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LasR of *Pseudomonas aeruginosa* Is a Transcriptional Activator of the Alkaline Protease Gene (*apr*) and an Enhancer of Exotoxin A Expression

MICHAEL J. GAMBELLO, SUSAN KAYE, AND BARBARA H. IGLEWSKI*

Department of Microbiology and Immunology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

Received 16 October 1992/Accepted 6 January 1993

The *lasR* gene of *Pseudomonas aeruginosa* is required for transcription of the genes for elastase (*lasB*) and LasA protease (*lasA*), two proteases associated with virulence. We report here that the alkaline protease gene (*apr*) also requires the *lasR* gene for transcription. Alkaline protease mRNA was absent in the *lasR* mutant PAO-R1 and present when an intact *lasR* gene was supplied in *trans* as determined by Northern (RNA) analysis. The *lasR* gene also enhances exotoxin A production. Exotoxin A activity in supernatants of PAO-R1 were 30% less than in supernatants of the parental strain, PAO-SR. Multiple copies of *lasR* in *trans* in PAO-R1 increased toxin A activity to twice the parental levels. Analysis of PAO-R1 containing the *toxA* promoter fused to β-galactosidase suggests that LasR acts at the *toxA* promoter or at upstream *toxA* mRNA sequences. β-Galactosidase activity was approximately 40% lower in PAO-R1 than in the parental strain, PAO-SR. Furthermore, the effect of LasR on the *toxA* promoter is not due to the stimulation of transcription of *regA*, a transcriptional activator of *toxA*. No difference in chloramphenicol acetyltransferase (CAT) activity was noted between PAO-SR and PAO-R1 containing transcriptional *regA* promoter-CAT gene fusions. These results broaden the regulatory dominion of *lasR* and suggest that the *lasR* gene plays a global role in *P. aeruginosa* pathogenesis.

Pseudomonas aeruginosa is an opportunistic pathogen of significant medical importance. It has gained notoriety in a number of clinical settings: chronic pulmonary infections in cystic fibrosis patients, wound infections, malignant otitis externa, and generalized systemic infections (26). The success of this organism as a pathogen stems in part from its ability to secrete a variety of toxic substances, including exotoxin A (ETA), phospholipases, and several proteases (21). These exoproducts are not produced constitutively but are regulated in response to various environmental and cellular stimuli (2, 16, 17, 34). Recently, the isolation of the *lasR* gene has shed some light on the regulation of two virulence-associated protease genes, *lasB* and *lasA* (11, 32). The *lasR* gene encodes a 26,618-Da protein required for the transcription of both of these genes. Neither *lasB* nor *lasA* mRNA is detected in the strain PAO-R1, a *lasR* chromosomal deletion mutant of PAO1. However, supplying *lasR* in *trans* restores transcription of these genes.

The present study was undertaken to determine if two other exoproducts associated with virulence in *P. aeruginosa*, alkaline protease and ETA, are regulated by LasR. Alkaline protease (encoded by *apr*) is a 49-kDa enzyme with optimal activity at alkaline pH (19, 23). Its primary amino acid sequence is 55% identical to the *Serratia* protease. ETA, an enzyme which transfers the ADP-ribose moiety of NAD to mammalian elongation factor 2, inhibits protein synthesis and thus causes cell death (15). Several genes appear to be involved in the regulation of ETA expression (2, 22, 30). To date, two such genes have been isolated, *regA* and *regB* (13, 35). The *regA* gene product is a transcriptional activator of ETA and controls, at least in part, the response of *toxA* to the phase of bacterial growth and the concentration of iron in the medium (10, 33). The *regB* gene is located downstream of *regA* in the hypertoxigenic strain PA103 (35). It encodes a protein of 7,527 Da and is believed to partially account for the higher levels of ETA made in this strain compared with that made by PAO1, which lacks a functional *regB* open reading frame (35). The possibility that the *lasR* gene product regulates several unlinked virulence-associated genes is not without precedence. In *Vibrio cholerae*, the causative agent of Asiatic cholera, the *toxR* and *toxS* genes not only positively regulate expression of the cholera toxin operon but affect toxin-coregulated pilus, at least two outer membrane proteins, and about 15 other genes (8).

In this study we demonstrated that the transcription of *apr*, the alkaline protease gene, like *lasB* and *lasA*, requires the presence of an intact *lasR* gene. While the *lasR* gene is not absolutely required for ETA expression, more ETA is synthesized when *lasR* is present than when it is absent. LasR increases the activity of a *toxA* promoter fused to β-galactosidase but has no effect on the *regA* promoters.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. aeruginosa* strains and plasmids are listed in Table 1. The 1.8-kb stabilizing fragment from pRO1614 enables ColE1-based plasmids to replicate stably in *P. aeruginosa* (24).

