A GacS deficiency does not affect *Pseudomonas chlororaphis* PA23 fitness when growing on canola, in aged batch culture or as a biofilm

N. Poritsanos, C. Selin, W.G.D. Fernando, S. Nakkeeran, and T.R. de Kievit

Abstract: *Pseudomonas chlororaphis* PA23 is a biocontrol agent that protects against the fungal pathogen *Sclerotinia sclerotiorum*. Employing transposon mutagenesis, we isolated a *gacS* mutant that no longer exhibited antifungal activity. *Pseudomonas chlororaphis* PA23 was previously reported to produce the nonvolatile antibiotics phenazine 1-carboxylic acid and 2-hydroxyphenazine. We report here that PA23 produces additional compounds, including protease, lipase, hydrogen cyanide, and siderophores, that may contribute to its biocontrol ability. In the *gacS* mutant background, generation of these products was markedly reduced or delayed with the exception of siderophores, which were elevated. Not surprisingly, this mutant was unable to protect canola from disease incited by *S. sclerotiorum*. The *gacS* mutant was able to sustain itself in the canola phyllosphere, therefore, the loss of biocontrol activity can be attributed to a reduced production of antifungal compounds and not a declining population size. Competition assays between the mutant and wild type revealed equivalent fitness in aged batch culture; consequently, the *gacS* mutation did not impart a growth advantage in the stationary phase phenotype. Under minimal nutrient conditions, the *gacS*-deficient strain produced a tenfold less biofilm than the wild type. However, no difference was observed in the ability of the mutant biofilm to protect cells from lethal antibiotic challenge.

Key words: Pseudomonas, biocontrol, *gacS*, fitness, biofilms.

Résumé : La souche PA23 de *Pseudomonas chlororaphis* est un agent de lutte biologique au pathogène fongique *Sclerotina sclerotiorum*. En utilisant une mutagenèse par transposon, nous avons isolé un mutant *gacS* qui n'exerce plus d'activité antifongique. On a rapporté précédemment que *Pseudomonas chlororaphis* PA23 produisait des antibiotiques non volatiles : l'acide phénazine-1-carboxylique et le 2-hydroxyphénazine. Nous rapportons ici que PA23 produit des composés additionnels y compris une protéase, une lipase, du cyanure d'hydrogène et des sidérophores qui pourraient contribuer à ses propriétés de contrôle biologique. Dans un contexte *gacS*$^-$, la génération de ces produits est fortement réduite ou retardée, à l'exception des sidérophores, dont la production demeure élevée. Tel que prévu, ce mutant est incapable de protéger le canola contre la maladie causée par *S. sclerotiorum*. Le mutant *gacS* est capable de se suffire à lui-même dans la phyllosphère de canola; ainsi, la perte d'activité de contrôle biologique peut être attribuée à la réduction de la production de composés antifongiques et non pas à un déclin de la taille de la population. Des essais de compétition entre le mutant et la souche sauvage a révélé une compétence équivalente en culture avancée; en conséquence, la mutation *gacS* ne confère pas d'avantage de croissance en phase stationnaire. Sous des conditions nutritives minimales, la souche déficiente *gacS* produit 10 fois moins de biofilm que la souche sauvage. Cependant, aucune différence n'a été trouvée dans la capacité du biofilm mutant à protéger les cellules contre un traitement antibiotique létal.

Mots clés : Pseudomonas, lutte biologique, *gacS*, compétence, biofilms.

[Traduit par la Rédaction]

Introduction

Pseudomonas chlororaphis PA23 is a biocontrol agent that was first isolated from the root tips of soybean plants. Our interest in this strain derives from its ability to protect canola from disease caused by the fungal pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary in both greenhouse and field studies (Savchuk and Fernando 2004; Zhang 2004; Fernando et al. 2006). *Sclerotinia sclerotiorum* is a ubiquitous, soil-borne fungus capable of infecting more than 400 plant hosts,

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N. Poritsanos,[1] **C. Selin, and T.R. de Kievit.**[2] Department of Microbiology, University of Manitoba, Winnipeg, MB R3T 2N2, Canada. **W.G.D. Fernando, and S. Nakkeeran.**[3] Department of Plant Science, University of Manitoba, Winnipeg, MB R3T 2N2, Canada.

[1]Present address: Department of Physiology, University of Manitoba, Winnipeg, MB, Canada.
[2]Corresponding author (email: dekievit@ms.umanitoba.ca).
[3]Present address: Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore 641003, India.

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causing significant loss of economically important crops (Purdy 1979). Because there is no known resistance against *S. sclerotiorum* in commercially grown canola cultivars, management of this pathogen is essential. Public concern over the use of chemical pesticides together with the potential for acquiring resistance to these compounds has led to renewed interest in bacterial antagonists like PA23 for biocontrol. Biological approaches to disease management have previously shown inconsistent results in the field. It is therefore essential to elucidate the molecular mechanisms mediating biocontrol activity in strain PA23 so that in the environment, expression and activity of the pathogen-suppressing factor(s) can be optimized.

In many fluorescent pseudomonads, a mutation in either *gacS* or *gacA* encoding the GacS–GacA two-component signal transduction system eliminates biocontrol activity (Gaffney et al. 1994; Whistler et al. 1998; Chancey et al. 1999; Koch et al. 2002; van den Broek et al. 2003). Within this two-component system, the membrane-associated sensor kinase, GacS, is stimulated by a yet-to-be-identified signal resulting in autophosphorylation. The current working model suggests that phosphotransfer to the response regulator GacA results in activation, enabling it to induce expression of small regulatory RNA molecules (Heeb and Haas 2001; Haas and Défago 2005). These RNA molecules titrate translational repressor proteins, such as RsmA and RsmE, which block translation of target mRNAs by forming a complex at the ribosome binding site.

In fluorescent pseudomonads, a broad range of antibiotics and enzymes regulated by GacA–GacS contribute to biocontrol, including production of phenazines, pyrrolnitrin, 2,4-diacylphloroglucinol, pyoluteorin, hydrogen cyanide (HCN), protease(s), and chitinase (Heeb and Haas 2001). *Pseudomonas chlororaphis* PA23 was previously reported to produce the nonvolatile antibiotics phenazine 1-carboxylic acid and 2-hydroxyphenazine together with volatile compounds exhibiting antifungal activity (Fernando et al. 2005; Zhang et al. 2006). During our analysis, we discovered several additional compounds that likely contribute to the biocontrol capacity of this strain, including protease, lipase, HCN, and siderophores. Characterization of a PA23 *gacS* mutant revealed that all of these products are in some manner linked to the Gac two-component system of regulation.

In this paper, we describe the characterization of *P. chlororaphis* PA23 with respect to extracellular metabolite production. Furthermore, the effect of a *gacS* mutation on PA23 biocontrol as well as its ability to form antibiotic-resistant biofilms was determined. Finally, we investigated whether a *gacS* mutation would confer a growth advantage in stationary phase (GASP) phenotype.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultured at 37 °C on Lennox Luria Bertani (LB) agar (Difco Laboratories, Detroit, Mich.). *Pseudomonas chlororaphis* PA23 and its derivatives were cultured at 28 °C on LB agar, M9 minimal media (Difco), minimal M9 casamino acid (M9CA; Difco), Terrific broth (TB; Sambrook et al. 1989), or peptone tryptic soy broth (Ohman et al. 1980). As required, media were supplemented with the following antibiotics: tetracycline (Tc; 15 μg/mL), gentamicin (15 μg/mL), ampicillin (100 μg/mL) for *E. coli*; rifampicin (25 μg/mL), Tc (15 or 100 μg/mL), and gentamicin (25 μg/mL) for *P. chlororaphis*. All antibiotics were obtained from Research Products International Corp. (Mt. Prospect, Ill.).

