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Mutational Disruption of the Biosynthesis Genes Coding for the Antifungal Metabolite 2,4-Diacetylphloroglucinol Does Not Influence the Ecological Fitness of *Pseudomonas fluorescens* F113 in the Rhizosphere of Sugarbeets

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The ability of *Pseudomonas fluorescens* F113 to produce the antibiotic 2,4-diacetylphloroglucinol (DAPG) is a key factor in the biocontrol of the phytopathogenic fungus *Pythium ultimum* by this strain. In this study, a DAPG-producing strain (rifampin-resistant mutant F113Rif) was compared with a nearly isogenic DAPG-negative biosynthesis mutant (Tn*5*::*lacZY* derivative F113G22) in terms of the ability to colonize and persist in the rhizosphere of sugarbeets in soil microcosms during 10 plant growth-harvest cycles totaling 270 days. Both strains persisted similarly in the rhizosphere for 27 days, regardless of whether they had been inoculated singly onto seeds or coinoculated in a 1:1 ratio. In order to simulate harvest and resowing, the roots were removed from the soil and the pots were resown with uninoculated sugarbeet seeds for nine successive 27-day growth-harvest cycles. Strains F113Rif and F113G22 performed similarly with respect to colonizing the rhizosphere of sugarbeet, even after nine cycles without reinoculation. The introduced strains had a transient effect on the size of the total culturable aerobic bacterial population. The results indicate that under these experimental conditions, the inability to produce DAPG did not reduce the ecological fitness of strain F113 in the rhizosphere of sugarbeets.

Certain fluorescent *Pseudomonas* strains can inhibit fungal pathogens of plant roots, and these have been proposed as potential biocontrol agents of root disease in the field (5, 8, 16, 22, 30, 31, 33). The production of antifungal secondary metabolites is recognized as an important trait in the biocontrol of fungal plant diseases (4, 7, 11–15, 19). Production of these metabolites in the rhizosphere has been documented for phenazine (25) and 2,4-diacetylphloroglucinol (DAPG [11]).

Pythium ultimum is a fungus that mediates damping-off of sugarbeet seedlings when sowing is carried out under conditions of cool temperature and high soil water content. Previous work in this laboratory has shown that DAPG produced by *Pseudomonas fluorescens* F113 inhibits the growth of *P. ultimum* in vitro (23). The strain suppresses the damping-off of sugarbeets in soil microcosms prepared with soil naturally infested by *Pythium* spp. (9). A mutant derivative, F113G22, is a Tn*5*::*lacZY* biosynthesis DAPG-negative F113 mutant (23). F113G22 does not inhibit *P. ultimum* under in vitro conditions, nor does it protect sugarbeets from damping-off disease in soil microcosms, thus establishing the importance of DAPG in the ability of F113 to inhibit *Pythium* spp. (9, 23).

A prerequisite for effective biocontrol of root disease is the aggressive colonization of the rhizosphere by biocontrol *Pseudomonas* spp. introduced as a seed inoculant (28, 29). Factors likely to influence the ecological competence of pseudomonads in the rhizosphere have been reviewed on several occasions (1, 3, 6, 16, 26, 32). It has been hypothesized that secondary metabolites involved in biocontrol may also contribute to the ability of *Pseudomonas* spp. to colonize and persist in soil and in the rhizosphere (18).

F113.T1 is a Tn*5*::*lacZY*-marked derivative of F113 that displays the same biocontrol ability as the wild-type strain (9). In a preliminary study, strains F113.T1 and F113G22 were shown to survive similarly in the rhizosphere of sugarbeets grown in natural soil for 3 weeks after inoculation of seeds, suggesting that the ability to produce DAPG may not influence the fitness of the strain in the rhizosphere (9). Similar results were obtained with *Pseudomonas aureofaciens* 30-84, which produces antifungal phenazine antibiotics, and a biosynthesis phenazine-negative 30-84 mutant monitored for 40 days in soil (18). However, the wild type survived at higher cell numbers when strains were studied for another 30-day period, suggesting that the autecological effect of antibiotic-producing genes may require prior establishment of the strain in soil (18). Whether the ability to produce DAPG influences the long-term persistence of *Pseudomonas* spp. in the rhizosphere of sugarbeets is unknown.

