ppGpp Controlled by the Gac/Rsm Regulatory Pathway Sustains Biocontrol Activity in *Pseudomonas fluorescens* CHA0

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In *Pseudomonas fluorescens* CHA0 and other fluorescent pseudomonads, the Gac/Rsm signal transduction pathway is instrumental for secondary metabolism and biocontrol of root pathogens via the expression of regulatory small RNAs (sRNAs). Furthermore, in strain CHA0, an imbalance in the Krebs cycle can affect the strain's ability to produce extracellular secondary metabolites, including biocontrol factors. Here, we report the metabolome of wild-type CHA0, a *gacA*-negative mutant, which has lost Gac/Rsm activities, and a *retS*-negative mutant, which shows strongly enhanced Gac/Rsm-dependent activities. Capillary electrophoresis-based metabolomic profiling revealed that the *gacA* and *retS* mutations had opposite effects on the intracellular levels of a number of central metabolites, suggesting that the Gac/Rsm pathway regulates not only secondary metabolism but also primary metabolism in strain CHA0. Among the regulated metabolites identified, the alarmone guanosine tetraphosphate (ppGpp) was characterized in detail by the construction of *relA* (for ppGpp synthase) and *spoT* (for ppGpp synthase/hydrolase) deletion mutants. In a *relA spoT* double mutant, ppGpp synthesis was completely abolished, the expression of Rsm sRNAs was attenuated, and physiological functions such as antibiotic production, root colonization, and plant protection were markedly diminished. Thus, ppGpp appears to be essential for sustaining epiphytic fitness and biocontrol activity of strain CHA0.

Pseudomonas fluorescens CHA0 is an effective biocontrol bacterium, which suppresses plant diseases by producing several extracellular enzymes and secondary metabolites with antibiotic activity in the rhizosphere. These exoproducts importantly contribute to plant protection by strain CHA0 and other root-colonizing *Pseudomonas* spp. having biocontrol activity. Mutants defective in the Gac/Rsm signal transduction pathway have a reduced ability to produce such biocontrol factors and to suppress plant diseases (Haas and Défago 2005; Kim et al. 2011).

The Gac/Rsm signal transduction pathway is initiated by the GacS/GacA two-component system (Lapouge et al. 2008). At

high cell population densities, the phosphorylated GacS sensor kinase activates the cognate GacA response regulator, which promotes the transcription of two or more noncoding small RNAs (sRNAs) in *Pseudomonas* spp. In *P. fluorescens* CHA0, GacA positively controls the expression of three sRNAs termed RsmX, RsmY, and RsmZ. These sRNAs have a high affinity for the RNA-binding protein RsmA and, additionally, for the RsmA paralog RsmE (Heeb et al. 2002; Kay et al. 2005; Reimmann et al. 2005; Valverde et al. 2003). The RsmA and RsmE proteins repress the translation of genes involved in secondary metabolism during the trophophase (i.e., during rapid growth). When the RsmX/Y/Z sRNAs are induced in the idiophase (i.e., during restricted growth), they relieve translational repression of target genes by sequestering the RsmA and RsmE proteins, thereby allowing the synthesis of secondary metabolites (Kay et al. 2005, 2006). In *P. fluorescens* and *P. aeruginosa*, two additional sensors termed RetS and LadS provide input into the Gac/Rsm pathway (Goodman et al. 2009; Humair et al. 2009). RetS inhibits and LadS activates the activity of the Gac/Rsm pathway and, in this way, both sensors affect the expression of target genes and biocontrol factors (Humair et al. 2009). In *P. fluorescens* CHA0, evidence for physical interaction between RetS and GacS has been obtained (Workentine et al. 2009). The RetS–GacS interaction is influenced by temperature: at 35°C, the maximum growth temperature of strain CHA0, RetS is turned into a strong GacS antagonist and biocontrol factor production is almost completely arrested (Humair et al. 2009).

In a previous study, we have shown that an imbalance in Krebs (tricarboxylic acid [TCA]) cycle functions can affect biocontrol factor production by strain CHA0 through altered *rsmX/Y/Z* gene expression (Takeuchi et al. 2009). In that study, two enzymes involved in the function of the TCA cycle (i.e., fumarase [*fumA*] and pyruvate carboxylase [*pycAB*]), were found to be key factors in the regulation of the Gac/Rsm signal transduction pathway. Furthermore, a metabolic analysis of the *fumA* and *pycAB* mutants revealed changes in the levels of TCA cycle intermediates, suggesting that regulatory links exist between primary and secondary metabolism via TCA cycle function and GacA-dependent sRNAs.

In the present study, we ask to what extent the metabolomic profile of strain CHA0 will be affected in a *gacA*-negative mutant, which has lost Gac/Rsm activities, and in *retS*- and *fumA*-negative mutants, which show strongly enhanced Gac/Rsm-dependent activities (Humair et al. 2009; Takeuchi et al. 2009). To this end, we have used a capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS)-based approach, which

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provides a sensitive and reproducible analysis of a broad range of compounds (Soga et al. 2003). Among the metabolites found to be strongly regulated by the Gac/Rsm system, the intracellular alarmone guanosine tetraphosphate (ppGpp) was characterized in detail for its role in the signaling pathway and biocontrol activity.

RESULTS

Metabolomic profiling of central metabolites in strain CHA0 and in *gacA*, *retS*, and *fumA* mutants.

To see the metabolic consequences of the loss of genes involved in the Gac/Rsm cascade, we determined the intracellular metabolome by CE-TOFMS in the wild-type CHA0 and in *gacA*, *retS*, and *fumA* mutants. These strains were grown in a diluted and modified glycerol-casamino acids medium (GCM), which minimizes the effects of the medium on metabolite analysis, as described previously (Takeuchi et al. 2009). In this medium, the wild type and the mutants grew equally well and showed the typical pattern of differential *rsmZ-lacZ* expression (i.e., enhanced expression in the *retS* and *fumA* mutants and downregulation in the *gacA* mutant) (Supplementary Fig. S1), in agreement with previous studies (Heeb et al. 2002; Humair et al. 2009; Takeuchi et al. 2009).

