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# GidA Posttranscriptionally Regulates *rhl* Quorum Sensing in *Pseudomonas aeruginosa*▽†

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**The opportunistic pathogen *Pseudomonas aeruginosa* utilizes two interconnected acyl-homoserine lactone quorum-sensing (acyl-HSL QS) systems, LasRI and RhlRI, to regulate the expression of hundreds of genes. The QS circuitry itself is integrated into a complex network of regulation by other factors. However, our understanding of this network is still unlikely to be complete, as a comprehensive, saturating approach to identifying regulatory components has never been attempted. Here, we utilized a nonredundant *P. aeruginosa* PA14 transposon library to identify additional genes that regulate QS at the level of LasRI/RhlRI. We initially screened all 5,459 mutants for loss of function in one QS-controlled trait (skim milk proteolysis) and then rescreened attenuated candidates for defects in other QS phenotypes (LasA protease, rhamnolipid, and pyocyanin production) to exclude mutants defective in functions other than QS. We identified several known and novel genes, but only two novel genes, *gidA* and *pcnB*, affected all of the traits assayed. We characterized *gidA*, which exhibited the most striking QS phenotypes, further. This gene is predicted to encode a conserved flavin adenine dinucleotide-binding protein involved in tRNA modification. Inactivation of the gene primarily affected *rhlR*-dependent QS phenotypes such as LasA, pyocyanin, and rhamnolipid production. GidA affected RhlR protein but not transcript levels and also had no impact on LasR and acyl-HSL production. Overexpression of *rhlR* in a *gidA* mutant partially restored QS-dependent phenotypes. Taken together, these results indicate that GidA selectively controls QS gene expression posttranscriptionally via RhlR-dependent and -independent pathways.**

*Pseudomonas aeruginosa* is a ubiquitous environmental bacterium commonly found in soil and freshwater. It is also an opportunistic human pathogen that infects immunocompromised individuals, including those suffering from cystic fibrosis. It utilizes a quorum-sensing (QS) mechanism to regulate and coordinate virulence gene expression (2, 11, 37, 46). There are two complete acyl-homoserine lactone (acyl-HSL) QS systems in *P. aeruginosa*, the LasR-LasI (*las*) system and the RhlR-RhlI (*rhl*) system. LasI and RhlI produce the signals 3-oxo-dodecanoyl ($3OC_{12}$)-HSL and butanoyl ($C_4$)-HSL, respectively; these signals bind to and activate their cognate transcriptional regulators, LasR and RhlR. There is a third, orphan regulator, QscR, that also responds to $3OC_{12}$-HSL (24). Under standard growth conditions, the *las* system activates the *rhl* system (23, 36), and the two systems together control the expression of hundreds of genes (12, 42, 51). A large portion of these QS-controlled genes encode secreted virulence factors such as proteases (LasA protease, LasB elastase, and alkaline protease), biosurfactants (rhamnolipid), and secondary metabolites (hydrogen cyanide and pyocyanin).

*P. aeruginosa* QS is embedded in a complex network of global regulation (18, 41, 49). Most QS-controlled genes are not induced until the stationary phase of growth even when exogenous acyl-HSL signals are present early in growth (8, 42, 53, 54). Thus, additional factors are required for expression of these QS-controlled genes. Several global regulators and regulatory pathways (e.g., Vfr, GacA/GacS, RsaL, RelA, RpoS, and PqsR [MvfR]/PQS) have already been identified (18, 41). Although much remains to be learned about this complex network of global regulation, it is clear that control of QS is multifaceted and will require an integrative approach to understanding its complexity.

In this study, we employed a saturation mutagenesis approach, making use of a recently constructed nonredundant transposon mutant library of *P. aeruginosa* strain PA14 (25), to identify additional regulators of QS gene expression. We characterized one such novel regulator, GidA, which shares homology with conserved flavin adenine dinucleotide-binding proteins involved in tRNA modification (27, 55). Initially isolated as a factor involved in a glucose-inhibited cell division phenotype in *Escherichia coli* (50), more recent studies have established that GidA, together with MnmE, is responsible for the addition of a carboxymethylaminomethyl group to uridine 34 of a subset of tRNAs (55). This process is important for the appropriate decoding of two-family box triplet codons. Despite the potential to indiscriminately affect cellular gene expression, GidA homologs rather specifically regulate virulence gene expression in *Aeromonas hydrophila* (45), *Pseudomonas syringae* (21), *Shigella flexneri* (9), and *Streptococcus pyogenes* (5), with little to no impact on growth. In *Myxococcus xanthus*, GidA is involved in fruiting-body development (52). Our data show that GidA specifically controls *rhl*-dependent QS gene expression in *P. aeruginosa* by modulating RhlR expression posttranscriptionally.