The objective of this study was to determine whether the ability to produce DAPG has an effect on the ecological fitness of introduced *P. fluorescens* F113 in the rhizosphere of sugarbeets and its ability to subsequently colonize roots of uninoculated sugarbeets sown repeatedly in the same soil. Rifampin-resistant mutant F113Rif and DAPG-negative F113G22 were inoculated singly onto seeds or in combination so that both strains competed with one another in the rhizosphere. Sugarbeets were grown in soil microcosms for 27 days, after which roots and shoots were removed and the soil was resown with uninoculated seeds for nine subsequent cycles of growth of sugarbeet seedlings.

MATERIALS AND METHODS

Strains. *P. fluorescens* F113 was isolated from the rhizosphere of sugarbeets (23). The strain produces DAPG and its precursor, monoacetylphloroglucinol.

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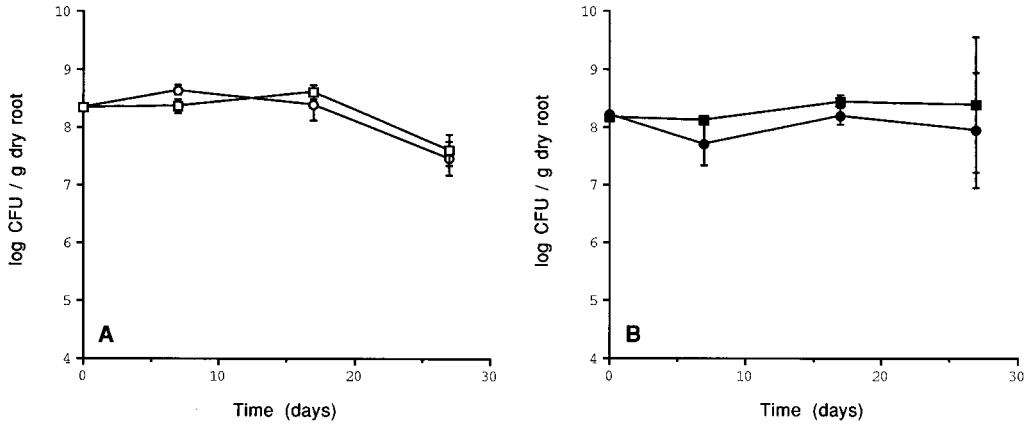

FIG. 1. Population size of *P. fluorescens* F113Rif (○, ●) and DAPG-negative F113G22 (□, ■) in the rhizosphere of inoculated sugarbeets. Strains were inoculated singly (A) or coinoculated in a 1:1 ratio (B) onto sugarbeet seeds. Vertical bars represent standard deviations. There was no significant difference between treatments at any of the sampling times.

Derivative F113Rif is a spontaneous rifampin-resistant F113 mutant which produces DAPG and displays the same growth rate as the wild type when cultured in vitro. Strain F113G22 is a Tn*5*::*lacZY* DAPG-negative derivative of F113 (23). The transposon is inserted within the DAPG biosynthetic locus, and the insertion is stable under in vitro conditions (23). F113G22 is resistant to kanamycin and cleaves the chromogenic compound 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) to produce blue-pigmented colonies. The strain colonizes the rhizosphere of sugarbeets similarly to a DAPG-positive Tn*5*::*lacZY*-marked derivative of F113 for 3 weeks after inoculation of seeds (9).