We identified 218 metabolites by matching their migration times in CE and molecular masses (m/z) to those of the metabolic standards (Supplementary Table S1). For each strain, the intracellular metabolite concentrations were calculated from MS peak intensities and the number of cells in the medium. Concentrations were compiled for 85 primary metabolites (Supplementary Table S2) and sorted in descending order of the ratio *gacA*/CHA0 or *retS*/CHA0. Several metabolites classified as “amino acids” (e.g., arginine, tyrosine, tryptophan, and homoserine) or “purine and pyrimidine” (e.g., the purine degra-

dation products hypoxanthine, urate, and allantoate) were more abundant in the *gacA* mutant but less abundant in the *retS* and *fumA* mutants, by comparison with the wild type (Fig. 1A). Conversely, more than half of the metabolites whose levels were higher in the *retS* mutant were lower in the *gacA* mutant, always by comparison with the wild type (Fig. 1B). These results indicate that GacA and RetS regulate central metabolism in strain CHA0 divergently and to a considerable extent. Overall, the metabolomic pattern of the *retS* mutant was similar to that of the *fumA* mutant, reflecting their phenotypic similarities, although several deviating responses could also be seen (Fig. 1AB). Such similarities and differences of metabolic states between the strains were also visualized by a principal component analysis using a two-dimensional score plot. Again, the *retS* and *fumA* mutants were found to be in close positions, whereas the *gacA* mutant revealed an obvious metabolic separation from the other three strains (Supplementary Fig. S2). The fumarate pool was increased in the *fumA* mutant, supporting our results obtained previously (Takeuchi et al. 2009), but not in the *retS* mutant, suggesting that fumarate and lack of RetS can have distinct effects on the activation of the Gac/Rsm signal transduction pathway.

ppGpp analysis and construction of *relA* and *spoT* mutants.

Among the metabolites that differentially accumulated in the *gacA* and *retS* mutants, the intracellular alarmone ppGpp caught our attention because i) ppGpp is the metabolite that was most strongly regulated by GacA (Fig. 1A) and ii) ppGpp is a signaling molecule involved in the LetS/LetA and BarA/UvrY systems (which are homologous to GacS/GacA) in *Legionella pneumophila* and *Escherichia coli*, respectively (Edwards et al. 2011; Molofsky and Swanson 2004). Moreover, ppGpp is a regulator of virulence in various bacteria, besides mediating

A Sorted by *gacA*/CHA0

Metabolite	Pathway	Ratio to WT		
		<i>gacA</i>	<i>retS</i>	<i>fumA</i>
ppGpp, divalent	Others (ppGpp biosynthesis)	6.5	0.4	0.3
GABA	Amino acids (Glu, Gln, His, Pro, Arg)	4.3	0.4	0.7
Sarcosine	Amino acids (Gly, Ser, Cys)	4.2	5.3	ND
2-Methylserine	Others	3.9	1.6	0.8
Hypoxanthine	Purine, Pyrimidine	3.2	0.2	0.7
Adipic acid	Others	2.9	0.8	0.7
3-Phosphoglyceric acid	Glucose degradation, Pentose-P, Krebs cycle	2.8	1.7	1.0
Uric acid	Purine, Pyrimidine	2.5	0.4	0.8
Ribulose 5-phosphate	Glucose degradation, Pentose-P, Krebs cycle	2.4	0.8	0.8
Phosphoenolpyruvic acid	Glucose degradation, Pentose-P, Krebs cycle	2.3	1.3	0.9
Homoserine	Others (Homoserine biosynthesis)	2.2	1.0	0.4
Arg	Amino acids (Glu, Gln, His, Pro, Arg)	2.2	0.4	0.7
N-Acetylglutamic acid	Amino acids (Glu, Gln, His, Pro, Arg)	2.0	0.5	0.3
Tyr	Amino acids (Aromatic amino acids)	2.0	0.8	0.7
β-Ala	Amino acids (Asp, Ala, Lys)	2.0	1.0	0.5
N-Acetylglucosamine	Nucleotide sugar, Amino sugar	1.9	0.9	0.8
Quinic acid	Others (Shikimic acid pathway)	1.9	0.7	0.9
2-Phosphoglyceric acid	Glucose degradation, Pentose-P, Krebs cycle	1.9	1.7	1.0
Trp	Amino acids (Aromatic amino acids)	1.8	0.5	1.0
4-Guanidinobutyric acid	Amino acids (Glu, Gln, His, Pro, Arg)	1.7	0.4	1.2
Pipecolic acid	Amino acids (Asp, Ala, Lys)	1.7	0.5	0.9
2,6-Diaminopimelic acid	Others	1.7	1.0	1.0
Urea	Amino acids (Glu, Gln, His, Pro, Arg)	1.7	0.8	0.7
2-Deoxyglucose 6-phosphate	Others	1.7	0.6	0.6
Isobutyryl CoA, divalent	Amino acids (Branched chain amino acids)	1.6	0.4	1.0
UDP-glucuronic acid	Nucleotide sugar, Amino sugar	1.6	0.8	0.8
Cysteine glutathione disulphide	Others	1.6	1.1	1.1
dCTP	Purine, Pyrimidine	1.6	0.9	0.9
Allantoic acid	Purine, Pyrimidine	1.6	0.8	1.0
N-Acetylmethionine	Amino acids (Glu, Gln, His, Pro, Arg)	1.5	0.4	0.3
Acetyl CoA, divalent	Glucose degradation, Pentose-P, Krebs cycle	1.5	1.6	1.0
dCDP	Purine, Pyrimidine	1.5	0.6	0.7

B Sorted by *retS*/CHA0

Metabolite	Pathway	Ratio to WT		
		<i>gacA</i>	<i>retS</i>	<i>fumA</i>
3-Hydroxyaspartic acid	Others	ND	9.6	0.4
Sarcosine	Amino acids (Gly, Ser, Cys)	4.2	5.3	ND
N,N-Dimethylglycine	Amino acids (Gly, Ser, Cys)	0.15	4.4	0.2
2,4-Diaminobutyric acid	Others	ND	3.7	1.9
N-Acetylaspartic acid	Amino acids (Asp, Ala, Lys)	0.7	2.3	0.6
Nicotinamide	Nicotinic acid, Nicotinamide	0.3	2.3	2.6
Hydroxyproline	Amino acids (Glu, Gln, His, Pro, Arg)	0.2	1.9	0.3
3-Phosphoglyceric acid	Glucose degradation, Pentose-P, Krebs cycle	2.8	1.7	1.0
Betaine	Amino acids (Gly, Ser, Cys)	0.2	1.7	0.5
Phe	Amino acids (Aromatic amino acids)	0.6	1.7	1.3
2-Phosphoglyceric acid	Glucose degradation, Pentose-P, Krebs cycle	1.9	1.7	1.0
Phenylhydrazine	Others	ND	1.6	1.6
Acetyl CoA, divalent	Glucose degradation, Pentose-P, Krebs cycle	1.5	1.6	1.0
2-Methylserine	Others	3.9	1.6	0.8
Malonyl CoA, divalent	Glucose degradation, Pentose-P, Krebs cycle	0.8	1.5	1.3
2-Amino-2-(hydroxymethyl)-1,3-propanediol	Others	ND	1.5	1.8
3-Aminoisobutyric acid	Amino acids (Branched chain amino acids)	0.8	1.5	1.8

