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Pleiotropic Effects of GacA on *Pseudomonas fluorescens* Pf0-1 *In Vitro* and in Soil

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***Pseudomonas* species can exhibit phenotypic variation resulting from *gacS* or *gacA* mutation. *P. fluorescens* Pf0-1 is a *gacA* mutant and exhibits pleiotropic changes following the introduction of a functional allele. GacA enhances biofilm development while reducing dissemination in soil, suggesting that alternative Gac phenotypes enable *Pseudomonas* sp. to exploit varied environments.**

Two-component regulators, consisting of a sensor kinase and response regulator pair, mediate bacterial adaptation to environmental perturbations. The GacS/GacA system in *Pseudomonas* spp., with orthologs spread across diverse members of the class *Gammaproteobacteria*, is a key regulator of environmentally relevant phenotypes, including motility, biofilm formation, and secondary-metabolite production. Despite the role of Gac in important and varied phenotypes, mutations in *gacS* or *gacA* arise with high frequency in a range of pseudomonads both in laboratory culture and in natural environments (1–5). The frequency with which such mutations arise suggests positive selection for loss of Gac, perhaps because of a reduced metabolic load compared to that of Gac^+ species. Consistent with this hypothesis, Gac^- members of the population often have a growth advantage over their Gac^+ counterparts and spontaneous Gac-deficient mutants become the majority population following extended growth in nutrient-rich laboratory cultures (4, 6). Gac-deficient mutants are additionally found at high frequency in the rhizosphere; however, Gac^- mutants do not displace their wild-type counterparts in nature. On the contrary, wild-type populations can benefit from the presence of Gac-deficient neighbors in mixed populations. For example, mixed-strain biofilms consisting of Gac^+ and Gac^- populations are more robust than those with the Gac^+ wild type alone. In addition, Gac^- strains are more likely to be found spatially near Gac^+ neighbors, perhaps allowing exogenous "common good" metabolites produced by the wild type to be utilized by Gac-deficient members of the community (3). Such apparent mutualism may select for the maintenance of mixed-Gac populations in natural environments.

Mutations in *gacS* and *gacA* arise at high frequency in culture and soil and on plant roots (1, 2, 4). The characterization of such mutants reveals point mutations, small insertions and deletions, larger insertions of greater than 200 bp, and reversible mechanisms of phenotypic switching (5, 7). It was recently reported (8) that heterologous expression of the *Pseudomonas protegens* Pf-5 *gacA* allele in Pf0-1 induces Gac-controlled phenotypes, including swarming motility, exoprotease and chitinase activities, and hydrogen cyanide and biosurfactant production. These results suggest that Pf0-1, often used as a model of pseudomonad behavior in soil, is Gac deficient. Indeed, *in silico* analysis reveals an apparent N109P substitution within the signal receiver domain of Pf0-1 GacA, perhaps rendering the protein nonfunctional. DNA sequencing of the *gacA* region reveals that the N109P substitution is present in all of the Pf0-1 strains tested, including archived stocks spanning 2 decades and those collected from several research groups. Thus, we sought to generate Pf0-1 strains with a functional *gacA* allele and define Gac-related phenotypes exhibited by Pf0-1 *in vitro* and in the soil environment. We additionally explored the ability of Gac^+ cells to compensate for Gac-deficient neighbors in mixed populations. Given the extensive use of the reference strain Pf0-1 in models of environmental persistence, biofilm formation, and biocontrol, the influence of the *gacA* mutation on the observed phenotypes will be crucial to consider in future research.

