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Characterization of spontaneous *gacS* and *gacA* regulatory mutants of *Pseudomonas fluorescens* biocontrol strain CHA0

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Abstract

In *Pseudomonas fluorescens* strain CHA0, the response regulator gene *gacA* controls expression of extracellular enzymes and antifungal secondary metabolites, which are important for this strain's biocontrol activity in the plant rhizosphere. Two Tn*5* insertion mutants of strain CHA0 that had the same pleiotropic phenotype as *gacA* mutants were complemented by the *gacS* sensor kinase gene of *P. syringae* pv. *syringae* as well as that of *P. fluorescens* strain Pf-5, indicating that both transposon insertions had occurred in the *gacS* gene of strain CHA0. This conclusion was supported by Southern hybridisation using a *gacS* probe from strain Pf-5. Overexpression of the wild-type *gacA* gene partially compensated for the *gacS* mutation, however, the overexpressed *gacA* gene was not stably maintained, suggesting that this is deleterious to the bacterium. Strain CHA0 grown to stationary phase in nutrient-rich liquid media for several days accumulated spontaneous pleiotropic mutants to levels representing 1.25% of the population; all mutants lacked key antifungal metabolites and extracellular protease. Half of 44 spontaneous mutants tested were complemented by *gacS*, the other half were restored by *gacA*. Independent point and deletion mutations arose at different sites in the *gacA* gene. In competition experiments with mixtures of the wild type and a *gacA* mutant incubated in nutrient-rich broth, the mutant population temporarily increased as the wild type decreased. In conclusion, loss of *gacA* function can confer a selective advantage on strain CHA0 under laboratory conditions.

Abbreviations: DAPG – 2,4-diacetylphloroglucinol; HCN – hydrogen cyanide

Introduction

Fluorescent pseudomonads produce an array of antimicrobial secondary metabolites, some of which are important for the biological control of plant root diseases (Thomashow & Weller 1995; Handelsman & Stabb 1996; Haas et al. 2000). *Pseudomonas fluorescens* strain CHA0 has become a model organism to study plant-associated pseudomonads used for biological control of soilborne fungal pathogens (Voisard et al. 1994). Strain CHA0 produces the antibiotics 2,4-diacetylphloroglucinol (DAPG), pyoluteorin and pyrrolnitrin, as well as the biocide hydrogen cyanide (HCN) (Keel & Défago 1997). Mutational analysis has demonstrated a role for DAPG, pyoluteorin and HCN in the biocontrol of root diseases caused by *Thielaviopsis basicola, Fusarium oxysporum, Gaeumannomyces graminis* var. *tritici* and *Pythium ultimum*

(Voisard et al. 1994; Keel & Défago 1997; Duffy & Défago 1998). The relative importance of these compounds in disease control varies depending on the plant-pathogen combination (Maurhofer et al. 1994; Keel & Défago 1997). *In vitro*, the antibiotics and HCN are produced by strain CHA0 primarily during the transition from exponential to stationary phase (Voisard et al. 1989; Laville et al. 1992; Schnider-Keel et al. 2000).

A functional *gacA* gene (*g*lobal control of antibiotics and *c*yanide) in *P. fluorescens* is required for the biosynthesis of the antifungal compounds mentioned and certain enzymes, i.e., tryptophan side-chain oxidase, extracellular protease, and phospholipase C (Laville et al. 1992; Sacherer et al. 1993). Mutations in *gacA* virtually abolish biological control of tobacco black root rot caused by *T. basicola* (Laville et al. 1992). The GacA response regulator is a member of the FixJ family of bacterial two-component regulation systems. These are composed of a histidine protein kinase that acts as an environmental sensor and a cytoplasmic response regulator. Signal-dependent autophosphorylation of the sensor and subsequent activation of the response regulator by phosphate transfer provide transcriptional control of target genes (Albright et al. 1989; Perraud et al. 1999; Pirrung 1999). The cognate sensor of GacA, that is, GacS, has been characterized mainly in the biocontrol strain *P. fluorescens* Pf-5 and in the plant pathogen *P. syringae* pv. *syringae* where this sensor is required for antibiotic production and lesion manifestation, respectively (Rich et al. 1994; Corbell & Loper 1995; Kitten et al. 1998). The GacS/GacA system occurs in many Gram-negative bacteria associated with plants and animals (Gaffney et al. 1994; Frederick et al. 1997; Reimmann et al. 1997; Blumer et al. 2000).