Fig. 1. Comparison of the metabolomic profiles in *Pseudomonas fluorescens* CHA0 and its *gacA*, *retS*, and *fumA* mutants. **A**, Metabolites upregulated in the *gacA* mutant and **B**, metabolites upregulated in the *retS* mutant were compared with the wild-type CHA0. Metabolite levels were sorted in descending order of fold-change values in each mutant. Red color increments indicate enhanced levels and green indicates attenuated levels.

the classical stringent response (Braeken et al. 2006; Dalebroux et al. 2010; Potrykus and Cashel 2008). The cellular ppGpp concentration is controlled by two enzymes, RelA (ppGpp synthase) and SpoT (ppGpp synthase/hydrolase, bifunctional) (Potrykus and Cashel 2008). In the fully sequenced strain Pf-5 of *P. fluorescens*, which is very closely related to strain CHA0, the genes of PFL_4446 and PFL_6063 are annotated as *relA* and *spoT*, respectively (Pseudomonas Genome Database). To investigate the role of ppGpp in CHA0, we generated chromosomal *relA* (PFL_4446) and *spoT* (PFL_6063) deletion mutants (Supplementary Fig. S3). We also constructed a *relA spoT* double mutant, a *gacA relA* double mutant, and a *gacA relA spoT* triple mutant.

Intracellular ppGpp levels were determined in each strain by HPLC (Table 1). The concentration of ppGpp was 7.5 ± 3.6 pmol per optical density at 600 nm (OD_{600}) in the wild-type CHA0 grown in diluted and modified GCM to $OD_{600} = 1.7$ (one OD_{600} unit corresponds to approximately 1.0×10^9 cells/ml). In all *relA*-negative mutants, ppGpp accumulation was below the detection limit of 0.5 pmol/ OD_{600} , suggesting that RelA is likely to be the major source of ppGpp under these conditions. By contrast, the *spoT* single mutant showed a slightly higher level of ppGpp than that in the wild type; this effect was significant at $P < 0.1$ but not at $P < 0.05$ (Table 1).

In *E. coli*, a completely ppGpp-defective mutant is unable to grow on glucose minimal media because of auxotrophic requirements (Murphy and Cashel 2003). When the *relA* mutant and the *relA spoT* double mutant of *P. fluorescens* were compared for growth on an M9 glucose plate, a clear-cut difference was observed. Whereas the *relA* mutant could grow on minimal medium with glucose, the *relA spoT* double mutant was strongly handicapped (Supplementary Fig. S4), suggesting that the *relA spoT* mutant was a ppGpp null mutant, whereas the *relA* mutant might contain residual amounts of ppGpp, which could not be detected by high-performance liquid chromatography (HPLC). We also tested glycerol, mannitol, and fructose as a sole carbon source and found that the *relA spoT* double mutant had a growth handicap in each case (data not shown); thus, the mutant phenotype was not specific to glucose. We should also point out that ppGpp (guanosine pentaphosphate), which is a minor product of RelA in *E. coli* and *Pseudomonas* spp. (Boes et al. 2008; Manuel et al. 2011; Potrykus and Cashel 2008), was below detection in our nonradioactive assays.

Confirming the metabolomic profiles (Fig. 1A), we detected an elevated ppGpp concentration in the *gacA* mutant CHA89 and a lower concentration in the *retS* mutant CHA1202, by

Table 1. Comparison of intracellular guanosine tetraphosphate (ppGpp) concentrations in *Pseudomonas fluorescens* CHA0 and its mutants

Strain	Genotype ^y	ppGpp (pmol/ OD_{600}) ^z
CHA0	Wild type	7.5 ± 3.6
CHA89	<i>gacA::Km^r</i>	$25.9 \pm 11.3^*$
CHA0relA	$\Delta relA$	<0.5
CHA0spoT	$\Delta spoT$	$10.2 \pm 1.8^{**}$
CHA0relAspoT	$\Delta relA \Delta spoT$	<0.5
CHA89relA	<i>gacA::Km^r relA</i>	<0.5
CHA89relAspoT	<i>gacA::Km^r relA spoT</i>	<0.5
CHA19	$\Delta gacS$	$15.0 \pm 6.8^*$
CHA1202	$\Delta retS$	$5.2 \pm 2.6^{***}$
CHA1009	<i>rsmA::Ω-Km rsmE::Ω-Hg</i>	$2.5 \pm 1.6^*$
CHA1008	<i>rsmA::Ω-Km rsmE::Ω-Hg ΔgacS</i>	$2.1 \pm 1.9^*$

^y Km^r indicates resistant to kanamycin.

^z Data are presented as averages of three replicates $\pm$ standard deviation, except for those of CHA0 (nine replicates) and CHA0spoT (six replicates). OD_{600} = optical density at 600 nm; *, **, and *** indicate values significantly different from those in CHA0 at $P < 0.05$, 0.1, and 0.3, respectively (t test).

comparison with the wild-type CHA0; the effect of the *retS* mutation was significant at $P < 0.3$ but not at $P < 0.05$ (Table 1). To answer the question of whether ppGpp accumulation occurs in a Gac/Rsm-dependent manner, we measured ppGpp in the *gacS* mutant CHA19, in the *rsmA rsmE* double mutant CHA1009, and in the *gacS rsmA rsmE* triple mutant CHA1008. The *gacS* mutant showed an increased level of ppGpp compared with the wild type, which is consistent with GacS being the cognate sensor of the GacA response regulator, although the effect of the *gacS* mutation was weaker than that of the *gacA* mutation (Table 1). The *rsmA rsmE* null mutations resulted in a decreased ppGpp level and canceled the positive effect of the *gacS* mutation in strain CHA1008 (Table 1), suggesting that the GacS/GacA system negatively regulates intracellular ppGpp concentrations via the RsmA/RsmE proteins.

A *gacA* mutant exhibits increased expression of a *relA* λ *lacZ* fusion.

To determine whether the elevated level of ppGpp observed in the *gacA* mutant may be due to a different expression level of *relA*, we constructed a translational *relA* λ *lacZ* fusion (Supplementary Fig. S5) and compared its expression in the wild type and in *gacA* and *retS* mutants (Fig. 2). While the *retS* mutation had little effect, we observed an increased level of *relA* expression in the *gacA* mutant at high cell densities, suggesting that GacA-dependent regulation of *relA* may account, at least in part, for the increased ppGpp concentration in the *gacA* mutant.

