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Rhizosphere Selection of Highly Motile Phenotypic Variants of *Pseudomonas fluorescens* with Enhanced Competitive Colonization Ability

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Phenotypic variants of *Pseudomonas fluorescens* F113 showing a translucent and diffuse colony morphology show enhanced colonization of the alfalfa rhizosphere. We have previously shown that in the biocontrol agent *P. fluorescens* F113, phenotypic variation is mediated by the activity of two site-specific recombinases, Sss and XerD. By overexpressing the genes encoding either of the recombinases, we have now generated a large number of variants (mutants) after selection either by prolonged laboratory cultivation or by rhizosphere passage. All the isolated variants were more motile than the wild-type strain and appear to contain mutations in the *gacA* and/or *gacS* gene. By disrupting these genes and complementation analysis, we have observed that the Gac system regulates swimming motility by a repression pathway. Variants isolated after selection by prolonged cultivation formed a single population with a swimming motility that was equal to the motility of *gac* mutants, being 150% more motile than the wild type. The motility phenotype of these variants was complemented by the cloned *gac* genes. Variants isolated after rhizosphere selection belonged to two different populations: one identical to the population isolated after prolonged cultivation and the other comprising variants that besides a *gac* mutation harbored additional mutations conferring higher motility. Our results show that *gac* mutations are selected both in the stationary phase and during rhizosphere colonization. The enhanced motility phenotype is in turn selected during rhizosphere colonization. Several of these highly motile variants were more competitive than the wild-type strain, displacing it from the root tip within 2 weeks.

Pseudomonas fluorescens F113 is a biocontrol agent isolated from the sugar beet rhizosphere (11) and capable of protecting this crop against the pathogenic fungus *Pythium ultimum* (17, 31). In addition, derivatives of this strain with the ability to degrade polychlorinated biphenyls have been constructed by the integration of the *Burkholderia* sp. strain LB400 *bph* operon under the control of different regulatory elements (4, 38). *P. fluorescens* F113 is a good rhizosphere colonizer and can colonize the rhizospheres of different plants such as alfalfa (37), tomato (33), and pea (23).

During alfalfa rhizosphere colonization, F113 undergoes phenotypic variation (27) characterized by the appearance of variants with a translucent and diffuse colony morphology. These variants were more prevalent in distal parts of the root (1, 27). Phenotypic variation in this strain appears to be mediated by the activity of two site-specific recombinases, Sss and XerD, since mutants with mutations in either of the genes encoding these recombinases show a severe reduction in the appearance of phenotypic variants after rhizosphere colonization and prolonged laboratory cultivation (22). Phenotypic variation seems to be an important trait for rhizosphere colonization, and mutants of different *Pseudomonas* strains affected in the *sss* (10, 22) or *xerD* (22) genes are severely impaired for competitive rhizosphere colonization. Furthermore, the introduction of additional copies of a cloned *sss* gene improves the colonization abilities of several *Pseudomonas* strains, including F113 (9).

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The overexpression of the *sss* or *xerD* genes in F113 results in an important increase in the production of phenotypic variants after prolonged cultivation in the laboratory or after rhizosphere passage (22). These variants harbored mutations in the *gacA* and/or the *gacS* genes (22) encoding a two-component system that regulates the production of multiple secondary metabolites (21), including some important for biocontrol, such as exoprotease (3, 32), pyoverdin (22), and hydrogen cyanide (26). Mutations in the Gac system have been shown to accumulate after prolonged cultivation of *P. fluorescens* CHA0 (15) and have been reported to be the basis of phenotypic (phase) variation in *Pseudomonas* sp. strain PCL1171 (35).

Motility is one of the most important traits for competitive rhizosphere colonization, and mutants incapable of chemotactic motility are among the most defective colonization mutants tested (13, 33). Even mutants that are still motile, but show decreases compared to the wild-type level of motility, are totally displaced from the root tip in competition experiments (6). Furthermore, in vivo transcription experiments have shown rhizosphere induction of the *fliO* gene (25), implicated in flagellum assembly.

