GacS Sensor Domains Pertinent to the Regulation of Exoproduct Formation and to the Biocontrol Potential of *Pseudomonas fluorescens* CHA0

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In the root-colonizing biocontrol strain CHA0 of *Pseudomonas fluorescens*, cell density-dependent synthesis of extracellular, plant-beneficial secondary metabolites and enzymes is positively regulated by the GacS/GacA two-component system. Mutational analysis of the GacS sensor kinase using improved single-copy vectors showed that inactivation of each of the three conserved phosphate acceptor sites caused an exoproduct null phenotype (GacS⁻), whereas deletion of the periplasmic loop domain had no significant effect on the expression of exoproduct genes. Strain CHA0 is known to synthesize a solvent-extractable extracellular signal that advances and enhances the expression of exoproduct genes during the transition from exponential to stationary growth phase when maximal exoproduct formation occurs. Mutational inactivation of either GacS or its cognate response regulator GacA abolished the strain's response to added signal. Deletion of the linker domain of the GacS sensor kinase caused signal-independent, strongly elevated expression of exoproduct genes at low cell densities. In contrast to the wild-type strain CHA0, the *gacS* linker mutant and a *gacS* null mutant were unable to protect tomato plants from crown and root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* in a soil-less microcosm, indicating that, at least in this plant-pathogen system, there is no advantage in using a signal-independent biocontrol strain.

Pseudomonas fluorescens CHA0 is a root-colonizing soil bacterium that protects a range of crop plants from diseases caused by fungal root pathogens (Haas et al. 2000; Keel and

Défago 1997; Voisard et al. 1994). This biological control is mediated to a considerable extent by antifungal secondary metabolites which are produced by strain CHA0 and certain other fluorescent pseudomonads under conditions of slow, nonexponential growth and high cell densities (Bloemberg and Lugtenberg 2001; Haas et al. 2000; Keel and Défago 1997; Pierson et al. 1998; Thomashow 1996; Walsh et al. 2001). Secondary metabolites of strain CHA0 mediating biological control include hydrogen cyanide (HCN), 2,4-diacetylphloroglucinol, and pyoluteorin (Haas et al. 2002; Keel and Défago 1997; Voisard et al. 1994). The production of these metabolites and some exoenzymes such as the AprA protease is positively regulated by the global activator GacA (Laville et al. 1992; Sacherer et al. 1994). GacA is the response regulator of a two-component regulatory system in which GacS (formerly LemA) serves as a membrane-bound sensor kinase (Bull et al. 2001; Chancey et al. 2002; Heeb and Haas 2001; Hrabak and Willis 1992; Rich et al. 1994; Sánchez-Contreras et al. 2002; Whistler et al. 1998).

The primary sequences of the GacS and GacA proteins are well conserved in many gram-negative bacteria. In a variety of animal- or plant-pathogenic strains, including *Pseudomonas*, *Vibrio*, *Salmonella*, *Legionella*, and *Erwinia* spp., mutational loss of GacS or GacA markedly attenuates the expression of certain virulence factors (Hammer et al. 2002; Heeb and Haas 2001). In strain CHA0 and other fluorescent pseudomonads, the GacS/GacA system is of central importance for biocontrol in that *gacA* mutants have lost the ability to protect a range of dicotyledonous plants from fungal root pathogens (Gaffney et al. 1994; Laville et al. 1992; Ligon et al. 1999; Schmidli-Sacherer et al. 1997). Sensor proteins similar to GacS also have been found in eukaryotes; they include the osmosensor SLN1 in yeast (Tao et al. 1999), regulators of hyphal formation in several fungi (Brinkman et al. 2001), and the ethylene receptor ETR1 in *Arabidopsis* (Bleecker et al. 1998).

A current model postulates that, in gram-negative bacteria, the GacS/GacA system upregulates the production of one or several small, noncoding regulatory RNA molecules (e.g., RsmZ; Fig. 1) which lift posttranscriptional repression exerted by a repressive complex formed at or near the ribosome binding site (RBS) of target genes. RsmA is believed to be a key component of this repressive complex (Blumer et al. 1999; Heeb and Haas 2001). This model is inspired by the finding that, in enteric bacteria, the RsmA protein (=CsrA) can be sequestered by the regulatory RNA RsmB (=CsrB), whereby the negative effect of RsmA is relieved (Baker et al. 2002; Romeo 1998). In *Escherichia coli* and the plant pathogen *Erwinia carotovora*, homologs of the GacS/GacA system positively

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control the expression of RsmB (CsrB) (Cui et al. 2001; Hyytiäinen et al. 2001; Suzuki et al. 2002). Furthermore, Altier and associates (2000) have demonstrated that, in *Salmonella typhimurium*, a *barA* (*gacS*) mutation can be suppressed by overexpression of a regulatory RNA homologous to CsrB of *Escherichia coli*. In *P. fluorescens* strains F113 and CHA0, the small regulatory RNAs PrtB and RsmZ, respectively, are positively controlled by GacA and, when overexpressed, can phenotypically suppress a *gacA* mutation (Aarons et al. 2000; Heeb et al. 2002).

GacS has two transmembrane segments and consists of three putative phosphotransfer domains: a transmitter, a receiver, and a C-terminal secondary transmitter (termed Hpt for histidine phosphotransfer) (Heeb and Haas 2001). By analogy with other unorthodox sensor kinases (Kwon et al. 2000; Perraud et al. 1999; Uhl and Miller 1996), it can be hypothesized that a phosphorelay mechanism operates in GacS, whereby the conserved amino acid residues His-294 in the transmitter, Asp-717 in the receiver, and His-863 in the Hpt domain, in this order, would be phosphorylated (Fig. 1). Phosphotransfer then occurs from GacS to GacA. This has been demonstrated in vitro for the GacS/GacA homologs BarA/UvrY of *E. coli* (Pernestig et al. 2001). Phosphorylated GacA is assumed to be the transcriptional activator of the signal transduction pathway controlling exoproduct formation. Several sensor kinases are known to interact in their periplasmic domain with signal molecules (Galperin et al. 2001). Another sensor domain that can be important for signal perception and transduction is the linker domain (Williams and Stewart 1999), which is located between the second transmembrane segment and the transmitter (Fig. 1). Upon interaction with an activating signal, sensor kinases undergo conformational changes favoring autophosphorylation (Williams and Stewart 1999). There is evidence that culture supernatants of dense *P. fluorescens* CHA0 cultures contain a low molecular weight signal molecule that stimulates the expression of the *rsmZ* gene (Heeb et al. 2002). This signal, which has not yet been identified chemically, is not related to quorum-sensing signals of the *N*-acylhomoserine-lactone (AHL) type (Heeb et al. 2002), and it is not known where in the GacS/GacA signal transduction pathway this molecule interacts.

