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Transcription of Quorum-Sensing System Genes in Clinical and Environmental Isolates of *Pseudomonas aeruginosa*

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Quorum sensing (QS)-based transcriptional responses in *Pseudomonas aeruginosa* have been defined on the basis of increases in transcript levels of QS-controlled genes such as *lasB* and *aprA* following the hierarchical transcriptional increases of central controllers such as the *lasR* gene. These increases occur at high bacterial concentrations such as early-stationary-phase growth in vitro. However, the extent to which the increases occur in a variety of clinical and environmental isolates has not been determined nor is there extensive information on allelic variation in *lasR* genes. An analysis of the sequences of the *lasR* gene among 66 clinical and environmental isolates showed that 81% have a sequence either identical to that of strain PAO1 or with a silent mutation, 15% have nucleotide changes resulting in amino acid changes, and 5% have an insertion sequence in the *lasR* gene. Using real-time PCR to quantify transcript levels of *lasR*, *lasB*, and *aprA* in the early log and early stationary phases among 35 isolates from bacteremia and pneumonia cases and the environment, we found most (33 of 35) strains had increases in *lasR* transcripts in early stationary phase but with a very wide range of final transcript levels per cell. There was a strong correlation ($r^2 = 0.84$) between early-log- and early-stationary-phase transcript levels in all strains, but this finding remained true only for the 50% of strains above the median level of *lasR* found in early log phase. There were significant ($P < 0.05$) but weak-to-modest correlations of *lasR* transcript levels with *aprA* ($r^2 = 0.2$) and *lasB* ($r^2 = 0.5$) transcript levels, but again this correlation occurred only in the 50% of *P. aeruginosa* strains with the highest levels of *lasR* transcripts in early stationary phase. There were no differences in distribution of *lasR* alleles among the bacteremia, pneumonia, or environmental isolates. Overall, only about 50% of *P. aeruginosa* strains from clinical and environmental sources show a *lasR*-dependent increase in the transcription of *aprA* and *lasB* genes, indicating that for about 50% of clinical isolates this regulatory system may not play a significant role in pathogenesis.

Pseudomonas aeruginosa is a ubiquitous environmental organism able to colonize and infect humans with underlying genetic susceptibilities (14) or under opportunistic conditions. Ventilated patients hospitalized in intensive care units are often highly susceptible to *P. aeruginosa* colonization in the upper respiratory tract (throat and/or trachea) (1, 8). The transition from colonization to infection by *P. aeruginosa* is often seen in the setting of immunosuppression of host defenses such as occurs in cancer patients undergoing chemotherapy, when the blood levels of polymorphonuclear neutrophils are below 100/mm^3 (26). In addition, controlled transcription and synthesis of genes and proteins involved in virulence are thought to be instrumental in sensing the host environment and producing appropriate bacterial responses.

The transcription of genes encoding several virulence factors of *P. aeruginosa* is controlled by the two quorum-sensing (QS) systems, *las* and *rhl* (19), which are regulated by autoinducers (30). Our knowledge of these two QS systems in *P. aeruginosa* has rapidly progressed in the last decade (for a recent review, see reference 4). The *las* system controls the expression of virulence genes such as *lasA*, *lasB*, *aprA*, *toxA*, and *lasI* (6, 7, 17, 23, 28). The *rhl* system controls, for example, the expression of *lasA*, *lasB*, and *rhlAB* (2, 3, 11, 12, 15, 18, 30). The *las* and *rhl* QS systems are hierarchically linked. The *las* system positively regulates the expression of both *rhlR* and *rhlI* (11, 19).

In vitro studies with primarily laboratory strains and virulence studies in animals with these same strains have suggested a role for QS systems in pathogenesis. However, the importance of QS in clinical isolates from typical human *P. aeruginosa* infections is not clearly known (21). A recent study (25) showed a positive correlation between the accumulation of *lasR* transcripts and those of *lasA*, *lasB*, and *toxA* in sputum samples from cystic fibrosis (CF) patients infected with *P. aeruginosa*, suggesting that the *las* system controls virulence gene expression during the course of this specific infection. The autoinducers have also been detected in sputum from patients with CF that also had high levels of *lasI* and *lasR* transcripts (5, 24, 25). However, detection of the autoinducers and *lasI* and *lasR* transcripts remained low for several CF patients infected by *P. aeruginosa* (5, 25), and due to the high immune responses of CF patients to virulence factors, it is unclear in this setting of chronic infection whether QS-controlled proteins remain effective virulence factors. As there has been little investigation into the role of QS in *P. aeruginosa* strains outside of CF, it may be that some isolates do not

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possess or do not express the QS systems yet remain capable of causing serious human infections.

In order to determine the presence and function of the QS gene *lasR* among non-CF clinical isolates, we analyzed the sequence of most of the *lasR* gene from a collection of 66 *P. aeruginosa* strains isolated from cases of nosocomial pneumonia in ventilated patients hospitalized in an intensive care unit, from cases of bacteremia in cancer patients with neutropenia, and from water from swimming pools and rivers (20). A subset of these strains selected on the basis of genetic diversity of the *lasR* gene sequence and of the origin of strains was further used to quantify the amount of mRNA transcribed by *lasR*, *lasB*, and *aprA* genes during the growth of these strains by using real-time reverse transcription-PCR.