Soil microcosm. Soil material was collected from the sandy loam surface horizon of a brown podzolic soil cropped with sugarbeets (Bandon, County Cork, Ireland). The soil was dried at room temperature to 28% (wt/wt) water content and was passed through a 0.5-cm-mesh screen. A quantity of 140 g of soil (i.e., about 100 g of dry soil) was put into pots 7.5 cm in diameter and 8 cm deep (9). Distilled water was used to adjust the water content of the soil to 32% (wt/wt), which corresponds to 70% saturation of the soil porosity. The pots were put in a growth chamber set at 12°C with a 16-h light and 8-h dark photoperiod. Water content was adjusted every other day throughout the duration of the experiment.

Inoculum preparation. Strains F113Rif and F113G22 were grown in Luria-Bertani broth (LB [17]) containing 100 μg of rifampin per ml (Rif100) and 50 μg of kanamycin per ml (Km50), respectively. Cultures were incubated overnight at 30°C with shaking. Cells were harvested by centrifugation in a Denley BS400 centrifuge (3,000 rpm for 10 min) and washed twice in one-fourth-strength Ringer solution (Oxoid). Cell numbers in the suspensions were determined by dilution plating onto LB agar supplemented with Rif100 (for F113Rif) or Km50 (for F113G22). The suspensions were stored at 4°C overnight and were adjusted to about 6.0 log CFU/ml prior to seed inoculation. Cell suspensions of F113Rif and F113G22 were mixed in a 1:1 ratio for the competition experiment.

Seed inoculation, plant growth conditions, and treatments. Sugarbeets (*Beta vulgaris* cv. 'Rex') were inoculated by soaking of the seeds in cell suspensions for five min (5 g of seeds in a 10-ml cell suspension) prior to sowing. A total of nine seeds were sown per pot, at a depth of 1.5 cm (9). Plants were grown for 27 days, after which roots and shoots were removed. A carbon- and nitrogen-free rooting solution (27) was added to the soil (1 ml per pot), which was mixed throughout and put back into the pots, prior to sowing with uninoculated sugarbeet seeds. The procedure was repeated every 27 days for a total of 10 cycles of sugarbeet growth.

The first two treatments corresponded to strains F113Rif and F113G22 inoculated singly onto seed. The two strains were also coinoculated in a 1:1 ratio. A fourth treatment, in which seeds were not inoculated, was studied.

Sampling procedures and plate counts. For the first cycle of sugarbeet growth, plants were sampled 7, 17, and 27 days after sowing. On days 7 and 17, two plants were sampled from each of three pots for each treatment. The soil and pots were discarded. Colony counts were performed with the germinating seed (i.e., seed and emerging radicle) on day 7 and the root system on days 17 and 27. The soil from pots used on day 27 was retained for resowing with uninoculated seeds, as described above.

At each sampling time, plants were shaken to remove loosely adhering soil. The shoots were excised. Root systems and their closely adhering soil were transferred to 10 ml of one-fourth-strength Ringer solution in McCartney bottles and were vortexed for 2 min. A 10-fold serial dilution of the extract was prepared. Strain F113Rif was enumerated on LB agar containing Rif100. Strain F113G22 was recovered on LB agar containing Km50 and 20 μg of X-Gal per ml. The numbers of total culturable aerobic bacteria were determined on LB agar supplemented with 100 μg of cycloheximide per ml. Colony counts were expressed per gram of dry root.

Statistical design and analyses. The experiment was run in triplicate. Nine pots were used per treatment. The location of each pot in the growth chamber was chosen at random. At each sampling time, a total of three pots were used per treatment. Two root systems were sampled per pot, and the results from analysis of the two root systems were used as one replicate. Plate count data were $\log_{10}$ transformed. Analysis of variance was carried out at each sampling time ($P = 0.05$) with the General Linear Model procedures of the Statistical Analysis System (SAS Institute Inc.). Multivariate analyses of variance (MANOVA) were also conducted, with Wilks' lambda, to compare treatments throughout the experiments (2, 20, 21). Samplings on days 7, 17, and 27 were used as variables for seed-inoculated sugarbeets in the MANOVA, and data from the first, third, fifth, seventh, and ninth resowings (five variables) were used for uninoculated sugarbeets.