The aim of this study using *P. fluorescens* CHA0 as a model is to investigate the roles of various GacS domains (Fig. 1) in the activation of the GacA regulon specifying exoproduct synthesis. In particular, we present evidence for a signal-blind *gacS* mutant that expresses exoproduct genes at low cell densities irrespective of the presence of the signal. We then tested the biocontrol properties of this mutant in a microcosm with tomato and *Fusarium oxysporum* f. sp. *radicis-lycopersici* as a pathogen, to see if this *gacS* mutation would lead to improved plant protection.

RESULTS

Essential conserved amino acid residues in the sensor kinase GacS.

Phosphotransfer from the GacS homolog BarA to the GacA homolog UvrY has been demonstrated in *E. coli* (Pernestig et al. 2001), but the amino acid residues of GacS involved in phosphotransfer have not been determined experimentally. The GacS model (Fig. 1) predicts that His-294 is the site of autophosphorylation and that Asp-717 and His-863 are involved in phosphotransfer, by analogy, e.g., with the ArcB sensor of *E. coli* (Kwon et al. 2000). Point mutations were introduced into the *gacS* gene of *P. fluorescens* CHA0, creating either a His-294 to Arg, an Asp-717 to Asn, or a His-863 to Arg change

(discussed below). These alleles as well as the *gacS*⁺ wild-type gene were integrated as single copies into the *P. fluorescens* chromosome of a mutant deleted for *gacS*, resulting in strains CHA19.3 [*gacS*(H294R)], CHA19.4 [*gacS*(D717N)], CHA19.5 [*gacS*(H863R)], and CHA19.1 (*gacS*⁺), respectively. All three mutants had a *gacS* null phenotype (i.e., they did not produce detectable amounts of HCN, AprA protease, 2,4-diacetylphloroglucinol, and tryptophan side chain oxidase) (data not shown). By contrast, the reconstructed *gacS*⁺ strain CHA19.1 was indistinguishable from the wild-type CHA0. Thus, a phosphorelay typical of tripartite unorthodox sensor kinases (Perraud et al. 1999) is likely to operate in GacS.

GacS and GacA are essential for transduction of an extracellular inducing signal.

We have shown previously that supernatants of CHA0 cultures grown to high cell density contain a solvent-extractable signal that activates the transcription of the *rsmZ* gene and, hence, increases the posttranscriptional expression of exoproduct genes such as *hcnA* (for HCN synthase) (Heeb et al. 2002). We verified that this signal requires a functional GacS/GacA system to bring about the activation of a translational *hcnA*'-lacZ reporter fusion carried by the plasmid pME3219 (Blumer et al. 1999). In the wild-type CHA0, the addition of signal stimulated the expression of the reporter two- to threefold (Fig. 2A), whereas in the *gacS* deletion mutant CHA19, the signal had no significant effect on the low-level expression of the reporter (Fig. 2B). Interestingly, the *gacS*(Δ 128) mutant CHA19.6 deleted for codons 34 to 161 ap-

Fig. 1. Model of the GacS/GacA signal transduction pathway in *Pseudomonas fluorescens*. The postulated phosphorelay indicated by P and arrows is based on a comparison with the other two-component systems involving a tripartite sensor kinase (e.g., ArcB/ArcA in *Escherichia coli*) and is supported by the in vitro demonstration of phosphotransfer from BarA (GacS) to UvrY (GacA) in *E. coli* (Kwon et al. 2000; Pernestig et al. 2001; Perraud et al. 1999) as well as by the mutational analysis performed in this study. Accordingly, GacS is assumed to be autophosphorylated at His-294. The phosphate then could be transferred to Asp-717 in the receiver domain and to His-863 in the Hpt domain of GacS and, ultimately, to Asp-54 in the receiver domain of GacA. RsmZ, regulatory RNA similar to PrtB (Aarons et al. 2000; Heeb et al. 2002); RsmA, RNA binding protein (Blumer et al. 1999); (+) postulated positive control; (–) postulated negative control.

Fig. 2. GacS is required for induction of *hcnA'*-*lacZ* expression and modulation by signal. Strains were grown in nutrient yeast broth. β -Galactosidase activities are mean values of three experiments $\pm$ standard deviation. **A**, Expression of the *hcnA'*-*lacZ* fusion carried by pME3219 in CHA0 (*gacS*⁺) with extract from CHA0 (filled circles) or without extract (empty circles); **B**, expression in CHA19 (*gacS*⁻) with extract (filled circles) or without extract (empty circles); **C**, expression in CHA19.6 (*gacS*(Δ 128)) with extract (filled circles) or without extract (empty circles).

Fig. 3. The 5' leader region of the *hcnA* gene is required for responsiveness to signal. β -Galactosidase activities were determined in triplicate in strain CHA0 carrying pME6531 (*ptac-hcnA'*-*lacZ*) or pME3856 (*ptac-lacZ*). Both reporter strains were grown in nutrient yeast broth with 1 mM isopropyl- β -D-thiogalactoside. Empty squares, pME6531 without extract; filled squares, pME6531 with extract; empty circles, pME3856 without extract; filled circles, pME3856 with extract.

peared to be essentially unaffected in the response to signal and in the expression of the reporter gene (Fig. 2C). In this *gacS*(Δ 128) mutant, 128 out of 135 amino acid residues located in the periplasmic loop of the GacS protein are missing (Fig. 1), suggesting that this GacS domain is not essential for signal transduction. As noted before (Heeb et al. 2002), growth rates of *P. fluorescens* strains were similar with or without added extract and only a slight growth lag was observed for the reporter cells in media containing added extract (data not shown).