MATERIALS AND METHODS

Bacterial strains and growth conditions. We studied 68 strains of *P. aeruginosa*, including PAO1, a wild-type prototroph (9) sensitive to chloramphenicol and thus not overexpressing the MexEF-oprN multidrug efflux pump (10); PAOR, a *lasR* mutant derived from PAO1 (11); 30 strains isolated from patients with nosocomial pneumonia; 20 strains isolated from patients with bacteremia; and 16 strains isolated from water in swimming pools and rivers. The strains were stored frozen at −80°C in individual aliquots in peptone with 10% glycerol. The clinical and environmental strains were part of a collection of strains previously studied for their genetic diversity (20). Some strains were retrieved from six closely related clusters of infection, and we did not use all of the strains likely to be closely related in this analysis. Criteria for inclusion of clinical strains were clearly identified (20), and details of the properties of the clinical and environmental strains are presented at the website http://www.chez.com/rruimy. On the basis of the results of *lasR* gene sequencing and the origin of strains, we selected 13 strains from the two clinical groups and 9 strains from the environmental group to quantify the amount of mRNA of *lasR*, *lasB*I, and *aprA* genes in early log phase after 120 min of growth in broth and in early stationary phase after 300 min of growth in broth.

DNA extraction, amplification, and sequencing of *lasR*, *lasB*, and *aprA*. DNA extraction was performed by using MagnaPure LC (Roche, Mannheim, Germany) according to the manufacturer's recommendations with slight modifications. Bacteria from the frozen stocks were inoculated onto Mueller-Hinton agar (Sanofi-Pasteur, Marnes-la-coquette, France). After overnight growth at 37°C, one colony was suspended in 60 μl of DNase-RNase-free water (Sigma-Aldrich, Saint Quentin Fallavier, France) with 2 μl of lysozyme at 20 mg/ml (Sigma-Aldrich) and incubated for 5 min at 37°C. One hundred thirty microliters of bacterial lysis buffer supplied in isolation kit III (Roche) was added to the mixture, and the mixture was incubated for 10 min at 65°C. Twenty microliters of proteinase K was also added to the mixture. After the mixture was incubated for 10 min at 95°C, DNA was extracted by using the MagNA Pure LC and the other products of kit III (Roche). DNA was quantified at an optical density of 260 nm with a Gene Quant II spectrophotometer (Amersham-Pharmacia Biotech, Orsay, France).

Internal fragments of 665 bp of the *lasR* gene, 441 bp of the *lasB* gene, and 728 bp of the *aprA* gene were amplified in an I-cycler (Bio-Rad, Marnes-la-coquette, France). An internal fragment of the *lasR* gene from each of the 66 strains was amplified and sequenced by using primers that encompassed the 5′ and 3′ ends of the gene to ensure that most of the gene from each strain would be amplified. We did not use primers outside of the coding sequence for *lasR* because the variability of the nucleotide sequence around the *lasR* gene was unknown. The nucleotide sequence recovered 92.4% of the *lasR* gene, which was a good reflection of the overall variation in the *lasR* gene; the remaining 7.6% of the *lasR* sequence was from the primers used for PCR amplification. Internal fragments of *lasB* and *aprA* genes from the 35 strains selected for real-time PCR were amplified and sequenced in order to choose pairs of primers for amplification and fluorogenic probes that encompassed the region of the genes with the fewest nucleotide changes among all 35 strains. The primers used for amplification are listed in Table 1.

The amplification mixture for each gene fragment contained 100 ng of bacterial DNA, two primers at 400 nM each, 250 μM (each) deoxynucleoside triphosphate (Boehringer GmbH, Mannheim, Germany), and 1× reaction buffer supplied by the manufacturer with 1.5 mM $MgCl_2$ and 1 U of AmpliTaq DNA polymerase (Applera, Courtaboeuf, France) in a final volume of 50 μl. DNA was amplified by using the following protocol: 94°C for 4.5 min, then 27 cycles of 94°C for 30 s, 57°C for 1 min, and 72°C for 1 min, followed by 72°C for 10 min. The PCR products were electrophoresed through agarose gels (2%, wt/vol) containing ethidium bromide (0.5 μg ml^{-1}) and visualized under UV irradiation.

The PCR products were purified by using the PCR purification kit Qiaquick (Qiagen, Courtaboeuf, France) and then quantified following electrophoresis on agarose gels by using visual comparisons with markers of known quantity (Low DNA Mass Ladder; Boehringer GmbH). The PCR products were sequenced according to the manufacturer's recommendations for the ABI Prism sequencing kit (Applera) by using the same two primers that were used in amplification (Table 1). The non-primer-derived sequences were aligned and compared to those of the published PAO1 strain by using the Sequence Navigator program (Applera).

RNA extraction and purification. RNA was extracted from 35 of the strains, which were chosen based on their origin and their *lasR* gene sequences, which are representative of each category of clinical isolate, and from PAO1 and its mutant in the *lasR* gene, PAOR. The 35 strains included the following: (i) 11 strains with *lasR* gene sequences identical to that of PAO1 and 11 strains with silent *lasR* mutations, both groups having a similar distribution in terms of clinical source and comprising 4 strains isolated from patients with pneumonia, 4 strains from patients with bacteremia, and 3 strains from environmental water isolates; (ii) 10 strains with mutations in the *lasR* gene compared with the PAO1 *lasR* sequence and with a similar distribution in regard to clinical source; and (iii) three strains with an inserted sequence in *lasR*.