RNA biochemistry and analysis. RNA from various strains was isolated by centrifugation through 5.7 M CsCl and analyzed in Northern (RNA) blots as previously described (11). Approximately 7 μg of RNA from each strain was fractionated on a 0.66 M formaldehyde–1.2% agarose gel, transferred to nylon, and hybridized to a ^{32}P-labeled 25-bp oligonucleotide representing the N-terminal region of mature alkaline protease.

ETA activity. The ETA activity of supernatants was determined by assaying for ADP-ribosyltransferase activity with wheat germ elongation factor 2 as previously reported

* Corresponding author.

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TABLE 1. Strains and plasmids

Strain or plasmid	Description	Reference or source
P. aeruginosa strains		
PAO1	Prototroph	14
PAO-SR	PAO1 Sm^r	11
PAO-R1	PAO-SR Δ*lasR*::*tet*	11
Plasmids		
pRO1614	Ap^r; source of 1.8-kb stabilizing fragment	24
pUC18	Ap^r; general cloning vector	18
pSW200	pUC18 containing 1.8-kb stabilizing fragment from pRO1614	This laboratory
pMJG1.7	pSW200 containing *lasR* on a 1.7-kb *Sac*II-*Eco*RI fragment	11
pMLB1034	Ap^r; promoterless *lacZ* gene	28
pSW205	pMLB1034 containing the 1.8-kb stabilizing fragment	This laboratory
pSW228	pSW205 carrying *Pvu*II-*Bam*HI *tox* promoter fragment	This laboratory; 12
pQF26	Broad-host-range vector with promoterless *cat* gene	9
pP11	P1 promoter of *regA* fused to promoterless CAT gene in pQF26	31
pP21	P2 promoter of *regA* fused to promoterless CAT gene in pQF26	31

(5). *P. aeruginosa* strains were grown overnight in deferrated TSBD (Trypticase soy broth dialysate) containing 50 mM monosodium glutamate, 1% glycerol, 10 μg of $FeCl_3$ per ml, and 200 μg of carbenicillin per ml at 32°C. The overnight cultures were subcultured into 25 ml of TSBD containing all of the above additives except iron. The inoculations were such that the starting A_{540} was ca. 0.02; secondary cultures were harvested at 18 h.

β-Galactosidase activity of PAO-SR and PAO-R1. Bacteria were grown under the same culture conditions described above for assaying ETA production, except that culture samples for quantitation of β-galactosidase were taken at 10 h. β-Galactosidase was assayed as previously described (25).

Activity of *regA* promoter CAT gene fusions in PAO-R1. Strains were grown under the same conditions for optimal ETA production as those described above. Sample volumes were chosen to yield similar numbers of cells at each time point so that all samples would be in the linear range of the chloramphenicol acetyltransferase (CAT) assay. Cells were centrifuged, washed once in 1 ml of 100 mM Tris-HCl, pH 7.8, centrifuged again, and stored as dry pellets at −20°C. To prepare lysates, cells were resuspended in 500 ml of 100 mM Tris-HCl, pH 7.8, and sonicated on ice. Debris was removed by centrifugation in a microcentrifuge at 4°C for 10 min. Aliquots of supernatant were stored at −20°C. CAT assays were performed at room temperature according to the method of Neumann et al. (20). Protein was measured by the Bradford assay (3).

RESULTS

The *lasR* gene is required for transcription of the *apr* gene. We have previously demonstrated that the *lasR* gene is required for transcription of the *lasB* and *lasA* genes (11, 32). Transcriptional control of these two proteases by *lasR* suggests that *lasR* exerts global control over protease expression and therefore might also be required for expression of a third *P. aeruginosa* protease, alkaline protease. Total RNA from PAO1, PAO-R1, PAO-R1(pMJG1.7), and PAO-R1(pSW200) was probed for the presence of *apr* message. Figure 1 demonstrates that the *apr* probe hybridized to a 1.77-kb RNA species isolated from PAO1. No *apr* mRNA was detected in PAO-R1, which lacks a functional *lasR* gene (Fig. 1, lane 2); however, when *lasR* was supplied to PAO-R1 on the multicopy plasmid pMJG1.7, the 1.77-kb *apr* mRNA was restored (Fig. 1, lane 3). No signal was detected in the vector control lane, as expected (Fig. 1, lane 4). To demonstrate that RNA was present in those lanes in which the *apr* probe failed to hybridize, blots were probed with a 290-bp fragment of the PAO1 pilin gene (pil). In each lane, a 700-bp mRNA that is the predicted size of the pilin message was observed, indicating that the failure to detect *apr* message in PAO-R1 was not due to degradation of RNA during the isolation procedure. These results indicate that the *apr* gene, like *lasA* and *lasB*, requires the *lasR* gene in *trans* for efficient transcription.