Several controls were performed. i) A single-copy, chromosomal *hcnA'*-*lacZ* reporter fusion in a wild-type background (CHA207) showed the same two- to threefold stimulation of expression by added signal (discussed below) as did the *hcnA'*-*lacZ* plasmid pME3219 (Fig. 2A), which has approximately six copies (Heeb et al. 2000), indicating that the copy number of the reporter construct is not a critical parameter. ii) A *gacA*

mutation in the *hcnA'*-*lacZ* reporter strain CHA89.207 also abolished the response to the signal entirely (data not shown) and the expression of *hcnA'*-*lacZ* was very low, as noted before (Blumer et al. 1999). iii) Addition of signal to strain CHA0 carrying the GacS/GacA-controlled *phlA'*-*lacZ* reporter pME6259, which monitors the expression of 2,4-diacetylphloroglucinol biosynthetic genes (Schnider-Keel et al. 2000), also caused a two- to threefold stimulation of *phlA* expression (data not shown), iv) Activation of target gene expression by the GacS/GacA regulatory system occurs at a posttranscriptional level (Blumer et al. 1999); therefore, we also tested an *hcnA'*-*lacZ* translational fusion construct (pME6531) in which the native *hcn* promoter had been replaced by the *tac* promoter (*ptac*). As expected, expression was markedly induced by added signal in strain CHA0 (Fig. 3), confirming that the native *hcn* promoter is not required for signal transduction. By contrast, the expression of a construct lacking the 5' *hcnA* leader region (i.e., *ptac-lacZ* [pME3856]) was insensitive to the addition of signal (Fig. 3), in agreement with the previous finding that the repressive RsmA complex acts near the *hcnA* ribosome binding site (Blumer et al. 1999). Collectively, these results support the model shown in Figure 1 and they establish that an extracellular signal produced by strain CHA0 can act only when both GacS and GacA are functional.

Construction of a signal-independent *gacS* mutant.

To evaluate the importance of the GacS linker region located between the second transmembrane domain and the transmitter domain (Fig. 1), we deleted a segment of 79 amino acids, which contains two putative amphipathic helices (Williams and Stewart 1999). Three new amino acid residues (EAF) were inserted in this process (Fig. 4A). The resulting *gacS*(Δ 76) allele also was placed as a single copy in the CHA0 chromosome previously deleted for *gacS*. In the resulting strain CHA207.8 containing a chromosomal translational *hcnA'*-*lacZ* fusion, β -galactosidase expression was insensitive to the addition of signal and enhanced four- to fivefold at low cell densities (i.e.,

Fig. 4. Deletion of the GacS linker domain and effect of the *gacS*(Δ76) mutation on the expression of the *hcn* biosynthetic genes. **A**, Amino acid sequence of the linker region, with two postulated amphipathic helices (ASI, ASII) (Williams and Stewart 1999). TM, transmembrane segment; bold face, conserved residues in sensor kinases (Williams and Stewart 1999). The Δ76 deletion removes the 79 amino acid residues from Gly191 to Ile 269 of the linker region and inserts three (EAF) at the junction. **B**, Expression of an *hcnA*'-'*lacZ* fusion in strain CHA207 (*gacS*⁺, *hcnA*'-'*lacZ*) with signal from CHA0 (filled squares) or without signal (empty squares) and in strain CHA207.8 (*gacS*(Δ76), *hcnA*'-'*lacZ*) with signal (filled circles) or without signal (empty circles). **C**, Hydrogen cyanide (HCN) production by strain CHA0 with signal (filled squares) or without signal (empty squares) or by strain CHA19.8 (*gacS*(Δ76)) with signal (filled circles) or without signal (empty circles) growing in glycine minimal medium. Experiments were done three times. Mean values are given in B; C shows a typical experiment. HCN was quantified as previously described (Laville et al. 1998).

below optical density at 600 nm [OD₆₀₀] = 1.5), compared with β-galactosidase expression in the parental *gacS*⁺ reporter strain CHA207 (Fig. 4B). Thus, the *gacS*(Δ76) mutation locks the system in the “on” position. This was confirmed by following the production of the secondary metabolite HCN itself. In standard nutrient yeast broth (NYB), the production of HCN is low; therefore, the experiment was performed in a synthetic medium optimized for HCN formation (Castric 1975). Note that, in this medium, growth stops at OD₆₀₀ ≈ 1.0. In the *gacS*⁺ strain CHA0, HCN was synthesized above OD₆₀₀ = 0.7. Addition of signal enhanced and advanced HCN production (Fig. 4C). In the *gacS*(Δ76) strain CHA19.8, HCN synthesis started early at OD₆₀₀ = 0.4 and was not further stimulated by signal (Fig. 4C). Thus, in the *gacS*(Δ76) mutant strain, the need for an activating signal was by-passed.

To show that the effects of signal and the *gacS*(Δ76) mutation were not restricted to HCN production, we also examined the expression of *aprA* encoding the major extracellular protease of strain CHA0. Indeed, the expression of a translational *aprA*'-'*lacZ* fusion in the *gacS*(Δ76) mutant CHA806.8 was signal independent, and β-galactosidase activities were higher than in the wild-type background (CHA805) at low cell densities (Fig. 5). Enhanced and advanced expression in the *gacS*(Δ76) background CHA19.8 also was noted for the *phlA*'-'*lacZ* reporter pME6259 (Fig. 6A). In the mutant CHA19.8 grown in glycerol-casamino acids medium (GCM), the metabolite 2,4-diacetylphloroglucinol was overproduced strongly at low cell densities and also was ele-

vated significantly at high cell densities (Fig. 6B). Thus, it is likely that the *gacS*(Δ76) mutation globally affects GacS/GacA-dependent expression of exoproduct genes. We noted in these experiments that the growth kinetics of the *gacS*(Δ76) mutants were similar to those of the *gacS*⁺ wild type, except that the mutants had an extended lag phase when stationary phase cells were inoculated into fresh medium.