In this study, we have generated a large number of phenotypic variants by overexpression of the genes encoding the site-specific recombinases and selection after prolonged laboratory cultivation and after rhizosphere colonization. Analysis of these variants has shown that enhanced motility is selected in the rhizosphere, while a *gac* mutant phenotype is selected both in the rhizosphere and in the stationary phase. We have also shown that overexpression of site-specific recombinases followed by a single rhizosphere passage allows the isolation of more competitive strains.

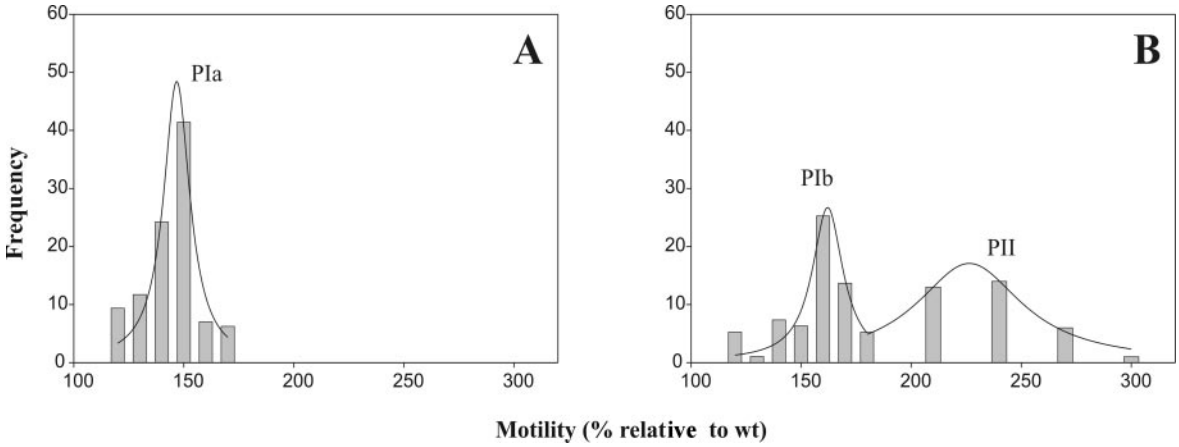

FIG. 1. Frequency distribution of motility phenotypes among phenotypic variants produced after overexpression of the *sss* or *xerD* gene and selection by prolonged cultivation in SA medium (A) or alfalfa rhizosphere passage (B). Swimming haloes in SA plates were measured and were assigned to frequency intervals. Bar diagrams were shown to fit a normal distribution for the three detected peaks, PIa, PIb, and PII. wt, wild type.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All the *Pseudomonas fluorescens* strains used here are derivatives of the biocontrol strain F113, which was isolated from the sugar beet rhizosphere (31). Plasmids were mobilized into *P. fluorescens* by triparental matings using pRK2013 as the helper plasmid (18).

P. fluorescens strains were grown in SA medium (29) overnight at 28°C; solid growth medium contained 1.5% (wt/vol) purified agar. *Escherichia coli* strains were grown overnight in Luria-Bertani (LB) medium (2) at 37°C. Site-specific recombinase genes were overexpressed under the control of the nptII promoter in the pFAJ1709 plasmid (14), which contains a tetracycline resistance gene. *gacA* and *gacS* mutants were obtained by single homologous recombination of amplified internal fragments from *gacA* and *gacS* genes cloned into the suicide vector p*K18mobsac* (28). For the prolonged laboratory growth experiments, bacteria were grown for 1 week in SA liquid medium supplemented with tetracycline shaking at 28°C, and the cultures were plated in SA tetracycline medium. The following antibiotics were used, when required, at the indicated concentrations: rifampin, 100 μg/ml; tetracycline, 10 μg/ml for *E. coli* or 70 μg/ml for *P. fluorescens*; and kanamycin, 25 μg/ml for *E. coli* or 50 μg/ml for *P. fluorescens*.