It has long been suggested, but little studied, that handling of biocontrol strains in the laboratory can lead to the accumulation of undesirable mutations with subsequent loss of biocontrol activity (Weller 1988). Our preliminary observations (Voisard et al. 1994; Duffy & Défago 1995) have suggested that pleiotropic, antibiotic-negative mutants of strain CHA0 occur at high frequency under certain cultural conditions. In *Streptomyces* spp., instability of secondary metabolism has also been observed and is due to gene amplification or deletion (Aigle et al. 1996). The principal aim of this study was to elucidate the basis of genetic instability adversely affecting antibiotic production in strain CHA0.

Materials and methods

Bacterial strains, plasmids, media 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 nutrient agar or in nutrient yeast broth (NYB; 25 g Oxoid nutrient broth; 5 g Difco yeast extract per liter) with aeration (Stanisich & Holloway 1972). *P. fluorescens* strains were grown at 30 °C on nutrient agar, minimal medium E (MME; Vogel & Bonner 1956), 2% Oxoid malt agar, King's medium B (KB; King et al. 1954) or in either NYB or nutrient broth (NB; 8 g Difco nutrient broth per liter). Protease production was detected on skim milk agar (Gerhardt et al. 1981). Nutrient agar was supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; 40 μg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG; 100 μg/ml) in cloning experiments when required. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 20 μg/ml; chloramphenicol, 20 μg/ml; and kanamycin, 50 μg/ml.

Determination of secondary metabolites and enzyme activities

DAPG, pyoluteorin and HCN were determined qualitatively and quantitatively as previously described (Voisard et al. 1989; Keel et al. 1992; Maurhofer et al. 1994; Duffy & Défago 1999). Tryptophan side-chain oxidase and protease activities were assessed qualitatively on agar plates (Oberhänsli et al. 1991; Sacherer et al. 1993). Pyoverdin production was scored as growth on MME containing ethylene-diamine-di-(*o*-hydroxyphenylacetate) (EDDHA) at 50 μg/ml (Keel et al. 1989).

Generation of pleiotropic mutants

Tn*5*-induced *gacS* mutants were isolated by a technique described previously (Maurhofer et al. 1994). Spontaneous pleiotropic mutants were generated by growing *P. fluorescens* CHA0 in 108 separate tubes each containing 3 ml NYB. Half of the tubes were agitated during incubation and the other half were not. After 22, 42 and 51 days of incubation at 30 °C, 50 colonies grown from 10 to 20 tubes chosen at random were tested for pleiotropic mutations by screening for tryptophan side-chain oxidase activity and production of protease and HCN. In addition, auxotrophy and

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype[1]	Source or reference
Pseudomonas fluorescens		
CHA0	wild-type	Voisard et al. 1994
CHA89	*gacA* mutant of CHA0, Km^r	Laville et al. 1992
CHA500	spontaneous *gacA* mutant of CHA0	Laville et al. 1992
CHA510	*gacS*::Tn*5* mutant of CHA0	This study
CHA511	*gacS*::Tn*5* mutant of CHA0	This study
CHAS5	spontaneous *gacA* mutant of CHA0	This study
CHAS9	spontaneous *gacA* mutant of CHA0	This study
CHAS17	spontaneous *gacS* mutant of CHA0	This study
CHAS33	spontaneous *gacA* mutant of CHA0	This study
CHAS38	spontaneous *gacA* mutant of CHA0	This study
CHAS61	spontaneous *gacS* mutant of CHA0	This study
Escherichia coli		
DH5α	*recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1* Δ(*lacZYA-argF*)*U169* (ϕ80d*lacZ*Δ*M15*)	Sambrook et al. 1989
JM109	*supE44 hsdR17 thi gyrA96 relA1*Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	Yanisch-Perron et al. 1985
Plasmids		
pVK100	Mob^+, IncP replicon, Km^r, Tc^r	Voisard et al. 1989
pLAFR3	Mob^+, IncP replicon, Tc^r	Staskawicz et al. 1987
pBluescript II KS+	ColE1 replicon, Ap^r	Stratagene
pME497	Tra^+, Mob^+, IncP replicon, Ap/Cb^r	Voisard et al. 1988
pME3008	contains *gacA*(Y49) and *uvrC* of strain CHA0, Tc^r	Laville et al. 1992
pME3066	contains *gacA*(Y49) of strain CHA0, Tc^r	Laville et al. 1992
pME3067	contains *gacA*(Y49) of strain CHA0 in pBluescript II KS^+, Ap^r	Laville et al. 1992
pJEL5680	contains *gacS* (*apdA*) of *P. fluorescens* Pf-5, Ap^r	Corbell & Loper 1995
pJEL5771	contains *gacS* (*apdA*) of *P. fluorescens* Pf-5, Tc^r	Corbell & Loper 1995
pEMH97	contains *gacS* (*lemA*) of *P. syringae* pv. *syringae* B728a, Tc^r	Hrabak & Willis 1992
pME3250	19-kb *Hin*dIII fragment of CHA0, containing *gacA*(D49) in pVK100, Tc^r	This study
pME3254	1.7-kb *Bam*HI-*Pst*I fragment from pME3250, containing *gacA*(D49) inserted into the *Bam*HI site of pLAFR3[1]	This study
pME3255	1.7-kb *Bam*HI-*Pst*I fragment from pME3250, containing *gacA*(D 49) in pLAFR3 [2]	This study