A point mutation in *gacA* renders the Gac system of *P. fluorescens* Pf0-1 nonfunctional. Site-specific mutagenesis was used to generate a P109N amino acid change within the Pf0-1 *gacA* allele on a plasmid via overlap extension PCR (9) with primers gacAMUT1 and gacAMUT4 (Table 1). The engineered plasmid, designated pSS115 (Table 1), includes an approximately 1,800-bp DNA fragment spanning the GacA coding region and its native promoter. The introduction of pSS115 into Pf0-1 elicited the onset of multiple Gac-associated phenotypes not typical of the parent strain, including profound changes in motility, antifungal activity, and biofilm development (Fig. 1 and 2; also described below). To generate a Pf0-1 strain with a stable *gacA* allele and to eliminate multicopy effects, the Pf-5 *gacA* gene under the control of its native chromosomal promoter was cloned into the mini-Tn*7* element carried by pHRB2 (Table 1) and introduced into the chromosome of Pf0-1 as described previously (10). Correct integration of the transposon into the chromosomal *glmS* locus was verified by PCR (11), and DNA sequencing confirmed the *gacA* sequence of the final strain. This strain, designated Pf0-$gacA^+$ (Table 1), shows motility and biofilm phenotypes identical to those of the Pf0-1 strain harboring pSS115 (not shown) and was used for all additional experiments. Pf0-$gacA^+$ exhibits a range of phenotypes consistent with proper GacS/GacA function; however, it is important to note that the strain harbors two *gacA* alleles—the

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TABLE 1 Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description or sequence[a]	Source or reference
P. fluorescens strains		
Pf0-1	Parental strain, Ap^r	37
Pf0-2X	Pf0-1 *adnA*::ΩSmSp^r; nonmotile mutant	18
Pf0-2X *gacA*$^+$	Pf0-2X::mini-Tn*7* *gacA* Km^r	This study
Pf0-*gacA*$^+$	Pf0-1::mini-Tn*7* *gacA* Km^r	This study
Pf0-*gacA*$^+$ *hcn*$^-$	Pf0-1 *hcnAB*-ΩSmSp^r mini-Tn*7* *gacA* Km^r	This study
Pf0-*gacA*$^+$ *aprA*$^-$	Pf0-1 *aprA*-ΩSmSp^r mini-Tn*7* *gacA* Km^r	This study
Pf0-*gacA*$^+$ 2211ΔS	Pf0-1 partial Δ2211-ΩSmSp^r mini-Tn*7* *gacA* Km^r	This study
Plasmids		
pJEL5965	*gacA* gene and promoter region from Pf-5 cloned into pME6000	8
pHRP315	pMB1 origin, Ap^r, source of ΩSmSp^r cassette	38
pSR47S	Suicide vector, R6K origin, *sacB*, Km^r	39
pBBRMCS2	Broad-host-range cloning vector, Km^r	40
pHRB2	Km^r carried on mini-Tn*7* element	10
pSS100	*gacA* from Pf-5 cloned into mini-Tn*7* Km^r element of pHRB2	This study
pSS102	pSR47S with Pf0-1 *hcnAB* fragment (3995538–3996732) interrupted by ΩSmSp^r	This study
pSS104	pSR47S with Pf0-1 *aprA* fragment (3078319–3079638) interrupted by ΩSmSp^r	This study
pSS110	pSR47S with Pf01 Pfl_2211ΔΩSmSp^r fragment	This study
pSS115	Pf0-1 *gacA*(P109N) allele cloned into pBBRMCS2	This study
Primers		
gacAMUT1	GAT GGA CAC AGG CGC CAC CC	
gacAMUT2	GAA GGG TGC CGG ATT G**AA C**GA AAT GGT TCA GGC C	
gacAMUT3	GGC CTG AAC CAT TTC **GTT** CAA TCC GGC ACC CTT C	
gacAMUT4	GAG CCG AGG CAC CGT TCA CC	
AprA_1	TTC CAG CTG AAC GCG GCT CCC T	
AprA_2	GCC GCC CGA TGA CGT CAG AAC C	
Hcn_1	AGT TGC GTG CGC GGG ATG AA	
Hcn_2	ACG AGC CGG CGA GGT GTA GT	
2211_1	TAC CAG CAC CGT CCC GAG CCG	
2211_2	GGG ATT CAG CCG CTA CCG CG	
2211_3	GAT CTG TGT CGA ACG CAG CCT GG	
2211_4	GGT TGG TCC GGT TCC AGT GTC	

