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Timothy Robert Gobble for the degree of Honors Baccalaureate of Science in Microbiology presented on May 16, 2008. Title: A Genome-Wide, Phenotypic Screen for Genes Regulating Quorum Sensing in *Pseudomonas aeruginosa*.

Abstract approved:

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Martin Schuster

The opportunistic pathogen *Pseudomonas aeruginosa* possesses two LuxR-LuxI type quorum-sensing systems that use diffusible acyl-homoserine lactone molecules to autoinduce and coordinate expression of multiple virulence factors. We conducted a high-throughput screen for mutants deficient in skim-milk proteolysis—a quorum-sensing dependent phenotype—using a nonredundant transposon-insertion library representing most nonessential genes in *P. aeruginosa* strain PA14. Of 75 mutants with decreased skim-milk proteolysis, a mutant with a transposon insertion in *gidA* grew sufficiently and displayed significant deficiencies in three other quorum-controlled phenotypes—LasA staphylolytic activity, pyocyanin production, and rhamnolipid production. These deficiencies were restored by complementation. Moreover, overexpression of *gidA* in PA14 wild-type cells led to a significant increase in pyocyanin production. The *gidA* gene, which has already been identified in other bacteria as a regulator of virulence, encodes an enzyme involved in tRNA modification that promotes codon-anticodon interaction. Thus, *gidA* may play an important role as a posttranscriptional super regulator of virulence, and additional investigation may show a new means to interfere with *P. aeruginosa* virulence.

Key Words: *Pseudomonas aeruginosa*, quorum sensing, *gidA*, virulence, regulation, gene

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A Genome-Wide, Phenotypic Screen for Genes Regulating Quorum Sensing in *Pseudomonas aeruginosa*

By

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TABLE OF CONTENTS

Page

INTRODUCTION 1

Pseudomonas aeruginosa. 1
Quorum Sensing..... 2
Introduction to Research. 5

MATERIALS AND METHODS..... 6

Bacterial Strains and Plasmids. 6
PA14 Non-Redundant Set. 7
Enzymes, Chemicals, and Recombinant DNA Techniques. 7
Skim-Milk Proteolysis. 8
Growth Curve Analysis..... 8
Rhamnolipid Production. 9
Staphylolytic Activity. 10
Pyocyanin Production. 10
Localization of Transposon Insertion. 11
Complementation Analysis. 11

RESULTS 13

Identification of Mutants with Reduced Skim-Milk Proteolysis. 13
Identifying Mutants with Multiple Altered QS-Dependent Phenotypes..... 14
High-Throughput Growth Curve. 15
Characterization of PA14 *gidA*::MAR2xT7. 18
Complementation with pCF430 Plasmid Vector. 19

DISCUSSION..... 24

BIBLIOGRAPHY..... 29

LIST OF FIGURES

Figure	Description	Page
1	Integration of QS into global regulatory networks	4
2	High-throughput screen for skim-milk proteolysis	13
3	Gel electrophoresis confirming transposon location	18
4	Growth curve comparison of PA14 *gidA*::MAR2xT7 and parent	19
5	Complementation of skim-milk proteolysis	20
6	Complementation of staphylolytic activity (raw data)	21
7	Complementation of staphylolytic activity (ΔOD_{600} / minute)	21
8	Complementation of pyocyanin production (normalized)	22
9	Complementation of rhamnolipid production	23

LIST OF TABLES

Table	Description	Page
1	Bacterial strains and plasmids	6
2	Summary phenotypic data for *P. aeruginosa* PA14 mutants	16

A Genome-Wide, Phenotypic Screen for
Genes Regulating Quorum Sensing in *Pseudomonas aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa.

The resilient Gram-negative bacterium *Pseudomonas aeruginosa* flourishes throughout various terrestrial and aquatic environments. When given a chance, *P. aeruginosa* can successfully invade and infect vulnerable hosts ranging from lower eukaryotes to plants and humans. This opportunistic pathogen is characterized by its formidable antibiotic resistance and is responsible for over ten percent of all hospital-acquired infections [1]. Severe burns, surgical sites, and the lungs of cystic fibrosis patients are areas particularly vulnerable to infection; moreover, the number of immunocompromised patients has risen enormously as improvements are made in treating cancer, organ failure, and other serious medical problems. The rise of vulnerable patients and multi-antibiotic resistance pathogens has made hospital-acquired infections a very serious and emerging issue [2]. Consequently, an enormous effort has been made to understand *P. aeruginosa* and discover a more effective means of combating its lethal infections. An intriguing area of research has been investigating the pathogen's use of quorum sensing (QS)—a mechanism used to regulate and coordinate the gene expression of many important virulence factors.

Quorum Sensing.

In bacterial QS, cells sense and respond to the concentration of a small self-produced signal molecule or autoinducer; the concentration of this autoinducer reflects cell density, spatial distribution and the ability of the signal to defuse or be washed away from its source. Accordingly, by sensing the autoinducer concentration, bacteria can sense the efficacy of secreting effectors; thus this concept has also been referred to as efficiency sensing [3]. Autoinducer-producing cells benefit themselves by sensing whether circumstances warrant spending the additional energy and resources required to express and secrete the many virulence factors required for pathogenesis. Additionally, the population of cells also benefits by coordinating gene expression to more effectively invade and thrive while up against host defenses [4].

QS systems are diverse among bacteria. In Gram-positive bacteria, the autoinducer is often an oligopeptide that is sensed by the membrane receptor portion of a two-component signal-transduction cascade [5]. Some of the most well-described cell-to-cell signaling molecules in Gram-negative bacteria are acylated homoserine lactones (acyl-HSL) which can freely diffuse in and out of each cell. Acyl-HSL QS was first described in the LuxI-LuxR system controlling bioluminescence in the Gram-negative bacterium *Vibrio fischeri* [6]. In the cytoplasm of *V. fischeri*, acyl-HSL signals are produced by the synthase LuxI and sensed by the transcriptional regulator LuxR. Once activated, LuxR stimulates transcription of the bioluminescence genes and the cognate autoinducer-synthase LuxI, which results in a positive feedback loop [7].

P. aeruginosa possesses two LuxR-LuxI type QS systems, the LasR-LasI (*las*) system and the RhlR-RhlI (*rhl*) system. LasI and RhlI produce the signals 3-oxododecanoyl (3OC12) HSL and butanoyl (C4) HSL respectively; these signals modulate the activity of their respective transcriptional regulator LasR or RhlR [8]. The *las* system activates the *rhl* system and the two systems modulate two large overlapping regulons that together compose 2-5% of the *P. aeruginosa* genome [9-11]. A large portion of these QS-controlled genes encode secreted virulence factors such as the extra-cellular enzymes LasA protease, elastase (LasB), alkaline protease, lipase, and the secondary metabolites hydrogen cyanide and pyocyanin [12]. *P. aeruginosa* QS also plays a significant role in biofilm formation [13]. These surface adherent populations consist of organized layers and clusters of cells embedded within a self-produced extracellular matrix. Biofilms are essential to *P. aeruginosa's* resiliency in chronic infections. Consequently, a great deal of effort has gone into understanding the details involved in the *las* and *rhl* systems in hopes of developing anti-virulence therapies which may be essential for treating serious multi-drug resistant infections.