Protection of tomato from crown and root rot caused by *F. oxysporum* f. sp. *radicis-lycopersici*.

The *gacS*(Δ76) strain CHA19.8 starts expressing biocontrol traits at lower cell densities than does the wild-type CHA0; therefore, we wondered whether the mutant would have altered, possibly improved biocontrol properties. In a soil-less rockwool system, strain CHA0 protected tomato partially from crown and root rot caused by *F. oxysporum* f. sp. *radicis-lycopersici*, as was evident from a significantly lower disease index compared with a control without biocontrol bacteria added (Table 1). The *gacS* null mutant CHA19 did not afford any protection, and, surprisingly, the *gacS*(Δ76) mutant CHA19.8 also was unable to protect tomato plants from disease (Table 1). Thus, at least in this gnotobiotic plant–pathogen system, there was no advantage in using signal-independent biocontrol bacteria. Importantly, as observed in other interactions between dicotyledonous plants, root pathogens, and fluorescent pseudomonads, the GacS/GacA function clearly was required for biocontrol.

DISCUSSION

From a sequence comparison of approximately 20 *gacS* genes from various pathogenic or beneficial gram-negative bacteria, several features of the GacS sensor kinase have been deduced (Heeb and Haas 2001). In the present study, we have investigated experimentally some of these traits in *P. fluorescens* CHA0, using mutational analysis. First, the three highly conserved amino acid residues and predicted phosphoacceptor sites located in the primary transmitter, the primary receiver, and the carboxy-terminal Hpt domains (Fig. 1) were all found to be essential for GacS function, as predicted for tripartite sensor kinases (Perraud et al. 1999). Second, the periplasmic domain comprising approximately 135 amino acid residues turned out to be nonessential for GacS activity and for activation of the GacS/GacA pathway by the extracellular signal. This finding is supported by the observation that the periplasmic domain is poorly conserved in GacS from various microorganisms (Heeb and Haas 2001). Third, the GacS linker domain, which contains two relatively well-conserved amphipathic sequences (Heeb and Haas 2001; Williams and Stewart 1999), proved to have an important regulatory function as indicated by the *gacS*(Δ 76) mutation, which locked the GacS protein in an active state, irrespective of the presence of the signal. Similarly, mutations affecting the linker domain of the VirA sensor kinase of *Agrobacterium tumefaciens* have revealed that

Fig. 5. Elevated, signal-independent expression of an *aprA'*-*lacZ* fusion in a *gacS*(Δ 76) mutant. Expression of an *aprA'*-*lacZ* fusion in strain CHA805 (*gacS*⁺, *aprA'*-*lacZ*) growing in nutrient yeast broth with signal (filled squares) or without signal (empty squares) and in strain CHA806.8 (*gacS*(Δ 76), *aprA'*-*lacZ*) with signal (filled circles) or without signal (empty circles). Measurements were carried out in triplicate.

this domain is required for sensing phenolic compounds and low pH (Heath et al. 1995; Toyoda-Yamamoto et al. 2000).

At this stage and in the absence of structural information on the inducing signal of *P. fluorescens* CHA0, two interpretations of the *gacS*(Δ 76) phenotype can be proposed. The signal might directly interact with GacS and induce a conformational change of the cytoplasmic C-terminal part, thereby favoring autophosphorylation. In this activated state, the amphipathic helices ASI and ASII of the linker domain are proposed to interact (Heeb and Haas, 2001). It is possible that, due to the absence of ASI and ASII in GacS Δ 76, the protein can no longer undergo this conformational switch and has adopted a structure which constantly favors autophosphorylation. Alternatively, the signal might activate some downstream element in the GacS/GacA pathway and deletion of the GacS linker domain might constitutively activate GacS, such that the signal would no longer be able to further stimulate target gene expression. It should be pointed out here that the model shown in Figure 1 is still incomplete. For instance, RsmA is only one of several repressive elements in the GacS/GacA system, in that inactivation of *rsmA* suppresses a *gacS* defect only partially (Blumer et al. 1999; Haas et al. 2002). Small regulatory RNAs in addition to RsmZ also may exist in this regulatory network. In principle, any of these components could interact with the activating signal.

In some *Pseudomonas* strains, the GacS/GacA system has a positive effect on the synthesis of AHL signal molecules (Chancey et al. 1999; Kinscherf and Willis 1999; Reimann et al. 1997). For instance, in *Pseudomonas aeruginosa* PAO, GacA-dependent regulation of HCN formation involves *N*-(3-oxo-dodecanoyl)-homoserine lactone and *N*-butanoyl-homoserine lactone as signals (Pessi and Haas 2000; Whiteley et al. 1999) and in *P. aureofaciens* 30-84, *N*-hexanoyl-homoserine lactone participates in GacA-regulated formation of phenazine compounds (Chancey et al. 1999). However, it has been noted that GacA control of target gene expression in these *Pseudomonas* strains also can occur in the absence of AHL synthesis (Chancey et al. 1999; Kinscherf and Willis 1999; Pessi and Haas 2001). In *P. fluorescens* CHA0, no evidence for the production of AHL-type signals has been found (Heeb et al. 2002). This suggests that, in the GacS/GacA pathway, the AHL signals can have a subordinate or lateral role.