[a] Ap^r, ampicillin resistant; $Sm Sp^r$, spectinomycin and streptomycin resistant; Km^r, kanamycin resistant. Nucleotides in bold are those that were engineered to introduce the desired mutation.

inactive allele at the native locus and the allele from Pf-5 at a second site—and thus may show *gacA* expression different from that of a true wild-type strain.

GacA alters Pf0-1 motility and antifungal activity. The GacS/GacA regulatory cascade directs motility in diverse bacterial genera (12, 13); however, specific effects vary from organism to organism. The *P. protegens* Pf-5 Gac system, for instance, is required for swarming but does not significantly alter swimming motility (13). Swimming by *P. fluorescens* F113, however, is under the negative control of the Gac system, and mutants lacking *gacS* or *gacA* produce larger swimming halos than the wild type (14, 15). We assessed the role of *gacA* in Pf0-1 swimming and swarming motility by using a minimal-salts medium solidified with 0.3 and 0.6% agar, respectively. Pf0-*gacA*$^+$ exhibits enhanced swarming compared to that of the Pf0-1 parent (Fig. 1A). Increased swarming is abrogated by the deletion of a gene (*Pfl01_2211*) that encodes a nonribosomal peptide synthetase (NRPS) similar to that which specifies orfamide production in *P. protegens* Pf-5, confirming that its *gac*-dependent swarming is associated with the production of an orfamide-like cyclic lipopeptide (CLP). Swimming motility is also altered in the Pf0-*gacA*$^+$ strain, with swimming halos showing an irregular shape (Fig. 1B) rather than the concentric-ring pattern exhibited by the Pf0-1 parent (16–18). While the overall sizes of the swimming halo of Pf0-1 and Pf0-*gacA*$^+$ are similar, excretion of a surfactant ring (Fig. 1B) ahead of the cellular front is apparent in Pf0-*gacA*$^+$ and absent from the parent strain, as well as the strain with NRPS-encoding *Pfl01_2211* deleted (Fig. 1B).

P. fluorescens strains produce an array of secondary metabolites with antibiotic activity, many of which are under the control of GacS/GacA. In the case of Pf0-1, biosynthetic genes for many of the major antibiotics (phenazine, 2,4-diacetylphloroglucinol, pyrrolnitrin) are absent and the organism exhibits little *in vitro* antibiotic activity. Because the GacA/GacS system is closely linked to antibiosis in related pseudomonads, we tested whether *gacA* promotes antibiosis in Pf0-1. An *in vitro* assay was used to assess the activity of Pf0-1 and Pf0-*gacA*$^+$ against the phytopathogenic fungus *Rhizoctonia solani*. The introduction of *gacA* increased the ability of Pf0-1 to inhibit the growth of fungal hyphae, compared to the parent (Fig. 1C). To pinpoint the *gac*-dependent antifungal metabolite(s) induced in Pf0-*gacA*, genes known for their antibiotic activity were inactivated, including *hcnA* (necessary for hydrogen cyanide production), *aprA* (encodes an extracellular protease), and *Pfl01_2211* (required for CLP production, as described above). Genes of interest and flanking DNA regions were PCR amplified, interrupted via the insertion of an antibiotic resistance cassette, and cloned into the suicide vector pSR47S. Mutated al-