P. aeruginosa QS is embedded in a complex network of global regulation (Fig. 1). Most QS-controlled genes delay expression until the stationary phase despite exogenous acyl-HSL signals [9, 14-16]. Thus, additional factors may be required for expression of these QS-controlled genes. Schuster and Greenberg (2006) describe seven regulators that have already been identified as involved in *P. aeruginosa* QS. These include: 1) Vfr, a catabolite repressor homolog that directly induces *lasR* transcription. 2) GacA/GacS, a two-component regulatory system that regulates QS posttranscriptionally through activation of RsmZ—a small RNA that inhibits the RNA-binding protein RsmA which represses autoinducer synthesis. 3) The stringent response protein RelA which is involved

in the early generation of many QS-controlled processes. 4) RsaL, a protein activated by LasR-3OC12-HSL that represses *lasI* transcription. 5) The stationary phase sigma factor RpoS which affects expression of almost half of QS-controlled genes. 6) QscR—a third LuxR-type transcriptional regulator that lacks a cognate autoinducer synthase—delays the activation of several QS-controlled genes perhaps by forming heterodimers with LasR and RhlR. 7) MvfR, which is under the regulation of LasR-3OC12-HSL, activates the transcription machinery required for synthesis of Pseudomonas quinolone signal (PQS)—a third signal also identified as controlling QS gene expression [8]. Much remains to be learned regarding this complex network of global regulation, but it is clear that control of QS is multifaceted and will require an integrative approach to understand such a complex regulatory network.

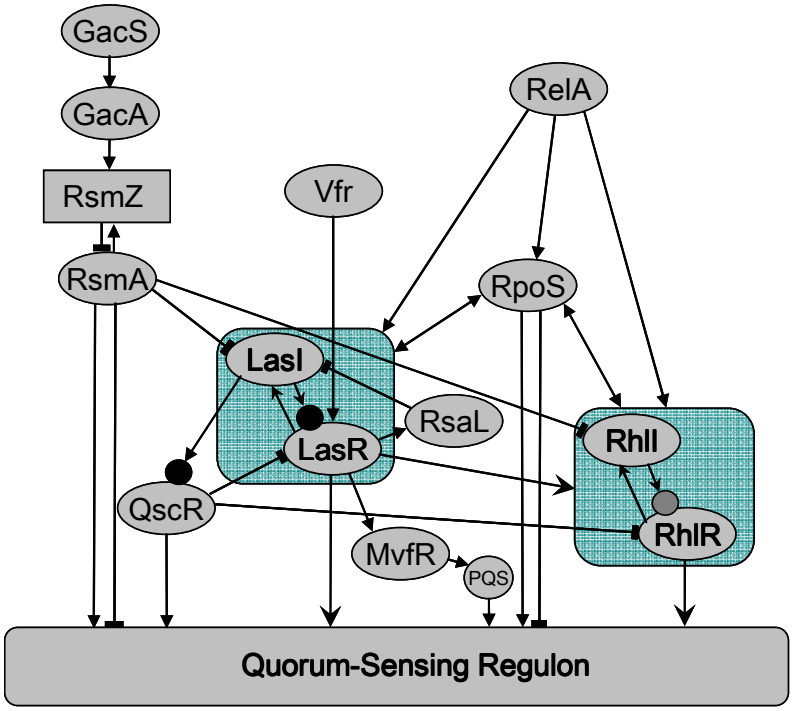

Fig. 4. Integration of QS into global regulatory networks. The QS regulon is defined as the group of genes activated directly or indirectly by the *las* and/or *rhl* systems.

Introduction to Research.

In order to identify additional genes that regulate QS and consequently multiple QS-dependent virulence phenotypes, we conducted a high-throughput phenotypic screen of a non-redundant library of 5,459 *P. aeruginosa* strain PA14 transposon insertion mutants [17], representing almost all nonessential genes in the PA14 genome.

An initial screen of the entire PA14 mutant library identified 75 mutants with a deficiency in proteolysis of casein on skim-milk agar. With these 75 mutants, we conducted multiple high-throughput assays studying three additional QS-dependent phenotypes. These assays identified several mutants with deficiencies in multiple phenotypes. Most intriguing, however, is the mutant with a transposon insertion in the *gidA* ORF that exhibits phenotypes similar to both the *lasR*$^{-}$ and *rhlR*$^{-}$ mutants. Consequently, *gidA*, which has been identified in other bacteria as an important regulator of virulence [18-20], is an important candidate for additional investigation towards finding a means to interfere with *P. aeruginosa* virulence.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Reference or source
Strains		
P. aeruginosa		
PA14	Wild type	17
PA14 Δ*lasR*	Tn*phoA lasR* mutant derived from PA14	21
PA14 *lasI*::MAR2xT7	MAR2xT7 *lasI* mutant derived from PA14	17
PA14 *rhlR*::MAR2xT7	MAR2xT7 *rhlR* mutant derived from PA14	17
PA14 *rhlI*::MAR2xT7	MAR2xT7 *rhlI* mutant derived from PA14	17
PA14 *gidA*::MAR2xT7	MAR2xT7 *gidA* mutant derived from PA14	17
PA14 *suhB*::MAR2xT7	MAR2xT7 *suhB* mutant derived from PA14	17
PA14 *gidA*::MAR2xT7/pCF430	PA14 *gidA*::MAR2xT7 transformed with pCF430	This study
PA14 *gidA*::MAR2xT7/pCF*gidA*	PA14 *gidA*::MAR2xT7 transformed with pCF*gidA*	This study
PA14/pCF430	PA14 wild type transformed with pCF430	This study
PA14/pCF*gidA*	PA14 wild type transformed with pCF*gidA*	This study
E. coli		
DH5α	F-, Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *deoR recA*1 *endA*1 *hsdR*17 (rk-, mk+) *phoA supE*44 λ- *thi*-1 *gyrA*96 *relA*1	Invitrogen
Plasmids		
pCF430	Broad host-range vector (tetracycline resistant)	22
pCF*gidA*	2-kb HindIII/XbaI PA14 DNA insert (*gidA*) in pCF430	This study

MATERIALS AND METHODS

Bacterial Strains and Plasmids.

The initial skim-milk proteolysis assay was conducted with all 5,459 mutants in the PA14 non-redundant set. Mutants in the secondary screen consisted of: 75 mutants that initially displayed a deficiency in skim-milk proteolysis; 15 mutants whose mutated genes have already been identified as QS modulators [8]; and, the PA14 parent and PA14 *lasR*::Tn*phoA* [21] as positive and negative controls, respectively (Table 2). Unless stated otherwise, strains were grown at 37°C in Luria-Bertani (LB) broth with agitation (250 rpm). Overnight cultures in 2.0 ml Deep 96-well Titerblocks were generated by inoculating wells containing 600 µl of LB broth buffered with 50 mM MOPS (pH 7.0)

and incubating for 18h at 37°C with agitation (250 rpm). The broad-host-range expression vector pCF430 [22] (Table 1) contains the *araC-*P_{BAD} regulatory system and *tetA* for tetracycline resistance. The plasmid pCF*gidA* was formed and stored with competent *Escherichia coli* DH5α cells (Invitrogen, CA) (Table 1). Liquid cultures of *P. aeruginosa* strains containing pCF430 or pCF*gidA* were grown in LB broth containing 50 mM L-arabinose, 50 mM MOPS (pH 7.0), and 50 μg/ml tetracycline.

