GABA, A Primary Metabolite Controlled by the Gac/Rsm Regulatory Pathway, Favors a Planktonic Over a Biofilm Lifestyle in *Pseudomonas protegens* CHA0

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In *Pseudomonas protegens* CHA0 and other fluorescent pseudomonads, the Gac/Rsm signal transduction pathway is crucial for the expression of secondary metabolism and the biological control of fungi, nematodes, and insects. Based on the findings of a previous metabolomic study, the role of intracellular γ -aminobutyrate (GABA) as a potential signal in the Gac/Rsm pathway was investigated herein. The function and regulation of a *gabDT* (c01870-c01880) gene cluster in strain CHA0 were described. The *gabT* gene encoded GABA transaminase (GABAT) and enabled the growth of the bacterium on GABA, whereas the upstream *gabD* gene (annotated as a gene encoding succinic semialdehyde dehydrogenase) had an unknown function. A *gacA* mutant exhibited low GABAT activity, leading to the markedly greater intracellular accumulation of GABA than in the wild type. In the *gacA* mutant, the RsmA and RsmE proteins caused translational *gabD* repression, with concomitant *gabT* repression. Due to very low GABAT activity, the *gabT* mutant accumulated GABA to high levels. This trait promoted a planktonic lifestyle, reduced biofilm formation, and favored root colonization without exhibiting the highly pleiotropic *gacA* phenotypes. These results suggest an important role of GABA in the Gac/Rsm-regulated niche adaptation of strain CHA0 to plant roots.

Pseudomonas protegens CHA0 is an effective biocontrol bacterium that has adapted to the rhizosphere, in which it suppresses plant diseases by producing several extracellular enzymes and secondary metabolites with antibiotic activity. These exoproducts contribute to plant protection by strain CHA0 and other root-colonizing *Pseudomonas* species exhibiting biocontrol activity. The expression of these biocontrol factors depends on the Gac/Rsm signal transduction pathway (Kidarsa et al. 2013; Lapouge et al. 2008). Mutants defective in the Gac/Rsm signal transduction pathway have a reduced ability to synthesize biocontrol factors, suppress plant diseases, and produce insecticides (Haas and Défago 2005; Kim et al. 2011; Kupferschmied et al. 2013). The Gac/Rsm signal transduction pathway also affects other phenotypes of multicellular behavior, such as biofilm formation and swarming motility (Kidarsa et al. 2013; Lapouge et al. 2008; Song et al. 2016). The Gac/Rsm system is involved in a switch between a planktonic and biofilm mode of growth (Valentini

and Filloux 2016). In the case of biocontrol strains of *Pseudomonas* spp., this switch enables bacteria to proliferate on plant roots and, subsequently, to compete effectively with other rhizosphere organisms during a sessile mode of growth (Barahona et al. 2011; Lalaouna et al. 2012). This switch is positively regulated by guanosine tetraphosphate (ppGpp), an intracellular signal characteristic of nutrient limitation and stress (Takeuchi et al. 2012). Furthermore, the implementation of a sessile lifestyle typically requires bis-(3'-5')-cyclic dimeric GMP (c-di-GMP) as an important intracellular signal in pseudomonads as well as in other bacteria (Valentini and Filloux 2016).

The Gac/Rsm signal transduction pathway is initiated by the GacS/GacA two-component system (Lapouge et al. 2008; Valentini and Filloux 2016). At high cell population densities, the GacS sensor kinase is autophosphorylated; this enables activation of the cognate GacA response regulator, leading to the transcription of two or more noncoding small RNAs (sRNAs) in *Pseudomonas* spp. (Kay et al. 2005, 2006; Lalaouna et al. 2012). In *P. protegens* strains CHA0 and Pf-5 as well as in *P. brassicacearum*, GacA positively controls the expression of three sRNAs termed RsmX, RsmY, and RsmZ (Kay et al. 2005; Kidarsa et al. 2013; Lalaouna et al. 2012). These sRNAs exhibit high affinity for the RNA-binding protein RsmA and its paralogue 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 having a conserved ANGGAN motif in or near the Shine-Dalgarno sequence; in vitro, this repression typically occurs during rapid growth (Lapouge et al. 2008; Reimmann et al. 2005). When RsmX/Y/Z sRNAs are induced during restricted growth at a high cell population density, they relieve the translational repression of target genes by sequestering the RsmA and RsmE proteins. Thus, the biofilm state and secondary metabolism are favored (Lapouge et al. 2008; Valentini and Filloux 2016). A multitude of regulatory proteins, including the antagonistic sensor RetS and agonistic sensor LadS, modulate the output of the Gac/Rsm pathway (Goodman et al. 2009; Humair et al. 2009; Valentini and Filloux 2016). In strain CHA0, evidence has been obtained for the physical interaction between RetS and GacS (Workentine et al. 2009). A direct and specific interaction between RetS and GacS has been demonstrated in *Pseudomonas aeruginosa* PAK, in which RetS forms heterodimers with GacS, thereby preventing the phosphorylation of GacA (Goodman et al. 2009).

In a previous metabolomic study (Takeuchi et al. 2012), we noted that *gacA* and *retS* mutations exerted opposite effects on the intracellular levels of some central metabolites including ppGpp. In a *gacA* mutant, ppGpp accumulates to high levels, which appears to prevent the overshooting of GacA-dependent functions by

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*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary figures and four supplementary tables are published online.

a feedback mechanism. Another metabolite that accumulates in a *gacA* mutant and is relatively depleted in a *retS* mutant is γ -aminobutyrate (GABA) (Takeuchi et al. 2012). This finding is of particular interest because GABA is a potential signaling molecule in the rhizosphere (Badri et al. 2013) and is known to down-regulate quorum sensing in the plant pathogen *Agrobacterium tumefaciens* (Chevrot et al. 2006). Moreover, GABA has a negative effect on the ability of *Pseudomonas syringae* pv. *tomato* DC3000 to elicit the hypersensitive reaction in tobacco (Park et al. 2010). These findings imply that GABA plays important roles as a signal molecule in plant-bacteria interactions. In bacteria, GABA originates from polyamines, mostly from putrescine (Bartsch et al. 1990; Chou et al. 2008; Dasu et al. 2006). GABA is catabolized to succinate in two steps. GABA transaminase (GABAT) encoded by the *gabT* gene generates succinic semialdehyde. The *gabD* gene specifies an NAD(P)⁺-dependent succinic semialdehyde dehydrogenase (SSDH), which oxidizes succinic semialdehyde to succinate (Bartsch et al. 1990; Chou et al. 2008).