The *lasR* gene is required for optimal ETA production. Table 2 illustrates the effect of *lasR* on the production of ETA as measured by the ADP-ribosyltransferase assay. Ablation of *lasR* in PAO-R1 reduced toxin activity to 66% of the parental level (PAO-SR). When multiple copies of *lasR* were supplied in *trans* in PAO-R1(pMJG1.7), there was a threefold increase in ADP-ribosyltransferase activity above that in the vector control PAO-R1(pSW200) and a twofold increase above the PAO-SR level. These data suggest that a functional *lasR* gene, though not absolutely required for expression of ETA, contributes significantly to the ETA phenotype. Moreover, providing multiple copies of *lasR* to the *lasR* mutant, PAO-R1, increases extracellular ETA

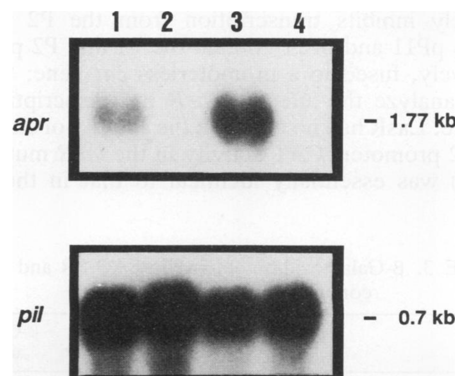

FIG. 1. Northern blot analysis of total cellular RNA hybridized to a ^{32}P-labeled *apr*-specific 25-bp oligonucleotide (top) and a ^{32}P-labeled 290-bp fragment of the pilin gene (bottom). Lanes: 1, PAO-SR; 2, PAO-R1; 3, PAO-R1(pMJG1.7); 4, PAO-R1(pSW200). The approximate molecular sizes (in kilobases) are indicated to the right.

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TABLE 2. ADP-ribosyltransferase activity of PAO-SR, PAO-R1, and PAO-R1 complemented in *trans* with the *lasR* gene

Strain	Plasmid carried	ETA activity (cpm/10 μl of supernatant)[a]
PAO-SR		1,500 ± 80
PAO-R1		1,000 ± 60
PAO-R1	pMJG1.7	2,900 ± 160
PAO-R1	pSW200	1,000 ± 60

[a] Values are averages of three measurements. The experiment was repeated three times with similar results.

activity above the parental levels of PAO-SR, which contains one chromosomal copy of *lasR*.

The *lasR* gene affects the ETA phenotype at upstream *toxA* sequences but not via an effect on the *regA* promoter. The decrease in ADP-ribosyltransferase activity seen in supernatants of the *lasR* mutant strain PAO-R1 compared with that in supernatants of the parental *lasR*$^+$ strain PAO-SR could result from an effect of LasR on one or several biological processes leading to the presence of ETA in the culture supernatants, that is, transcription or translation of the toxin gene and/or processing and secretion. In order to determine whether *lasR* affects the activity of the *toxA* promoter, we analyzed the expression of β-galactosidase from the *toxA*::*lacZ* fusion plasmid pSW228 in strains PAO-SR and PAO-R1. Thirty-eight percent less β-galactosidase was produced in PAO-R1(pSW228) than in PAO-SR(pSW228) (Table 3). The relative decrease in β-galactosidase expression in PAO-R1 is similar to the decrease in ETA activity in the supernatant. These experiments suggest that LasR alters ETA production by acting at upstream *toxA* sequences rather than later steps of secretion or processing. Since the promoter fragment used to construct pSW228 included the first 24 bp of *toxA* coding sequence as well as promoter sequences, we cannot distinguish whether LasR affects transcription or translation or both.

Since RegA controls *toxA* transcription, it was plausible that *lasR* might affect *toxA* expression indirectly by affecting *regA* expression (33). The *regA* gene has two promoters, P1 and P2 (31). Transcription utilizing the P1 promoter occurs early in the growth curve and is independent of the concentration of iron in the medium (10). Later in the growth curve, transcription from the P2 promoter is initiated, but only if the iron concentration is low; an iron concentration of 10 μM completely inhibits transcription from the P2 promoter. Plasmids pP11 and pP21 contain the P1 and P2 promoters, respectively, fused to a promoterless *cat* gene; they were used to analyze the affect of *lasR* on transcription of the *regA* gene. LasR had no effect on the activity of either the P1 or the P2 promoter. CAT activity in the *lasR* mutant PAO-R1(pP11) was essentially identical to that in the parental control PAO-SR(pP11) throughout the growth curve (Fig. 2A). Similar results were obtained with the pP21 construction (Fig. 2B). Thus, the *lasR* gene product does not alter ETA expression by acting at the *regA* promoters.