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TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Relevant genotype or phenotype | Reference or source |
|---|---|---|
| Strains | | |
| *P. aeruginosa* | | |
| PA14 | Wild type | 25 |
| PA14 *gidA*7 | MAR2 × T7 *gidA* mutant derived from PA14 (mutant 44643) | 25 |
| PA14 *lasR* | Tn*phoA lasR* mutant derived from PA14 | 47 |
| PA14 *rhlR* | MAR2 × T7 *rhlR* mutant derived from PA14 (mutant 37943) | 25 |
| PA14_73400 | MAR2 × T7 *mnmE* mutant derived from PA14 (mutant 38726) | 25 |
| Additional MAR2 × T7 mutants | See Table S1 in the supplemental material and http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi | 25 |
| PA14 Δ*gidA* | Markerless in-frame deletion mutant derived from PA14 | This study |
| *E. coli* DH5α | $F^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17* ($r_K^-$ $m_K^+$) *phoA supE44* $\lambda^-$ *thi-1 gyrA96 relA1* | Invitrogen |
| Plasmids | | |
| pCF430 | Broad-host-range vector containing *araC-*$P_{BAD}$ promoter (tetracycline resistant) | 28 |
| pCF430G | *gidA* open reading frame and ribosome-binding site of PA14 in HindIII/XbaI of pCF430 | This study |
| pCF430R | NheI/SacI fragment containing *rhlR* open reading frame and ribosome-binding site of PAO1 subcloned from pJN105.*rhlR* | This study |
| pEX19Tc | Gene replacement vector with *sacB* counterselectable marker (tetracycline resistant) | 15 |
| pEX19Tc.Δ*gidA* | pEX19Tc containing *gidA* with a markerless in-frame deletion | This study |

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The strains used in this study are listed in Table 1. The MAR2 × T7 mutants used in the study were taken from a subset of the parental PA14 mariner transposon insertion library (25). The mutants were handled and stored as described in the library's user manual (25). Strains were initially plated from frozen stocks onto Luria-Bertani (LB) agar plates and grown for 24 h at 37°C. Unless noted otherwise, *P. aeruginosa* liquid cultures were grown at 37°C with agitation (250 rpm) in Lennox LB broth buffered with 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.0. *Escherichia coli* DH5α (Invitrogen, CA) was cultured in LB at 37°C. Where appropriate, the antibiotics gentamicin and tetracycline (10 μg/ml for *E. coli* and 100 μg/ml for *P. aeruginosa*) were added to the growth media.

For high-throughput assays, colonies grown on rectangular LB plates in a 12-by-8 format were replicate plated using a 96-pin replicator. Subsequent assays were either plate based or liquid based (see below). For the latter, 600 μl of LB-MOPS in 96-deep-well titer blocks were inoculated from plates and grown for 18 h. For liquid culture assays performed with few individual strains, including assays for LasA protease activity, pyocyanin production, Western blotting, $3OC_{12}$-HSL and $C_4$-HSL quantitation, and real-time PCR, experimental *P. aeruginosa* cultures were started by inoculating 25 ml LB-MOPS in 250-ml Erlenmeyer flasks with mid-logarithmic-phase bacteria to an optical density at 600 nm ($OD_{600}$) of 0.02. Culture aliquots were harvested after 7 to 8 h of growth (stationary phase). For real-time PCR analysis, culture aliquots were also harvested after 4 to 5 h of growth (transition from logarithmic to stationary phase). The culture supernatants were filter sterilized (pore size, 0.2 μm) for individual phenotypic assays.

For complementation analysis, LB-MOPS medium containing L-arabinose (50 mM final concentration) and tetracycline was inoculated with a colony from a freshly streaked culture. The cultures were grown for 24 h (stationary phase) prior to analysis.

**DNA manipulations.** General molecular cloning techniques were based on standard protocols (38). Chromosomal DNA was isolated using the Puregene Core Kit A (Gentra Systems). Plasmids used for complementation were constructed as follows. A 1,893-bp DNA fragment containing the *gidA* coding sequence was amplified from PA14 chromosomal DNA using primers 5′-$N_6$AAGCTTCGCTAAATCCTTATACTGTCCG-3′ and 5′-$N_6$TCTAGAGGTGTTGGGTTACCGCAGAC-3′. The fragment was then digested with HindIII and XbaI (the respective restriction enzyme recognition sites are underlined) and ligated to HindIII/XbaI-digested pCF430 to generate pCF430G (Table 1). Proper construction was verified by DNA sequencing. Plasmid pCF430R (Table 1) was generated by subcloning a 700-bp NheI/SacI fragment from pJN105.*rhlR* (40).

The location of the transposon (MAR2 × T7) insertion in the PA14 *gidA* mutant was confirmed by PCR and subsequent agarose gel electrophoresis. Primers were designed based on the presumed location of the transposon 1,716 bp downstream of the *gidA* translational start (25). Primers 5′-CATTACAGTTTACGAACCGAACAG-3′ (forward) and 5′-GCAAGGCAAGGACAGCTGGTG-3′ (reverse) amplified a fragment of the appropriate size (661 bp in length; 179 bp of MAR2 × T7 and 482 bp of *gidA*).

**Construction of a *gidA* deletion mutant.** The gene deletion strategy of Hoang et al. was used to construct a markerless *gidA* deletion mutant of *P. aeruginosa* strain PA14 (15, 44). Splicing by overlap extension-PCR was used to construct a 981-bp in-frame deletion in *gidA*. The resulting protein contains the first 14 amino acids fused in frame with the last 289 amino acids. The following primers were used (sequences in the 5′ to 3′ direction): primer 1, $N_6$TCTAGAACATGGGATGGATATCCACCTGG; primer 2, ACCGCCGATCACGATTACGTC; primer 3, GACGTAATCGTGATCGGCGGTGCGATCGAGTACGACTTCTTC; and primer 4, $N_6$AAGCTTCCATTTGATCAGCAGGGCCAAG. Primers 1 and 4 are flanking primers containing engineered XbaI and HindIII restriction sites, respectively (underlined), and primers 2 and 3 are partially overlapping, internal primers to create the in-frame deletion. The fusion product was digested with XbaI and HindIII and ligated to the equally digested allelic exchange plasmid pEX19Tc (15). The resulting construct, pEX19Tc.Δ*gidA*, was sequenced prior to transformation into *E. coli* SM10. Mating with *P. aeruginosa* PA14 and appropriate selection steps yielded a *gidA* deletion mutant. Proper construction was confirmed by PCR amplification of chromosomal DNA.

