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The Two-Component Regulators GacS and GacA Influence Accumulation of the Stationary-Phase Sigma Factor σ^S and the Stress Response in *Pseudomonas fluorescens* Pf-5

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Three global regulators are known to control antibiotic production by *Pseudomonas fluorescens*. A two-component regulatory system comprised of the sensor kinase GacS (previously called ApdA or LemA) and GacA, a member of the FixJ family of response regulators, is required for antibiotic production. A mutation in *rpoS*, which encodes the stationary-phase sigma factor σ^S, differentially affects antibiotic production and reduces the capacity of stationary-phase cells of *P. fluorescens* to survive exposure to oxidative stress. The *gacA* gene of *P. fluorescens* Pf-5 was isolated, and the influence of *gacS* and *gacA* on *rpoS* transcription, σ^S levels, and oxidative stress response of Pf-5 was determined. We selected a *gacA* mutant of Pf-5 that contained a single nucleotide substitution within a predicted α-helical region, which is highly conserved among the FixJ family of response regulators. At the entrance to stationary phase, σ^S content in *gacS* and *gacA* mutants of Pf-5 was less than 20% of the wild-type level. Transcription of *rpoS*, assessed with an *rpoS-lacZ* transcriptional fusion, was positively influenced by GacS and GacA, an effect that was most evident at the transition between exponential growth and stationary phase. Mutations in *gacS* and *gacA* compromised the capacity of stationary-phase cells of Pf-5 to survive exposure to oxidative stress. The results of this study provide evidence for the predominant roles of GacS and GacA in the regulatory cascade controlling stress response and antifungal metabolite production in *P. fluorescens*.

Certain strains of fluorescent pseudomonads inhabit root and seed surfaces, where they suppress plant diseases caused by soilborne plant pathogens. Antifungal metabolites produced by *Pseudomonas* spp. in situ contribute to the suppression of plant disease (47). *Pseudomonas fluorescens* Pf-5 suppresses plant diseases caused by the fungal pathogens *Pythium ultimum* (20) and *Rhizoctonia solani* (19) and produces at least four antifungal secondary metabolites: pyoluteorin (20), pyrrolnitrin (19), 2,4-diacetylphloroglucinol (37), and hydrogen cyanide (HCN) (26). Secondary metabolite production by *Pseudomonas* spp. does not occur uniformly in all environments but is subject to regulation by genes responding to unknown environmental or physiological signals. Mutations in regulatory genes that alter antifungal metabolite production can improve or diminish biological control by *P. fluorescens* (5, 12, 30, 44). Therefore, elucidation of molecular mechanisms regulating antifungal metabolite production of *P. fluorescens* is likely to provide opportunities for enhancement of biological control.

In *P. fluorescens*, antifungal metabolite production and biological control are controlled by a two-component regulatory system comprised of GacS and GacA, which are highly conserved among *Pseudomonas* spp. (25, 41). *gacA* (12, 30) encodes a response regulator in the FixJ family, and *gacS* (also called *apdA*, *lemA*, *repA*, or *pheN*) (6, 25) encodes the cognate sensor kinase. GacS (for global activator sensor kinase) was renamed recently to reflect the high degree of deduced amino acid sequence similarity and functional conservation among homologues present in various species of *Pseudomonas* (25). *gacS* and *gacA* are required for production of pyrrolnitrin, pyoluteorin, 2,4-diacetylphloroglucinol, HCN, extracellular protease(s), and tryptophan side chain oxidase (TSO) by strains of *P. fluorescens*. *gacS* and *gacA* mutants produce none of these secondary metabolites or exoenzymes (6, 12, 30) and are less effective than wild-type strains in suppressing disease (5, 12, 30, 39). Mutants with nucleotide substitutions in *gacA* accumulate in late-stationary-phase cultures of *P. fluorescens* (7, 10). The functional *gacA* allele *gacA*(Y49), which specifies a tyrosine residue at position 49 (30), apparently was isolated from such a mutant of *P. fluorescens* CHA0. The wild-type *gacA* gene from strain CHA0, termed *gacA*(D49), encodes an aspartate residue at position 49 (3, 40).

The stationary-phase sigma factor σ^S is a third regulator of antibiotic production in *P. fluorescens*. In *Escherichia coli*, σ^S directs the transcription of many genes expressed upon entry into stationary phase (31) and in response to starvation (34, 38) or osmotic stress (17). Some genes transcribed by the σ^S-RNA polymerase holoenzyme confer stress tolerance on stationary-phase cells of *E. coli* (34, 42). In *P. fluorescens* Pf-5, an *rpoS* mutation is pleiotropic, reducing the bacterium's capacity to survive oxidative stress and altering the spectrum of secondary metabolite production (44). An *rpoS* mutant of Pf-5 overproduces pyoluteorin and 2,4-diacetylphloroglucinol but produces no pyrrolnitrin (39, 44). Characterization of σ^S-regulated phenotypes of Pf-5 provided the first evidence that a single