Altered Rsm sRNA levels in *relA* and *spoT* mutants.

To determine whether ppGpp affects the function of the Gac/Rsm cascade, we determined the transcriptional expression of the *rsmY* and *rsmZ* sRNA genes by introducing the transcriptional fusions *rsmY-lacZ* (chromosomal) or *rsmZ-lacZ* (on a plasmid) into *relA* and *spoT* mutants. The expression of both fusions was markedly reduced in the *relA spoT* double mutant but to a lesser extent than in the *gacA* mutant. In the *spoT* mutant, the expression levels were slightly increased, whereas those in the *relA* mutant were slightly decreased (Fig. 3A and B). In the rich medium used (nutrient yeast broth [NYB]), all the mutants grew at wild-type rates (Fig. 3E). These data suggest that, in a *gacA*⁺ background, ppGpp itself has a positive, probably indirect effect on the expression of the Rsm sRNAs.

Fig. 2. Expression of a *relA* λ *lacZ* fusion on pME6014relA in CHA0, the *gacA* mutant CHA89, and the *retS* mutant CHA1202 grown in full-strength glycerol-casamino acids medium. CHA0 harboring the empty vector pME6014 was included as vector control. The symbols indicate averages of triplicate cultures and bars indicate standard deviations. OD_{600nm} = optical density at 600 nm.

A *relA spoT* double mutant exhibits decreased *gacS* and *gacA* expression.

Because the *relA spoT* double mutant of CHA0 showed attenuated levels of *rsmY* and *rsmZ*, we wondered whether this effect would be due to an effect of ppGpp on the expression of the GacS/GacA system. Indeed, the expression of translational *gacS*'-*lacZ* and *gacA*'-*lacZ* fusions was downregulated in the *relA spoT* mutant by comparison with the wild type (Supple-

mentary Fig. S6); however, this effect was less drastic than that on the sRNA genes (Fig. 3A and B).

A *relA spoT* double mutant exhibits decreased *aprA* and *rpoS* expression.

In *P. fluorescens* CHA0, the GacS/GacA system positively controls the translational expression of *aprA*, the gene for the major exoprotease, and *rpoS*, the gene for the stationary phase

Fig. 3. Effects of *relA* and *spoT* mutations on the expression of the *rsmY*, *rsmZ*, *aprA*, and *rpoS* genes. Transcriptional expression of **A**, a chromosomal *rsmY*-*lacZ* fusion and **B**, a plasmid-borne *rsmZ*-*lacZ* fusion on pME6091 was determined in *Pseudomonas fluorescens* CHA0 (wild type, open circles), *relA* mutant (closed triangles), *spoT* mutant (closed diamonds), *relA spoT* mutant (closed squares) and *gacA* mutant CHA89 (open squares). Expression of **C**, a translational *aprA*'-*lacZ* fusion on pME6060 and **D**, an *rpoS*'-*lacZ* fusion on pME6355 was followed similarly in the *relA* and *spoT* mutants. **E**, Growth of each strain in the same medium (nutrient yeast broth) was monitored in parallel. Incubation was carried out in Erlenmeyer flasks. Measurements were carried out in triplicate. Symbols indicate averages and the error bars indicate standard deviations. OD_{600nm} = optical density at 600 nm.

and stress σ factor (Heeb et al. 2005; Takeuchi et al. 2009). Therefore, we followed the expression of a translational *aprA*-*lacZ* fusion (Fig. 3C) and an *rpoS*-*lacZ* fusion (Fig. 3D) in the *relA* and *spoT* mutants. The effects of these mutations were consistent with the ppGpp accumulation levels and the *rsmY* and *rsmZ* expression data. In particular, the *relA spoT* double mutant gave low *aprA* (Fig. 3C) and *rpoS* (Fig. 3D) expression, which is in agreement with the positive activity of ppGpp on the function of the Gac/Rsm pathway.

A *relA spoT* double mutant exhibits decreased antibiotic activity, swarming motility, and biocontrol efficacy.

We also wondered whether the *relA spoT* system would affect antibiotic activity and swarming motility of *P. fluorescens* CHA0. For the detection of antibiotic activity, strains were grown at 30°C on a glucose medium (NBGlucose), which is conducive to 2,4-diacetyl-phloroglucinol (DAPG) production, and on a glycerol medium (NBGlycerol), which favors pyoluteorin production in *P. fluorescens* Pf-5 (Kidarsa et al. 2011). The *relA spoT* double mutant had no detectable antibiotic activity on the NBGlucose plate and reduced activity on the NBGlycerol plate (Fig. 4A). The *relA* single mutant showed an attenuated level of antibiotic activity on the NBGlucose plate, whereas this strain was not affected on the NBGlycerol plate. These results suggest that ppGpp can affect the production of DAPG and, to a lesser extent, that of pyoluteorin. We also performed the same antibiotic assay at 26°C, which is the temperature used in the biocontrol assays described later, and found the same inhibition pattern (data not shown).

The *relA spoT* double mutant showed a decreased level of swarming motility (Fig. 4B) relative to that of the wild type. We also note that the double mutant formed a thicker edge of the colony on a swarming plate (Fig. 4B). With respect to the swarming phenotype, the single *relA* mutant was similar to the wild-type CHA0 (Fig. 4B), suggesting that perhaps the residual (and undetectable) level of ppGpp in the *relA* mutant may be sufficient for this function.

The results obtained with the Rsm sRNA genes, antibiotic activity tests, and motility tests indicate that ppGpp influences the biocontrol capacity of *P. fluorescens* CHA0. To investigate the influence of the *relA* and *spoT* mutations in a natural habitat, we adopted a cucumber-*Pythium ultimum* pathosystem, which enabled us to evaluate the plant protection efficacy by measuring the root weight and shoot weight. The *relA spoT* double mutant was less effective as a biocontrol agent than the wild type, with $\geq 95\%$ confidence (Table 2). Furthermore, this mutant also had a decreased root colonization capacity in both the presence and absence of *Pythium ultimum* (Table 2). In conclusion, these results are consistent with an impaired function of the Gac/Rsm pathway in the double mutant.