[1]The vector *lac* promoter and the *gacA*(D49) gene are in opposite orientation.
[2]The vector *lac* promoter reads into the *gacA*(D49) gene.

production of fluorescent siderophore (pyoverdin) was monitored.

DNA manipulations

Plasmids and cosmids were isolated from *E. coli* and *P. fluorescens* strains by an alkaline lysis procedure (Sambrook et al. 1989) and purified using Qiagen Tip 100 (Qiagen Inc.). Genomic DNA was isolated by the cetyl-trimethyl-ammonium-bromide (CTAB) method (Del Sal et al. 1988). T4 DNA ligase and restriction endonucleases were used as recommended by the suppliers (GIBCO-BRL Life Technologies or Boehringer Mannheim).

Identification of genes complementing the pleiotropic mutants

A genomic library of strain CHA0 constructed in the vector pVK100 (Voisard et al. 1989) was used in this study. Mobilisation of recombinant cosmids from *E. coli* into inherently chloramphenicol resistant *P. fluorescens* strains was accomplished by triparental matings with the helper plasmid pME497 (Voisard

et al. 1988). Transconjugants, selected for resistance to chloramphenicol and tetracycline, were screened qualitatively for tryptophan side-chain oxidase activity and production of HCN and protease. Transconjugants which were positive for these traits were further tested for production of antibiotics (DAPG, pyoluteorin).

Southern hybridisation

Probes for Southern blots were the *gacA* gene of *P. fluorescens* strain CHA0 on the 1.6-kb *Bgl*II fragment of pME3067 (Laville et al. 1992) and the *gacS* gene of strain Pf-5 on the 6.7-kb *Kpn*I fragment of pJEL5680 (Corbell & Loper 1995). These fragments were purified from low melting agarose gels (SeaKem GTG, FMC BioProducts) using Geneclean Kit (Bio 101 Inc.). Probes (0.25 μg/ml) were labeled by the random-primed DNA with digoxigenin-11-dUTP (Boehringer Mannheim). DNA fragments to be probed were transferred from agarose gels to nylon membranes (Hybond N, Amersham) by standard methods (Sambrook et al. 1989). Conditions of low stringency at 44 to 55 °C with 0.2 × SSC were used for hybridisation (Sambrook et al. 1989).

PCR and nucleotide sequence analysis of the [gacA] *gene*

PCR reactions used 0.2 μg of genomic DNA from *P. fluorescens*, 0.5 μg of each primer (*Bam*HI: 5′CCTT*GGATCC*CGATCCAG3′ prime upstream of the *gacA* promoter and *Pst*I: 5′GCGGCCA*CTGCAG*G TAGA3′ downstream of *gacA* [Laville et al. 1992]), 1 unit Taq DNA polymerase (Boehringer Mannheim) in 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3, 0.2 mM of each deoxyribonucloside triphosphate (dNTP) in a total volume of 100 μl. DNA was amplified for 35 cycles (94 °C, 60 s; 50 °C, 45 s; 72 °C, 120 s) using a DNA Thermal Cycler (Appligene, Heidelberg, Germany). The 1-kb PCR products were gel-purified, digested with *Bam*HI and *Pst*I, and cloned into M13 mp18/19 digested with *Bam*HI and *Pst*I. The host strain for transfection was JM109 (Yanisch-Perron et al. 1985). Sequencing was conducted by the dideoxy terminator method (Sanger et al. 1977) using Sequenase Version 2.0 (United States Biochemical, Cleveland, OH). Sequencing reactions were primed with standard M13 and two primers internal to *gacA*. The chromosomal *gacA* alleles were sequenced from four separate group 1 mutants (CHAS5, CHAS9, CHAS33 and CHAS38) and two group 2 mutants (CHAS17 and CHAS61), as well as the *gacS* Tn*5* mutant CHA510, and CHA0 from two separate frozen stocks.