PA14 Non-Redundant Set.

We used a subset of the parental PA14 transposon insertion library [17]. This collection consists of 5,459 single insertion mutants representing 4,596 predicted PA14 genes; some genes are represented by more than one mutant. To minimize cross-contamination of wells, each 96-well storage plate was handled and stored as described in the library's User Manual [23].

Enzymes, Chemicals, and Recombinant DNA Techniques.

Gentamicin was used at 15 μg/ml with *P. aeruginosa* for the maintenance of MAR2xT7 mutants [23]. To maintain pCF430 and pCF*gidA* plasmids, tetracycline was used at 15 μg/ml and 50 μg/ml with *E. coli* and *P. aeruginosa*, respectively. Chromosomal-DNA isolation, PCR-product purification, gel extraction, and plasmid purification were conducted using spin centrifugation with the PUREGENE^{TM} DNA Purification System (Gentra Systems, US), QIAquick: PCR Purification Kit, Gel Extraction Kit, and QIAprep Spin Miniprep Kit (Qiagen, US), respectively.

Skim-Milk Proteolysis.

To assess mutants of the PA14 nonredundant set for skim-milk proteolysis**,** strains from each 96-well microtiter plate were grown overnight on a *Nunc* (#267060) rectangular dish (Thermo Fisher Scientific, US) containing LB agar, then transferred to a second rectangular dish with skim-milk agar [24] and grown for 18h at room temperature. A 96-pin replicator was used to simultaneously transfer strains from storage wells to LB plates and from LB plates to skim-milk plates. *P. aeruginosa* PA14 (wild type) and the Tn*phoA*-insertion *lasR* mutant [21] (Table 1) were used as positive and negative controls, respectively. Strains with no halo or one visibly smaller than the wild-type halo were chosen for additional screening.

For a more quantitative assessment of skim-milk proteolysis, we used an assay suitable for small sample sizes that assessed stationary-phase (~16h) liquid-culture supernatant instead of cells growing of plates. In this case, 100 µl of supernatant were added to holes cut out (using bulb end of Pasteur pipette) in solid media consisting of 10% wt/vol skim-milk and 1.5% wt/vol granulated agar. Plates were incubated at 37°C for 24 hours and proteolysis was assessed from the relative size of the halo around each cutout.

Growth Curve Analysis.

Growth analysis of each strain listed in Table 2 was conducted simultaneously in a 96-well microplate using an Infinite 200 multimode microplate reader (Tecan, US) to grow strains at 37°C with shaking in 200 µl of LB medium buffered with 50 mM MOPS

buffer (pH 7.0) and sealed with 50 µl of mineral oil. The OD_{600} was determined every 15min for 8h, and the doubling time for each strain was calculated with respective data. Uninoculated wells were used as both blanks and negative controls. Growth of PA14 *gidA*::MAR2xT7 and the parent PA14 wild type was also quantified in standard culture flasks. Flasks with 20 ml of LB broth were inoculated with mid-log 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 hours.

Rhamnolipid Production.

Rhamnolipid production was assayed by inoculating strains from LB agar onto M9-based rhamnolipid-detection agar prepared with 0.2% glucose, trace elements, 2 mM $MgSO_4$, 0.05% glutamate as the nitrogen source, 0.0005% methylene blue, and 0.02% cetyltrimethylammonium bromide [25]. Plates were incubated at 37°C for 24h followed by 24h at room temperature or until a purple halo appeared. Relative rhamnolipid production was determined by the size of the blue/purple halo around each colony. The PA14 wild type and PA14 *rhlR*::MAR2xT7 strains were used as positive and negative controls, respectively.

A more stringent method was required for assessing rhamnolipid production in strains transformed with pCF430 or pCF*gidA*. Instead, 100 µl of stationary-phase liquid culture (~16h) was added to holes cut out (using bulb end of Pasteur pipette) in rhamnolipid-detection agar supplemented with tetracycline and 50 mM L-arabinose. Plates were incubated at 37°C for 24 hours then at 4°C for another 24 hours. Rhamnolipid production was assessed from the relative size of the halo around each hole.

Staphylolytic Activity.

Staphylolytic activity was established by measuring the rate of *Staphylococcus aureus* cell lysis by culture supernatant [14, 26]. Overnight cultures (~16h) of respective PA14 strains in 2.0 ml Deep 96-well Titerblocks were centrifuged at 4000 rpm for 10 minutes; 20 µl of culture supernatant was transferred into microplate wells 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 minutes for 1h. PA14 wild-type cultures and uninoculated broth were used as positive and negative controls, respectively.

Pyocyanin Production.

Absorbance of culture supernatant at 310 nm was used to assess pyocyanin production in a high-throughput format [27]. Overnight cultures in 2.0 ml Deep 96-well Titerblocks were centrifuged at 4000 rpm for 10 minutes; 200 µl of supernatant was transferred to a 96-well microtiter plate and the OD_{310} was determined with a microplate reader. PA14 wild-type cultures and uninoculated broth were used as positive and negative controls, respectively.

To more precisely quantify pyocyanin production in PA14 *gidA*::MAR2xT7/pCF430, PA14 *gidA*::MAR2xT7/pCF*gidA*, PA14/pCF430, and PA14/pCF*gidA*, pyocyanin was extracted from filtered liquid-culture supernatant and quantified by measuring absorbance at 520 nm (OD_{520}) [28]. Filtered supernatant (3.5 ml) from a stationary-phase liquid culture (~16h) was mixed with 2.1 ml of chloroform.

Pyocyanin was extracted from the chloroform phase into 1 ml of 0.2 N HCL. In 0.2 N HCL, pyocyanin formed a pink to deep red color.

Localization of Transposon Insertion.

The location of MAR2xT7 insertion in PA14 *gidA*::MAR2xT7 was confirmed by polymerase chain reaction (PCR) and gel electrophoresis. Primers were designed from the sequences of MAR2xT7 and the PA14 *gidA* ORF using the Transposon Insertion Site Map found at the PA14 Transposon Insertion Mutant Library website [29]. According to the online insertion-site map [29], primers 5' CATTACAGTTTACGAACCGAACAG 3' (forward) and 5' GCAAGGCAAGGACAGCTGGTG 3' (reverse) amplify a section 661 base pairs long. The PCR product was assessed using standard gel-electrophoresis technique.

Complementation Analysis.