In the present study, the mechanisms by which the Gac/Rsm system regulates GABA accumulation in *P. protegens* CHA0 were investigated. GABA accumulation in the *gacA* mutant of strain CHA0 suggests i) GABA production is negatively regulated by the Gac/Rsm pathway or ii) GABA catabolism is positively regulated by the Gac/Rsm pathway. Since the Gac/Rsm pathway is known to play important roles in the positive regulation of many genes (Lapouge et al. 2008), the latter is more plausible. Therefore, the hypothesis that the expression of one or both the *gabT* and *gabD* gene is positively regulated by the Gac/Rsm pathway was examined. Furthermore, to evaluate the function of GABA in interactions with plants, the role of GABA in the switch from a planktonic and to a biofilm mode of growth was examined in vitro and during root colonization.

RESULTS

Construction of *gabD* and *gabT* mutants in strain CHA0.

In the genome of strain CHA0, the genes PFLCHA0_c01880, PFLCHA0_c48650, and PFLCHA0_c49850 are annotated as GABAT genes, termed *gabT1*, *gabT2*, and *gabT3*, respectively (*Pseudomonas* Genome database; Winsor et al. 2011). Among the products of these three genes, the homolog of GabT1 has been functionally characterized in the most detail as GABAT in *Escherichia coli* K-12 (Schneider et al. 2002). The deduced amino acid sequence of GABAT in *E. coli* K-12 shared 74.2, 28.5, and 41.2% identities with the *gabT1*, *gabT2*, and *gabT3* gene products, respectively. The *gabD* locus (PFLCHA0_c01870) encoding a putative SSDH is located upstream of *gabT1* (Fig. 1). Furthermore, only the homolog of *gabT1* in *P. syringae* pv. *tomato* DC3000, named *gabT2* in that strain and sharing 91.3% amino acid identity, has the ability to restore the GABAT-negative phenotype of the triple mutant *gabT2/T3/T1*, whereas the other two homologs cannot (Park et al. 2010). Therefore, the *gabDT1* cluster in strain CHA0 was characterized and chromosomal *gabD* and *gabT1* (hereafter called *gabT*) deletion mutants were generated (Fig. 1).

GABAT activity was tested with GABA as the substrate and 2-oxoglutarate as the amino-accepting oxoacid in the wild type and in the mutants. Strains were grown in defined mannitol-glutamate (MG) medium amended with 5 mM GABA to ensure the induction of GABA catabolic functions and were harvested during the late exponential phase. The *gabT* mutant had strongly decreased GABAT-specific activity (Table 1), demonstrating that the *gabT* gene encodes the major 2-oxoglutarate-dependent GABAT in *P. protegens* CHA0. The expression of *gabDT* genes from the upstream region of *gabD* in trans restored GABAT activity, indicating that this phenotype was due to the inactivation of the *gabT* gene (Supplementary Table S1).

SSDH activity was also measured. The wild-type strain and the *gabD* mutant showed similar activities under the conditions tested (Table 1), suggesting that the *gabD* gene either specifies a minor SSDH or is incorrectly annotated as an SSDH gene. In the *gacA* mutant, both enzyme activities were lower than that of the wild type (Table 1), indicating that the levels of both enzymes are under the control of the Gac/Rsm system.

In order to confirm the function of the *gabT* gene in GABA catabolism, the ability of the mutant to grow on a minimal medium agar plate supplemented with 20 mM GABA as the sole carbon and nitrogen source was analyzed (Supplementary Fig. S1). GABA supported the growth of the wild type, whereas the *gabT* mutant did not grow, confirming that the *gabT* gene is necessary for GABA catabolism. The *gabD* mutant grew on the same plate, similarly to the wild type, which is consistent with SSDH data (Table 1). The *gacA* mutant did not have any detectable growth handicap, suggesting that its reduced GABAT activity was sufficient for growth. Complementation of the *gabT* mutant was found to restore growth. Putrescine, a precursor of GABA in *P. aeruginosa* (Yao et al. 2011), was also examined as the sole carbon and nitrogen source and the growth patterns of all strains were similar to those found on the GABA plate. Thus, putrescine catabolism also depends on the function of the *gabT* gene in strain CHA0.

The Gac/Rsm system regulates *gabDT* expression at the translational level.

The *gabDT1* genes of strain CHA0 may be cotranscribed and coordinately induced by putrescine and GABA, similarly to the *gabDT* genes of *Pseudomonas aeruginosa* PAO1 (Chou et al. 2008). In order to clarify how defective GacA decreases GABAT activity and, hence, increases intracellular GABA levels (Table 1), a translational *gabD*'-'*lacZ* fusion was constructed and its expression was measured. The *gacA* mutant CHA89 as well as the *rsmX rsmY rsmZ* triple mutant CHA1144 expressed markedly lower levels of *gabD*'-'*lacZ* throughout growth than the wild type (Fig. 2A). The *retS* mutant CHA1202 and the *rsmA rsmE* double mutant CHA1009 showed higher levels of expression than the wild type (Fig. 2A). This expression pattern is typical of post-transcriptional control by the GacA cascade in strain CHA0 (Humair et al. 2009). When GacA is absent, the repressor RsmA/E will cause translational repression of this fusion, and the *retS* mutation may have the opposite effect; the absence of RetS results in the overexpression of *rsmX rsmY rsmZ*, all of which sequester the repressor RsmA/E. The removal of RsmA/E renders the ribosome-binding site of the *gabDT* operon accessible for the translational machinery.

The predicted Shine-Dalgarno sequence of *gabD* has a potential RsmA/E binding site (UAAGGAAG) (Fig. 1A), in which the central ribonucleotides (AAGGAA) may form a hexa-loop on a U-G base pair (underlined) and allow the binding of RsmA and RsmE (Lapouge et al. 2008). No such motif was found upstream of *gabT*. Assuming that *gabD* and *gabT* are cotranscribed and cotranslated, two versions of a *gabD-gabT*'-'*lacZ* fusion were constructed; one had the original UAAGGAAG sequence, whereas the other contained a conventional Shine-Dalgarno sequence (AGGA) (Ma et al. 2002) (Fig. 1B). As shown in Figure 2B, *gabD-gabT*'-'*lacZ* expression was significantly weaker in the *gacA* mutant than in the wild type (Fig. 2B), with a 25% reduction being observed at an optical density at 600 nm (OD₆₀₀) of approximately 1.7 (Miller units of CHA0 and the *gacA* mutant were 800 ± 18 and 601 ± 12, respectively). In contrast, the construct that had a conventional *gabD* Shine-Dalgarno sequence essentially lost the regulation of *gabD-gabT*'-'*lacZ* by GacA (Fig. 2B), with a 9% reduction being observed at the same growth phase (Miller units of CHA0 and the *gacA* mutant were 639 ± 18 and 579 ± 8, respectively). This noncanonical translational control of the downstream *gabT* gene

may be attributed to translational coupling; however, this was not examined in detail in the present study. The mutation introduced into the RsmA/E binding site reduced the expression of the *lacZ* reporter in the wild type, and a possible reason for this is that the mutant Shine-Dalgarno sequence is weaker than the wild-type sequence. Another explanation is that the wild-type leader sequence appears to be optimal for mRNA stability, which is consistent with previous findings on *hcnA* expression (Blumer and Haas 2000; Lapouge et al. 2007).