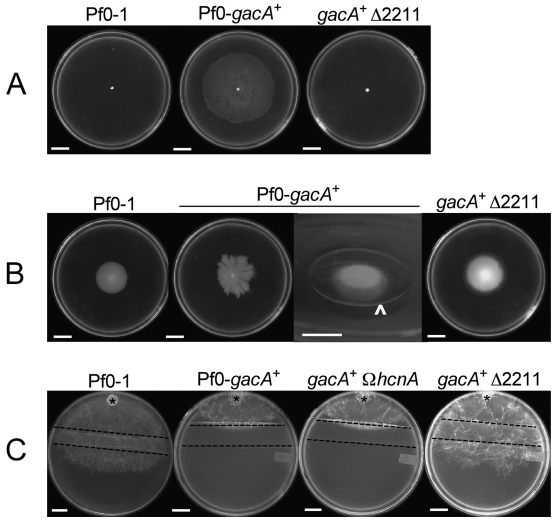

FIG 1 Motility and antifungal activity. (A) The impact of *gacA*- and *gacA*-dependent CLP production on Pf0-1 swarming (A) and swimming (B) motility was assessed on 0.6 and 0.3% agar, respectively. In all cases, the size marker indicates 1 cm and the arrowhead in panel B delineates a surfactant front seen only in Pf0-*gacA*$^+$. (C) An *in vitro* assay was used to assess the antifungal activity of Pf0-1 and its derivatives. A plug of agar containing *R. solani* (*) was placed adjacent to the strain of interest (between the dashed lines), and the extent of hyphal growth was used as an indirect measure of antifungal activity. Photos were taken following 4 days of fungal growth.

leles cloned into pSR47s were transferred to *P. fluorescens* Pf0-1 by conjugation as previously described (19), and subsequent allelic exchange generated Pf0-*gacA*$^+$ strains that do not produce hydrogen cyanide, AprA protease, or the orfamide-like CLP (Table 1). An *in vitro* antifungal assay revealed that Pf0-*gacA*$^+$ still inhibits fungal growth in the absence of *hcnA* (Fig. 1C) and *aprA* (not shown), indicating that these products are not essential for *in vitro* activity against *Rhizoctonia*. However, antifungal activity is reduced in the *Pfl01_2211* mutant, highlighting a role for *gac*-dependent CLP production in antibiosis under our *in vitro* conditions (Fig. 1C). Although the absence of *Pfl01_2211* renders Pf0-*gacA*$^+$ less able to inhibit *R. solani*, the altered appearance of the mycelium compared to that seen in the presence of parental Pf0-1 suggests the production of at least one compound that interferes with fungal growth. It is likely that a cocktail of Gac-controlled factors, including as-yet-unidentified compounds, are produced *in situ* to thwart competitors. The construction of a Gac$^+$ variant of Pf0-1 thus provides a new avenue for the discovery of novel antifungal metabolites by approaches such as the genomisotopic method used successfully with Pf-5 (20).

Effects of GacA on Pf0-1 growth and colonization of soil. To probe the role of *gacA* in the environmental fitness of Pf0-1, we assessed the abilities of Pf0-1 and Pf0-*gacA*$^+$ to persist and compete in soil. Soil persistence assays were done essentially as described previously (19), with an irradiated sandy loam soil of known composition (17). Five-gram soil columns were inoculated with ~200 CFU of Pf0-1 (marked with SpSm^r) or Pf0-*gacA*$^+$ (marked with Km^r), and viable bacteria were monitored for 30 days. Despite the growth advantage of the *gac*-deficient parent strain in laboratory medium (Fig. 2A), the growth of the two strains was indistinguishable in soil (Fig. 2B). Similarly, competitive fitness was assayed by using soil columns inoculated with a 1:1 mixture of Pf0-1 and Pf0-*gacA*$^+$. No significant difference in fitness during head-to-head competition under the conditions tested was observed (Fig. 2C). The role of *gacA* in survival in natural environments varies greatly, depending on the specific test conditions. Some reports indicate a selective advantage for Gac$^+$ populations, especially in the presence of competing indigenous rhizosphere microbiota (2, 21). Others report a neutral (15, 22, 23) or even negative effect (15), suggesting that the Gac regulon controls traits that are beneficial under specific environmental conditions or within a particular ecological niche. Here, we assessed colonization potential in an irradiated loam soil, thus eliminating factors such as competition with indigenous bacterial populations, predation by nematodes and amoeba, and rhizosphere-dependent factors. Thus, while we see no defects in the ability of *gac*-deficient Pf0-1 to colonize or persist, we acknowledge that the GacA/GacS system may offer a selective advantage in more complex environments.