One colony of each strain was inoculated into 5 ml of Luria-Bertani broth and grown overnight at 37°C with shaking. Five hundred microliters of this overnight culture was inoculated into 200 ml of fresh broth and incubated under the same growth conditions. For PAOR cultures, 300 μg of carbenicillin (Sigma-Aldrich, Saint Quentin Fallavier, France) per ml was added. After 120 min of growth, the optical density of the culture at 600 nm was measured with a Spectronic 301 spectrophotometer (Bioblock Scientific, Paris, France). After 120 and 300 min of growth, a volume of culture corresponding to 2×10^8 bacteria was centrifuged for 5 min at 6,000 × *g* at 4°C. The bacterial pellet was immediately suspended in 100 μl of Tris-EDTA lysis buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) containing 400 μg of lysozyme (Sigma-Aldrich) per ml and incubated for 3 min at room temperature. Three hundred fifty microliters of RLT buffer (Qiagen) containing 0.145 μM β-mercaptoethanol was added, and the mixture was vigorously shaken, immediately frozen in liquid nitrogen, and stored at −80°C until RNA extraction.

The bacterial lysates were defrosted and centrifuged for 2 min at 6,000 × *g* at 4°C. Supernatant samples of 450 μl were transferred to microcentrifuge tubes, and 250 μl of absolute ethanol was added to each tube to precipitate nucleic acids. The samples were loaded onto RNeasy columns (Qiagen) and centrifuged for 15 s at 6,000 × *g*, washed with 700 μl of RW1 buffer, and centrifuged for 15 s at 20,200 × *g*. Eighty microliters of RD buffer per sample containing 0.34 U of RNase-free DNase (Qiagen) per μl was added, the samples were left to stand for 20 min at 37°C, and the column was then washed twice with 500 μl of RPE buffer and centrifuged for 15 s at 6,000 × *g* for the first wash and 2 min for the second. RNA was then eluted twice in 50 μl of RNase-DNase-free water (Sigma-Aldrich) by centrifugation for 1 min at 6,000 × *g* and quantified at an optical density of 260 nm with a Gene Quant II spectrophotometer (Amersham-Pharmacia Biotech, Orsay, France). Any RNA breakdown was detected by ethidium bromide staining after electrophoresis (0.5× Tris-acetate-EDTA) on agarose gel (1.4%, wt/vol). RNAs were judged undamaged when only two sharp bands corresponding to the large and small subunits of rRNA were visible.

Reverse transcription. Immediately after extraction, reverse transcription was performed on 500 ng of RNA by using the Taqman RT reagents kit (Applera) according to the manufacturer's recommendations.

Real-time PCR. The amounts of cDNA obtained by reverse transcription were quantified with the real-time fluorogenic 5′ nuclease assay by using an ABI Prism 7000 sequence detector (Applera). We determined the level of transcripts of the *lasR* gene, which controls both of the QS systems, of the *lasB* and *aprA* genes, which are regulated by these QS systems, and of the small-subunit rRNA gene. The probes and primers (Table 1) used to quantify their expression were designed by using the Primer Express ABI Prism program (Applera) and considering the published sequences of *lasR*, *lasB*, and *aprA*. The probes were obtained from Applera and labeled 5′ with the 6-carboxyfluorescein fluorescent dye as reporter and 3′ with the 6-carboxytetramethylrhodamine as quencher. Amplification was performed in a final volume of 20 μl in MicroAmp Optical plates (Applera). The 20-μl reaction mixture contained 10 μl of cDNA sample diluted 1:20, 1× Taqman buffer A, 5.5 mM $MgCl_2$, 200 μM (each) dATP, dCTP, and dGTP, 400 μM dUTP, 200 nM forward primer, 200 nM reverse primer, 100 nM probe, 0.01 U of uracil-*N*-glycosylase (AmpErase UNG) per μl, and 0.025 U of

TABLE 1. Primers used for amplification and sequencing and fluorogenic probes used for real-time PCR

Gene target	GenBank no.	Forward and reverse primers[a]		Fluorogenic probe		Internal primer[a]	
		Position[b]	Sequence	Position[b]	Sequence	Position[b]	Sequence
lasR	D30813	4 (F)	5′-GCCTTGGTTGACGGTTTTCTTG-3′			473	5′-GCGTAGTCCTTGAGCATCCA-3′
		713 (R)	5′-GTAATAAGACCCAAATTAACGGC-3′			454	5′-TGGATGCTCAAGGACTACGC-3′
						271	5′-TCCATCTACCAGACGCGAAAG-3′
		532 (F)	5′-ACCAGCTGGGAGAAGGAAG-3′	572	5′-CCGATGGCGCACCAC-3′		
		595 (R)	5′-CCGATATCTCCCAACTGGTCTTG-3′				
lasB[c]	M19472	363 (F)	5′-TCGTTGCGATCATGGGTGT-3′				
		842 (R)	5′-CGGGAATCAGGTAGGAGACG-3′				
		588 (F)	5′-GCGAAGCCATCACCGAAGT-3′	646	5′-CCATTTCGTCGCCAACA-3′		
		713 (R)	5′-CCTGCTCGGCGGATACC-3′				
		588 (F)	5′-CGAGGCCATCACCGAAGT-3′	646	5′-CCATTTCGTCGCCAACA-3′		
		713 (R)	5′-ACCTGCTCGGCGGAGAC-3′				
aprA[c]	X64558	5042 (F)	5′-TTGCATTGAAAGGTCGTAGCGATG-3′			5270	
		5813 (R)	5′-GTGCGAGTGGTCAGGTTGGC-3′			5373	5′-GGTGACGTCCGACCAGGAT-3′
		5495 (F)	5′-GGCAATCCTGGTACCTGATCAA-3′	5526	5′-CAGCGCCAACGTCAA-3′		
		5554 (R)	5′-AGGGTCTGGCGTCCGTAGTT-3′				
		5495 (F)	5′-GCCAGTCCTGGTACCTGATCAA-3′	5526	5′-CAGCGCCAACGTCAA-3′		
		5554 (R)	5′-AGCGTCTGGCGTCCGTAGTT-3′				
		5495 (F)	5′-GGCAATCCTGGTACCTGATCAA-3′	5526	5′-CAGCGCCAACGTCAA-3′		
		5554 (R)	5′-AGCGTCTGGCGCCCGTAGTT-3′				
Small-subunit rDNA	X06684	485 (F)	5′-CAACAGAATAAGCACCGGCTAA-3′	525	5′-CGCGGCTGCTGGCACGAA-3′		
		547 (R)	5′-ACGCTTGCACCCTTCGTATTA-3′				

