Comparative Analysis of Antibiotic Resistance, Immunofluorescent Colony Staining, and a Transgenic Marker (Bioluminescence) for Monitoring the Environmental Fate of a Rhizobacterium

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Field releases of the wild-type plant growth-promoting rhizobacterium *Pseudomonas fluorescens* 89B-27, its bioluminescent derivative GEM-8 (89B-27::Tn*4431*), and a spontaneous rifampin-resistant variant (R34) were established on cucumber in 1994 and 1995 to examine the efficiency of these marker systems for estimating the wild-type population. Seed and root samples were taken 0, 7, 14, 21 or 28, 35 or 42, and 70 days after planting in each year and processed for enumeration by spiral plating or immunofluorescent colony staining (IFC). In both years, the populations of 89B-27, R34, and GEM-8, as measured by IFC, were not significantly different ($P > 0.05$) from each other at each sampling time. However, the populations of R34 and GEM-8, as measured by spiral plating and differentiation based on their respective phenotypes, were significantly lower ($P < 0.05$) than the wild-type populations and their IFC-determined populations. These data indicate that traditional marker systems may underestimate populations and hence the survival and colonization of genetically marked bacteria.

Monitoring of introduced bacteria in the natural environment requires rapid differentiation of the introduced strain from a background of genetically similar and dissimilar indigenous bacteria (13, 14). In addition, investigations into the population dynamics of introduced bacteria add the extra burden of accurate quantification.

Antibiotic-marked strains frequently have pleiotropic phenotypes (14) and may not express the resistance phenotype upon growth on selective media (18). The use of introduced genetic markers, such as *lacZY*, *lux*, and *xylE*, has been promoted as a solution to the pleiotropic effects associated with antibiotic resistance (13, 14). However, the insertion of foreign DNA into a single site on the chromosome or as a plasmid-borne character does not necessarily preclude pleiotropic changes (1, 3, 8). Additionally, these genetically marked strains usually are derivatives of spontaneous antibiotic-resistant variants of the wild type (9, 13, 14, 24) and could still have pleiotropic effects associated with antibiotic resistance in addition to those associated with the introduced DNA. The use of the above markers without an assessment of how they affect the fitness of the marked bacterium could result in false conclusions about population dynamics and survival of the introduced strain.

A major limitation with the currently available techniques for monitoring bacteria is the need for a method to monitor the unaltered wild type. In some cases, rapid differentiation and accurate quantification have been accomplished by using intrinsic antibiotic resistance (11, 21) or culturing on selective media or under selective environmental conditions (20, 31). However, these methods may stress bacterial cells, causing them to be viable but nonculturable on selective media (10,

18). Counts based on such techniques would underestimate the population size and survival of introduced strains.

Immunological techniques, such as immunofluorescence colony staining (IFC), also take advantage of intrinsic characteristics to differentiate and quantify introduced bacteria without using selective media (27–29). Detection limits as low as 20 CFU in a background of 10^{10} CFU/g of cattle slurry have been reported with IFC (28). The technique uses immunoglobulin G antibodies specific for a bacterial strain, conjugated with fluorescein isothiocyanate (FITC), to distinguish target from nontarget colonies. Bacterial colonies remain viable, and IFC-positive colonies can be subcultured to confirm their identity by other methods (e.g., fatty acid analysis or biochemical reactions) (29). Since the use of IFC does not require alteration of the wild type, this technique allows the comparison of an unaltered wild type to its genetically modified derivatives.

The objective of this study was to compare three different marking systems, spontaneous resistance to rifampin, genetic modification for bioluminescence, and IFC, for their ability to measure the population dynamics of an introduced plant growth-promoting rhizobacterium strain under field conditions.

Generation of variants. The wild-type strain *Pseudomonas fluorescens* 89B-27 was used as a model rhizobacterium. In previous studies, 89B-27 induced systemic resistance in cucumber against several pathogens (15) and colonized plant roots endophytically (16). *Escherichia coli* HB101 was the donor strain for plasmid pUCD623, a suicide vector that contains the transposon Tn*4431* and encodes chloramphenicol and ampicillin resistance. Tn*4431* carries the promotorless luciferase operon of *Vibrio fischeri* and encodes tetracycline resistance (25). The promotorless operon was chosen over a constitutive promoter because a constitutive promoter might cause more of an energy drain in the marked strain.

The bioluminescent marked derivative of 89B-27 was generated through biparental matings between HB101(pUCD623)

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and 89B-27 (25) mixed at a ratio of 2:3 (vol/vol) and plated (100 μl) onto Luria-Bertani agar. After incubation at 25°C for 48 h, a loop of bacteria from each mating was streaked onto Pseudomonas agar F (PAF) (Difco) amended with 15 μg of tetracycline per ml to recover bioluminescent transconjugants that fluoresced under UV light. One such colony from each mating was selected, streaked for purity on PAF plus tetracycline, and examined for light production with a liquid nitrogen-cooled charge-coupled device camera (Photometrics Ltd., Tucson, Ariz.). Selected bioluminescent transconjugants were examined for colony development and morphology in comparison to the wild-type strain on 5% TSBA (1.5 g of tryptic soy broth [TSB], 20 g of agar per liter) and for generation time in 5% TSB in 250-ml flasks shaken (250 rpm) at 25°C. Generation times were calculated based on growth during the exponential phase of three replications. Any transconjugant with a reduced generation time was not considered further. Each transconjugant was compared to the wild type, 89B-27, for carbohydrate utilization and fatty acid composition by cluster analysis of Biolog-GN (2) and gas chromatography fatty acid methyl ester (GC-FAME) (22, 23) profiles, respectively. Loss of the suicide vector was verified by streaking each bioluminescent derivative on PAF amended with 20 μg of chloramphenicol per ml and examining the plasmid profile in comparison to the wild type by the methods of Kado and Liu (12).