An inverse relationship between biofilm formation and dissemination in soil. A role for the Gac system in the modulation of

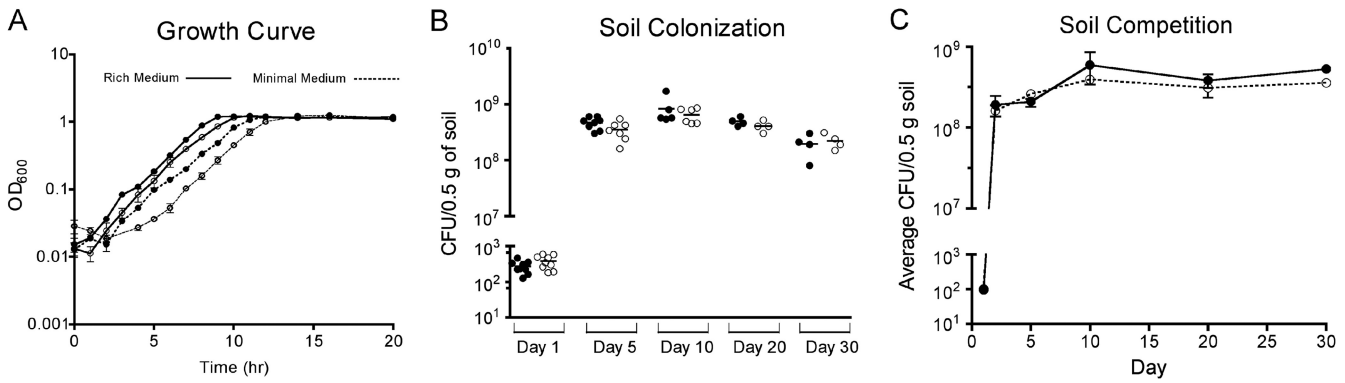

FIG 2 Growth and competition in laboratory medium and soil. (A) Growth curves of Pf0-1 and Pf0-*gacA*$^+$ in rich medium (solid line) and a defined minimal medium (dashed line). (B) Pf0-1 and Pf0-*gacA*$^+$ were inoculated into soil, and viable bacteria were monitored over a 30-day period. Data points represent individual soil columns, and horizontal lines represent median values. Differences between the parent and the Gac$^+$ derivative are not statistically significant ($P >$ 0.05 for all time points), as calculated with the Mann-Whitney nonparametric statistical hypothesis test. For competition assays (C), a 1:1 ratio of each strain was used to inoculate a single soil column. Cell numbers for each strain were assessed over a 30-day period, and average CFU counts are plotted. In all three panels, Pf0-1 and Pf0-*gacA*$^+$ data points are represented as closed and open circles, respectively.