DISCUSSION

In the present study, we established metabolomic profiles of *gacA*, *retS*, and *fumA* mutants of *Pseudomonas fluorescens* CHA0 in a rich, albeit dilute medium. At the time of sampling for metabolome analysis, cells had not exhausted all nutrients, although some preferred carbon and nitrogen sources originally present in the medium had been consumed (e.g., gluta-

Fig. 4. Effects of *relA* and *spoT* mutations on antibiotic activity toward *Bacillus subtilis* and swarming motility. **A**, Antibiotic activities of *Pseudomonas fluorescens* strains grown on glucose medium (NBGlucose, left) and glycerol medium (NBGlycerol, right) plates were evaluated by the size of the growth inhibition zone of *B. subtilis*. Antibiotic activities of *P. fluorescens* CHA0 wild type, *relA* mutant, *spoT* mutant, and *relA spoT* mutant were compared on each plate incubated at 30°C for 16 h. **B**, Swarming motility of *P. fluorescens* CHA0 and the *relA* and *spoT* mutants on Luria-Bertani plates containing 0.5% agar. Photographs were taken 24 h after inoculation.

mate, proline, histidine, and aspartate) (Takeuchi et al. 2009). Thus, although cells were not growing optimally, they did not experience drastic amino acid limitation, a condition that typically elicits the stringent response. Therefore, we were surprised to find that the intracellular metabolite that was most strongly regulated by GacA under these conditions was ppGpp (Fig. 1A). The ppGpp concentration was elevated in the *gacA* mutant and reduced in the *retS* mutant, relative to that in the wild type (Fig. 1A; Table 1). Our methods did not allow us to quantify pppGpp, a minor RelA product; and, in this respect an uncertainty remains. Furthermore, the temperature (30°C) chosen for our metabolomic analysis and the in vitro expression studies differs from the temperature used for the biocontrol assay (26°C) and from the temperature range that root-colonizing pseudomonads normally encounter in nature. It is quite possible that the metabolomic profiles of the wild-type and the mutants might be different at temperatures around 20°C.

Among 218 metabolites detected, we also found that several compounds related to arginine metabolism were markedly affected by the *gacA* and *retS* mutations in an opposite manner (Fig. 1). The intracellular pools of arginine, its precursors *N*-acetyl-glutamate and *N*-acetyl-ornithine, and its degradation products 4-guanidinobutyrate, 4-aminobutyrate (GABA), and urea (Haas et al. 1990) all went up significantly in the *gacA* mutant (Fig. 1A). These effects are not understood at present. Arginine has many functions in *Pseudomonas* spp. and more work will be required to figure out the significance of regulation by the Gac system. Furthermore, the *gacA* mutation affected purine and pyrimidine metabolism, primarily by increasing the levels of the purine degradation products hypoxanthine, urate, allantoin, and urea (Fig. 1A). In this context, there is a precedent that deserves to be mentioned: *N*-acyl homoserine lactones (quorum-sensing signals) positively control purine degradation in *P. aeruginosa* (Heurlier et al. 2005). Strain CHA0 does not have homoserine lactones but the Gac/Rsm pathway can be regarded as a quorum-sensing regulatory mechanism. It is possible that, during the transition from fast growth to slow growth (i.e., during activation of the Gac system), some RNAs and, hence, purines and pyrimidines may be degraded. If the Gac system were involved in positively controlling purine degradation in strain CHA0, then the observed accumulation of intermediates of purine degradation in the *gacA* mutant could, indeed, be expected. Some effects of *gacA* and *retS* mutations on pyrimidine metabolism (UDP-glucuronate, dCTP, and dCDP) were seen (Fig. 1A) but are difficult to rationalize at present.

Three further metabolomic findings deserve a comment. First, in *E. coli*, the RsmA homologue CsrA regulates central carbon metabolism (Babitzke and Romeo 2007). We find no clear-cut evidence for an analogous role in *P. fluorescens* (i.e., opposite effects of *gacA* and *retS* mutations on metabolites of central C metabolism) (Fig. 1). Second, two nonprotein amino acids, 3-hydroxyaspartate and 2,4-diaminobutyrate, were undetectable in the *gacA* mutant and overproduced in the *retS* mutant (Fig. 1). Although 2,4-diaminobutyrate is a building block in pyoverdine biosynthesis, we are not aware of the function of 3-hydroxyaspartate in strain CHA0. Third, the fact that GABA accumulated in the *gacA* mutant and was relatively depleted in the *retS* mutant (Fig. 1) is particularly intriguing, because GABA has been reported to be a signal capable of modulating quorum sensing in *Agrobacterium tumefaciens* (Chevrot et al. 2006). More work is needed to find out whether GABA may also have a function as a signal molecule in *P. fluorescens* CHA0. In conclusion, the metabolic changes found in the *gacA* and *retS* mutants imply that the Gac/Rsm signal transduction pathway has a profound impact on primary metabolism in strain CHA0, a fact that was previously not appreciated.

In the remainder of the present study, we focused on the role of ppGpp in the Gac/Rsm pathway and in biocontrol of *P. fluorescens* CHA0. By comparison with the wild type, the *relA spoT* mutant of strain CHA0 showed markedly attenuated expression of RsmY and RsmZ (Fig. 3A and B) and, as a consequence, lower expression of traits regulated by these sRNAs: the AprA protease (Fig. 3C), the RpoS σ factor (Fig. 3D), antibiosis (Fig. 4), and biocontrol efficacy (Table 2). The salient features of this regulation are summarized in a model (Fig. 5). The stimulating effect of ppGpp on the function of the Gac/Rsm pathway in *P. fluorescens* has similar counterparts in *L. pneumophila* and *E. coli*. In the latter two organisms, ppGpp has been found to exert indirect positive effects on the signal transduction pathways initiated by the GacS/GacA homologues LetS/LetA and BarA/UvrY, respectively (Edwards et al. 2011; Molofsky and Swanson 2004). In all three organisms, there is circumstantial evidence that the effect of ppGpp may be due, at least in part, to induction or activation of the GacS/GacA two-component system; however, other mechanisms are not excluded. The fact that the *spoT* mutant of strain CHA0 showed increased expression levels of the *rsmY*, *rsmZ*, *aprA*, and *rpoS* genes compared with the wild-type levels (Fig. 3) supports the model according to which ppGpp activates the

Table 2. Effects of *relA* and *spoT* mutations on the suppression of *Pythium* damping-off and root rot of cucumber by *Pseudomonas fluorescens* CHA0^x

Bacterial strain	<i>Pythium</i> added	Per flask ^y			
		Surviving plants (%)	Shoot fresh weight (g)	Root fresh weight (g)	Colonization (log ₁₀ CFU/g) ^z
None	–	100 a	0.99 a	0.22 ab	ND
CHA0 (wild type)	–	100 a	1.08 a	0.25 a	7.56 ± 0.25 c
$\Delta relA$	–	100 a	0.99 a	0.22 ab	7.12 ± 0.28 c
$\Delta spoT$	–	100 a	1.00 a	0.22 ab	7.42 ± 0.27 c
$\Delta relA spoT$	–	100 a	1.07 a	0.23 ab	6.15 ± 0.26 d
None	+	17 d	0.41 d	0.10 c	ND
CHA0 (wild type)	+	71 b	0.74 b	0.20 ab	8.68 ± 0.23 a
$\Delta relA$	+	77 b	0.78 b	0.17 b	8.69 ± 0.29 a
$\Delta spoT$	+	75 b	0.74 b	0.19 b	8.52 ± 0.29 ab
$\Delta relA spoT$	+	52 c	0.59 c	0.12 c	7.77 ± 0.45 bc

^x *P. fluorescens* strains were added at 10⁷ CFU/g of soil contained within 100-ml flasks (30 g of soil/flask), after planting three 92-h-old, sterile-grown cucumber seedlings per flask. *Pythium ultimum* was added as a millet-seed inoculum at 2.5 g/kg of soil before planting. Plants were harvested after 7 days.