Competition between wild-type CHA0 and a [gacA] *mutant over time*

A strain mixture was prepared by combining starter cultures grown in NB to an OD at 600 nm of 1.6, at a ratio of 9.9-ml of wild type CHA0 and 0.1-ml of the *gacA* mutant CHA89. Test cultures of 20 ml NB per 100-ml flask were inoculated with 10 μl of the strain mixture. Total CFU/ml and percent mutants at each sampling time were determined by plating serial dilutions on 10% tryptic soy agar (Difco). The number of pleiotropic mutants, expressed as percent of total CFU, was determined with 1500 to 2500 colonies per replicate. Plates were incubated for 2 days at 24 °C and mutant colonies were identified based on the lack of tryptophan side-chain oxidase activity, using an indicator agar overlay technique described previously (Oberhänsli et al. 1991). Treatments consisted of six replicate test cultures. Bacterial CFU data were $\log_{10}$ transformed and percent mutant values were arcsine-transformed prior to analysis.

Results

Isolation of Tn5 insertion mutants of strain CHA0 defective in the [gacS] *gene*

Two pleiotropic mutants, CHA510 and CHA511, lacking production of DAPG, pyoluteorin, HCN, extracellular protease and tryptophan side-chain oxidase were isolated after Tn*5* mutagenesis of strain CHA0. These mutants were phenotypically indistinguishable from *gacA* mutants of strain CHA0 (Laville et al. 1992; Sacherer et al. 1993), but not complemented for any of their phenotypes by the *gacA* allele present on plasmids pME3008 and pME3066, indicating that a pleiotropic mutation had occurred outside *gacA*. The mutants, namely CHA510 and CHA511, were functionally complemented by the *gacS* (formerly *apdA*) gene from *P. fluorescens* strain Pf-5 on plasmid pJEL5771 and by *gacS* (formerly *lemA*) from *P. syringae* pv. *syringae* strain B728a on plasmid pEMH97 (Table 2). The *gacS* gene of strain Pf-5 hybridised to a 9.4-kb *Bam*HI fragment and to two *Eco*RI (7.9-kb and 6.8-kb) fragments in genomic DNA from strain CHA0. In the mutant strain CHA510, the *gacS* gene hybridised to two *Bam*HI bands (9.5-kb and 5.7-kb) and to two *Eco*RI bands (13.6 kb and 6.8 kb) (data not

Table 2. Complementation of pleiotropic mutations by plasmids containing *gacA*(D49), *gacA*(Y49), *apdA/gacS* or *lemA/gacS*

Strain	Relevant genotype	Antibiotic production (μg/ml medium)		Protease[3]	TSO[4]	HCN production[5] (μM)
		DAPG[1]	PLT[2]			
CHA0	wt	3.2 ± 0.6	4.0 ± 0.2	++	++	30.9 ± 3.7
CHA510	*gacS*$^-$	< 0.1	< 0.1	–	–	< 5
CHA510(pME3250)	*gacA*(D49)	< 0.1	2.1 ± 1.1	+	+	9.3 ± 1.0
CHA510(pME3254)	*gacA*(D49)	< 0.1	6.5 ± 0.9	+	+	6.9 ± 2.8
CHA510(pME3066)	*gacA*(Y49)	< 0.1	NT[6]	–	–	< 5
CHA510(pJEL5771)	*gacS*$^+$ (*apdA*$^+$)	NT	NT	++	++	NT
CHA510(pEMH97)	*gacS*$^+$ (*lemA*$^+$)	3.1 ± 3.0	4.1 ± 0.4	++	++	11.7 ± 3.5
CHA89	*gacA*$^-$	< 0.1	< 0.1	–	–	< 5
CHA89(pME3250)	*gacA*(D49)	6.1 ± 3.1	6.3 ± 1.1	++	+	21.0 ± 0.6
CHA89(pME3254)	*gacA*(D49)	1.4 ± 0.7	3.7 ± 1.2	++	+	14.5 ± 2.1
CHA89(pME3008)	*gacA*(Y49)	0.7 ± 1.2	5.0 ± 0.7	++	+	33.9 ± 7.7
CHA89(pME3066)	*gacA*(Y49)	3.7 ± 2.3	3.4 ± 0.4	++	+	27.9 ± 0.8
CHA89(pEMH97)	*gacS*$^+$ (*lemA*$^+$)	< 0.1	< 0.1	++	+	< 5

