Secondary Metabolism and Interspecific Competition Affect Accumulation of Spontaneous Mutants in the GacS-GacA Regulatory System in *Pseudomonas protegens*

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ABSTRACT Secondary metabolites are synthesized by many microorganisms and provide a fitness benefit in the presence of competitors and predators. Secondary metabolism also can be costly, as it shunts energy and intermediates from primary metabolism. In *Pseudomonas* spp., secondary metabolism is controlled by the GacS-GacA global regulatory system. Intriguingly, spontaneous mutations in *gacS* or *gacA* (Gac⁻ mutants) are commonly observed in laboratory cultures. Here we investigated the role of secondary metabolism in the accumulation of Gac⁻ mutants in *Pseudomonas protegens* strain Pf-5. Our results showed that secondary metabolism, specifically biosynthesis of the antimicrobial compound pyoluteorin, contributes significantly to the accumulation of Gac⁻ mutants. Pyoluteorin biosynthesis, which poses a metabolic burden on the producer cells, but not pyoluteorin itself, leads to the accumulation of the spontaneous mutants. Interspecific competition also influenced the accumulation of the Gac⁻ mutants: a reduced proportion of Gac⁻ mutants accumulated when *P. protegens* Pf-5 was cocultured with *Bacillus subtilis* than in pure cultures of strain Pf-5. Overall, our study associated a fitness trade-off with secondary metabolism, with metabolic costs versus competitive benefits of production influencing the evolution of *P. protegens*, assessed by the accumulation of Gac⁻ mutants.

IMPORTANCE Many microorganisms produce antibiotics, which contribute to ecologic fitness in natural environments where microbes constantly compete for resources with other organisms. However, biosynthesis of antibiotics is costly due to the metabolic burdens of the antibiotic-producing microorganism. Our results provide an example of the fitness trade-off associated with antibiotic production. Under noncompetitive conditions, antibiotic biosynthesis led to accumulation of spontaneous mutants lacking a master regulator of antibiotic production. However, relatively few of these spontaneous mutants accumulated when a competitor was present. Results from this work provide information on the evolution of antibiotic biosynthesis and provide a framework for their discovery and regulation.

KEYWORDS interspecific competition, *Pseudomonas*, secondary metabolism, spontaneous mutations, GacS-GacA

Secondary metabolites play important roles in physiological adaptation and ecologic fitness of the organisms producing secondary metabolites (1, 2). These metabolites vary widely in structure and biological activity, with some having valuable pharmaceu-

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tical and agricultural applications (3, 4). Diverse classes of secondary metabolites are produced by plants, animals, and microorganisms, but knowledge of the natural functions of secondary metabolites is still limited. Secondary metabolites are thought to represent important adaptive characters of the organisms producing secondary metabolites in their native environments (2, 5). For example, a secondary metabolite with antimicrobial activity may allow organisms to inhibit competitors that occupy the same niches (6). On the other hand, secondary metabolism poses metabolic burdens to the producer by diverting precursors, cofactors, and energy from primary metabolism (7). Consequently, unnecessary expression of secondary metabolites can be costly to organisms producing secondary metabolites. To balance the benefits and costs, secondary metabolism is controlled by complex regulatory networks that regulate production in response to environmental factors and physiological status of the organism producing secondary metabolites (8, 9). These networks involve both global and pathway-specific regulators operating to coordinate secondary metabolite biosynthesis pathways at transcriptional and posttranscriptional levels (10–12).

The production of a large spectrum of secondary metabolites is a characteristic of bacteria in the genus *Pseudomonas* (13). Secondary metabolism in *Pseudomonas* spp. requires the GacS-GacA two-component regulatory system, composed of the sensor kinase GacS and the cognate cellular response regulator GacA (14). In the presence of an unknown signal(s), the sensor kinase GacS autophosphorylates and transfers a phosphate group to the response regulator GacA, which governs a complex signal transduction pathway involving regulatory RNAs and translational repression (14). The GacS-GacA system controls the expression of hundreds of genes, including those required for the production of secondary metabolites and extracellular enzymes (15–19). Accordingly, mutation of *gacA* and/or *gacS* has pleiotropic effects on the bacterial cell, influencing motility, iron acquisition, biofilm formation, aspects of primary metabolism, and many other phenotypes (15, 16, 20). Given the broad influence of the GacS-GacA system on bacterial physiology, it is striking that spontaneous *gacS* and *gacA* mutants (i.e., Gac⁻ mutants) emerge and accumulate in cultures of *Pseudomonas* spp. (17, 21–27). Colonies of these mutants can be distinguished from those of the wild type visually by their expanded colony size, flattened appearance, increased fluorescence under UV light, and lack of extracellular protease production (21, 24).

In this study, we compared several species of *Pseudomonas* spp. for accumulation of spontaneous Gac⁻ mutants in a nutrient-rich broth medium. One strain, *Pseudomonas protegens* Pf-5, was selected for further studies. Strain Pf-5 is a soil bacterium known for its capacity to inhibit the growth of phytopathogenic bacteria and fungi, suppress plant diseases, and kill certain insects through production of a large spectrum of secondary metabolites and toxins (10, 28–32). Here, we evaluated the role of secondary metabolism in the relative fitness of Gac⁻ mutants versus wild-type Pf-5 in culture. We found that secondary metabolism contributes to the evolution of *P. protegens* Pf-5 in culture, assessed as the proportion of spontaneous mutants in this important regulatory system. More importantly, the accumulation of Gac⁻ mutants was significantly affected by both biotic and abiotic environmental factors, including medium composition and interspecific competition, suggesting that spontaneous mutation of *gacS* or *gacA* can be an approach by which bacteria adapt to different environmental conditions.

RESULTS

***P. protegens* Pf-5 accumulates a high proportion of spontaneous Gac⁻ mutants in culture.** Certain strains of *Pseudomonas* spp. are known to accumulate spontaneous Gac⁻ mutants in culture. We compared the frequency with which spontaneous Gac⁻ mutants accumulate in four species of *Pseudomonas*, including *Pseudomonas protegens* Pf-5, *Pseudomonas fluorescens* SS101, *Pseudomonas aeruginosa* PAO1, and *Pseudomonas chlororaphis* 30-84. These strains were cultured in NYB (nutrient broth supplemented with 0.5% yeast extract), a medium in which *P. protegens* CHA0 was previously shown to accumulate Gac⁻ mutants (21). Bacterial cultures were diluted and spread on LMA (litmus milk agar) plates at 6 days postinoculation (dpi) and assessed for exoprotease
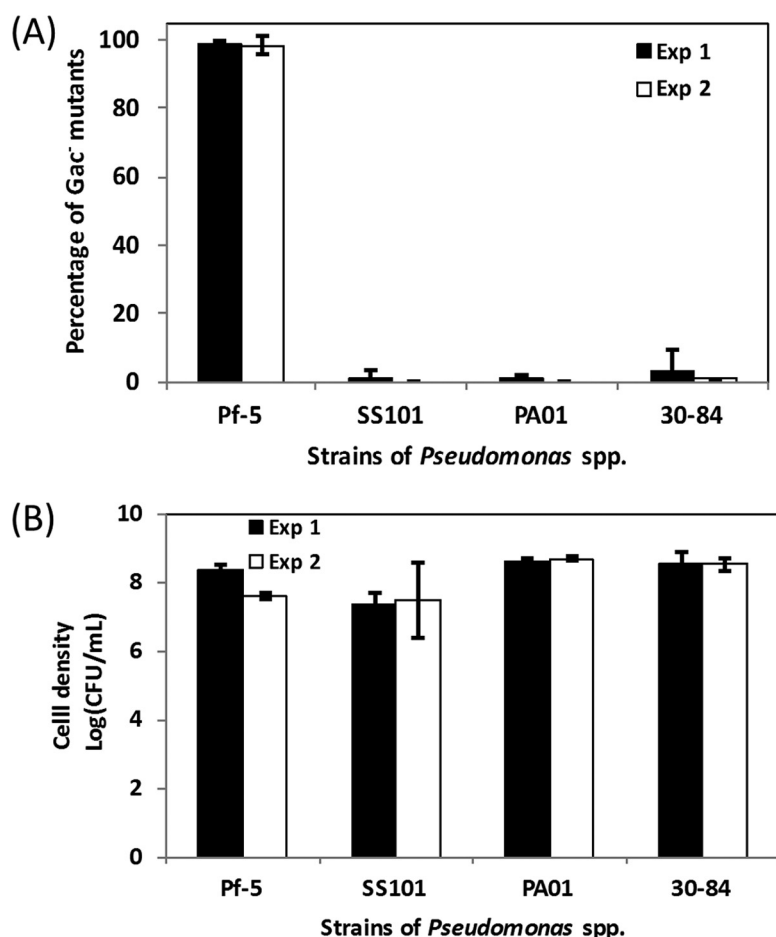
FIG 1 Accumulation of spontaneous Gac⁻ mutants of *Pseudomonas* spp. in NYB cultures. (A) Four strains, *P. protegens* Pf-5, *P. fluorescens* SS101, *P. aeruginosa* PAO1, and *P. chlororaphis* 30-84, were grown independently in NYB for 6 days, and dilutions of cultures were spread on LMA to assess exoprotease production. The percentage of the Gac⁻ mutants in the bacterial population was estimated from counts of exoprotease-deficient colonies relative to total colonies on LMA. At least 400 colonies were screened for each strain. The results of two experiments are shown. (B) Total cell density of the four strains in NYB cultures at 6 dpi. For both panels A and B, results are from two independent experiments (experiment 1 [Exp 1] and Exp 2). Each experiment had at least three replicates. Means ± standard deviations (error bars) are shown.

activity. Production of exoprotease requires the GacS-GacA system (33) and is a readily visualized phenotype (27) that has been used reliably to estimate the proportion of Gac⁻mutants in cultures of *P. protegens* Pf-5 (17). As such, colonies deficient in exoprotease activity are referred to as Gac⁻ mutants throughout this report.