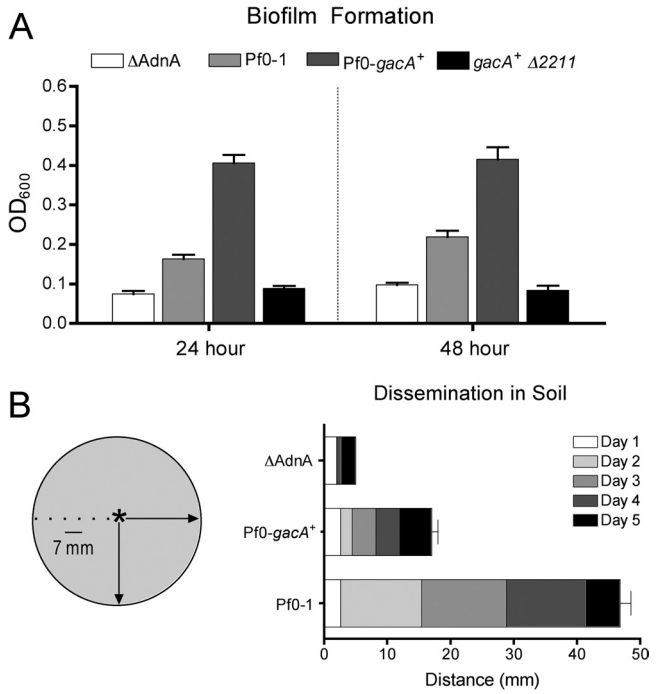

FIG 3 GacA enhances biofilm formation and decreases soil dissemination. (A) The impact of GacA on biofilm formation was assessed on borosilicate glass by the staining method described by O'Toole and Kolter (21). Biofilm data are reported as the average of 8 to 12 replicates. (B) The motility of Pf0-1 and Pf0-*gacA*$^+$ was assessed in soil microcosms. Radial dissemination from a central inoculation point was measured daily over a 5-day time course. A nonflagellated mutant lacking ΔAdnA is known to have a defect in dissemination in the soil environment (14) and was thus used as a negative control. Results are the average of four to six independent microcosms for each strain tested, with duplicate samples taken at each time point as an internal replicate.

biofilm formation has been reported in several *Pseudomonas* spp. In *P. aeruginosa* PA14 and *P. fluorescens* F113, a defect in *gacA* leads to 10- and 5-fold reductions in biofilm density, respectively (23, 24). *P. fluorescens* Pf0-1 has been used as a model of biofilm development, and multiple factors have been identified as important for biofilm formation by this strain, including the *lap* genes, *sadB*, and *pstC*, among others (10, 17, 25). The discovery that Pf0-1 is a *gacA* mutant led us to investigate the potential role of the Gac system in biofilm formation by this strain. Biofilm formation was assessed on borosilicate glass by the method of O'Toole and Kolter (26). Briefly, *P. fluorescens* strains were grown to mid-log phase, diluted to an optical density at 600 nm (OD_{600}) of 0.025 in *Pseudomonas* minimal medium (PMM) supplemented with 0.2% glucose, and incubated at 30°C without shaking. Biofilm density was measured at 24 and 48 h by crystal violet staining as described previously (17, 18). Despite the array of *gac*-independent factors known to play a role in Pf0-1 biofilm formation, our results indicate that the ability of Pf0-1 to form a biofilm is enhanced by the presence of functional *gacA* (Fig. 3A). As Gac-dependent CLP production is linked to biofilm formation by *Pseudomonas* strains (27, 28), we additionally assessed biofilm density in the *gacA*$^+$ derivative harboring the *Pfl01_2211* deletion. Indeed, the enhanced-biofilm phenotype exhibited by Pf0-*gacA*$^+$ is not apparent in a strain deficient in CLP production (Fig. 3A), indicating that the *gac*-dependent increase in biofilm formation is due, at least in part, to the production of the orfamide-like CLP.

Our *in vitro* assays show that in Pf0-1, GacA enhances both motility and biofilm formation. In contrast, others have suggested that phenotypic switching through Gac mutation may allow bacteria to oscillate between motile and biofilm behaviors, perhaps suggesting a transition between exploratory and plant-associated lifestyles. To test this hypothesis for Pf0-1 in a nonlaboratory environment, we assessed the abilities of Pf0-1 and Pf0-*gacA*$^+$ to disseminate in natural soil. A modification of an assay described previously (29) was developed to measure the dissemination of *P. fluorescens* strains away from a central inoculation point in soil microcosms. Microcosms were constructed in 150-mm-diameter petri dishes and consisted of 100-g soil samples wetted to ~50% of their water-holding capacity. Mid-log-phase cultures of Pf0-1, Pf0-*gacA*$^+$, and a nonflagellated mutant (ΔAdnA) were standardized to an OD_{600} of 0.1, and 20 μl was dispensed into the center of the microcosm. Dissemination from the inoculation point was measured for 5 days with a sterilized sampling device with eight glass capillary tubes 7 mm apart. The results reveal that the rate of dissemination in soil is inversely correlated with functional *gacA* (Fig. 3B), despite the fact that Pf0-*gacA*$^+$ exhibits increased swarming motility *in vitro*.