[a] For each gene target, two primers, forward (F) and reverse (R), were used for amplification and sequencing, while the internal primer was used only for sequencing.
[b] The numbers indicate the position in the gene sequence, as published in GenBank (see the accession numbers in the GenBank column), corresponding to the nucleotide at the 5′ end of each forward, reverse, and internal primer and of each fluorogenic probe.
[c] For the *lasB* and *aprA* genes, different primers were chosen according to the strain to be amplified by real-time PCR.

TABLE 2. Comparison of the *lasR* sequence of 66 *P. aeruginosa* strains and a subset of 35 strains with the internal fragment of the *lasR* gene of PAO1[b]

Identity or difference	Origin of strain		
	Pneumonia[a] (n = 30)	Bacteremia[a] (n = 20)	Environment[a] (n = 16)
Full identity			
All strains	15 (50)	8 (40)	7 (44)
RT-PCR strains	4 (13)	4 (20)	3 (19)
Silent mutations			
All strains	11 (37)	7 (35)	6 (38)
RT-PCR strains	4 (13)	4 (20)	3 (19)
Nucleotide changes resulting in amino acid changes			
All strains	3 (10)	4 (20)	2 (12)
RT-PCR strains	4 (13)	4 (20)	2 (12)
Insertions			
All strains	1 (3)	1 (5)	1 (6)
RT-PCR strains	1 (3)	1 (5)	1 (6)

[a] The numbers in parentheses indicate the percentages of *lasR* sequence retrieved from all strains from each origin.
[b] The strains were studied by using real-time PCR (RT-PCR). The internal fragment used for comparison was 665 bp; the *lasR* gene of PAO1 is 720 bp.

AmpliTaq Gold DNA polymerase per ml. Before amplification, the PCR mixtures were heated at 50°C for 2 min to prevent carryover of PCR products and then to 95°C for 10 min to denature nucleic acids. All PCRs were run in duplicate and included 40 cycles (95°C for 15 s and 60°C for 1 min). The evolution of the fluorescent intensity of each dye was recorded continuously by using the ABI Prism 7000 sequence detection system (PE Applied Biosystems). The amount of DNA in the sample was calculated by comparing it with the values obtained with standards comprising 10-fold dilutions of *P. aeruginosa* DNA ranging from 25 to 0.025 ng/ml (corresponding approximately to 3.8×10^6 to 3.8×10^1 copies per ml of each amplified target). Negative controls consisting of distilled water or total RNA were included in each test to detect DNA contamination. Data were analyzed with Sequence Detector version 16 application software (Applera) on a personal computer linked directly to the ABI Prism 7000 sequence detection system, as recommended by the manufacturer. The mRNA of *lasB*, *aprA*, and *lasR* genes extracted from each strain was normalized on the basis of the small-subunit rRNA levels, which were determined in each of the real-time PCR experiments. Normalization consisted of dividing the number of copies of the *larR*, *aprA*, or *lasB* transcripts by the number for the small-subunit rRNA and multiplying by 1,000. The PCRs for each strain were repeated three times. Analysis of our data indicated that small-subunit rRNA levels were essentially identical at 120 and 300 min of growth for every strain.

Statistical analysis. All analyses were carried out by using the Prism software package (GraphPad Prism software).

RESULTS

Sequences of the internal fragments of *lasR* genes. An internal fragment (665 bp) of the 720-bp *lasR* gene (92.4% of the sequence) was detected in all 66 strains isolated from pneumonia, bacteremia, and environmental sources. The distribution of strains, based on the change in the *lasR* sequences of the 66 strains, is given in Table 2. Nucleotide changes resulting in amino acid changes were found in nine strains in various regions of the *lasR* gene. Details of the amino acid changes resulting from the nucleotide substitutions in the *lasR* protein from these nine strains are given in Table 3. Finally, one strain in each group had an inserted sequence of roughly 1,300 bp that was always in the same region. These sequences were compared with insertion sequence elements from the database available at http://www-is.biotoul.fr/page-is.html and were found to be members of the IS*5* family group of insertion sequences, IS*427*, IS*5*, and IS*6* for the pneumonia, bacteremia, and environmental strains, respectively. Characteristics of the three insertion sequences can be found at http://www.chez.com/rruimy. By chi-square analysis there were no significant associations between changes in the *lasR* gene and the source of isolation of the strain.

Transcriptional analysis of *lasR* genes in early log and stationary phase. To explore the transcriptional manifestations of QS activity in each strain, the mRNA levels of *lasR*, *lasB*, and *aprA* genes were assayed by real-time PCR at 120 and 300 min of growth, corresponding to the beginning of early exponential phase and entry into stationary phase, respectively. The small-subunit rRNA gene was assayed in parallel to normalize the transcript levels of *lasR*, *lasB*, and *aprA* genes. The results for each strain are in Table 3. The resultant amounts of mRNA transcripts were then compared by paired t tests, analysis of variance, correlation analysis, and linear regression.