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference
P. fluorescens		
Pf-5	Rhizosphere isolate	19
JL3985	Derivative of Pf-5, *rpoS*::Tn*5*	44
JL4135	Derivative of Pf-5, *gacS*::Tn*5*	6
JL4477	Derivative of Pf-5, *gacA*(V203)	This study
JL4489	Derivative of Pf-5, *rpoS*::*lacZ*	This study
JL4491	Derivative of JL4135, *gacS*::Tn*5* *rpoS*::*lacZ*	This study
JL4492	Derivative of JL4477, *gacA*(V203) *rpoS*::*lacZ*	This study
E. coli DH5α	F^- *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96 relA1* ϕ80d*lacZ*ΔM15 λ^-	43
Plasmids		
pUC19	ColE1 replicon, Amp^r	43
pRK2013	Mobilizing plasmid, Tra^+ Km^r	11
pRK415	IncP1 replicon, polylinker of pUC19, Mob^+ Tc^r	23
pME3066	1.65-kb *Bam*HI-*Bgl*II fragment containing *gacA*(Y49) from *P. fluorescens* CHA0 cloned in pLAFR3, Mob^+ Tc^r	30
pMini-Tn*5lacZ1*	Mini-Tn*5* containing promoterless *lacZ* on a 4.1-kb *Sma*I fragment cloned in pUT, Km^r Tc^r	8
pJEL01	Stably maintained in *E. coli* or *Pseudomonas* spp., replicons from pVSP1 and pACYC184, Mob^+ Tc^r	44
pJEL5649	2.9-kb *Eco*RI fragment containing *rpoS* from Pf-5 cloned in pJEL01, Mob^+ Tc^r	44
pJEL5926	*rpoS-lacZ* transcriptional fusion cloned in pRK415, Mob^+ Tc^r	This study
pJEL5937	1.65-kb *Bam*HI-*Bgl*II fragment containing *gacA* from Pf-5 cloned in pUC19, Amp^r	This study

[a] Abbreviations: Amp^r, Km^r, and Tc^r, resistance to ampicillin, kanamycin, and tetracycline, respectively.

regulatory gene can control both antibiotic production and stress response in *P. fluorescens* (44).

The research described here was undertaken to determine if the GacS-GacA two-component regulatory system and σ^S interact or operate through independent regulatory circuits in Pf-5. In this study, we describe the nucleotide sequence of the *gacA* gene of Pf-5 and demonstrate that GacS and GacA influence σ^S accumulation and *rpoS* transcription in Pf-5. We also demonstrate that *gacS* and *gacA*, like *rpoS*, are required for optimal survival of stationary-phase cells of Pf-5 when exposed to oxidative stress.

(Portions of this work were published earlier as abstracts [7, 48].)

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids are listed in Table 1. *P. fluorescens* was grown at 27°C, with shaking at 200 rpm, in King's medium B broth (KMB) (24) for routine culturing; in KMB broth amended with glycine (4.4 g/liter) for HCN assays; in Luria-Bertani (LB) medium (43) for transcriptional fusion studies and Western analysis; in nutrient broth (Difco Laboratories, Detroit, Mich.) supplemented with 2% (wt/vol) glucose or 1% (wt/vol) glycerol for antibiotic extractions; in nutrient broth supplemented with 1% (wt/vol) glycerol for TSO assays; or in M9 minimal medium (M9) supplemented with 0.4% glucose (43) for Western analysis and oxidative stress tests. Cells of *P. fluorescens* were enumerated by spreading serial dilutions of bacterial suspensions on KMB. Cultures of *E. coli* were routinely grown in LB medium at 37°C.

Recombinant DNA techniques. Methods for transformations, digestions with restriction enzymes, and gel electrophoresis were standard (43). Blunt-end ligation was performed by the thermal cycling method (33). Enzymes were from Gibco BRL Life Technologies (Gaithersburg, Md.). Plasmids were purified by an alkaline lysis procedure (43). Plasmids were mobilized from *E. coli* DH5α donors into Pf-5 in triparental matings with helper plasmid pRK2013 (11). Transconjugants were selected on KMB containing 200 μg of tetracycline per ml.

Derivation of a *gacA* mutant of Pf-5. Strain JL4477, a derivative of Pf-5 containing a point mutation in *gacA*, was selected by the method described by Duffy and Défago (10). Pf-5 was grown in nutrient broth amended with 0.5% yeast extract at 27°C. After 6 days, dilutions of cultures were spread onto LB agar. Colonies that appeared orange in comparison to the wild-type strain after several days incubation at 27°C (a characteristic of *gacA* mutants [10]) were screened for loss of extracellular protease activity on Bacto Litmus milk agar (Difco). Protease-deficient mutants were evaluated for antibiotic production by reverse-phase thin-layer chromatography as described previously (26).