RESULTS

Survival of seed-inoculated F113Rif and F113G22 in the rhizosphere of sugarbeets. The role of DAPG genes in the survival of strain F113 in the rhizosphere of sugarbeets was assessed by comparing DAPG-producing F113Rif and DAPG-negative mutant F113G22. First, the two strains were inoculated singly onto sugarbeet seeds. Both were recovered at similar levels from the emerging radicle and the rhizosphere of sugarbeets during the 27-day period following inoculation of seeds (Fig. 1A and Table 1). They persisted at about 7.5 log CFU/g of dry root 27 days after inoculation, when all sugarbeet plants were removed from the pots.

F113Rif and F113G22 were also coinoculated onto sugarbeet seeds, in a 1:1 ratio, to provide conditions in which the two strains could compete with one another. F113Rif and F113G22 survived at essentially similar levels for 27 days after inoculation (Fig. 1B and Table 1). No difference between F113Rif and F113G22 in terms of survival during the 27-day period following inoculation of seeds was found, regardless of whether strains were inoculated singly or in combination.

Effect of seed inoculation with F113Rif and F113G22 on total culturable aerobic bacteria. Single inoculation of seeds with F113Rif or F113G22 increased the number of culturable aerobic bacteria in the rhizosphere of sugarbeets by about 0.5 log CFU/g of dry root compared with the uninoculated treatment on days 7 and 17 (Fig. 2A). The difference between the uninoculated treatment and the two inoculated ones was statistically significant on days 7 and 17 but not on day 27. At each sampling time, single inoculations with F113Rif or F113G22 led to similar levels of total culturable aerobic bacteria in the rhizosphere (Fig. 2A). Coinoculation of seeds with a 1:1 mixture of F113Rif and F113G22 also resulted in an increase in

TABLE 1. Results of MANOVA with Wilks' lambda

Comparison	MANOVA result for[a]: Seed inoculated sugarbeets (first sowing)		Uninoculated sugarbeets (resowings)	
	F ratio	*P* > *F*	*F* ratio	*P* > *F*
Introduced strains				
F113Rif singly vs F113G22 singly	0.93	0.547	0.11	0.976
F113Rif coinoculated vs F113G22 coinoculated	2.35	0.254	6.65	0.288
Total culturable aerobic bacteria				
Inoculation vs no inoculation	16.9	0.010*	7.91	0.116
F113Rif singly vs no inoculation	4.08	0.104	8.92	0.104
F113G22 singly vs no inoculation	22.1	0.006*	3.66	0.228
F113Rif singly vs F113G22 singly	18.9	0.008*	1.62	0.425
Introduced strains as % of total culturable aerobic bacteria				
F113Rif singly vs F113G22 singly	1.04	0.467	2.68	0.294
F113Rif coinoculated vs F113G22 coinoculated	12.5	0.017*	3.00	0.268

[a] Samplings on days 7, 17, and 27 were used as variables for seed-inoculated sugarbeets (first sowing), and data from the first, third, fifth, seventh, and ninth resowings were used for uninoculated sugarbeets (MANOVA with five variables). Asterisks indicate which *F* ratios were statistically significant at $P = 0.05$.

the number of culturable aerobic bacteria in the rhizosphere of sugarbeets compared with the uninoculated treatment on days 7 and 17 but not on day 27 (Fig. 2B).

F113Rif and F113G22 inoculated singly onto seeds represented about 90% of the total culturable aerobic population of bacteria at 7 days but dropped to less than 20% by 27 days after inoculation. In the competition experiment, F113Rif and F113G22 comprised 24 and 56%, respectively, of the total culturable aerobic bacteria on day 7 after inoculation. Again, both strains represented a small fraction of the total culturable aerobic bacteria 27 days after inoculation (data not shown). Seed inoculation had a transient effect on culturable aerobic bacteria in the rhizosphere of sugarbeets, but the particular strain(s) used for inoculation (i.e., F113Rif, F113G22, or a combination of both) had no significant influence on the number of total culturable aerobic bacteria or on the percentage of the latter that corresponded to the introduced strain(s).

