolactone (or analog) hydrochlorides in water, employing 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (Aldrich Chemical) as the coupling reagent. When not commercially available, the requisite acids were generally prepared from Meldrum's acid by a previously published procedure (19). The amino pyrrolidinone precursor to compound N was prepared by the method of Adamson (1).

Preparation of radiolabelled PAI. Tritiated PAI (³H]PAI) was prepared commercially in a custom tritiation by DuPont-NEN (Boston, Mass.). Briefly, 25 mg of *N*-3-oxo-7-dodecenoyl homoserine lactone (compound G) was reacted with a large excess of tritium gas in the presence of a palladium catalyst. The reaction allows for the reduction of the alkene to form *N*-3-oxo-(6,7-³H₂)-dodecanoyl homoserine lactone (³H]PAI). One microcurie of ³H]PAI was equivalent to 40 nM PAI as determined through the previously described standard PAI bioassay (22) and demonstrated levels of β-galactosidase which were consistent with those reported previously (data not shown) (22).

Assay for agonist activity. Various concentrations (0 to 3 μM) of PAI or the analogs to be tested (dissolved in ethyl acetate) were added to culture tubes (13 by 100 mm). The solvent was evaporated under a stream of nitrogen gas and fresh supplemented A medium (22) containing 100-μg/ml ampicillin was added. Cultures were inoculated (0.01% inoculum) from overnight cultures of *E. coli* MG4(pKDT17) or *E. coli* MG4(pKDT20) (22). The tubes were incubated at 30°C, and culture aliquots were analyzed for β-galactosidase production as previously described (22). The value for half-maximal induction was determined as previously described (27).

³H]PAI binding and competition assay. A modified version of the binding assay described by Hanzelka and Greenberg (10) was used. Briefly, cultures of *E. coli* MG4 containing pKDT37 or the control vector pKDT33 were tested for their ability to bind ³H]PAI. In addition, cultures containing pGroESL (10) in combination with either pKDT37 or pKDT33 were also tested. The plasmid pKDT37 is essentially the same as pKDT17 (22) except that *lasR* is under the control of the much stronger *tac* promoter. The control plasmid pKDT33 lacks the *lasR* structural gene. The cultures were grown in Luria broth (15) for 3 h at 30°C. IPTG (5-bromo-4-indol-3-chloro-isopropyl β-D-galactopyranoside) was added to a final concentration of 1 mM, and the cultures were allowed to grow for 1 h further. Following growth, 1-ml aliquots of the culture to be tested were added to 1.5-ml microcentrifuge tubes containing either 200 nM ³H]PAI alone for binding assays or in combination with 2 μM (10-fold excess) analog to be tested (antagonist assays) contained in 30 μl of distilled water. The contents of the tube were mixed and incubated at 25°C for 15 min. In the case of competition assays, the cells were allowed to incubate in the presence of the analog at 25°C over a range (0, 15, 30, 60, 120, and 180 min) of times prior to the addition of ³H]PAI. After the addition of the ³H]PAI the reaction mixtures were allowed to incubate for another 15 min. Following incubation cells were pelleted by centrifugation at full speed (15,000 × g) at 4°C in a microcentrifuge. The cell pellet was resuspended in 1 ml of phosphate-buffered saline (PBS) (15) and repelleted as before. The cells were washed twice more and all liquid was removed. All manipulations were done on ice where possible. The cell pellet was resuspended in 30 μl of PBS and placed into a scintillation vial containing 5 ml of EcoScintA (National Diagnostics, Atlanta, Ga.). The samples were then counted in a liquid scintillation counter.