Motility is an important trait for rhizosphere colonization, but bacterial dissemination in natural environments is mediated by complex factors both intrinsic (motility, adherence to soil and plant matter, chemotaxis) and external (soil type, packing, moisture content, rainfall). Few studies have addressed a link between motility *in vitro* and dissemination in soil. Of note, Natsch and colleagues (30) report that a *gac*-deficient mutant of *P. fluorescens* CHA0 shows decreased translocation in a vertical soil column and is less abundant than the wild type in percolated water collected from the column after simulated rainfall. However, such results are complicated by the fact that the mutant also exhibits decreased survival under the conditions tested, making it difficult to determine if the difference in translocation is linked to motility *per se* or merely to survival. Other studies have addressed the ability of Gac$^+$ and Gac$^-$ populations to adhere to sand, seeds, and roots (31, 32) but have done little to link this information to dissemination of the organism in natural soil. Our radial dissemination assay, while simplified, offers a link between motility phenotypes exhibited *in vitro* on agar and translocation in bulk soil. Results highlight the importance of swimming, but not swarming, in dissemination in soil microcosms and suggest that these two forms of motility are regulated differently by the GacS/GacA system. Our results are consistent with and extend those reported for F113, Gac-deficient mutants of which exhibit a hypermotile swimming phenotype *in vitro* and are more competitive than the wild-type strain in rhizosphere colonization (15). Of note, Martinez-Granero et al. (14) have suggested that the Gac-dependent repression of motility occurs during exponential growth, which is unlikely to be the physiological state of Pf0-1 in the soil dissemination assay. Our data suggest a more complex situation *in situ* than has been defined *in vitro*.

GacA-deficient strains can benefit from GacA$^+$ neighbors in mixed populations. Secreted public-good compounds can sometimes be used by members of a population that do not produce those compounds, for example, in the case of siderophore-negative *Pseudomonas* mutants that show increased survival in soil when wild-type, siderophore-producing neighbors are also present (33). Many Gac-controlled metabolites are extracellular products with the potential to be shared as public goods in heteroge-