Intracellular GABA concentrations in strain CHA0 and its mutants.

Intracellular GABA levels were assessed by high-performance liquid chromatography (HPLC) (Table 1). The concentration of GABA was 94.5 ± 4.3 pmol per optical density at 600 nm (OD_{600nm}) unit (one OD_{600nm} unit corresponds to approximately 1.0×10^9 cells per milliliter) in wild-type CHA0 grown in MG + GABA medium to the late exponential phase (at an OD_{600} of approximately 1.7) (Supplementary Fig. S2). In the *gabT* mutant, GABA accumulation was markedly greater than that in the wild type, as expected from the GABAT assays and growth tests (Table 1). The *gabD* and *gacA* mutants showed higher levels of GABA than the wild type, which is consistent with GABAT data (Table 1). The elevated GABA level observed in the *gabD*

mutant may be due to a polar effect caused by the deletion of *gabD* on the expression of the downstream *gabT* gene. It is important to note that, in the original metabolomic study (Takeuchi et al. 2012), the *gacA* mutant also showed elevated GABA levels after growth in medium not amended with GABA. Although the medium used (diluted and modified glycerol-casamino acid medium) was different from that in the present study, the concentration of GABA was 34 ± 2.5 pmol per OD_{600nm} unit in wild-type CHA0 and 147 ± 47 pmol per OD_{600nm} unit in the *gacA* mutant at the late exponential phase (Takeuchi et al. 2012).

In the medium used (MG + GABA), the *gabT* and *gabD* mutants grew at wild-type rates. In this experiment, the *gacA* mutant resumed growth without a lag phase, giving it a growth advantage over the other strains. This growth advantage in the *gacA* mutant was also observed in MG medium without amended GABA (data not shown).

GABA affects planktonic growth, biofilm formation, and root colonization.

It has not yet been clarified whether the accumulation of GABA affects bacterial phenotypes involved in rhizosphere colonization, specifically, the switch between the motile planktonic and sessile modes of growth. In vitro, the *gacA* mutant had a reduced ability to form biofilms and remained longer in a planktonic phase than

Fig. 1. Physical map and constructs involving *gabD* and *gabT* genes of *Pseudomonas protegens* CHA0. **A**, Construction of *gabD* and *gabT* mutants. Δ represents the regions deleted in *gabD* or *gabT*. The 380-bp intergenic region between PFLCHA0_c01860 and *gabD* is shown as a hatched box and its nucleotide sequence is indicated below. A putative RsmA/E binding site is shown in a box upstream of the predicted ATG start codon (underlined) of *gabD* (according to the annotation of strain Pf-5). Inset, predicted secondary structure of the *gabD* mRNA leader sequence near the translation start site when complexed with RsmA/E. The typical GGA motif is shown in bold face. **B**, Construction of *gabD*'-'*lacZ* and *gabDT*'-'*lacZ* fusions. The plasmids pME6014*gabD*, pME6014*gabDT*, and pME6014*gabDTgga* each contain a translational '*lacZ*' fusion in the vector pME6014. The triangle shows the site at which the predicted RsmA/E binding site of pME6014*gabDT* was replaced by a conventional AGGA Shine-Dalgarno sequence in pME6014*gabDTgga*.

the wild type (Fig. 3), which is consistent with previous findings obtained in strain Pf-5 (Kidarsa et al. 2013) and *P. aeruginosa* (Parkins et al. 2001). The behavior of the *gabD* mutant was similar to that of wild-type CHA0 (Fig. 3), except that it showed intermediate behavior between the wild type and *gabT* mutant in the absence of amended GABA in planktonic cell growth (Fig. 3B and C). Phenotypically, the *gabT* mutant was intermediate between the wild type and *gacA* mutant in this assay (Fig. 3). Biofilm formation by the *gabT* mutant was not significantly weaker than that by the wild type in the absence of amended GABA at a $P < 0.05$ level (Fig. 3A). Although the intracellular GABA level in each strain without amended GABA has yet to be assessed, a threshold intracellular GABA level may be required to exert effects on the planktonic to biofilm switch. The addition of GABA to the medium enhanced the coefficient of planktonic cells over the biofilm cell mass in all strains in a dose-dependent manner (Fig. 3C), supporting the function of GABA as a signal molecule.

Although the *gacA* mutant had lost swarming motility (Supplementary Fig. S3), which is known to depend on Gac/Rsm function in strains CHA0 and Pf-5 (Kay et al. 2005; Kidarsa et al. 2013), the *gabT* mutant exhibited the ability to swarm, similarly to the wild type and *gabD* mutant. Unlike the biocontrol-defective *gacA* mutant (Kay et al. 2005), the *gabT* mutant had conserved biocontrol ability against the oomycete *Pythium ultimum* on cucumber under natural soil conditions (Supplementary Table S2), suggesting that the *gabT* mutant had an unabated ability to produce biocontrol factors. We then performed a gnotobiotic cucumber root colonization assay (Table 2), in which the CHA0 inoculum was 10^4 -fold lower than that used in the biocontrol assay to avoid the saturation of colonization. The *gabT* mutant displayed a significantly greater capacity for the colonization of cucumber roots than the wild type; however, these differences were small (Table 2), without showing a difference in the fresh weight of host plants (data not shown). These results suggest that GABA promotes root colonization by sustaining a planktonic mode of growth following root inoculation.

DISCUSSION

Pseudomonads have two possible modes of growth, i.e., they adopt a planktonic or sessile lifestyle. During planktonic growth, cells prioritize primary metabolism for the rapid production of cell mass and motility, whereas, in the sessile (biofilm) lifestyle, cells favor the production of extracellular matrix molecules, secreted enzymes, and secondary metabolites. This dual behavior is observed in plant-beneficial *Pseudomonas* spp., such as *P. protegens*, as well

as in plant- or animal-pathogenic species such as *P. aeruginosa* (Kay et al. 2005; Kidarsa et al. 2013; Lapouge et al. 2008; Laville et al. 1992; Parkins et al. 2001; Valentini and Filloux 2016). The switch from planktonic to sessile growth is a highly complex, structured process that requires, in addition to an appropriate surface, the function of a plethora of *Pseudomonas* genes. In the hierarchy of master regulators that orchestrate this switch, the GacS/GacA two-component system occurs at the top (Valentini and Filloux 2016), resulting in the highly pleiotropic phenotypes of *gacS/gacA* mutants (Lapouge et al. 2008). Numerous extra- and intracellular signals (or cues) were expected to participate in

Table 1. Specific enzyme activities and intracellular γ -aminobutyrate (GABA) concentrations in *Pseudomonas protegens* CHA0 and its mutants grown in mannitol-glutamate medium with 5 mM GABA^a

Strain	Genotype	Specific activity		
		GABAT ^b	SSDH ^c	GABA ^d
CHA0	Wild type	38.7 ± 9.8	16.7 ± 3.0	94.5 ± 4.3
CHA0gabD	Δ <i>gabD</i>	14.8 ± 6.7*	14.2 ± 4.0	173.0 ± 13.6†
CHA0gabT	Δ <i>gabT</i>	2.9 ± 5.0**	14.9 ± 0.2	329.7 ± 16.7††
CHA89	<i>gacA::Km^r</i>	9.4 ± 0.4*	5.7 ± 3.8*	163.6 ± 15.2†

^a Data are presented as averages of three replicates ± standard deviation.