RESULTS AND DISCUSSION

A previous study demonstrated that each LuxR-like protein requires the cognate-paired AI for maximal levels of activity (8). The unique features of the AIs which make them specific for their own quorum-sensing system suggests that there exist moieties in PAI which are important for its interaction with LasR. Both *V. fischeri* (3) and *E. carotovora* (2) utilize VAI as an AI in quorum sensing; however, analog studies of the AIs for each organism (2, 3) provided conflicting results as to the

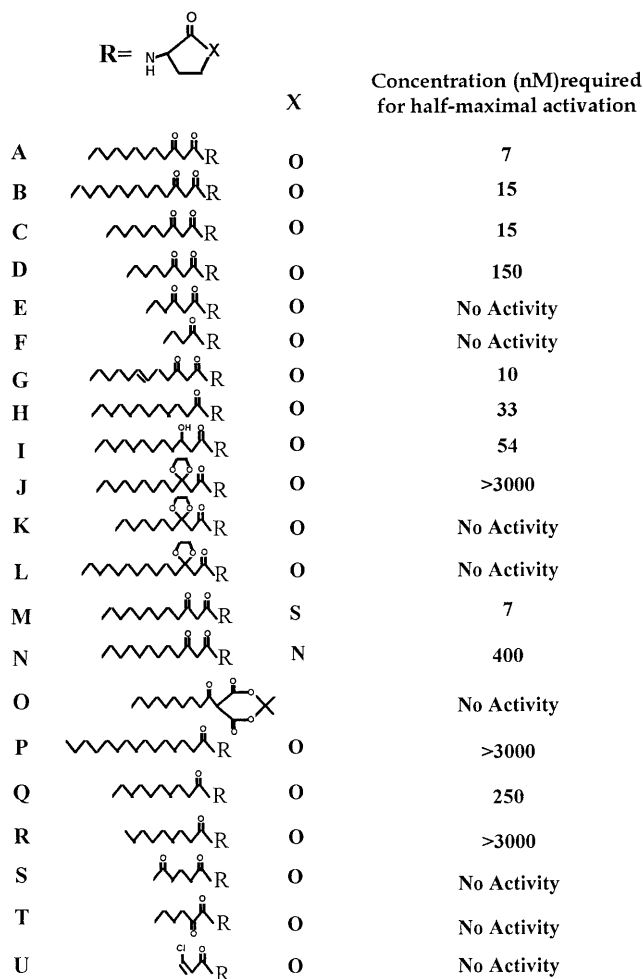


FIG. 1. Agonistic activity of PAI and analogs. The generalized structures of PAI (A) and the various (B to U) analogs are shown. The ability of each compound to stimulate expression of *lasB* is shown as the concentration (nM) required to induce half-maximal activation (50% expression).

critical features of the molecule. Studies of AI activity in *V. fischeri* suggested that the homoserine lactone ring component was important for activity while the exact nature of the acyl chain was not as critical. However, chain length was instrumental in identifying putative inhibitors of the VAI-LuxR interaction (26). The studies with *E. carotovora* suggested that the acyl side chain was the more critical moiety in determining the activity of the AI molecule. In order to address these questions for PAI, a series of structural analogs of PAI were obtained and screened for their ability to act as agonists in our previously described bioassay system (22). The analogs were also tested for antagonist activity in a newly developed assay which makes use of radiolabelled PAI to demonstrate association with LasR.

Agonistic activity of analogs. All the analogs synthesized were tested for their ability to stimulate the expression of a *lasB-lacZ* translational fusion carried on pKDT17 as described previously (22). A range of concentrations (0 to 3 μM) of each compound were tested, and from the resulting dose-response curve the amounts required for half-maximal induction (K_{ind}) (27) were determined. The results of the agonist assays are shown in Fig. 1.