In biocontrol of root diseases, one of the most important parameters is the level of root colonization by the biocontrol agent (Paulitz 2000). The production of plant-beneficial secondary metabolites by *P. fluorescens* in vitro requires relatively high cell densities: for efficient expression of the *hcn* and *phl*

Table 1. Deletion in the linker domain of GacS in *Pseudomonas fluorescens* CHA0: Effect on the protection of tomato against root and crown rot caused by *F. oxysporum* f. sp. *radicis-lycopersici*^a

Microorganisms added ^w		Disease index ^x	Shoot weight (g) ^y	Colonization (log ₁₀ CFU/g of root) ^z
<i>P. fluorescens</i>	<i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>			
None	—	0 c	0.47 b	0 c
CHA0 (wild type)	—	0 c	0.48 b	7.3 b
CHA19 (Δ <i>gacS</i>)	—	0 c	0.57 a	7.4 b
CHA19.8 (<i>gacS</i> (Δ 76))	—	0 c	0.52 ab	7.5 b
None	+	2.6 a	0.28 d	0 c
CHA0	+	1.3 b	0.39 c	8.1 a
CHA19	+	2.4 a	0.40 c	7.8 ab
CHA19.8	+	2.3 a	0.36 c	7.2 b

^a Means within the same column followed by the same letter do not differ significantly at *P* = 0.05, according to Student's *t* test. Data represent three individual repetitions of the same experimental setup. Treatments consisted of 12 plants and were replicated three times in each experiment.

^w *P. fluorescens* strains and *F. oxysporum* f. sp. *radicis-lycopersici* were added at 10⁸ CFU and 10⁶ conidia per seedling, respectively. Plants were grown in a soil-less rockwool system for 14 days.

^x Disease severity was scored on a scale of 0 to 4 in which 0 = symptomless and 4 = dead or nearly so.

^y Shoot fresh weight.

^z Root colonization by *P. fluorescens*.

genes in liquid cultures $\geq 10^9$ CFU/ml are needed, considering that $OD_{600} = 1$ corresponds to approximately 10^9 CFU/ml (Figs. 4B and 6). Although these cell densities in liquid media cannot be directly correlated with bacterial densities on roots (expressed as CFU per gram of root), it is well established that biocontrol effects are observed above threshold levels of typically $>10^5$ to 10^7 CFU/g (Bull et al. 1991; Paulitz 2000; Raaijmakers et al. 1995). For these reasons, we were interested to test the biocontrol efficacy of the *gacS*($\Delta 76$) strain CHA19.8, which shows an approximately 10-fold increased expression of the *hcn* and *phl* genes at $OD_{600} \leq 1.0$, compared with the wild type (Figs. 4B and 6). However, in the soil-less plant–pathogen system used (Table 1), the *gacS*($\Delta 76$) mutant's biocontrol ability was largely abolished compared with that of the wild type. As shown in Figure 6B, 2,4-diacetylphloroglucinol was produced constitutively by the *gacS*($\Delta 76$) mutant and accumulated to high levels, compared with the wild-type CHA0. *F. oxysporum* f. sp. *radicis-lycopersici* is highly sensitive to this antibiotic in vitro (Duffy and Dfago 1997; Keel et al. 1992; Maurhofer et al. 1995) and, therefore, loss of biocontrol activity in strain CHA19.8 may seem surprising. In the presence of *F. oxysporum* f. sp. *radicis-lycopersici*, strain CHA19.8 gave significantly lower root colonization than did the wild type (Table 1). However, colonization by strain CHA19.8 still exceeded 10^7 CFU/g of root and, therefore, should be above the typical threshold level. Moreover, the complemented *gacS* mutant CHA19.1, which was used as a control in the same experiment, gave wild-type protection at a root colonization of 10^7 CFU/g of root (data not shown). Therefore, we assume that reduced root colonization was not the major reason for the failure of CHA19.8 to suppress disease. Overproduction of 2,4-diacetylphloroglucinol by this strain might be toxic to the host plant in the presence of *F. oxysporum* f. sp. *radicis-lycopersici*, such that toxicity might cancel a positive biocontrol effect. It has been shown previously that tomato is, indeed, sensitive to low concentrations of 2,4-diacetylphloroglucinol, ranging from 3 to 50 μ M (Keel et al. 1992; Maurhofer et al. 1995). There are other examples of antibiotic overproduction by biocontrol strains that have not resulted in improved biocontrol. For instance, overproduction of 2,4-diacetylphloroglucinol and pyoluteorin by strain CHA0 containing additional copies of the housekeeping sigma factor *rpoD* has provided improved biocontrol of *Pythium ultimum* on cucumber (Schnider et al. 1995), but this overproducing strain has been less efficient than the wild type in suppressing disease in other plant–pathogen systems, such as *Pythium ultimum*–cress, *Pythium ultimum*–sweet corn, and *Gaeumannomyces graminis*–wheat (Maurhofer et al. 1992, 1995). In vitro, strain

CHA19.8 showed a longer lag phase, compared with the wild-type CHA0. Whether this situation also applies to the biocontrol assay is not certain, because only one point in time (at 14 days) was measured. However, a kinetic analysis of antibiotic production and of *F. oxysporum* f. sp. *radicis-lycopersici* proliferation in the rhizosphere would have been warranted if the signal-independent derivative had given superior biocontrol effects.

Care was taken to study the effects of *gacS* mutations at the single-copy level. To achieve this objective, we used an improved mini-Tn7 delivery system described in Figure 7, which allows the transpositional insertion of modified mini-Tn7 constructs into the unique Tn7 attachment site in the chromosome of many gram-negative bacteria (Bao et al. 1991; Hjberg et al. 1999; Koch et al. 2001). These singly-copy delivery vectors also are proving to be useful in *P. aeruginosa* (unpublished data).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

P. fluorescens strains and plasmids used are listed in Table 2. Strains were routinely grown in NYB or on nutrient agar (Stanisich and Holloway 1972) at 30C (*P. fluorescens*) or 37C (*E. coli*). Strain CHA0 was cultivated in GCM containing 100 μ M FeCl₃ 6H₂O and 100 μ M ZnCl₂ (Schnider-Keel et al. 2000) for signal extraction, in glycine minimal medium (Castric 1975) for HCN determinations, or in Ornston Stanier (OS) minimal medium with glucose (Schnider-Keel et al. 2000) for *phlA* expression experiments. Antibiotic concentrations have been detailed before (Blumer et al. 1999; Heeb et al. 2002; Schnider-Keel et al. 2000).