Rhizosphere colonization experiments. Alfalfa seeds were sterilized in 70% ethanol for 2 min and diluted bleach (1:5) for 15 min and rinsed thoroughly with sterile distilled water. Seed vernalization was performed at 4°C overnight, and germination was for 1 day at 28°C. Germinated alfalfa seeds were sown in Leonard jar gnotobiotic systems (37) using perlite as the solid substrate and 8 mM KNO_3-supplemented FP (16) as the mineral solution. After 2 days, alfalfa seeds were inoculated with ca. 10^8 cells of the appropriate strains. In competition experiments, strains were inoculated at a 1:1 ratio. Plants were maintained under controlled conditions (16 h in the light at 25°C and 8 h in the dark at 18°C) for 2 weeks. Bacteria were recovered from the rhizosphere by vortexing the root tips (last centimeter of the main root) for 2 min in a tube containing 5 ml of 0.9% NaCl and plating the appropriate dilutions on SA plates. Every experiment was performed three times with three replicates each time, and every replicate contained at least 20 plants.

Swimming assays. SA medium plates containing 0.3% purified agar were used to test swimming abilities. Swimming assays were done with variants obtained after independent overexpression of site-specific recombinases (*sss* and *xerD*) either after long laboratory culture conditions or after rhizosphere colonization. The selected variants were cured from the site-specific recombinase overexpression plasmids after several platings without selection. The swimming ability of these derivatives was tested in comparison with the wild-type strain. Complementation assays were done using the plasmid-cloned *gacA* gene from *Pseudomonas fluorescens* CHA0(pME3066) (21) (tetracycline resistant) and the *gacS* gene from *Pseudomonas syringae* (pEMH97) (19) (tetracycline resistant). A wild-type strain harboring the empty pFAJ1709 plasmid was used as a control. The cells from exponentially growing cultures were inoculated in the middle of the plate in triplicate using a toothpick. Swimming haloes were measured after 18, 24, and 42 h of inoculation. Every assay was done at least three times.

Statistical analysis. Statistical analysis was done with Sigma Plot 4 and SigmaStat 3.1 software. The Lorentzian (three parameters) equation was used to represent motility distribution, and the Kolmogorov-Smirnov test was used to determine the normality ($P > 0.05$) of the estimated underlying population.

RESULTS

Phenotypic variants of *P. fluorescens* F113 are hypermotile. Overexpression of the genes encoding the Sss and XerD site-specific recombinases in *P. fluorescens* F113 results in the generation of a large number of phenotypic variants after both prolonged laboratory cultivation and rhizosphere colonization (22). Plasmids pBG1457 (pnptII::*sss*) and pBG1442 (pnptII::*xerD*) (22) were independently introduced into strain F113 cells that were either cultivated in SA medium or applied to alfalfa seedlings. After 1 week of cultivation or 2 weeks of colonization, cells were plated on SA and phenotypic variants, characterized by a translucent colony morphology (27), were randomly picked. One hundred fifty variants from the culture experiments and 200 variants from the rhizosphere experiments were isolated and tested for swimming motility. All the isolated phenotypic variants showed enhanced motility when compared with the wild-type strain, ranging from 120% to 300% (considering 100% the motility of the wild-type strain). These results indicate that hypermotility is a general trait of phenotypic variants.

Rhizosphere selects hypermotile variants. Figure 1 shows the distribution of motility phenotypes among the variants isolated from liquid culture (Fig. 1A) and rhizosphere (Fig. 1B). All variants were classified in intervals according to their swimming halo diameter compared with the wild-type strain. Clear differences can be observed between variants isolated from liquid culture and those isolated from the rhizosphere. The variants isolated from liquid culture are all grouped in a narrow peak (PIa) indicating that they form a single population with an average motility of 150%. The variants isolated from the rhizosphere showed a wider distribution, and they are grouped in two peaks, suggesting the presence of more than one population. The first peak (PIb) is also narrow and overlaps with the peak observed in the variants isolated from liquid culture, suggesting that they represent the same population. The second peak observed in the variants isolated from the