All four tested strains grew well in NYB medium and accumulated spontaneous Gac⁻ mutants by 6 dpi (Fig. 1). However, large variations in the proportion of spontaneous Gac⁻ mutants that accumulated in cultures were observed among the four evaluated strains. Strains SS101 and PAO1 accumulated spontaneous Gac⁻ mutants to a level of <2% of the total CFU in the culture. Strain 30-84 was more variable between experimental replicates, but on average, cultures were composed of <5% Gac⁻ mutants. *P. protegens* Pf-5 was strikingly different, with 99% of total CFU on average being spontaneous Gac⁻ mutants. Due to the high proportion of Gac⁻ mutants in cultures of strain Pf-5, we focused on this strain to identify possible causes for the accumulation of mutants.

To understand the rate at which Gac⁻mutants accumulate in cultures of strain Pf-5, we estimated the proportion of mutants daily over 6 days. No Gac⁻ mutant was observed at 1 dpi, whereas 2% of the population were Gac⁻ mutants at 2 dpi (Fig. 2A).
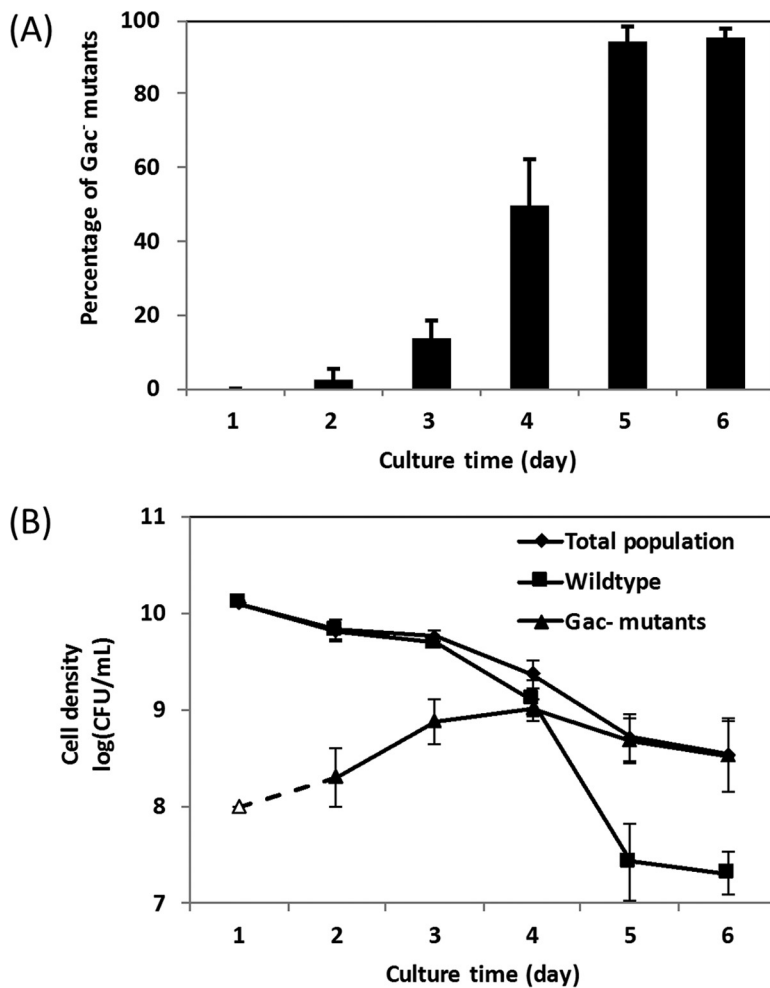
FIG 2 Accumulation of spontaneous Gac⁻ mutants in NYB cultures of *P. protegens* Pf-5 over time. Wild-type *P. protegens* Pf-5 was cultured in NYB for 6 dpi. (A and B) The percentage of spontaneous Gac⁻ mutants in the bacterial population (A) and the bacterial cell density (B) were recorded daily. In panel B, the cell density of Gac⁻ mutants at 1 dpi is shown as an open triangle, which represents the detection limit, as no Gac⁻ mutant was detected by screening more than 400 colonies. Each experiment had at least three replicates. Means ± standard errors (error bars) are shown.

The population size of the wild type decreased after 1 dpi, while the population size of Gac⁻ mutants increased gradually between 1 and 4 dpi (Fig. 2B). At 4 dpi, the Gac⁻ mutant population size also began to decline, but the population size of the wild type declined at a higher rate than the Gac⁻ mutants. By 5 dpi, the Gac⁻ mutants dominated the cultures (94% on average).

Secondary metabolism affects the accumulation of spontaneous Gac⁻ mutants. A prominent feature of *P. protegens* Pf-5 is the production of a large number of secondary metabolites (32, 34). Importantly, the production of these secondary metabolites is known to be positively regulated by the GacS-GacA system (15). We hypothesized that secondary metabolism contributes to the accumulation of spontaneous Gac⁻ mutants. To test this hypothesis, we evaluated the accumulation of Gac⁻ mutants of a Pf-5 derivative strain that contains mutations in *hcnB*, *ofaA*, *phlA*, *pltA*, *prnC*, and *rzxB* (referred to as the 6-fold mutant hereafter) (35). This 6-fold mutant does not produce any of the six secondary metabolites: hydrogen cyanide, orfamide A, 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, or rhizoxin (35). After 6 days of growth, the proportion of Gac⁻ mutants was significantly lower in cultures of this 6-fold mutant relative to wild-type Pf-5 (Fig. 3A), which supports our hypothesis that secondary metabolism plays a role in the accumulation of spontaneous Gac⁻ mutants in strain Pf-5.
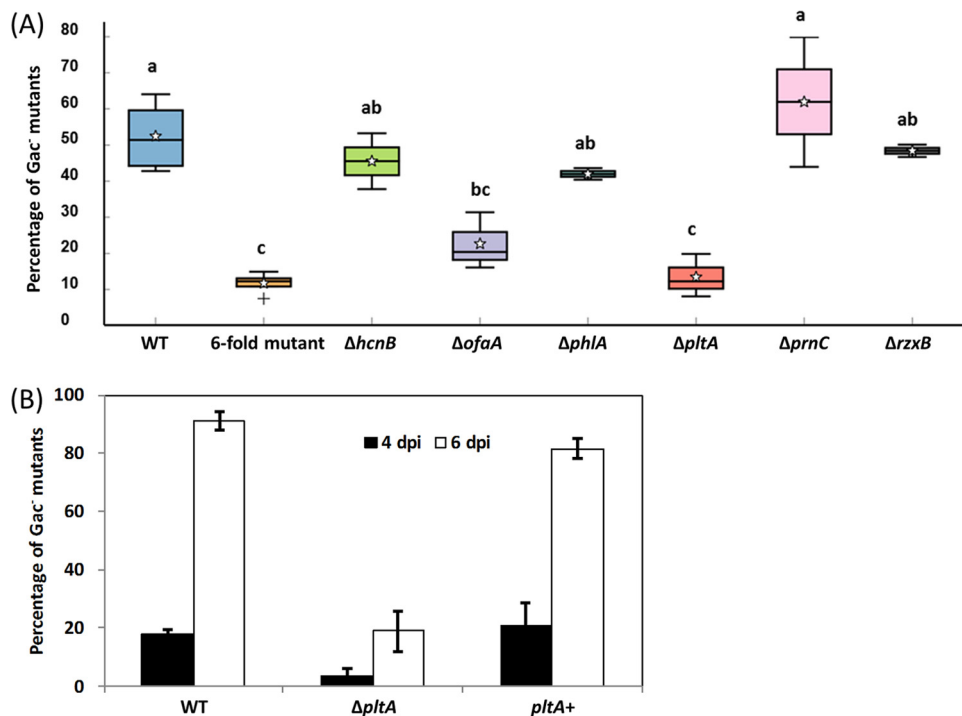
FIG 3 Accumulation of spontaneous Gac⁻ mutants of *P. protegens* Pf-5 and its secondary metabolism knockout mutants. Wild-type *P. protegens* Pf-5 and derivatives that contain mutations in the biosynthetic genes for six secondary metabolites (A) and the complemented Δ*pltA* mutant (B) were cultured in NYB. The percentage of Gac⁻ mutants in the bacterial population was calculated at 4 dpi (A and B) and 6 dpi (B). WT, wild-type Pf-5; 6-fold mutant, Pf-5 mutant containing mutations in *prnC*, *rzxB*, *pltA*, *hcnB*, *ofaA*, and *phlA*; *pltA*+, Δ*pltA* complemented strain. Each experiment had at least three replicates. In panel A, different letters above the boxes indicate that the mean values differ significantly ($P < 0.05$) according to Tukey's pairwise comparison test. In panel B, means ± standard errors are shown.