Nucleic acid manipulation

Cloning, purification, electrophoresis, and other manipulations of nucleic acid fragments and constructs were performed using standard techniques (Sambrook et al. 1989). To clone the PA23 *gacS* gene, oligonucleotide primers GacS-F and GacS-R (Table 1) were designed from the *P. chlororaphis* PCL1391 *gacS* gene sequence (accession No. AF502252; Chin-A-Woeng et al. 2005). A TOPO® kit (Invitrogen Life Technologies, Burlington, Ont.) was used to clone the 3.0 kb *gacS* polymerase chain reaction (PCR) product into the pCR®2.1-TOPO® vector following the manufacturer's instructions. The 3.0 kb *gacS* insert was then excised with *Eco*RV and *Hin*dIII and cloned into the *Sma*I-*Hin*dIII sites of pUCP23 to generate pUCP23-*gacS*. To clone *rpoS* from PA23, primers RpoS-F and RpoS-R (Table 1) were designed from the sequence of the *rpoS* gene of PCL1391 (accession No. AY586457; Girard et al. 2006). The 1.3 kb PCR product was cloned into pCR®2.1-TOPO®. The *rpoS* gene was excised as a *Kpn*1-*Xba*I fragment and cloned into the same sites of pUCP22, generating pUCP22-*rpoS*.

PCR

PCR was performed under standard conditions as suggested by Invitrogen Life Technologies data sheets supplied with their *Taq* polymerase.

Tn*5*-OT182 transposon mutagenesis

Bacterial conjugations were performed to introduce Tn*5*-OT182 into *P. chlororaphis* PA23 by biparental mating following the method of Lewenza et al. (1999). For each mating, 5–10 Tc^r colonies were screened by PCR to ensure that transconjugants contained a Tn*5* insertion using TNP5-F and TNP5-R primers (Table 1). To determine the site of Tn*5*-OT182 insertion, rescue cloning was performed following previously described methods (Lewenza et al. 1999).

Sequence analysis

Plasmids isolated from Tc^r *Xho*I clones were sent for sequencing using oligonucleotide primer Tn*5*-ON82 (Table 1), which anneals to the 5′ end of Tn*5*-OT182. *Bam*HI or *Cla*I rescue plasmids were sequenced using primer Tn*5*-OT182 right (Table 1), which anneals to the 3′ end of the transposon. All sequencing was performed at the University of Calgary Core DNA Services facility (Calgary, Alberta). Sequences were analyzed with blastn and blastx databases.

Nucleotide sequence accession number

The GenBank accession numbers for the sequences of the *P. chlororaphis* PA23 *gacS* and *rpoS* genes are DQ386697 and DQ525801, respectively.

Table 1. Bacterial strains, plasmids, and primers used in this study.

Strain, plasmid, primer	Relevant genotype or phenotype	Source or reference
Strains		
Pseudomonas chlororaphis		
PA23	Phz$^+$ Rif^r wild type (soybean plant isolate)	Savchuk and Fernando 2004
PA23-314	Phz$^-$ Rif^r *gacS*::Tn*5*-OT182 genomic fusion	This study
PA23-314 (pUCP23-*gacS*)	Phz$^-$ Rif^r *gacS*::Tn*5*-OT182 genomic fusion, *gacS* complemented strain	This study
Escherichia coli		
DH5α	*supE44 ΔlacU169* (Φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Gibco
SM10	Mobilizing strain; RP4 *tra* genes integrated in chromosome; Km^r Tc^r	Simon et al. 1983
Pseudomonas aeruginosa		
PAO1	Wild type	Holloway et al. 1979
Pseudomonas fluorescens		
Pf-5	Wild type	Howell and Stipanovic 1980
Chromobacterium violaceum		
CV026	Autoinducer synthase (*cviI*) mutant from *C. violaceum* ATCC 31532, autoinducer biosensor	Latifi et al. 1995
Plasmids		
pOTI82	pSUP102(GM)::Tn*5*-OT182 Cm^r Gm^r Amp^r Tc^r	Merriman and Lamont 1993
pOT182-314 (*Xho*I)	pOT182 containing *gacS*::Tn*5*-OT182 genomic fusion	This study
pCR®2.1-TOPO®	Cloning vector for PCR products	Invitrogen
pUCP23	Broad-host-range vector; IncP OriT, Amp^r Gm^r	West et al. 1994
pUCP23-*gacS*	pUCP23 containing *gacS* from *P. chlororaphis* PA23	This study
pUCP22	Broad-host-range vector; IncP OriT, Amp^r Gm^r	West et al. 1994
pUCP22-*rpoS*	pUCP22 containing *rpoS* from *P. chlororaphis* PA23	This study
pRPOS-*lacZ*	pLP170 containing the *rpoS* promoter region from *P. chlororaphis* PA23	This study
pLP170	Promoterless *lacZ* transcriptional fusion vector	Preston et al. 1997
pME3219	pME6010 containing an *hcnA-lacZ* translational fusion	Laville et al. 1998
Primers		
GacS-F	5′-gggattcattagcttctgcaa-3′	This study
GacS-R	5′-tggctgctgaagagaatcgt-3′	This study
RpoS-F	5′-tacgtcagtgcttacggcca-3′	This study
RpoS-R	5′-cagcagggttttatccgaat-3′	This study
RpoS-R short	5′-cagcagggttttatccgaat-3′	This study
TNP5-F	5′-accatttcaacggggtctcac-3′	This study
TNP5-R	5′-tgactccatgtgacctccta-3′	This study
Tn*5*-ON82	5′-gatcctggaaaacgggaaagg-3′	This study
Tn*5*-OT182 right	5′-atgttaggaggtcacatg-3′	This study

Note: Rif, rifampicin; Km, kanamycin; Tc, tetracycline; Cm, chloramphenical; Gm, gentamicin.

Antifungal assays

Radial diffusion assays were performed to assess fungal inhibition in vitro. Aliquots (5 μL) of overnight bacterial cultures were spotted onto potato dextrose agar (Difco) plates, 0.5 cm away from the edge of the plate. The bacteria were allowed to grow for 16 h at 28 °C before a 0.6 cm fungal plug of *S. sclerotiorum* was placed on the center of the plate. Plates were incubated at room temperature and antifungal activity was assessed after 3–4 days by measuring the distance between the edges of the colony and the fungal mycelium. Four replicates were analyzed for each strain and assays were repeated three times.

Quantification of phenazine

Overnight cultures of *P. chlororaphis* PA23 (pUCP23), PA23-314 (pUCP23), and PA23-314 (pUCP23-*gacS*) grown in LB were subjected to phenazine extraction and quantification by ultraviolet-visible light spectroscopy, following the method of Chancey et al. (1999). Phenazine analysis was performed three times.

HCN analysis

Production of HCN was determined qualitatively using Cyantesmo paper (Machery-Nagel GmbH & Co., Düren, Germany). To monitor expression of the genes encoding HCN, plasmid pME3219 containing an *hcnA-lacZ* translational fusion was transformed into PA23, PA23-314, and the positive control strain *Pseudomonas fluorescens* Pf-5. Strain Pf-5 was included because the *hcnA* promoter was isolated from *P. fluorescens*. Cultures were grown in peptone tryptic soy broth until they reached stationary phase (OD_{600} = 2.5–3.0), at which point *hcnA* expression was assessed with β-galactosidase assays (Miller 1972). Samples were analyzed in triplicate and experiments were repeated three times.