**Growth curves.** Growth analysis of each strain listed in Table S1 in the supplemental material was conducted simultaneously in a 96-well format using a microplate reader (Infinite M200; Tecan). Strains were grown at 37°C with shaking in 200 μl of LB-MOPS medium and sealed with 50 μl of mineral oil. The $OD_{600}$ was determined every 15 min for 8 h, and the doubling time in exponential phase for each strain was calculated with the respective data. The presence of mineral oil did not restrict growth during logarithmic phase (data not shown). Growth of PA14 *gidA*::MAR2 × T7 and the PA14 wild-type parent was also quantified in standard culture flasks. Twenty-five milliliters of LB-MOPS medium (in 250-ml flasks) was inoculated with mid-logarithmic-phase cells to an $OD_{600}$ of 0.02 and incubated at 37°C with shaking (250 rpm). The $OD_{600}$ was measured every hour for 8 h.

**Skim milk proteolysis, rhamnolipid production, and adenosine utilization.** Skim milk proteolysis (39), rhamnolipid production (22) and adenosine utilization (13, 39) were determined through the use of agar plate assays. Isolated colonies, freshly grown on LB plates, were patched individually with a toothpick or replica plated onto the respective agar test plates. Rhamnolipid plates were incubated at 37°C for 24 h, followed by 24 h at 4°C or until a blue halo appeared. Relative rhamnolipid production was determined by the size of the blue halo around each colony. Skim milk plates contained not only 4% (wt/vol) skim milk but also one-quarter-strength LB. The addition of LB allowed QS-deficient mutants to grow as well as the wild-type parent.

**Pyocyanin production and LasA protease activity.** Pyocyanin production was measured in two different ways. For high-throughput analysis, 200 μl of culture supernatant was transferred to a 96-well microtiter plate and the OD was determined at 310 nm with a microplate reader (31). For accurate quantitation of QS-dependent products from individual cultures grown in flasks, pyocyanin was

extracted from liquid culture supernatant and absorbance was read at 520 nm as described previously (10). LasA protease activity was determined by measuring the rate of *Staphylococcus aureus* cell lysis by *P. aeruginosa* culture supernatant (decrease in $OD_{600}$ per minute) (8, 20). Twenty microliters of culture supernatant was transferred to microtiter plates containing 180 μl of boiled *S. aureus* suspension ($OD_{600}$ of 0.8) buffered with 10 mM $K_2HPO_4$ (pH 7.5). $OD_{600}$ readings were taken in a microplate reader every 4 min for 1 h.

**Assays for $3OC_{12}$-HSL and $C_4$-HSL.** Two milliliters of ethyl acetate-extracted *P. aeruginosa* culture supernatant was used to measure $3OC_{12}$-HSL and $C_4$-HSL levels with *E. coli* bioassays as previously described (32–34). Synthetic $3OC_{12}$-HSL and $C_4$-HSL were used to generate standard curves.

**Western blot analysis.** Western blotting was performed as described previously (40). Culture aliquots were harvested by centrifugation. Cell pellets were resuspended in LasR protein buffer and sonicated. The resulting lysates were centrifuged to remove insoluble material. Protein concentrations in the soluble fraction were determined by Bradford assay. Equal amounts of protein were separated by 12.5% polyacrylamide gel electrophoresis. The separated proteins were blotted onto a nylon membrane and probed with polyclonal anti-LasR and anti-RhlR rabbit antibodies as described previously (40).

**Complementation and suppression analysis.** Complementation and suppression of the PA14 *gidA* transposon mutant was carried out with the low-copy-number plasmids pCF430G and pCF430R, which express *gidA* and *rhlR* from the arabinose-inducible *araBAD* promoter. L-Arabinose was added to the cultures at a concentration of 50 mM. The *rhlR* allele was from *P. aeruginosa* PAO1 rather than PA14. Both alleles differ by only a single silent mutation at position 717; however, the affected codon is not in a mixed-codon family box and is therefore not recognized by a GidA-modified tRNA.

**Real-time PCR.** Total RNA isolation and cDNA synthesis with semirandom primers were performed as described previously (42). RNA quality was determined using an Agilent Bioanalyzer 2100. Real-time PCR was performed as described previously (40) with a 7300 real-time PCR system (Applied Biosystems) using specific primers for *rhlA* and *rhlR*, 1 ng of purified cDNA as measured by Nanodrop (Thermo Scientific), and Power SYBR green PCR master mix reagents (Applied Biosystems). The primers were designed using Primer Express software (Applied Biosystems). Serially diluted genomic DNA was used as the template to obtain a relative standard curve. Instead of normalizing transcript levels to those of a calibrator gene, as is commonly done in one-step real-time reverse transcription-PCRs, we chose a two-step reaction and calibrated the amount of input cDNA prior to PCR (39). Thus, data are normalized to the total amount of cDNA.