Cloning of *gacA* from Pf-5. An extant genomic library of Pf-5 (39) was screened by colony hybridization (14) to identify cosmids that hybridized to *gacA*(Y49) of *P. fluorescens* CHA0 (30). The *gacA*(Y49) probe, a 1.65-kb *Bam*HI-*Bgl*II fragment of pME3066, was labeled with [^{32}P]dCTP or biotinylated dATP by using a nick translation kit (Gibco BRL Life Technologies) and purified over a D50 column (International Biotechnologies Inc., New Haven, Conn.). Southern analysis identified restriction fragments in cosmids that hybridized to the *gacA*(Y49) probe. A 1.65-kb *Bam*HI-*Bgl*II fragment that hybridized to the probe was cloned into pUC19 to construct pJEL5937.

Sequence analysis of *gacA* alleles. DNA sequencing and oligonucleotide syntheses were done at the Center for Gene Research and Biotechnology at Oregon State University, Corvallis. Sequencing of double-stranded templates was done on an ABI model 373A automated DNA sequencer using a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, Calif.) according to the manufacturer's protocol. Oligonucleotide primers were synthesized on an ABI model 380B DNA synthesizer using phosphoramidite chemistry (1). Sequencing of the *gacA* gene of Pf-5 was done with primers complementary to pUC19 DNA on either side of the polylinker and by oligonucleotide primers complementary to regions within the 1.65-kb fragment of pJEL5937 containing *gacA*. Sequencing of an allele of *gacA* with a point mutation [termed *gacA*(V203)] was performed directly on the PCR product amplified from the genome of JL4477 with primers designed from the sequence of the *gacA* gene cloned in pJEL5937. Analyses of DNA and deduced protein sequences and comparisons with sequences in the GenBank database were accomplished with software from the Genetics Computer Group, Inc., Madison, Wis. (9). Theoretical secondary structures of proteins encoded by alleles of *gacA* were predicted by PepPlot and PlotStructure programs (Genetics Computer Group).

Antibiotic quantification. Antibiotics were extracted from cells and spent media of cultures grown in triplicate as described previously (37). Pyoluteorin and pyrrolnitrin concentrations were quantified from cultures grown for 2 days at 20°C in 5 ml of nutrient broth containing 1% glycerol, a medium that favors their production. The concentration of 2,4-diacetylphloroglucinol was quantified from cultures grown for 4 days in 5 ml of nutrient broth containing 2% glucose, a medium that favors its production. Restoration of antibiotic production in JL4477 harboring plasmid pME3066 was assessed in the absence of tetracycline, which decreased the growth rate of the strain. Culture supernatants were extracted twice with ethyl acetate, and excess water was removed with anhydrous $MgSO_4$. The bacterial pellet was extracted with acetone. Extracts dissolved in methanol were analyzed by C_{18} reverse-phase high-performance liquid chromatography (0.8- by 10-cm Waters Nova-Pak radial compression cartridge; 45% water–30% acetonitrile–25% methanol [vol/vol]; 1.5 ml/min). Antibiotics were detected with a UV photodiode array detector at 225 (pyrrolnitrin), 310 (pyoluteorin), and 278 (2,4-diacetylphloroglucinol) nm and quantified against authentic standards. Quantification was done twice, with similar results.

Exoenzyme production. Extracellular protease was assessed visually as a cleared zone around bacterial colonies on Bacto Litmus milk agar (Difco) following incubation at 27°C for 48 h.

TSO production was quantified from duplicate cultures of *P. fluorescens* grown at 27°C for 48 h with shaking. Cells from 1 ml of culture were harvested, washed, and suspended in 100 μl of ice-cold 50 mM potassium phosphate, pH 6.0. Cells were lysed by two sequential cycles of rapid freezing in liquid nitrogen followed by thawing at 45°C. Cell debris was harvested at 4°C, and the supernatant was incubated at room temperature in 1.0 mM acetyl-L-tryptophanamide–50 mM potassium phosphate, pH 6.0. The production of *N*-acetyl-α,β-didehydrotryptophanamide was monitored spectrophotometrically at 333 nm (ε_{mM} of 19.8 cm^{-1}) at 5-min intervals for 30 min and at 60, 120, and 180 min (36). The rate of production (amount of *N*-acetyl-α,β-didehydrotryptophanamide produced per

TABLE 2. Secondary metabolite and exoenzyme production by *P. fluorescens* Pf-5 and derivatives