RpoS expression

To monitor *rpoS* transcription levels, a transcriptional fusion to *lacZ* was constructed. For cloning the *rpoS* promoter, primers RpoS-F and RpoS-R short (Table 1) were used to PCR amplify a 1010 bp fragment from PA23 genomic DNA. The PCR product was cloned into pCR®2.1-TOPO®. The insert was excised using *Eco*RI and *Sal*I and then subcloned into the same sites of pLP170 (Preston et al. 1997), generating pRPOS-*lacZ*. β-Galactosidase assays were performed in triplicate after cultures had reached an optical density (OD) at 600 nm of 1.0. For assaying RpoS protein levels, sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot analysis were performed as specified by the manufacturer (Bio-Rad Laboratories, Hercules, Calif.). Late-logarithmic-phase and stationary-phase cells were obtained from cultures grown to an OD at 600 nm of 1.0 and 2.0, respectively, in LB medium. The cultures were adjusted, based on OD, to enable the same number of cells from each culture to be analyzed. Cells were pelleted and resuspended in cracking buffer (50 mmol/L Tris–HCL pH 6.8, 1% SDS, 2 mmol/L EDTA, 10% *v*/*v* glycerol, 0.01% bromophenol blue, 1% β-mercaptoethanol) and then boiled for 3 min. A 100 μg protein sample, determined by the Bradford assay (Bradford 1976), was loaded and separated on a 12% SDS–PAGE gel. The blots were hybridized to RpoS-specific antibodies, generously supplied by K. Tanaka (Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan), and then to alkaline-phosphatase-conjugated goat anti-rabbit antiserum (Amersham Biosciences, Baie d'Urfé, Que.). Antibodies were detected using the chemiluminescence detection reagent CDP-Star® as specified by the manufacturer (Roche Diagnostics, Mannheim, Germany). RpoS was quantified with a Fluorochem 2000 Phosphoimager using Fluorochem Stand-Alone software, version 2.0. Western analysis was performed twice with similar findings.

Exoproduct analyses

The production of homoserine lactone autoinducer molecules was assessed qualitatively by spotting 5 μL of an overnight culture onto plates seeded with *Chromobacterium violaceum* CV026. CV026 is an autoinducer-deficient strain that turns purple in the presence of exogenous C_4, C_6, or C_8 homoserine lactones because of the production of the quorum-sensing-controlled pigment violacein. Extracellular protease activity was determined by inoculating 5 μL of an overnight culture onto 2% skim milk agar plates. Proteolysis was observed as zones of lysis around the colony after 24–36 h at 28 °C. Lipase activity was detected by using the protocol of Lonon et al. (1988). Lipase activity was indicated by a zone of fatty acid precipitation around the colony after 24–72 h. Siderophore production was assayed by spotting a 5 μL aliquot of overnight culture onto Chrome azurol S (CAS) agar plates (Schwyn and Neilands 1987) and then incubating for 16 h at 28 °C. Data represent the average of four replicates, and assays were repeated three times.

Growth analysis

Growth rate analysis of the wild-type strain PA23 (pUCP23) and strains PA23-314 (pUCP23), PA23-314 (pUCP23-*gacS*), PA23-314 (pUCP22-*rpoS*), and PA23 (pUCP22-*rpoS*) was performed in rich and minimal media with a Bioscreen® C automated turbidometer. Overnight cultures were adjusted to an OD_{600} of 0.1 by diluting with the same media and 100 μL of the culture was inoculated into each well of a Bioscreen® C microtiter plate. The control wells contained an equal volume of sterile media. Growth of the cultures at 28 °C was monitored every 15 min over a 45 h period. Samples were analyzed in triplicate and the growth rate analysis was repeated four times.

Competition experiments

Competition assays were carried out in M9 minimal media (0.2% glucose; 1 mmol/L $MgSO_4$) to minimize spontaneous *gacS* accumulation. Overnight cultures of PA23 and PA23-314 were used to inoculate 20 mL of media, such that competing strains were present in equal numbers (10^8 cfu/mL). The mixed culture was grown at 28 °C with shaking for a period of 8 days. Colony forming units (cfu) of the wild-type and *gacS* mutant populations were monitored daily by plating serial dilutions onto LB agar plates with and without antibiotics (PA23-314 is Tc^r due to the Tn*5*-OT182 insertion). Cultures were analyzed in triplicate and the experiment was repeated twice.

Motility analysis

Flagellar (swimming) motility was assayed by inoculating 5 μL of an overnight bacterial culture into either LB or M9CA media solidified with 0.3% agar. After 20 and 36 h incubation at 28 °C, the diameter of the swim zone was measured. Swarming motility was assayed by inoculating bacterial cells with an applicator stick onto the surface of a Swarm media plate (0.5% peptone, 0.3% yeast extract, and 0.5% agar) previously air-dried for 2 h. Results were obtained after 16–30 h incubation at 28 °C. Twitching motility was assessed on LB and M9CA plates containing 1% agar. Bacterial cultures were stabbed to the bottom of the plates, and after 72 h incubation, twitch zones were measured. For motility assays, five replicates were analyzed and the experiment repeated three times.

Minimum inhibitory concentration determination

For minimum inhibitory concentration (MIC) assays, cultures were adjusted to a concentration of 1×10^7 cfu/mL in M9CA (0.2% glucose, 1 mmol/L $MgSO_4$). Aliquots of 100 μL were added to the wells of a 96-well microtiter plate containing a range of concentrations of either ciprofloxacin or tobramycin. The MIC was defined as the lowest concentration of antibiotic resulting in no growth. Cultures were analyzed in triplicate and MIC determinations were repeated three times.

Biofilm development and antibiotic susceptibility

We employed a highly reproducible 96-well plate assay (O'Toole and Kolter 1998*a*) to assess the ability of PA23 (pUCP23), PA23-314 (pUCP23), and PA23-314 (pUCP23-*gacS*) to form biofilms. Biofilm antibiotic susceptibility profiles were determined by the method of Parkins et al. (2001), using a microtiter plate with a lid containing 96 pegs that allows formation of biofilms on the peg surface (Nunc, Rochester, N.Y.). For the minimal biofilm eradication concentration (MBEC) determinations, ciprofloxacin was tested

Table 2. Phenotypic characterization of *Pseudomonas chlororaphis* PA23, Tn*5* mutant PA23-314, and complemented strain PA23-314 (pUCP23-*gacS*).

	Extracellular metabolite activity				
Strain	Antifungal[a]	Protease[a]	Autoinducer[a]	Siderophore[a]	Lipase
PA23 (pUCP23)	8 (0.4)	5 (0.2)	12 (0.8)	6 (0.7)	+/+
PA23-314 (pUCP23)	0 (0.0)[b]	0 (0.0)[b]	0 (0.0)[b]	16 (3.5)[c]	–/+[d]
PA23-314 (pUCP23-*gacS*)	8 (0.5)[e]	5 (0.5)[e]	12 (0.6)[e]	8 (0.5)[c]	+/+

[a]Mean (SD) of the zones of activity (mm) obtained from four replicates.
[b]Significantly different from the wild type ($p < 0.0001$).
[c]Significantly different from the wild type ($p < 0.01$).
[d]Lipase activity at 24 h (–) and 72 h (+).
[e]Not significantly different from the wild type.

at a concentration range of 0.0125–31.25 μg/mL (0.1× – 250× MIC) and tobramycin was tested at 0.025–162.5 μg/mL (0.1× – 650× MIC). The MBEC was defined as the lowest concentration of antibiotic resulting in no growth in the recovery plate. For the antibiotic susceptibility analysis, samples were analyzed in triplicate and the experiment was repeated three times.