To pinpoint the biosynthetic pathway(s) that influences the accumulation of spontaneous Gac⁻ mutants of *P. protegens* Pf-5, we evaluated six independent mutants, each containing a single mutation in a biosynthetic gene for one of the six secondary metabolites. Of the six mutants, only the Δ*pltA* mutants and Δ*ofaA* mutants, which are unable to produce pyoluteorin and orfamide A, respectively, significantly differed from wild-type Pf-5 in the proportion of Gac⁻ mutants that accumulated in culture (Fig. 3A). The proportions of Gac⁻ mutants in cultures of wild-type Pf-5 and Δ*pltA* and Δ*ofaA* mutants were also assessed in another independent experiment, in which the production of secondary metabolites was quantified over time (see Fig. S1 in the supplemental material). Again, cultures of the Δ*pltA* and Δ*ofaA* mutants accumulated a smaller proportion of Gac⁻ mutants than a culture of the wild type in NYB medium. Results also confirmed that strain Pf-5 produced pyoluteorin and orfamide A in the NYB cultures, whereas the Δ*pltA* and Δ*ofaA* mutants did not produce pyoluteorin and orfamide A, respectively. Additionally, 2,4-diacetylphloroglucinol and rhizoxin were not detected in the cultures of wild-type Pf-5, the Δ*pltA* mutant, or the Δ*ofaA* mutant, which is consistent with results that the Δ*phlA* and Δ*rzxB* mutants accumulated proportions of Gac⁻ mutants similar to those accumulated by the wild type when cultured in NYB medium (Fig. 3A). We also noticed that pyrrolnitrin was produced by Pf-5 in NYB cultures (Fig. S1), but the Δ*prnC* mutant did not differ significantly from the wild type in the accumulation of Gac⁻ mutants (Fig. 3A). Therefore, it appears that the biosynthesis of some, but not all, secondary metabolites influences the accumulation of Gac⁻ mutants of Pf-5.

Most spontaneous Gac⁻ mutants have point mutations in *gacS* or *gacA*. We sequenced the *gacS* and *gacA* alleles from Gac⁻ mutants that accumulated in cultures of the following four strains: wild-type Pf-5, Δ*pltA* mutant, Δ*ofaA* mutant, and 6-fold

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TABLE 1 Sites of mutations in spontaneous Gac⁻ mutants sampled from cultures of wild-type *P. protegens* Pf-5 and derivatives[a]

Strain	Replicate	Colony	gacS[b] M1A	T254S	T307P	A447V	A463D	L476F	Q727*	2419^2420 insG*	Δ500-504	Δ2422-2426	gacA[b] RBS	V4E	V4G	I29N	T103I	Q155R	K190R
Pf-5	1	1								X									
	1	2																X	
	2	1						X											
	2	2				X													
	3	1																X	
	3	2						X											
ΔofaA	1	1			X														
	1	2			X														
	2	1[c]																	
	2	2							X										
	3	1										X							
	3	2					X												
ΔpltA	1	1	X																
	1	2	X																
	2	1												X					
	2	2															X		
	3	1													X				
	3	2													X				
LK147	1	1																	X
	1	2																X	
	2	1									X								
	2	2		X															
	3	1											X						
	3	2															X		

[a]*P. protegens* Pf-5 and derivatives (ΔofaA, ΔpltA, and the 6-fold mutant LK147) were each grown in three replicate flasks containing NYB for 4 days, dilutions of the cultures were spread on LMA, and two exoprotease-deficient colonies were selected from each replicate. *gacS* and *gacA* were PCR amplified and sequenced to map mutations.

[b]Abbreviations of amino acids show the results of point mutations in the *gacS* or *gacA* gene. RBS indicates a mutation in the predicted ribosome binding site. An asterisk indicates a stop codon introduced by a point mutation or by an insertion.

[c]No mutation in *gacS* or *gacA* was found in this isolate.

mutant. A total of 24 exoprotease-deficient colonies were evaluated: two colonies were randomly selected from LMA plated with three independent populations of each strain, for a total of six colonies per strain. Of the 24 exoprotease-deficient colonies, 23 have a single missense or nonsense mutation, an insertion, or a short deletion in the open reading frame (ORF) or ribosome binding site (RBS) of *gacA* or *gacS* (Table 1). This result substantiates that counting colonies lacking exoprotease production provided an accurate estimate of the number of *gacS* and *gacA* mutants in a culture of *P. protegens* Pf-5, as shown earlier (17). The mutations of 23 colonies mapped to 17 different sites of *gacS* or *gacA*. Ten colonies were affected in *gacA*, with nine having a point mutation in the ORF, and one having a point mutation in the predicted RBS. Thirteen colonies have mutations in *gacS*, of which two have small deletions that cause a frameshift and one has a single-nucleotide insertion that introduces a stop codon (Table 1). Two colonies were sampled from each of the 12 cultures of this experiment; 3 of the 12 pairs of colonies had identical mutant alleles and are probably siblings (Table 1). The observation that most pairs from the same population have different mutations, and the identification of 17 different mutation sites show that different mutants were selected within populations over the course of the experiment.

Biosynthesis of pyoluteorin contributes to the accumulation of spontaneous Gac⁻ mutants. To further investigate the relationship of secondary metabolism to the accumulation of spontaneous Gac⁻ mutants, we focused on the pyoluteorin biosyn-

thetic pathway because it had the greatest effect on the accumulation of Gac⁻ mutants in our experiments (Fig. 3A). To confirm the role of pyoluteorin biosynthesis in the accumulation of spontaneous Gac⁻ mutants, we complemented the ΔpltA mutant with a wild-type pltA gene. Data showed that the complemented mutant accumulated spontaneous Gac⁻ mutants at a level similar to that of the wild type (Fig. 3B). We also evaluated cultures of P. protegens Pf-5 growing in NBG (nutrient broth supplemented with 2% glucose). This medium supports bacterial growth but not pyoluteorin biosynthesis by strain Pf-5 (36). No spontaneous Gac⁻ mutant was detected when strain Pf-5 was cultured in NBG (193, 155, and 148 colonies were screened in three independent cultures), which further supports the conclusion that pyoluteorin biosynthesis contributes to accumulation of spontaneous Gac⁻ mutants of Pf-5.

Accumulation of spontaneous Gac⁻ mutants is associated with the expression of pyoluteorin biosynthetic genes, but not the product pyoluteorin. We then hypothesized that the presence of the compound pyoluteorin increased the accumulation of Gac⁻ mutants in cultures of P. protegens Pf-5. To test this hypothesis, we cultured the ΔpltA mutant in NYB broth spiked with different concentrations of purified pyoluteorin. Our data showed that pyoluteorin did not increase the proportion of Gac⁻ mutants in cultures of the ΔpltA mutant (Fig. 4). To the contrary, the proportion of Gac⁻ mutants decreased with the increased concentration of pyoluteorin added to the ΔpltA mutant cultures. The addition of pyoluteorin had no significant effect on the growth of the ΔpltA mutant (Fig. 4B). Because pyoluteorin is present in cultures of wild-type Pf-5 grown in NYB (Fig. S2), reduced sensitivity of Gac⁻ mutants to pyoluteorin could enhance their fitness relative to the wild-type strain in this medium. To test this possibility, we compared the ΔgacA mutant to the wild-type Pf-5 in their sensitivity to pyoluteorin. No significant difference was observed between the ΔgacA mutant and the wild-type Pf-5 in sensitivity to pyoluteorin either in NYB broth or on NYA (NYB with 1.5% agar) plates (Fig. S3). Furthermore, the addition of pyoluteorin to the wild-type Pf-5 cultures also resulted in a decreased proportion of Gac⁻ mutants (Fig. 4C). These data indicate that pyoluteorin, the product of the biosynthesis pathway, is not responsible for the accumulation of Gac⁻ mutants in cultures of Pf-5.