^b GABAT = GABA transaminase; SSDH = succinic semialdehyde dehydrogenase. Measured as nanomoles glutamate per minute per milligram of protein. Asterisks (*) and (**) indicate values significantly different from those in CHA0 at $P < 0.05$ and 0.01 , respectively (*t*-test).

^c Measured as nanomoles NADH per minute per milligram of protein. An asterisk (*) indicates a value significantly different from that in CHA0 at $P < 0.05$ (*t*-test).

^d Measured as picomoles per optical density at 600 nm unit. Daggers († and ††) indicate values significantly different from those in CHA0 at $P < 0.005$ and 0.00005 , respectively (*t*-test).

Fig. 2. Expression of *gabD'*-*lacZ* and *gabDT'*-*lacZ* fusions in CHA0 and in Gac/Rsm mutants. **A**, Expression of *gabD'*-*lacZ* on pME6014*gabD* in CHA0 and in *gacA*, *retS*, *rsmAE*, and *rsmXYZ* mutants grown in nutrient yeast broth (NYB). The Student's *t* test was performed to compare each mutant with CHA0 in the late exponential phase (at an optical density at 600 nm [OD₆₀₀] of approximately 1.7). Asterisks (*) indicate values significantly different at $P < 0.05$. **B**, Expression of *gabD-gabT'*-*lacZ* on pME6014*gabDT* and of *gabD-gabT'*(*gga*)-*lacZ* on pME6014*gabDTgga* in CHA0 and the *gacA* mutant grown in NYB. The symbols indicate the averages of triplicate cultures and error bars indicate standard deviations. The Student's *t* test was performed to compare the *gacA* mutant with CHA0 in each construct in the late exponential phase (at an OD₆₀₀ of approximately 1.7). An asterisk (*) indicates values significantly different at $P < 0.05$ (with an arrow).

the switch under GacS/GacA control. However, limited information is currently available on these low-molecular weight effectors. As described above, the intracellular starvation and stress signal ppGpp has a positive effect on the function of the Gac/Rsm cascade in *P. protegens* (Takeuchi et al. 2012) and there is ample evidence for intracellular c-di-GMP playing an executive role in the Gac/Rsm cascade and, hence, in the establishment of biofilms of *P. aeruginosa* (Kulasakara et al. 2006; Valentini

and Filloux 2016). Furthermore, a positive correlation has been reported between extra- and intracellular levels of Krebs cycle intermediates such as fumarate, succinate, and 2-oxoglutarate and the regulation of secondary metabolism via the expression of GacA-dependent sRNAs in *P. protegens* (Takeuchi et al. 2009). However, other signals may exist. This study focused on GABA, which is overproduced in a *gacA* mutant but is under-represented in a *retS* mutant of strain CHA0 (Takeuchi et al. 2012).

Using simple in-vitro culture systems, the function of the *gabT* gene was found to be instrumental for intracellular GABA levels in *P. protegens*, i.e., the *gabT* gene encoded GABAT, which catabolized GABA (Table 1). High GABA levels prolonged planktonic growth and diminished biofilm formation (Fig. 3). A root colonization assay under gnotobiotic conditions revealed that accumulated intracellular GABA promoted root colonization by strain CHA0 (Table 2). This result may be rationalized by assuming that GABA delays the action of the Gac/Rsm system during the biofilm phase by negatively affecting the GacS/GacA two component system; however, the underlying mechanisms remain unclear. With a delay in Gac/Rsm activity, i.e., under conditions in which the costly production of secondary metabolites and biofilm formation are attenuated, cells may engage more extensively in proliferation and planktonic modes of growth, which is preferable for root colonization. Previous studies reported that the *gacS/gacA* mutants of *P. fluorescens* F113 and *P. brassicacearum* exhibited better root colonization abilities than the wild type under gnotobiotic conditions (Achouak et al. 2004; Lalaouna et al. 2012; Sánchez-Contreras et al. 2002).

GABA concentrations in the rhizosphere in natural soil are an important factor that needs to be considered from a biocontrol point of view. GABA is naturally present in the rhizosphere, and, although few studies have examined its concentration, a low micromolar level is plausible (Moe 2013). In the present study, GABA was used at concentrations of 2 and 5 mM to investigate its function as an intracellular signal; however, the potential of GABA to function as an extracellular signal cannot be excluded. The identification of GABA sensors or receptors that may respond to exogenous GABA at lower concentrations will be a challenge for future studies.

In nutrient-rich, liquid, or semisolid laboratory media and on gnotobiotic plant roots, *P. protegens*, *P. brassicacearum*, *P. fluorescens* F113, and related strains showed signs of unusual genetic instability, with the frequent selection of spontaneous *gacS* and *gacA* mutants (Achouak et al. 2004; Bull et al. 2001; Lalaouna et al. 2012; Sánchez-Contreras et al. 2002; Song et al. 2016; Whistler et al. 1998). Although driving selection is not yet fully understood, the *gacS* and *gacA* mutants may benefit from an extended phase of cell mass proliferation. In the defined medium used, the *gacA* mutant did not grow more extensively

Fig. 3. Effects of γ -aminobutyrate (GABA) on the growth of planktonic cells and biofilm formation by *Pseudomonas protegens* CHA0 and its mutants. Cells were grown in mannitol-glutamate medium without added GABA (white bar), with 2 mM GABA (light gray bar), or with 5 mM GABA (dark gray bar). **A**, Biofilm formation on polypropylene, as assessed by crystal violet staining. **B**, Planktonic cell growth, as assessed by measuring optical density at 600 nm in a liquid culture. **C**, Ratio of planktonic cell growth to biofilm formation. The means and standard deviations from triplicate experiments are shown. The Student's *t* test was performed to compare each strain treated with 5 mM GABA with its respective control strain without added GABA; asterisks (*) and (**) indicate values significantly different at $P < 0.05$ and 0.01 , respectively. The Student's *t* test was also performed to compare each mutant with CHA0 at 0 mM added GABA; daggers († and ††) indicate values significantly different at $P < 0.05$ and 0.01 , respectively.