Biocontrol under greenhouse conditions

The bacterial strains PA23 (pUCP23), PA23-314 (pUCP23), and PA23-314 (pUPC23-*gacS*) were assessed for their efficiency in suppressing stem rot of canola under greenhouse conditions. *Brassica napus* 'Westar' plants were grown in pots (21 cm × 20 cm) under a 24 °C : 16 °C 16 h : 8 h light:dark regime. The plants were sprayed at 30% and 50% flowering (double spray) with bacterial strains (2.0×10^8 cfu/mL) suspended in 100 mmol/L phosphate buffer (pH 7.0) containing 0.02% Tween 20 and were kept in a humidity chamber (24 °C : 16 °C 16 h : 8 h light:dark regime). Twenty-four hours after bacterial inoculation, canola petals were sprayed with ascospores of *S. sclerotiorum* (8×10^4 spores/mL) suspended in 100 mmol/L phosphate buffer (pH 7.0) containing 0.02% Tween 20. The pathogen control plants were inoculated with ascospores, while the healthy control plants were sprayed with phosphate buffer. All plants were incubated in a humidity chamber. Fourteen days after inoculation of *Sclerotinia* ascospores, symptom development was observed and recorded using a 0–7 scale (0, no lesions on the stem; 1, leaf lesion with no stem symptoms; 2, 1–20 mm stem lesion; 3, 21–40 mm stem lesion; 4, 41–60 mm stem lesion; 5, 61–80 mm stem lesion; 6, 81–100 mm stem lesion; 7, >100 mm stem lesion or plant death). Based on symptom development, percent leaf incidence by *Sclerotinia*, percent stem rot incidence, and disease severity was calculated. Eight plants were used for each treatment. For assessing infection on leaves, the first 10 leaves, from top to bottom, were scored for the presence or absence of the symptom per plant.

$$\%\ \text{leaf incidence} = (\text{no. of leaves infected with } \textit{Sclerotinia} \text{ / no. of leaves observed}) \times 100$$

$$\%\ \text{stem rot incidence} = (\text{no. of stems infected by } \textit{Sclerotinia} \text{ / no. of stems observed}) \times 100$$

$$\text{disease severity} = \text{total points for all plants using a 0–7 scale / no. of plants observed}$$

Bacterial sustainability

To determine the efficiency of the bacterial strains to colonize the canola plant, the population of PA23 (pUCP23), PA23-314 (pUCP23), and PA23-314 (pUCP23-*gacS*) was analyzed over a period of 5 days, starting 2 days after the second application of bacteria. For each test sample, 10 canola petals were resuspended in 10 mL of phosphate-buffered saline and mixed with a vortex for 30 s. The bacterial suspension was serially diluted and 1 mL aliquots were plated onto LB agar supplemented with selective antibiotics. After 30 h of incubation at 28 °C, bacteria were enumerated.

Statistical analysis

An unpaired Student's *t* test was used for statistical analysis of antifungal, protease, autoinducer, and siderophore activity; flagellar motility; and bacterial sustainability on canola.

Results

Isolation of a *P. chlororaphis gacS* mutant

Approximately 4000 transconjugants were screened in radial diffusion plate assays to identify mutants exhibiting increased or decreased antifungal activity by comparison with the wild type. One mutant, PA23-314, exhibited no antifungal activity (Table 2). Sequence analysis revealed a Tn insertion in a gene exhibiting >90% identity to the *gacS* genes from two strains of *P. chlororaphis*, namely PCL1391 (GenBank accession No. AF502252) and O6 (GenBank accession No. AF192795).

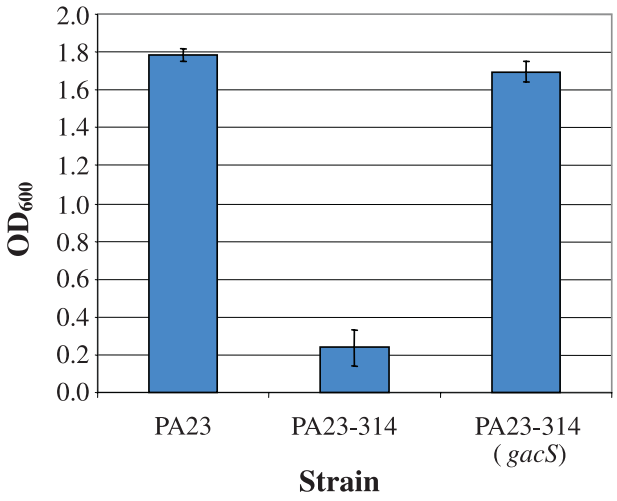
gacS cloning and complementation

To ensure that the phenotype observed for PA23-314 was caused by the Tn insertion in *gacS* and not by polar effects on downstream genes, complementation analyses were undertaken. The PA23 *gacS* gene was PCR amplified and ultimately cloned into pUCP23. Transformation of pUCP23-*gacS* into PA23-314 restored antifungal activity to wild-type levels (Table 2), indicating that the PA23-314 phenotype results from inactivation of *gacS*.

Effect of *gacS* on phenazine, HCN, protease, lipase, siderophore, and autoinducer production

Wild-type strain PA23 is orange due to the production of the compounds phenazine 1-carboxylic acid and 2-hydroxyphenazine (Zhang et al. 2006). PA23-314, on the other hand, is white, suggesting phenazines are not produced by this mutant. Spectral analysis of culture extracts confirmed that

Fig. 1. Biofilm formation by *Pseudomonas chlororaphis* PA23 (wild type; pUCP23), PA23-314 (*gacS* mutant; pUCP23), and PA23-314 (*gacS* mutant; pUCP23-*gacS*). Cultures were grown in 96-well polyvinyl chloride microtiter plates containing M9CA (0.2% glucose) for 16 h at 28 °C. Biofilm formation, indicated by crystal violet staining, was measured at an absorbance of 600 nm.



phenazines are not produced by PA23-314 (data not shown). A deficiency in antibiotic production undoubtedly contributed to decreased antifungal activity; however, GacS is part of a global regulatory system, and a mutation in this allele is expected to exert pleiotropic effects. Accordingly, we examined PA23 and PA23-314 for the production of a number of extracellular metabolites, including HCN, protease, lipase, autoinducer molecules, and siderophores. Until now, PA23 had not been investigated for the presence of these compounds.

We discovered that PA23 produces the volatile antibiotic HCN; whereas, the *gacS* mutant does not (data not shown). These findings were further substantiated by *hcnA-lacZ* expression studies. Expression of an *hcnA* translational fusion was negligible in PA23-314 (261 ± 11 Miller units) compared with that of the PA23 wild-type strain (18 658 ± 3 509 Miller units) and positive control strain, *P. fluorescence* Pf-5 (19 803 ± 2 582 Miller units). Whether HCN production contributes to PA23 biocontrol activity has yet to be established, but its expression is clearly dependent on GacS.