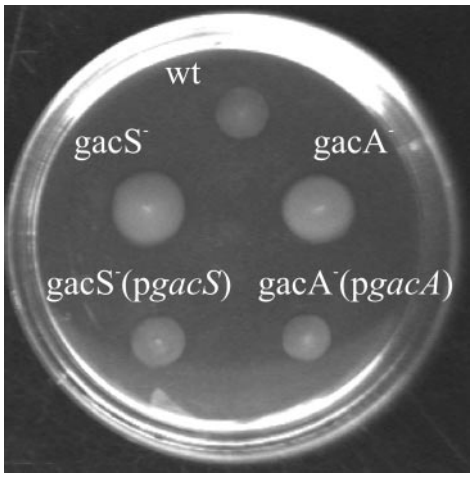

FIG. 2. Swimming motility haloes produced by *P. fluorescens* F113 and derivatives harboring insertion mutations in the *gacA* and *gacS* genes. Complementation analysis was performed with mutant strains containing the cloned *gac* genes (p*gacA*/*S*). The empty vectors (not shown) had no effect on halo formation. Cells were inoculated on SA plates and observed after 24 h. wt, wild type.

rhizosphere (PII) is wider and represents one or several populations not appearing in liquid culture. Statistical analysis showed that the frequencies within each of the peaks follow a normal distribution ($P = 0.187$ for peak PIa, $P = 0.153$ for peak PIb, and $P > 0.200$ for peak PII). The *t* test and chi-square test showed that the peak obtained from liquid culture is not different from the first peak obtained from rhizosphere ($P < 0.001$), but both peaks are significantly different from peak PII ($P < 0.001$).

Peaks PIa and PIb variants are Gac mutants. Since most variants presented a mutation in the *gacA*/*gacS* genes (22), F113 derivatives harboring mutations in either gene were constructed. As shown in Fig. 2, *gacA* and *gacS* mutants are more motile than the wild-type strain, forming a swimming halo of 147% compared with the 100% halo of the wild-type strain. Introduction of the cloned *gacA* and *gacS* genes complemented the hypermotility phenotype of the mutants, restoring motility to the wild-type level. These results clearly show that the Gac system modulates motility by a repression pathway.

Thirty-two variants from peak PIa were selected for complementation analysis, plasmids expressing either the *gacA* or *gacS* genes (22) were introduced by triparental mating, and the motility was compared to those of the wild-type strain and the noncomplemented variant. Wild-type motility was restored in 30 variants: 24 by the cloned *gacS* gene and 6 by the *gacA* gene. The remaining two variants were not complemented by any gene and are likely to be affected in both genes. Figure 3A shows a typical complementation of one of these variants. The same experiment was performed with 51 variants from peak PIb. Wild-type motility was restored in 41 of the variants: 34 by *gacS* and 7 by *gacA*. Figure 3B shows the results of one of the complementation assays. These results indicate that the first peak population is affected in the Gac system and that *gac* mutants are selected both during the late stationary phase and during rhizosphere colonization.

Peak PII variants harbor other mutations besides Gac mutations. Complementation analysis of 14 variants from peak PII showed that none of them had motility restored to wild-type levels by either of the *gac* genes. However, as shown in Fig. 4, the motility phenotype of all the variants tested was partially complemented by the cloned *gacS* gene. These variants showed a halo of 229% ± 12% compared to the wild-type level (100%); no significant differences were found when complementation was done with the *gacA* gene (204% ± 13%). Conversely, the cloned *gacS* gene partially complemented the motility phenotype (137% ± 14%). These results indicate that these rhizosphere-isolated variants harbor mutations in the *gacS* gene and at least one additional mutation leading to increased motility. Therefore, the rhizosphere selects for these hypermotile variants with multiple mutations that are not selected or generated by the late stationary phase.