Colonization of the rhizosphere of uninoculated sugarbeets by F113Rif and F113G22 present in soil. Sugarbeet plants were harvested 27 days after inoculation. A diligent effort was made to remove all roots from soil before resowing with uninoculated seeds. The procedure was repeated at the end of each subsequent 27-day period of sugarbeet growth. Both F113Rif and F113G22 were capable of recolonizing the rhizosphere of sugarbeets even after nine sugarbeet growth cycles without reinoculation (Fig. 3A). No difference between the two strains in terms of the level of colonization at 27 days after each resowing was observed. Furthermore, the results of MANOVA performed with data from five resowings indicated that both strains were found at similar population levels throughout the experiment (Table 1). Cell numbers for F113Rif and F113G22 at day-27 samplings declined in time from the first to third growth cycles of uninoculated sugarbeets, after which they remained stable at about 6.0 log CFU/g of dry root (Fig. 3A).

In pots initially sown with seeds coinoculated with F113Rif and F113G22, cell numbers for each strain were similar to each other at the end of each sugarbeet growth cycle but one (Fig. 3B). At eight of nine sampling times, the results of rhizosphere colonization for both F113Rif and F113G22 were similar in the coinoculation treatment to those obtained with soil in which

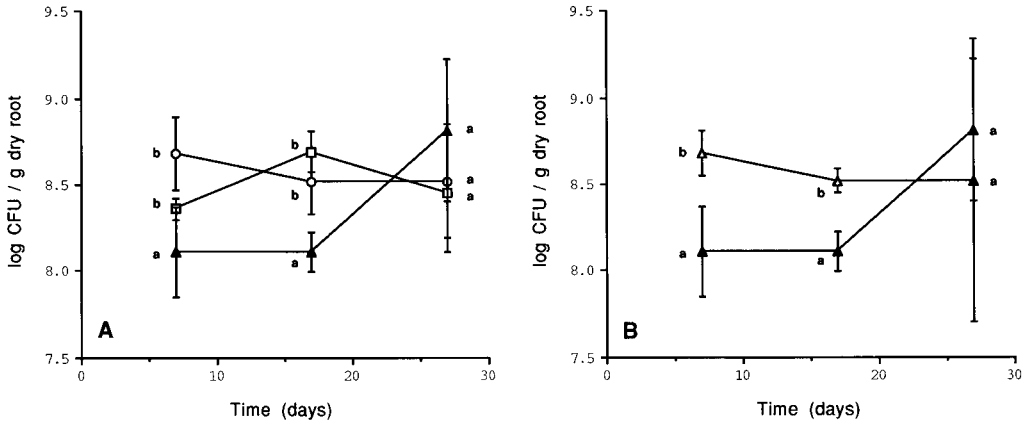

FIG. 2. Counts of total culturable aerobic bacteria in the rhizosphere of sugarbeets inoculated with *P. fluorescens* F113Rif alone (○), DAPG-negative F113G22 alone (□), or a combination of both (△). Results for strains inoculated singly (A) or coinoculated onto seeds in a 1:1 ratio (B) are shown together with those for the uninoculated control treatment (▲). Vertical bars represent standard deviations. At each sampling time, letters (a and b) were used to discriminate between treatments when differences were statistically significant.

