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Microarray Analysis of *Pseudomonas aeruginosa* Quorum-Sensing Regulons: Effects of Growth Phase and Environment†

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Bacterial communication via quorum sensing (QS) has been reported to be important in the production of virulence factors, antibiotic sensitivity, and biofilm development. Two QS systems, known as the *las* and *rhl* systems, have been identified previously in the opportunistic pathogen *Pseudomonas aeruginosa*. High-density oligonucleotide microarrays for the *P. aeruginosa* PAO1 genome were used to investigate global gene expression patterns modulated by QS regulons. In the initial experiments we focused on identifying *las* and/or *rhl* QS-regulated genes using a QS signal generation-deficient mutant (PAO-JP2) that was cultured with and without added exogenous autoinducers [*N*-(3-oxododecanoyl) homoserine lactone and *N*-butyryl homoserine lactone]. Conservatively, 616 genes showed statistically significant differential expression ($P \leq 0.05$) in response to the exogenous autoinducers and were classified as QS regulated. A total of 244 genes were identified as being QS regulated at the mid-logarithmic phase, and 450 genes were identified as being QS regulated at the early stationary phase. Most of the previously reported QS-promoted genes were confirmed, and a large number of additional QS-promoted genes were identified. Importantly, 222 genes were identified as being QS repressed. Environmental factors, such as medium composition and oxygen availability, eliminated detection of transcripts of many genes that were identified as being QS regulated.

Many bacteria have been shown to communicate by using a process known as quorum sensing (QS) to coordinate population behavior in response to environmental cues. Small molecules, termed autoinducers, are produced by the bacterial cell and accumulate in the environment at a high population density. Once an intracellular threshold level of an autoinducer is reached, the autoinducer binds to its cognate transcriptional regulator protein to activate or repress target genes. This behavior was first identified in *Vibrio fischeri* as a mechanism regulating the *lux* genes required for bioluminescence (41). Since this discovery, QS has been described in both gram-negative and gram-positive microorganisms (41) and has been implicated in the regulation of cellular behavior and virulence (10).

Pseudomonas aeruginosa is a highly adaptable bacterium that can colonize various environmental niches, including soil and marine habitats, plants, animals, and humans (10). *P. aeruginosa* possesses one of the best-studied models of QS, and two complete *lux*-like QS systems, *las* and *rhl*, have been identified (28). The *las* system consists of the transcriptional regulatory protein LasR and its cognate signaling molecule, *N*-(3-oxododecanoyl) homoserine lactone (3O-C_{12}-HSL), whose production is directed by the autoinducer synthase encoded by *lasI*. The *rhl* system consists of the RhlR protein and an autoinducer synthase (RhlI), which is involved in production of the cognate autoinducer *N*-butyryl homoserine lactone (C_4-HSL). These systems are intertwined in a hierarchical manner, and the *las* system controls the *rhl* system at both the transcriptional and posttranslational levels. QS has been shown to regulate the production of *P. aeruginosa* virulence factors (such as proteases, exotoxin A, rhamnolipids, and pyocyanin) and to be involved in biofilm formation and development, and it has been implicated in antibiotic resistance (7, 10). Given these findings, it has been suggested that QS may contribute to the ability of *P. aeruginosa* to initiate infection and to persist in a host. Data from many models of both acute infection and chronic infection have supported the hypothesis that QS is important in *P. aeruginosa* pathogenesis (10, 30, 32, 37).

Recent technological advances have made it possible to study global gene expression in both prokaryotic and eukaryotic organisms by using high-density oligonucleotide microarrays. Completion of the entire wild-type *P. aeruginosa* PAO1 genome sequence (36), as well as the availability of high-density oligonucleotide arrays (Affymetrix *P. aeruginosa* GeneChip arrays), permit global gene expression profiling of strain PAO1. The ability of *P. aeruginosa* to colonize and thrive in diverse environments is reflected by its relatively large genome (6.3 Mbp) and genetic complexity (5,570 open reading frames [ORFs]) (36). Compared to known sequenced bacterial genomes, the genome of *P. aeruginosa* possesses a greater overall number of genes, as well as a larger number of genes encoding outer membrane proteins, resistance-nodulation-cell division efflux systems, and multiple, complex chemotaxis systems (36), which may contribute to its pathogenesis. More than 9% of assigned ORFs are classified as transcriptional regulators and two-component systems, reflecting the ability of *P. aeruginosa* to respond and adapt to myriad environments (36). In previous studies workers have identified 50 *P. aeruginosa* genes that are

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† For a commentary on this article, see page 2061 in this issue.

positively regulated by the *las* and/or *rhl* QS systems (8, 18, 19, 28, 29, 38, 39, 44, 46). In this study, *P. aeruginosa* DNA microarrays were utilized to identify QS-controlled genes by using a mutant deficient in production of the autoinducers 3O-C_{12}-HSL and C_4-HSL. Most of the previously described QS-promoted genes were confirmed by this technique, thereby establishing the utility of the DNA microarrays. Furthermore, numerous novel QS-regulated genes were identified, including genes that are repressed by QS in *P. aeruginosa*. A systems approach to understanding QS regulons may elucidate the mechanism(s) that modulates phenotypic changes in response to different environmental stimuli.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. *P. aeruginosa* wild-type strain PAO1 and the Δ*lasI* Δ*rhlI* double-null, autoinducer-deficient strain PAO-JP2 (28) were used for expression analysis studies. The strains were maintained at 37°C on PTSB (26) agar plates. Overnight aerobic cultures (PAO1 or PAO-JP2) were grown with agitation in 125-ml Erlenmeyer flasks at 37°C in 20 ml of modified FAB [0.1 mM $CaCl_2$, 0.01 mM Fe-EDTA, 0.15 mM $(NH_4)_2SO_4$, 0.33 mM Na_2HPO_4, 0.2 mM KH_2PO_4, 0.5 mM NaCl, 1 mM $MgCl_2$, 40 mM KNO_3, 10 mM glucose; pH 7.0 (9)]. When appropriate, exogenous 3O-C_{12}-HSL (1 μM) and C_4-HSL (2 μM) were added. The autoinducers were synthesized and purified as previously reported at the University of Rochester, Rochester, N.Y. (21), and were used as received. For other studies, PAO1 cultures were grown in NY (nutrient broth-yeast extract [mass ratio, 5:1]; pH 7.0) or NY supplemented with KNO_3 at a final concentration of 100 mM (pH 7.0). For anaerobic studies, overnight cultures were grown at 37°C in an anaerobic chamber (85% nitrogen, 5% CO_2, 10% H_2; Coy Laboratory Products Inc., Grass Lake, Mich.) in 20-ml portions of NY containing KNO_3 in 50-ml culture bottles; each bottle contained a magnetic stir bar for agitation. All medium components other than autoinducers were reagent grade, were obtained from Sigma (St. Louis, Mo.), and were used as received.

Aerobic overnight cultures were used to inoculate 200-ml portions of medium in 500-ml Erlenmeyer flasks to obtain a starting optical density at 660 nm (OD_{660}) of 0.05, and the resulting cultures were grown at 37°C with agitation to the mid-logarithmic or early stationary phase. The mid-logarithmic phase was defined as the OD_{660} at the midpoint of the linear range of exponential growth (OD_{660}, 0.4 to 0.5), and the early stationary phase was defined as the OD_{660} at which the culture exhibited a growth rate close to zero (OD_{660}, 1.0 to 1.4). Anaerobic overnight cultures were subcultured in 200 ml of NY containing KNO_3 in 250-ml culture bottles, agitated with a magnetic stir bar, and grown to the mid-logarithmic phase (OD_{660}, 0.5) or the early stationary phase (OD_{660}, 1.4) in an anaerobic chamber. All experiments were performed in triplicate.

RNA extraction, cDNA probe generation, and microarray processing. Culture samples were collected at the mid-logarithmic and early stationary growth phases. Bacterial cells were pelleted by centrifugation at 4°C for 5 min at 10,000 × *g*, and the supernatant was removed. GramCracker reagent (Ambion, Austin, Tex.) was used to degrade the bacterial cell walls, and total RNA was isolated by using RNAwiz (Ambion) according to the manufacturer's instructions. RNA was purified from residual DNA by using a DNA-Free kit (Ambion), and RNA purity was assessed by reverse transcriptase PCR by using RETROscript and SuperTaq kits (Ambion) with primers specific for *pilA* (sense primer 5′-GAATCGCAGA AGGGCTATTG-3′ and antisense primer 5′-CCTAACGCCTGAACCTCAAC-3′). Synthesis of cDNA, target hybridization, staining, and scanning were performed at the Microarray Core Facilities in the Functional Genomics Center at the University of Rochester. Briefly, cDNA was generated by using random hexamers as primers for reverse transcription (Invitrogen Life Technologies, Carlsbad, Calif.). The primers were annealed (70°C for 10 min, followed by 25°C for 10 min) to total RNA (10 μg) and to exogenous transcripts (described in the Affymetrix technical manual for *P. aeruginosa* expression products [www.affymetrix.com]) (130 pM) that were added to each sample, which served as a control for monitoring transcriptional efficiency and array performance, and they were extended with SuperScript II reverse transcriptase (Invitrogen) (25°C for 10 min, 37°C for 60 min, 42°C for 60 min, and 70°C for 10 min). Residual RNA was removed by alkaline treatment followed by neutralization, and cDNA was purified with a QIAquick PCR purification kit (QIAGEN, Valencia, Calif.). Purified cDNA was fragmented with DNase I (Amersham Pharmacia Biotech, Piscataway, N.J.) and end labeled with Biotin-ddUTP by using an Enzo BioArray terminal labeling kit (Affymetrix, Santa Clara, Calif.). Target hybridization, staining, and scanning were performed by using an Affymetrix GeneChip system (workstation with Microarray Suite [MAS] software, version 5.0, a hybridization oven, a fluidics workstation, and a Hewlett-Packard GeneArray scanner). Individual microarrays were scanned at two intensity levels to provide a greater dynamic range of gene expression (www.affymetrix.com). Data were stored in an Affymetrix LIMS database for analysis.