Hypermotile variants from rhizosphere peak PII are more competitive than the wild-type strain for rhizosphere colonization. The three variants isolated from the rhizosphere and belonging to peak PII (V5, V12, and V35) that showed the

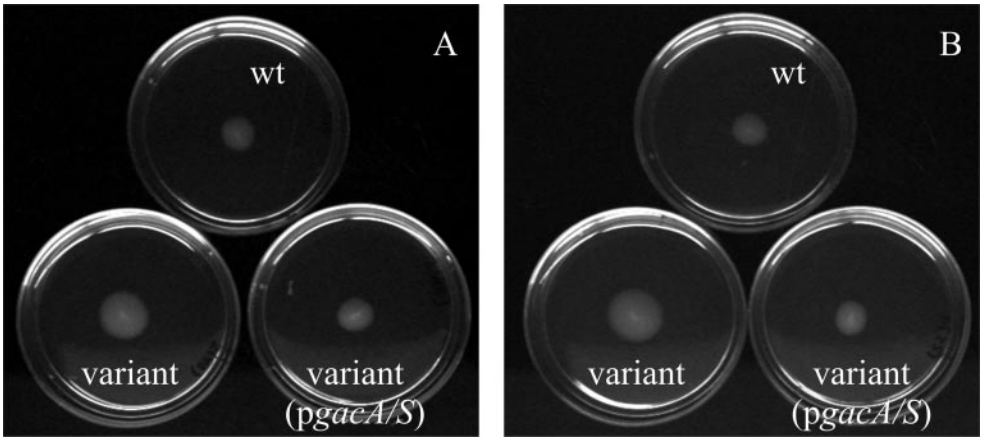

FIG. 3. Swimming motility phenotype and complementation analysis of phenotypic variants belonging to peaks PIa (A) and PIb (B), respectively, isolated after prolonged cultivation and rhizosphere passage. The figure presents a typical complementation experiment in which either the cloned *gacA* or *gacS* (p*gacA*/*S*) genes totally complemented the motility phenotype of the phenotypic variants. wt, wild type.

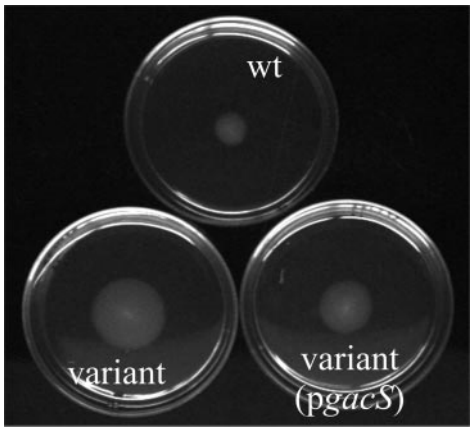

FIG. 4. Swimming motility phenotype of phenotypic variants belonging to peak PII isolated after rhizosphere selection. The figure presents a typical complementation experiment in which a cloned *gacS* gene (p*gacS*) complemented only partially the motility phenotype of the phenotypic variants. wt, wild type.

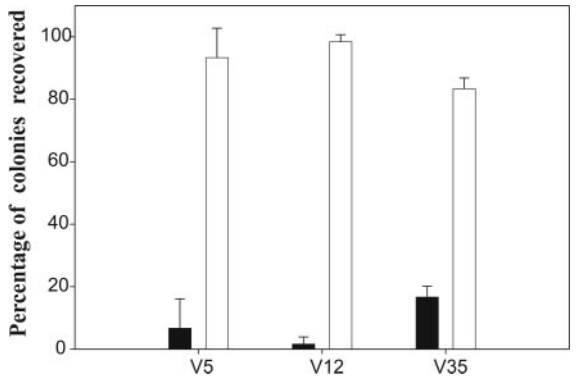

FIG. 5. Competitive root colonization by *P. fluorescens* F113 and phenotypic variants V5, V12, and V35 isolated after rhizosphere colonization. The wild-type strain was used as the competitor for the phenotypic variants. Plants were inoculated 1:1 with the test strain and the competitor, and after 2 weeks, root tips were collected and the bacteria present were plated. White bars represent the percentage of colonies recovered from the tested strains; black bars represent the percentage of colonies recovered from the competitor (wild-type) strain. Arithmetic means and standard deviations are presented.

highest motility were selected for competitive colonization experiments. The variants were cured for the plasmids expressing the site-specific recombinases, since it has been previously shown that *P. fluorescens* F113 overexpressing the *sss* gene shows enhanced competitive colonization (9). As shown in Fig. 5, the three variants were more competitive than the wild-type strain, being able to displace wild-type *P. fluorescens* F113 from the last centimeter of the root within 2 weeks.