These results (showing that deletion of pltA led to a decreased accumulation of Gac⁻ mutants, whereas addition of pyoluteorin failed to restore the proportion of mutants to the wild-type Pf-5 level) imply that the process and the product of pyoluteorin biosynthesis contribute differentially to the accumulation of Gac⁻ mutants. Therefore, we hypothesized that the differential expression of pyoluteorin biosynthetic genes in the wild-type Pf-5 and the ΔpltA mutant drives the selection favoring Gac⁻ mutants. To test this hypothesis, a double mutant, the pltR* ΔpltA mutant, was constructed. The pltR gene encodes a positive transcriptional regulator of pyoluteorin biosynthesis genes, and the pltR* allele is an engineered variant in which all rare codons are substituted with optimized codons that increase its expression as well as that of its regulon (11). As expected, the promoter activity of pltL, the first gene in the plt biosynthetic gene cluster, increased significantly in the pltR* ΔpltA mutant versus the ΔpltA mutant (Fig. 5A). More importantly, the proportion of Gac⁻ mutants accumulated in cultures of the pltR* ΔpltA mutant was significantly higher than that of the ΔpltA mutant (Fig. 5B), indicating that overexpression of pyoluteorin biosynthetic genes increased the accumulation of spontaneous Gac⁻ mutations.

Collectively, our results suggest that the expression of pyoluteorin biosynthetic genes, but not the pyoluteorin product, contributes to accumulation of spontaneous Gac⁻ mutants in strain Pf-5.

Interspecific competition reduced the accumulation of spontaneous Gac⁻ mutants. The high numbers of spontaneous Gac⁻ mutants in P. protegens Pf-5 cultures prompted us to ask the question: does competitive pressure affect the accumulation of spontaneous Gac⁻ mutants? Bacillus spp. and Pseudomonas spp. are commonly identified from the same environments, and secondary metabolites are known to mediate interactions between these bacteria (37). To test whether interspecific competition occurs between P. protegens Pf-5 and Bacillus subtilis strain 168, these two strains were
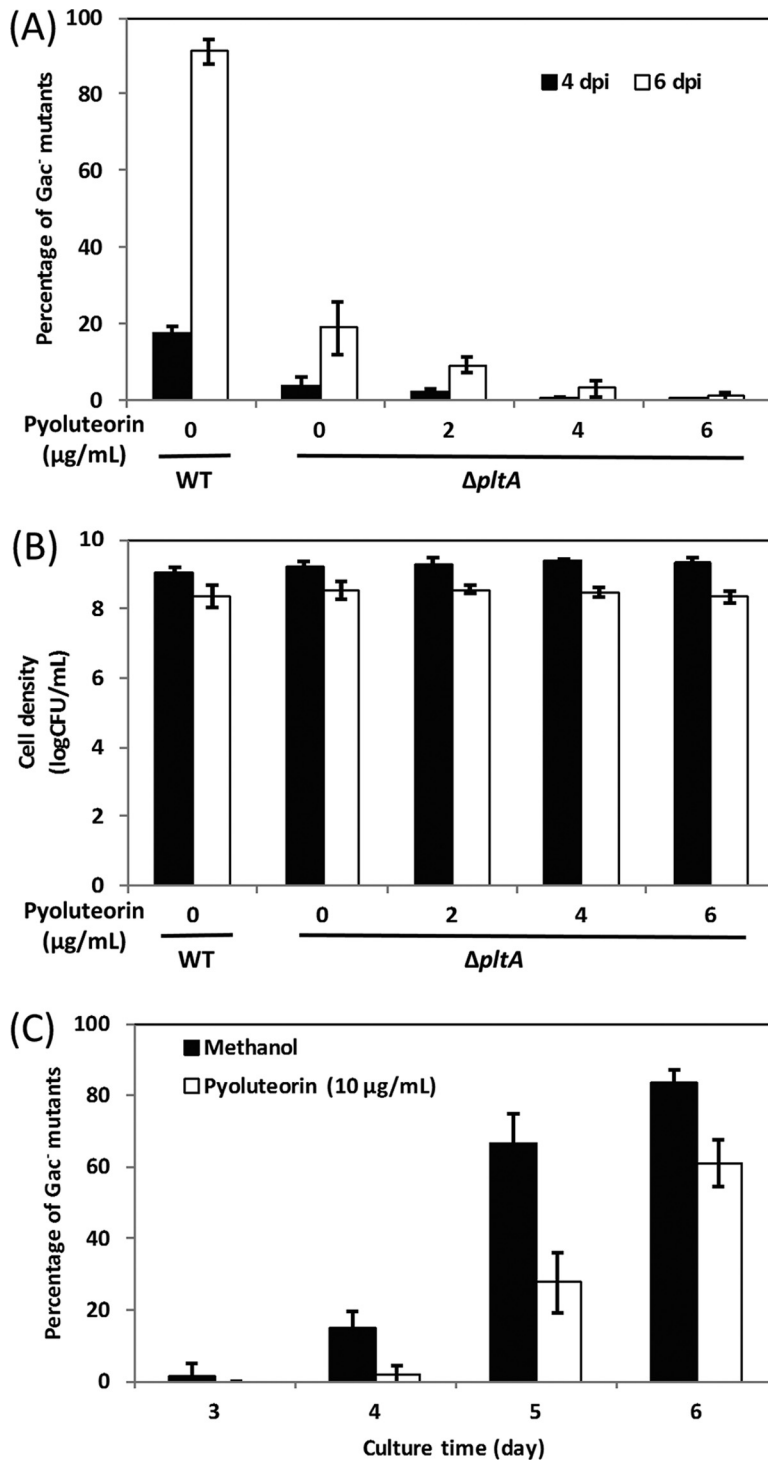
FIG 4 Influence of purified pyoluteorin on accumulation of Gac⁻ mutants of *P. protegens* Pf-5. Wild-type Pf-5 (WT) and the Δ*pltA* mutant were cultured in NYB with or without the addition of purified pyoluteorin at different concentrations. (A and B) The percentage of Gac⁻ mutants in the bacterial population (A) and the density of the total bacterial population (B) were recorded at 4 dpi and 6 dpi. (C) Wild-type Pf-5 was cultured in NYB with the addition of purified pyoluteorin at 10 μg/ml compared to the equivalent methanol control. The percentage of Gac⁻ mutants was estimated at different time points. Each experiment had least three replicates. Means ± standard deviations are shown.
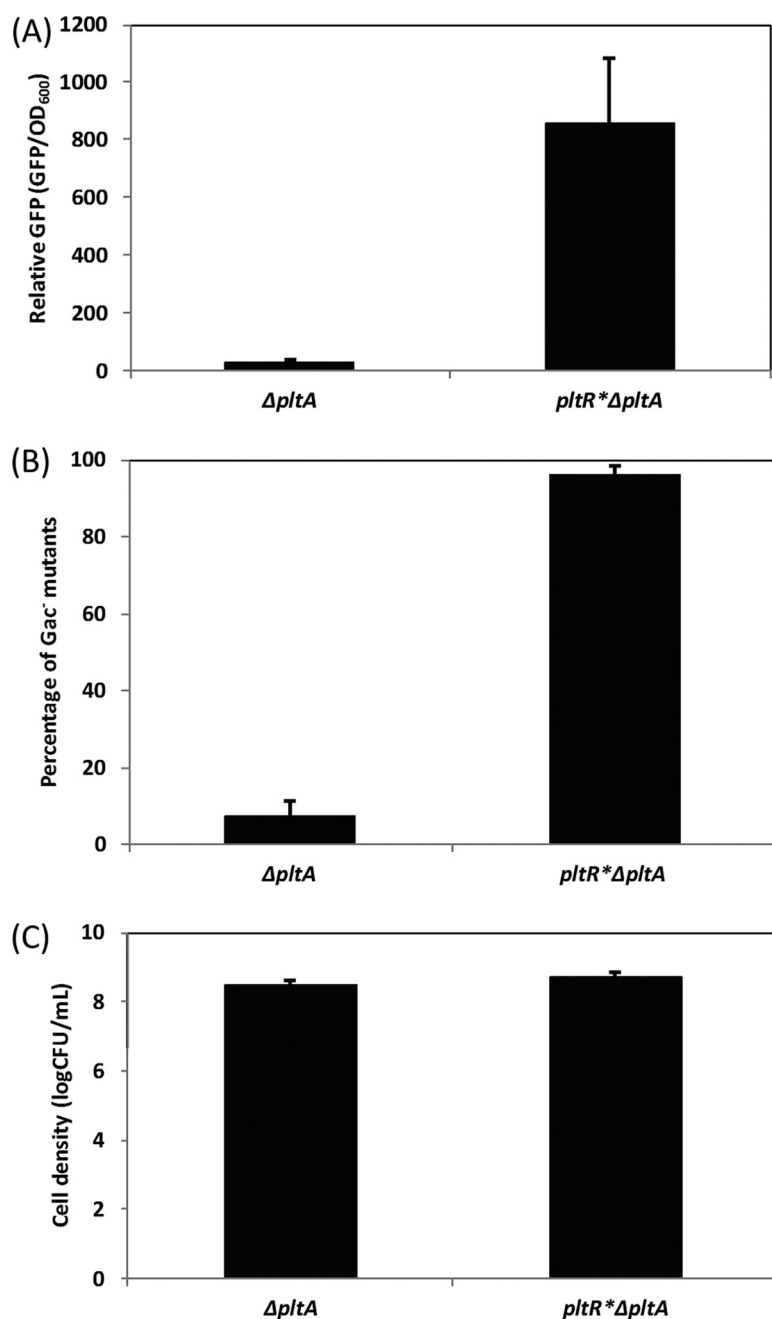
FIG 5 Effect of overexpression of the pyoluteorin biosynthetic genes on the accumulation of spontaneous Gac⁻ mutants of *P. protegens* Pf-5. *P. protegens* Pf-5 derivative Δ*pltA* and *pltR** Δ*pltA* strains were cultured in NYB. To test the expression level of pyoluteorin biosynthetic genes, plasmid ppltL-gfp containing a transcriptional fusion *pltL::gfp*, in which the promoter of *pltL* was fused with a promoterless *gfp* (11), was transformed into both strains, and the GFP level was assayed at 1 dpi (A). The accumulation of Gac⁻ mutants (B) and the total bacterial cell density (C) were recorded at 4 dpi. Each experiment had at least three replicates. Means ± standard deviations are shown.