Microarray data analysis. Microarray data were analyzed by using protocols described by Affymetrix (www.affymetrix.com). All data were globally scaled to a target intensity of 500, and absolute expression levels of transcripts were generated for each chip. Briefly, in this procedure three metrics (detection, call change, signal log ratio) were used to identify genes whose transcripts were different in an individual control sample and an experimental sample. Transcript expression for a particular gene (presence or absence) was detected by using default parameters in the MAS software (version 5.0; Affymetrix). We performed pairwise comparisons with Data Mining Tools (DMT) (version 3.0; Affymetrix) to determine whether a transcript was present (positive call) and, if it was present, whether the transcript changed. We defined a positive call as 66% present across the pairwise comparisons (six of nine pairwise comparisons for three chips) since we performed three biological replicate experiments for each condition. Saturating probes which may have incorrectly appeared to be absent when they were scanned at a higher intensity level were identified by a comparison of microarray scans performed at two intensity levels. Transcripts that were absent under both control and experimental conditions were eliminated from further consideration. We then used statistical tools available in DMT, version 3.0, to perform *t* tests with absolute data to identify genes that showed significant changes in transcript levels ($P \leq 0.05$) when the results obtained under control and experimental conditions were compared. Comparative analyses of experimental and control chips were also performed by using MAS, version 5.0, to generate $\log_2$ signal ratio values in order to quantify the direction and magnitude of differential transcript expression, as well as the statistical significance of the difference (change call). We performed pairwise comparisons of change call data for each condition and defined a positive call as a 66% increase or decrease among the nine pairwise comparisons for the three chips. Transcripts that exhibited statistically significant change calls (increases or decreases) were combined with transcripts that showed significant changes by using a *t* test ($P \leq 0.05$) to obtain a final list of genes whose transcripts exhibited differential expression under the two conditions. Log_2 signal ratio values were converted and expressed as fold changes.

Data were also analyzed by using Significance of Analysis of Microarrays (SAM) software (version 1.13) (44) to determine the possible number of false positives in each data set (the false discovery rate). Tuning parameters were adjusted to minimize the false discovery rate for each data set. Additionally, lists of transcripts obtained by using SAM were compared to lists of transcripts generated by using MAS (version 5.0) and DMT (version 3.0) software tools to obtain an estimate of the difference between the two statistical approaches and to provide greater confidence in the transcripts that were determined to be differentially expressed under the different conditions. Further information about these statistical tests is available elsewhere (44; www.affymetrix.com).

Microarray validation: QRT-PCR and data analysis. Differential expression of genes was examined by quantitative real-time PCR (QRT-PCR) by using the MGB Eclipse expression system chemistry with probes and primers designed by using sequences from the *Pseudomonas* database associated with the probe designations in the Affymetrix library files. Probes and primers were designed by using the Epoch Biosciences MGB Eclipse gene expression design software in the custom mode to allow for the development of assays that have efficiencies commensurate with a comparative threshold cycle (C_t) analysis (www.epochpharm.com). The following dye combination for probe generation was used for detection and data normalization: 6-carboxyfluorescein (FAM) (3′ reporter) and MGB-Q (minor grove binder-quencher) for genes of interest and for normalization control genes. The following probe and primer sequences were used: for PA0122, sense primer 5′-TCGTCGTCCTTGTTGGTGTA-3′, antisense primer 5′-ATGGATCGCAGTGAAGGT-3′, and probe (MGB-Q)CA ATGGGGCAAGTTCTA(FAM); for PA1897, sense primer 5′-GTAGGTGAC GAAGGTGTTA-3′, antisense primer 5′-CCTGGAACAAATGGATGA-3′, and probe (MGB-Q)TGCCCTGCCACCACTG(FAM); for PA1978, sense primer 5′-GATGCCGTGTTCTTCGTG-3′, antisense primer 5′-AAGCCATC GAGCAGATCCT-3′, and probe (MGB-Q)TGCCCTCGGACGTGAT(FAM); for PA3391, sense primer 5′-GTACAGGGTGAAGGCGTT-3′, antisense primer 5′-CCGCTACGTGTTCTGCAA-3′, and probe (MGB-Q)ATCCCCC ATCCCAACGA(FAM); for PA3874, sense primer 5′-TGTTGAACCAGGCG TACT-3′, antisense primer 5′-GTGCTGAACCTCGACAAA-3′, and probe (MGB-Q)CCACACCTGCTCGATC(FAM); for PA3877, sense primer 5′-GGT

AGGAGAGGAAGAACA-3′, antisense primer 5′-ACCGCATCCTTCATCAC CAT-3′, and probe (MGB-Q)TTCTGGGTCTGCCTGG(FAM); for PA3904, sense primer 5′-CCGACGCTACCTACCA-3′, antisense primer 5′-CGTCTTC TTCGACCATCT-3′, and probe (MGB-Q)CCACCCTCAGCGCCAA(FAM); for PA4651, sense primer 5′-ACCGAATAGCGCATCTGGAA-3′, antisense primer 5′-GTGCTGATCGACGAAGTG-3′, and probe (MGB-Q)ACGCAAC GAAGCCCCG(FAM); and for 16S RNA, sense primer5′-GCTTGCACCCTT CGTATT-3′, antisense primer 5′-CCTTGCTGTTTTGACGTTACC-3′, and probe (MGB-Q)GCACCGGCTAACTTCGT(FAM). In short, the MGB Eclipse probe system incorporates a minor groove binder, dark quenchers, and modified bases which work in concert to generate probes for difficult templates. In a nonhybridized state MGB Eclipse probes assume a random coil structure in solution, which causes a quenching of the reporter due to the proximity of the quencher molecule to the reporter molecule (for specific thermodynamic and physical characteristics of the quencher and its ability to efficiently quench reporters in the nonhybridized state, see the MGB Eclipse technical manual [www.epochpharm.com]). When hybridized to complementary targets at lower annealing temperatures, the quencher and the fluorophore are spatially separated, which allows the reporter to be accessible to excitation and emission from a light source. During the extension phase of the PCR, the forward primer causes the MGB Eclipse probe to be removed from the target, which returns the probe to a nonfluorescent state. Data are collected only during the annealing phase of the assays, and a melting curve is completed following each reaction to confirm that no template-independent amplification was present. Although the chemistry is slightly different, it is important to note that the principle of this approach is the same as that of Taqman chemistry used for quantitative PCR and that in fact, this approach is more sensitive and allows for the design of probes for thermodynamically undesirable regions (A. I. Brooks, unpublished observations). Prior to comparative analysis each probe and primer pair were tested to determine their relative efficiency compared with that of the probe and primer pair for 16S RNA (referred to as the normalizer gene) to ensure that the C_t data analysis approach could be employed (for a detailed description of the procedure used to measure efficiency see Applied Biosystems technical bulletin 2, Relative Gene Expression Analysis). The absolute value of the slope of log input amount versus ΔC_t was less than 0.1 for all comparisons, which allowed us to use the $\Delta\Delta C_t$ calculation to determine the relative levels of gene expression in all experimental cultures compared to the levels in controls. De novo cDNAs from RNA used in microarray target preparation were employed for all analyses. Following probe and primer optimization all cDNAs were diluted to a concentration of 2 ng/μl, and 5 μl was used for each 10-μl PCR mixture containing each deoxynucleoside triphosphate at a concentration of 400 μM, 1× MGB Eclipse buffer, 1× primer mixture, 1× MGB Eclipse probe mixture, and 0.4 U of JumpStart *Taq* (Sigma). All reactions were performed in triplicate, and the experiment was replicated three times; therefore, the data presented below are a reflection of nine individual reactions for each sample tested. All reactions were performed with an ABI 7900 HT with the following cycle parameters: one cycle of 95°C for 2 min, followed by 50 cycles of 95°C for 5 s, annealing at 56°C for 20 s, and extension at 76°C for 20 s. Data were collected only during the annealing phase, and melting curves were obtained following completion of the reaction. Raw data were analyzed by using the Sequence Detection software (ABI, Foster City, Calif.), while relative quantitation with the C_t method was performed by using Microsoft Excel.

Identification of *las* boxes upstream of QS-regulated genes. The DNA regions (nucleotides −500 to 50 relative to the translational start site) surrounding differentially expressed genes were searched for the presence of putative activator protein-autoinducer complex binding elements (*las* boxes [43]) by using RSA tools (http://bioinformatics.bmc.uu.se/~jvanheld/rsa-tools). The search was conducted by using a consensus sequence based on known QS-regulated promoters, and the total number of matches, sequence identity, and position are reported below.

RESULTS AND DISCUSSION

Identification of QS-regulated genes in PAO-JP2 in the presence and absence of exogenous autoinducers. To determine the contribution of QS in regulating *P. aeruginosa* gene expression, samples of total RNA were extracted at the mid-logarithmic and early stationary phases from three independent cultures of a mutant (PAO-JP2) deficient in 3O-C_{12}-HSL and C_4-HSL production grown aerobically in modified FAB with or without exogenously added autoinducers (1 μM 3O-C_{12}-HSL and 2 μM C_4-HSL). RNA samples were processed and hybridized to *P. aeruginosa* DNA microarrays. An average of 3,628 (65%) of the 5,570 PAO1 ORFs were expressed in PAO-JP2 at the mid-logarithmic phase, and 4,204 ORFs (75%) were expressed in PAO-JP2 at the early stationary phase. When exogenous autoinducers were added to culture media, 4,168 ORFs (75%) were expressed at the mid-logarithmic phase, and 4,333 ORFs (78%) were expressed at the early stationary phase. The gene expression pattern of PAO-JP2 was compared to profiles generated from PAO-JP2 grown in the presence of both autoinducers, and transcripts which exhibited significant differential expression ($P \leq 0.05$) under the two culture conditions were defined as QS regulated. A comparison of the gene expression profiles of the PAO-JP2 mutant cultivated with and without exogenous autoinducers eliminated possible erroneous interpretations of QS regulation that might have been due to secondary mutations in PAO-JP2 that may have arisen to compensate for disruption of QS regulons (2, 3). The effect of secondary mutations would confuse direct comparisons of PAO-JP2 with the PAO1 parent strain. By using this approach, 616 genes (more than 10% of the known ORFs) were identified as being QS regulated. This value is more than double the 200 to 300 genes predicted previously (44). Table 1 shows genes that were identified as being QS regulated ($P \leq 0.05$) and that also showed a change of fivefold or more (QS promoted) or threefold or more (QS repressed). A complete list of all genes whose expression was identified as being QS regulated that exhibited a statistically significant change ($P \leq 0.05$) is available online (http://www.urmc.rochester.edu/smd/mbi/bhi/). Using SAM, version 1.1.3, we determined that our data sets have a false discovery rate of approximately 9%. Lists of genes whose expression was determined to exhibit statistically significant changes in response to autoinducer signals generated by MAS (version 5.0) and DMT (version 3.0) were compared to similar lists of genes generated by SAM (version 1.13). Overall, only 24 genes (4%) generated by using SAM (version 1.13) did not match the 616 genes generated by using MAS (version 5.0) and DMT (version 3.0), which substantiated our statistical analysis.