^y Data represent the averages of 11 replicates (flasks containing three cucumber plants) per treatment without *Pythium ultimum* and 22 replicates per treatment with *Pythium ultimum*. The control (uninfested plants not treated with *P. fluorescens*) was done in 20 replicates. Data represent the means from two individual repetitions of the same experiment. Means within the same column followed by different letters are significantly different ($P < 0.05$) according to Fisher's protected least significant difference (LSD) test.

^z Colonization by *P. fluorescens* in CFU/g of root. The rhizosphere-stable plasmid pME6031 containing a tetracycline resistance determinant (Heeb et al. 2000) was introduced as a selective marker into the bacterial strains to determine their root colonization capacity in soil. ND = not detected. Means within the same column followed by different letters are significantly different ($P < 0.05$) according to Fisher's protected LSD test.

Gac/Rsm pathway (Fig. 5) and suggests that SpoT functions mainly as a ppGpp hydrolase when RelA is present.

Our observation that ppGpp levels were enhanced in the *gacS* and *gacA* mutants and attenuated in the *rsmA rsmE* mutant (Table 1) points to a positive regulatory effect of the RsmA/E proteins in strain CHA0 (Fig. 5). However, the slight derepressing effect of the *gacA* mutation on *relA'-lacZ* expression (Fig. 2) cannot adequately explain these findings, and more work will be needed to elucidate this aspect of the regulatory cascade. By contrast, a *gacS* mutant of the biocontrol agent *Pseudomonas* sp. DF41 produced less (p)ppGpp (Manuel et al. 2011) and a *csrA* (homologue of *rsmA*) mutant of *E. coli* produced more (p)ppGpp than did the wild type (Edwards et al. 2011). It appears that different organisms have evolved different ways to control ppGpp levels via the Gac/Rsm pathway.

The *relA spoT* double mutant of strain CHA0 showed decreased root colonization in nonsterile soil in both the presence and absence of *Pythium ultimum* (Table 2). This mutant also exhibited decreased swarming motility (Fig. 4B). There are precedents for these observations. In many pathogenic bacteria, ppGpp contributes to their virulence such as invasiveness and persistence in the host (Braeken et al. 2006; Dalebroux et al. 2010). In *L. pneumophila*, for instance, a ppGpp-negative mutant shows decreased expression of its flagellar subunit gene *flaA* (Zusman et al. 2002). In *P. aeruginosa*, whose Gac/Rsm system is similar to that of CHA0, ppGpp has a positive effect on swarming and virulence determinants such as pyocyanin and siderophores (Vogt et al. 2011). In *P. putida*, a strain overexpressing a diguanylate cyclase was simultaneously affected in swarming motility and root colonization

(Matilla et al. 2011). Likewise, in the case of CHA0, it is conceivable that the *relA spoT* double mutant was handicapped in the rhizosphere, in which bacterial motility is important for root colonization and in which fluctuating nutrient conditions result in a risk of depletion of vital molecules. Therefore, the compromised plant protection efficacy of the *relA spoT* mutant may be due not only to the decreased antibiotic activity but also to the relatively poor epiphytic fitness.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacterial strains and plasmids used are listed in Table 3. Strains of *E. coli* and *P. fluorescens* were routinely grown in NYB (2.5% [wt/vol] nutrient broth and 0.5% [wt/vol] yeast extract) and Luria-Bertani (LB) medium with shaking, or on nutrient agar plates (4% [wt/vol] blood agar base and 0.5% [wt/vol] yeast extract) amended with the following antibiotics when required: ampicillin, 100 µg/ml (only for *E. coli*); gentamicin, 50 µg/ml; kanamycin, 25 µg/ml; or tetracycline, 25 µg/ml (125 µg/ml for selection of *P. fluorescens*). For metabolome analyses, bacteria were grown in diluted and modified GCM to minimize the effects of the medium on the analysis (Takeuchi et al. 2009). The modified medium contained, per liter: casamino acids (Bacto), 1.5 g; glycerol, 1.0 g; K₂HPO₄, 0.038 g; MgSO₄ · 7H₂O, 0.15 g; and FeSO₄ · 7H₂O, 1 mg. When relevant, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside was added to plates at a final concentration of 0.02%. The inoculation temperatures were 30°C for *P. fluorescens* and 37°C for *E. coli*. *P. fluorescens* was grown at 35°C to improve

Fig. 5. Model for gene regulation by guanosine tetraphosphate (ppGpp) in the Gac/Rsm system of *Pseudomonas fluorescens* CHA0. Evidence for this pathway comes from this work and from previous studies (Heeb et al. 2002, 2005; Kay et al. 2005; Reimmann et al. 2005; Valverde et al. 2003). The new finding of this study is that ppGpp has a positive effect on the Gac/Rsm system. Nutrient limitation and stress are the major factors inducing ppGpp synthesis. Thus, when nutrients become scarce and under stress conditions, ppGpp activates the Gac/Rsm system. This will allow cells to expand their metabolic repertoire by excreting proteases and lipases and to restrict access of competing microorganisms by forming antibiotic compounds. The negative effect of the Gac/Rsm system on ppGpp formation has a modulating function and, presumably, avoids overshooting of secondary metabolism and exoenzyme production. Thus, the model postulates a negative (self-limiting) loop. Symbols: ↓, positive effect; ⊥, negative effect; dotted lines, indirect effects; superscript a, it remains to be determined in detail how the Gac/Rsm pathway controls ppGpp levels via *relA* and, presumably, *spoT* expression.

its capacity to accept heterologous DNA (e.g., in electrotransformation or in matings with *E. coli*). For some other assays, bacteria were grown in the normal GCM (Maurhofer et al. 1998). M9 glucose medium plates (Na₂HPO₄, 12.8 g/liter; KH₂PO₄, 3.0 g/liter; NaCl, 0.5 g/liter; NH₄Cl, 1.0 g/liter; MgSO₄ · 7H₂O, 0.494 g/liter; thiamine, 0.01 g/liter; CaCl₂ · 2H₂O, 0.015 g/liter; and glucose, 2.0 g/liter) were used to test growth of *relA* and *spoT* mutants of *P. fluorescens*. For detection of antibiotic activity, strains were grown on Difco Nutrient Broth (Becton, Dickinson and Company, Sparks, MD, U.S.A.) supplemented with either 1% (wt/vol) glucose (NBGlu) or 1% (vol/vol) glycerol (NBGly) (Kidarsa et al. 2011).