Strain	Characteristics	Concn (mean ± SD)					Extracellular protease(s)[d]
		Pyrrolnitrin (μg/ml)[a]	2,4-Diacetylphloroglucinol (μg/ml)[a]	Pyoluteorin (μg/ml)[a]	HCN (μmol/10^{10} CFU)[b]	TSO (U/10^{10} CFU)[c]	
Pf-5	Wild type	3.6 ± 0.4	26.2 ± 0.5	9.4 ± 1.5	30 ± 1	2,290 ± 290	+
JL4135	*gacS*::Tn*5*	−	−	−	−	−	−
JL4477	*gacA*(V203)	−	−	−	9 ± 1	4 ± 3	−
JL4477(pME3066)	*gacA*(V203) *gacA*(Y49)	1.1 ± 0.4	32.3 ± 1.4	28.4 ± 1.1	38 ± 13	390 ± 3	+
JL3985	*rpoS*::Tn*5*	−	60.5 ± 15.6	30.9 ± 0.1	88 ± 15	−	+
JL4489	*rpoS*::*lacZ*	0.5 ± 0.1	29.0 ± 1.1	27.8 ± 0.1	110 ± 34	59 ± 18	+

[a] Mean from three replicate cultures; −, below the detection limit of 0.1 μg/ml.
[b] Mean from two replicate cultures; −, below the detection limit of 1 μM.
[c] Mean from two replicate cultures; −, below the detection limit of 0.1 U. One unit is defined as the amount of enzyme that catalyzed the formation of 1 μmol of *N*-acetyl-α,β-didehydrotryptophanamide/min (36).
[d] +, detected; −, not detected.

minute) was determined from the linear portion of a curve relating absorbance at 333 nm to time. TSO production was normalized to CFU and reported as enzymatic units per 10^{10} CFU. One unit of TSO is defined as the amount of enzyme that catalyzed the formation of 1 μmol of *N*-acetyl-α,β-didehydrotryptophanamide/min (36). Quantification was done twice, with similar results.

HCN production. To quantify HCN production (15, 16), duplicate cultures were grown at 27°C for 48 h with shaking. A sample from each culture was incubated in the presence of 0.1 N NaOH at room temperature for 3 h in a chamber sealed with paraffin. The NaOH fraction was diluted with 0.1 N NaOH to a concentration within the linear range of a standard curve relating the concentration of an NaCN standard to absorbance at 578 nm; 0.04 ml of the diluted NaOH fraction was added to 1.0 ml of a solution comprised of 2 parts 0.2 M 4-nitrobenzaldehyde in ethylene glycol monomethyl ether, 2 parts 0.1 M *o*-dinitrobenzene in ethylene glycol monomethyl ether, and 1 part 0.088 N NaOH. After 25 min of incubation in the dark at room temperature, absorbance at 578 nm was measured. HCN was quantified against an NaCN standard curve and normalized to CFU. Quantification was done twice, with similar results.

Western analysis of σ^S and GacS. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transblotting for Western analysis were performed as specified by the manufacturer (Bio-Rad Laboratories, Hercules, Calif.). Exponential-phase cells were obtained from cultures grown to an optical density at 600 nm (OD_{600}) of 0.2 to 0.4 (for reference, see Fig. 3A, $t = 0$ to 1 h). Early-stationary-phase cells were obtained from cultures grown until the optical density stopped increasing exponentially (see Fig. 3A, $t = 2$ to 3 h), and an additional stationary-phase sample was obtained from cultures 4 h later (see Fig. 3A, $t = 6$ to 7 h). Optical densities were used to estimate the volume of each culture that would provide an equivalent number of bacterial cells. Cells from that volume were harvested, immediately frozen in an ethanol–dry-ice bath, and extracted by boiling in protein sample buffer (Bio-Rad) containing 5% 2-mercaptoethanol. Proteins were separated by SDS-PAGE on a 12% gel and transferred onto a nitrocellulose membrane (Bio-Rad) for Western analysis. Blots were incubated with polyclonal antibodies to *E. coli* σ^S, generously supplied by K. Tanaka (46), and the antibodies were detected by enhanced chemiluminescence as specified by the manufacturer (Amersham Life Science Inc., Arlington Heights, Ill.). Blots were stripped at 65°C for 30 min in a solution of 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7. Blots were then incubated with polyclonal antibodies to GacS (previously LemA) from *Pseudomonas syringae*, generously supplied by T. Kitten and D. K. Willis (41), and the antibodies were detected by enhanced chemiluminescence. σ^S and GacS were quantified by using a Molecular Dynamics model SI personal densitometer and ImageQuant software, version 4.1 (Molecular Dynamics, Sunnyvale, Calif.), and the results were normalized based on Bradford assays for total protein (Bio-Rad). In the absence of a purified standard for quantification, the linear range of σ^S detection was determined by using a dilution series of Pf-5 protein extracts. Samples quantified were within the linear range of detection. Cell content of σ^S and GacS is reported relative to the amount in stationary-phase Pf-5 cells. Each experiment was done twice, with similar results.