Table 2. Effects of a *gabT* mutation on the ability of *Pseudomonas protegens* CHA0 to colonize cucumber roots

Bacterial strain added ^a	Colonization by <i>P. protegens</i> (log ₁₀ CFU per gram of root) ^b			
	6 days	8 days	10 days	12 days
CHA0 (wild type)	7.16 ± 0.11	6.99 ± 0.06	7.70 ± 0.08	7.22 ± 0.10
<i>ΔgabT</i>	7.05 ± 0.12	7.32 ± 0.02*	7.86 ± 0.14*	7.46 ± 0.19*

^a *P. protegens* strains were added at 1×10^3 CFU per seedling in 50-ml Falcon tubes, each containing 20 ml of one-fifth strength Murashige and Skoog medium with 0.6% agar and 0.05% morpholineethanesulfonic acid buffer (pH 6.0), after planting two 48-h-old, sterile-grown cucumber seedlings per tube. Plants were harvested after the days indicated. Asterisks (*) denote values that are significantly different from that in CHA0 at the $P < 0.05$ level (*t* test).

^b Data represent the averages of four to six replicates per treatment, and, thus, a total of eight to 12 plants.

but had a markedly shorter lag phase than the wild type. Under the in-vitro conditions, *gacA* mutants (temporarily) dominated by optimizing the planktonic growth phase. In contrast, under natural soil conditions, in which this selection has not been observed, the developmental process leading to biofilm formation will dominate.

In *A. tumefaciens*, the quorum-sensing signal *N*-(3-oxo-octanoyl)-homoserine lactone positively regulates virulence, i.e., plant tumor formation. GABA induces AttM lactonase, which degrades *N*-acyl-homoserine lactones. Thus, GABA—of plant or bacterial origin—quenches quorum sensing and, thus, modulates virulence (Chevrot et al. 2006). *P. protegens* and several other fluorescent pseudomonads (but not *P. aeruginosa*) lack *N*-acyl-homoserine lactone-dependent quorum-sensing machinery; however, at high cell population densities, the Gac/Rsm system provides a quorum sensing-like response (Haas and Keel 2003). It currently remains unclear whether GABA acts on some component of the Gac/Rsm cascade in strain CHA0. In contrast, as shown in the present study, the Gac/Rsm system affects the accumulation of GABA in a similar manner to its effects on secondary metabolism and the production of extracellular macromolecules.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacterial strains and plasmids used are listed in Supplementary Table S3. Strains of *E. coli* and *P. protegens* were routinely grown in nutrient yeast broth (NYB) (2.5% [wt/vol] nutrient broth, 0.5% [wt/vol] yeast extract) and Luria-Bertani medium with shaking or on nutrient agar plates (4% [wt/vol] blood agar base, 0.5% [wt/vol] yeast extract) amended with the following antibiotics, when required: ampicillin, 100 µg/ml (only for *E. coli*); kanamycin, 25 µg/ml; or tetracycline, 25 µg/ml (125 µg/ml for selection of *P. protegens*). To evaluate the GABA utilization abilities of the strains tested, we used modified M9 minimal medium agar plates (per liter: Na₂HPO₄, 6.0 g; KH₂PO₄, 3.0 g; NaCl, 0.5 g; MgSO₄·7H₂O, 0.494 g, and CaCl₂·2H₂O, 0.015 g) supplemented with 20 mM GABA as the sole carbon and nitrogen source. In other assays, bacteria were grown in MG medium containing (per liter): mannitol, 10 g; L-glutamic acid, 2.0 g; KH₂PO₄, 0.5 g; NaCl, 0.2 g; MgSO₄·7H₂O, 0.494 g, final pH 7 (Park et al. 2010). Incubation temperatures were 30°C for *P. protegens* and 37°C for *E. coli*. *P. protegens* was grown at 35°C to improve its capacity to accept heterologous DNA (e.g., in electro-transformation or in mating with *E. coli*).

Construction of *gabD*'-'*lacZ*- and *gabDT*'-'*lacZ*- fusions.

A 420-bp *EcoRI*-*Bam*HI fragment from the upstream region of PFLCHA0_c01870 (*gabD*), including the first five codons of the *gabD* gene and presumably containing the *gabD* promoter, was amplified using the primers GabDProFEcoRI and GabD-ProR (Supplementary Table S4), and the amplified fragment was cloned into pCR-Blunt II-TOPO (Invitrogen). The insert obtained was confirmed by sequencing and was digested with *EcoRI* and *Bam*HI. This fragment was fused in-frame to the eighth codon of the *lacZ* gene in vector pME6014 to obtain pME6014*gabD*, containing a translational *gabD*'-'*lacZ* fusion (Fig. 1A).

A 2.0-kb *Bam*HI-*Pst*I fragment containing the region from 0.4 kb upstream of PFLCHA0_c01870 (*gabD*) to the 5' region of PFLCHA0_c01880 (*gabT*) including the first six codons of the *gabT* gene was amplified, using the primers GabDProFBamHI and GabTProR, and was inserted into pBluescript II KS (Stratagene). The insert obtained was confirmed by sequencing, and, for the mutational introduction of a conventional Shine-Dalgarno sequence (i.e., the modification of TAAGGAAG to AGGA), this construct was used as a template plasmid for polymerase chain

reaction (PCR) with the mutagenic primers GabDggaF and GabDggaR designed for the amplification of a circular product. The mutation was confirmed by sequencing. The native and mutated regions were fused to the eighth codon of the *lacZ* gene in the vector pME6014 (at the *Bam*HI and *Pst*I sites) to obtain pME6014*gabDT* and pME6014*gabDTgga*, respectively, containing a translational *gabDT*'-'*lacZ* fusion (Fig. 1B).

Generation of *gabD*- and *gabT*-negative mutants.

In-frame deletions in the chromosomal *gabD* and *gabT* genes of *P. protegens* CHA0 were created as follows. Fragments of approximately 800 to 850 bp located on each side of the *gabD* or *gabT* gene were amplified by PCR with the primer pairs GabDUF/GabDUR and GabDDF/GabDDR for *gabD* and GabTUF/GabTUR and GabTDF/GabTDR for *gabT*. Each of the two corresponding fragments were ligated and amplified as 1.6 to 1.7-kb fragments, using the primers GabDUF/GabDDR or GabTUF/GabTDR, respectively. The amplified fragments were cloned into pCR-Blunt II-TOPO. After confirmation by sequencing, these fragments were subcloned into pME3087 cut with *Bam*HI and *Hind*III, to give pME3087*gabD* and pME3087*gabT*. These plasmids were mobilized from *E. coli* DH5α to *P. protegens* CHA0 by triparental mating with *E. coli* HB101/pME497 and were selected for tetracycline resistance. Excision of the vector via a second crossing-over was performed after enrichment for tetracycline-sensitive cells (Humair et al. 2009), generating the *gabD* or *gabT* mutant.

Complementation of the *gabT*-negative mutant.