Complementation analysis of PA14 *gidA*::MAR2xT7 was accomplished by introducing wild-type *gidA* using the broad-host-expression vector pCF430 (Table 1). A 2-kb DNA fragment containing the *gidA* gene from PA14 chromosomal DNA was amplified using primers 5' NNNNNN<u>AAGCTT</u>CGCTAAATCCTTATACTGTCCG 3' and 5' NNNNNN<u>TCTAGA</u>GGTGTTGGGTTACCGCAGAC 3'; HindIII and Xbal sites are underlined respectively. HindIII/XbaI-digested PCR product and pCF430 vector were ligated to generate the recombinant plasmid pCF*gidA* (Table 1). After transforming this new plasmid into competent *E. coli* DH5α cells, we verified the presence of the *gidA*

insert by plasmid isolation, restriction-enzyme analysis, and sequencing of the *gidA* insert using primers of the respective sequence. Purified pCF*gidA* and pCF430 were transformed into chemically competent PA14 *gidA*::MAR2xT7 and parent wild type cells to create strains PA14 *gidA*::MAR2xT7/pCF*gidA*, PA14 *gidA*::MAR2xT7/pCF430, PA14/pCF*gidA* and PA14/pCF430 respectively. Strains PA14 *gidA*::MAR2xT7/pCF430 and PA14/pCF430 were used a negative controls.

RESULTS

Identification of Mutants with Reduced Skim-Milk Proteolysis.

We screened all 5,549 mutants in the non-redundant PA14 transposon insertion library [17] for altered skim-milk proteolysis (Fig. 2) and found 75 mutants with reduced proteolysis. Among these 75 mutants, we rediscovered the autoinducer-synthase gene *lasI*, and QS-regulators *vfr* and *gacA* [8]. Also, identified were 6 genes of the Xcp type-II secretion system *xcpT*, *xcpX*, *xcpW*, *xcpZ*, *xcpQ*, and *xcpR* [30]. For our negative control, we used the proteolysis deficient *lasR* mutant [21] (Table 2). In each subsequent high-throughput study, we included these 75 mutants as well mutants of genes already described as QS modulators [8] (Table 2).

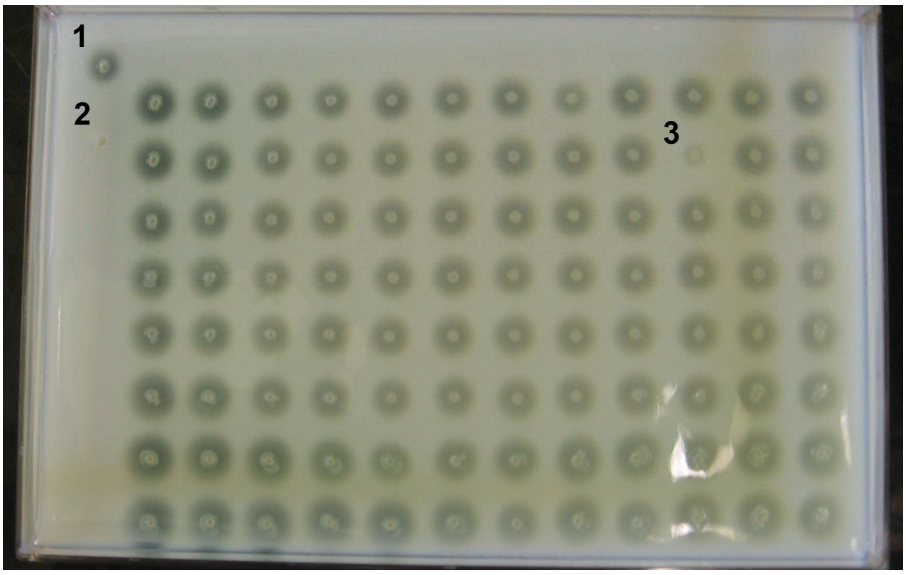

Fig. 2. High-throughput screen for skim-milk proteolysis in mutants of PA14 nonredundant set. PA14 (**1**) and PA14 Δ*lasR* (**2**) were used as positive and negative controls, respectively. This example represents one of the 62 plates assayed and includes PA14 *lasI*::MAR2xT7 (**3**).

Identifying Mutants with Multiple Altered QS-Dependent Phenotypes.

The set of 75 mutants was assayed for three additional QS-dependent phenotypes—staphylolytic activity, rhamnolipid production and pyocyanin production. This was done to 1) discover genes that control multiple QS-dependent phenotypes, and 2) to exclude genes that are involved in processes other than QS, such protease secretion. (Table 2)

By assessing staphylolytic activity of culture supernatants [26], we deduced extracellular levels of the QS-controlled extracellular protease LasA [12]. Mutants of *lasR, lasI, rhlR, rhlI, vfr*, *gacA* and those in the *xcp* secretion system all showed little or no staphylolytic activity (Table 2). Mutants of the genes *gidA*, *lepA*, *suhB*, *fis*, *tpiA* and a gene for a hypothetical protein (PA14_45710) also demonstrated little or no staphylolytic activity (Table 2).

Rhamnolipid production varied widely between mutants assayed on a specific M9-based minimal media [25]. Notably, the *rhlR* mutant showed the greatest decrease in rhamnolipid production; whereas the *lasR* mutant had a slightly smaller halo than the PA14 wild type, and the mutants for the two autoinducer-synthase genes *lasI* and *rhlI* showed little distinguishable differences from the wild type. Additionally, some 26 of the 75 mutants originally identified mutants had little to no growth on this minimal media. However, 20 mutants, including those for genes *gidA*, *suhB*, *fis*, *rsaL*, and *dksA*, clearly had smaller halos than the PA14 wild type (Table 2).

To estimated relative pyocyanin production in a high-throughput format, we simply measured the absorbance of culture supernatant at 310 nm. Overall, 28 mutants consistently had a low OD_{310}. Among these were mutants for genes: *lasR*, *lasI*, *rhlR*, *rhlI*,

vfr and *gacA*—genes already identified as QS regulators; 17 of the 26 genes whose mutants could not grow on the minimal media used in the rhamnolipid assay; and the genes *gidA* and *suhB* (Table 2). PA14 *gidA*::MAR2xT7 and PA14 *suhB*::MAR2xT7 were the only mutants of the 75 identified initially with severe deficiencies in all four phenotypes assess. PA14 *suhB*::MAR2xT7 did not grow well at room temperature (data not shown). Thus, additional work focused on characterizing PA14 *gidA*::MAR2xT7.

High-Throughput Growth Curve.

We measured growth of each mutant in 96-well plates to determine if the observed phenotypes were simply a consequence of impaired growth. Table 2 shows the doubling times based on this experiment. Although mutants of the genes for TpiA and a putative transcriptional regulator (PA14_38380) had significantly lower growth rates, most mutants exhibited growth rates similar to that of the PA14 wild type and *lasR* mutant. This indicates that with most of these mutants, distinct phenotypic variations in liquid cultures (pyocyanin production and staphylolytic activity) are not likely attributed to differences in growth.