cocultured on NYA and NYB. Our results showed that wild-type *P. protegens* Pf-5 strongly inhibited the growth of *B. subtilis* 168 in both solid and broth cultures (Fig. 6A and B). Importantly, a functional GacS-GacA system is essential for *P. protegens* Pf-5 to inhibit *B. subtilis* strain 168, as indicated by a strong inhibitory effect of the wild-type Pf-5, but a much lower effect of the Δ*gacA* mutant, on the growth of *B. subtilis* 168 (Fig. 6A and B). The role of the GacS-GacA system in the interspecific competition is further validated by the result that, relative to the single culture, the Δ*gacA* mutant had
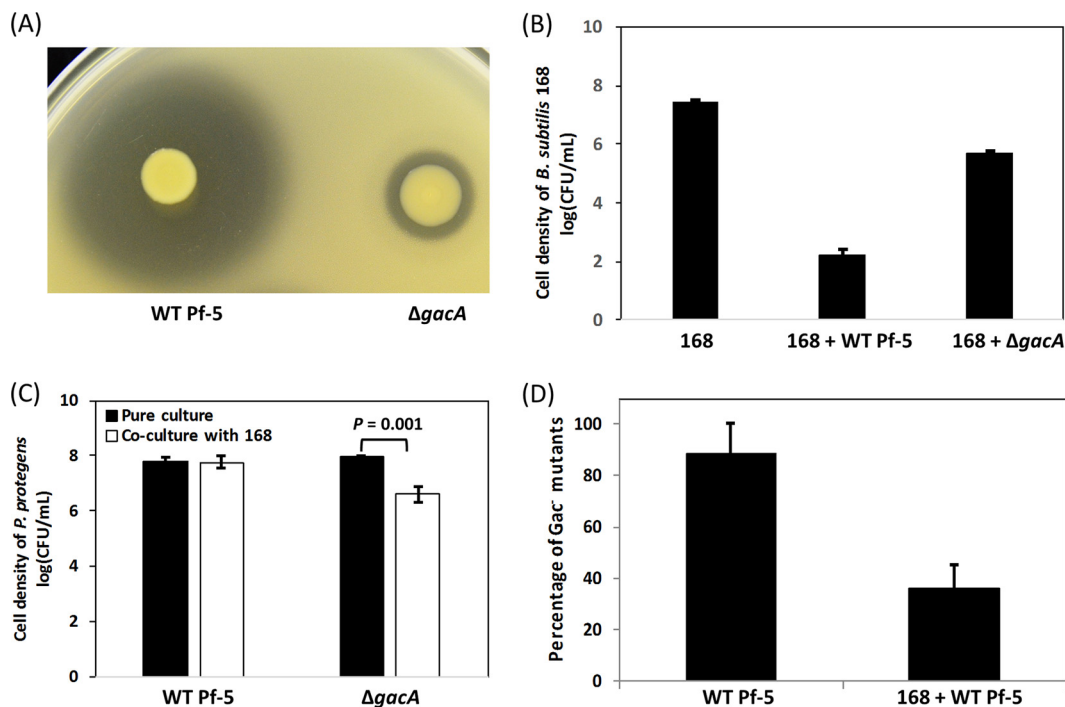
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FIG 6 Influence of interspecific competition on the accumulation of spontaneous Gac⁻ mutants of *P. protegens* Pf-5. Different inhibitory effects of the wild-type Pf-5 strain and the Δ*gacA* mutant of strain Pf-5 on the growth of *B. subtilis* strain 168 on NYA (A) and NYB (B). In panel B, strain 168 was inoculated in NYB or coinoculated with Pf-5 strains with an initial ratio 1:1 in NYB, and the cell density of strain 168 was tested at 6 dpi. (C) Cell density of *P. protegens* Pf-5 and the Δ*gacA* mutant grown in pure culture or in coculture with *B. subtilis* strain 168 in NYB at 6 dpi. *P* value was calculated by a Student's *t* test. (D) The percentage of Gac⁻ mutants that accumulated in the pure culture of wild-type Pf-5 or the coculture of wild-type Pf-5 and strain 168 in NYB at 6 dpi is shown. Each experiment had at least three replicates. Means ± standard deviations are shown.

reduced survival in cocultures with *B. subtilis* 168 (Fig. 6C). However, coculturing with *B. subtilis* 168 had no significant effect on the growth of wild-type Pf-5 (Fig. 6C).

We then compared the proportion of spontaneous Gac⁻ mutants that accumulated in pure cultures started with *P. protegens* Pf-5 to those in the cocultures started with equal densities of *P. protegens* Pf-5 and *B. subtilis* 168 to test whether the presence of a competitor influenced the accumulation of the mutants. Our data showed that coculturing with *B. subtilis* 168 reduced the proportion of Gac⁻ mutants of strain Pf-5 (36% on average) relative to pure cultures of strain Pf-5 (88% on average) (Fig. 6D).

Overall, these data show that a functional GacS-GacA system is important for strain Pf-5 to compete with *B. subtilis* and that interspecies competition led to a decreased accumulation of spontaneous Gac⁻ mutants of *P. protegens* Pf-5.

DISCUSSION

It has been known for decades that spontaneous Gac⁻ mutants accumulate in nutrient-rich cultures of some *Pseudomonas* spp. (17, 21, 22, 25, 26, 38–41), but the reasons for this phenomenon have remained obscure. In this study, we focused on *P. protegens* Pf-5, which accumulated a very high proportion of Gac⁻ mutants in NYB, outnumbering wild-type cells by at least 9:1 in 6-day cultures (Fig. 2A). *P. protegens* Pf-5 is also known for the production of a large number of secondary metabolites (32, 34, 42). The results of this study showed that secondary metabolism influences the accumulation of spontaneous Gac⁻ mutants in cultures of *P. protegens* Pf-5. The study also showed that secondary metabolism does not have a uniform role in the evolution of *P. protegens* Pf-5. Of six secondary metabolic pathways possessed by strain Pf-5, only two (pyoluteorin and orfamide A pathways) had a significant influence on the accumulation of Gac⁻ mutants, determined by evaluating a series of mutants deficient in production of one or six of the secondary metabolites. Rhizoxin and 2,4-diacetyl-

phloroglucinol were not produced to detectable levels in NYB medium, so it is possible that the production of these secondary metabolites could influence the proportion of Gac⁻ mutants of Pf-5 in a different medium. However, no Gac⁻ mutants were detected when strain Pf-5 was cultured in NBG, which is conductive to 2,4-diacetylphloroglucinol production (36), indicating that production of 2,4-diacetylphloroglucinol may not influence the accumulation of Gac⁻ mutants. Pyrrolnitrin was produced by strain Pf-5 on NYB (see Fig. S1C in the supplemental material), but the ΔprnC mutant did not differ from the wild type in the accumulation of Gac⁻ mutants (Fig. 3A). These results suggest that secondary metabolism does not have a uniform role in the accumulation of Gac⁻ mutants. Of the two secondary metabolites that influenced the accumulation of Gac⁻ mutants of Pf-5 in NYB medium, our study focused on pyoluteorin.