Transcription of *rpoS*. A transcriptional fusion of *lacZ* to *rpoS* of Pf-5 was constructed by inserting a 4.1-kb blunt-ended *Sma*I fragment from pMini-Tn*5lacZ1* (8) into a blunted *Xho*I site, located 36 nucleotides from the 3′ end of *rpoS*. The *rpoS*::*lacZ* transcriptional fusion cloned in pJEL5926 was exchanged with the genomic copy of *rpoS* in Pf-5 to derive JL4489, in JL4135 to derive JL4491, and in JL4477 to derive JL4492 by marker exchange mutagenesis as described previously (27). The *rpoS*::*lacZ* mutation in each strain was complemented with pJEL5649, a multicopy plasmid carrying the wild-type *rpoS* gene. From duplicate cultures of each strain grown in LB medium, β-galactosidase activity was determined at 1-h intervals for 8 h as described by Miller (35). Cells were made permeable with SDS and $CHCl_3$ and then incubated for 10 min at 28°C, after which *o*-nitrophenyl-β-D-galactopyranoside was added to a final concentration of 0.66 mg/ml. β-Galactosidase was expressed as Miller units (35), and numbers of CFU were determined to verify that optical density was an accurate representation of cell density in all strains. The experiments were done twice, with similar results.

Stress response. Survival of *P. fluorescens* following exposure to H_2O_2 was determined as previously described (44), with slight modifications. Cultures were grown in M9 with 0.4% glucose, and stationary-phase cells were harvested at 4 and 8 h after the optical density of cultures stopped increasing. Harvested cells were washed once and suspended in 5 ml of M9 without glucose to obtain an OD_{600} of 0.8. Suspended cells were exposed to 15 mM H_2O_2 and incubated with shaking at 27°C for 1 h; then CFU were enumerated at 20-min intervals. Three replicate cultures were evaluated for each treatment. The experiment was done twice, with similar results.

Nucleotide sequence accession number. The GenBank accession number for the DNA sequence of the *gacA* gene of *P. fluorescens* Pf-5 is AF065156.

RESULTS

Sequence analysis of *gacA* alleles. A 1.65-kb *Bam*HI-*Bgl*II fragment that hybridized to *gacA* from *P. fluorescens* CHA0 (30) was identified from a genomic library of Pf-5. The deduced amino acid sequence of a 639-bp open reading frame present on the fragment is identical to that of the wild-type GacA(D49) of *P. fluorescens* CHA0 (40), and the open reading frame was therefore identified as *gacA* of Pf-5. JL4477, a spontaneous mutant of Pf-5 exhibiting the colony morphology described for *gacA* mutants of CHA0 (10), had an allele of *gacA* with a T rather than a C at nucleotide 607. Consequently, the deduced amino acid sequence of the mutant allele, heretofore called *gacA*(V203), has a valine rather than an alanine at position 203. The 314 nucleotides immediately upstream of *gacA* in JL4477 were identical to those upstream of *gacA* in Pf-5.

Phenotypic analysis of *gacS*::Tn*5*, *gacA*(V203), *rpoS*::Tn*5*, and *rpoS*::*lacZ* derivatives of Pf-5. Pf-5 produced pyrrolnitrin, 2,4-diacetylphloroglucinol, pyoluteorin, extracellular protease(s), HCN, and TSO (Table 2). JL4135 (*gacS*::Tn*5*) produced no detectable antibiotics, extracellular protease(s), HCN, or TSO. JL4477 [*gacA*(V203)] produced no detectable antibiotics or extracellular protease(s), and it produced less HCN and TSO than Pf-5 produced. Production of pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol, HCN, TSO, and extracellular protease(s) by JL4477 [*gacA*(V203)] was restored with plasmid pME3066, containing a functional *gacA*(Y49) allele from CHA0.

Two derivatives of Pf-5 containing insertions in *rpoS* produced more pyoluteorin and HCN and less pyrrolnitrin than was produced by the wild-type strain (Table 2). JL3985 (*rpoS*::Tn*5*) overproduced 2,4-diacetylphloroglucinol, whereas JL4489 (*rpoS*::*lacZ*) produced wild-type levels of this antibiotic. Both JL3985 and JL4489 produced an extracellular protease(s). JL3985 produced no detectable TSO, but JL4489 produced trace levels of TSO. In JL4489, the *lacZ* insertion is located 36 nucleotides from the 3′ terminus of *rpoS*, corresponding to domain 4.2 of σ^S, which is involved in recognition of the −35