To restore *gabT* function in the *gabT* mutant, a 3.8-kb fragment containing the region from 0.4 kb upstream of *gabD* to 0.5 kb downstream of *gabT* was amplified with the primers GabD-ProFBamHI and GabTDR. The amplified fragment was cloned into pCR-Blunt II-TOPO and the insert obtained was confirmed by sequencing and was digested with *Bam*HI and *Hind*III. This fragment was subcloned into pME6031 cleaved at the *Bam*HI and *Hind*III sites. The resultant plasmid, pME6031*gabT*, was introduced into the *gabT* mutant by electroporation.

β-Galactosidase assays.

β-Galactosidase activities were quantified by the Miller method (Miller 1972). *P. protegens* 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.

GABA extraction and quantification by HPLC.

Intracellular levels of GABA were assessed in *P. protegens* strains cultured in MG medium supplemented with GABA at a concentration of 5 mM (MG + GABA). For the preculture, the strains were grown overnight in NYB medium and were rinsed twice with MG medium without GABA. The strains were then inoculated at a starting OD of 0.3 in 10 ml fresh MG + GABA medium in 50-ml flasks and were grown at 180 rpm and 30°C for 15 h. In each strain, three cultures were incubated in parallel. Cells were harvested by centrifugation at 5,000 × *g* for 10 min, and the pellet was washed twice with MG medium without GABA and was preserved at -80°C for GABA extraction. The pellets were homogenized in 750 µl of 70% methanol and centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant was dried and preserved at -80°C.

Regarding the detection of GABA, the metabolite extract was labeled with 4-dimethyl-aminoazobenzene-4'-sulfonyl (dabsyl) chloride (Thermo Fisher Scientific K.K.), following the method described by Abe et al. (1998). The metabolite extract was dissolved in 200 µl of 0.1 M sodium hydrogen carbonate buffer (pH 9.0), and 200 µl of 2 mM dabsyl chloride solution in acetone was added. The dabsylation reaction was performed at

70°C for 20 min. After vacuum evaporation, the dried samples were dissolved in 100 µl of 100% ethanol. The concentration of GABA was assessed by HPLC on a Waters system (Waters) consisting of a photodiode array detector 996 and pump 616, equipped with a normal phase column (TSKgel ODS-80Ts column, 4.6 × 250 mm, Tosoh). Routine chromatography was performed at 28°C, using a solvent system consisting of 50 mM sodium acetate (pH 4.15) (solvent A) and acetonitrile (solvent B). Elution was performed with the concentration of solvent B as follows: 0 to 40 min, linear gradient from 35 to 40%; 40 to 80 min, isocratic at 40%. The column was then washed with a solution of 5% solvent A and 95% solvent B for 20 min and was reconditioned with a solution of 75% solvent A and 35% solvent B. Dabsyl derivatives of amino acids were detected at 430 nm. The concentration of GABA was expressed relative to cell density (OD_{600nm} is approximately 1.0/ml). Regarding the standard, GABA was obtained from Wako and was labeled with dabsyl chloride in the same manner described above.

Enzyme assays.

To prepare cell extracts, cultures of *P. protegens* CHA0 or the mutant strains were grown on MG medium supplemented with GABA at 5 mM, under the same conditions as described above. Cells were harvested at 4°C by centrifugation at 5,000 × g for 10 min and the pellet was suspended in 5 ml of 20 mM phosphate buffer (pH 7.2) and was centrifuged again. Cells were then resuspended in 1 ml of breaking buffer (40 mM HEPES, 1 mM dithiothreitol [DTT] at pH 7.0) and were sonicated on ice for 1 min. Cell debris was removed by centrifugation at 10,000 × g for 10 min. Protein concentrations were assessed by the Bradford method.

2-Oxoglutarate-dependent GABAT activity was measured with 150 mM GABA and 50 mM 2-oxoglutarate as substrates, as described earlier by Bartsch et al. (1990) and Prell et al. (2002). The assay was performed in a final volume of 300 µl, using 150 µl of the cell extract. Glutamate formation was assessed by the reduction of NAD⁺ in a coupled glutamate dehydrogenase (GDH) assay. In a final volume of 450 µl (40 mM hydrazine, 50 mM glycine [pH 7.6], 2.7 mM NAD⁺, 1 unit GDH), the complete GABAT sample (150 µl) was incubated at 30°C for 2 h. NADH production was measured at 340 nm in a spectrophotometer. Assays were performed with a control lacking GDH for each extract. Results were compared with a standard curve (with 0–1 µmol glutamate per sample).

SSDH was measured with 2.5 mM succinate semialdehyde and 1 mM NAD⁺ as substrates. The assay was performed in 50 mM Tris-HCl buffer at pH 8.0 and 1 mM DTT. The reduction of NAD⁺ was measured spectrophotometrically at 340 nm. Assays were performed with a control lacking succinate semialdehyde for each extract.

Biofilm assay.

Cells from overnight cultures grown in NYB were washed once in saline (0.9% NaCl) and were resuspended to an OD₆₀₀ of 3.0 in saline. One-microliter aliquots were inoculated into 96-well polypropylene plates filled with MG medium (200 µl per well) without GABA or supplemented with GABA at 2 or 5 mM. Cultures were incubated statically at room temperature, with a cover, in a container with a water-soaked paper towel to prevent evaporation. After 24 h, 100 µl of the planktonic cell cultures was used to measure OD₆₀₀ values. Fifty microliters of a 0.5% solution of crystal violet was added to each well to stain the biofilm, the plates were incubated at room temperature for 15 min, and were then rinsed thoroughly and repeatedly with water. Regarding quantification, the stained biofilm of each well was suspended in 400 µl of 95% ethanol, followed by the

addition of 600 µl of water, and absorbance was measured at 600 nm.

Swarming motility assay.

Cells from overnight cultures grown in NYB were washed once in saline (0.9% NaCl) and were resuspended to an OD₆₀₀ of 3.0 in saline. A sterile toothpick was then dipped into the cell suspension and was used to inoculate by stabbing vertically into the center of semisolid MG agar (0.5%) plates containing 0, 2, or 5 mM GABA. The plates were incubated at room temperature for 60 h, and swarming motility was assessed by examining the circular lawn formed by the motile bacterial cells.

Plant disease suppression and root-colonization assays.

Flasks containing 20 g of nonsterile soil (vermiculite) were each planted with three seedlings of cucumber (*Cucumis sativus* L. cv. Tokiwajibai) and were treated with one or both *Pythium ultimum* MAFF425494 and *P. protegens*. *P. protegens* strains were added to soil as a suspension (4 ml per flask) of cells washed twice in sterile distilled water, to give 2 × 10⁷ CFU per gram 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 containing 60% relative humidity at 26°C with light for 16 h, followed by an 8-h dark period. No watering was necessary. After a 7-day incubation, the biocontrol activity of each strain was assessed by counting the number of surviving plants and measuring shoot and root fresh weights per flask. The root colonization of each strain was also simultaneously assessed; whole roots in each flask were rinsed thoroughly and repeatedly with sterile, ultrapure water and were collected in 50-ml conical tubes containing 15 ml of saline. After vigorous agitation, bacteria were enumerated by plating dilutions. Data of both experiments were first analyzed for trial-by-treatment interactions by an analysis of variance, which indicated that the data from the two independent trials could be pooled. Means were separated using Tukey's highly significant difference test (at *P* ≤ 0.05). Statistical analyses were performed using R (version 3.0.1).