The production of other extracellular metabolites is summarized in Table 2. Protease assays revealed that while the wild type tested positive for protease activity, the *gacS* mutant was protease deficient. Similarly, PA23-314 did not produce autoinducer molecules. Lipase production by the *gacS* mutant could be detected at 72 h vs 24 h for the wild type, indicating that lipase is produced at a delayed rate. Siderophore detection on CAS agar plates showed that the *gacS* mutant produced an orange halo 2.5 times larger than that of the wild-type strain. Although siderophore levels were significantly increased, PA23-314 exhibited no antifungal activity in plate assays; therefore, enhanced siderophore production alone is not sufficient for *S. sclerotiorum* biocontrol. For all of the assays described above, addition of *gacS* in trans restored production of extracellular metabolites to near wild-type levels (Table 2).

Table 3. Flagellar motility analysis of *Pseudomonas chlororaphis* PA23, PA23-314, and complemented strain PA23-314 (pUCP23-*gacS*).

	Swim zone diameter (mm)	
Strain	20 h	36 h
PA23 (pUCP23)	36 (1.3)[a]	68 (1.6)
PA23-314 (pUCP23)	21 (0.8)[b]	48 (1.1)[b]
PA23-314 (pUCP23-*gacS*)	36 (1.9)[c]	70 (3.2)[c]

[a]Mean (SD) of swim zones from five replicates.
[b]Significantly different from the wild type ($p < 0.0001$).
[c]Not significantly different from the wild type.

Effect of *gacS* on biofilm formation and susceptibility to antibiotics

As illustrated in Fig. 1, under minimal media conditions, biofilm formation by the *gacS* mutant was almost tenfold less than that of the PA23 wild type. Therefore, it appears that *P. chlororaphis* is capable of forming robust biofilms on an abiotic surface as long as a functional copy of GacS is present.

We chose to investigate resistance of the PA23 wild type, the *gacS* mutant, and the complemented mutant to two antibiotics, ciprofloxacin and tobramycin. No differences in planktonic MIC values were observed among the three strains for either ciprofloxacin (0.125 μg/mL) or tobramycin (0.250 μg/mL). Intriguingly, the MBEC values were equivalent among PA23 (pUCP23), PA23-314 (pUCP23), and PA23-314 (pUCP23-*gacS*) for ciprofloxacin (12.5 μg/mL) and tobramycin (162.5 μg/mL). The results indicate that for all three strains, the biofilm populations exhibited 100-fold increased resistance to ciprofloxacin compared with planktonic cultures. With tobramycin, a 650-fold increase in resistance was observed when grown as a biofilm.

Effect of *gacS* on motility

We investigated whether altered motility contributes in some manner to the diminished biofilm formation and biocontrol activity of PA23-314. Three forms of translocation were investigated; twitching motility, flagellar motility, and swarming. Our twitching motility assays revealed a zone of twitching for the positive control strain *P. aeruginosa* PAO1. In contrast, no twitch zones were detected for PA23 (pUCP23), PA23-314 (pUCP23), or PA23-314 (pUCP23-*gacS*), independent of whether rich (LB agar) or minimal (M9 agar) media plates were used (data not shown). These findings led us to conclude that under the conditions tested, *P. chlororaphis* does not employ type IV twitching motility.

Flagellar motility was assessed at 20 and 36 h. Although the *gacS* mutant was capable of swimming, it did so at a significantly reduced rate compared with the wild type and complemented *gacS* mutant (Table 3). Thus, reduced flagellar motility may, in part, mediate the defect in biofilm formation and biocontrol activity observed with the *gacS* mutant.

Because of the irregular pattern of swarming, we did not perform quantitative analyses. We did, however, consistently

observe that compared with the PA23 wild-type strain and complemented strain PA23-314 (pUCP23-*gacS*), the *gacS* mutant was delayed in swarming initiation. At the onset of swarming, fork-like tendrils are observed extending out from the colony. The PA23-314 swarming pattern was also more uniform than that of the *gacS*-sufficient strains (PA23 and PA23-314 (pUCP23-*gacS*)), with a portion of the motility zone resembling swimming more than swarming motility. Collectively these results indicate that reduced swimming and swarming motility may contribute to the *gacS* biofilm defect.

gacS influences growth rate of *P. chlororaphis* in liquid culture

We performed growth analysis of PA23, PA23-314, and the complemented mutant in four different media: M9 minimal supplemented with 0.2% glucose or 0.2% glycerol, LB, and TB. In M9 media containing either glucose (Fig. 2*a*) or glycerol (data not shown) as a carbon source, the *gacS* mutant had a reduced lag phase compared with the wild-type and complemented strains (approximately 4 vs 8 h). In the comparatively rich LB medium and the ultra-rich TB medium, the three strains exhibited similar entry into logarithmic growth phase (Figs. 2*b* and 2*c*).

A second major difference in the growth profile of the *gacS* mutant appeared at a later point in the growth curve. At approximately 20 h, in all media tested, the *gacS* mutant population started to increase at a much slower rate than the wild-type and complemented strain populations. The most marked difference in population density was observed in TB where the *gacS* mutant strain reached an OD of 1.0 only after 45 h growth (Fig. 2*c*). In comparison, by 15 h the population density of the *gacS*-sufficient strains had exceeded an OD of 1.5 (Fig. 2*c*). Thus, it appears that a *gacS* mutation dramatically reduces the total achievable population size, and this phenomenon is most pronounced under nutrient-rich conditions.

Altered RpoS expression does not affect growth profiles

RpoS is a sigma factor that controls expression of the genes involved in the transition into stationary phase, thereby enabling cells to cope with environmental stresses and starvation (Hengge-Aronis 1993). In other bacterial strains, a deficiency in *gacS* results in decreased *rpoS* expression (Whistler et al. 1998; Kang et al. 2004; Heeb et al. 2005). We wondered whether expression of *rpoS* was repressed in PA23-314, and if so, could this account for the decreased cell numbers observed in stationary phase. We discovered that expression of an *rpoS-lacZ* transcriptional fusion was greatly decreased in the PA23 a *gacS*-deficient background (389 ± 97 Miller units) compared with the wild type (1421 ± 73 Miller units). Furthermore a decrease in RpoS protein levels in Western blots was observed (Fig. 3). These findings led us to postulate whether constitutive expression of *rpoS* under control of the *lac* promoter (pUCP22-*rpoS*) would restore the growth phenotype of PA23-314 to that of the wild type. As illustrated in Fig. 3, increased expression of RpoS was observed in PA23-314 (pUCP22-*rpoS*). However, growth analysis of PA23-314 (pUCP22-*rpoS*) and PA23 (pUCP22-*rpoS*) revealed that *rpoS* had no impact on total

Fig. 2. Growth rate analysis of *Pseudomonas chlororaphis* wild-type strain PA23 (pUCP23), PA23-314 (pUCP23), and PA23-314 (pUCP23-*gacS*). Cultures were standardized to the same optical density (OD) and were inoculated into wells of a Bioscreen C microtiter plate. Growth was monitored every 15 min over a 45 h period at 28 °C with a Bioscreen® C automated turbidometer. (A) Growth in minimal M9 casamino acid (M9) + 0.2% glucose, (B) growth in Lennox Luria Bertani agar, and (C) growth in Terrific broth. Control wells contained an equal volume of sterile media. The growth rate analysis was repeated four times and the same trend consistently observed. A representative set of data is shown.