TABLE 3. β-Galactosidase activity in PAO-SR and PAO-R1 containing a *toxA*::*lacZ* fusion

Strain	Plasmid carried	β-Galactosidase activity (U)[a]
PAO-SR	pSW228	1,600 ± 29
PAO-SR	pSW205	0
PAO-R1	pSW228	1,000 ± 38
PAO-R1	pSW205	0

[a] Values are averages of three measurements. The experiment was repeated three times with similar results.

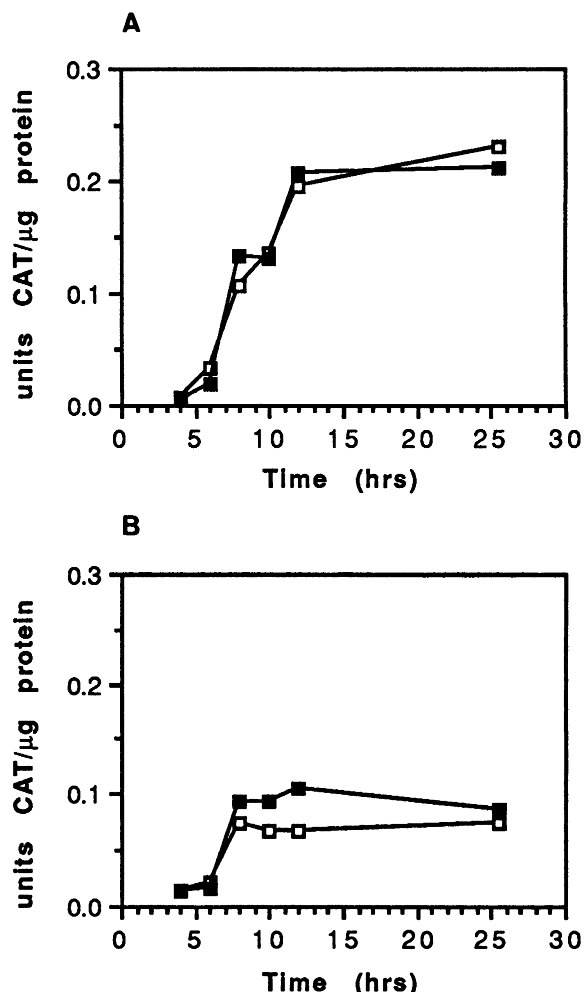

FIG. 2. CAT activity throughout the growth cycle of PAO-SR (open squares) and PAO-R1 (closed squares) containing pP11 (A) and pP21 (B) grown in iron-deficient medium.

DISCUSSION

The results of the experiments reported in this communication suggest that *lasR* is a global regulator of proteases in *P. aeruginosa*. This may arise from the need to be able to respond efficiently to environmental stimuli. Northern analysis has clearly demonstrated the requirement for an intact *lasR* gene for transcription of the alkaline protease gene (this report) and both the *lasB* and *lasA* genes (11, 32). These protease genes are widely dispersed on the *P. aeruginosa* PAO1 chromosome and thus appear to be part of a protease regulon that is under the control of *lasR* (29).

The absolute requirement for *lasR* for transcription of these three proteases raises many questions regarding the regulation of *lasR* itself. The regulation of the proteases and *lasR* must be understood in relation to the metabolic needs of the cell in different environmental settings. Protease production occurs in late-logarithmic and stationary-phase cultures, suggesting that nutritional deprivation might be a

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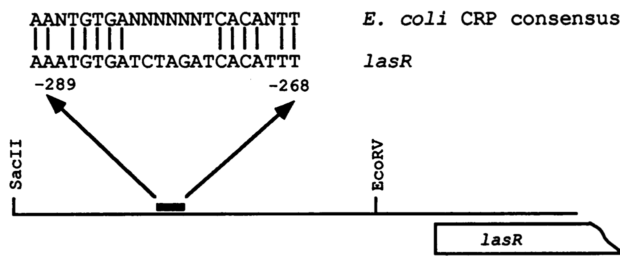

FIG. 3. Sequence comparison of a putative CRP-binding site in the *lasR* upstream sequence and the consensus sequence as described by Berg and von Hippel (1). The start of translation is at position 0.