Table 2. Summary phenotypic data for *P. aeruginosa* PA14 mutants

Locus[a] (Mutated Gene)	QS-Dependent Phenotypes[d]				
	Doubling Time	Skim-Milk Proteolysis	Staphylolytic Activity	Rhamnolipid Production	Pyocyanin Production
PA14 wild type	24.1	+++	+++	+++	+++
PA14_45960 *(lasR)*[b]	31.2	-	-	++	-
PA14_45940 *(lasI)*[c]	27.1	+	-	++	-
PA14_19120 *(rhlR)*[c]	30.7	+++	-, ++, -[f]	-	-
PA14_19130 *(rhlI)*[c]	25.9	+++	-, +++, -[f]	++	-
PA14_08370 *(vfr)*[c]	25.4	++	-, ++, -[f]	++	+
PA14_30650 *(gacA)*[c]	28.8	++	-, +++, -[f]	+++	-
PA14_52180 *(relA)*[c]	28.3	+++	+++	++	+++
PA14_51340 *(mvfR)*[c]	27.0	+++	+++	++	+++
PA14_45950 *(rsaL)*[c]	29.1	+++	+++	++	+++
PA14_52570 *(rsmA)*[c]	27.9	+++	+++	+++	+++
PA14_39980 *(qscR)*[c]	30.8	+++	+++	+++	++
PA14_62490 *(dksA)*[c]	29.5	++	+++	-	+++
PA14_56070 *(mvaT)*[c]	30.8	+++	+++	++	+++
PA14_62530 *(cbrA)*[c]	27.5	+++	+++	++	+++
PA14_17480 *(rpoS)*[c]	28.1	+++	++	+++	++
PA14_57940 *(rpoN)*[c]	28.5	+++	++	+++	+++, ++, +[f]
PA14_66710 *(rpmE)*	34.0	-	+++	++	++
PA14_26890 *(pyrF)*	27.8	++	+++	NG	++
PA14_07700 *(apaH)*	28.9	-	++	++	+++
PA14_69670 *(lysA)*	27.5	++	+++	NG	+++, +, ++[f]
PA14_05250	24.8	++	+++	NG	+
PA14_62930 *(carA)*	27.2	-	+++	NG	+
PA14_67530	28.9	++	+++	++	++
PA14_49010	27.7	++	+++	+++	+++
PA14_07620 *(cca)*	33.8	-	+++	+	+++
PA14_57940 *(rpoN)*	25.6	++	+++	NG	+++
PA14_70370 *(pyrE)*	32.5	-	+++	NG	++
PA14_30290 *(ftsK)*	34.3	++	++	++	+++
PA14_05260 *(pyrB)*	25.6	++	+++	NG	-
PA14_41240 *(clpP)*	26.6	++	+++	+	+++
PA14_05620 *(sahH)*	24.6	+	++	NG	+
PA14_25110 *(topA)*	30.8	++	+++	+	+++
PA14_05250	34.1	+	+++	NG	+
PA14_62930 *(carA)*	26.1	-	++	NG	+
PA14_05260 *(pyrB)*	27.2	++	+++	NG	+
PA14_62570 *(folK)*	35.2	+	-, +++, +++[f]	NG	+
PA14_14700 *(cysE)*	28.3	-	+++	NG	++
PA14_14690	27.6	-	++	NG	+
PA14_70370 *(pyrE)*	29.7	-	+++	NG	+
PA14_45710	32.8	-	+	NG	-
PA14_66600 *(aroB)*	33.0	++	++	NG	-
PA14_70370 *(pyrE)*	33.6	+	+++	NG	++
PA14_51790 *(ruvA)*	26.2	++	+++	++	++
PA14_16930	25.5	++	+++	+++	++
PA14_69670 *(lysA)*	26.0	++	+++	NG	++
PA14_58600	25.8	++	+++	+++	+++
PA14_51690	28.4	++	+++	++	+++
PA14_12080 *(sltB1)*	27.7	++	+++	++	++
PA14_64190 *(fis)*	34.1	++	++	++	++

Table 2. Summary phenotypic data for *P. aeruginosa* PA14 mutants (Continued)

Locus[a] (Mutated Gene)	QS-Dependent Phenotypes[d]				
	Doubling Time	Skim-Milk Proteolysis	Staphylolytic Activity	Rhamnolipid Production	Pyocyanin Production
PA14_15600	25.5	++	+++	+++	++
PA14_64220 *(purD)*	28.4	++	+++	NG	++
PA14_72490 *(polA)*	29.9	+, ++, +++[f]	++	+++	++
PA14_12490	26.9	+, ++, +++[f]	+++	+++	+++, ++, +[f]
PA14_24020 *(xcpT)*	31.4	-	-	+++	++
PA14_44070 *(gltA)*	27.5	++	+++	++	+++
PA14_51240 *(purC)*	30.0	++	+++	NG	+++
PA14_25110 *(topA)*	23.8	-	+++	-	+++
PA14_67560 *(typA)*	32.4	++	+++	+++	++, +++, +[f]
PA14_14680 *(suhB)*	31.9	-[e]	-	+	+
PA14_24070 *(xcpX)*	26.1	-	-	+++	++
PA14_54370 *(lepA)*	28.5	-	-	+++	++
PA14_24060 *(xcpW)*	25.8	-	-	+++	+++, ++, -[f]
PA14_68370 *(cysQ)*	27.2	++	+++	++	++
PA14_66940 *(hisI)*	30.8	++	+++	++	++
PA14_38380	41.5	++	+++	++	+++
PA14_24100 *(xcpZ)*	26.7	-	-	+++	++
PA14_62560 *(pcnB)*	27.3	++	++	++	++
PA14_23970 *(xcpQ)*	28.6	-	-	+++	++
PA14_09520 *(mexI)*	31.1	++	++	+++	++
PA14_60280 *(fimU)*	27.7	++	+++, ++, -[f]	++	+++, ++, -[f]
PA14_73370 *(gidA)*	30.1	++[g]	-	-	-
PA14_27950	27.2	++	+++	++	+++
PA14_64200 *(purH)*	33.3	+	+++	NG	+++
PA14_52040 *(purM)*	32.5	++	+++	NG	++
PA14_23990 *(xcpR)*	27.6	-	-	+++	+++, +++, +[f]
PA14_08370 *(vfr)*	29.0	++	-, +++, -[f]	++	+
PA14_22620 *(cyaB)*	29.0	++	+++, +++, -[f]	++	+++, ++, -[f]
PA14_22020 *(minD)*	23.0	++	+++	++	+++
PA14_05380 *(pilK)*	26.0	++	+++	++	++
PA14_66110	26.9	++	+++	++	+++
PA14_23920 *(purF)*	27.5	++	+++	+	+++
PA14_56300	**	++	+++	++	+++
PA14_57940 *(rpoN)*	24.9	++	+++	NG	+++
PA14_62830 *(tpiA)*	38.7	++	-	NG	-
PA14_41570 *(oprF)*	23.4	++	+++	++	++
PA14_20010 *(hasR)*	25.8	++	++	+++	+++, +++, +[f]
PA14_15740 *(purL)*	26.8	++	+++	NG	+, +++, ++[f]
PA14_13220	26.1	+++	+++	+++	+++

[a] Unless labeled otherwise, all mutants have a MAR2xT7 transposon insertion in the respective ORF.
[b] The *lasR* mutant contains Tn*phoA* insertion in codon 154.
[c] Mutants included in the high-throughput assays because the corresponding genes have been shown to be involved in QS regulation.
[d] +++, ++, +, -, NG, and ** indicate a phenotype resembling the wild type, slightly down-regulated, substantially down-regulated, absent, no growth, and well contamination, respectively.
[e] The *suhB* mutant is cold-sensitive; there was little growth at room temperature so no halo appeared; however proteolysis did occur when assay was repeated at 37°C (data not shown).
[f] Due to inconsistent results, phenotypes from each replication are given.
[g] Skim-milk proteolysis was completely absent when liquid culture supernatant was assessed (Fig. 5).