DISCUSSION

Motility is an important trait for competitive rhizosphere colonization. Here we show that the rhizosphere selects for hypermotile mutants and that these mutants present enhanced competitive colonization. The fact that all the phenotypic variants isolated from the rhizosphere are more motile than the wild-type strain highlights the importance of motility for rhizosphere colonization.

Phenotypic (phase) variation has been frequently associated with rhizosphere colonization by pseudomonads. It has been shown that phenotypic variants arise during alfalfa root colonization by *P. fluorescens* (27) and during *Arabidopsis thaliana* root colonization by *Pseudomonas brassicacearum* (1), indicating that this might be a general fact of rhizosphere colonization by these bacteria. We have previously shown that overexpression of either of two rhizosphere-induced genes, *sss* and *xerD*, encoding site-specific recombinases accounts for a large increase in the number of variants obtained, especially after rhizosphere colonization (22). Considering that a *P. fluorescens* mutant affected in the *sss* gene is impaired in rhizosphere colonization of a variety of crops (10) and that the rhizosphere colonization ability of several pseudomonads can be increased by ectopic expression of this gene (9), it can be concluded that phenotypic variation is an important trait for rhizosphere competitive colonization, especially after the finding, reported here, that all the variants show enhanced motility.

We have made use of the increase in the number of variants obtained after overexpression of either of the genes encoding site-specific recombinases (22) to analyze a large number of phenotypic variants. Besides enhanced motility, we have observed that a vast majority of these variants are affected in the Gac system, a two-component system that regulates multiple traits, including the formation of a variety of secondary metabolites (21). The results presented here show that the Gac system also regulates swimming motility through a repression pathway. We are currently investigating this regulatory circuit, since for most traits, the Gac system acts as a posttranscriptional activator (26). Our previous results (22) showed a linkage between phenotypic variation and the Gac system that we have confirmed here by analyzing a larger number of phenotypic variants. It is interesting to note that different types of mutations in the *gacA* and -*S* genes (point mutations, tandem repeats, insertions, inversions, and short and long deletions) have been proposed as a mechanism for phenotypic (phase) variation in *Pseudomonas* sp. strain PCL1171 (34–36). The fact that different types of mutations are observed in these genes (35), together with the locations of the *gacA* and *gacS* genes in different parts of the genome and the observation reported here of a mutation bias toward the larger *gacS* open reading frame, points out that the role of site-specific recombination in the appearance of these mutants is indirect and, according to van den Broek et al. (34, 36), is possibly related to the activity of the *mutS* and *rpoS* genes.

We have observed a different pattern of hypermotile variants between stationary-phase cultures and rhizosphere colonization. All the phenotypic variants isolated after prolonged laboratory cultivation show a motility that is about 150% of the wild-type motility. All these phenotypic variants cluster in a narrow peak when a frequency distribution of motility is plotted, suggesting that they all belong to the same population. In fact, the increase in motility corresponds to the increase observed for *gac* mutants (Fig. 2), and their motility phenotype is complemented by the cloned *gac* genes (Fig. 3A), indicating that *gac* mutants are selected during the stationary phase. This complementation analysis has been done with the cloned *gacA* gene from *P. fluorescens* CHA0 (20), being the GacA protein

from strain CHA0 97% identical to F113 GacA. To complement the *gacS* mutants, we have used the cloned gene from *P. syringae* (18). This GacS protein presents 78% identity and 88% homology to its *P. fluorescens* F113 counterpart (data not shown). The total complementation of both insertion mutants with these heterologous genes shows that they are functionally equivalent to the F113 genes. Duffy and Defago (15) showed that *gac* mutants arose during prolonged cultivation of *P. fluorescens* CHA0. The characterization of several of these mutants showed that they were caused by independent point and deletion mutations in different parts of the *gacA* gene (5). It is possible that induction of genes encoding site-specific recombinases (22) is a mechanism for generating diversity, *gac* mutants being selected because of their increased fitness under stationary-phase conditions.