[1]Production of 2,4-diacetylphloroglucinol (DAPG) on malt agar incubated at 24 °C for 3 days.
[2]Production of pyoluteorin (PLT) on KB agar incubated at 24 °C for 3 days.
[3]A clear zone around colonies grown for 3 to 7 days on milk agar (++, zone present after 3 days; +, zone present after 7 days; –, no zone present after 7 days).
[4]Qualitative evaluation of tryptophan side-chain oxidase (TSO) activity: ++, black; +, brown; –, white.
[5]Quantitative evaluation of hydrogen cyanide (HCN) production by strains grown for 48 h at 27°C under semi-anaerobic conditions.
[6]NT, not tested.

shown), indicating that an insertion of approximately 5.7 kb, i.e. Tn*5*, was in a locus hybridising to the *gacS* gene. According to analysis of restriction fragments to which the *gacS* gene hybridised, the Tn*5* insertions in CHA510 and CHA511 were close to each other (data not shown). Taken together, these data suggest that the mutations in CHA510 and CHA511 affect a homolog of *gacS*. Therefore, these mutations were designated *gacS*.

High frequency of spontaneous pleiotropic mutations in strain CHA0

Under laboratory conditions, strain CHA0 was genetically unstable giving rise to a high frequency of spontaneous pleiotropic mutants. Over 4000 colonies derived at random from 108 independent broth cultures of strain CHA0 grown to late stationary phase in nutrient-rich media (NYB) were screened for production of HCN, extracellular protease, tryptophan side-chain oxidase, and a fluorescent siderophore (pyoverdin) and for auxotrophy. One (unidentified) auxotrophic mutant and no siderophore-negative mutants were found in this experiment. In contrast, 1.25% of all colonies tested were negative for HCN production, extracellular protease and tryptophan side-chain oxidase. These spontaneous mutants arose with similar probabilites in shaken and unshaken cultures incubated for up to 51 days, although there was considerable fluctuation of mutant – to – wild-type ratios among individual cultures. Forty-four pleiotropic mutants, which came from separate cultures and therefore represented individual mutational events, were separated into two groups based on complementation with *gacA* or *gacS*. Twenty-two mutants (group 1) were complemented for HCN and protease production, and tryptophan side-chain oxidase activity by the *gacA* gene on plasmids pME3066 and pME3008, indicating that these mutants were *gacA* mutants. Additionally, group 1 mutants were not complemented by plasmids pJEL5771 or pEMH97, which carry *gacS* from *P. fluorescens* and *P. syringae* pv. *syringae*, respectively. Complementation of the other mutants (group 2) for HCN and protease production, and tryptophan side-chain oxidase activity by pEMH97 or pJEL5771 indicated that these mutants were *gacS* mutants. Group 2 mutants were not complemented by pME3008 and pME3066, carrying *gacA*. All spontaneous mutants were insensitive to UV irradiation, like the wild-type

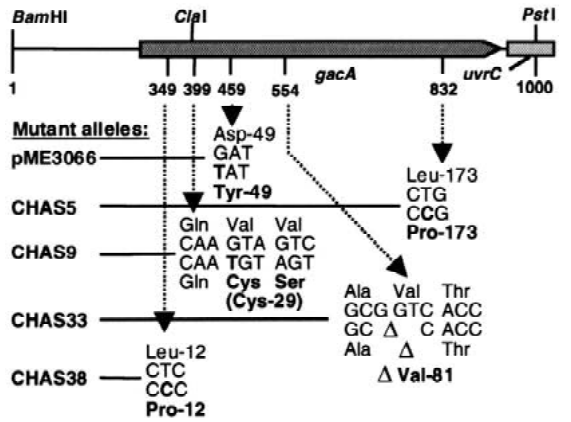

Figure 1. Spontaneous mutations in *gacA*. Nucleotide numbers refer to the sequence published by Laville et al. (1992). Amino acids printed in bold represent changes in mutants compared to the wild-type allele of *gacA*(D49). Arrows from the cloned DNA fragment indicate the location of the mutations in the *gacA* (D49) gene; Δ designates deletion.

strain CHA0, but unlike some *gacA* insertion mutants which are UV-sensitive because the insertion mutations have a polar effect on the downstream *uvrC* gene (Laville et al. 1992). We conclude from the mutant analysis that, when strain CHA0 is grown in nutrient-rich media, *gacA* and *gacS* mutants appear to be specifically selected in that mutants affected in biosynthetic pathways for amino acids or siderophores were rarely recovered.