Gnotobiotic plant root-colonization assay.

Two 48-h-old, sterile-grown cucumber seedlings were planted in 20 ml of fifth-strength Murashige and Skoog agar medium (0.6%) (Murashige and Skoog 1962) supplemented with 0.05% morpholineethanesulfonic acid buffer (pH 6.0) in 50-ml conical tubes (Becton Dickinson/Falcon). Bacterial cells from overnight cultures grown in NYB were washed once in sterile, ultrapure water and were adjusted to a final concentration of approximately 1.0 × 10⁴ CFU/ml. Two hundred-microliter aliquots were inoculated into seedlings to give 1.0 × 10⁵ CFU per seedling, and the tubes were loosely capped. As a control, six plants were treated with 200 µl of sterilized water.

Seedlings were incubated in a growth chamber at 26°C with light for 16 h, followed by an 8-h dark period. After 6, 8, 10, and 12-day incubations, whole roots were rinsed thoroughly and repeatedly with sterile, ultrapure water and were collected in 50-ml conical tubes containing 15 ml of saline. After vigorous agitation, bacteria were enumerated by plating dilutions. In water-control plants, no microorganism was confirmed to be colonized on the roots after a 12-day inoculation.

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LITERATURE CITED

- Abe, T., Kurozumi, Y., Yao, W. B., and Ubuka, T. 1998. High-performance liquid chromatographic determination of β -alanine, β -aminoisobutyric acid and γ -aminobutyric acid in tissue extracts and urine of normal and (aminooxy)acetate-treated rats. *J. Chromatogr. B Biomed. Sci. Appl.* 712:43-49.
- Achouak, W., Conrod, S., Cohen, V., and Heulin, T. 2004. Phenotypic variation of *Pseudomonas brassicacearum* as a plant root-colonization strategy. *Mol. Plant-Microbe Interact.* 17:872-879.
- Badri, D. V., Chaparro, J. M., Zhang, R., Shen, Q., and Vivanco, J. M. 2013. Application of natural blends of phytochemicals derived from the root exudates of *Arabidopsis* to the soil reveal that phenolic-related compounds predominantly modulate the soil microbiome. *J. Biol. Chem.* 288:4502-4512.
- Barahona, E., Navazo, A., Martínez-Granero, F., Zea-Bonilla, T., Pérez-Jiménez, R. M., Martín, M., and Rivilla, R. 2011. *Pseudomonas fluorescens* F113 mutant with enhanced competitive colonization ability and improved biocontrol activity against fungal root pathogens. *Appl. Environ. Microbiol.* 77:5412-5419.
- Bartsch, K., von Johnn-Marteville, A., and Schulz, A. 1990. Molecular analysis of two genes of the *Escherichia coli* *gab* cluster: Nucleotide sequence of the glutamate:succinic semialdehyde transaminase gene (*gabT*) and characterization of the succinic semialdehyde dehydrogenase gene (*gabD*). *J. Bacteriol.* 172:7035-7042.
- Blumer, C., and Haas, D. 2000. Iron regulation of the *hcnABC* genes encoding hydrogen cyanide synthase depends on the anaerobic regulator ANR rather than on the global activator GacA in *Pseudomonas fluorescens* CHA0. *Microbiology* 146:2417-2424.
- Bull, C. T., Duffy, B., Voisard, C., Défago, G., Keel, C., and Haas, D. 2001. Characterization of spontaneous *gacS* and *gacA* regulatory mutants of *Pseudomonas fluorescens* biocontrol strain CHA0. *Antonie van Leeuwenhoek* 79:327-336.
- Chevrot, R., Rosen, R., Haudecoeur, E., Cirou, A., Shelp, B. J., Ron, E., and Faure, D. 2006. GABA controls the level of quorum-sensing signal in *Agrobacterium tumefaciens*. *Proc. Natl. Acad. Sci. U.S.A.* 103:7460-7464.
- Chou, H. T., Kwon, D. H., Hegazy, M., and Lu, C. D. 2008. Transcriptome analysis of agmatine and putrescine catabolism in *Pseudomonas aeruginosa* PAO1. *J. Bacteriol.* 190:1966-1975.
- Dasu, V. V., Nakada, Y., Ohnishi-Kameyama, M., Kimura, K., and Itoh, Y. 2006. Characterization and a role of *Pseudomonas aeruginosa* spermidine dehydrogenase in polyamine catabolism. *Microbiology* 152:2265-2272.
- Goodman, A. L., Merighi, M., Hyodo, M., Ventre, I., Filloux, A., and Lory, S. 2009. Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen. *Genes Dev.* 23:249-259.
- Haas, D., and Défago, G. 2005. Biological control of soil-borne pathogens by fluorescent pseudomonads. *Nat. Rev. Microbiol.* 3:307-319.
- Haas, D., and Keel, C. 2003. Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. *Annu. Rev. Phytopathol.* 41:117-153.
- Heeb, S., Blumer, C., and Haas, D. 2002. Regulatory RNA as mediator in GacA/RsmA-dependent global control of exoproduct formation in *Pseudomonas fluorescens* CHA0. *J. Bacteriol.* 184:1046-1056.
- Humair, B., González, N., Mossialos, D., Reimmann, C., and Haas, D. 2009. Temperature-responsive sensing regulates biocontrol factor expression in *Pseudomonas fluorescens* CHA0. *ISME J.* 3:955-965.
- Kay, E., Dubuis, C., and Haas, D. 2005. Three small RNAs jointly ensure secondary metabolism and biocontrol in *Pseudomonas fluorescens* CHA0. *Proc. Natl. Acad. Sci. U.S.A.* 102:17136-17141.
- Kay, E., Humair, B., Dénervaud, V., Riedel, K., Spahr, S., Eberl, L., Valverde, C., and Haas, D. 2006. Two GacA-dependent small RNAs modulate the quorum-sensing response in *Pseudomonas aeruginosa*. *J. Bacteriol.* 188:6026-6033.
- Kidarsa, T. A., Shaffer, B. T., Goebel, N. C., Roberts, D. P., Buyer, J. S., Johnson, A., Kobayashi, D. Y., Zabriskie, T. M., Paulsen, I., and Loper, J. E. 2013. Genes expressed by the biological control bacterium *Pseudomonas protegens* Pf-5 on seed surfaces under the control of the global regulators GacA and RpoS. *Environ. Microbiol.* 15:716-735.
- Kim, Y. C., Leveau, J., McSpadden Gardener, B. B., Pierson, E. A., Pierson, L. S., 3rd, and Ryu, C. M. 2011. The multifactorial basis for plant health promotion by plant-associated bacteria. *Appl. Environ. Microbiol.* 77:1548-1555.