Of the 616 genes whose expression was identified as being QS regulated, 394 were promoted and 222 were repressed. In addition, nine intergenic regions (six promoted regions and three repressed regions) responded to the presence of exogenous autoinducers. The expression of 43 genes was increased more than 16-fold, and five genes exhibited increases of more than 64-fold (Table 1 and Fig. 1). In comparison, only one gene (PA2261) showed a decrease in expression of more than 16-fold, and the expression of 27 genes decreased more than fourfold. Approximately equivalent numbers of genes exhibited changes in expression between 1.3- and 4-fold (the expression of 223 genes increased, while the expression of 197 genes decreased). The temporal patterns of QS-regulated gene expression were also compared at the mid-logarithmic and early stationary phases. The expression of 244 genes was found to be QS regulated at the mid-logarithmic phase, and 450 genes were identified as being QS regulated at the early stationary phase. Seventy-eight genes were identified as being QS regulated at both phases. Interestingly, the transcripts of four genes (PA1760, PA1988, PA4520, and PA4912) exhibited a biphasic response (expression was QS promoted at the mid-logarithmic

TABLE 1. Genes whose expression was identified as being QS regulated by using microarrays[a]

ORF or intergenic region (ig)	Gene name	Change in PAO-JP2 (induced to uninduced) (fold)[b]	Growth stage[c]	Protein description[d]	*las* box[e]
QS-promoted genes					
PA0052		6.7	ES	Hypothetical protein	
PA0105	*coxB*	5.7	ES	Cytochrome *c* oxidase subunit II	ACCTGGCTGATCTGATAGCC (R) (−456 to −437)
PA0107		6.1	ES	Conserved hypothetical protein	
PA0122		29	ES	Conserved hypothetical protein	GCCTACCAGATCTGGCAGGC (D), GCCTGCCAG ATCTGGTAGGC (R) (−166 to −147)
PA0144		17	ES	Hypothetical protein	
PA0399		5.2	ML	Cystathionine beta-synthase	
PA0447	*gcdH*	7.8	ML	Glutaryl-coenzyme A dehydrogenase	
PA0572		7.8	B	Hypothetical protein	
PA0588		7.4	ML	Conserved hypothetical protein	
PA0744		7.0	ML	Probable enoyl-coenzyme A hydratase/isomerase	
PA0745		8.2	ML	Probable enoyl-coenzyme A hydratase/isomerase	
PA0852[f]	*cpbD*	18	ES	Chitin-binding protein CbpD precursor	
PA0997		5.9	B	Hypothetical protein	
PA0998		7.2	B	Hypothetical protein	
PA0999	*fabH1*	5.1	B	3-Oxoacyl-(acyl carrier protein) synthase III	
PA1130[f]		7.4	B	Hypothetical protein	
PA1131[f]		5.4	B	Probable MFS transporter	
PA1212		6.9	ES	Probable MFS transporter	
PA1214		5.0	ES	Hypothetical protein	TCCTGGGTGTTCAGCCAGCC (D) (−381 to −362)
PA1217		30	ES	Probable 2-isopropylmalate synthase	GCCTACCCGATGTTCAAGTG (D) (−438 to −419)
PA1219		5.3	ES	Hypothetical protein	
PA1246	*aprD*	6.3	ML	Alkaline protease secretion protein AprD	
PA1249[f]	*aprA*	6.9	B	Alkaline metalloproteinase precursor	
PA1250	*aprI*	12	B	Alkaline proteinase inhibitor AprI	
PA1318	*cyoB*	5.7	ES	Cytochrome *o* ubiquinol oxidase subunit I	
PA1431[f,g]	*rsaL*	7.7	B	Regulatory protein RsaL	AACTAGCAAATGAGATAGAT (D) (−77 to −58)[h]
PA1657		6.6	ML	Conserved hypothetical protein	
PA1784		7.3	ES	Hypothetical protein	CCCTATCCGTTCTGGGAGCT (R) (−140 to −121)
PA1869[f]		79	B	Probable acyl carrier protein	CACTGCCAGATCTGGCAGTT (D), AACTGCCAG ATCTGGCAGTG (R) (−90 to −71)
PA1871[f]	*lasA*	29	ES	LasA protease precursor	
PA1874		9.9	ES	Hypothetical protein	
PA1875		9.1	ES	Hypothetical protein	
PA1894[f]		25	ES	Hypothetical protein	
PA1895		11	ES	Hypothetical protein	
PA1896[f]		11	ES	Hypothetical protein	
PA1897[g]		33	B	Hypothetical protein	ACCTGCCCGGAAGGGCAGGT (D), ACCTGCCC GGAAGGGCAGGT (R) (−288 to −269)
PA1901[f]		16	ES	Phenazine biosynthesis protein PhzC	
PA1902		42	ES	Phenazine biosynthesis protein PhzD	
PA1903		26	B	Phenazine biosynthesis protein PhzE	
PA1904		51	B	Probable phenazine biosynthesis protein	
PA1905		22	B	Probable pyridoxamine 5-phosphate oxidase	
PA1927	*metE*	20	ES	Methyltetrahydropteroyltriglutamate-homocysteine *S*-methyltransferase	
PA1999		32	ML	Probable coenzyme A transferase subunit A	
PA2000		46	ML	Probable coenzyme A transferase subunit B	
PA2001	*atoB*	23	ML	Acetyl-coenzyme A acetyltransferase	
PA2014		7.5	ML	Probable acyl-coenzyme A carboxyltransferase beta chain	
PA2030		7.3	B	Hypothetical protein	
PA2068		8.6	ES	Probable MFS transporter	
PA2069		8.8	ES	Probable carbamoyl transferase	GCCTGCGAAATCTGGCAGGG (D) (−109 to −90)
PA2193[f]	*hcnA*	210	B	Hydrogen cyanide synthase HcnA	ACCTACCAGAATTGGCAGGG (D), CCCTGCCAA TTCTGGTAGGT (R) (−112 to −93)[h]
PA2194[f]	*hcnB*	52	B	Hydrogen cyanide synthase HcnB	
PA2195	*hcnC*	30	B	Hydrogen cyanide synthase HcnC	
PA2196		5.3	ML	Probable transcriptional regulator	
PA2250	*lpdV*	7.8	ML	Lipoamide dehydroger ase-Val	
PA2300	*chiC*	5.1	ES	Chitinase	ACCTACCACAATTGGCAGAG (D) (−193 to −174)
PA2302[f]		27	ES	Probable nonribosomal peptide synthetase	
PA2303		10	B	Hypothetical protein	
PA2304		14	ES	Hypothetical protein	
PA2305		28	B	Probable nonribosomal peptide synthetase	
PA2306		13	ES	Conserved hypothetica protein	
PA2331		9.4	ES	Hypothetical protein	
PA2366		7.5	ES	Conserved hypothetical protein	

Continued on following page

TABLE 1—*Continued*

ORF or intergenic region (ig)	Gene name	Change in PAO-JP2 (induced to uninduced) (fold)[b]	Growth stage[c]	Protein description[d]	*las* box[e]
PA2367		6.3	ES	Hypothetical protein	
PA2423		12	B	Hypothetical protein	
PA2552		10	ML	Probable acyl-coenzyme A dehydrogenase	
PA2553		14	ML	Probable acyl-coenzyme A thiolase	
PA2554		11	ML	Probable short-chain dehydrogenase	
PA2555		6.2	ML	Probable AMP-binding enzyme	
PA2564		11	ES	Hypothetical protein	
PA2565		5.5	ES	Hypothetical protein	
PA2566		10	ES	Conserved hypothetical protein	CCCTGCCAGTTCTGGTAGGC (D), GCCTACCAG AACTGGCAGGG (R) (−157 to −138)
PA2570[f]	*palL*	60	ES	PA-I galactophilic lectin	
PA2587[f]		16	B	Probable FAD-dependent monooxygenase	CCCTATCAGAAGATCGAGTT (D) (−126 to −107)
PA2588		6.9	B	Probable transcriptional regulator	
PA2591[f]		5.7	B	Probable transcriptional regulator	AACTACCAGTTCTGGTAGGT (D) (−115 to −96)
PA2592[f,g]		5.6	B	Probable periplasmic spermidine-putrescine-binding protein	AACTACCAGTTCTGGTAGGT (D) (−59 to −40)
PA2939		16	ES	Probable aminopeptidase	
PA3032[f]	*snr-1*	10	B	Cytochrome *c*	
PA3326[f]		8.7	B	Probable Clp family ATP-dependent protease	ACCTAACAGATTTGTAAGTT (D) (−241 to −222)
PA3328[f]		15	ES	Probable FAD-dependent monooxygenase	
PA3329[f]		55	ES	Hypothetical protein	
PA3330[f]		38	ML	Probable short-chain dehydrogenase	
PA3331[f]		16	B	Cytochrome P450	
PA3332		15	B	Conserved hypothetical protein	
PA3333[f]	*fabH2*	32	B	3-Oxoacyl-(acyl carrier protein) synthase III	
PA3334		23	B	Probable acyl carrier protein	
PA3335		5.1	B	Hypothetical protein	
PA3361		51	ES	Hypothetical protein	
PA3477[f]	*rhlR*	5.9	ES	Transcriptional regulator RhlR	
PA3478[f]	*rhlB*	37	ES	Rhamnosyltransferase chain B	
PA3479[f]	*rhlA*	100	ES	Rhamnosyltransferase chain A	TCCTGTGAAATCTGGCAGTT (D) (−288 to −269)[h]
PA3519		16	ES	Hypothetical protein	
PA3520		70	ES	Hypothetical protein	
PA3709		5.2	ML	Probable MFS transporter	
PA3721		5.8	ES	Probable transcriptional regulator	
PA3724[f]	*lasB*	39	B	Elastase LasB	ACCTGCCAGTTCTGGCAGGT (D), ACCTGCCAG TTCTGGCAGGT (R) (−194 to −175)[h]
PA3904		18	ML	Hypothetical protein	
PA3906		5.8	B	Hypothetical protein	
PA3908		5.9	B	Hypothetical protein	
PA3923		12	ML	Hypothetical protein	
PA4078[f]		7.6	ES	Probable nonribosomal peptide synthetase	
PA4129		14	B	Hypothetical protein	
PA4130		12	B	Probable sulfite or nitrite reductase	
PA4131		6.8	ES	Probable iron-sulfur protein	
PA4132		8.1	ES	Conserved hypothetical protein	
PA4133		46	ES	Cytochrome *c* oxidase subunit (cbb3 type)	
PA4134		22	ES	Hypothetical protein	
PA4141		25	B	Hypothetical protein	
PA4142		9.6	B	Probable secretion protein	
PA4175		5.5	ES	Probable endoproteinase Arg-C precursor	
PA4205	*mexG*	8.0	ES	Hypothetical protein	
PA4206	*mexH*	5.5	ES	Probable RND efflux membrane fusion protein precursor	
PA4207[f]	*mexI*	6.2	ES	Probable RND efflux transporter	
PA4208	*opmD*	6.2	ES	Probable outer membrane efflux protein precursor	
PA4209		14	B	Probable *O*-methyltransferase	ACCTACCAGATCTTGTAGTT (D) (−327 to −308)
PA4211		74	B	Probable phenazine biosynthesis protein	
PA4217[f]		19	ES	Probable FAD-dependent monooxygenase	
PA4293		6.6	ES	Probable two-component sensor	
PA4294		8.6	ES	Hypothetical protein	
PA4297		7.5	ES	Hypothetical protein	GCCTGGCTGGCCATCCAGGC (R) (−190 to −171)
PA4298		6.8	ES	Hypothetical protein	
PA4299		8.9	ES	Hypothetical protein	
PA4300		7.7	ES	Hypothetical protein	
PA4302		9.6	ES	Probable type II secretion system protein	
PA4303		5.2	ES	Hypothetical protein	
PA4304		6.7	ES	Probable type II secretion system protein	
PA4305		7.3	ES	Hypothetical protein	
PA4306		28	ES	Hypothetical protein	