## RESULTS

**High-throughput screening for QS regulators identifies *gidA*.** We used a *P. aeruginosa* PA14 nonredundant transposon insertion library (25) to screen for mutants deficient in QS-dependent phenotypes. The library is a collection of 5,459 mutant strains where virtually every strain has a unique gene disabled by transposon insertion. The library allowed us to perform a comprehensive genome-wide search for genes that regulate QS in *P. aeruginosa*. To identify global regulators that affect multiple QS-regulated traits, we employed a two-step screening procedure. Screen 1 involved screening of all mutants for defects in skim milk proteolysis. Screen 2 involved screening the proteolysis-deficient mutants identified in screen 1 for additional QS-controlled phenotypes, namely, staphylolytic (LasA) activity, rhamnolipid production, and pyocyanin production. This second, more focused screen allowed us to eliminate mutants from screen 1 that are defective in processes other than QS, such as protease production or protease secretion. The different QS phenotypes assayed are dependent on the LasRI and/or RhlRI systems to various degrees (see below).

Screen 1 identified 59 mutants with reduced skim milk proteolysis (see Table S1 in the supplemental material). This number excludes mutants with severely reduced growth on skim milk plates and mutants whose doubling time in liquid LB culture, assayed in microtiter plate format, was >30% of that of the PA14 wild type. We rediscovered several genes known to control QS in *P. aeruginosa* PAO1. These were *vfr*, *gacA*, *rpoN*, *dksA*, in addition to the QS core genes *lasRI* and *rhlRI*. Vfr is a catabolite repressor homolog that directly induces *lasR* transcription (1). GacA is part of a regulatory pathway that controls QS posttranscriptionally (14, 19). RpoN is an alternative sigma factor that regulates *rhlI* expression (48), and DksA also controls expression of several QS-dependent phenotypes posttranscriptionally (17). Several other known QS regulatory genes were not rediscovered (see Table S1 in the supplemental material) but were nevertheless included in the second screen for comparison. Screen 2 revealed that many of the 59 mutants were also deficient in additional QS-controlled phenotypes (see Table S1 in the supplemental material). However, only three mutants were at least partially deficient in all of the QS phenotypes tested (several others were defective in skim milk proteolysis, LasA activity, and pyocyanin production but did not grow on rhamnolipid minimal medium, suggesting a QS-independent pleiotrophic defect such as amino acid auxotrophy). The three mutants harbor transposon insertions in *vfr* and in two genes not previously known to be involved in QS regulation, *pcnB* and *gidA*. *pcnB* encodes poly(A) polymerase, which is involved in destabilization of mRNA in bacteria, a property that in some cases can directly regulate gene expression (16). In this study, we chose the *gidA* mutant for further characterization as it exhibited the most compelling QS phenotypes, being completely deficient in three of the four traits assayed. We confirmed the location of the transposon insertion in the *gidA* mutant by PCR as described in Materials and Methods.

Previous work indicated a role for *gidA* in virulence gene regulation in several bacterial pathogens (5, 9, 21, 45). A recent mechanistic study demonstrated its involvement in tRNA modification in association with *mnmE* in *E. coli* (4). To investigate the contribution of *mnmE* to the observed QS phenotypes in *P. aeruginosa*, we attempted to characterize a PA14 mutant with an insertion in a gene that is 67% identical to *E. coli mnmE*. However, this mutant exhibited severe growth defects in all of the standard media employed, thus not allowing us to investigate QS-specific deficiencies.

**A *gidA* mutant is defective primarily in *rhl*-dependent QS.** As mentioned above, high-throughput screening revealed that *gidA* causes defects in several QS-dependent phenotypes. To confirm and extend the results from high-throughput screening, we repeated assays for the *gidA* mutant and compared the results to those for the wild type, a *lasR* mutant, and a *rhlR* mutant. We also included a new assay, growth on adenosine, to better determine the relative contributions of *las* and *rhl* QS systems in *gidA*-mediated regulation. Utilization of adenosine as the sole carbon source requires expression of nucleoside hydrolase (Nuh), which is dependent solely on the *las* system (13, 39). We found no adenosine utilization defect in the *gidA* mutant (Fig. 1A), indicating that *las* QS is not affected by *gidA*. Consistent with the high-throughput data, the *gidA* mutant also had only a modest defect in skim milk proteolysis (Fig. 1C). Skim milk proteolysis is primarily *las* dependent (involving *las*-specific alkaline protease and *las*- and *rhl*-dependent LasB elastase), because a *rhlR* mutant exhibited only a slight proteolytic deficiency. However, the *gidA* mutant was more strongly affected in two different *rhl*-specific QS phenotypes,