- Kulasakara, H., Lee, V., Brenic, A., Liberati, N., Urbach, J., Miyata, S., Lee, D. G., Neely, A. N., Hyodo, M., Hayakawa, Y., Ausubel, F. M., and Lory, S. 2006. Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence. *Proc. Natl. Acad. Sci. U.S.A.* 103:2839-2844.
- Kupferschmied, P., Maurhofer, M., and Keel, C. 2013. Promise for plant pest control: Root-associated pseudomonads with insecticidal activities. *Front. Plant Sci.* 4:287.
- Lalaouna, D., Fochesato, S., Sanchez, L., Schmitt-Kopplin, P., Haas, D., Heulin, T., and Achouak, W. 2012. Phenotypic switching in *Pseudomonas brassicacearum* involves GacS- and GacA-dependent Rsm small RNAs. *Appl. Environ. Microbiol.* 78:1658-1665.
- Lapouge, K., Schubert, M., Allain, F. H.-T., and Haas, D. 2008. Gac/Rsm signal transduction pathway of γ -proteobacteria: From RNA recognition to regulation of social behaviour. *Mol. Microbiol.* 67:241-253.
- Lapouge, K., Sineva, E., Lindell, M., Starke, K., Baker, C. S., Babiszke, P., and Haas, D. 2007. Mechanism of *hcnA* mRNA recognition in the Gac/Rsm signal transduction pathway of *Pseudomonas fluorescens*. *Mol. Microbiol.* 66:341-356.
- Laville, J., Voisard, C., Keel, C., Maurhofer, M., Défago, G., and Haas, D. 1992. Global control in *Pseudomonas fluorescens* mediating antibiotic synthesis and suppression of black root rot of tobacco. *Proc. Natl. Acad. Sci. U.S.A.* 89:1562-1566.
- Ma, J., Campbell, A., and Karlin, S. 2002. Correlations between Shine-Dalgarno sequences and gene features such as predicted expression levels and operon structures. *J. Bacteriol.* 184:5733-5745.
- Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Moe, L. A. 2013. Amino acids in the rhizosphere: From plants to microbes. *Am. J. Bot.* 100:1692-1705.
- Murashige, T., and Skoog, F. 1962. A revised medium for the rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant.* 15:473-497.
- Park, D. H., Mirabella, R., Bronstein, P. A., Preston, G. M., Haring, M. A., Lim, C. K., Collmer, A., and Schuurink, R. C. 2010. Mutations in γ -aminobutyric acid (GABA) transaminase genes in plants or *Pseudomonas syringae* reduce bacterial virulence. *Plant J.* 64:318-330.
- Parkins, M. D., Ceri, H., and Storey, D. G. 2001. *Pseudomonas aeruginosa* GacA, a factor in multihost virulence, is also essential for biofilm formation. *Mol. Microbiol.* 40:1215-1226.
- Prell, J., Boesten, B., Poole, P., and Priefer, U. B. 2002. The *Rhizobium leguminosarum* bv. *viciae* VF39 γ -aminobutyrate (GABA) aminotransferase gene (*gabT*) is induced by GABA and highly expressed in bacteroids. *Microbiology* 148:615-623.
- Reimmann, C., Valverde, C., Kay, E., and Haas, D. 2005. Posttranscriptional repression of GacS/GacA-controlled genes by the RNA-binding protein RsmE acting together with RsmA in the biocontrol strain *Pseudomonas fluorescens* CHA0. *J. Bacteriol.* 187:276-285.
- Sánchez-Contreras, M., Martín, M., Villaceros, M., O'Gara, F., Bonilla, I., and Rivilla, R. 2002. Phenotypic selection and phase variation occur during alfalfa root colonization by *Pseudomonas fluorescens* F113. *J. Bacteriol.* 184:1587-1596.
- Schneider, B. L., Ruback, S., Kiupakis, A. K., Kasbarian, H., Pybus, C., and Reitzer, L. 2002. The *Escherichia coli* *gabDTPC* operon: Specific γ -aminobutyrate catabolism and nonspecific induction. *J. Bacteriol.* 184:6976-6986.
- Song, C., Kidarsa, T. A., van de Mortel, J. E., Loper, J. E., and Raaijmakers, J. M. 2016. Living on the edge: Emergence of spontaneous *gac* mutations in *Pseudomonas protegens* during swarming motility. *Environ. Microbiol.* 18:3453-3465.
- Takeuchi, K., Kiefer, P., Reimmann, C., Keel, C., Dubuis, C., Rolli, J., Vorholt, J. A., and Haas, D. 2009. Small RNA-dependent expression of secondary metabolism is controlled by Krebs cycle function in *Pseudomonas fluorescens*. *J. Biol. Chem.* 284:34976-34985.
- Takeuchi, K., Yamada, K., and Haas, D. 2012. ppGpp controlled by the Gac/Rsm regulatory pathway sustains biocontrol activity in *Pseudomonas fluorescens* CHA0. *Mol. Plant-Microbe Interact.* 25:1440-1449.
- Valentini, M., and Filloux, A. 2016. Biofilms and cyclic di-GMP (c-di-GMP) signaling: Lessons from *Pseudomonas aeruginosa* and other bacteria. *J. Biol. Chem.* 291:12547-12555.
- Valverde, C., Heeb, S., Keel, C., and Haas, D. 2003. RsmY, a small regulatory RNA, is required in concert with RsmZ for GacA-dependent expression of biocontrol traits in *Pseudomonas fluorescens* CHA0. *Mol. Microbiol.* 50:1361-1379.
- Whistler, C. A., Corbell, N. A., Sarniguet, A., Ream, W., and Loper, J. E. 1998. The two-component regulators GacS and GacA influence accumulation of the stationary-phase sigma factor σ^S and the stress response in *Pseudomonas fluorescens* Pf-5. *J. Bacteriol.* 180:6635-6641.

Winsor, G. L., Lam, D. K., Fleming, L., Lo, R., Whiteside, M. D., Yu, N. Y., Hancock, R. E., and Brinkman, F. S. 2011. Pseudomonas Genome database: Improved comparative analysis and population genomics capability for *Pseudomonas* genomes. Nucleic Acids Res. 39:D596-D600.

Workentine, M. L., Chang, L., Ceri, H., and Turner, R. J. 2009. The GacS-GacA two-component regulatory system of *Pseudomonas fluorescens*: A bacterial two-hybrid analysis. FEMS Microbiol. Lett. 292:50-56.

Yao, X., He, W., and Lu, C. D. 2011. Functional characterization of seven γ -glutamylpolyamine synthetase genes and the *bauRABCD* locus for polyamine and β -alanine utilization in *Pseudomonas aeruginosa* PAO1. J. Bacteriol. 193:3923-3930.

AUTHOR-RECOMMENDED INTERNET RESOURCE

Pseudomonas Genome database: <http://www.pseudomonas.com>