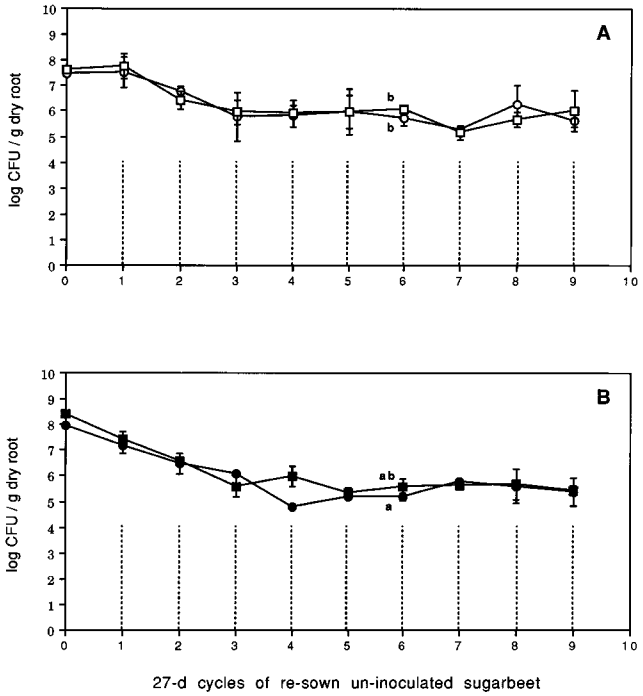

FIG. 3. Population size of *P. fluorescens* F113Rif (○, ●) and DAPG-negative F113G22 (□, ■) in the rhizosphere of uninoculated sugarbeets 27 days (d) after each resowing. Strains had been inoculated singly (A) or coinoculated in a 1:1 ratio (B) onto sugarbeet seeds at the start of the experiment. Vertical bars represent standard deviations. There was no significant difference between treatments at any of the sampling times, except in the sixth resowing, in which strains inoculated singly were found at higher levels than F113Rif in the coinoculation treatment (as indicated by letters a and b).

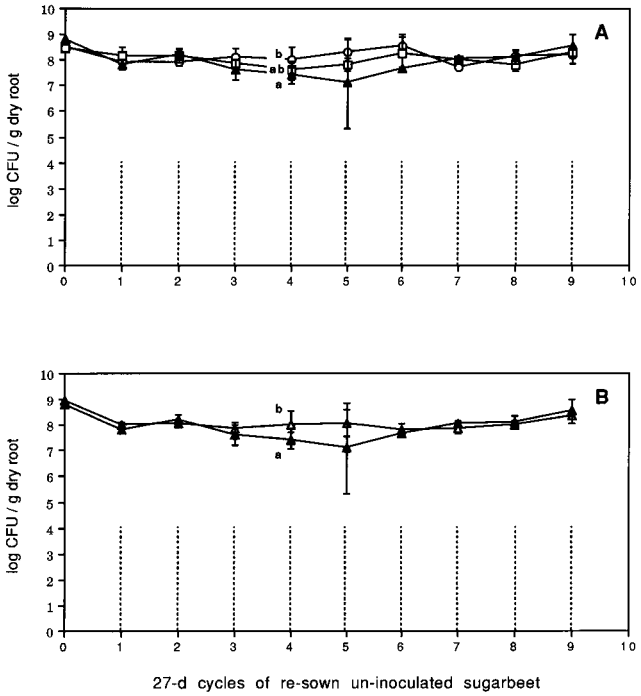

FIG. 4. Counts of total culturable aerobic bacteria in the rhizosphere of uninoculated sugarbeets 27 days (d) after each resowing. (A) Results are shown for treatments in which soil had previously been planted with seeds inoculated singly with *P. fluorescens* F113Rif (○) or DAPG-negative F113G22 alone (□). (B) Results are shown for the treatment in which soil had previously been planted with seeds inoculated with both F113Rif and F113G22 in a 1:1 ratio (△). Results for the uninoculated control (▲) are shown in panels A and B. Vertical bars represent standard deviations. There was no significant difference between treatments at any of the sampling times, except in the fourth resowing, in which total counts were lower in the uninoculated control treatment than in soil that had been inoculated with F113Rif alone or with a combination of F113Rif and F113G22 (as indicated by letters a and b).

seeds inoculated with single strains had been used. Overall, there was no difference between F113Rif and F113G22 in terms of colonization of the rhizosphere of uninoculated sugarbeets, regardless of whether the strains were initially present singly or together in the soil.