Characterization of PA14 *gidA*::MAR2xT7.

Fig. 3. Gel electrophoresis confirming MAR2xT7 localization in PA14 *gidA*::MAR2xT7.

According to the Transposon Insertion Map available on the PA14 Transposon Insertion Mutant Library website [29], MAR2xT7 inserted 227 base pairs from the C-terminus of *gidA*. We confirmed the Mar2xT7-insertion location in *gidA*::MAR2xT7 using PCR with a forward primer 179 base pairs within the MAR2xT7 sequence and a reverse primer derived from a part of the *gidA* gene 482 base pairs upstream of the assigned insertion site (661 base pairs upstream of the forward primer.) Figure 3 shows that the PCR product was 600-800 base pairs long; this is consistent with the assigned insertion location.

Figure 4 reveals that PA14 *gidA*::MAR2xT7 grew 30% slower than the parent PA14 wild type. However, since discrepancies in stationary-phase optical density were not proportional to phenotypic changes (Figs. 5-9), hindered growth could not have been solely responsible from deficiencies in the four QS-dependent phenotypes that were assessed.

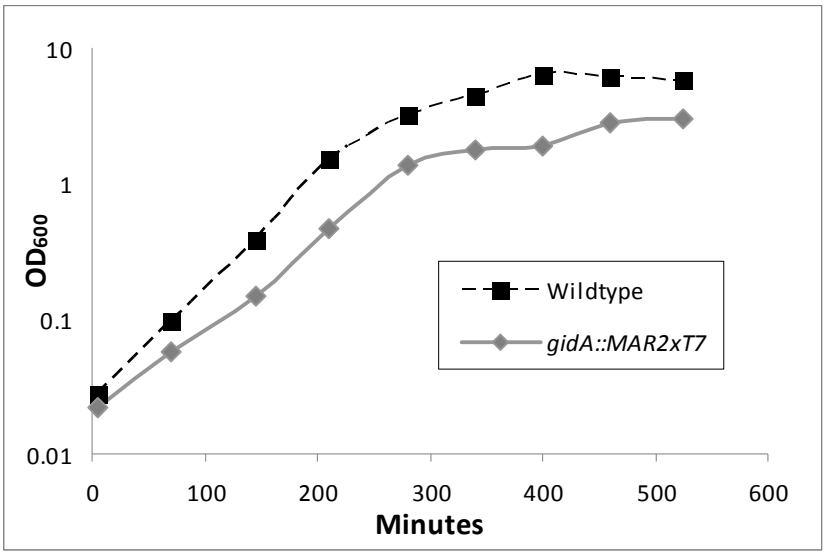

Fig. 4. Growth curve comparison of PA14 *gidA*::MAR2xT7 and parent. PA14 wild type (▪) doubled in 36 minutes while the insertion mutant (♦) doubled in 46.5 minutes (30% slower). Data represent a single experiment.

Complementation with pCF430 Plasmid Vector.

To confirm whether or not the transposon insertion in *gidA* was responsible for the QS-dependent phenotypes observed in the high-throughput screen, we used the broad-host-range expression vector pCF430 [22] to reintroduce *gidA* and attempt to 1) restore wild-type phenotypes, and 2) exclude polar effects on downstream genes.

In our initial milk-plate assays, proteolysis with the *gidA* transposon-insertion mutant was only slightly reduced (Table 2). However, we found that this effect was much more pronounced in liquid-culture supernatant. So for complementation analysis, it was more suitable to assess skim-milk proteolysis in liquid-culture supernatant. In 10% skim-milk agar, we observed halo formation after 24 hours at 37°C around cutouts filled with respective culture supernatant. PA14 *gidA*::MAR2xT7/pCF430 was still proteolysis

deficient, while proteolysis was complemented in PA14 *gidA*::MAR2xT7/pCF*gidA*, which produced a large halo around its respective cutout (Fig. 5). Supernatant from both wild-type transformants created large halos.

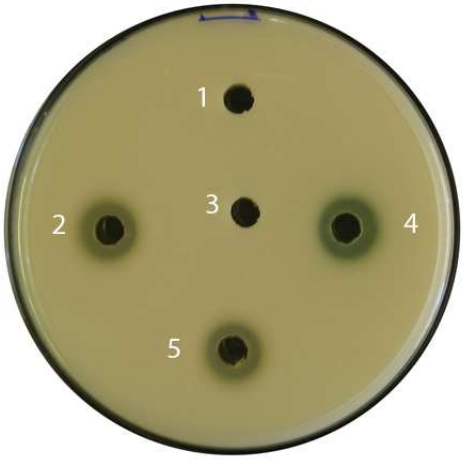

Fig. 5. Complementation of skim-milk proteolysis in PA14 *gidA*::MAR2xT7 and PA14 parent. Cutouts represent: (**1**) PA14 *gidA*::MAR2xT7/pCF430; (**2**) PA14/pCF*gidA*; (**3**) sterile media; (**4**) PA14 *gidA*::MAR2xT7/pCF*gidA*; (**5**) PA14/pCF430. This plate is representative of three biologically independent repetitions.

LasA staphylolytic activity was measured in quadruplicate for all four transformants. Supernatant from PA14 *gidA*::MAR2xT7/pCF430 was unable to lyse *S. aureus* cells. However, lysis was restored in PA14 *gidA*::MAR2xT7/pCF*gidA* to a level similar to PA14/pCF430 and PA14/pCF*gidA* (Figs. 6-7).