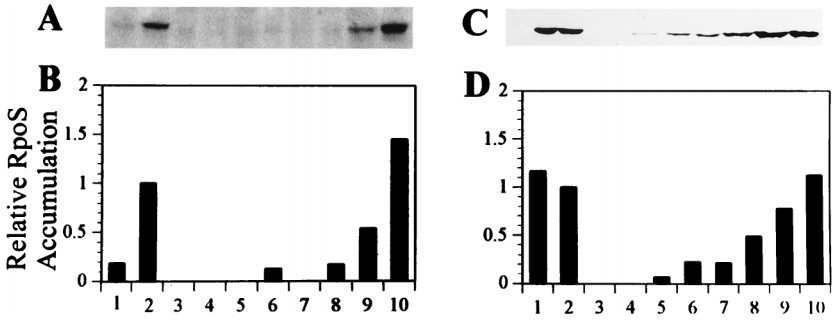

FIG. 1. Relative σ^S accumulation. σ^S was visualized with antibodies to *E. coli* σ^S (46) from Western blots of protein extracted from cultures grown in LB medium (A and B) or M9 containing 0.4% glucose (C and D). σ^S content in each lane was estimated by scanning Western blots with a densitometer. For each sample, σ^S content was normalized to total protein content, determined from Bradford assays. The normalized σ^S content (σ^S content divided by total protein content) for each sample is reported relative to the normalized σ^S content of stationary-phase cells of Pf-5 grown in the corresponding medium (B and D, lanes 2). Sample numbers correspond to extracts from cells growing exponentially (first of each pair; lanes 1, 3, 5, 7, and 9) or in early stationary phase (second of each pair; lanes 2, 4, 6, 8, and 10): 1 and 2, Pf-5; 3 and 4, JL3985 (*rpoS*::Tn*5*); 5 and 6, JL4135 (*gacS*::Tn*5*); 7 and 8, JL4477 [*gacA*(V203)]; 9 and 10, Pf-5 harboring pJEL5649, a multiple-copy plasmid containing *rpoS* cloned from Pf-5. Scanned images were reproduced for publication by using Adobe Photoshop version 4.0 (Adobe Systems Incorporated, San Jose, Calif.).

region of target promoters (32). Like JL4489, mutants of *E. coli* that produce σ^S proteins with altered lengths, due to insertions or deletions in the C-terminal region adjacent to domain 4.2, confer phenotypes that differ quantitatively from the wild-type phenotype (21, 49).

σ^S accumulation. In LB medium, σ^S was detected at a low level in exponentially growing cells of Pf-5. Upon entry into stationary phase, the cellular content of σ^S increased by 500% (Fig. 1A and B, lanes 1 and 2). σ^S was not detected in exponentially growing cells of JL4135 (*gacS*::Tn*5*) or JL4477 [*gacA*(V203)] (Fig. 1A and B, lanes 5 and 7). At the entrance to stationary phase, σ^S content in JL4135 and JL4477 was less than 20% of the wild-type level (Fig. 1A and B, lanes 2, 6, and 8). Four hours after exponential growth ceased, the σ^S content in JL4135 and JL4477 was 50% of that observed in Pf-5 (data not shown). Multiple copies of *rpoS* enhanced levels of σ^S in derivatives of Pf-5 grown in LB medium (Fig. 1A and B, lanes 1, 2, 9, and 10).

σ^S was detected both in exponentially growing cells and in early-stationary-phase cells of Pf-5 in M9 containing 0.4% glucose (Fig. 1C and D, lanes 1 and 2). In contrast, the content of σ^S in exponentially growing cells of JL4135 (*gacS*::Tn*5*) and JL4477 [*gacA*(V203)] was less than 20% of the wild-type level (Fig. 1C and D, lanes 1, 5, and 7). The contents of σ^S in early-stationary-phase cells of JL4135 and JL4477 were 20 and 50%, respectively, of the wild-type level (Fig. 1C and D, lanes 2, 6, and 8). Four hours after exponential growth ceased, the σ^S content increased in JL4135 and JL4477 to 40 and 50%, respectively, of that observed in Pf-5 (data not shown).

GacS accumulation. The level of GacS increased slightly (by 20%) in Pf-5 during the transition from exponential growth to stationary phase (Fig. 2, lanes 1 and 2). The cellular GacS content was less in JL4477 [*gacA*(V203)] than in Pf-5, for both growth phases and culture media (Fig. 2, lanes 1, 2, 7, and 8). In exponentially growing cells, the GacS content in JL3985 (*rpoS*::Tn*5*) was greater than that in Pf-5 in one experiment (Fig. 2, lanes 1 and 3), but no difference was observed in a second experiment (data not shown). Multiple plasmid-borne copies of *rpoS* decreased GacS levels in stationary-phase cells of Pf-5 (Fig. 2, lanes 2 and 10) in both experiments.

Transcription of *rpoS* assessed with a *lacZ* fusion. β-Galactosidase activity conferred by a chromosomal *rpoS-lacZ* transcriptional fusion increased by 300% within a 1-h period when strain JL4489 (*rpoS*::*lacZ*) began the transition from exponential to stationary phase (Fig. 3A), reflecting an increase in *rpoS* transcription. In JL4491 (*gacS*::Tn*5* *rpoS*::*lacZ*) (Fig. 3B) and JL4492 [*gacA*(V203) *rpoS*::*lacZ*] (Fig. 3C), induction of *rpoS* transcription occurred more gradually and to a smaller magnitude than in strains with functional GacS and GacA proteins (Fig. 3A). Multiple plasmid-borne copies of *rpoS* decreased β-galactosidase activity of stationary-phase cells of JL4489 by 50% (data not shown).