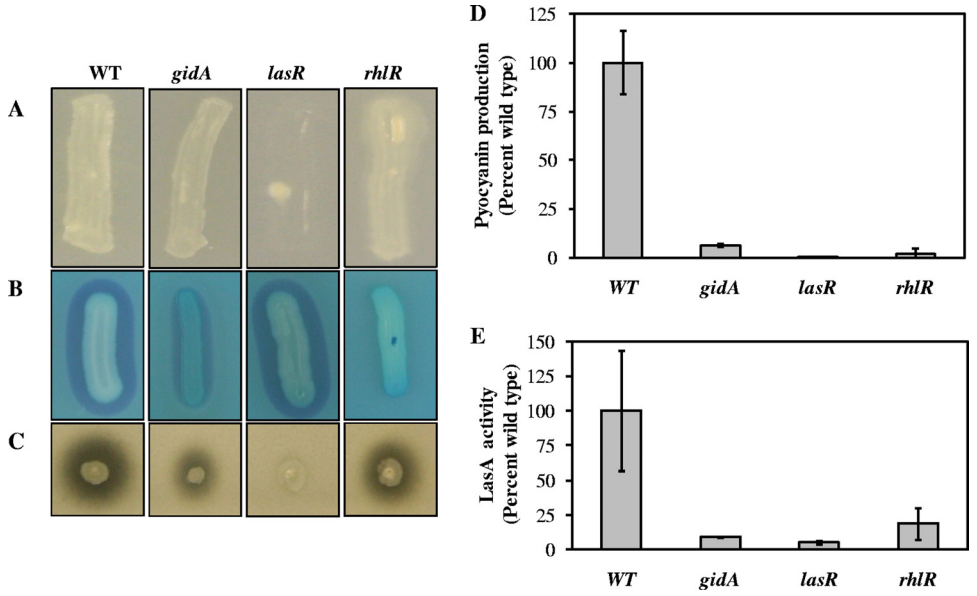


FIG. 1. QS-dependent phenotypes of a *P. aeruginosa* PA14 *gidA* mutant. Assays were performed with the wild type (WT) and the *gidA*, *lasR*, and *rhlR* mutants. (A) Growth on adenosine. (B) Rhamnolipid production. The presence of a blue halo surrounding the bacterial streak indicates rhamnolipid production. (C) Skim milk proteolysis. Zones of clearance surrounding the bacterial streak indicate proteolytic activity. (D) Pyocyanin production, expressed as percentages of the wild-type level. (E) LasA activity, expressed as percentages of the wild-type level. Each experiment was performed at least three times. Plate images depict representative experiments. Values shown in graphs are means from three independent biological experiments, normalized to culture density ($OD_{600}$). Error bars indicate ±1 standard deviation.

rhamnolipid and pyocyanin production (Fig. 1B and D). LasA protease expression, which depends on both *las* and *rhl* systems for full activity, was also severely affected in the *gidA* mutant (Fig. 1E). We define a phenotype or gene as *las* specific if it is affected in a *lasR* (or *lasI*) mutant but not in a *rhlR* (or *rhlI*) mutant. On the other hand, we define a phenotype or gene as *rhl* specific if it is affected in a *rhlR* (or *rhlI*) mutant and it is affected to the same degree in a *lasR* (or *lasI*) mutant. Under most growth conditions, a *rhl*-specific trait is equally affected in a *lasR* (or *lasI*) mutant because the *las* system controls expression of the *rhl* system. However, under some conditions, for example, those employed for the rhamnolipid assay, the *rhl* system is independent of the *las* system, and a *lasR* (or *lasI*) mutant displays a wild-type phenotype.

Individual growth curves showed that the *gidA* mutant was attenuated in logarithmic growth compared with the wild type (doubling times of 45 and 36 min, respectively) and that it entered stationary phase at a slightly lower density (Fig. 2). While the *gidA* mutant grew more slowly, this relatively small difference alone is not sufficient to explain the dramatic QS phenotypes observed. In addition, these growth differences were taken into account for all assays performed in liquid culture. Taken together, our phenotypic data indicate that the *gidA* mutant is deficient primarily in *rhl*-dependent QS phenotypes.

**Complementation fully restores the wild-type phenotype in a *gidA* mutant.** We performed a complementation analysis to exclude the possibility of polar effects of the transposon insertion. This was important because *gidA* is the first gene in a four-gene operon and because two genes in the operon, *soj* and *spo0J*, are involved in chromosome partitioning and cell division. Introduction of an intact copy of the *gidA* gene in the mutant restored QS-dependent phenotypes to wild-type levels (Fig. 3). This indicates that QS-deficient phenotypes conferred by the transposon insertion in the *gidA* gene are solely due to loss of function of *gidA* itself.

Because the transposon insertion was near the 3′ end of the *gidA* gene, the resulting protein could have retained residual activity. To test whether this was in fact the case, we constructed a markerless in-frame *gidA* deletion mutant (see Materials and Methods). Its QS phenotypes and growth characteristics were indistinguishable from those of the transposon mutant (data not shown). This suggests that the transposon insertion completely inactivated GidA and further confirms that the insertion does not exert any polar effects on down-

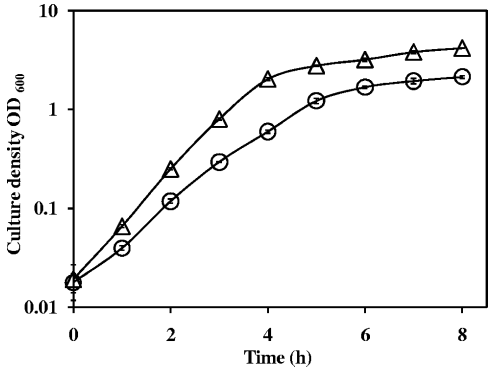


FIG. 2. Growth of the *P. aeruginosa* PA14 *gidA* mutant (circles) and the wild-type parent (triangles). Values are means from three biologically independent experiments. Error bars indicate ± 1standard deviation.