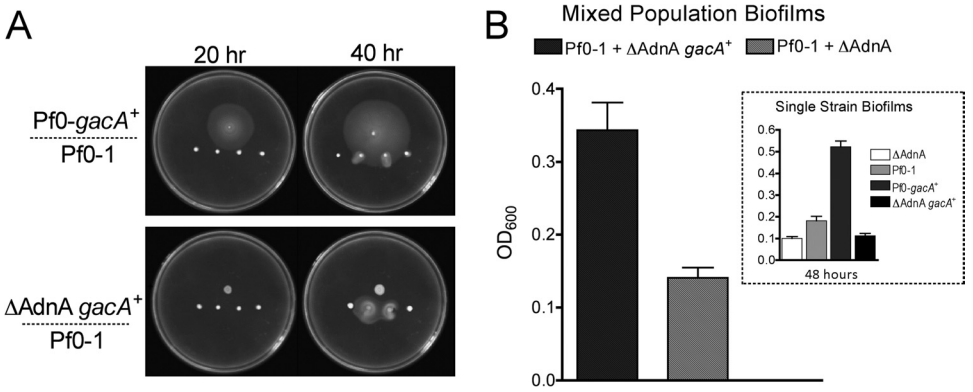

FIG 4 Phenotypes of mixed Gac populations. (A) Gac$^+$ strains induce swarming motility in nearby Gac$^-$ colonies, even when the Gac$^+$ strain is itself nonmotile (ΔAdnA *gacA*$^+$, bottom). Motility on PMM solidified with 0.6% agar following 20 (left) and 40 (right) h of incubation at 25°C is shown. (B) Biofilm formation in mixed Gac populations. The ΔAdnA *gacA*$^+$ mutant strain does not form a biofilm with or without a functional *gacA* allele (inset). However, the ΔAdnA *gacA*$^+$ mutant strain enhances biofilm formation when mixed with the *gac*-deficient Pf0-1 parent.

neous populations. Recent attention has therefore been dedicated to the ecology of mixed Gac populations, and interaction between Gac$^+$ and Gac$^-$ cells has, in some cases, proven mutually beneficial (3).

We tested whether exoproducts produced by Pf0-*gacA*$^+$ may alter the physiology of the *gac*-deficient parent in mixed populations. As described above, swarming motility is enhanced in Pf0-*gacA*$^+$ and is dependent on the production of an orfamide-like CLP. Interestingly, the presence of the Pf0-*gacA*$^+$ strain induces swarming in nearby GacA-deficient Pf0-1 colonies (Fig. 4A). This effect is also seen when Pf0-*gacA*$^+$ is replaced by a *gacA*$^+$ strain that lacks functional flagella (Fig. 4A), indicating that the induced motility is due to the diffusion of a secreted product and not some form of physical translocation of *gac*-deficient cells by the *gacA*$^+$ partner. We additionally assessed the impact of *gac*-dependent exoproducts in mixed-population biofilms. The experiment was designed as follows. A strain harboring functional *gacA* but lacking flagella (Pf0-*gacA*$^+$ *adnA*$^-$) was mixed 1:1 with the *gac*-deficient Pf0-1 parent strain, and biofilm formation was assessed as described above and elsewhere (18, 26). The former strain did not form robust biofilms (Fig. 4B, inset), presumably because of the absence of flagella, which aid in the physical attachment of bacteria to surfaces (18, 34). However, Pf0-*gacA*$^+$ *adnA*$^-$ is able to enhance biofilm production when mixed with the Pf0-1 parent (Fig. 4B), supporting the hypothesis that *gac*-dependent exoproducts can alter the physiology of Gac$^-$ derivatives in mixed populations.

Much remains to be understood about the ecology of mixed Gac populations; however, our data and those from other groups suggest that there are benefits to maintaining both Gac$^+$ and Gac$^-$ variants. Gac$^+$ cells, because of the production of antibiotics and exoproteases, kill or suppress competing organisms (35), are less susceptible to predation by nematodes and amoeba (21, 36), and are better able to form biofilms. Gac-deficient cells enjoy a reduced metabolic load and appear more motile in the soil environment, allowing faster proliferation and increased dissemination when local conditions become unfavorable. Populations showing both phenotypes, and in particular those able to undergo high-frequency phase variation (6), capitalize on the strengths of each strategy.

Conclusions. Phenotypic variation caused by mutation in *gacS* or *gacA* is an important driver of population heterogeneity in natural *Pseudomonas* populations, allowing the formation of specific subpopulations that may have increased success in the rhizosphere. Here, we confirm that the model fluorescent pseudomonad *P. fluorescens* Pf0-1 is a *gacA*-deficient variant. While this organism is well adapted to survival in natural soil, we observed the induction of multiple environmentally relevant phenotypes upon the introduction of a functional *gacA* allele. Thus, it is clear that the benefits of being Gac$^+$ or Gac$^-$ depend strongly on the ecological context, and the high rates of alteration of the GacA/GacS system observed in nature may represent an adaptive mechanism enabling soil pseudomonads to exploit and explore ever-changing environments.

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