Continued on following page

TABLE 1—*Continued*

ORF or intergenic region (ig)	Gene name	Change in PAO-JP2 (induced to uninduced) (fold)[b]	Growth stage[c]	Protein description[d]	*las* box[e]
PA4496		10	ML	Probable binding protein component of ABC transporter	
PA4648		14	ES	Hypothetical protein	
PA4651		8.8	ES	Probable pilus assembly chaperone	
PA4677		15	B	Hypothetical protein	
PA4878		8.8	B	Probable transcriptional regulator	
PA5059		11	ES	Probable transcriptional regulator	
PA5220		19	ML	Hypothetical protein	
ig 4713795-4713098		47	ES	Intergenic region between PA4280 and PA4281, 4713098-4713795, (−) strand	
QS-repressed genes					
PA0040		5.6	ES	Conserved hypothetical protein	
PA0509	*nirN*	4.7	ES	Probable *c*-type cytochrome	
PA0510		4.1	ES	Probable uroporphyrin-III c-methyltransferase	
PA0512		3.2	ES	Conserved hypothetical protein	
PA0714		3.2	ES	Hypothetical protein	
PA1978		6.1	ES	Probable transcriptional regulator	
PA1983	*exaB*	12	ES	Cytochrome c_{550}	
PA1984		5.5	ES	Probable aldehyde dehydrogenase	
PA2259	*ptxS*	3.6	ML	Transcriptional regulator PtxS	
PA2260		13	ML	Hypothetical protein	
PA2261		18	ML	Probable 2-ketogluconate kinase	
PA2321		3.2	ML	Gluconokinase	
PA2539		4.5	ML	Conserved hypothetical protein	
PA2540		3.0	ML	Conserved hypothetical protein	
PA2711		3.0	ES	Probable periplasmic spermidine-putrescine-binding protein	
PA2903	*cobJ*	3.3	ML	Precorrin-3 methylase CobJ	
PA3391	*nosR*	3.0	ES	Regulatory protein NosR	
PA3392	*nosZ*	3.8	ES	Nitrous oxide reductase precursor	TACTGCCTCGACTGCCAGAT (D) (−168 to −149)
PA3393	*nosD*	3.5	ES	NosD protein	
PA3394	*nosF*	4.4	ES	NosF protein	
PA3395	*nosY*	4.8	ES	NosY protein	
PA3396	*nosL*	4.3	ES	NosL protein	
PA3662		4.6	ES	Hypothetical protein	
PA3870	*moaA1*	5.1	ES	Molybdopterin biosynthetic protein A1	
PA3871		6.4	B	Probable peptidyl-prolyl *cis-trans* isomerase, PpiC type	
PA3872	*narI*	6.1	ES	Respiratory nitrate reductase gamma chain	
PA3873	*narJ*	6.1	B	Respiratory nitrate reductase delta chain	
PA3874	*narH*	8.5	ES	Respiratory nitrate reductase beta chain	
PA3875	*narG*	8.3	ES	Respiratory nitrate reductase alpha chain	
PA3876	*narK2*	11	ES	Nitrite extrusion protein 2	
PA3877	*narK1*	13	ES	Nitrite extrusion protein 1	ATCTGTCCCGCTGGTCAGCG (R) (−217 to −198 in ORF of *narX*)
PA3911		3.7	ES	Conserved hypothetical protein	
PA3912		5.1	ES	Conserved hypothetical protein	
PA3913		6.5	ES	Probable protease	
PA3914	*moeAI*	6.9	ES	Molybdenum cofactor biosynthetic protein A1	
PA3916	*moaE*	7.5	ES	Molybdopterin converting factor large subunit	
PA3917	*moaD*	8.0	ES	Molybdopterin converting factor small subunit	
PA3918	*moaC*	6.1	ES	Molybdopterin biosynthetic protein C	
PA4587	*ccpR*	3.9	ES	Cytochrome c_{551} peroxidase precursor	
PA4628	*lysP*	3.4	ML	Lysine-specific permease	
PA4918		3.0	ML	Hypothetical protein	
ig 2923366-2922568		6.4	ML	Intergenic region between PA2587 and PA2588, 2922568-2923366, (−) strand	

[a] Only genes identified as being QS regulated that were differentially expressed with a magnitude of change of ≥5-fold (QS promoted) or of ≥3-fold (QS repressed) are included. A list of all genes identified as being QS regulated exhibiting a statistically significant change ($P \leq 0.05$) is available online (http://www.urmc.rochester.edu/smd/mbi/bhi/). Genes are identified by ORF designation, gene name, and protein description (http://www.pseudomonas.com).

[b] The magnitude of transcript induction was calculated for PAO-JP2 grown with exogenous autoinducers (1 μM 3O-C_{12}-HSL and 2 μM C_4-HSL) compared to PAO-JP2 cultures grown aerobically in modified FAB. The values are reported for the early stationary phase when differential expression was observed in both growth phases.

[c] Growth stage(s) during which statistically significant differential transcript expression ($P \leq 0.05$) was exhibited. ML, mid-logarithmic phase; ES, early stationary phase; B, both mid-logarithmic and early stationary phases.

[d] FAD, flavin adenine dinucleotide; RND, resistance-nodulation-cell division; MFS, major facilitator superfamily; ABC, ATP-binding cassette.

[e] The orientation of the consensus sequence is indicated in parentheses (D, directly upstream of the ORF; R, opposite strand). The numbers in parentheses are sequence positions.

[f] Gene previously identified as being QS regulated (4, 8, 18, 27–29, 38, 39, 44).

[g] Gene possessing overlapping *las* boxes upstream of adjacent ORFs of currently identified QS-regulated genes.

[h] The *las* box that was directly upstream of the ORF was previously confirmed experimentally (11, 29, 43).

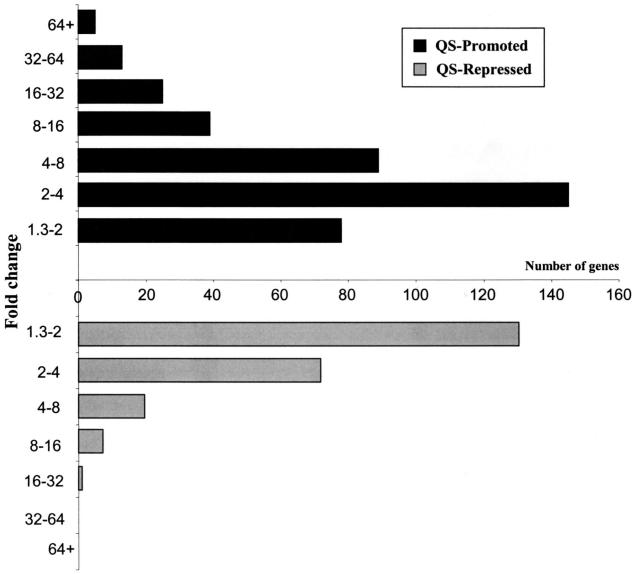

FIG. 1. Changes in the distribution of QS-regulated genes. The numbers of QS-regulated genes displaying specific magnitudes of differential expression (PAO-JP2 induced versus uninduced) are shown. Genes were placed in groups according to the magnitude of differential expression (1.3- to 2.0-, 2.0- to 4.0-, 4.0- to 8.0-, 8.0- to 16-, 16- to 32-, 32- to 64-, and more than 64-fold changes).

phase but QS repressed at the early stationary phase). Time dependence of induction of QS-regulated genes in response to exogenous autoinducers has been observed previously in QS mutants (44) and is discussed in an accompanying paper (34a).

In some instances, for genes that formed an operon, such as the *hcn* cluster (PA2193 to PA2195), the changes for the first gene in the operon were much greater than the changes for the downstream genes when transcript expression in induced PAO-JP2 (grown in the presence of 1 μM 3-O-C_{12}-HSL and 2 μM C_4-HSL) was compared with transcript expression in uninduced PAO-JP2 (Table 1) (http://www.urmc.rochester.edu/smd/mbi/bhi/). This phenomenon has also been noted by other workers and has been attributed to RNA degradation from the 3′ end of the transcript (25). Not all individual genes in known operons exhibited statistically significant changes in transcript expression. For example, at least three genes involved in type III secretion systems, *pscQ*, *pscI*, and *pcrH*, exhibited statistically significant decreases in transcript expression in induced PAO-JP2 compared to transcript expression in uninduced PAO-JP2, but other genes in these operons did not change. We observed some fluctuations in transcript levels in replicates which may have been due to biological variation, as well as RNA degradation, and which resulted in large standard deviations for some genes in operons that precluded their identification as being QS regulated in this study. Although standard deviations in transcript levels tended to be lower as the value for the transcript level decreased, the intensities of transcripts present at very low levels (<500 intensity units) were often close to the average background intensities (~200 intensity units), and thus the transcripts were defined as absent and were excluded from further analyses. These transcripts included some of the genes in type III secretion operons (data not shown), which precluded detection of such genes as genes that exhibit statistically significant changes in response to added autoinducers.

Correlation of microarray data to previously identified QS-regulated genes. Of 52 previously reported QS-regulated genes, 37 were confirmed by using microarrays. These 37 genes are PA0852 (*cpbD*), PA1130 (*rhlC*), PA1131, PA1249 (*aprA*), PA1431 (*rsaL*), PA1869, PA1871 (*lasA*), PA1894, PA1896, PA1901, PA2193 and PA2194 (*hcnAB*), PA2302, PA2570 (*pa1L*), PA2587 (designated *pqsH* [17]), PA2592, PA3032 (*snr-1*), PA3103 (*xcpR*), PA3104 (*xcpP*), PA3327 to PA3331, PA3333 (*fabH2*), PA3336, PA3477 (*rhlR*), PA3478 and PA3479 (*rhlAB*), PA3724 (*lasB*), PA3907, PA4078, PA4084 (*cupB3*), PA4207 (designated *mexI* [1]), PA4217 (*phzS*), PA4236 (*katA*), and PA4869 (Table 1) (http://www.urmc.rochester.edu/smd/mbi/bhi/) (8, 18, 19, 28, 29, 38, 39, 44, 46). Interestingly, PA4084 was previously reported to be QS promoted (44) but in this study was found to be QS repressed. In addition, we found that PA3622 (*rpoS*) is QS promoted (2.7-fold change[P = 0.002] for mid-logarithmic-phase samples and 1.4-fold change [P = 0.026] for early-stationary-phase samples), which agreed with several previous reports (22, 40) but differed from one study in which it was concluded that *rpoS* was not QS regulated (45). Two genes (PA0179 and PA3904) which were only recently identified as being QS promoted by using a fluorescence-activated cell sorting approach (J. Lamb and B. H. Iglewski, unpublished data) were also determined to be QS promoted by using microarrays.