The natural AI molecule PAI (compound A, *N*-3-oxo-dode-

canoyl-L-homoserine lactone) contains an acyl chain length of 12 carbons and exhibited a K_{ind} of 7 nM. These findings were consistent with previously published data (22). Increasing or decreasing the length of the fatty acyl chain by 2 carbons (compounds B and C, respectively [Fig. 1]) resulted in compounds which exhibited an increase in the K_{ind} . Decreasing the length of the fatty acyl side chain to 8 carbons (compound D, *N*-3-oxo-octanoyl-L-homoserine lactone [Fig. 1]) produces the molecule identified as the AI molecule in *Agrobacterium tumefaciens* (31). This molecule resulted in an approximate 21-fold increase in the K_{ind} . The ability of compound D to stimulate LasR to some degree has been previously observed (22). The *V. fischeri* AI molecule VAI (compound E, *N*-3-oxo-hexanoyl-L-homoserine lactone [Fig. 1]) did not exhibit any activity, an observation which is consistent with previous findings (8, 22). Factor 2 (compound F; *N*-butanoyl-L-homoserine lactone), the second identified AI molecule from *P. aeruginosa* (24), differs from PAI in that it has a chain length of 4 carbons and does not have a 3-oxo moiety. Factor 2 did not exhibit any activity as an agonist. Likewise, compound U (*N*-3-chloro-2-propenoyl-L-homoserine lactone [Fig. 1]) did not demonstrate any activity. The data from the above compounds indicate that the proper chain length is crucial for PAI to be capable of inducing expression of *lasB*. In addition, the ability of the acyl side chain to freely rotate, although not crucial, may also play a role in the ability to activate LasR. Modification of PAI to form an alkene derivative (compound G; *N*-3-oxo-7-dodecenoyl-L-homoserine lactone [Fig. 1]) resulted in a compound which was slightly less active than PAI. This finding suggests that specific placement of the last six carbons within the AI-binding site of LasR may be important. The fact that good activity is seen suggests that the presence of the sole double bond is not sufficient to severely hinder formation of the proper contacts with LasR.

The removal of the 3-oxo moiety in the acyl side chain results in a molecule (compound H; *N*-dodecanoyl-L-homoserine lactone [Fig. 1]) which demonstrates an approximate sixfold increase in K_{ind} . Replacement of the 3-oxo moiety with a hydroxyl group (compound I; *N*-3-hydroxy-dodecanoyl-L-homoserine lactone [Fig. 1]) further increases the K_{ind} to a level about eightfold greater than that of PAI. Replacement of the 3-oxo moiety with a bulkier ketal adduct (compounds J, *N*-3,3-ethylenedioxydodecanoyl-L-homoserine lactone; K, *N*-3,3-ethylenedioxytetradecanoyl-L-homoserine lactone; and L, *N*-3,3-ethylenedioxydecanoyl-L-homoserine lactone [Fig. 1]) results in a series of molecules that exhibit very little activity as agonists. Possibly the large size of this ketal moiety impedes activation by a steric hindrance effect such that the molecule cannot enter the proposed AI-binding region of LasR. However, the alternative that these compounds can actually enter the AI-binding region of LasR but do not bind efficiently cannot be discounted. The data from molecules with alterations at carbon 3 suggest that the 3-oxo group, although not critical for agonistic activity, plays an important role perhaps by providing a specific contact point for LasR. The importance of acyl chain length as described above is further supported by the findings derived from molecules which are missing the 3-oxo group and which have chain lengths either larger or smaller than PAI. Compound Q (*N*-decanoyl-L-homoserine lactone [Fig. 1]) lacks a 3-oxo moiety but otherwise is identical to compound C, a molecule which exhibits good agonistic activity. As shown above, compound H, which is identical to PAI except for the lack of a 3-oxo moiety, demonstrates good agonistic activity. In contrast, Q demonstrates a much-decreased ability to act as an AI, as illustrated by a much larger K_{ind} compared with compound C or H. In addition, removal of even one more carbon

from the chain results in a compound (compound R; *N*-3-nonaoyl-L-homoserine lactone [Fig. 1]) which demonstrates very little activity. Increase of the chain length to 15 carbons (compound P; *N*-pentadecanoyl-L-homoserine lactone [Fig. 1]) also results in a compound which demonstrates very little activity. This chain length, however, may inhibit appropriate entry of the molecule into the cell.