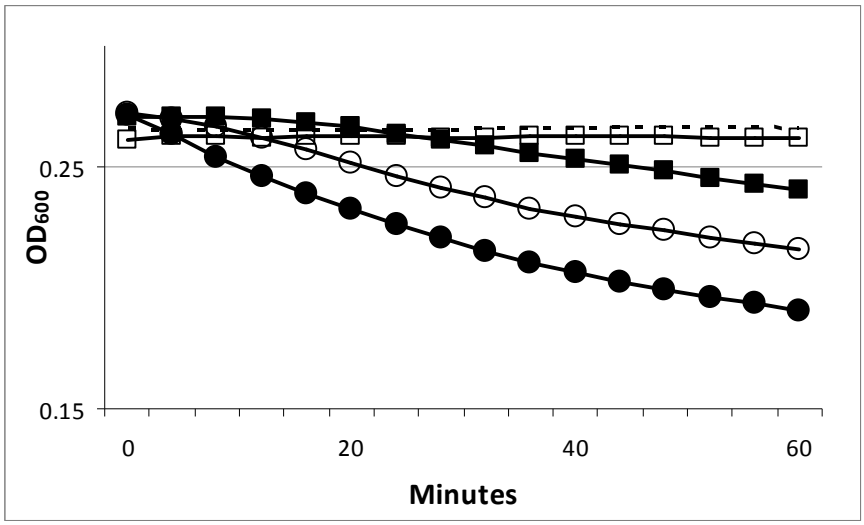

Fig. 6. Complementation of staphylolytic activity in PA14 *gidA*::MAR2xT7 and PA14 parent (raw data). Supernatant from stationary-phase cultures: (□) PA14 *gidA*::MAR2xT7/pCF430; (▪) PA14 *gidA*::MAR2xT7/pCF*gidA*; (○) PA14/pCF430; (●) PA14/pCF*gidA*; and control using sterile media (dashed trend).

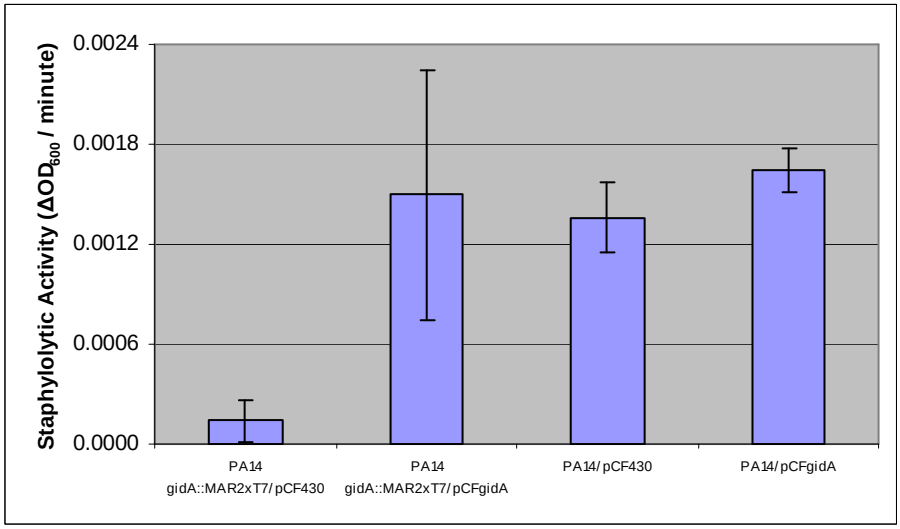

Fig. 7. Staphylolytic activity of complemented PA14 *gidA*::MAR2xT7 and parent expressed as decrease in OD_{600} per minute. Values are means of three biologically independent experiments, and normalized with respective culture OD_{600}. Error bars indicate ± 1 standard deviation.

We determined pyocyanin production for each transformant by extracting pyocyanin from culture supernatants. Pyocyanin production was restored in PA14 *gidA*::MAR2xT7/pCF*gidA* and overexpression of *gidA* in PA14/pCF*gidA* led to pyocyanin levels much greater than in PA14/pCF430 (Fig. 8). Rhamnolipid production was measured by inoculating circular cutouts in rhamnolipid-detection agar with 100 µl of stationary-phase liquid culture. In this case, PA14 *gidA*::MAR2xT7/pCF430 produced very little rhamnolipids while production was fully complemented in PA14 *gidA*::MAR2xT7/pCF*gidA* which produced a halo like that of the two wild-type transformants (Fig. 9). Overexpression of *gidA* in PA14/pCF*gidA* did not appear to significantly increase rhamnolipid production beyond levels seen in PA14/pCF430 (Fig. 9).

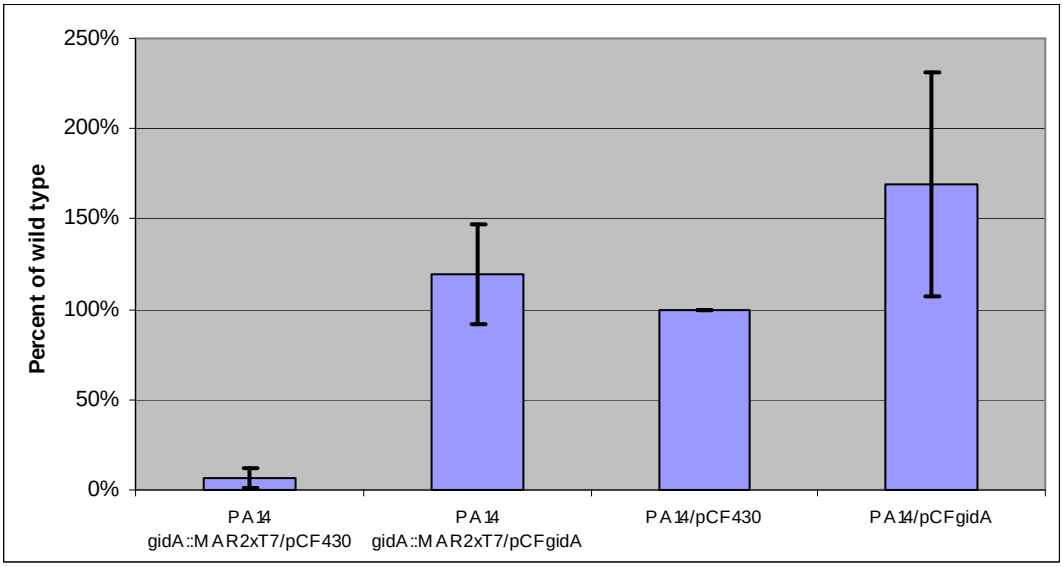

Fig. 8. Normalized average pyocyanin production of complemented PA14 *gidA*::MAR2xT7 and parent. Data are from three biologically independent experiments, normalized for OD_{600} of respective culture, and expressed as percent of pyocyanin production in PA14/ pCF430. Error bars indicate ± 1 standard deviation.

Fig. 9. Rhamnolipid production of complemented PA14 *gidA*::MAR2xT7 and parent. Cutouts represent: (**1**) PA14 *gidA*::MAR2xT7/pCF430; (**2**) PA14/pCF*gidA*; (**3**) sterile media; (**4**) PA14 *gidA*::MAR2xT7/pCF*gidA*; and (**5**) PA14/pCF430. This plate is representative of three biologically independent repetitions.

DISCUSSION

To identify genes required for QS-dependent phenotypes, we screened the entire nonredundant PA14 transposon insertion library [17] using a 96-pin replicator and rectangular LB and skim-milk plates. This method allowed one person to easily screen about sixty 96-well plates in a short two-four week period. Consequently, the plethora of data required an electronic database for analysis.