Effect of F113Rif and F113G22 remaining in soil on total culturable aerobic bacteria in the rhizosphere of uninoculated sugarbeets. In the uninoculated control treatment, total culturable aerobic bacteria were recovered at population levels of between 7.4 and 8.8 log CFU/g of dry root at the end of each 27-day growth cycle of uninoculated sugarbeets (Fig. 4). Essentially similar levels were found in the inoculated treatments (Table 1). Despite recolonizing the roots of sugarbeets, strains F113Rif and F113G22 had no quantitative effect on the numbers of total culturable aerobic bacteria in the rhizosphere of uninoculated sugarbeets resown nine times in the same soil (Fig. 4 and Table 1). There was no difference between treatments in which F113Rif and F113G22 had been inoculated singly (Fig. 4A) and that in which F113Rif and F113G22 had been inoculated in combination (Fig. 4B). In both cases, the introduced strains comprised between 0.1 and 3.0% of the total population of culturable aerobic bacteria on day 27 from the second to the ninth growth cycles of uninoculated sugarbeets (Fig. 5A and B). Unlike in the first experiment, F113Rif and F113G22 had no effect on culturable aerobic bacteria in the rhizosphere of sugarbeets.

DISCUSSION

P. fluorescens F113 produces DAPG, inhibits the fungal pathogen *P. ultimum* under laboratory conditions, and controls the incidence of *Pythium*-mediated damping-off disease of sugarbeets in soil naturally infested by *Pythium* spp. (9, 23). In contrast, DAPG-negative biosynthetic mutant F113G22 has no biocontrol potential under the same conditions (9, 23). Previous work has shown that the ability to produce DAPG did not influence root colonization by F113 for 21 days after inoculation of sugarbeet seeds (9). However, the results obtained by Mazzola et al. (18) showed that this type of short-term experiment favors the introduced strains and may be inappropriate to assess the autecological role of antibiotic-producing genes, especially when strains are inoculated at high cell numbers. The experimental design in the current study was chosen with the objective of subjecting the introduced strains to conditions of competition from the resident soil microbiota that are relevant to those that may be encountered under field conditions within the context of a sugarbeet rotation, as is practiced in Ireland. Therefore, the colonizing ability and persistence of DAPG-producing F113Rif and DAPG-negative biosynthetic mutant F113G22 were compared over a longer period (270 days). Untreated sugarbeet seeds were resown nine times in the same soil to expose F113Rif and F113G22 to increased competition from the resident microbiota, which were well adapted to the conditions of the sugarbeet rhizosphere.

F113Rif and F113G22 colonized the root system of sugarbeets at similar levels during the 27-day period after inoculation of seeds. Both strains were also coinoculated onto seeds to provide conditions in which they could compete with one an-

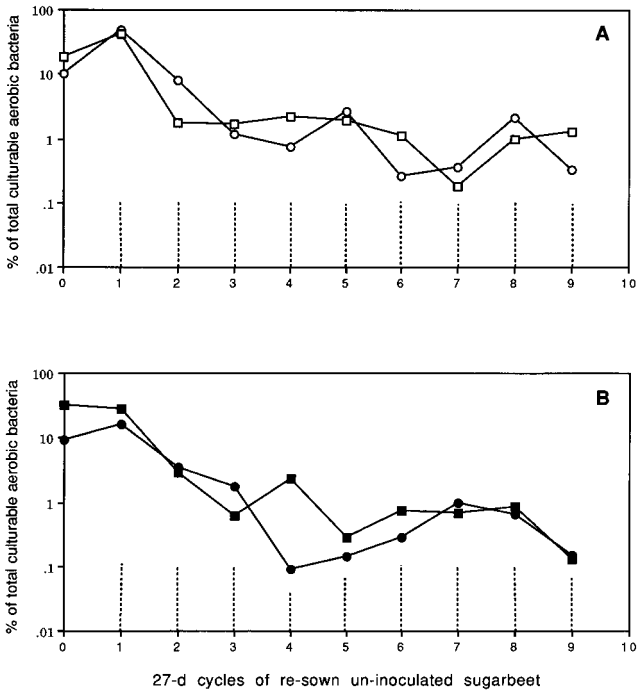

FIG. 5. *P. fluorescens* F113Rif (○, ●) and DAPG-negative F113G22 (□, ■) expressed as a percentage of the total culturable aerobic bacteria of the rhizosphere of uninoculated sugarbeets 27 days after each resowing. Strains had been inoculated singly (A) or coinoculated in a 1:1 ratio (B) onto sugarbeet seeds at the start of the experiment. There was no significant difference between F113Rif and F113G22 within each experiment at any of the sampling times.