A mutant deficient in pyoluteorin biosynthesis accumulated spontaneous Gac⁻ mutations to a level that is significantly lower than that of the wild type (Fig. 3), so we initially hypothesized that the compound pyoluteorin enhances the accumulation of Gac⁻ mutants. Contrary to this expectation, the addition of purified pyoluteorin to cultures of a ΔpltA mutant or wild-type Pf-5 decreased the proportion of Gac⁻ mutants in the culture (Fig. 4). These results suggest that the process of pyoluteorin production, instead of the pyoluteorin compound, contributes to the accumulation of spontaneous Gac⁻ mutants. One possible explanation for these results is that the biosynthesis of pyoluteorin imposes a metabolic load on the bacterial cell such that Gac⁻ mutants, relieved of that metabolic load, have a fitness advantage over the wild type in culture. In support of this explanation, a derivative of P. protegens Pf-5 (pltR* ΔpltA mutant) that overexpressed the pyoluteorin biosynthesis genes but did not synthesize pyoluteorin, accumulated a high proportion of Gac⁻ mutants (Fig. 5B). Therefore, increasing the expression of pyoluteorin biosynthesis genes, which presumably increased the metabolic load on the cell, resulted in enhanced accumulation of the Gac⁻ mutants. These results support the idea that the fitness disadvantage of the wild type to Gac⁻ mutants in culture (Fig. 2) is due to the metabolic load imposed by pyoluteorin synthesis in the wild type, which is absent in the mutants.

We recognize that several alternative explanations for our results exist. For example, we evaluated the possibility that the Gac⁻ mutants and wild-type Pf-5 differ in sensitivity to pyoluteorin, which could impose a selective pressure on the relative abundance of the two genotypes; however, no detectable difference was observed in pyoluteorin sensitivities of Gac⁻ mutants versus wild-type Pf-5 (Fig. S3). It is also possible that intermediates accumulated during pyoluteorin biosynthesis (Fig. S4) could influence the relative fitness of Gac⁻ mutants versus wild-type cells in culture. Pyoluteorin is composed of a pyrrole moiety, which is synthesized via a nonribosomal peptide synthetase (NRPS), and a resorcinol ring synthesized by a polyketide synthase (PKS). Of the eight enzymes (PltLABCDEFG [Fig. S4A]) required for the biosynthesis of pyoluteorin (30), two (PltE and PltF [Fig. S4B]) synthesize the pyrrolyl-2-thioester on the peptidyl carrier protein PltL (43). Two chlorines are added to the pyrrolyl substrate on the carrier protein scaffold by the FADH₂-dependent halogenase PltA (44). The dichloropyrrolyl-S-PltL is the likely substrate for the PKS steps catalyzed by PltB and PltC, in which malonyl-coenzyme A (CoA) monomers are added to an elongating carbon chain, released from the PKS by the thioesterase PltG, and cyclized to form the resorcinol ring (30). The mutation of pltA used in this study is not expected to interfere with the expression of downstream genes because a promoter is predicted upstream of pltB (45). The PltA substrate is pyrrolyl-S-PltL (Fig. S4B), which is tethered to the peptidyl carrier protein so is unlikely to accumulate in the cytosol of the ΔpltA mutant (44). Therefore, it is unlikely that the pltA mutation causes an increased concentration of an intermediate that influences the accumulation of Gac⁻ mutants in cultures of Pf-5. In wild-type Pf-5, the dichlorinated pyrrole is proposed to be transferred from PltL to the acyl carrier protein domains in the polyketide synthases PltB and PltC. The dichlorinated pyrrole, which is present in wild-type Pf-5 and absent in the ΔpltA mutant, is not thought to be released into the cytosol (44), so it is unlikely to influence the proportion of Gac⁻ mutants in cultures of P. protegens Pf-5. On the basis of the current model for pyoluteorin

biosynthesis, it seems unlikely that a pathway intermediate is responsible for the accumulation of Gac⁻ mutants in cultures of strain Pf-5, but the model has not yet been fully evaluated *in vivo*. Therefore, we cannot exclude the possibility that an intermediate(s) in pyoluteorin biosynthesis, which is not present in the ΔpltA mutant, contributes to the differential fitness of Gac⁻ mutants and wild-type Pf-5 in NYB cultures. Our recent work also showed that the intermediates of secondary metabolic pathways can function as signals regulating gene expression of *P. protegens* (10, 36, 46). Future experiments are needed to investigate the possible contribution of intermediates in pyoluteorin biosynthesis to the accumulation of spontaneous Gac⁻ mutants.

Bacteria living in natural environments must compete with other organisms for nutrients and space, and competition is often a strong selective pressure influencing bacterial evolution. In this study, the proportion of spontaneous Gac⁻ mutants in cultures of *P. protegens* Pf-5 was higher in pure culture than in coculture with *B. subtilis* (Fig. 6B). From these results, we speculate that phenotype(s) positively regulated by GacS-GacA were required for *P. protegens* Pf-5 to compete successfully with *B. subtilis*. Indeed, strain Pf-5 inhibited growth of *B. subtilis* on NYA, whereas a ΔgacA mutant showed much less inhibition (Fig. 6A). Production of pyoluteorin, which is positively controlled by GacS-GacA (15), is required for full inhibition of *B. subtilis* (Fig. S5). Also, the population size of strain Pf-5 was similar in pure culture or coculture with *B. subtilis*, whereas the population size of a ΔgacA mutant was significantly higher in pure culture than in coculture with *B. subtilis* (Fig. 6C). Consistent with our results, it has been reported that a ΔgacA mutant of *P. aureofaciens* 30-84 has a competitive advantage against the wild type in sterile rhizosphere soil, but the wild type has a competitive advantage in natural rhizosphere soil, where the bacteria must compete with other organisms (22). Taken together, these results indicate that competitive pressure influences the evolution of these *Pseudomonas* species, favoring wild-type cells and reducing the proportion of spontaneous Gac⁻ mutants, which show reduced fitness in the presence of competing organisms.

We commonly observe colony variants that exhibit a Gac⁻ phenotype (expanded colony size, flat, increased fluorescence under UV light, orange color following many days of incubation) (21, 24) in 3- to 5-day agar cultures of *P. protegens* Pf-5. These observations suggest that Pf-5 cultures typically consist of subpopulations of the wild type and Gac⁻ mutants that vary in proportion under different conditions. Populations of *Pseudomonas* spp. in the rhizosphere also contain variants with gacS or gacA mutations (24, 47, 48). The Gac⁻ mutant subpopulation may dominate in nutrient-rich conditions, such as NYB evaluated in this study, to achieve a maximum proliferation of the whole population. The wild-type subpopulation may dominate in other conditions, such as in the presence of competitors when GacS-GacA is needed to optimize fitness. Given the known role of GacS-GacA in controlling diverse cellular traits, including motility, biofilm formation, and secondary metabolism (15, 16, 20), spontaneous mutation of gacS or gacA provides a mechanism to regulate bacterial adaption at both cellular and population levels.

In this study, medium composition (NYB versus NBG) and competition with *B. subtilis* influenced the predominance of Gac⁻ mutants in culture in a manner that supports the role of secondary metabolism as a primary phenotype influencing bacterial fitness. We recognize, however, that the influence of the GacS-GacA system reaches beyond secondary metabolism, controlling the expression of approximately 10% of the genes in the genome of *P. protegens* Pf-5 (15). Accordingly, different GacS-GacA-regulated phenotypes are likely to influence the relative fitness of Gac⁻ mutants versus wild type in different conditions. For example, the motility of strain Pf-5 is influenced by the GacS-GacA system (16), and the predominance of Gac⁻ mutants at the advancing edge of swarming colonies of *P. protegens* Pf-5 has been attributed to their hyperflagellation (17). Biofilm formation is also influenced by the GacS-GacA system (16), and Driscoll et al. (23) reported evidence for a mutualism between Gac⁻ mutant and wild-type cells of *P. chlororaphis* 30-84 in laboratory biofilms. Duffy and Défago (24) proposed that the osmotic potential of the medium influenced the proportion of Gac⁻ mutants in cultures

of *P. protegens* CHA0, recognizing that GacS-GacA influences the response of bacterial cells to environmental stress (20, 27). Secondary metabolism influenced the proportion of Gac⁻ mutants in cultures of *P. protegens* Pf-5 in this study, but other GacS-GacA-controlled phenotypes are likely to mediate the proportion of Gac⁻ mutant versus wild-type cells in other *Pseudomonas* species and in other environments.