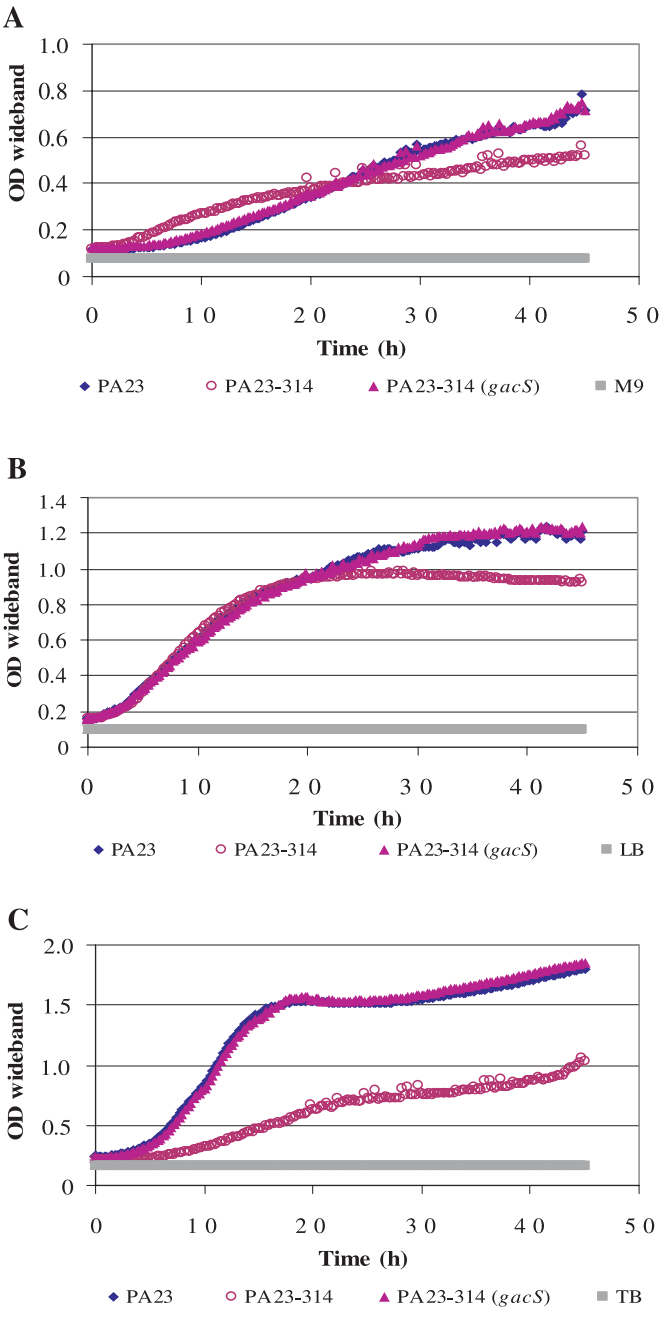
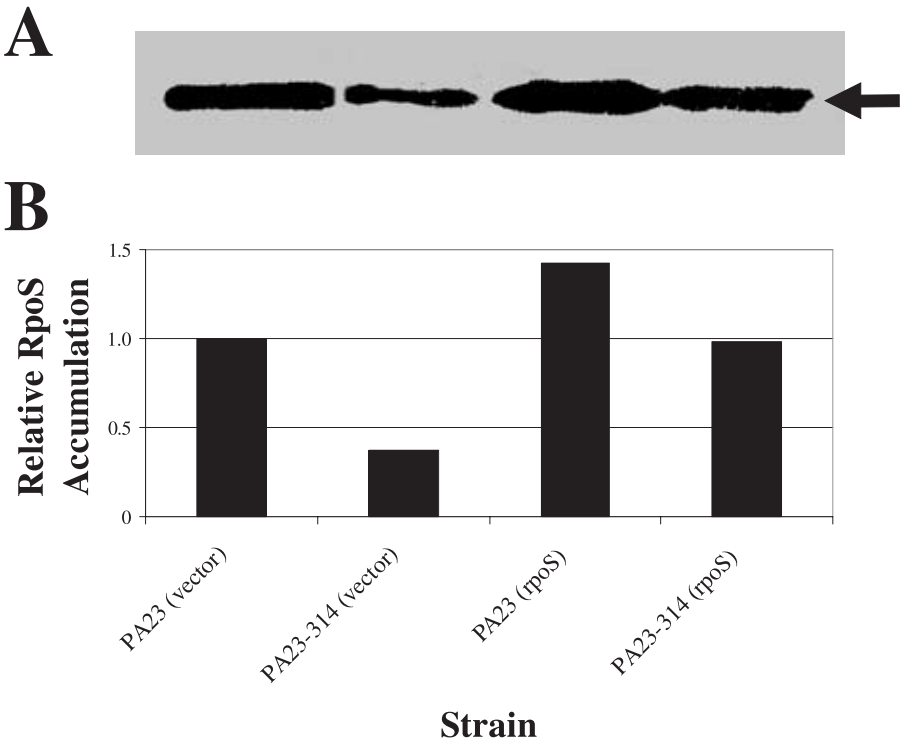


Fig. 3. Relative RpoS accumulation. (A) RpoS-specific antiserum was used to visualize σ^s (indicated with an arrow) from Western blots of protein extracted from cultures grown to late logarithmic phase. Each lane contains 100 μg of protein, determined from Bradford assays (Bradford 1976). (B) The amount of RpoS reported for each sample was normalized relative to the RpoS content of late-logarithmic-phase wild-type cells. The RpoS content in each lane was estimated by analyzing Western blots with a phosphoimager. Cells from both late-logarithmic-phase and stationary-phase cultures were analyzed for RpoS expression, but because the results were similar for both, only the logarithmic data is shown. Western blot analysis was independently replicated. A representative data set is shown.



achievable population size (data not shown) because the growth profiles were identical to those shown in Fig. 2.

A *gacS* mutation does not confer a GASP phenotype

It has been previously demonstrated that attenuated σ^s activity can confer a GASP phenotype on bacterial cells (Zambrano et al. 1993; Martínez-García et al. 2003). We sought to determine whether PA23-314, which also exhibits reduced RpoS expression, could successfully overtake the wild type during prolonged batch culture. A 1:1 mixed culture of PA23 and PA23-314 was established and allowed to grow for 8 days. Daily enumeration of culture viability revealed equivalent numbers of PA23 and PA23-314 survivors (data not shown). Hence, a *gacS* mutation does not impart a fitness advantage over the wild type during long-term batch culture.

gacS is required for biocontrol of *S. sclerotiorum* in greenhouse studies

The wild-type PA23, the *gacS* mutant, and the complemented mutant were evaluated for their antifungal action against canola stem rot disease caused by *S. sclerotiorum*. Three parameters were evaluated: (*i*) incidence of stem rot, (*ii*) incidence of leaf infection, and (*iii*) disease severity. Studies revealed that the wild-type PA23 afforded significant protection against fungal infection of both stems and leaves and dramatically reduced disease severity (Fig. 4). Conversely, the *gacS* mutant was markedly impaired in its biocontrol ability (Fig. 4). Complementation with the *gacS* gene in trans restored protection levels to those of the PA23 parent. Therefore, expression of GacS is essential for effective biocontrol of *S. sclerotiorum* infection in canola.