Genetic analysis of group 1 [gacA] *mutants*

The 1.65-kb *Bam*HI-*Bgl*II restriction fragment of pME3066 carrying the *gacA* gene (Laville et al. 1992) was present in all group 1 mutants examined by Southern hybridization (data not shown), indicating that the spontaneous *gacA* mutations are not caused by deletions of 50 bp or greater. After amplification by PCR, the *gacA* alleles from four group 1 mutants were sequenced and found to contain different mutations (Figure 1). Strain CHAS38 had a point mutation at position 349 (T ⇒ C) which resulted in a change of Leu-12 to Pro in the deduced amino acid sequence of GacA. Strain CHAS5 had a point mutation at position 832 (T ⇒ C) which produced a Leu-173 to Pro change. Strain CHAS33 had a 3-bp deletion starting at position 554 which resulted in loss of Val-81. The mutation in strain CHAS9, addition of a T at position 399, created a frameshift affecting the GacA protein downstream of Gln-28. Two group 2 strains, CHAS17 and CHAS61, analysed as controls, did not have any mutation in the *gacA* allele. However, the four *gacA* mutant alleles and the two *gacA* control alleles from the group 2 mutants all contained a G at position 459 (resulting in Asp-49), whereas the *gacA* gene on pME3066 previously sequenced (Laville et al. 1992) contains a T at this position (resulting in Tyr-49). Because of this unexpected difference, the *gacA* gene of the wild-type CHA0 was also sequenced and found to contain a G at position 459. Thus, the *gacA*(Y49) sequence reported by Laville et al. (1992) appears to be a functional mutant allele of the *gacA*(D49) wild-type gene.

Isolation of the [gacA] *(D49) wild-type gene from a cosmid library*

Four recombinant cosmids from a genomic library of strain CHA0, each containing the same 19-kb *Hin*dIII insert, complemented the *gacA* mutant CHA89 for HCN and protease production, and tryptophan side-chain oxidase activity. One representative cosmid (pME3250) was similar to the *gacA*(Y49) cosmid pME3008 (Laville et al. 1992) in terms of the restriction map. The 1.0-kb *Bam*HI-*Pst*I fragment of pME3250 encompassing the *gacA* gene (Figure 1) was identified by sequencing. At position 459 a G was found, implying that pME3250 carries the *gacA*(D49) wild-type gene. The 1.0-kb *Bam*HI-*Pst*I was inserted into the cloning site of pLAFR3, resulting in pME3255. In this construct, the *gacA*(D49) gene was under the control of the *lac* promoter of the vector. Although pME3255 was able to complement the *gacA* mutant CHA89 for HCN and protease production, and tryptophan side-chain oxidase activity, complementation was highly unstable, as evidenced by the rapid loss of tryptophan side-chain oxidase activity. Complementation of the chromosomal tryptophan side-chain oxidase marker by the recombinant plasmid could be complete or partial, with sectored colonies appearing. Therefore, the 1.0-kb fragment was cloned into pLAFR3 in the opposite orientation, producing pME3254, in which *gacA*(D49) was assumed to be expressed from its own promoter. Plasmid pME3254 complemented the *gacA* mutant CHA89 for all phenotypes, including the production of the antibiotics DAPG and pyoluteorin (Table 2). The *gacA*(D49) allele also conferred partial complementation of the *gacS* mutants, CHA510 and CHA511, in that tryptophan side-chain oxidase activity and pyoluteorin production were observed (Table 2). Protease activity, which was apparent in strain CHA0 after 1 day, was

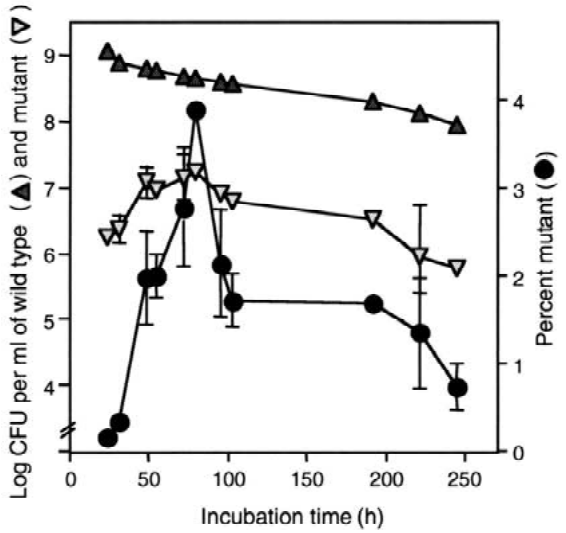

Figure 2. Competition between wild-type CHA0 and its *gacA* mutant CHA89 in 0.8% nutrient broth during stationary phase growth. Cultures were inoculated with a suspension of 1% mutant and 99% wild type to give approximately 2×10^6 CFU/ml. Log CFU/ml were determined by spreading culture dilutions onto 0.1 × tryptic soy agar. The number of mutants accumulating over time is expressed as the percentage of total colonies observed. Mutant colonies were identified based on lack of tryptophan side-chain oxidase activity. Values represent the means (± standard error) of six replicate cultures per sampling time.

not detected in the complemented CHA510 after 1 day, but could be detected after 1 week. HCN production was also restored partially (Table 2). Thus, the amplified *gacA*(D49) gene partly compensated for the absence of a functional GacS sensor, whereas the *gacA*(Y49) allele did not (Table 2).