Overall, there were increases in the levels of *lasR* transcripts in stationary phase compared with those in early log phase in 33 of the 35 strains ($P = 0.01$, paired t test), indicating that for most clinical isolates some type of QS response occurred, as reflected by an increase in the *lasR* transcript level. There was no correlation in the changes in *lasR* transcript levels based on the genotypic relationship of the *lasR* gene to the *lasR* gene of strain PAO1. Two strains isolated from patients with pneumonia had no increase in *lasR* transcript level. The geometric mean increase in the level of *lasR* transcripts between 120 and 300 min of growth was 7.1 transcripts per cell, but there was a wide range between a 0.54-fold geometric change (i.e., decrease) and a 17-fold increase. Thus, almost all of the strains showed an increase in *lasR* transcript levels going from early-log- to stationary-phase growth in vitro.

Interestingly, there were significant differences noted in the levels of *lasR* transcripts in isolates from different sources (Fig. 1). Overall, the pneumonia isolates had significantly higher levels ($P < 0.01$) of *lasR* transcripts at both 120 and 300 min of growth than those of the bacteremia strains. Isolates from the environment, which likely represents the source of most clinical *P. aeruginosa* isolates, were intermediate in their *lasR* transcript levels between the other two clinical sources and not significantly different from the strains isolated from these other two sources. It is unclear if this association of clinical source and *lasR* transcript level is of significance in regard to virulence, as there were no significant differences ($P > 0.5$) by clinical source in the levels of the LasR-regulated transcripts for *lasB* or *aprA* at either 120 or 300 min of growth.

To determine if there was any relationship between the levels of *lasR* transcripts in the early log and stationary phases, we carried out a correlation analysis of *lasR* transcript levels at 120 and 300 min of growth. There was a strong correlation between the level of *lasR* transcripts at 120 min and 300 min of growth ($r^2 = 0.84$, $P < 0.001$). However, for 18 strains below the median level of all *lasR* transcripts at 120 min (≤4.8 transcripts per cell), there was no real correlation with the transcript levels present in stationary phase ($r^2 = 0.2$, $P > 0.05$), as shown in Fig. 2A. In contrast, almost all of the correlation between the levels of *lasR* transcripts at 120 min and 300 min of growth occurred with the 17 strains above the initial median

TABLE 3. Comparisons of *lasB, aprA* and *lasR* transcript levels in early log phase (120 min) and stationary phase (300 min) for *P. aeruginosa* strains

Strain no. and origin[a]	Amt of transcript at indicated culture time[b]						Comparison with *lasR* gene of PAO1
	lasR transcript		*lasB* transcript		*aprA* transcript		
	120 min	300 min	120 min	300 min	120 min	300 min	
47, B	0.24	1.94	0.04	3.46	1.7	197.93	Full identity
63, E	0.24	4.09	0.6	1.74	0.09	3.23	C201 stop[c]
41, B	0.45	0.66	0.04	0.01	1.03	6.93	Full identity
45, B	0.59	4.14	1.51	13.41	0.17	0.57	Silent mutations
53, B	0.88	10.36	1.43	1.44	4.5	12.91	Insert
42, B	0.92	1.67	2.58	4.38	0.13	1	Q186P
58, E	1.09	2.59	0.27	18.94	0.1	11.78	Silent mutations
55, B	1.36	17.66	0.83	0.46	1.83	3.11	Full identity
43, B	1.64	7.77	0.16	0.17	0.23	1.81	A70E
37, B	2.26	8.09	1.59	20.92	2.85	64.95	Silent mutations
30, P	2.42	9.11	0.46	12.14	0.29	29.62	Silent mutations
67, E	2.76	3.21	1.27	0.58	1.08	3.64	Full identity
15, P	2.96	5.58	0.6	35.98	1.42	309.28	Silent mutations
51, B	3.19	7.38	2.47	3.35	15.59	17.21	Full identity
66, E	3.62	6.49	3.02	2.63	5.09	9.56	Insertions
24, P	3.93	44.01	0.87	27.61	0.88	111.67	Silent mutations
48, B	4.02	5.16	15.05	9.86	2.14	1.72	F210L
16, P	4.83	12.93	0.25	4.74	0.86	44.89	Full identity
39, B	5.15	27.22	0.96	7.73	0.88	21.5	Silent mutations
11, P	6.86	27.99	0.49	5.07	0.29	33.74	Multiple substitutions[d]
64, E	7.45	28.91	1.87	201.68	4.51	144.01	Silent mutations
23, P	9.54	88.21	3.01	87.95	4.25	141.91	Silent mutations
71, E	11.35	12.94	0.5	6.36	0.34	35.45	Multiple substitutions
20, P	11.55	6.2	2.59	0.61	6.95	2.01	Full identity
50, B	12.67	26.98	2.27	42.86	0.41	134.41	K25M
65, E	14.7	70.88	1.7	121.08	3.38	361.46	Full identity
69, E	15.24	19.47	7.51	119.05	7.69	197.64	Silent mutations
36, P	15.84	33.05	3.06	0.44	0.45	4.78	Insertions
19, P	18.57	44.88	10.01	8.84	3.31	31.67	A50G
38, B	21.24	35	1.73	155.55	2.12	203.25	Silent mutations
9, P	22.15	51.88	2.32	73.68	1.22	3.24	Full identity
14, P	34.27	37.36	1.53	3.14	0.94	9.12	Full identity
13, P	37.21	25.97	2.49	77.86	0.3	59.51	V226I
61, E	42.1	137.97	4.57	58.77	6.48	133.12	Full identity
27, P	106.29	414.39	5.02	258.05	16.84	269.79	Silent mutations
PAO1	8.39	12.23	8.22	129.07	5.12	210.15	Full identity