In addition to the *lasI* and *rhlI* genes, which could not be detected in this study because they are mutated in PAO-JP2, 13 genes previously identified as being QS regulated were not identified in this analysis (Table 2). Discrepancies between previously reported QS-regulated genes and the genes identified in the present study could be explained by several observations. Transcripts were not detected for 6 of the 13 genes (Table 2). A high coefficient of variation was observed for an additional 4 of the 13 genes (Table 2). One of these four genes, PA1148, encodes toxin A. This gene is known to be repressed in the presence of elevated iron concentrations, such as the concentration present in modified FAB (16). Although we previously reported that *lasR* is QS regulated, we did not find that expression of the *lasR* transcript was QS regulated under the conditions used in the present study, which were different from those used in the previous study (28). Inconsistencies between the results of the present study and the previously reported results for QS-regulated genes that were obtained by using transposon mutagenesis (44) might be explained by the fact that in some cases the orientation of the transposon insertions was found to be opposite that of the ORF, as shown in Table 2 for PA1129, PA2770, and PA3325. We could not detect the transcript for PA1129 under the experimental conditions used in this study; however, we detected transcripts for PA1130 and PA1131, which are immediately downstream of PA1129, which we identified as being QS promoted using microarrays. Interestingly, although we did not determine that PA2770 is QS regulated in the present study, we noted that the intergenic region between PA2770 and PA2771 that corresponded to the reported transposon insertion location (position 3128663 in the intergenic region from position 3122585 to position 3123598 on the opposite strand [44]) showed an increase in expression at the early stationary phase when PAO-

TABLE 2. Previously reported QS-regulated genes not identified by using microarrays[a]

Gene or ORF	Protein description	Transcript detection[b]	CV[c]	Comment	Reference
PA0051	Probable glutamine amidotransferase	A	NA	Late induction	44
PA0109	Hypothetical protein	P	High	Late induction	44
PA0855	Hypothetical protein	PL	High	Late induction	44
PA1129	Probable fosfomycin resistance protein	A	NA	Insertion in opposite orientation	44
PA1148	Exotoxin A precursor	PL	High	Iron regulated	16
PA1430	Transcriptional regulator LasR	P	Low		28
PA2385	Probable acylase	PL	Low	Late induction	44
PA2401	Probable nonribosomal peptide synthetase	A	NA	Late induction	44
PA2402	Probable nonribosomal peptide synthetase	A	NA	Late induction	44
PA2424	Probable nonribosomal peptide synthetase	A	NA	Late induction	44
PA2770	Hypothetical protein	P	Low	Insertion in opposite orientation	44
PA3325	Conserved hypothetical protein	A	NA	Insertion in opposite orientation	44
PA5356	Transcriptional regulator GlcC	P	High		44

[a] Genes are identified by ORF designation and protein description (http://www.pseudomonas.com).
[b] Transcript detection of genes by using microarrays. P, present; A, absent; PL, present at low levels. A low level of transcript abundance was defined as a transcript abundance of less than 500 intensity units.
[c] A high coefficient of variation (CV) was defined as greater than 20%. NA, not applicable.

JP2 and PAO-JP2 grown in the presence of both autoinducers were compared ($P = 0.054$). Similarly, even though we did not determine that PA3325 was QS regulated in the present study, we found that PA3326, the gene immediately downstream of PA3325 and on the opposite strand, was QS regulated. Additionally, the temporal expression of QS-regulated genes may influence their behavior under various growth conditions. For example, seven genes (Table 2) not identified as being QS regulated in this study reportedly showed very-late-phase QS regulation in studies performed with *lacZ* promoter fusions (44). Extraction of RNA from cultures in the early stationary phase, as performed in the present study, may have excluded determination of these genes as being QS regulated. Taking into account these explanations, we found that the results of the present study matched previously reported results for QS-regulated genes. The high degree of correlation between data obtained in this study and data for previously identified QS-regulated genes provided considerable validation for the use of microarrays.

Independent microarray validation by using QRT-PCR. In addition to the high correlation between previously identified QS-regulated genes and the results of the present study, we chose eight newly identified QS-regulated genes (four promoted genes and four repressed genes) that represented a range of changes observed in microarray studies (8.0- to 33-fold changes for QS-promoted genes and −3.0- to −13-fold changes for QS-repressed genes) to be validated by QRT-PCR. The direction of regulation for all of these genes matched the direction of regulation (promotion or repression) determined from the microarray data (data not shown). The magnitudes of the changes for the QS-promoted genes (PA0122, PA1897, PA3904, and PA4651) obtained from the QRT-PCR analysis agreed well with the magnitudes of the changes determined in the microarray analysis. For the QS-promoted genes, the greatest difference in changes was obtained for PA0122; the change determined in the QRT-PCR analysis was 34.7-fold ± 8.1-fold at the early stationary phase, compared to the change of 29-fold ± 1.4-fold determined in the microarray analysis. The QRT-PCR analysis resulted in larger changes (approximately three to four times larger) for the QS-repressed genes (PA1978, PA3391, PA3874, and PA3877). For example, PA3877 (*narK1*) was reported to show a change of −13-fold ± 1.3-fold when the microarrays were used, but the change was −50.3-fold ± 24.3-fold when QRT-PCR was used. This was not unexpected since a bias towards underestimating the magnitude of mRNA change has previously been observed for oligonucleotide microarray data (48). This suggests that our stringent analysis of the microarray data may have underestimated the true number of QS-repressed genes. The excellent agreement (100%) between the results obtained with the QRT-PCR and the results obtained with the microarrays further substantiated both the statistical analysis approach that we utilized in this study and the DNA microarray technology.

Identification of *las* boxes upstream of QS-regulated genes. A *las* box was detected upstream of 7% of the QS-regulated genes identified in this study (Table 1) (http://www.urmc.rochester.edu/smd/mbi/bhi/). Among the genes whose expression was QS regulated, at least 111 gene clusters were discerned that might form possible operons, and 17 of clusters these included a *las* box upstream of the first gene in the cluster. The *las* boxes identified exhibit homology with the palindromic *lux* box DNA elements identified in *V. fischeri*, which are located upstream of LuxR-regulated genes and have been proposed to act as binding sites for the regulatory protein-autoinducer complex (14). The consensus sequence used in the present study was based on alignment of several known *las* boxes (Fig. 2). Most boxes were found directly upstream of the ORF on the same strand, but a number of genes possessed *las* boxes upstream of the ORF but on the antisense strand. The presence of overlapping *las* boxes on both the sense and antisense strands upstream of some QS-regulated genes is reminiscent of the putatively shared *las* box of *lasI* and *rsaL* (8). Additionally, a *las* box was located within the first 50 bp internal to the coding region (ORF) of one QS-repressed gene (PA3811). This observation may hint that binding of the regulatory protein-autoinducer complex to this region might interfere with expression of this gene. However, some QS genes that are negatively regulated have *las* boxes upstream of their ORFs. It would be particularly interesting to determine where these *las* boxes are relative to the start of transcription. The

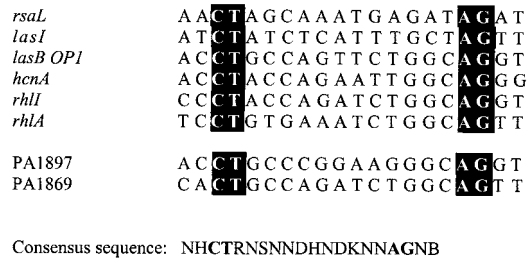

FIG. 2. Alignment of *las* boxes upstream of QS-regulated genes. Previously identified *las* boxes were aligned to form a consensus sequence (11, 29, 43). This sequence was used to search all upstream regions in front of the translational start site of all QS-regulated genes. The consensus sequence NHCTRNSNNDHNDKNNAGNB was derived from inspection, where H = C, T, or A; R = A or G; S = C or G; D = G, A, or T; K = G or T; B = G or T; and N = A, C, G, or T. Sequences that matched the consensus sequence were identified, and the position of the *las* box is shown in Table 1.

relative paucity of *las* boxes upstream of genes and operons that we found to be QS regulated suggests that regulation of a large number of these genes or operons may be indirect. In this regard, it is interesting that we found 37 QS-regulated genes known or thought to be transcriptional regulators or members of two-component systems (Table 1) (http://www.urmc.rochester.edu/smd/mbi/bhi/). Thus, target genes of these regulators would have been identified as being QS regulated in the present study, although their regulation may be via an indirect mechanism. We are currently constructing mutants with mutations in these known and putative transcriptional regulatory genes to determine the effects of such mutations on other genes that we identified as being QS regulated.

Characterization of QS-regulated genes and involvement in physiological processes. Expression of many genes involved in basic cellular processes, such as DNA replication, RNA transcription and translation, cell division, and amino acid biosynthesis, and genes involved in group behavior, such as chemotaxis and biofilm formation, were found to be QS regulated (Table 1 and Fig. 3) (http://www.urmc.rochester.edu/smd/mbi/bhi/). The large effect of QS on such diverse physiological functions has been observed previously in microarray studies with *Escherichia coli*, in which approximately 5.6% of the *E. coli* genome responded to the presence of autoinducer-2 in an autoinducer-2 signaling-deficient mutant (13). In the present study with *P. aeruginosa*, the largest percentage of QS-regulated transcripts (34%) encoded proteins having hypothetical, unclassified, and unknown functions (Table 1) (http://www.urmc.rochester.edu/smd/mbi/bhi/). Expression of 56 of these genes was QS repressed, and expression of 147 genes was QS promoted. Approximately 31% (62 genes) of the hypothetical proteins exhibited homology with known or similar hypothetical proteins in other microorganisms. Expression of 60 genes (31 QS-promoted genes and 29 QS-repressed genes) (Fig. 3), representing 40% of the 150 known or predicted ORFs encoding membrane proteins (36), was determined to be QS regulated. Membrane proteins are of particular interest due to their involvement in virulence factor secretion (36). Other functional classes with large numbers of QS-regulated genes included the genes encoding putative enzymes (14%), the genes encoding transcriptional regulators and two-component systems (9%), the genes encoding energy metabolism (9%), the genes encoding transport of small molecules (8%), and the genes encoding secreted factors and the protein secretion and/or export apparatus (7%) (Fig. 3). Interestingly, expression of 44 genes involved in nitrogen metabolism was identified as being QS regulated (Table 3) (49). Expression of the transcripts of several well-characterized genes involved in the denitrification system (49) was repressed by QS activation (*nar*, *nos*, *mod*, *moa*, and *moe* clusters), while expression of the molybdate-independent shared nitrate reductase (*snr-1*) gene, the