signal for *lasR* activation. Indeed, glucose as well as other substrates appear to modulate protease production (34). We have identified a sequence in the upstream region of *lasR* (−291 to −268) which is identical to the catabolite repressor protein (CRP) consensus binding site (1) of *Escherichia coli* (Fig. 3). DeVault et al. have shown that the *P. aeruginosa algD* gene, encoding a key enzyme in alginate biosynthesis, may be regulated in part by a CRP-like analog in *P. aeruginosa* (6). They have demonstrated that activation of *algD* in *E. coli* is dependent upon a functional CRP and that the *algD* promoter is sensitive to glucose repression in both *P. aeruginosa* and *E. coli*. A consensus CRP-binding site in the upstream region of *algD* binds CRP, as determined by the gel mobility shift assay. Furthermore, these investigators have preliminary evidence that a CRP-like protein exists in *P. aeruginosa*, although it is still unknown whether the CRP operator site of *algD* binds the putative CRP-like protein. Nonetheless, the identification of a *CRP*-binding site in *lasR* raises the interesting question of whether *lasR* and the three proteases are under the more global control of a CRP-like catabolite repression in *P. aeruginosa*. The various types of control mechanisms and biologic signals affecting *lasR* expression are currently being investigated in our laboratory by using a *lasR*-reporter gene fusion.

How *lasR* regulates protease gene expression remains unknown. LasR may act directly at the promoters of the protease genes or may be part of a regulatory cascade. LasR shows a high degree of identity to the putative DNA-binding carboxy terminus of LuxR, a regulatory protein of *Vibrio fischeri*, a species of luminous bacteria (4). Thus, like LuxR, LasR may be a DNA-binding protein which can activate protease gene expression by binding to the protease promoters (11). This hypothesis predicts that there may be regions of sequence similarity in the promoter regions of the three protease genes regulated by *lasR*. In fact, a 20-nucleotide sequence with dyad symmetry immediately upstream of the −35 site of the *lasB* gene resembles a dyad in the upstream regions of *lasA* and *apr* (7, 32). Although operator binding sites often show dyad symmetry, the significance of these sites is as yet unclear. Site-directed mutagenesis, promoter deletion, and DNA footprinting experiments will be required to determine if those sequences bind an activator. Experiments are under way in our laboratory to assess the extent of similarity of the regulation of protease expression in *P. aeruginosa* to the *lux* bioluminescence system of *V. fischeri*.

The regulation of ETA expression is complex. Two genes which form an operon, *regA* and *regB*, have been shown to affect ETA expression, but several studies strongly suggest that additional genes play a role in this complex genetic system (2, 13, 22, 30, 35). The *fur* gene, which controls expression of iron-repressible genes in *E. coli*, has been demonstrated to regulate both *toxA* and *regA* in *P. aeruginosa* (27). Furthermore, a Fur homolog has been detected in *P. aeruginosa* and may be involved in iron-mediated regulation of ETA expression. Our results add yet another gene, *lasR*, to the growing list of genes regulating ETA synthesis. Inactivation of the *lasR* gene resulted in less ETA activity in the supernatants of PAO-R1 than in the supernatants of the parental strain, PAO-SR. Interestingly, when multiple copies of *lasR* were provided to PAO-R1, ETA activity rose to twice that of PAO-SR, which contains only one copy of the *lasR* gene (Table 2). These results demonstrate that increasing cellular concentrations of LasR lead to increased expression of ETA.

Analysis of β-galactosidase activity in strains containing the *toxA*::*lacZ* fusion suggests that LasR acts either directly or indirectly at upstream *toxA* sequences. However, since the *toxA*::*lacZ* fusion contains the translational start of the *toxA* gene as well as 736 bp of upstream sequence, we cannot exclude an effect of *lasR* on translation of *toxA* mRNA. Since *regA* is known to affect the activity of the *toxA* promoter, we analyzed the effect of *lasR* on *regA* transcription. The results in Fig. 2 clearly demonstrate no difference in P1 or P2 promoter activity in *lasR* and *lasR*$^+$ strains. Thus, LasR does not mediate ETA expression via *regA* transcription. However, these experiments do not rule out an interaction between the RegA and LasR proteins in affecting *toxA* expression. That a deletion of *lasR* did not abolish *toxA* expression, as seen with the proteases, underscores the complexity of ETA regulation.

The transcriptional regulation of the protease genes by LasR and the enhancement of ETA production when *lasR* is present suggest that this gene may function as a global regulator of virulence in *P. aeruginosa*. A thorough understanding of the genetics and biochemistry of LasR-mediated gene regulation will undoubtedly provide a clearer picture of the pathogenic process of this opportunistic pathogen.

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