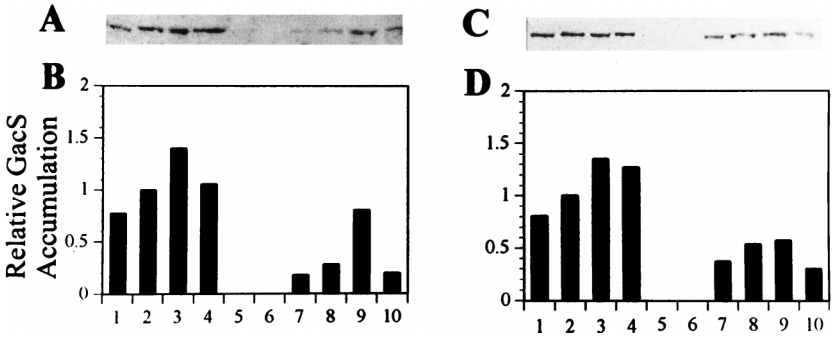

FIG. 2. Relative GacS accumulation. GacS was visualized with antibodies to GacS from *P. syringae* (41) on the Western blots used for quantification of σ^S (Fig. 1). Protein was extracted from cultures grown in LB medium (A and B) or M9 containing 0.4% glucose (C and D). GacS content in each lane was estimated and reported as normalized values (B and D, lanes 2). Sample numbers correspond to extracts from cells growing exponentially (first of each pair; lanes 1, 3, 5, 7, and 9) or in early stationary phase (second of each pair; lanes 2, 4, 6, 8, and 10): 1 and 2, Pf-5; 3 and 4, JL3985 (*rpoS*::Tn*5*); 5 and 6, JL4135 (*gacS*::Tn*5*); 7 and 8, JL4477 [*gacA*(V203)]; 9 and 10, Pf-5 harboring pJEL5649, a multiple-copy plasmid containing *rpoS* cloned from Pf-5. The truncated form of GacS was not quantified in JL4135 (*gacS*::Tn*5*). Scanned images were reproduced for publication by using Adobe Photoshop version 4.0 (Adobe Systems Incorporated).

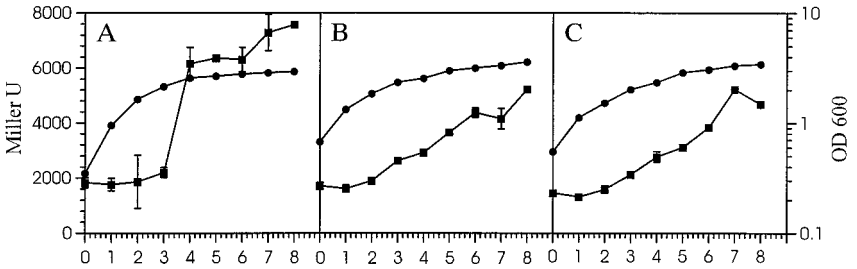

FIG. 3. Growth and β-galactosidase activity of Pf-5 derivatives containing chromosomal *rpoS-lacZ* transcriptional fusions. OD_{600} (●) and β-galactosidase activity expressed in Miller units (■) were determined at 1-h intervals from duplicate cultures grown in LB medium. Bacterial strains were JL4489 (*rpoS*::*lacZ*) (A), JL4491 (*gacS*::Tn*5* *rpoS*::*lacZ*) (B), and JL4492 [*gacA*(V203) *rpoS*::*lacZ*] (C). Error bars representing standard deviations may be obscured by symbols.

Survival of Pf-5 and *gacS*::Tn*5* and *gacA*(V203) derivatives when exposed to oxidative stress. In addition to influencing antibiotic and exoenzyme production, σ^S influences the capacity of Pf-5 to survive oxidative stress (44). Stationary-phase cells of JL3985 (*rpoS*::Tn5) are more sensitive than stationary-phase cells of Pf-5 to hydrogen peroxide (44). Similarly, JL4135 (*gacS*::Tn5) and JL4477 [*gacA*(V203)] harvested from cultures 4 h (Fig. 4) or 8 h (data not shown) after cell density stopped increasing were more sensitive than Pf-5 to exposure to oxidative stress.

DISCUSSION

This report provides the first evidence that the global regulators GacS and GacA influence the σ^S accumulation and stress response of stationary-phase cells of *Pseudomonas* spp. Mutations in *gacS* and *gacA* reduced σ^S accumulation in *P. fluorescens* Pf-5 and compromised the bacterium's capacity to survive exposure to oxidative stress. These data are consistent with the hypothesis that the two-component regulatory system comprised of GacS and GacA can regulate gene expression by influencing σ^S levels. Nevertheless, GacS and GacA are required for the expression of certain phenotypes (such as pyoluteorin and 2,4-diacetylphloroglucinol production) that are not positively regulated by *rpoS*, indicating that the two-component regulatory system also must function through a mechanism other than the control of σ^S.