P. protegens, *P. chlororaphis*, and other species in the *Pseudomonas fluorescens* group have characteristics, including their capacities to colonize plant surfaces and produce antifungal secondary metabolites, that make them well suited as biological control agents for the management of plant diseases (49–52). Bacteria in the *P. fluorescens* group are known to contribute to the disease suppressive characteristics of certain agricultural soils, and some strains have been developed as commercial products that can be applied to plant surfaces to suppress plant disease (53). The genetic instability of these bacteria is a concern in the development of biological control for use in agriculture: if Gac⁻ mutants predominate in cultures used to produce inoculum, biocontrol efficacy could be lost because secondary metabolites essential for their biological control activity are not produced (20, 21, 24). The results of this study substantiate previous conclusions that the emergence and accumulation of Gac⁻ mutants are context dependent (17, 24). Accordingly, media for inoculum development can be selected to minimize the possibility that Gac⁻ mutants will develop.

Secondary metabolite production is an adaptive characteristic subject to natural selection. It contributes to ecologic fitness of the producers (1, 2) but can be metabolically costly (7). Secondary metabolism in *Pseudomonas* spp. is controlled by intricate regulatory circuits that include pathway-specific and global regulators that respond to environmental cues and physiological status. For example, in addition to the positive regulation imposed by GacS-GacA (54) and the pathway-specific regulator PltR (30), synthesis of pyoluteorin is also kept in check by numerous negative regulators that operate at the transcriptional and posttranscriptional levels. Specifically, pyoluteorin production by *P. protegens* Pf-5 and other strains of *Pseudomonas* is moderated by the presence of nonpreferred codons in PltR (11), transcriptional repressor PltZ (55), RNA-binding protein RsmE (45), stationary-phase sigma factor RpoS (29), Lon protease (56), and phloroglucinol, an intermediate in the biosynthesis of 2,4-diacetylphloroglucinol (36, 46). The presence of multiple layers of negative regulation suggests that controlling pyoluteorin production to moderate levels is important for the fitness of the bacterial cell. When conditions allow relatively high levels of pyoluteorin production, such as the NYB cultures evaluated in this study, Gac⁻ mutants accumulate, thereby stopping secondary metabolism.

Overall, our data showed that *P. protegens* Pf-5 accumulates spontaneous Gac⁻ mutants in a nutrient-rich medium conducive to the synthesis of pyoluteorin and other secondary metabolites. We showed that secondary metabolism, specifically pyoluteorin synthesis, has an important role in the accumulation of Gac⁻ mutants. Furthermore, interspecific competition with *B. subtilis*, which requires a functional GacS-GacA system and its controlled secondary metabolism (pyoluteorin synthesis), influences the accumulation of Gac⁻ mutants. We propose that both the metabolic load and the fitness advantage associated with secondary metabolism are important factors contributing to the accumulation of Gac⁻ mutants. The results of this study support the contention that the accumulation of Gac⁻ mutants is an approach by which bacteria shut off secondary metabolism under conditions in which cells pay more in metabolic cost than gained in ecologic fitness to the population.

MATERIALS AND METHODS

Strains of *Pseudomonas* spp. and cultural conditions. Bacterial strains used in this study are listed in Table 2. Four species of *Pseudomonas* were evaluated for accumulation of spontaneous Gac⁻ mutants: *P. fluorescens* SS101 (57), *P. aeruginosa* PAO1 (58), *P. chlororaphis* 30-84 (50), and *P. protegens* Pf-5 (49). *Bacillus subtilis* strain 168 was evaluated for its impact on the growth and accumulation of Gac⁻ mutants of *P. protegens* Pf-5. All strains were stored at −80°C and grown in NYB (nutrient broth supplemented with 0.5% yeast extract), NYA (NYB with 1.5% agar), NBG (nutrient broth supplemented with 2% glucose), and/or KB (King's medium) (59). The bacteria were cultured at 27°C unless specifically indicated.

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TABLE 2 Bacterial strains, plasmids, and oligonucleotide primers used in this study

Bacterial strain, plasmid, or oligonucleotide primer	Description (genotype and/or relevant characteristics[a]) or sequence of oligonucleotide primer[b]	Reference or source
Bacterial strains		
P. protegens		
LK099	Wild-type Pf-5	49
LK147	6-fold mutant; contains mutations in *hcnB*, *ofaA*, *phlA*, *pltA*, *prnC*, and *rzxB* of strain Pf-5 described below; HCN⁻ Ofa⁻ DAPG⁻ MAPG⁻ Plt⁻ Prn⁻ Rzx⁻	35
JL4909	Δ*hcnB* mutant; contains a 239-bp deletion in *hcnB* of Pf-5; HCN⁻	52
JL4807	Δ*ofaA* mutant; contains 1,143-bp deletion in *ofaA* (PFL_2145) of Pf-5; contains FRT scar in deleted *ofaA* frame; Ofa⁻	15
LK023	Δ*phlA* mutant; contains a 639-bp deletion in *phlA* (PFL_5954) of Pf-5; MAPG⁻ DAPG⁻	36
JL4805	Δ*pltA* mutant; contains a 275-bp deletion in *pltA* (PFL_2787) of Pf-5; Plt⁻	60
LK415	*pltA*+ complemented mutant; contains a wild-type *pltA* gene replacing the mutated *pltA* in the chromosome of strain JL4805; Plt⁺	This study
JL4793	Δ*prnC* mutant; contains an 86-bp insertion of FRT site in *prnC* (PFL_3606) of Pf-5; Prn⁻	60
JL4808	Δ*rzxB* mutant; contains a 1,342-bp deletion in *rzxB* (PFL_2989) of Pf-5; Rzx⁻	60
LK298	*pltR** mutant; contains codon-modified *pltR* (PFL_2785) in the chromosome of Pf-5; overexpresses *plt* biosynthesis genes and overproduces pyoluteorin	11
LK417	*pltR** Δ*pltA* double mutant; contains a 275-bp deletion in *pltA* and the codon-modified *pltR** in the chromosome of Pf-5; overexpresses *plt* biosynthesis genes; Plt⁻	This study
JL4975	Δ*gacA* mutant; contains a 612-bp deletion in *gacA* (PFL_3563) of Pf-5; altered in the secondary metabolism and many other phenotypes regulated by GacA	60
P. fluorescens SS101	Wild-type strain	57
P. aeruginosa PAO1	Wild-type strain	58
P. chlororaphis 30-84	Wild-type strain	50
B. subtilis 168	Wild-type strain	61
Plasmids		
pEX18Tc 168	Gene replacement vector with MCS from pUC18; *sacB*⁺ Tcʳ	62
P18Tc-pltA	pEX18Tc containing wild-type *pltA* in a 1,359-bp BamHI fragment	This study
ppltL-gfp	pPROBE′-gfp (tagless) contains the intergenic region between *pltR* and *pltL*, including the promoter of *pltL* fused with a promoterless *gfp*	11
pEX18km-pltR-MCod3	pEX18Km with a 1,160-bp synthesized DNA fragment, containing *pltR* of Pf-5 with modifications in 35 types of rare codons	11
Oligonucleotide primers		
plt UpF-Bam	GTGTGGTAGT<u>GGATCC</u>TCCAGGACTGTCGAGCAAC	
plt DnR-Bam	GCAGAAGAGA<u>GGATCC</u>TACTTGTGCCAGAGGTGTTC	
gacA-seqF	CGGTCTTGCGGAAATAGCTG	
gacA-seqR	TAGGACCGTTATTGCGCCC	
gacS-5′F	CCAAGATCAGCCCCCGGCAA	
gacS-Reverse (1)	ATCCAGCTCCTGGCTGCCCA	
gacS-Middle Forward (2)	GCCGCACAATCAACAACCCGC	
gacS-Middle Reverse (2)	GCGCAGTTGCACGCTGTCTT	
gacS-Middle Forward (3)	CGCTGCGGCTCAAGCAGATTC	
gacS-Middle Reverse (3)	TCGACACACAGCACTCGCGG	
gacS-Forward (1)	CAGCCAGTTGCAGGCCAAGC	
gacS-3′R	AGCGCCGAGGAAACTCTCGC	

[a]Ofa, orfamide A; DAPG, 2,4-diacetylphloroglucinol; MAPG, monoacetylphloroglucinol; Plt, pyoluteorin; Prn, pyrrolnitrin; Rzx, rhizoxin analogs; HCN, hydrogen cyanide; Tox, toxoflavin; Smʳ, streptomycin resistance; Tpʳ, trimethoprim resistance; Tcʳ, tetracycline resistance; Kmʳ, kanamycin resistance; MCS, multiple-cloning site.
[b]Restriction sites used for cloning are underlined in oligonucleotide primer sequences.

Colonies of *Pseudomonas* spp. were transferred to LMA (litmus milk agar) to ensure that bacteria produced extracellular protease, indicating the integrity of the GacS-GacA regulatory systems (17, 27). The presence of a halo surrounding the colonies on the LMA plates means that the extracellular protease is produced and confirms the lack of mutations in the *gacS* or *gacA* gene. Strains were used in experiments only after their exoprotease production was confirmed.