A limited number of molecules with substitutions within the homoserine lactone ring were obtained. Conversion of the homoserine lactone to a homocysteine thiolactone (compound M; *N*-3-oxo-dodecanoyl-L-homocysteine thiolactone) resulted in no detectable difference in the molecule's ability to act as an AI. However, conversion of the homoserine lactone to a lactam ring [compound N; L-3-(3-oxododecanoyl)amino-2-pyrrolidinone (Fig. 1)] resulted in a molecule which exhibited reduced activity. Compound O [5-(3-oxodecanoyl)-2,2-dimethyl-1,3-dioxane-4,6-dione (Fig. 1)], an acylated version of Meldrum's acid, is an intermediate in the synthesis of several compounds. Similar to compound C it has a 10-carbon acyl side chain, which in the case of compound C results in little decrease in activity compared with that of PAI. However, in compound O a more complex ring structure is present in the place of the normally occurring homoserine lactone. This compound fails to demonstrate any activity. The limited number of ring substitutions indicates that some substitution is tolerated in the ring heteroatom but that changes which might result in altered size or polarity of the ring structure result in inactive molecules. More analogs with alterations in the homoserine ring structure need to be tested to more clearly elucidate the importance of the ring heteroatom.

Data presented in Fig. 1 demonstrate that the *V. fischeri* AI (compound E) cannot activate LasR. In an attempt to determine whether different placement of the 3-oxo moiety might convert VAI to a competent AI for LasR, two analogs which contain either a 5-oxo moiety (compound S; *N*-5-oxo-hexanoyl-L-homoserine lactone [Fig. 1]) or a 2-oxo moiety (compound T; *N*-2-oxo-hexanoyl-L-homoserine lactone [Fig. 1]) were obtained and tested. While both demonstrated agonistic activity in studies of *V. fischeri* analogs (3), neither demonstrated any activity in our system as would be expected on the basis of our findings with VAI (Fig. 1).

Those compounds which demonstrated little or no activity were further tested at concentrations as high as 10 μ M. None of these molecules exhibited any difference from the level of activity evidenced at 3 μ M (data not shown).

Ability of PAI to bind to LasR. Previous studies with *V. fischeri* demonstrated the association of radiolabelled VAI with cells overproducing LuxR and the chaperonin proteins GroESL (10). To study this association in the case of LasR and PAI, compound G was commercially tritiated (DuPont-NEN) to provide radiolabelled PAI {[3 H]PAI; *N*-3-oxo-(6,7- 3 H₂)-dodecanoyl homoserine lactone}. In this reaction the alkene substrate is reacted in the presence of a large excess of tritium gas and of a palladium catalyst. Reduction of the double bond within the substrate results in a yield of labelled product which approaches 100%. This procedure has been used to produce both tritiated VAI (12) and tritiated factor 2 (23). The [3 H]PAI was tested in our previously described bioassay (22) in order to ensure activity and to determine concentrations to be used for binding experiments. On the basis of a specific activity of 125 Ci/mmol, it was determined that 1 μ Ci of [3 H]PAI corresponded to 40 pmol of PAI (data not shown).

Utilizing a modified version of a published protocol (10), *E. coli* strains carrying *lasR* on an inducible expression vector (pKDT17 [22] or pKDT37) were incubated with [3 H]PAI after induction of *lasR* expression with IPTG. In our system the

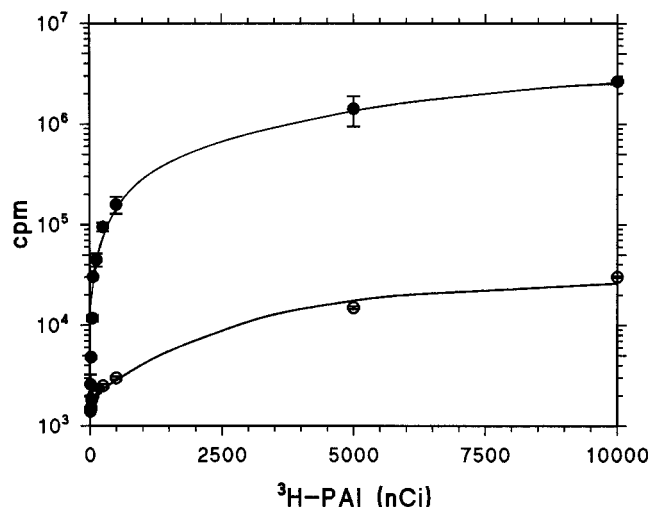


FIG. 2. Association of [^3H]PAI with cells expressing LasR. The binding of [^3H]PAI by *E. coli* MG4 containing pKDT37 (●, *lasR*⁺) or the control plasmid pKDT33 (○, *lasR*⁻ mutant) is shown.