Competition between the wild type CHA0 and a [gacA] *mutant during stationary phase growth in liquid culture*

In order to see whether a *gacA* mutant has a competitive advantage over the wild type during stationary growth phase, a reconstruction experiment was performed. NB was inoculated with 99% CHA0 wild-type cells and 1% CHA89 *gacA* mutant cells, resulting in a total inoculum of 2×10^6 CFU/ml. During an initial period of late-stationary growth the proportion of pleiotropic mutants (either the initial *gacA* mutant or new mutants formed spontaneously) increased markedly to a maximal level of about 4% of the total cell population (Figure 2). The concomitant drop of cultivable cells of CHA0 suggested that during this period the mutants grew at the expense of dying wild-type cells (Figure 2). Interestingly, the mutant population declined thereafter to levels below 1% of the total cell population (Figure 2), indicating that the selective advantage of the mutant over the wild type was only temporary and restricted to an early phase of stationary growth.

Discussion

We have shown here that *P. fluorescens* CHA0 undergoes, with equal probability, spontaneous mutations in the *gacS* or *gacA* genes. Other spontaneous mutations giving a similar pleiotropic phenotype (i.e., loss of antibiotics, HCN, extracellular protease, and tryptophan side-chain oxidase) were not detected. This finding provides further genetic evidence that GacS and GacA constitute a sensor kinase – response regulator pair. GacA is related to FixJ (Laville et al. 1992). FixJ can be activated by phosphorylation of Asp-54 (Reyrat et al. 1994). Asp-54 is conserved in GacA (Laville et al. 1992) and in many other response regulators (Hrabak & Willis 1992; Parkinson & Kofoid 1992; Moore et al. 1993). In wild-type GacA of *P. fluorescens* CHA0 (this study), *P. syringae* (Rich et al. 1994), *P. fluorescens* BL915 (Gaffney et al. 1994), *P. viridiflava* (Liao et al. 1996), *P. aeruginosa* (Reimmann et al. 1997), *Erwinia carotovora* (Ericksson et al. 1998) and *Salmonella typhimurium* (Johnston et al. 1996), there is another conserved aspartate residue (Asp-49) in the vicinity of Asp-54. We demonstrate here that the *gacA*(Y49) sequence reported before (Laville et al. 1992) is a functional mutant allele of *gacA*(D49). When the wild-type *gacA*(D49) gene was carried by oligo-copy plasmids in strain CHA0, spontaneous loss of *gacA* function was frequent and it proved difficult to maintain the intact plasmids. Instability was most pronounced when a strong vector promoter drove the expression of *gacA*(D49) in plasmid pME3255. Thus, it appears that over-expression of the wild-type *gacA*(D49) gene was poorly tolerated by strain CHA0. In contrast, the *gacA*(Y49) allele (Laville et al. 1992) has been carried by the same IncP plasmids (such as pME3008 or pME3066) in strain CHA0 for many generations. The *gacA*(Y49) allele is biologically active, as evidenced by quantitative complementation of the *gacA* mutant CHA89, and stable in strain CHA0. We suggest that *gacA*(Y49) has reduced regulator activity, compared with that of the wild-type *gacA*(D49), but that it can gain full activity by a gene dosage effect.

In the VirG response regulator of *Agrobacterium tumefaciens*, Asp-52 (corresponding to Asp-54 of

GacA and FixJ) is phosphorylated during signal transduction (Jin et al. 1990). A mutation near this conserved aspartate (Asn-54→Asp) causes a constitutive (high) phenotype, suggesting that the two closely linked aspartyl residues can mimic the phosphorylated form of Asp-52 (Scheeren-Groot et al. 1994; Gubba et al. 1995). In wild-type GacA, the postulated phosphorylation of Asp-54 might be positively influenced by Asp-49 located nearby.