DNA manipulation and plasmid constructions.

Standard methods were used for the preparation of chromosomal and plasmid DNA, electroporation and transformation, Southern blotting, and cloning procedures (Farinha and Kropinski 1990; Sambrook et al. 1989; Schnider et al. 1995). The *lacI*^q-*ptac* segment of pJF118EH, bordered by *Nru*I and *Kpn*I sites, was used to create pME6531, a *lacI*^q derivative of pME6530. Plasmid pME3856 (*lacI*^q *ptac*-*lacZ*) was constructed from the *lacI*^q-*ptac* segment of pJF118EH, the *lacZ* gene with its own RBS of pME3535, and vector pME6010. Construction of the mini-Tn7 carrier plasmids pME3280a and pME3280b was done in several steps. Polymerase chain reaction (PCR) amplification using the primers Gm1 (5'-GACGCACACCGTGGAAA-3') and Gm2 (5'-GCGGCGT-TGTGACAATTT-3') and the Gm-resistant plasmid pML8 as

Fig. 6. Effect of the *gacS*($\Delta 76$) mutation on the expression of the *phl* biosynthetic genes. **A**, Expression of the *phlA*'-'*lacZ* construct pME6259 was tested in strain CHA0 (empty squares) and in the mutant CHA19.8 (empty circles) growing in Ornston Stanier minimal medium with glucose; **B**, 2,4-diacetylphloroglucinol production by strain CHA0 (empty squares) or by strain CHA19.8 (empty circles) grown in glycerol-casamino acids medium. Measurements were carried out in triplicate.

a template produced a 0.85-kb fragment which was treated with T4 DNA polymerase and cloned into the *HincII* site of pBluescript. The Gm resistance cassette then was excised with *KpnI* and *SacI* and used to replace the *SalI* fragment of pUX-BF5 carrying the kanamycin resistance gene. The ends were made compatible with T4 DNA polymerase. The Gm resistance pUX-BF5 variant was linearized with *HindIII*, treated with T4 DNA polymerase, and cleaved with *SpeI* to give a fragment (X) of 5.9 kb. A second fragment of 0.35 kb (Y), which carries a translation stop signal (Ter; Fig. 7A) was obtained from pHP45 Ω Sm/Sp by cleavage with *SphI* and *HindIII*; the *SphI* end was made flush with T4 DNA polymerase. Ligation of fragments X and Y to the left half of the pUK21 polylinker (*SpeI*-*StuI*-*XhoI*-*SphI*-*NcoI*-*KpnI*-*SmaI*-*EcoRI*-*HindIII*) generated pME3280a, whereas ligation to the right half (*HindIII*-*PstI*-*MluI*-*BamHI*-*SpeI*) gave pME3280b (Fig. 7A).

The *gacS* gene of strain CHA0 was isolated from a partial genomic library (pBluescript KS containing *BamHI* fragments of 5 to 9 kb from strain CHA0) in *E. coli* DH5 α . A recombinant plasmid which hybridized with a 6.7-kb *KpnI* fragment containing *gacS* (= *apdA*) from pJEL5680 (Corbell and Loper 1995) carried the *gacS* gene of strain CHA0 on an 8.5-kb insert. Nucleotide sequencing (carried out by Microsynth, Balgach, Switzerland) was used to localize *gacS* to a 4.1-kb *BamHI*-*BsiWI* fragment. This fragment (Fig. 7B) was cloned into the vector pME6010, resulting in pME3258, which complemented the *gacS* mutant CHA510 but not the *gacA* mutant CHA89. A *gacS* null mutant (CHA19) was constructed by deleting the entire *gacS* gene (on a 3.2-kb *EcoRV*-*BsiWI* fragment) and by transferring this mutation to the CHA0 chromo-

some using the suicide vector pME3087, as previously described (Laville et al. 1998; Schnider et al. 1995); strain CHA19 was checked by Southern blotting (data not shown).

The *gacS*(Δ 76) allele was constructed by joining a PCR-amplified 5' *gacS* fragment (nucleotides 1 [being the first nucleotide specifying the *EcoRV* recognition site; Fig. 7B] to 971) to an amplified 3' *gacS* fragment (nucleotides 1,205 to 3,225) via an artificial *HindIII* linker sequence (CAAGCTT), creating in the GacS polypeptide a deletion of 79 amino acid residues between Met-190 and Glu-270, fused with a Glu-Ala-Phe linker (Figs. 1 and 4A). The resulting *gacS*(Δ 76) allele containing its own promoter was checked by sequencing and inserted into the suicide cloning vector pME3280a (Fig. 7A), which delivers the mini-Tn7 with passenger DNA into the unique Tn7 attachment site of the *P. fluorescens* chromosome in the presence of the Tn7 transposition helper pUX-BF13 (Bao et al. 1991; Højberg et al. 1999; Koch et al. 2001). Thus, strains CHA19.8 [Δ *gacS/gacS*(Δ 76) in *att*Tn7], CHA207.8 [*hcnA*'-'*lacZ gacS*::Tn5/*gacS*(Δ 76) in *att*Tn7], and CHA806.8 [*aprA*'-'*lacZ* Δ *gacS/gacS*(Δ 76) in *att*Tn7] were obtained and checked by Southern blotting (data not shown).

For the construction of *gacS*(Δ 128), the 5' region of *gacS* (nucleotides 1 [being the first nucleotide specifying the *EcoRV* recognition site; Fig. 7B] to 505) was PCR-amplified from an *EcoRV*-*BsiWI* *gacS* subclone using a vector primer and primer LoSph (5'-AGAGCTGCATGCAGGTGAAGT-3'), which, due to a single base change (written in bold face) introduces an artificial *SphI* site (underlined) and changes the 32nd codon from Trp to Cys. The PCR fragment was fused at its *SphI* site to a *SphI*-*BsiWI* fragment encompassing the 3' region of *gacS* (nucleotides 883 to 3,225). This created a

Fig. 7. Mini-Tn7 delivery vectors and *gacS* of *P. fluorescens*. **A**, The Gm-resistant mini-Tn7 vectors were derived from pUX-BF5 as described in Materials and Methods. MCS, multiple cloning site; Ter, transcription terminator; Tn7L, Tn7R, left and right borders, respectively, of Tn7. Unique restriction sites are underlined. **B**, Restriction map of the *gacS* region of *Pseudomonas fluorescens* CHA0, cloned in the pME6010-derivative pME3558.