labelled PAI binds LasR from pKDT37 with half-maximal saturation at 5 μCi (200 nM) (Fig. 2). This is 100-fold greater than the nonspecific binding seen with pKDT33. As a result of these findings, 5 μCi of [^3H]PAI was used in all subsequent binding and competition assays to ensure the observation of possible stimulatory or possible inhibitory effects.

Induction of *lasR* expression in *E. coli* MG4(pKDT37) which carries *lasR* under the control of the IPTG-inducible *tac* promoter as well as the *lasB-lacZ* fusion from pTS400 (20) causes a 45-fold increase in the amount of [^3H]PAI retained compared with that of either a strain carrying the expression vector control (pKDT33) or strains carrying no plasmid (Fig. 3). This finding indicates that the presence of LasR accounts for the increased retention of [^3H]PAI, which strongly supports a direct interaction between PAI and LasR.

In similar studies of VAI binding to LuxR, Hanzelka and Greenberg (10) demonstrated that the presence of the

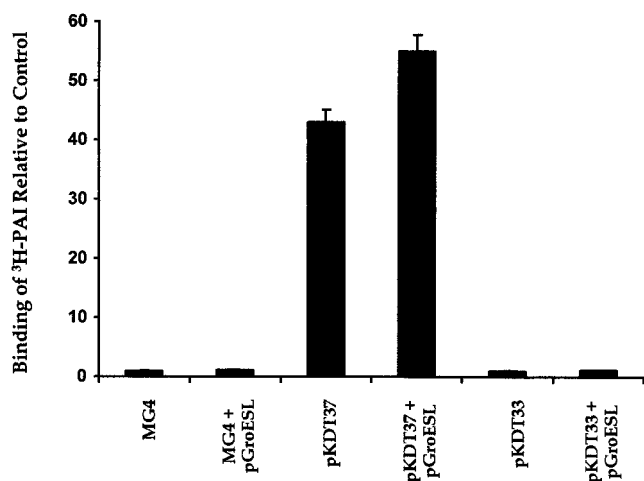


FIG. 3. Binding of [^3H]PAI. The ability of *E. coli* MG4 alone or carrying various genetic constructs (as described in Materials and Methods) to bind [^3H]PAI is illustrated. All data are shown relative to the binding of labelled PAI by *E. coli* MG4 alone.

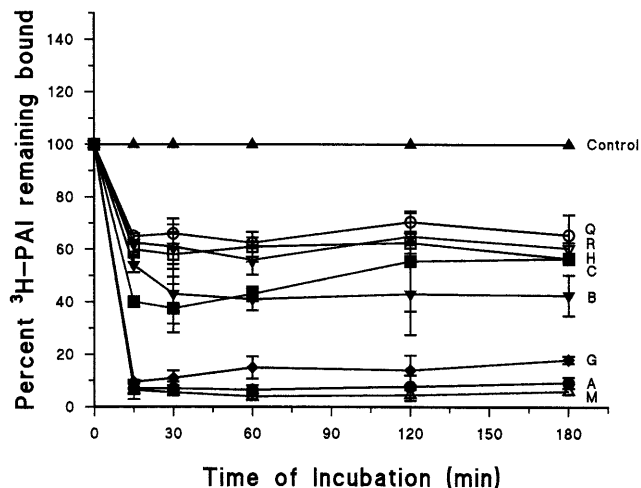


FIG. 4. Antagonistic activity of analogs. The ability of analogs to act as antagonists as determined by the assay described in Materials and Methods is shown. Data are presented as the percentages of [^3H]PAI retained compared with a control reaction in which no competitor was present and which is normalized to the 100% baseline for each datum point. Compounds not shown did not exhibit any activity as antagonists. The letters for each line correspond to the compounds as identified in Fig. 1.