DNA manipulation.

Small-scale plasmid extraction was carried out with a QIA-prep spin miniprep kit (Qiagen, Hilden, Germany); large-scale preparations were obtained with a Qiagen plasmid midi kit (Qiagen). Chromosomal DNA from *P. fluorescens* was prepared as previously described (Gamper et al. 1992). DNA fragments were purified from agarose gels with a QIAquick gel extraction kit (Qiagen). Oligonucleotides used are listed in Supplementary Table S3.

Construction of a *relA* Δ *lacZ* fusion.

A 420-bp *Pst*I fragment from the upstream region of PFL₄₄₄₆ was amplified using primers RelAProF and RelAProR, and inserted into pME6014 to obtain pME6014relA containing a translational *relA* Δ *lacZ* fusion.

Generation of *relA*- and *spoT*-negative mutants.

In-frame deletions in the chromosomal *relA* and *spoT* genes of *P. fluorescens* CHA0 were created as follows. Fragments of

approximately 750 bp located on each side of the *relA* or *spoT* gene were amplified by PCR with primer pairs RelAUF/RelAUR and RelADF/RelADR for *relA* or SpoTUF/SpoTUR and SpoTDF/SpoTDR for *spoT*. Each two corresponding fragments were annealed and amplified as 1.5-kb fragments using primers RelAUF/RelADR or SpoTUF/SpoTDR, respectively. After sequencing, these 1.5-kb fragments were cloned into pME3087 cut with *Bam*HI and *Hind*III to give pME3087relA and pME3087spoT. These plasmids were mobilized from *E. coli* DH5 α to *P. fluorescens* CHA0 by triparental mating with *E. coli* HB101/pME497. Excision of the vector via a second crossing-over was obtained after enrichment for tetracycline-sensitive cells (Humair et al. 2009), generating the *relA* or *spoT* mutant. To delete the *spoT* gene in the *relA* mutant, the pME3087spoT plasmid was mobilized to the *relA* mutant as recipient strain. Construction of a *gacA relA* double mutant and a *gacA relA spoT* triple mutant were created utilizing a *gacA* mutant as the parental strain.

β -Galactosidase assays.

β -Galactosidase activities were quantified by the Miller method (Miller, 1972). *P. fluorescens* strains were grown at 30°C in 50-ml flasks containing 20 ml of NYB amended with 0.05 % Triton X-100 with shaking at 180 rpm. Triton X-100 was required to prevent cell aggregation.

Metabolite extraction procedure.

P. fluorescens strains were grown in 20 ml of diluted and modified GCM in 50-ml Erlenmeyer flasks with shaking at 180 rpm and 30°C. For each strain, three cultures were incubated in parallel. Samples were taken from each culture during the idiophase (at OD₆₀₀ of approximately 1.7), when maximal

Table 3. Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^z	Source or reference
Strains		
<i>Bacillus subtilis</i> , M168	Wild type	C. Keel
<i>Escherichia coli</i> , DH5 α , HB101	Laboratory strains	Sambrook and Russell 2001
<i>Pseudomonas fluorescens</i>		
CHA0	Wild type	Stutz et al. 1986
CHA19	Δ <i>gacS</i>	Zuber et al. 2003
CHA89	<i>gacA</i> ::Km ^r ; Km ^r	Laville et al. 1992
CHA1008	<i>rsmA</i> :: Ω -Km <i>rsmE</i> :: Ω -Hg Δ <i>gacS</i>	Reimann et al. 2005
CHA1009	<i>rsmA</i> :: Ω -Km <i>rsmE</i> :: Ω -Hg	Reimann et al. 2005
CHA1202	Δ <i>retS</i>	Humair et al. 2009
CHA1322	Δ <i>fumA</i>	Takeuchi et al. 2009
CHA0relA	Δ <i>relA</i>	This study
CHA0spoT	Δ <i>spoT</i>	This study
CHA0relAspoT	Δ <i>relA</i> Δ <i>spoT</i>	This study
CHA89relA	<i>gacA</i> ::Km ^r Δ <i>relA</i>	This study
CHA89relAspoT	<i>gacA</i> ::Km ^r Δ <i>relA</i> Δ <i>spoT</i>	This study
Plasmids		
pBluescript II KS	Cloning vector, ColE1 replicon; Ap ^r	Stratagene
pME497	Mobilizing plasmid, IncP-1, Tra, RepA(Ts); Ap ^r	Voisard et al. 1988
pME3087	Suicide vector, ColE1 replicon, Mob; Tc ^r	Voisard et al. 1994
pME3087relA	pME3087 containing a <i>Bam</i> HI/ <i>Hind</i> III 1.5-kb <i>relA</i> region containing a deletion of 1.3 kb in the <i>relA</i> gene; Tc ^r	This study
pME3087spoT	pME3087 containing a <i>Bam</i> HI/ <i>Hind</i> III 1.5-kb <i>spoT</i> region containing a deletion of 1.3 kb in the <i>spoT</i> gene; Tc ^r	This study
pME3266	Translational <i>gacS</i> Δ <i>lacZ</i> fusion; Tc ^r	F. Carruthers
pME6014	Cloning vector for construction of translational <i>lacZ</i> fusions; Tc ^r	Schnider-Keel et al. 2000
pME6014relA	Translational <i>relA</i> Δ <i>lacZ</i> fusion under <i>ptac</i> ; Tc ^r	This study
pME6031	pACYC177-pVS1 shuttle vector; Tc ^r	Heeb et al. 2000
pME6060	Translational <i>aprA</i> Δ <i>lacZ</i> fusion; Tc ^r	Blumer et al. 1999
pME6091	Transcriptional <i>rsmZ-lacZ</i> fusion; Tc ^r	Heeb et al. 2002
pME6355	Translational <i>rpoS</i> Δ <i>lacZ</i> fusion; Tc ^r	Heeb et al. 2005
pME6827	Translational <i>gacA</i> Δ <i>lacZ</i> fusion; Tc ^r	C. Reimann
pME7699	Transcriptional <i>rsmY-lacZ</i> fusion in mini-Tn7 gene delivery vector pME6182; Gm ^r Ap ^r	Humair et al. 2009