In this study, amplification of the *gacA*(D49) gene by-passed, in part and transiently, the need for the sensor encoded by *gacS*. Overproduction of other response regulators has been observed to elicit a sensor-independent response (Albright et al. 1989). In particular, multicopy *gacA* (*expA*) suppresses a *gacS* (*expS*) mutation in *Erwinia carotovora* for virulence and cellulase production (Ericksson et al. 1998). From the point of view of biological control, a sensor-independent expression of secondary metabolites could be desirable. In *P. fluorescens* BL915 carrying a *gacA* overexpression construct enhanced biocontrol activity and antibiotic (pyrrolnitrin) production have been reported (Ligon et al. 1999). However, the lack of stability of plasmids such as pME3255 has precluded their use in biocontrol experiments with strain CHA0.

The first spontaneous *gacA* mutant of strain CHA0 reported by Laville et al. (1992) contained a 5-kb deletion encompassing the *gacA* gene and its upstream region. The spontaneous *gacA* mutants examined here appeared to be due to small genetic lesions such as the point mutations identified in Figure 1. One mutation (L173P) studied here as well as an additional mutation (T182I) reported by Gaffney et al. (1994) affect the putative helix-turn-helix motif of GacA. Another mutation (L12P) concerns a residue in the vicinity of the conserved aspartate residue at position 9 of GacA. However, none of the point mutations in GacA of strains CHA0 and BL915 (Gaffney et al. 1994) directly affects the DDK motif (Asp-9, Asp-54, Lys-104), which is characteristic of response regulators (Albright et al. 1989; Parkinson & Kofoid 1992). Two important conclusions of our work are that (i) there is no evidence for a genetic "hotspot" of mutation in *gacA* and (ii) the genetic instability of secondary metabolism in *P. fluorescens* appears to be unlike that of *Streptomyces* spp. where deletion and/or amplification of large genomic fragments are a major cause of genetic instability (Aigle et al. 1996).

Genetic instability of the *gacS*/*gacA* genes is probably not unique to *P. fluorescens* strain CHA0. For instance, *P. fluorescens* strain Pf-5 shows a similar phenomenon after growth in NYB for 6 days (JE Loper, pers. comm.). *P. tolaasii*, a mushroom pathogen, shows phenotypic switching between smooth and rough colony forms as a consequence of a reversible 661-bp duplication of a segment within the *gacS* (=*pheN*) gene, and inactivation of *gacS* appears to be favored by high cell densities (Grewal et al. 1995). A marked susceptibility of the *gacS* and *gacA* genes to spontaneous mutations has also been observed in *P. syringae* pv. *syringae* cultivated under conditions of high nutrient concentration (Rich et al. 1994). The common denominator in these experimental systems seems to be media containing more nutrients than needed for growth of the bacteria. It is not clear by which mechanism(s) these growth conditions might favor the *gacS*/*gacA* mutants over the wild type. It could be argued that the secondary metabolites and exoenzymes which are controlled by the *gacS*/*gacA* system may be of little use to bacteria when grown in nutrient rich, synthetic media. Lack of expression of these non-essential functions would lighten the metabolic burden on the bacteria, thereby resulting in better growth. This hypothesis may be correct but probably is too simple. For instance, the selective advantage of *gacS*/*gacA* mutants is not associated with the loss of DAPG and pyoluteorin production, because strain CHA0 produces negligible amounts of pyoluteorin (23 ng per 1×10^8 CFU) and no detectable DAPG in NYB where the advantage was observed. Also, the results from a competition experiment involving wild type CHA0 and a *gacA* mutant (Figure 2) indicate that the mutant has only a temporary selective advantage over the wild type during an initial period of stationary growth. The subsequent decline of the mutant population (Figure 2) could be due to a reduced resistance of the mutants to the stress conditions prevailing in late stationary phase culture, since stationary-phase cells of *gacA* and *gacS* mutants of the related *P. fluorescens* strain Pf-5 were found to be significantly more susceptible to environmental stress than the wild-type cells (Whistler et al. 1998). It will be difficult to propose a molecular mechanism that explains the population shifts occurring in strain CHA0 until the chemical signal(s) perceived by GacS and the primary target genes regulated by GacA are identified. A similar, possibly related, phenomenon has been observed in populations of *P. syringae* pv. *syringae* growing on bean leaves where a *gacS* mutant appeared to render the wild-type less fit to survive (Hirano et al. 1997).

In other studies, population shifts have been observed in starved, stationary phase cultures of *E. coli*. Sub-populations having a "growth advantage in stationary phase" (GASP) appear to be periodically selected (Zambrano & Kolter 1996). The emergence of *gacS* and *gacA* subpopulations in cultures of *P. fluorescens* CHA0 can be seen as an example of the GASP phenomenon. However, the *gacS* and *gacA* mutants have a selective advantage over the wild-type cells grown in nutrient rich conditions, not when starved.

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