[a] B, strain isolated from patient with bacteremia; P, strain isolated from patient with pneumonia; E, strain isolated from the environment.
[b] *lasB*, *aprA*, and *lasR* transcript levels were normalized by using the ratio of the amount of these transcripts to the small-subunit rRNA level and multiplying by 1,000.
[c] Amino acid substitution.
[d] Strains 11 and 71 had the following amino acid substitutions: R66K, N136S, A137N, and G172N.

level at early log phase ($r^2 = 0.83$, $P < 0.001$), as shown in Fig. 2B. The geometric mean level of *lasR* transcripts at 300 min of growth for the strains with ≤4.8 transcripts per cell at 120 min of growth was 5.5 (lower and upper standard error of the mean, 4.1, 7.1) ($P < 0.001$ compared with the level at 120 min, as determined by the paired *t* test), whereas for strains with >4.8 *lasR* transcripts per cell at 120 min of growth the geometric mean at 300 min of growth was over nine times higher, 38.4 (35.3, 41.6) ($P < 0.001$ compared with the level at 120 min, as determined by the paired *t* test; $P < 0.001$ for comparison of the geometric means at 300 min of both the initial 50% lowest and 50% highest levels of *lasR* transcripts). This finding indicates that the initial level of *lasR* transcripts in those strains with high constitutive levels in early log phase strongly influences the transcript level in stationary phase, whereas for strains with low constitutive levels the amount of *lasR* transcript produced in stationary phase was still low and not correlated with the initial transcript level.

Transcriptional analysis of *lasB* and *aprA* genes in early log and stationary phase. A similar analysis of the changes in transcript levels of the LasR-regulated *lasB* and *aprA* genes showed significant increases ($P \leq 0.001$, as determined by paired *t* tests) in transcript levels for both *lasB* and *aprA* genes among the 35 strains, but there was no correlation between levels at 120 and 300 min of growth. This finding may indicate that factors other than the initial level of *lasB* and *aprA* determined the level of transcripts in stationary phase.

Analysis of the correlation of *lasR* transcripts with *lasB* and *aprA* transcripts in early log and stationary phases. To determine if *lasR* transcription was one of the factors influencing levels of *lasB* and *aprA* transcripts in both the early log and stationary phases, we performed a variety of linear regression analyses. The level of *lasR* transcripts at 120 min in all 35 strains analyzed correlated significantly but very modestly with that of *aprA* in early log phase (Table 4, group A). At 300 min of growth, the level of *lasR* transcripts for all 35 strains actually

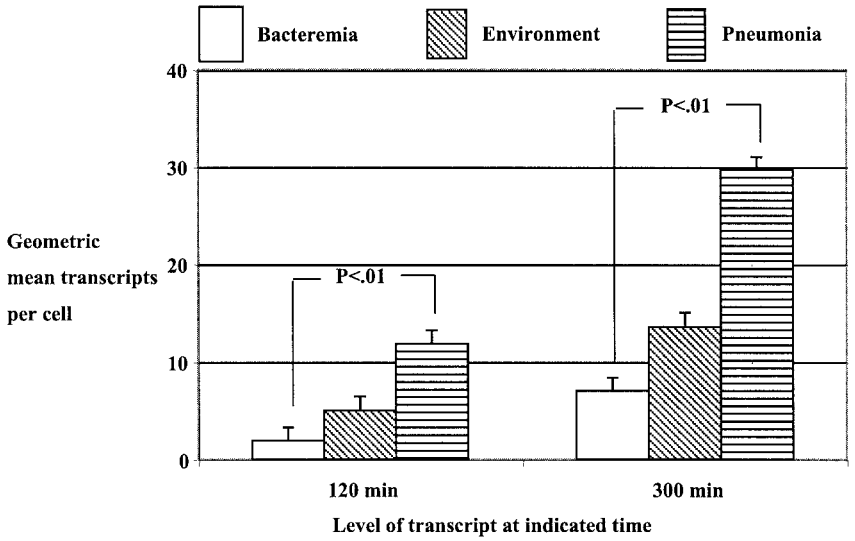

FIG. 1. Comparison by source of isolate of *lasR* transcript levels in the early log (120 min) and stationary (300 min) phases of growth. The bars represent the geometric means for the strains, and the error bars represent the standard errors of the means. There were 13 isolates from patients with bacteremia, 9 from the environment, and 13 from patients with pneumonia. The *P* values represent Fisher's probable least square differences for pair-wise comparisons. Values for the overall analysis of variance were as follows: at 120 min, $F = 6.317$, $P = 0.005$; at 300 min, $F = 4.44$, $P = 0.02$.

correlated less well, but still significantly, with that of *aprA* (Table 4, group A). There was no correlation between the transcript levels of *lasR* and *lasB* at early log phase for any of the 35 strains (Table 4, group A), but in stationary phase there was a reasonably good and significant correlation between these transcript levels (Table 4, group A). Thus, *lasB* transcript levels at 300 min seemed to be much more strongly influenced by *lasR* than were those of *aprA*.

Given that the correlation in early stationary phase between *lasR* and *lasB* transcript levels was only 0.5 and that the correlation in transcript levels for *lasR* and *aprA* was 0.2, we sought to determine if among the 35 strains there were those with a greater or lesser correlation between *lasR* and *lasB* or *aprA* transcript levels. For 17 strains below the median level for *lasR* transcripts at 300 min (12.3 transcripts per cell), there was no correlation with transcript levels for *lasB* or *aprA* (Table 4, group B) at either 120 min or 300 min of growth. For the 18 strains above the median level for *lasR* transcripts measured at 300 min of growth, the correlation with transcript levels for *lasB* were comparable to that for the entire population of strains, as was the correlation with transcript levels for *aprA* (Table 4, group C). This finding indicates that in only about one-half of *P. aeruginosa* strains that produce high levels of *lasR* transcripts does this transcript level correlate with that for *lasB* or *aprA* in stationary phase, suggesting that about one-half of strains do not have a LasR-dependent, QS-based regulation of *lasB* and *aprA* transcription.