To evaluate the accumulation of Gac⁻ mutants in cultures over time, bacteria were grown in culture tubes in NYB overnight with shaking (200 rpm). After that, optical densities (optical density at 600 nm [OD$_{600}$]) were measured, and cultures were adjusted to have equivalent starting OD$_{600}$ values. Then, 20 μl of the culture was transferred to 20 ml NYB in a 125-ml flask, which was incubated with shaking (200 rpm). For all strains tested, at least three replicate cultures were evaluated. At 1 day postinoculation (dpi) to 6 dpi, 10 μl was sampled from each flask and serially diluted, and dilutions were spread on KB and LMA plates. The numbers of CFU were counted on KB plates after 24-h incubation to estimate the population size in each flask. After 48-h incubation, the number of colonies with or without a halo on LMA was counted. Data were exported to the PAST software (University of Oslo, Norway), where an

analysis of variance (ANOVA) and Tukey pairwise comparison tests were used to analyze the existence of significant differences between the treatments.

Construction of Pf-5 derivative strains. To complement the ΔpltA mutant, the deleted pltA in the chromosome was repaired by the wild-type copy as follows. pltA was amplified from wild-type Pf-5 by using primers plt UpF-Bam/plt DnR-Bam (F stands for forward, R stands for reverse, and Dn stands for down) and then cloned into plasmid pEX18Tc, which is a suicide vector in Pf-5 (Table 2). The resultant plasmid, p18Tc-pltA, was then transformed into the ΔpltA mutant, and the repaired derivative strain was selected after two rounds of homologous recombination as described previously (11). The resultant pltA-repaired strain was identified by PCR and confirmed by sequence analysis.

To make the pltR* ΔpltA mutant, we used plasmid pEX18km-pltR-MCod3 (11), which contains pltR*, a derivative of pltR with all rare codons substituted with preferred synonymous codons. The approach described above was used to replace pltR with pltR* in the chromosome of the ΔpltA mutant.

Identification of the spontaneous mutation sites by PCR and DNA sequencing. P. protegens Pf-5 and three derivative strains (ΔofaA, ΔpltA, and 6-fold mutants) were grown in NYB as described above. At 4 dpi, 10 μl was sampled from each flask and serially diluted, and dilutions were spread on LMA agar. The plates were incubated for 2 days, and exoprotease-deficient colonies, identified by the lack of a halo on LMA, were selected randomly. Genomic DNA was isolated from six exoprotease-deficient colonies of each strain (two colonies from each of the three replicate flasks). The gacA and gacS ORFs and flanking sequences were amplified by using primers gacA-seqF/gacA-seqR, and gacS-5'F/gacS-3'R (Table 2), respectively. The amplified products were cleaned up using the ExoSAP-IT enzyme and submitted for Sanger sequencing. Primers gacA-seqF and gacA-seqR (seq stands for sequencing) were used to sequence the PCR products of the gacA gene. Multiple sequencing reactions and seven primers, including gacS-5'F, gacS-3'R, gacS-Reverse (1), gacS-Middle Forward (2), gacS-Middle Forward (3), gacS-Middle Reverse (3), gacS-Forward (1) (Table 2) were used to sequence the full length (2,754 bp) of gacS ORF. The DNA sequences were analyzed in the Geneious software (Newark, NJ) to detect single-nucleotide polymorphisms (SNPs), deletions or insertions in gacS or gacA.

Quantification of secondary metabolites. P. protegens Pf-5 and derivative strains were cultured in 20 ml NYB with shaking at 200 rpm as described above. Five milliliters of the culture were extracted twice with 2.5-ml ethyl acetate. The ethyl acetate extracts were dried under vacuum and solubilized in 100 μl methanol. A portion (10 μl) of the methanol solution was analyzed by high-performance liquid chromatography (HPLC) using an Agilent 1100 HPLC instrument consisting of a quaternary pump, autosampler, column heater (set at 30°C), and diode array detector. Separation was achieved using a Luna C_{18} column (4.6 mm by 150 mm; 5 μm; Phenomenex, Torrance, CA) with a flow rate of 1 ml/min with the following steps where solvent A was water plus 0.1% (vol/vol) formic acid, and solvent B was acetonitrile plus 0.1% (vol/vol) formic acid. The column was preequilibrated in 90% solvent A–10% solvent B, and upon injection, this composition was held for 2 min. The composition of the mobile phase was then changed to 0% solvent A–100% solvent B for 28 min utilizing a linear gradient. This composition was held for 6 min and then changed to 90% solvent A–10% solvent B for 2 min. The column was equilibrated in 90% solvent A–10% solvent B for 6 min prior to the next injection. Under these chromatographic conditions, pyoluteorin eluted at 15.1 min. The HPLC was operated with and data were viewed using ChemStation (version B.04.03; Agilent, Santa Clara, CA). Quantification was performed by integrating the area under the curve at 300 nm and comparing to a standard curve prepared by injection of purified pyoluteorin, 2,4-diacetylphloroglucinol, monoacetylphloroglucinol, orfamide A, rhizoxin WF-1360 F (the predominant rhizoxin analogue produced by P. protegens Pf-5), and pyrrolnitrin. Data were processed with GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

Transcriptional expression assay using a green fluorescent protein (GFP) reporter. To test the expression level of pyoluteorin biosynthetic genes, a gfp-based reporter plasmid ppltL-gfp (11) was transformed into P. protegens Pf-5 and its derivatives. The plasmid ppltL-gfp contains a promoterless gfp fused with the promoter of pltL, which is the first gene in the pyoluteorin biosynthetic gene cluster.

P. protegens Pf-5 strains containing ppltL-gfp were cultured overnight in NYB with kanamycin (50 μg/ml) with shaking at 200 rpm. The cells were washed once with NYB and inoculated in triplicate into 200 μl NYB in wells of a 96-well plate to a final optical density (OD_{600}) of 0.01. The plate was incubated in a 96-well plate reader (Tecan Infinite 200 Pro; Männedorf, Switzerland) with shaking at approximately 200 rpm. Growth of the bacteria was monitored by measuring the OD_{600}. The green fluorescence of bacteria was monitored by measuring the emission wavelength at 535 nm with an excitation wavelength at 485 nm and corrected for background by subtracting fluorescence emitted by the growth medium.

Assay for sensitivity to pyoluteorin. Wild-type Pf-5 and the ΔgacA mutant were cultured in NYB for 24 h with shaking (200 rpm). Cells were washed one time and suspended in fresh NYB broth to an OD_{600} of 1.0. The bacterial suspensions were diluted 1:100 vol/vol into melted, warm (45°C) NYA medium before pouring the medium into petri plates. The solidified agar was air dried for 1 h before use in the sensitivity assay. Purified pyoluteorin was dissolved in methanol to concentrations ranging from 20 μg/ml to 20 mg/ml. Three microliters of the pyoluteorin solution was placed on a sterilized filter paper disk (10-mm diameter). Dried filter paper disks were placed on the top of NYA seeded with bacterial cells of P. protegens Pf-5 and derivatives. The plates were incubated at 27°C. Results were recorded at 24 h after incubation. At least four replicate plates were evaluated in these experiments.

Assay for inhibition of P. protegens Pf-5 against B. subtilis strain 168. P. protegens Pf-5 (wild type and mutants) and B. subtilis 168 were cultured individually in NYB for 24 h. Cells were washed one time and suspended in fresh NYB broth to an OD_{600} at 1.0.

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To test the inhibitory effect of *P. protegens* Pf-5 and its derivatives on the growth of *B. subtilis* 168 on agar plates, the washed cell suspensions of *B. subtilis* 168 were diluted 1:100 (vol/vol) into melted, warm (45°C) NYA before the medium was poured into petri plates. The solidified agar was air dried for 1 h before use. Three-microliter portions of the washed wild-type Pf-5 or Δ*gacA*, Δ*pltA*, or *pltR** mutant cell suspensions were spotted onto the solidified plates. The plates were incubated at 27°C and imaged at 4 dpi. To test the inhibitory effect of *P. protegens* Pf-5 and its derivatives on the growth of *B. subtilis* 168 in liquid cultures, washed cells of *B. subtilis* 168 were inoculated alone or coinoculated (1:1) with strain Pf-5 (wild type or Δ*gacA* mutant) in NYB to an OD_{600} at 0.01. The bacterial cells were sampled at 6 dpi and diluted on NYA plates. The plates were incubated at 40°C which allows only the growth of *B. subtilis* 168 but not *P. protegens* Pf-5. The colonies were counted after 2-day incubation. At least three biological replicates were used in these experiments.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio .01845-17.

FIG S1, TIF file, 0.3 MB.
FIG S2, TIF file, 0.1 MB.
FIG S3, TIF file, 0.2 MB.
FIG S4, TIF file, 0.1 MB.
FIG S5, TIF file, 3.3 MB.

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