GroESL chaperonins expressed from a multicopy plasmid (pGroESL) resulted in a significant increase in the amount of [^3H]VAI retained by cells overexpressing LuxR. It was proposed (10) that the presence of the chaperonins would allow for proper folding and localization of the LuxR protein. No significant increase was seen when these experiments were carried out in the case of PAI. Fig. 3 illustrates that although the presence of GroESL results in a small increase in the radioactivity bound by cells, the effect was not nearly as dramatic as that reported for the LuxR-VAI system. Whether LasR protein is capable of proper folding and localization even in a heterologous host or whether it specifically requires the *P. aeruginosa* GroESL homologs is not clear. Regardless of the small effect, all subsequent binding studies were carried out using strains which carried pKDT37 and pGroESL or in control strains which carried both pKDT33 and pGroESL.

Antagonistic activity of analogs. The binding assay described above provided a convenient means of analyzing the ability of the PAI structural analogs to act as antagonists (i.e., competitors) of PAI for binding to LasR. In the case of antagonist studies the assays were carried out in the same manner as described above for the agonist studies except that in addition to the 200 nM [^3H]PAI, a 10-fold excess (2 μM) of the analog to be tested for antagonistic activity was also present. The results of such studies are shown in Fig. 4.

The PAI structural analogs could be placed into three broad categories on the basis of their antagonistic effects. The first category contained those analogs that exhibited a high degree of antagonism in the binding of [^3H]PAI to LasR. In general, those analogs which were the best agonists were also the best antagonists (Fig. 4). Antagonism is represented by the percentage of [^3H]PAI remaining bound compared with a control reaction culture [*E. coli* MG4 (pKDT37 + pGroESL)] in which no competitor analog was present.

Not surprisingly, PAI (compound A) exhibited the greatest level of competition, reducing the binding of [^3H]PAI by approximately 95%. Aside from PAI, the best of these antagonists was the homocysteine thiolactone derivative (compound M), whose effect was as potent as that of PAI. The alkene

derivative (compound G) was only slightly less effective as a competitor than was PAI or compound M and decreased binding of [^3H]PAI by approximately 80%.

The second category included those compounds which demonstrated a moderate level of inhibition. Compounds B (*N*-3-oxo-tetradecanoyl homoserine lactone) and C (*N*-3-oxo-decanoyl homoserine lactone) whose acyl side chains contain two more or two less methylene groups, respectively, than PAI exhibited inhibition of [^3H]PAI binding of between 50 and 60% (Fig. 4). Compounds R (*N*-nonanoyl homoserine lactone), H (*N*-dodecanoyl homoserine lactone), and Q (*N*-decanoyl homoserine lactone) also appear to be moderate antagonists and were able to reduce binding of the labelled PAI by 30 to 50% (Fig. 4). These findings support the conclusion that chain length is important for binding to LasR. Furthermore, the presence of the 3-oxo moiety appears to be critical for maximal binding, as evidenced by the dramatic difference in antagonism between PAI compound A and compound H.

The third category includes those analogs which demonstrated little or no antagonistic activity (data not shown). The most active of the group was compound P (*N*-pentadecanoyl homoserine lactone), which demonstrated a small but reproducible 5% decrease in the binding of [^3H]PAI to LasR. Interestingly, compound I (*N*-3-hydroxy-dodecanoyl homoserine lactone), which differs from PAI in that the 3-oxo group is substituted by a 3-hydroxyl group and which demonstrates good agonistic activity (Fig. 1), lacked antagonistic activity. All of the compounds (J, K, and L) which carried a ketal moiety substitution in place of the 3-oxo group also did not exhibit any antagonistic effect. These data further support the conclusion that the 3-oxo group is important for effective binding to LasR. This suggestion is further strengthened by the finding in the agonist studies that increased amounts, reflected by increased K_{ind} , of compounds H and I are required to stimulate LasR activation to the same level as that with PAI. Thus, it is postulated that these two compounds can only activate LasR when the compounds are present in excess. However, compared with PAI, their binding to LasR is proposed to be weak, and as a result they either do not bind at all or can be easily displaced from the AI-binding region of LasR when a compound with a higher binding affinity (e.g., PAI) is present. Furthermore, a compound such as Q, which is similar to C but lacks a 3-oxo group, demonstrated a decreased level ($K_{\text{ind}} = 250 \text{ nM}$) of agonistic activity compared with C ($K_{\text{ind}} = 15 \text{ nM}$) (Fig. 1).