384-bp deletion in *gacS*, removing the first 128 codons specifying the periplasmic loop. Thus, in the *GacS*(Δ 128) polypeptide, 128 amino acids located between Met-33 and Leu-162 (Fig. 1) were deleted. The *gacS*(Δ 128) mutant allele with its promoter was inserted into mini-Tn7 of pME3280a, as above, and transposed into the chromosome of CHA19 (*AgacS*), resulting in strain CHA19.6 [*AgacS/gacS*(Δ 128) in *attTn7*].

To generate the H294R mutation in *GacS*, a 1.9-kb *EcoRV*-*SalI* fragment carrying the 5' part of *gacS* was cloned into pBluescript KS and subjected to site-directed mutagenesis using the Chameleon double-stranded site-directed mutagenesis kit and the mismatch repair-defective *E. coli* strain XL-*mutS* (Stratagene Inc., La Jolla, CA, U.S.A.) (Deng and Nickoloff, 1992). The selection primer Sel (5'-GTGGCGGCCGCTCGCAAAGTAGTGGATC-3') and the mutagenic primer MutHis294 (5'-CCTGGCCAACATG-TCTCGAGAAATCCGTACACC-3') were used; nucleotides differing from the parental DNA are shown in bold face. Thus, the *gacS* wild-type sequence 5'-AGCCATGAA-3' (Ser His Glu) was changed to 5'-TCTCGAGAA-3' (Ser Arg Glu) containing an *XhoI* site (underlined) for screening. For the introduction of the D717N and H863R mutations, a 0.9-kb *ApaI*-*BsiWI* fragment carrying the 3' part of the *gacS* was inserted into pBluescript and subjected to mutagenesis, using the selection primer Sel and the mutagenic primers

MutAsp717 (5'-GACCTGGTAATGATGAACGTTTCAGAT-GCCCGGC-3') and MutHis863 (5'-GATCGAACGGGTCC-GTCTGACTGCACGGTGCCACC-3'), respectively; nucleotides differing from parental DNA are indicated in bold face. Thus, the *gacS* wild-type sequences 5'-ATGGACGTG-3' (Met Asp Val) and 5'-GTCCACCGCTG-3' (Val His Arg Leu) were changed to 5'-ATGAACGTT-3' (Met Asn Val) and 5'-GTCCGTCGACTG-3' (Val Arg Arg Leu), respectively. New restriction sites (underlined) for *AcI* and *SalI*, respectively, were created for screening. Each of the three mutations generated was verified by sequencing, integrated into the entire *gacS* gene (carried by the 3.2-kb *EcoRV*-*BsiWI* fragment), and moved to the chromosome of strain CHA19, using mini-Tn7 transposition as above. In this way, strains CHA19.3 (*gacS* H294R), CHA19.4 (*gacS* D717N), and CHA19.5 (*gacS* H863R) were obtained. As a control, the unmutated *gacS* gene also was introduced into the *attTn7* site of strain CHA19, resulting in strain CHA19.1 (*gacS*⁺). This strain was phenotypically indistinguishable from the wild-type CHA0. All constructs were checked by Southern blotting.

Extraction of signal from cell-free culture supernatants and biological assays for the presence of signal.

Strain CHA0 was grown in 500 ml of GCM in a 2-liter Erlenmeyer flask for 24 h, with shaking, to an OD₆₀₀ of 2.0 to 2.5. After cell removal by centrifugation, the supernatant