After examining these data, two mutants—PA14 *suhB*::MAR2xT7 and PA14 *gidA*::MAR2xT7—stood out as having sufficient growth yet clear deficiencies in all four phenotypes—skim-milk proteolysis, staphylolytic activity, pyocyanin production, and rhamnolipid production. Previous work also indicated *gidA* in virulence regulation [18, 19]; so we chose to further characterize PA14 *gidA*::MAR2xT7 through complementation analysis. Complementation revealed that expression of *gidA* via pCF*gidA* restored each phenotype deficiency. Additionally, in PA14/pCF*gidA,* pyocyanin production increased to levels above the wild type.

The initial high-throughput results were significantly dependent on growth conditions. For example, PA14 *gidA*::MAR2xT7 exhibited some skim-milk proteolysis when grown on skim-milk plates (Table 2). However, this mutant did not show any proteolysis when the respective liquid-culture supernatant was assayed (Fig. 5). This condition-dependent regulation, common in *P. aeruginosa,* may have caused us to overlook other QS-deficient mutants in our initial screen. With the rhamnolipid assay, both the *lasR* and *lasI* mutants showed little control over the *rhl* system (rhamnolipid production). However, in the assay for pyocyanin production, which used liquid culture, *lasR* and *lasI* mutants showed clear control of the *rhl* system (data not shown).

Additionally, the *gacA* and *vfr* mutants only lacked the respective QS-dependent phenotypes when the assay involved growth in liquid culture—staphylolytic activity and pyocyanin production (Table 2). This conditional dependency indicates that QS in *P. aeruginosa* involves greater hierarchical control when growth occurs in liquid culture compared to surface growth.

Results for other known QS regulators (Table 2) were largely consistent with their previously described roles. Both *rsmA, rslA* and *qscR* act to inhibit QS [8]. So, as expected, their respective insertion mutants showed no significant phenotypic changes from the wild-type strain. The genes *rpoS, relA*, and *mvfR* complement and/or play a role in the timing of QS initiation under specific conditions [8]; thus, their respective mutants also maintained wild-type phenotypes in each assay. Yet, the *rhlI*-insertion mutant showed levels of rhamnolipid production that were very similar to the PA14 wild-type (Table 2). Two situations can explain this phenomenon. First, the insertion mutation, located 236 base pairs from the N-terminus [29], may not completely inhibit C4 HSL production. Secondly, a mechanism may make up for the lack of C4-HSL signal. Perhaps the *Pseudomonas* quinolone signal (PQS)—a third signal involved in a parallel pathway that also controls *rhl*-targeted genes [8]—is able to induce rhamnolipid production. However, this secondary mechanism would likely still be *rhlR*-dependent due to the stringent control over rhamnolipid production by *rhlR* (Table 2).

In our genome-wide screen for milk-proteolysis deficiency, 75 mutants were identified and further screened for deficiencies in staphylolytic activity, rhamnolipid production, and pyocyanin production. Two mutants stood out as possible global, QS regulators. These mutants displayed deficiencies that were similar to deficiencies in *lasR*

and/or *rhlR* mutants with all four phenotypes. Additionally, these two mutants were still able to grow well in each assay and the high-throughput growth curve.

The first had a MAR2xT7 insertion in *suhB*—a putative inositol phosphatase. Mutations in the homolog (70% identity) in *Escherichia coli* cause a cold-sensitive phenotype [31]. The PA14 *suhB*::MAR2xT7 mutant similarly displayed cold sensitivity; it could not grow on skim-milk agar at room temperature. Thus, it was initially recorded as lacking skim-milk proteolysis. However, repeating the assay at 37°C revealed ample skim-milk proteolysis (data not shown). Inada *et al.* propose that the *suhB* gene product in *E. coli* possesses anti-RNase III activity thereby increasing dsRNA stability; this would be especially important at lower temperatures, explaining the cold-sensitive phenotype [31]. This phenotype could not be restored with exogenous inositol-1-phosphatase activity, so the mode of action for this anti-RNase III activity remains unclear [32]. This activity may be critical for RNAs essential to QS; yet, more work needs to been done to confirm that *suhB* is actually required for QS in *P. aeruginosa*.

We also found that PA14 *gidA*::MAR2xT7 showed clear deficiency in all four phenotypes. Deficiency in skim-milk-proteolysis was most pronounced in liquid-culture. Homologous forms of *gidA* are found throughout Bacteria and Eukarya in two distinct sizes; there is a larger form (~600 amino acids) and a shorter form (~480 amino acids) which is truncated on the C-terminal end [33]. The gene in question is the larger and sole form of *gidA* in *P. aeruginosa*.

Most recent data indicate that *gidA* is involved in tRNA modification [34]. More specifically, *gidA* along with *mnmE* are responsible for adding the carboxymethylaminomethyl (cmnm) group to position 5 of the U34 of tRNAs that read

codons ending with A or G [34]. This modification is important for preventing frameshifts and translation pausing or jamming [33, 34], but the exact roles played by *gidA* and *mnmE* have yet to be elucidated. Still, *gidA* was identified as a global regulator in *Pseudomonas syringae* [18] and as essential for translation of *act*, a significant virulence factor in *Aeromonas hydrophila* [19].

To confirm our results with PA14 *gidA*::MAR2xT7, we first verified that PA14 *gidA*::MAR2xT7 was accurately designated in the PA14 nonredundant transposon-insertion set by confirming transposon localization through PCR (Fig. 3). Additionally, we carried out complementation analysis where wild-type *gidA*, carried on a plasmid, was introduced into PA14 *gidA*::MAR2xT7 and its parent. Expression of plasmid-borne *gidA* was controlled by the arabinose-inducible P_{BAD} promoter. Pyocyanin production, skim-milk proteolysis, staphylolytic activity, and rhamnolipid production were all restored in the complemented mutant. In addition, the wild-type with pCF*gidA* showed elevated pyocyanin production. This complementation rules out possible polar effects as the source of the altered phenotypes in PA14 *gidA*::MAR2xT7.

While there is good evidence that these phenotypic changes arose from insertion mutation in the *gidA* ORF, some additional work will be necessary for establishing the importance of *gidA* to QS regulation. First, the respective assays (including an additional one measuring autoinducer levels) need to be repeated until clear statistical significance can be established. Also, by assaying the *mnmE*::MAR2xT7 mutant which may have been overlooked in the initial high-throughput screen, we could demonstrate whether the phenotypic deficiencies observed in PA14 *gidA*::MAR2xT7 are actually a result of insufficient tRNA modification. Additionally, translational-fusion experiments could be

used to look for inhibited translation of specific transcripts like *lasR* or *rhlR* mRNA, and Western-blot analysis of LasR and RhlR could demonstrate variation in overall expression of these key QS proteins.

Taken together, we demonstrate use of the PA14 transposon insertion library [17] for a genome-wide screen of QS-deficient phenotypes and show the importance of understanding condition-dependent phenotypes. Finally, our data suggest that *gidA* plays an essential role in the complex QS gene regulatory network of the opportunistic pathogen *P. aeruginosa*.

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