Also of interest is the fact that although compound D can activate LasR, albeit much less effectively than PAI, it cannot compete significantly with PAI for binding to LasR. This finding would support the idea that acyl chain length is also critical for effective binding to LasR. Shorter-chain-length compounds (six carbons or less) such as compounds E, F, and U also provide support for this argument since none of them are able to effectively compete with PAI. The chain length requirements appear to be finite since removing (compound C) or adding (compound B) two methylene units to the acyl chain also reduced the ability of the compounds to be effective competitors of PAI binding. As shown above, those compounds which maintain a chain length of 6 carbons or greater, regardless of the presence of the 3-oxo group, appear to demonstrate antagonistic activity.

The role of the ring structure in determining binding is not yet clear. As reported above the homocysteine derivative (compound M) was an effective antagonist while the lactam ring derivative (compound N) or the acylated Meldrum's acid derivative (compound O) exhibited no antagonistic activity. In the case of compound N one may speculate that a decreased affinity is to blame since this compound does demonstrate

agonistic activity (Fig. 1). In the case of compound O, which lacks agonistic activity, it is difficult to ascertain whether the lack of antagonistic activity is due to a decreased ability to bind to LasR or to steric hindrance because of the large ring structure.

Neither compound S or T exhibited antagonistic activity. Taken together with the fact that they also do not exhibit agonistic activity (Fig. 1), these data suggest that the compounds do not bind effectively to LasR.

In the case of antagonist assays, various modifications of the standard assay procedure were attempted for those compounds which did not demonstrate antagonistic activity. Modifications included incubation with increased concentrations (up to 50 μM , 250-fold excess) of the analog, carrying out binding reactions at 30°C, and increasing the time of incubation from 15 to 30 min. None of these modifications resulted in any significant change from the results reported with the standard assay (data not shown).

Quorum-sensing systems are proposed to act as communication mechanisms to allow a given population of a certain species to react to population density by expressing genes which confer some advantage at this point in the growth of the population. However, the ability of various analogs which are known AIs (e.g., compound D) to act as agonists raises the intriguing possibility of cross-species communication. Given the increasing numbers of gram-negative organisms which have been demonstrated to carry components of quorum-sensing systems, it is not unreasonable to assume that some interspecies communication may occur. Indeed, the demonstration in this and other reports (22) that LasR will accept AI molecules with shorter and longer chain lengths, especially AAI which is the AI for *A. tumefaciens* (11, 31), supports such an interaction between species. It would be assumed that if quorum-sensing systems were species specific that each organism would specify strict requirements for exact selection of AIs. The prospect of interspecies communication has also been suggested by others (9, 16). The initial observation (9) was based on the finding that AIs from other marine bacteria were able to stimulate luminescence gene expression in *Vibrio harveyi*. Those AIs were termed alloinducers, and the process was termed alloinduction. Recent studies (16) have shown that concentrated cell-free extracellular material of *P. aeruginosa* PAO1 was capable of stimulating virulence factor production in cultures of *Burkholderia cepacia*. *B. cepacia* has received increased attention because of its association with cystic fibrosis patients, a disease in which *P. aeruginosa* has been firmly established as a pathogen. The findings described in a recent publication (26) demonstrate more specifically that AI molecules may inhibit the function of the R protein from another species via a mechanism termed alloinhibition.

The elucidation of compounds which can act as true antagonists may provide a practical method for therapy of patients infected by organisms which utilize quorum-sensing mechanisms to regulate gene expression. Such studies are currently being actively pursued in our laboratory.

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