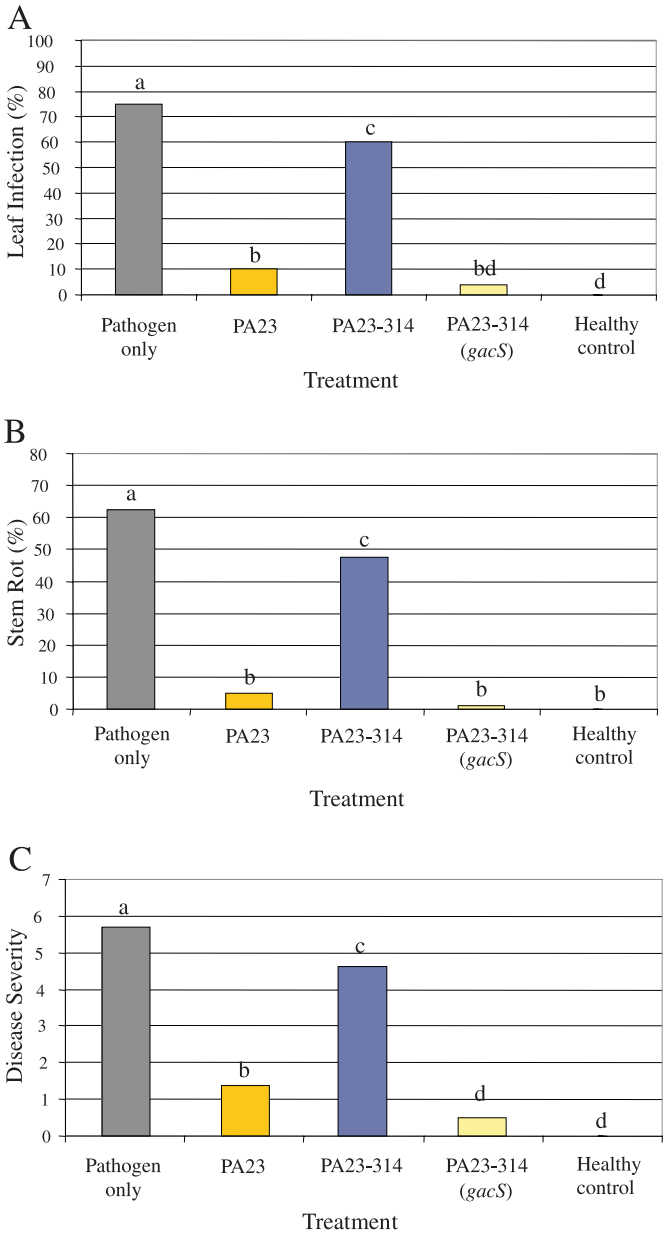
Bacterial sustainability on canola petals

To assess whether poor sustainability contributed to a lack of biocontrol by the *gacS* mutant, bacteria isolated from canola petals were enumerated over 5 days. Random sampling of petals was performed on days 1 through 5 from the inoculated plants. There was some fluctuation in the population sizes of the three strains over the 5 day period (Fig. 5); however the PA23-314 cells did not decline over time, rather the population size increased. By the end of the 5 days, PA23-314 cell numbers were significantly greater than those of either the wild type or complemented *gacS* mutant.

Discussion

The research described in this study was undertaken to characterize *P. chlororaphis* PA23 with respect to compounds that may contribute to its antifungal capacity. Isolation and characterization of a *gacS* mutant revealed that several of these compounds, including phenazines, protease, lipase, and HCN, are subject to GacS regulation. In greenhouse assays, unlike the wild type and complemented mutant, the *gacS*-deficient strain was unable to protect canola from disease

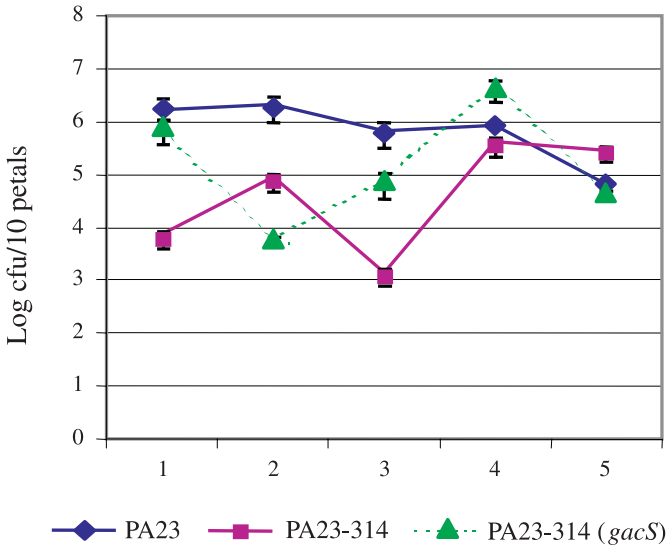
Fig. 4. Efficiency of *Pseudomonas chlororaphis* PA23 (wild type, pUCP23), *gacS*-mutant PA23-314 (pUCP23), and *gacS*-complemented strain PA23-314 (pUCP23-*gacS*) in managing *Sclerotinia sclerotiorum* ascospore infection on canola plants. (A) Percent incidence of leaf infection, (B) percent incidence of stem rot, and (C) disease severity on stem. In all treatments, except the healthy control, plants were sprayed with *S. sclerotiorum* ascospores. The healthy control plants were sprayed with phosphate buffer. Column means labeled with the same letter do not differ significantly by Duncan's multiple range test ($P > 0.05$).



caused by *S. sclerotiorum*, indicating that GacS is essential for PA23 biocontrol of this fungal pathogen.

In nature, the bulk of bacterial biomass exists as an attached community of cells known as a biofilm (Costerton et al. 1995). A previous investigation of a *P. chlororaphis* O6 *gacS* mutant revealed that GacS is involved in biofilm formation (Anderson et al. 2005). However, these studies were performed with King's B media, which is relatively rich and not reflective of the nutrient status in the environment. Furthermore, neither the ability of the *gacS* mutant biofilm to restrict penetration of harmful substances nor the nature of the biofilm defect was examined (Anderson et al. 2005). In the present study, we observed that in minimal media, a *gacS* mutation resulted in a significant reduction in biofilm formation on the surface of polyvinyl chloride microtiter plates. In an attempt to seek out the nature of the *gacS* biofilm defect, we examined three forms of motility: flagellar, swarming, and twitching motility. In *P. aeruginosa* and *P. fluorescens*, flagellar motility is important during the initial stages of biofilm formation (O'Toole and Kolter 1998*a*, 1998*b*). We discovered reduced swimming by the *gacS* mutant compared with the parent (Table 3). Although PA23-314 was capable of swarming motility, initiation was delayed and the pattern of swarming was distinct from that of the PA23 parent. Thus reduced swimming and aberrant swarming motility may contribute to the *gacS* biofilm defect. In *E. coli*, functional type I pili are required for attachment to surfaces and play a critical role in biofilm initiation (Pratt and Kolter 1998). Likewise in *P. aeruginosa* and *P. fluorescens,* mutations affecting type IV pili interfere with normal biofilm development (O'Toole and Kolter 1998*a*, 1998*b*; Pratt and Kolter 1998). We were unable to observe twitching motility in either the *gacS* mutant or the PA23 wild type, so we cannot conclude whether this mode of translocation contributes to the *gacS* biofilm defect. Studies of biofilm-defective mutants have revealed several gene products important for initiation and development of biofilms, including exopolysaccharides, motility, and various cell surface struc-

Fig. 5. Sustainability of *Pseudomonas chlororaphis* PA23 (wild type, pUCP23), *gacS* mutant PA23-314 (pUCP23), and *gacS*-complemented strain PA23-314 (pUCP23-*gacS*) on canola petals. Petals were harvested from plants each day for a total of 5 days. The bacterial population present on 10 petals was quantified by serial dilution plating. By day 5, the PA23-314 population was significantly greater than the populations of the wild-type and complemented mutant strains. Assays were performed in triplicate. Note: for some data points, error bars representing standard deviation are too small to show on the graph.



tures (O'Toole and Kolter 1998*a*, 1998*b*; Pratt and Kolter 1998; Hinsa et al. 2003). Since a detailed molecular analysis of biofilm formation in *P. chlororaphis* has not been undertaken, it is unclear exactly what components are involved in biofilm development. Considering the pleiotropic nature of *gacS* mutations, it would not be surprising to find that multiple facets of biofilm development are affected.