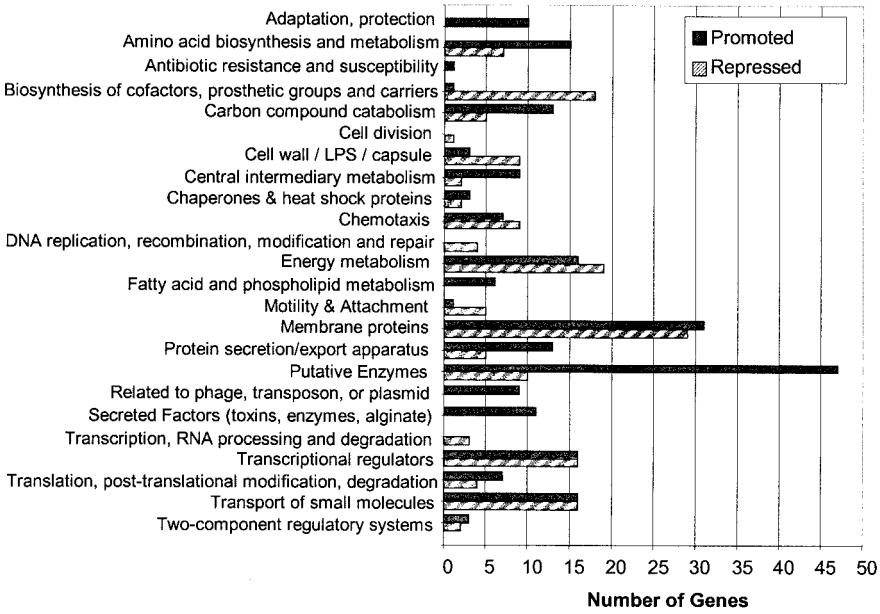

FIG. 3. Functional classes of QS-regulated genes. QS-regulated genes were grouped according to a functional classification. The number of genes either promoted or repressed for each category is shown. LPS, lipopolysaccharide.

TABLE 3. QS-regulated genes involved in nitrogen metabolism

Gene(s) or operon[a]	Description	Change (fold)[b]
PA0509-PA5018 (*nirNJLFCM*)	Nitrite respiration and heme D_1 synthesis	−4.7
PA0520-PA0522 (*nirQ, nirS*)	Denitrification	3.9
PA0523-PA0525 (*norBD*)	Nitrous oxide (NO) respiration	2.2
PA1172-PA1173 (*napCB*)	Periplasmic nitrate reduction	1.5
PA1861-PA1863 (*modCBA*)	Molybdenum transporters	1.8
PA3032	*snr-I* (shared nitrate reduction)	10
PA3391-PA3396 (*nosRZDFYL*)	Nitrate and N_2O respiration	−3.0
PA3872-PA3877 (*narK1K2IJHG*)	Nitrate respiration	−13
PA3870 (*moaA1*)	Molybdenum cofactor biosynthesis	−5.1
PA3914-PA3918 (*moeA1, moaEDC*)	Molybdenum cofactor biosynthesis	−6.9
PA4129-PA4130	Probable sulfite of nitrite reductase	14
PA4131-PA4134	Probable nitrogen fixation proteins	6.8

[a] Genes are identified by ORF designation, gene name, and protein description or known metabolic pathway (http://www.pseudomonas.com).

[b] The magnitude and direction of gene expression change are indicated for PAO-JP2 grown with exogenous autoinducers (1 μM 3O-C_{12}-HSL and 2 μM C_4-HSL) compared to PAO-JP2 cultures grown aerobically in modified FAB. The values are the values for the first gene in the known or probable operons.

napBC genes, and a probable gene cluster involved in nitrite reduction (PA4129 and PA4130) was promoted by QS (Table 3). This highlights the importance of QS as a switch for preferential usage of cellular pathways and is important when the design of therapeutic agents that disrupt a particular metabolic pathway(s) is considered. It also suggests that QS plays a pivotal role in the anaerobic growth of *P. aeruginosa*.

Microbial pathogenesis has been intimately linked to QS regulatory circuits, and several *P. aeruginosa* virulence factors are known to be QS regulated (10). More than 50 probable or known virulence genes (10), such as those encoding proteins involved in attachment to and colonization of host tissues and surfaces (type IV pili, PA-I galactophilic lectin) or dissemination (chemotaxis) or those that may be involved in tissue destruction (PA4142 to PA4144, PA1877), were identified as being QS regulated (Table 4). Interestingly, we identified three genes (PA3337, PA4996, and PA5450) important in lipopolysaccharide biosynthesis whose expression was repressed by QS. We also identified genes involved in type II secretion of virulence factors previously reported to be QS regulated (PA3095 to PA3103, PA3104, and PA3105 [4]), as well as a novel type II secretion gene (PA4304). In addition to type II secretion genes, we found that type III secretion genes (PA1694, PA1707, and PA1722) were QS regulated. Expression of type III secretion systems has been previously reported to be QS promoted in *E. coli* (35). Interestingly, expression of genes associated with the general secretion pathway and the type II pathway (e.g., *xcp*) was upregulated, and expression of genes involved in type III secretion (e.g., *pcrH*) was downregulated by QS. Likewise, gene products known to be secreted by the type II pathway (e.g., elastase [LasB] and the staphylolytic protease LasA) were also upregulated (38, 39), while the exoenzyme S synthesis protein B, which is involved in production of exoenzyme S, a known substrate for the type III secretion system, was downregulated (Table 4) (47). We also found that *tatC*, a gene involved in the twin-arginine translocation system that has only recently been implicated in virulence (23), was QS regulated.

Intrinsic antibiotic and biocide resistance of *P. aeruginosa* is associated with the ability of this organism to persist as an infectious agent. We found that genes involved in three resistance-nodulation-cell division efflux systems (PA0158, PA0425 and PA0426 [*mexAB*], and PA4205 to PA4208 [*mexGHI-opmD*]) were upregulated by QS (Table 4). The MexAB-OprM efflux pump is constitutively expressed in PAO1 and has been correlated with increased antibiotic resistance (12). MexAB-OprM is also required for active efflux of 3O-C_{12}-HSL from the cell (15). *P. aeruginosa* readily forms biofilms, both in vitro and in vivo (6). Even though in this study planktonic cultures were used exclusively, 15 of 73 genes previously demonstrated to be differentially expressed in PAO1 biofilms (42) were identified as being QS regulated in our analysis (Table 4). This is not surprising since it has been reported that QS plays a role in the formation of PAO1 biofilms (7). Interestingly, previous proteomic analysis of biofilm development suggested that there are distinct phases of biofilm initiation and maturation, and cells that are ready to disperse from a biofilm tend to have proteomic profiles similar to those of a planktonic state (34). In the present study, the expression profiles for genes identified as biofilm-regulated genes and the expression profiles for genes that are QS regulated were generally inversely correlated (11 of 15 genes). For example, genes that were upregulated in biofilms were downregulated by QS (PA0713, PA0714, PA0722, PA0971, and PA4765). The converse was also true (PA0105, PA0107, PA0108, PA3418, PA3622, and PA4296). Interestingly some genes identified as being QS and biofilm regulated are primarily involved in resistance to anionic detergents and/or antibiotics (e.g., low expression of *tolA* [31] and low expression of *omlA* [24]) or in the production and secretion of virulence factors (e.g., high expression of *exsB* [47]). Intriguingly, this suggests that QS is decreased during biofilm maturation, which agrees with the results of previously reported biofilm studies that showed that *lasI* expression and *rhlI* expression decrease after 4 to 6 days of biofilm development (9).

Effect of culture conditions on QS-regulated gene expression. The few discrepancies between the present study and previous studies of QS-regulated genes could be due to the growth media and culture conditions employed in the present study. Therefore, we investigated the influence of media and culture conditions on expression of QS-regulated genes. PAO1 was grown aerobically in modified FAB, NY, or NY containing KNO_3 and anaerobically in NY containing KNO_3, and the growth curves are available online (http://www.urmc.rochester.edu/smd/mbi/bhi/). The logarithmic growth rates of PAO1 grown aerobically in the three media were equivalent (doubling times, 59 ± 0.1 min) but were ~40% lower than the growth rates obtained when the strain was grown anaerobically in NY containing KNO_3 (doubling time, 92 ± 4.7 min). Mid-logarithmic- and early-stationary-phase samples were obtained by using the criteria described above. Early-stationary-phase growth occurred at an OD_{660} of 1.0 for PAO1 grown in modified FAB or in NY and at an OD_{660} of 1.4 for PAO1 cultivated aerobically or anaerobically in NY containing KNO_3. Samples

TABLE 4. Genes involved in virulence and genes differentially expressed in biofilms identified as being QS regulated by using microarrays[a]