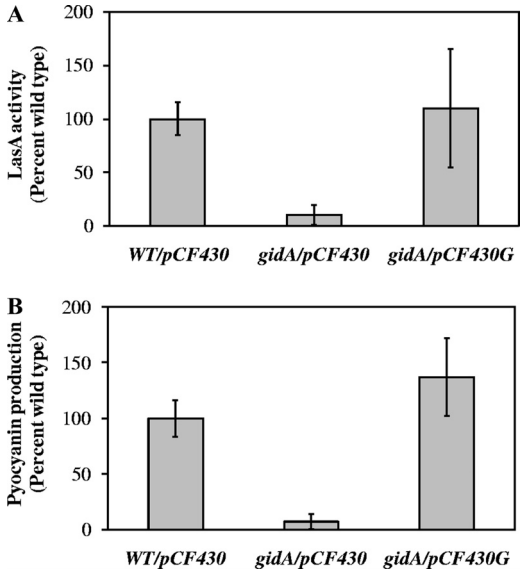


FIG. 3. Restoration of the wild-type phenotype in a *P. aeruginosa* PA14 *gidA* mutant. Wild-type (WT) and *gidA* mutant (*gidA*) strains were supplied with a plasmid expressing *gidA* from $P_{BAD}$ (pCF430G) or with empty vector (pCF430). Cultures were grown in the presence of 50 mM arabinose to induce gene expression. (A) LasA activity. (B) Pyocyanin production. Values are means from three independent biological experiments, normalized to culture density ($OD_{600}$) and expressed as percentages of the wild-type value. Error bars indicate ±1 standard deviation.

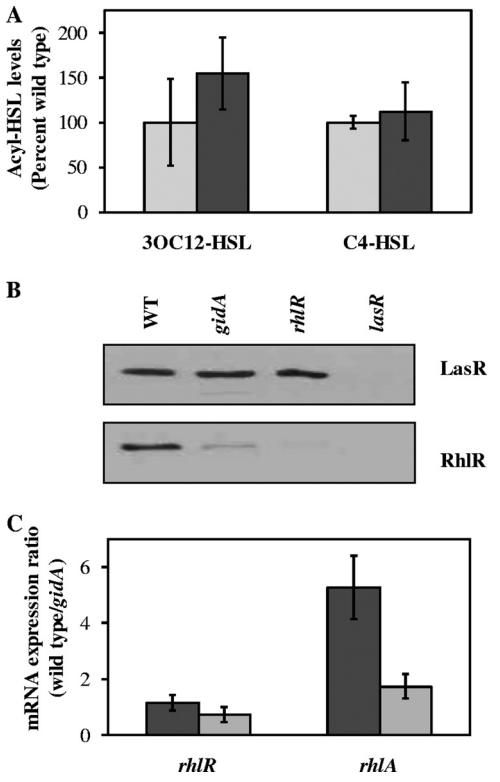


FIG. 4. Acyl-HSL levels and QS gene expression in the *P. aeruginosa* PA14 *gidA* mutant and the wild-type parent. (A) $3OC_{12}$-HSL and $C_4$ HSL levels, expressed as percentages of the wild-type level. Dark gray bars represent the *gidA* mutant, and light gray bars represent the wild-type (WT) parent. (B) Western blot analysis of LasR (top panel) and RhlR (bottom panel) proteins. For comparison, protein levels in the *lasR* and *rhlR* mutants are shown. Equal amounts of clarified lysates were loaded for each strain. Smaller bands, indicative of protein degradation products, were not detected. (C) Real-time PCR. mRNA levels of the *rhlA* and *rhlR* genes were measured in the *gidA* mutant and the wild-type parent at the transition from logarithmic to stationary phase (4 to 5 h, dark gray) and in stationary phase (7 to 8 h, light gray). Values represent ratios of the transcript levels for the wild type versus the *gidA* mutant. Equal amounts of cDNA were used for each amplification reaction. Values are means from three independent biological experiments. Error bars indicate ±1 standard deviation.

stream genes. As the properties of the deletion and the transposon insertion mutants were identical, we performed further experiments with the transposon insertion mutant.

**GidA controls RhlR expression at the posttranscriptional level.** Next, we sought to obtain further insights into how GidA controls QS. Based on the presumed function of GidA, we predicted that it might affect the expression of central QS regulatory proteins posttranscriptionally, in particular, RhlI or RhlR. To investigate whether GidA affects acyl-HSL synthase levels, we measured $3OC_{12}$-HSL and $C_4$-HSL levels in the wild type and the *gidA* mutant. We found no differences in the levels of either acyl-HSL (Fig. 4A). Next, we evaluated LasR and RhlR protein levels by Western blotting. Protein levels of RhlR were greatly reduced in a *gidA* mutant, while LasR levels were comparable to those in the wild type (Fig. 4B). RhlR protein levels were restored to wild-type levels in the *gidA* mutant when complemented with *gidA* in *trans* (data not shown). These results indicate that GidA influences the expression of RhlR, which is consistent with our phenotypic data.