In our antibiotic susceptibility analyses, we were intrigued to discover that a protective matrix was established despite the presence of very little PA23-314 biofilm. There are two main hypotheses to explain the inherent resistance of biofilms to antimicrobial agents. The first is based on the fact that the biofilm provides a protective matrix that restricts diffusion of harmful agents (Costerton et al. 1995). The second postulates that biofilm bacteria undergo phenotypic changes, entering a state of dormancy, which renders them more resistant to antimicrobials (Costerton et al. 1995). We chose to investigate resistance to two antibiotics that differ in their modes of action as well as their ability to penetrate biofilms. Susceptibility to ciprofloxacin, which readily diffuses through biofilms (Shigeta et al. 1997), was equivalent between the PA23 and PA23-314 biofilms. As restricted penetration is not a factor, the *gacS* and wild-type cells apparently underwent phenotypic changes during transition to the biofilm mode of growth, rendering them ciprofloxacin resistant. Tobramycin, on the other hand, is a bulky, negatively charged drug known for poor biofilm penetration (Davis 1987; Shigeta et al. 1997). Both the wild-type and *gacS*-deficient strains exhibited 650 times increased resistance to tobramycin when growing as a biofilm. These findings clearly demonstrate that althoug the *gacS* mutant did not form a robust biofilm, the exopolysaccharide matrix was sufficient for aminoglycoside exclusion. In the environment, *P. chlororaphis* would likely be exposed to antimicrobial agents elicited by both co-colonizing microbes and plants. For example, sweet basil roots secrete the antibiotic rosmarinic acid (RA), which exhibits antibacterial activity against phytopathogenic *P. aeruginosa* (Walker et al. 2004). Studies have shown that if *P. aeruginosa* biofilms are allowed to form on the basil root surface, the bacteria become resistant to RA and ultimately cause plant mortality (Walker et al. 2004). Upon induction of elevated RA levels in the plants or exogenous RA application, *P. aeruginosa* biofilm formation is prevented, resulting in bacterial death and plant survival (Walker et al. 2004). Findings such as these highlight the fact that the capacity to form biofilms will greatly impact bacterial sustainability in a given environment. Our studies suggest that for a number of antibacterial compounds, *P. chlororaphis gacS* mutants would exhibit resistance equivalent to the parent. Since *gacS* and *gacA* spontaneous mutants arise at such a high frequency (Chancey et al. 1999, 2002; Duffy and Défago 2000), it is not surprising that on many levels their fitness is on par with that of the wild-type strains.

Previous studies revealed that *Pseudomonas aureofaciens gacS*- and *gacA*-deficient strains have an early growth advantage in LB but not AB minimal media (Chancey et al. 2002). Schmidt-Eisenlohr and coworkers (2003) found the exact opposite trend. Analysis of a *P. chlororaphis gacS* mutant revealed a reduced lag phase in M9 minimal media; whereas this growth advantage was lost in LB. To determine whether *gacS* influenced growth, we performed a growth analysis of PA23, PA23-314, and the complemented mutant in four different media: M9 minimal supplemented with 0.2% glucose or 0.2% glycerol, LB, and TB. Our results are consistent with those of Schmidt-Eisenlohr et al. (2003). In M9 media containing either glucose (Fig. 2*a*) or glycerol (data not shown) as a carbon source, the *gacS* mutant had a reduced lag phase compared with the wild-type and complemented strains (approximately 4 vs 8 h). In the comparatively rich media LB and ultra-rich TB media, the three strains exhibited similar entry into logarithmic growth phase (Figs. 2*b* and 2*c*). Because nutrient availability would be limited in the rhizosphere and phyllosphere, a reduced lag phase may enable *gac* mutants to get a head start on colonization.

A second major difference in the growth profile of the *gacS* mutant appeared at a later point in the growth curve. At approximately 20 h, in all media tested, the *gacS* mutant population began increasing at a much slower rate that the wild-type and complemented strains. The most marked difference in population density was observed in TB where the *gacS* mutant strain only reached an OD of 1.0 after 45 h growth (Fig. 2*c*). In comparison, by 15 h the population density of the *gacS*-sufficient strains had exceeded an OD of 1.5 (Fig. 2*c*). Thus, it appears that a *gacS* mutation dramatically reduces the total achievable population size, and this phenomenon is most pronounced under nutrient-rich conditions. Consistent with previous findings (Whistler et al. 1998; Kang et al. 2004; Heeb et al. 2005), we observed that expression of an *rpoS-lacZ* transcriptional fusion and RpoS protein levels were greatly reduced in a PA23 *gacS* background. As RpoS is important for activation of stress-response genes, decreased levels of this sigma factor may render cells more sensitive to oxidative stress, etc. The addition of constitutively expressed *rpoS* in trans to PA23-314 had no impact on total achievable population size, indicating that decreased *rpoS* expression can not account for the *gacS* growth anomaly. Alternatively, a growth-limiting secondary metabolite might be produced by the *gacS*-deficient strain that accumulates to a high concentration in rich media. If this is the case, however, why would high-nutrient conditions select for *gac* mutant bacteria, as previously described by Défago and Duffy (2000)? Clearly, there are complex physiological changes associated with *gac* mutations. Early entry into stationary phase and arrested cell division under nutrient-rich conditions represent another twist in the *gac* story.

Bacterial cells that undergo prolonged growth in batch culture exhibit increased fitness referred to as a GASP phenotype (Zambrano and Kolter 1996; Zambrano et al. 1993; Vulić and Kolter 2001; Farrell and Finkel 2003). While the rest of the wild-type population remains in a nondividing state, GASP mutants scavenge nutrients released by dying cells, enabling them to reinitiate growth (Vulić and Kolter 2001). Recently, a *P. aureofaciens* GASP mutant was identified with a mutation in the LysR-type regulatory gene *finR* (Silby et al. 2005). It is important to note that the *finR* mutant lacked the antifungal phenotype of the parent. Because many GASP mutations result in attenuated RpoS activity (Zambrano et al. 1993; Martínez-García et al. 2003), we wondered if reduced RpoS expression would confer a GASP phenotype upon PA23-314. Our findings revealed that the

wild-type PA23 was not displaced by the *gacS* mutant during prolonged culture, rather both strains exhibited equivalent fitness (Fig. 4). In an *E. coli* background, GASP mutants were found to attain a higher cell density than the wild type when growing as a monoculture in LB medium (Vulić and Kolter 2001). Considering the fact that our *P. chlororaphis gacS* mutant exhibited premature entry into stationary phase in LB and achieved a lower total population density, it is not surprising that the *gacS* mutant did not overtake the wild type.

Towards commercial application of PA23, our findings underscore the need to prevent *gacS* mutant accumulation during inoculum preparation. Consistent with studies of other *gac* mutants (Gaffney et al. 1994; Whistler et al. 1998; Chancey et al. 1999; Koch et al. 2002; van den Broek et al. 2003), a *gacS*-deficient strain of PA23 exhibited no biocontrol activity. Furthermore, we have demonstrated that a *gacS* deficiency does not effect formation of antibiotic-resistant biofilms or persistence in the canola phyllosphere. Accordingly, natural selection against these mutants is expected to be very low.

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