ORF or operon	Gene name	Protein description or function	Change (fold)[b]
Motility and attachment			
PA0411	*pilJ*	Twitching motility protein PilJ	−1.5 (ES)
PA0413		Still frameshift probable component of chemotactic signal transduction system	−1.7 (B)
PA2570[c]	*palL*	PA-I galactophilic lectin	60 (ES)
PA4527	*pilC*	Still framshift type 4 fimbrial biogenesis protein PilC	−1.9 (ES)
PA4651		Probable pilus assembly protein	8.8 (ES)
PA5040-PA5043	*pilNOQ*	Type 4 fimbrial biogenesis proteins	−1.4 (ES)
Cell wall synthesis, LPS and capsule			
PA3337	*rfaD*	ADP-L-glycero-D-mannoheptose 6-epimerase	−1.7 (ES)
PA4996	*rfaE*	LPS biosynthesis protein RfaE	−1.8 (ML)
PA5161	*rmlB*	dTDP-D-glucose 4,6-dehydratase	1.5 (ES)
PA5163	*rmlA*	Glucose-1-phosphate thymidylyltransferase	1.6 (ES)
PA5450	*wzt*	ABC subunit of A-band LPS efflux transporter	−1.6 (ES)
PA5451	*wzm*	Membrane subunit of A-band LPS efflux transporter	2.2 (ML)
Chemotaxis			
PA0174-PA0179		Probable chemotaxis cluster	1.9 (ES)
PA1458		Probable two-component sensor	−1.3 (ES)
PA1608		Probable chemotaxis transducer	−2.2 (ES)
PA2573		Probable chemotaxis transducer	2.7 (ES)
PA2867		Probable chemotaxis transducer	−1.6 (ES)
PA4307	*pctC*	Probable chemotaxis transducer	−2.4 (ML)
PA4309	*pctA*	Probable chemotaxis transducer	−1.6 (ES)
PA4520		Probable chemotaxis transducer	2.1 (ML), −1.5 (ES)
Protein secretion export apparatus, and secreted factors			
PA1901-PA1906		Probable phenazine biosynthesis cluster	16 (ES)
PA4209, PA4211, PA4217		Probable phenazine biosynthesis cluster	14 (B), 74 (B), 19 (ES)
PA1250	*aprI*	Alkaline proteinase inhibitor AprI	12 (B)
PA1249[c]	*aprA*	Alkaline metalloproteinase precursor	6.9 (B)
PA1246-PA1248	*aprDEF*	Alkaline protease secretion proteins AprDEF	6.3 (ML)
PA1245	*aprX*	Membrane protein	4.1 (B)
PA1871[c]	*lasA*	LasA protease precursor	29 (ES)
PA2193-PA2195[c]	*hcnABC*	HCN biosynthesis genes	210 (B)
PA2300[c]	*chiC*	Chitinase	5.1 (ES)
PA3724[c]	*lasB*	Elastase LasB	39 (B)
PA3478-PA3479[c]	*rhlAB*	Rhamnosyltransferase chain A, chain B	100 (ES)
PA4142-PA4144		Probable toxin secretion cluster	9.6 (B)
PA4302		Probable type II secretion system protein	9.6 (ES)
PA4304		Probable type II secretion system protein	6.7 (ES)
PA1877		Probable secretion protein	4.3 (ES)
PA3096-PA3103[c]	*xcpRSTUVWXY*	General secretion pathway	3.5 (ML)
PA3104-PA3105[c]	*xcpPQ*	General secretion pathway	2.0 (ES)
PA1694	*pscQ*	Translocation protein in type III secretion	−1.7 (ML)
PA1722	*pscI*	Type III secretion protein PscI	−1.8 (ML)
PA1712	*exsB*	Exoenzyme S synthesis protein B	−2.4 (ES)
PA4559	*lspA*	Prolipoprotein signal peptidase	−1.4 (ES)
PA5070	*tatC*	Transport protein TatC	−1.5 (ES)
RND efflux transporters or drug efflux transporter			
PA0158		Probable RND efflux transporter	2.5 (ML)
PA0425-PA0426	*mexAB*	RND multidrug efflux system	1.9 (B)
PA4205-PA4208	*mexGHI-opmD*	Probable RND efflux transporter system	8.0 (ES)
PA5160		Drug efflux transporter	2.2 (ES)
Transcriptional regulators and sigma factors			
PA0764	*mucB*	Negative regulator for alginate biosynthesis MucB	−1.6 (ES)
PA0762	*algU*	Sigma factor AlgU	−1.5 (ES)
PA1003	*mvfR*	Regulator of multiple virulence factors	3.0 (B)
PA1707	*pcrH*	Regulatory protein PcrH	−2.2 (B)
PA1898[c]	*qscR*	Quorum-sensing control repressor	3.0 (ML)
PA2259	*ptxS*	Transcriptional regulator PtxS	−3.6 (ML)
PA3477[c]	*rhlR*	Regulatory protein RhlR	5.9 (ES)
Genes expressed in biofilms			
PA0105-PA0108		Cytochrome *c* oxidases	5.7 (ES)
PA0713		Hypothetical protein	−2.4 (ES)
PA0714		Hypothetical protein	−3.4 (ES)
PA0722		Hypothetical protein of bacteriophage Pf1	−1.2 (ES)
PA0971	*tolA*	TolA protein	−1.4 (ES)
PA3038		Probable porin	−2.3 (ES)
PA3234		Probable sodium:solute symporter	−2.8 (ES)
PA3235		Conserved hypothetical protein	−2.8 (ES)
PA3418	*ldh*	Leucine dehydrogenase	2.0 (ES)
PA3622[c]	*rpoS*	Stationary-phase sigma protein RpoS	2.7 (B)
PA4296		Probable two-component response regulator	3.4 (ES)
PA4765	*omlA*	Outer membrane lipoprotein OmlA precursor	−1.6 (ES)

[a] Genes are identified by ORF designation, gene name, and protein description (http://www.pseudomonas.com). LPS, lipopolysaccharide; RND, resistance-nodulation-cell division.

[b] The magnitude and direction of gene expression change, as well as the growth phase (ML,mid-logarithmic phase; ES, early stationary phase; B, both mid-logarithmic and early stationary phases), are indicated for PAO-JP2 grown with exogenous autoinducers (1 μM 3O-C_{12}-HSL and 2 μM C_4-HSL) compared to PAO-JP2 cultures grown aerobically in modified FAB.

[c] Gene previously identified as being QS regulated (4, 8, 18, 27–29, 38, 39, 44).

TABLE 5. QS-regulated transcripts detected at the early stationary phase in PAO1 grown under various nutrient and oxygen conditions[a]

ORF or intergenic region	Gene name	Presence in[b]:			Protein description[c]
		NY, aerobic	NY + KNO_3 aerobic	NY + KNO_3, anaerobic	
PA0007		+	−	−	Hypothetical protein
PA0040		+	−	+	Conserved hypothetical protein
PA0103		+	−	−	Probable sulfate transporter
PA0105	*coxB*	+	+	−	Cytochrome *c* oxidase subunit II
PA0107		−	−	−	Conserved hypothetical protein
PA0108	*coxIII*	+	−	−	Cytochrome *c* oxidase subunit III
PA0111		+	−	−	Hypothetical protein
PA0144		−	−	−	Hypothetical protein
PA0196	*pntB*	+	−	+	Pyridine nucleotide transhydrogenase beta subunit
PA0228	*pcaF*	−	−	−	Beta-ketoadipyl-coenzyme A thiolase PcaF
PA0239		+	−	−	Hypothetical protein
PA0244		+	−	−	Hypothetical protein
PA0365		+	+	−	Hypothetical protein
PA0524	*norB*	−	+	+	Nitric oxide reductase subunit B
PA0996		+	+	−	Probable coenzyme A ligase
PA0997		+	+	−	Hypothetical protein
PA0998		+	+	−	Hypothetical protein
PA1063		+	−	+	Hypothetical protein
PA1212		+	−	−	Probable MFS transporter
PA1214		+	−	−	Hypothetical protein
PA1217		+	−	−	Probable 2-isopropylmalate synthase
PA1221		+	−	+	Hypothetical protein
PA1318	*cyoB*	+	−	−	Cytochrome *o* ubiquinol oxidase subunit 1
PA1327		+	+	−	Probable protease
PA1356		−	−	−	Hypothetical protein
PA1608		+	+	−	Probable chemotaxis transducer
PA1679		−	+	+	Hypothetical protein
PA1694	*pscQ*	+	−	−	Translocation protein in type III secretion
PA1771		+	−	−	Probable esterase/lipase
PA1869		+	+	−	Probable acyl carrier protein
PA1875		+	−	−	Hypothetical protein
PA1877		−	−	−	Probable secretion protein
PA1892		+	+	−	Hypothetical protein
PA1893		+	−	−	Hypothetical protein
PA1894		+	+	−	Hypothetical protein
PA1895		+	+	−	Hypothetical protein
PA1896		−	−	−	Hypothetical protein
PA1897		+	+	−	Hypothetical protein
PA1898		+	+	−	Probable transcriptional regulator
PA1901		+	+	−	Phenazine biosynthesis protein PhzC
PA1902		+	−	−	Phenazine biosynthesis protein PhzD
PA1903		+	+	−	Phenazine biosynthesis protein PhzE
PA1904		+	+	−	Probable phenazine biosynthesis protein
PA1927	*metE*	−	−	+	5-Methyltetrahydropteroyltriglutamate-homocysteine *S*-methyltransferase
PA1976		+	−	−	Probable two-component sensor
PA1978		+	+	−	Probable transcriptional regulator
PA1983	*exaB*	−	−	−	Cytochrome c_{550}
PA1992		−	−	−	Probable two-component sensor
PA2068		+	−	−	Probable MFS transporter
PA2081		+	−	−	Hypothetical protein
PA2190		+	+	−	Conserved hypothetical protein
PA2194	*hcnB*	+	+	−	Hydrogen cyanide synthase HcnB
PA2196		+	+	−	Probable transcriptional regulator
PA2238		+	+	−	Hypothetical protein
PA2260		+	−	+	Hypothetical protein
PA2261		+	−	−	Probable 2-ketogluconate kinase
PA2262		+	−	−	Probable 2-ketogluconate transporter
PA2266		+	−	−	Probable cytochrome *c* precursor
PA2300	*chiC*	+	+	−	Chitinase
PA2303		+	+	−	Hypothetical protein
PA2331		+	+	−	Hypothetical protein
PA2369		+	−	−	Hypothetical protein
PA2414		+	+	−	L-Sorbosone dehydrogenase
PA2415		+	+	−	Hypothetical protein
PA2454		+	−	+	Hypothetical protein
PA2539		+	−	−	Conserved hypothetical protein
PA2540		+	−	+	Conserved hypothetical protein
PA2564		+	−	−	Hypothetical protein
PA2566		+	−	−	Conserved hypothetical protein