other, and they were recovered at similar levels. These results indicate that the presence of functional DAPG genes did not influence short-term (27 days) root colonization by the strain and strengthen previous observations derived from an experiment in which strains were only inoculated singly (9).

The experiments were carried out under conditions promoting *Pythium*-mediated damping-off of sugarbeets and in which DAPG-producing F113 was shown to effectively control the incidence of the disease (9). Whether inoculated singly onto seeds or coinoculated, the introduced strains F113Rif and F113G22 represented a large fraction of the culturable aerobic bacteria on day 7 but lost their quantitative prevalence by day 27. The biocontrol ability of the strain requires its establishment in the rhizosphere, but apparently this did not result in a lasting perturbation of the resident culturable bacterial microbiota, at least from a quantitative point of view. It is also possible that introduction of the DAPG-producing F113 affects the composition and assemblage of the resident bacterial microbiota of the rhizosphere rather than its population size. Inoculation of soybeans with the biocontrol *Bacillus cereus* UW85n resulted in a qualitative switch in the structure of the resident bacterial community at the surface of the root (10). Further work in this laboratory will address this issue.

At the end of the first 27-day cycle, all roots were removed and the soil was resown with uninoculated seeds. This process of harvesting and resowing was repeated nine times. The introduced strains were present at low cell densities in the soil once roots were removed, and the soil was mixed thoroughly prior to resowing. For instance, both strains were found at about 2.8 log CFU/g of dry soil at the end of the last cycle. However, F113Rif and F113G22 were capable of colonizing the seedlings of uninoculated sugarbeets despite not being favored for root colonization from inoculation as in the previous experiment. Results from analysis of individual samplings and from MANOVA of several samplings (Table 1) show that both strains were recovered at similar levels throughout the experiment. The ability to produce DAPG, which did not confer a selective advantage to the strain in the short term (27 days), did not influence the persistence of F113 in the long term either (10 resowings over a 270-day period).

Our results are consistent with those from previous short-term studies showing that loss of antibiotic-producing ability did not influence colonization of seeds and roots by introduced pseudomonads. For instance, *P. fluorescens* Hv37aR2 and its isogenic derivatives deficient in the biosynthesis of the antifungal compound oomycin A were found at similar cell numbers in the rhizosphere of cotton for 12 days after inoculation of seeds (14). Essentially similar results were obtained with the phenazine-producing strain *P. fluorescens* 2-79 and a phenazine-negative mutant in the rhizosphere of wheat in a 13-day-long experiment in which strains were inoculated singly onto seeds (24). Mazzola et al. (18) worked with the same strain used by Thomashow and Weller (24) but found different results in experiments in which soil was resown with uninoculated seeds over five 20-day cycles. Under these conditions, the ability of *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 to produce phenazine-1-carboxylic acid clearly contributed to their ecological fitness in the rhizosphere of wheat. Successive resowings of wheat in the same soil were thought to progressively enrich resident wheat rhizosphere microorganisms and to result in increased competition between them and the introduced strains (18). The experiments reported in this study were carried out over ten 27-day growth cycles of sugarbeets and demonstrate that under conditions in which the ability of *P. fluorescens* F113 to produce DAPG is involved in the biocontrol of *Pythium*-mediated damping-off of sugarbeets, the same ability to produce DAPG did not contribute to the ecological competence of F113 in the rhizosphere of sugarbeets. Further work in this laboratory will target expression of the DAPG genes in situ with gene fusion technology.

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