Analysis of the correlation of *lasB* and *aprA* transcripts in early log and stationary phases. Since LasR regulation of the transcription of *lasB* and *aprA* in stationary phase was limited to about one-half of the strains as determined by correlation analysis, we sought to determine if there were other factors regulating transcription of *lasB* or *aprA* by analyzing the correlation between *lasB* and *aprA* transcripts. An analysis of the entire group of 35 isolates revealed a reasonable and signifi-

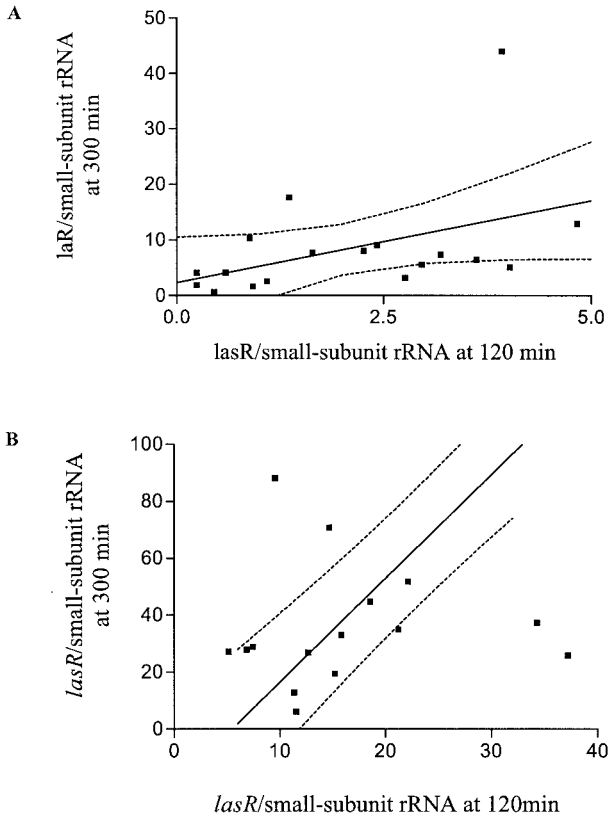

FIG. 2. Correlation between the level of *lasR*/small-subunit rRNA transcripts at 120 min and 300 min of growth for 18 strains below the median level of all *lasR*/small-subunit rRNA transcripts at early log phase ($r^2 = 0.2$, $P > 0.05$) (A) and for the other 17 strains above the median level of all *lasR*/small-subunit rRNA transcripts at early log phase ($r^2 = 0.83$, $P < 0.0001$) (B).

TABLE 4. Correlation analysis of *lasR*, *lasB* and *aprA* transcript levels in early log phase and stationary phase for 35 *P. aeruginosa* strains and for strains below the median level of *lasR* at 300 min or above the median level of *lasR* at 300 min

Group[a]	120 min						300 min					
	lasR				*aprA*		*lasR*				*aprA*	
	lasB		*aprA*		*lasB*		*lasB*		*aprA*		*lasB*	
	r^2	*P*	r^2	*P*	r^2	*P*	r^2	*P*	r^2	*P*	r^2	*P*
A	0.06	NS[b]	0.3	<0.001	0.1	NS[b]	0.5	<0.001	0.2	<0.01	0.5	<0.001
B	0.06	NS[b]	0.2	NS[b]	0.02	NS[b]	0.0006	NS[b]	0.002	NS[b]	0.4	<0.01
C	0.1	NS[b]	0.6	<0.001	0.3	<0.05	0.4	<0.01	0.2	<0.05	0.6	<0.001

[a] A, 35 *P. aeruginosa* strains; B, strains below the median level for *lasR* transcripts at 300 min; C, strains above the median level for *lasR* at 300 min.
[b] NS, not significant.

cant correlation between the transcript levels of *lasB* and *aprA* in stationary phase (Table 4, group A). The correlation at 300 min of growth was present both for strains with *lasR* transcript levels below the median for the entire population in stationary phase (Table 4, group B) and for strains with *lasR* transcript levels above the population median at stationary phase (Table 4, group C). Also, there was a modest correlation ($r^2 = 0.3$, $P < 0.05$) between *aprA* and *lasB* transcript levels at 120 min in those strains with *lasR* transcript levels above the median measured at 300 min. This correlation of *lasB* and *aprA* transcript levels with each other for all strains regardless of the *lasR* level indicates that LasR does not always play a major role in regulating transcription of *lasB* and *aprA*.

Other analyses indicated no difference in transcript levels or in correlations for *lasR*, *lasB*, or *aprA* among bacteremia, pneumonia, or environmental isolates. These individual analyses gave essentially the same results as did the analysis done on the entire group of 35 strains.

DISCUSSION

QS responses in *P. aeruginosa* have been principally defined by using transcriptional analysis of the *las* and *rhl* regulators and the regulated genes, such as *lasA*, *lasB*, *aprA*, and *toxA* (for a recent review, see reference 4). However, almost all of these measurements have been carried out with a limited number of mostly laboratory strains, with little data available for clinical isolates from different infections (16, 22, 29). Although animal studies in models of burn wound infection and pneumonia (13, 21, 27, 31) indicate a role for QS-regulated genes in pathogenesis, these studies were also carried out by using single laboratory strains. Here, we analyzed the relationship between transcription of one of the key QS regulators, *lasR*, and two important virulence factors whose transcription is controlled by LasR, *lasB*, and *aprA*. We found overall modest correlations in transcript levels between *lasR* and the other two genes, but this correlation was found for only one-half of the clinical and environmental isolates studied. For the other half, those with low, constitutive levels of *lasR*, there was no correlation of transcript levels for this regulator with those of the target genes. Thus, only about one-half of *P. aeruginosa* strains seem to depend upon *lasR* transcription for regulating transcription of other virulence factors.