Transcription of *rpoS*, assessed with a *lacZ* fusion, was positively influenced by GacS and GacA in Pf-5, which is consistent with the pattern of *rpoS* transcription in *Pseudomonas aeruginosa* (29, 40). In that species, GacA positively controls the production of the autoinducer *N*-butyryl-homoserine lactone (40), which, through its interaction with the response regulator RhlR, positively influences the expression of *rpoS* (29). An autoinducer involved in quorum sensing has not been found in Pf-5, but it could be among the unknown components of regulatory circuits controlling antibiotic production and stress response in *P. fluorescens*.

GacS accumulation was diminished in the *gacA*(V203) mutant of Pf-5 and in the presence of multiple plasmid-borne copies of *rpoS*. Positive regulation of GacS content by GacA may be one mechanism by which the relative concentration of the two proteins is controlled. The proper stoichiometric balance of other response regulators within the FixJ family and their cognate sensor kinases is required for normal function of these two-component regulatory systems (18). In *P. fluorescens* CHA0 (40), multiple copies of *gacA*(D49) are not tolerated, and in Pf-5, multiple copies of *gacA* can partially compensate for *gacS* mutations (7), indicating that the system is sensitive to relative GacS and GacA contents. The regulatory mechanisms through which GacA and σ^S influence GacS accumulation are not known, but the findings of this study highlight the complexity of interactions among the three global regulators.

Based on sequence similarities to better-characterized response regulators within the FixJ family, GacA contains two functional domains, an amino-terminal phosphorylation-induced activator domain and a carboxy-terminal output domain characterized by a helix-turn-helix DNA-binding motif (22). Amino acid substitutions within these functional domains typically destroy GacA function, manifested in the loss of multiple phenotypes controlled by the GacS-GacA two-component regulatory system (2). The *gacA*(V203) mutant evaluated in this study differed from those described previously because it lost only a subset of phenotypes controlled by the two-component regulatory system. Analysis of the theoretical secondary structures of GacA(V203) and GacA indicated that the valine substitution may interrupt an α-helical region, which is downstream of the helix-turn-helix motif and highly conserved within the FixJ family (22). This possibility is consistent with valine's assignment as a strong β-sheet-forming residue, whereas the replaced alanine residue is a strong α-helix-forming residue (4, 13). Stibitz (45) demonstrated that mutations within this α-helical region in the response regulator BvgA eliminate expression of two genes but have little effect on the expression of a third gene under the control of the BvgS-BvgA two-component system. Thus, the differential effect of the *gacA*(V203) mutation on phenotypes regulated by GacS and GacA in Pf-5 is not unprecedented and may reflect the importance of the α-helical region as a specificity determinant for recognition of various promoters by GacA. The binding site(s) of GacA has not been described, however, and further exploration of this

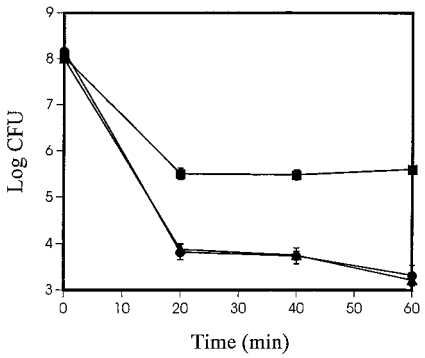

FIG. 4. Survival of oxidative stress. Stationary-phase cells of *P. fluorescens* Pf-5 (■), JL4135 (*gacS*::Tn5) (●), and JL4477 [*gacA*(V203)] (▲) were exposed to 15 mM H_2O_2, and the numbers of culturable cells were estimated over time. Presented values are means of three replicate cultures; error bars representing standard deviations may be obscured by symbols.

possibility would be facilitated by the identification of such target sequences.

The effects of *gacS* and *gacA* mutations on *rpoS* transcription and σ^S accumulation were greater during the transition from exponential growth than later in stationary phase. Nevertheless, the σ^S-mediated stress response of *gacS* and *gacA*(V203) mutants was diminished well into stationary phase. Induction of *rpoS* transcription at the transition between exponential and stationary phases may be critical to the process through which cells develop resistance to environmental stress. Consequently, the stress-resistant state could fail to develop fully if the level of σ^S increases gradually or later in stationary phase. Although the present study focused on *rpoS* transcription and σ^S accumulation, GacS and GacA also could influence *rpoS* translation or σ^S stability. Indeed, posttranscriptional regulation plays a prominent role in controlling levels of σ^S in *E. coli* (28, 50). In the event that GacS and GacA affect *rpoS* translation or σ^S stability, the influence of the two-component regulatory system would likely persist beyond the transition period when *rpoS* transcription was most notably influenced.

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