To distinguish between transcriptional and posttranscriptional control, we measured transcript levels of *rhlR* by real-time PCR (Fig. 4C). We also measured transcript levels of the RhlR-dependent gene *rhlA*, which is involved in rhamnolipid biosynthesis (29, 30). We expected downregulation of the *rhlA* gene in a *gidA* mutant, as it has low RhlR protein levels. In the *gidA* mutant, *rhlR* mRNA levels were near wild-type levels, whereas *rhlA* mRNA levels, as expected, were significantly decreased, resulting in an elevated ratio of wild-type to *gidA* mutant transcripts (Fig. 4C). Taken together, these results indicate that *gidA* modulates expression of RhlR posttranscriptionally and that this in turn affects transcription of RhlR-dependent genes.

**Induction of *rhlR* expression partially restores the wild-type phenotype.** Because GidA affected RhlR protein levels, we asked whether RhlR-dependent phenotypes could be rescued by increased expression of RhlR in a *gidA* mutant. We introduced a plasmid carrying *rhlR* under the control of an arabinose-inducible promoter into a *gidA* mutant. When induced fully, this strain expressed RhlR protein at levels slightly higher than those of the wild type and showed partial restoration of RhlR-dependent phenotypes (Fig. 5). This partial suppression suggests that *gidA*, in addition to affecting RhlR expression, also regulates these phenotypes in an RhlR-independent fashion.

## DISCUSSION

*P. aeruginosa* QS signaling is highly complex and involves a network of interconnected pathways (18, 41, 49). Several fac-

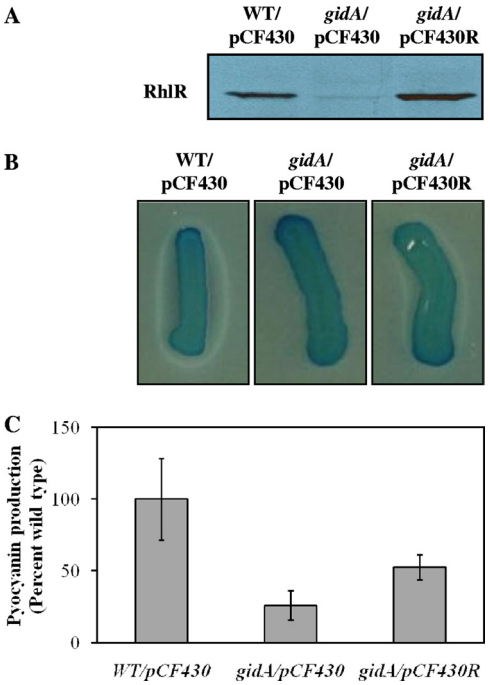


FIG. 5. Suppression of *gidA* mutant phenotypes by overexpression of *rhlR*. *P. aeruginosa* PA14 wild-type (WT) and *gidA* mutant strains were supplied with a plasmid expressing *rhlR* from $P_{BAD}$ (pCF430R) or with empty vector (pCF430). Cultures were grown in the presence of 50 mM arabinose to induce gene expression. (A) Western blot analysis of RhlR protein. Equal amounts of clarified lysates were loaded for each strain. Smaller bands, indicative of RhlR degradation products, were not detected. (B) Rhamnolipid production. (C) Pyocyanin production, expressed as percentages of the wild-type value. Values are means from three independent biological experiments, normalized to culture density ($OD_{600}$). Error bars indicate ±1 standard deviation.

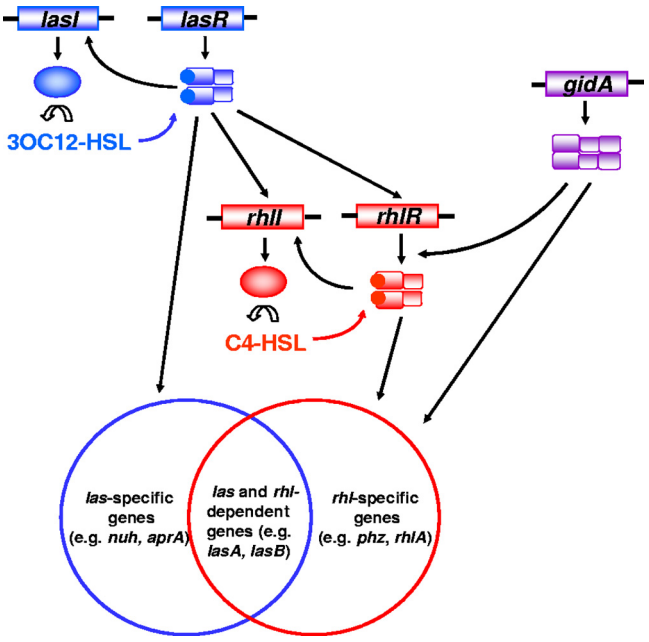


FIG. 6. Model depicting the position of GidA in the QS circuitry. See the text for details.

tors that regulate QS have been identified, but a comprehensive screen, such as a saturation mutagenesis, has not been attempted. In this study, we utilized a nonredundant *P. aeruginosa* transposon library to identify global regulators of QS gene expression. We rediscovered several known regulators but missed others (see Table S1 in the supplemental material). The fact that *rsaL* was missed is not surprising as it is a repressor, not an activator, of QS (6). However, it is not clear why other regulators that function as activators were not identified. One reason for this could be strain-specific regulatory differences. We used *P. aeruginosa* PA14 in our study, while most of the QS regulators have been identified and characterized in strain PAO1. Overall, the fact that not many new regulatory genes conferring multiple QS deficiencies were discovered suggests that the capacity for global regulation of QS, at the level of LasRI and/or RhlRI, is limited.