^z Km^r, Ap^r, Tc^r, and Gm^r indicate resistance to kanamycin, ampicillin, tetracycline, or gentamicin, respectively.

rsmZ-lacZ expression was observed. Cells were harvested by vacuum filtration through a 0.4- μ m pore size filter. The cells on the filter (calculated as $OD_{600} \times$ sampling volume of culture in milliliters of approximately 24) were washed twice with 5 ml of Milli-Q water. The filter was immersed in 2 ml of methanol, including 10 μ M Internal Standard Solution 1 (Human Metabolome Technologies, Tsuruoka, Japan). The filter was sonicated for 30 s using a Branson ultrasonic bath to suspend the cells completely. A 1.6-ml portion of the methanol cell suspension was transferred to a 15-ml centrifuge tube (Falcon Blue Max Jr. 352097; Becton Dickinson & Co., Franklin Lakes, NJ, U.S.A.) and mixed with 1.6 ml of chloroform and 640 μ l of Milli-Q water. After vortexing well, the mixture was centrifuged at $2,300 \times g$ at 4°C for 5 min. The aqueous layer was distributed to three Amicon Ultrafree-MC ultrafilter tips (Millipore Co., Bedford, MA, U.S.A.) and centrifuged at $9,100 \times g$ and 4°C for approximately 2 h. The filtrate was dried and preserved at -80°C until CE-TOFMS analysis. Prior to analysis, the sample was dissolved in 25 μ l of Milli-Q water.

CE-TOFMS.

CE-TOFMS was carried out using a capillary electrophoresis system equipped with a TOF mass spectrometer (Agilent Technologies, Waldbronn, Germany). Separations and detections of metabolites were basically performed as described previously for cationic metabolites (Soga and Heiger 2000; Soga et al. 2003) and anionic metabolites (Soga et al. 2002, 2003).

Cationic metabolites were analyzed with a fused silica capillary, with cation buffer solution (Human Metabolome Technologies) as the electrolyte. The sample was injected at a pressure of 50 mbar for 10 s. The applied voltage was set at 27 kV. Electrospray ionization (ESI)-MS was conducted in the positive ion mode, and the capillary voltage was set at 4,000 V.

Anionic metabolites were also analyzed with a fused silica capillary, with anion buffer solution (Human Metabolome Technologies) as the electrolyte. The sample was injected at a pressure of 50 mbar for 25 s. The applied voltage was set at 30 kV. ESI-MS was conducted in the negative ion mode, and the capillary voltage was set at 3,500 V.

For all analytical modes, the inner diameter and total length of capillary were 50 μ m and 80 cm, respectively. Exact mass data were acquired over a range of 50 to 1,000 m/z . Raw data were processed using the KEIO MasterHands software, ver. 2.1.0.1 (Sugimoto et al. 2010). Metabolites in the samples were identified by comparison of the migration time and m/z ratio with those of authentic standards, in which a difference of ± 0.5 min and ± 10 ppm, respectively, was permitted and quantified by comparing peak areas with those of the authentic standards using ChemStation software (Agilent Technologies). Principal component analyses were performed with MATLAB 2007a (The Math Works, Natick, MA, U.S.A.).

HPLC assay of ppGpp.

Intracellular levels of ppGpp were determined in *P. fluorescens* strains cultured in diluted and modified GCM. Samples were prepared from each culture with the same method of metabolite extraction procedure as described above. The intracellular concentration of ppGpp was determined by HPLC on a Waters system (Waters, Milford, MA, U.S.A.) consisting of photodiode array detector 996 and pump 616, equipped with a normal phase column (TSKgel Amide-80, 4.6 by 250 mm; Tosoh, Tokyo). Isocratic elution was performed with 65% (vol/vol) acetonitrile and 35% (vol/vol) 75 mM potassium phosphate buffer (pH 4.5) at a flow rate of 1.0 ml/min. Nucleotides were detected at 254 nm. ppGpp was detected with a retention time of 29.0 ± 0.5 min and its concentration was

expressed relative to cell density (OD_{600} of approximately 1.0/ml). The ppGpp standard (>95% pure) was obtained from TriLink Biotechnologies (San Diego, CA, U.S.A.).

Detection of antibiotic activity.

Antibiotic activity of *P. fluorescens* strains grown on NBGLy or NBGLy plates containing 1.8% agar was determined with *Bacillus subtilis* M168 as the reporter. Cultures of *P. fluorescens* were adjusted to $OD_{600} = 1.5$ and 5- μ l samples were spotted onto the plate. After overnight incubation at 30°C, cells were killed by UV irradiation on a transilluminator for 5 min. An overlay of *B. subtilis* revealed antibiotic production by growth-inhibition zones.

Swarming motility assay.

Cells from overnight cultures grown in NYB were adjusted to an $OD_{600} = 1.0$ with water and 2- μ l aliquots were spotted onto semisolid LB agar (0.5%) plates. The plates were incubated at room temperature for 24 h and swarming motility was assessed qualitatively by examining the circular halo formed by the motile bacterial cells.

Plant disease suppression and root colonization assays.

Flasks containing 30 g of a 1:1 mixture of nonsterile horticultural soil (Kureha, Tokyo) and vermiculite were planted with three cucumber seedlings each and treated with either *Pythium ultimum* MAFF425494, *Pseudomonas fluorescens*, or both. *P. fluorescens* strains were added to soil as a suspension (3 ml/flask) of cells washed twice in sterile distilled water to give 1×10^7 CFU/g of soil. Control flasks received the same amount of sterile water. Seedlings were covered with 5 g of nontreated soil and flasks were sealed with aerated silicon caps. The microcosms were incubated in a growth chamber at 60% relative humidity and 26°C with light for 16 h, followed by an 8-h dark period. No watering was necessary. After 7 days of incubation, the biocontrol activity and root colonization of each strain were assessed. Data in Table 2 represent the means from two individual repetitions of the same experiment. Data of both experiments were first analyzed for trial-by-treatment interaction by analysis of variance, which indicated that the data from the two independent trials could be pooled. Means were separated with Fisher's protected least significant difference test ($P \leq 0.05$). Data for CFU counts were $\log_{10}$ -transformed prior to statistical analysis. Statistical analysis was performed with the SPSS Statistics software (IBM, Tokyo).

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