Table 2. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics ^z	Source
Strains		
<i>Escherichia coli</i>		
DH5 α	<i>recA1 endA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1 Δ(lacZYA-argF) U169 (ϕ80dlacZΔM15)</i>	Sambrook et al. 1989
XL- <i>mutS</i>	<i>Δ(mcrA)183 Δ(mcrCB hsdSMR mrr)173 endA1 supE44 thi-1 gyrA96 relA1 lac mutS::Tn10 (Tc^r) F⁺ proAB lacI^QΔM15 Tn5; Km^r</i>	Stratagene
<i>Pseudomonas fluorescens</i>		
CHA0	Wild type	Voisard et al. 1994
CHA19	<i>AgacS</i>	This study
CHA19.1	<i>AgacS</i> /mini-Tn7 Gm ^r <i>gacS</i> ⁺	This study
CHA19.3	<i>AgacS</i> /mini-Tn7 Gm ^r <i>gacS</i> (H294R)	This study
CHA19.4	<i>AgacS</i> /mini-Tn7 Gm ^r <i>gacS</i> (D717N)	This study
CHA19.5	<i>AgacS</i> /mini-Tn7 Gm ^r <i>gacS</i> (H863R)	This study
CHA19.6	<i>AgacS</i> /mini-Tn7 Gm ^r <i>gacS</i> (Δ 128); periplasmic loop mutant	This study
CHA19.8	<i>AgacS</i> /mini-Tn7 Gm ^r <i>gacS</i> (Δ 76); linker domain mutant	This study
CHA89	<i>gacA</i> :: Km ^r	Laville et al. 1992
CHA89.207	<i>gacA</i> :: Km ^r <i>hcnA'</i> -' <i>lacZ</i>	Blumer et al. 1999
CHA207	<i>hcnA'</i> -' <i>lacZ</i>	Blumer et al. 1999
CHA207.8	<i>gacS</i> ::Tn5/mini-Tn7 Gm ^r <i>gacS</i> (Δ 76) <i>hcnA'</i> -' <i>lacZ</i>	This study
CHA510	<i>gacS</i> ::Tn5	Bull et al. 2001
CHA805	<i>aprA'</i> -' <i>lacZ</i>	Blumer et al. 1999
CHA806	<i>AgacS aprA'</i> -' <i>lacZ</i>	Heeb et al. 2002
CHA806.8	<i>AgacS</i> /mini-Tn7 Gm ^r <i>gacS</i> (Δ 76) <i>aprA'</i> -' <i>lacZ</i>	This study
Plasmids		
pBLS II KS	Cloning vector, Ap ^r	Stratagene
pHP45 Ω Sm/Sp	Source of transcription/translation stop cassette; Ap ^r , Sm ^r , Sp ^r	Prentki and Krisch 1984
pJEL5680	<i>gacS</i> ⁺ (=apdA ⁺), Ap ^r	Corbell and Loper 1995
pJF118EH	Expression vector; <i>ptac lacI</i> ^Q , Ap ^r	Fürste et al. 1986
pME3087	Suicide vector, MCS, Tc ^r	Voisard et al. 1994
pME3219	<i>hcnA'</i> -' <i>lacZ</i> , Tc ^r	Blumer et al. 1999
pME3258	<i>gacS</i> ⁺ , Tc ^r , pME6010 derivative	This study (Fig. 7B)
pME3280a,b	Chromosomal integration vectors, mini-Tn7 Gm ^r , MCS	This study (Fig. 7A)
pME3535	Transcriptional fusion vector, <i>lacZ</i> , MCS, Ap ^r	Højberg et al. 1999
pME3856	<i>ptac-lacZ lacI</i> ^Q , Tc ^r , pME6010 derivative	This study
pME6010	Cloning vector, MCS, Tc ^r	Heeb et al. 2000
pME6259	<i>phlA'</i> -' <i>lacZ</i> , Tc ^r	Schnider-Keel et al. 2000
pME6530	<i>ptac-hcnA'</i> -' <i>lacZ</i> , Tc ^r	Blumer et al. 1999
pME6531	<i>ptac-hcnA'</i> -' <i>lacZ</i> , <i>lacI</i> ^Q , Tc ^r	This study
pML8	Source of Gm ^r cassette, Ap ^r , Gm ^r	Labes et al. 1990
pUK21	Cloning vector, MCS, Km ^r	Vieira and Messing 1991
pUX-BF5	mini-Tn7 Km ^r , Ap ^r	Bao et al. 1991
pUX-BF13	Helper plasmid encoding Tn7 transposition functions, Ap ^r	Bao et al. 1991

^z Ap = ampicillin; Gm = gentamicin; Km = kanamycin; Sm = streptomycin; Sp = spectinomycin; Tc = tetracycline; and MCS = multiple cloning site.

was processed and extracted with dichloromethane to provide the CHA0 signal, as previously described (Heeb et al. 2002). *P. fluorescens* reporter strains carrying a translational *hcnA'*-*'lacZ*, *aprA'*-*'lacZ*, or *phlA'*-*'lacZ* fusion either in the chromosome or on a plasmid were grown in 50-ml Erlenmeyer flasks with 20 ml of NYB containing 0.05% Triton X-100, with shaking (180 rpm). Routinely, an equivalent of 50 ml of extracted supernatant was dissolved in 200 µl of 50% (vol/vol) acetonitrile and added to the medium at the beginning of the experiment. Controls had 200 µl of 50% (vol/vol) acetonitrile alone. Cultures were inoculated with washed cells (1:200) to give an initial OD₆₀₀ of 0.01 and assayed for β-galactosidase at different times by the Miller method (Sambrook et al. 1989). The amount of signal present in 50 ml of culture supernatant gave a maximal (saturating) response in the bioassays.

Detection of GacS/GacA-dependent phenotypes.

HCN, AprA protease, 2,4-diacetylphloroglucinol, and tryptophan side chain oxidase were tested qualitatively as previously described (Laville et al. 1992; Sacherer et al. 1994; Schnider et al. 1995; Voisard et al. 1989). Quantitative HCN and 2,4-diacetylphloroglucinol assays also were performed as described before (Keel et al. 1992; Laville et al. 1998; Schnider-Keel et al. 2000).

Biocontrol assay.

The ability of *P. fluorescens* CHA0 and its mutants to protect tomato against Fusarium crown and root rot was studied in a noncirculating hydroponics system. Seeds of tomato (*Lycopersicon esculentum* cv. Supermarmande) were surface-disinfested in 1% sodium hypochlorite for 30 min, rinsed in sterile distilled water, and germinated on 0.85% water agar at 24°C for 3 days. The seedlings were transferred to rockwool cubes (type A0 36/40-6/15; Grodania A/S, Hedehusene, Denmark) placed in individual trays (12 cubes per tray, 1 cube per plant). The cubes were saturated with sterile Knop nutrient solution (Duffy and Défago 1999) throughout the experiment. Two hours after planting, 1 ml of a suspension with 1×10^6 microconidia plus mycelial fragments of *F. oxysporum* f. sp. *radicis-lycopersici* strain 22 (obtained from C. Alabouvette, INRA, Dijon, France) was added per plant. The inoculum was prepared from a 2% Difco malt extract culture of the fungus grown with orbital shaking (120 rpm) at 24°C for 4 days as detailed elsewhere (Duffy and Défago 1997). One hour later, 1 ml of a suspension of washed bacterial cells (1.5×10^8 CFU/ml) was added per plant. The trays were placed in a randomized block design in a growth chamber at 80% relative humidity and 22°C with light (300 µmol/sec/m²; ratio of 1.37 of light at 660 nm to 730 nm) for 16 h, followed by an 8-h dark period at 18°C. Disease severity was assessed after 14 days, using the following scale: 0 = plant healthy; 1 = one or two necrotic lesions on the roots or the crown; 2 = extensive crown necrosis; 3 = extensive crown and root necrosis, but plant still alive; and 4 = plant dead or nearly so. Each experiment was repeated three times, with three replicates per treatment in each experiment and with each replicate consisting of 12 plants.

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