One such gene, whose inactivation conferred substantial deficiencies in several QS phenotypes, was *gidA*. A *P. aeruginosa* PA14 *gidA* mutant was primarily deficient in *rhl*-dependent phenotypes (Fig. 1). GidA in *P. aeruginosa* is 630 amino acids in length and is 70% identical to the *E. coli* protein. Two forms of the protein exist, a long form and a short form. Both forms contain an N-terminal flavin adenine dinucleotide-binding domain, but only the long form contains a C-terminal tRNA-binding domain required for tRNA modification (27). *P. aeruginosa gidA* is the long form. GidA requires another protein, MnmE, for tRNA modification (4). Unfortunately, we were unable to investigate the effect of an *mnmE* mutation on QS gene expression, thus precluding further evidence for a synergistic role of the two proteins in *P. aeruginosa*. In *E. coli*, both proteins are required for the addition of carboxymethylaminomethyl groups to the C5 carbon of uridine at position 34 of tRNAs that read codons ending with A or G in mixed-codon family boxes, including Glu, Gln, Lys, Leu, and Arg codons (4, 55). This modification enhances base-pairing specificity at the wobble position, allowing pairing with G and A, while restricting pairing with C and U. This prevents both misincorporation and +2 frameshifting (3, 4). It has been suggested that tRNA modification could function as a regulatory mechanism to adjust gene expression in response to nutrient deprivation through alteration of pools of cofactors required for the tRNA modification reactions (35).

In principle, GidA-mediated tRNA modification could affect the expression of all proteins in the cell. However, certain proteins will be more susceptible than others, if the respective genes contain a high proportion of codons that require decoding by tRNAs with U34 modifications. This includes many transcriptional regulators that contain clusters of positively charged Lys and Arg residues in their DNA-binding domains. Global, yet specific, regulation is consistent with previous studies. GidA controls virulence gene expression in *A. hydrophila*, *P. syringae*, *S. flexneri*, and *S. pyogenes* (5, 9, 21, 45) and fruiting-body development in *M. xanthus* (52), with little to no effect on growth. Post-transcriptional regulation has been shown in *A. hydrophila*, *S. flexneri*, and *S. pyogenes*, and involvement of a transcriptional regulator has been demonstrated in *S. flexneri* and *S. pyogenes*.

In this study, we have shown that GidA rather specifically controls RhlR expression in *P. aeruginosa*, also at the post-transcriptional level (see the model in Fig. 6). GidA affected RhlR

protein but not transcript levels (Fig. 4), and introduction of *rhlR* into a *gidA* mutant partially restored *rhl*-dependent phenotypes (Fig. 5). Such phenotypic suppression is plausible, because overexpression of *rhlR* mRNA in a *gidA* mutant would result in a higher fraction of correctly translated RhlR, thereby compensating for the decreased level of functional protein translated from native mRNA. In *S. flexneri* and *S. pyogenes*, overexpression of the transcriptional regulators VirF and RopB, respectively, results in full suppression of *gidA* mutant phenotypes (5, 9). The fact that there was no full restoration to wild-type levels in *P. aeruginosa* suggests that GidA also affects *rhl*-dependent phenotypes independent of RhlR. It cannot be excluded, however, that a significant portion of the overexpressed RhlR, although soluble, is nonfunctional due to translation errors in the absence of GidA, resulting in only partial, rather than full, suppression. Despite the modest growth defect (Fig. 2), our results argue against a general gene expression defect in the *gidA* mutant: compared with the wild type, the *gidA* mutant did not show any deficiency in a *las*-specific phenotype (growth on adenosine as the sole carbon source), in LasR protein levels, and in acyl-HSL production. The observation that $C_4$-HSL levels were not significantly altered in a *gidA* mutant is consistent with *rhlI* expression being primarily *las* dependent (7). While RhlR activates *rhlI* expression in the heterologous host *E. coli* (23), LasR rather than RhlR is the dominant regulator in *P. aeruginosa* (7).

Interestingly, the coding regions of LasR and RhlR both contain roughly equal numbers of codons (17 and 18, respectively) that require decoding by GidA/MnmE-modified tRNAs, which appears to be inconsistent with the observation that GidA affected RhlR but not LasR protein levels. However, the frequency of two-family box triplet codons alone does not determine the impact on protein function. Although none of the affected amino acid residues are highly conserved, the contribution of individual, less conserved residues to protein stability and activity may well be different in LasR and RhlR. Both proteins are only 33% identical and function quite differently. While LasR requires its acyl-HSL ligand for proper folding (43), RhlR does not (26). In addition, frameshifts, which tend to be much more deleterious than misincorporations, are also strongly dependent on the base composition surrounding the respective codons, although the precise mechanism is not clear (4). It is therefore plausible that GidA-mediated tRNA modification could have a selective effect on RhlR but not LasR protein levels and consequently could affect *rhl*-dependent but not *las*-dependent phenotypes.

Further experiments will be needed to elucidate the precise molecular mechanism of GidA function in *P. aeruginosa*. Taking our results together, we have identified an additional regulatory component of the QS network in *P. aeruginosa* that affects *rhl*-dependent gene expression through posttranscriptional control of RhlR.

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