The phenotypic variants isolated after rhizosphere colonization clustered in two groups. The first group (peak PIb) seems to be identical to the variants isolated after prolonged cultivation and therefore correspond to *gac* mutants. The cloned *gac* genes were able to complement the motility phenotype of most of these variants (Fig. 3B), confirming that they are mutated in the Gac system. Since the *sss* and *xerD* genes are also induced in the rhizosphere (22), a similar mechanism as for stationary-phase variation can be suggested, with *gac* mutants being selected because of their increased fitness in the rhizosphere. Chancey et al. previously showed (7) that *gac* mutants of *Pseudomonas aureofaciens* arise in the wheat rhizosphere in soil microcosms, comprising up to 36% of the recovered cells. They also showed that *gac* mutants did not appear through a conserved mutational mechanism, in agreement with the results obtained by others (5, 35) in laboratory cultivation of different pseudomonads. It is interesting to note that although *gac* mutants do not seem to be impaired in rhizosphere persistence and colonization in soil microcosms (24, 30), they do not displace wild-type populations (7).

The second group of phenotypic variants isolated from the rhizosphere (peak PII) form a wider peak, with higher motility than *gac* mutants. It is important to note that all the tested variants from this group harbor a mutation that is partially complemented by the cloned *gacS* gene and are therefore affected in the Gac system. The higher motility of these variants indicates that besides the *gac* mutation, they harbor additional mutations that derepress motility. It cannot be excluded that other phenotypes are also selected in the rhizosphere. The width of the peak probably means that it is a mixed population with several additive mutations, suggesting the presence of multiple regulatory circuits repressing motility. Our preliminary unpublished results that show that about 1% of transposon insertions result in more motile mutants support this hypothesis that implies that motility in *P. fluorescens* is severely limited. We are currently investigating the nature of these mutations in order to clarify the genetic constraints responsible for regulating motility. The isolation of these hypermotile variants from the rhizosphere and not from prolonged laboratory cultivation indicates that the enhanced motility phenotype is advantageous during rhizosphere colonization and is therefore selected under these conditions. To strengthen this observation, we have shown here that several of these hypermotile variants are more competitive than the wild-type strain, displacing it from the root tip (Fig. 5). Dekkers et al. (9) showed that the competitive colonization ability of several pseudomonads can be increased by overexpression of the *sss* gene. In the experiments reported here, the phenotypic variants were cured of the plasmids overexpressing the site-specific recombinases, indicating that the enhancement of competitive colonization is not directly related to the production of the recombinases but to the mutations generated after site-specific recombinase activity and subsequent rhizosphere selection. The fact that *gac* mutants are not more competitive than the wild type (7) points out that these additional mutations are the basis for increased competitiveness.

The results presented here provide a new method to generate more competitive strains for rhizosphere colonization. Since efficient rhizosphere colonization is a requirement for biotechnological applications in biocontrol and rhizoremediation (8, 20), engineering of competitiveness can be rendered in more effective strains. It has been previously shown that after three enrichment cycles in the rhizosphere, a *P. fluorescens* strain harboring a mutation in the *mutY* gene showed enhanced competitive colonization (12). Since the MutY protein is implicated in DNA repair and a mutant is therefore prone to accumulate mutations that can be selected by the rhizosphere environment, a similar mechanism can be inferred for the overexpression of the recombinases and selection of hypermotile strains after a single rhizosphere passage. It is important to note that after curing the plasmids encoding the recombinases, the resulting more-competitive strains are genetically stable. Although *gac* mutations affect several traits important for biocontrol, the finding that additional mutations conferring increased motility result in enhanced competitive colonization could allow, through uncoupling of this trait from the Gac system, the design of improved biocontrol strains.

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