Even among comparable laboratory strains of *P. aeruginosa* there are differences in QS-regulated transcriptional responses. When mutant strains of PAO1 unable to produce endogenous homoserine lactone (HSL) due to deletion of both the *lasI* and *rhlI* genes (29) or *rhlR* and *lasR* (22) were given exogenous HSL, both studies reported increased transcription of *lasB* but with a large difference in the magnitude of the transcriptional response. Shuster et al. (22) reported a 110-fold increase in *lasB* transcription in response to 2 μM *N*-3-oxododecanoyl-HSL (3O-C12-HSL) and a 180-fold increase in response to a combination of 2 μM 3O-C12-HSL and 10 μM *N*-butanoyl-HSL (C4-HSL). Wagner et al. (29) reported only a 39-fold increase in *lasB* transcription in response to 1 μM 3O-C12-HSL plus 2 μM C4-HSL. Both studies also reported increases in *aprA* transcript levels, with Wagner et al. (29) reporting a 6.9-fold change in response to 1 μM 3O-C12-HSL plus 2 μM C4-HSL, while Shuster et al. (22) found a 25-fold increase in the response of their mutant strain to 2 μM 3O-C12-HSL and a 27-fold increase in response to a combination of 2 μM 3O-C12-HSL and 10 μM C4-HSL. Note that another important difference between these two PAO1 variants is that the strain used by Wagner et al. (29) has also been found to harbor a mutation in the *nfxC* gene, leading to overexpression of the MexEF-OprN efflux pump (10), which increases antibiotic resistance, decreases virulence, alters cell-to-cell signaling, and decreases transcription of *lasB*, the latter result being apparent when comparing the transcriptional responses reported by Wagner et al. (29) and Shuster et al. (22).

Since the differences in transcript levels between two otherwise closely related strains with a difference in a gene outside of the QS system were large, it is clear that even small genetic differences that likely occur among the wide variety of clinical and environmental strains of *P. aeruginosa* can also impact the QS response in many ways. Therefore, it is not surprising that among about one-half of the strains we studied there was very little impact of the QS response that occurs in early stationary phase on the transcription of *lasB* and *aprA*.

A functional link between *lasR* gene transcription and the consequent transcription of the QS-activated genes has been suggested in lung infections associated with CF (5, 25). This link was made by using an approach based on the detection and correlation of gene transcripts in samples of CF sputa. The difficulty with analysis of transcript levels in sputum is that different strains of bacteria in the sputum may be responding to different concentrations of QS signals and thus transcribing different sets of genes at the same time (5, 25). Also, the rapid degradation of mRNA resulting from the interactions between bacterial mRNA and human products present in infected sputa

was problematic for quantifying the transcript levels. Thus, it is difficult to use such results for accurate analysis of QS responses during human infection.

Further support for the idea that a QS-based transcriptional response of virulence factor genes modulated by LasR may be present in only about half of *P. aeruginosa* clinical and environmental isolates came from the analysis of the correlation of the levels of *lasB* and *aprA* transcripts. Here, we found that the levels of these transcripts in early stationary phase correlated significantly with each other regardless of the strain's initial or final level of *lasR* transcripts. Thus, factors other than the *lasR* transcript level affected transcription of *lasB* and *aprA*, and these other factors appeared to have a more common effect among the isolates than did the *lasR* transcript level. While we cannot fully exclude the possibility that some of these factors may be related to the levels or functions of LasR that are independent of the *lasR* transcript level, these findings do point out that for about 50% of *P. aeruginosa* isolates conclusions regarding the role of LasR-dependent QS responses cannot be inferred from *lasR* transcript levels.

Overall, an extensive analysis of genetic sequences and transcriptional responses of a large set of *P. aeruginosa* clinical and environmental isolates indicates that the *lasR*-dependent QS transcriptional response of the *lasB* and *aprA* genes may be present in about one-half of the strains. There was no indication of a large amount of allelic variation among the *lasR* genes, with the vast majority of strains having a nucleotide sequence very close or identical to that of PAO1. Differences were noted principally between the strains with low and high constitutive levels of *lasR* transcripts in early log phase. Strains with levels above the median for 35 isolates tended to produce more *lasR* transcripts in stationary phase, with a strong correlation between the initial and final levels. These strains also had the better correlation of *lasR* transcripts with *lasB* and *aprA*, although for the latter, the correlation, while significant, was weak. For strains with *lasR* transcript levels below the median for the population, there was little increase in *lasR* transcripts in stationary phase and no correlation with the transcription of *lasB* or *aprA* genes. Given that many of these strains were, nonetheless, clinical isolates, it appears that among non-CF isolates of *P. aeruginosa* the *lasR*-dependent transcriptional activation of *lasB* and *aprA* is present in only one-half of strains and thus unlikely to contribute significantly to virulence in these strains. Finally, as we did not analyze protein levels, we cannot exclude the possibility that there was, nonetheless, production of LasB and AprA among the strains with low transcript levels that was comparable to that of strains with high transcript levels. But as the molecular aspects of the LasR-QS system have been defined by microarray analysis (22, 29) and correlative analysis of transcript levels, particularly in CF (5, 25), this study shows that correlations among the *lasR*, *lasB*, and *aprA* transcripts may be found in only about 50% of clinical strains of *P. aeruginosa*.

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