Continued on following page

TABLE 5—*Continued*

ORF or intergenic region	Gene name	Presence in[b]: NY, aerobic	NY + KNO_3 aerobic	NY + KNO_3, anaerobic	Protein description[c]
PA2570	*palL*	+	+	−	PA-I galactophilic lectin
PA2571		+	+	−	Probable two-component sensor
PA2572		+	+	−	Probable two-component response regulator
PA2588		+	+	−	Probable transcriptional regulator
PA2699		−	+	−	Hypothetical protein
PA2763		+	−	−	Hypothetical protein
PA2862	*lipA*	+	+	−	Lactonizing lipase precursor
PA2903	*cobJ*	+	−	+	Precorrin-3 methylase CobJ
PA2939		+	+	−	Probable aminopeptidase
PA2974		+	+	−	Probable hydrolase
PA3242		+	+	−	Probable lauroyl acyltransferase
PA3311		+	−	−	Conserved hypothetical protein
PA3328		+	+	−	Probable FAD-dependent monooxygenase
PA3329		+	+	−	Hypothetical protein
PA3330		+	+	−	Probable short-chain dehydrogenase
PA3332		+	+	−	Conserved hypothetical protein
PA3333	*fabH2*	+	+	−	3-Oxoacyl-(acyl carrier protein) synthase III
PA3334		+	+	−	Probable acyl carrier protein
PA3335		+	+	−	Hypothetical protein
PA3336		+	+	−	Probable MFS transporter
PA3391	*nosR*	−	+	+	Regulatory protein NosR
PA3394	*nosF*	−	+	+	NosF protein
PA3395	*nosY*	−	+	+	NosY protein
PA3478	*rhlB*	+	+	−	Rhamnosyltransferase chain B
PA3479	*rhlA*	+	+	−	Rhamnosyltransferase chain A
PA3519		+	+	−	Hypothetical protein
PA3709		−	−	−	Probable MFS transporter
PA3719		+	+	−	Hypothetical protein
PA3870	*moaAI*	−	+	+	Molybdopterin biosynthetic protein A1
PA3877	*narKI*	−	+	+	Nitrite extrusion protein 1
PA3914	*moeAI*	−	+	+	Molybdenum cofactor biosynthetic protein A1
PA4078		+	+	−	Probable nonribosomal peptide synthetase
PA4142		+	−	−	Probable secretion protein
PA4175		+	−	−	Probable endoproteinase Arg-C precursor
PA4209		+	+	−	Probable *O*-methyltransferase
PA4211		+	+	−	Probable phenazine biosynthesis protein
PA4293		+	−	−	Probable two-component sensor
PA4297		−	−	−	Hypothetical protein
PA4298		+	+	−	Hypothetical protein
PA4300		+	−	+	Hypothetical protein
PA4302		−	−	−	Probable type II secretion system protein
PA4303		+	+	−	Hypothetical protein
PA4304		+	−	−	Probable type II secretion system protein
PA4305		+	−	−	Hypothetical protein
PA4307	*pctC*	+	−	−	Chemotactic transducer PctC
PA4384		+	−	−	Hypothetical protein
PA4498		+	−	+	Probable metallopeptidase
PA4648		+	−	−	Hypothetical protein
PA4649		+	−	−	Hypothetical protein
PA4651		+	+	−	Probable pilus assembly chaperone
PA4681		+	−	+	Hypothetical protein
PA4908		+	+	−	Hypothetical protein
PA4910		+	+	−	Probable ATP-binding component of ABC transporter
PA4911		−	−	−	Probable permease of ABC branched-chain amino acid transporter
PA4912		+	−	−	Probable permease of ABC branched-chain amino acid transporter
PA4979		−	−	−	Probable acyl-coenzyme A dehydrogenase
PA5059		+	+	−	Probable transcriptional regulator
PA5097		−	−	−	Probable amino acid permease
PA5181		+	−	+	Probable oxidoreductase
PA5375	*betTI*	+	−	−	Choline transporter BetT
PA5411		+	−	+	Probable ferredoxin
PA5543		+	−	+	Hypothetical protein
ig 1427453-1428080		+	−	−	Intergenic region between PA1372 and PA1373, 1427453-1428080, (+) strand
ig 4713795-4713098		+	+	−	Intergenic region between PA4280 and PA4281, 4713098-4713795, (−) strand
ig 5820909-5820113		+	−	+	Intergenic region between PA5321 and PA5322, 5820113-5820909, (−) strand

[a] Genes are identified by ORF designation, gene name, and protein description (http://www.pseudomonas.com).

[b] The presence of each transcript was analyzed by using MAS (version 5.0) and DMT (version 3.0) software for duplicate samples. Transcripts identified as absent on at least two of three chips were considered absent. +, present; −, absent.

[c] FAD, flavin adenine dinucleotide.

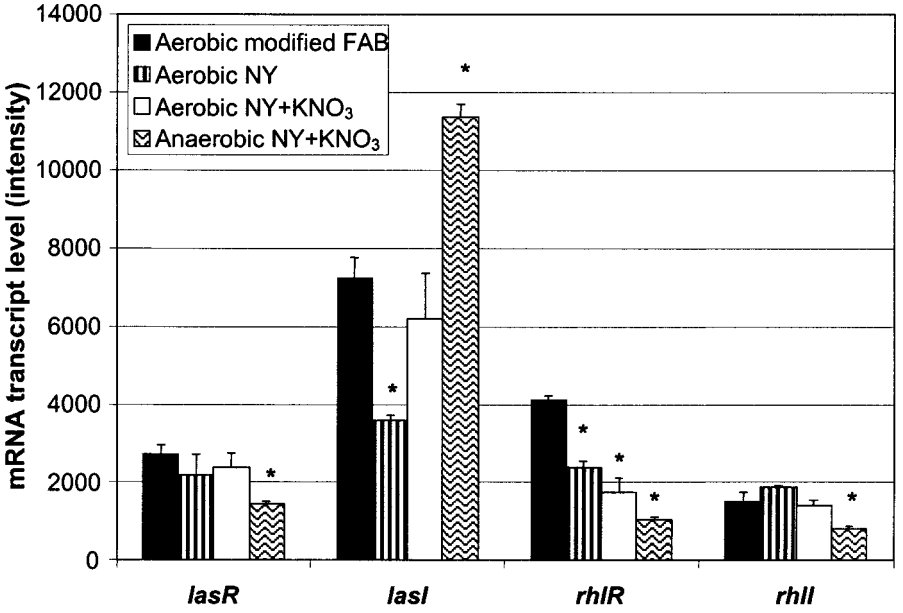

FIG. 4. Effect of media and oxygen conditions on *lasR*, *lasI*, *rhlR*, and *rhlI* expression. The levels of transcript expression for mRNA encoding the known key elements of the *las* and *rhl* regulatory system are shown for PAO1 grown to the early stationary phase. Statistically significant differences between the transcript levels for PAO1 grown in modified FAB and the transcript levels for PAO1 grown under other conditions are indicated by an asterisk ($P \leq 0.05$, as determined by one-way analysis of variance with the Tukey post-hoc test).

from three individual cultures of PAO1 grown aerobically in modified FAB, NY, or NY containing KNO_3 expressed averages of 66, 86, and 77% of the ORFs, respectively, at the mid-logarithmic and early stationary phases. PAO1 grown anaerobically in NY containing KNO_3 expressed an average of 68% of the total ORFs at the mid-logarithmic and early stationary phases. These values are similar to those obtained in this study with PAO-JP2 cultures, as well as to those previously reported for PAO1 grown in the presence of low or high iron concentrations (25). The presence of QS-regulated gene transcripts was determined at the early stationary phase in PAO1 when it was grown either aerobically in modified FAB, NY, or NY containing KNO_3 or anaerobically in NY containing KNO_3 (Table 5). As expected, all 616 genes found to be QS regulated by using the PAO-JP2 mutant grown in modified FAB were expressed in PAO1 grown aerobically in modified FAB (data not shown). However, growth in a rich medium (NY) eliminated the expression of 24 QS-regulated genes (4% of the QS-regulated genes). Aerobic growth of PAO1 in NY containing KNO_3 reduced the number of detectable QS-regulated gene transcripts by 11% (65 of 616 genes), and anaerobic growth in NY containing KNO_3 eliminated detectable transcripts for 18% of the QS-regulated genes identified by using the microarrays (110 of 616 genes). These observations suggest that there may be more than 616 QS-regulated genes that could be identified by using different experimental conditions. Likewise, other studies in which different growth conditions are used may not identify all of the genes that we found to be QS regulated using modified FAB. For example, medium components (KNO_3 or glucose) that specifically induce expression of certain genes, such as the *nar* genes and the glucose-inducible *oprB* gene, preclude detection of these QS-regulated genes in studies in which these constituents are absent from the medium (33, 49). Interestingly, we observed that three of the previously reported QS-regulated genes for which we did not detect transcripts (PA2401, PA2402, and PA2424 [44]) in modified FAB supplemented with nitrate were detected in medium without nitrate (NY). This most likely explains why we failed to detect these genes as QS-regulated genes in our experiments, since in our study QS-regulated genes were identified exclusively in modified FAB supplemented with nitrate.

Variable responses in the expression of QS-regulated genes to media and environments may be due to alterations in the expression of QS regulators themselves. Thus, we examined the absolute mRNA transcript abundance of *lasR*, *lasI*, *rhlR*, and *rhlI* in PAO1 under different growth conditions (Fig. 4). The levels of mRNA transcript for *lasR* at the early stationary phase in modified FAB did not differ significantly from the levels observed during aerobic cultivation in NY or NY containing KNO_3, but the level was significantly decreased (46%) when PAO1 was grown anaerobically in NY containing KNO_3 (Fig. 4). Of the four regulatory genes, *rhlR* was the most sensitive to media and oxygen conditions. The level of the *rhlR* transcript in PAO1 grown to the early stationary phase decreased by 42% when the organism was grown in NY aerobically, by 57% when it was cultivated aerobically in NY containing KNO_3, and by 75% when it was grown in anaerobic cultures in NY containing KNO_3 compared to the levels in samples obtained at the early stationary phase from cells cultivated aerobically in modified FAB. The large decrease (75%) in *rhlR* transcript abundance in anaerobic cultures may account for the absence of several transcripts whose genes are known to be primarily *rhl* regulated, such as *rhlAB* and *pa1L* (28, 46). No significant difference in *lasI* transcript level was observed between PAO1 grown aerobically in NY containing KNO_3 and PAO1 grown in modified FAB. However, growth in rich me-

dium (NY) decreased the expression of the *lasI* transcript by 50%, while anaerobic growth in NY containing KNO_3 increased the level of the *lasI* transcript by 57% compared to the level after growth in modified FAB. Interestingly, a statistically significant decrease in expression of PA1898 (*qscR*) ($P < 0.01$, as determined by one-way analysis of variance with the Tukey post-hoc test) (data not shown), a purported negative regulator of *lasI* (5), was also observed at the early stationary phase for PAO1 cultivated anaerobically in NY containing KNO_3 compared with the expression in PAO1 grown aerobically in modified FAB, NY, and NY containing KNO_3. QscR is believed to inhibit a premature increase in 3O-C_{12}-HSL production, thereby preventing activation of the QS modulon at low cell densities. The decrease in *qscR* expression may have caused the concomitant increase in *lasI* expression observed in PAO1 at the early stationary phase when the organism was grown anaerobically.

Conclusions. The *P. aeruginosa* DNA microarrays permitted a glimpse into gene regulation mediated by the *las* and/or *rhl* QS modulons. The large percentage of the genome (>10%) identified in this study as being QS regulated reflects the global level at which QS influences cellular behavior. Many novel activated genes (394 genes), as well as 222 repressed genes, were discovered. To our knowledge, no QS-repressed genes have been reported previously for *P. aeruginosa*. The high correlation between the QS-regulated genes identified in this study and those previously identified by alternate approaches provided a measure of confidence for both the *P. aeruginosa* microarray and data analysis methods and demonstrated the potential of this technology for investigating other regulons or modulons in *P. aeruginosa*. This conclusion was further supported by independent validation with QRT-PCR. Numerous novel genes identified as being QS regulated are themselves known or putative transcriptional regulators, and we are currently investigating their position in the QS hierarchy. The important effect of the bacterial environment on expression and detection of QS-regulated genes was highlighted by the study of PAO1 cultivated aerobically and anaerobically in various media, and the results underscore the importance of such variables when results from different laboratories are compared. This effect may account for conflicting reports concerning QS regulation of genes, such as *rpoS*, or concerning group behavior, such as biofilm formation (20). The variation in expression of QS-regulated genes dependent upon culture conditions emphasizes the need to clearly understand and mimic the environment that *P. aeruginosa* encounters in vivo when therapies are designed. The large number of QS-regulated genes that encode known or probable virulence genes identified in this study further emphasizes the role of QS in *P. aeruginosa* pathogenesis.

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