The ability of eight bioluminescent transconjugants to colonize roots was examined. Experiments were 2×9 factorials consisting of two sampling times with nine treatments, i.e., eight transconjugants and a methylcellulose control (no inoculum), arranged in a randomized complete block. Cucumber seed (cultivar Straight 8) was treated with a bacterial suspension in 2% methylcellulose (72 μl/g of seed) that resulted in approximately 10^6 CFU of bacteria per seed. The bacterial suspensions were prepared from confluent cultures grown on 5% TSBA at 28°C for 48 h and suspended in 10 ml of 0.02 M potassium phosphate buffer (pH 7.0) (PPB). Suspensions were centrifuged (8,000 × g for 10 min) and washed in 10 ml of PPB, and the pellet was resuspended in 2% methylcellulose. Three treated seeds were planted in 10- by 10- by 10-cm pots filled with soil (taken from the field described below), and the pots were then placed in a growth chamber at 25°C with a 14-h/10-h light-dark cycle. Rhizosphere populations were determined 2 and 4 weeks after planting. Root systems were removed from soil and gently shaken to remove any loosely adhering soil. The roots were weighed and placed in Tekmar sterile lab bags (Tekmar Co., Cincinnati, Ohio) with 10 ml of PPB. Each sample was then blended with a Stomacher Lab Blender (Tekmar) for 1 min, serially diluted in PPB, and plated onto 50% TSBA (15 g of TSB, 20 g of agar per liter) amended with 200 μg of cycloheximide per ml (to reduce fungal and yeast growth) with a spiral plater. The plates were incubated for 24 h at 25°C and then counted in the dark after addition of 1 μl of n-decyl aldehyde to the lid of each plate (24).

Rifampin-resistant derivatives of 89B-27 were selected from over 300 spontaneous resistant variants obtained by the gradient plate technique (4). Resistant variants were compared to the wild type for colony development, morphology, generation time, and Biolog and GC-FAME profiles. Nine variants were selected for further evaluation. Root colonization was examined as above, except that samples were plated on 5% TSBA amended with 100 μg of rifampin per ml and 200 μg of cycloheximide per ml.

Statistical analysis. All population data were converted to log CFU per gram (fresh weight) of tissue. Plates with fewer than five colonies were considered below detection limits and scored as 0 for calculation of means (13). Analysis of variance

was done by using the MANOVA procedure in PC-SAS 6.09 (SAS Institute, Cary, N.C.), and significant differences ($P <$ 0.05) were determined by using single-degree-of-freedom contrasts and Fisher's protected least significant difference. Cluster analysis of fatty acid profiles was performed by the unweighted two-dimensional dendrogram procedure of Sherlock Software (MIDI, Newark, Del.). Biolog-GN profiles were analyzed with NTSYS-pc Software (Exeter Software, Setauket, N.Y.) with the SIMQUAL and UPGMA routines. Only variants grouping with the wild type, 89B-27, at a Euclidean distance of less than 2.0 in both analyses were processed further.

Characterization of marked derivatives. For both genetic markers, derivatives of 89B-27 that were indistinguishable from the wild type by generation time and Biolog-GN or GC-FAME profiles were obtained (data not shown). In growth chamber colonization experiments, rifampin-resistant variant R34 had the largest rhizosphere population, 4.7 and 3.2 log CFU/g (fresh weight) of root 2 and 4 weeks after planting, respectively. Populations of GEM-8 were the largest, 4.6 and 3.1 log CFU/g (fresh weight) of root, of the bioluminescent transconjugants examined. Thus, GEM-8 and R34 were selected as marked variants for further analysis.

Field experiments. A randomized complete block design, consisting of four treatments and six blocks with one replication of each treatment per block, was established in a sandy-loam field soil at the E. V. Smith substation of the Alabama Agricultural Experiment Station in 1994 and 1995. Each experimental unit consisted of two rows with 10 mounds per row spaced 90 cm apart in raised beds under plastic mulch with drip irrigation. Plants to be sampled over the course of the experiment were randomly assigned for each experimental unit prior to planting. The field was maintained as recommended for commercial cucumber production by the Alabama Cooperative Extension Service. Treatments were a methylcellulose control, wild-type strain 89B-27, R34, and GEM-8. A 5-g portion of seed was treated for each experimental unit, and three seeds were planted in each mound. The mounds were thinned to two plants per mound 7 days after planting (DAP) prior to sampling plots.

Samples were taken from field trials 0, 7, 14, 21, 35, and 70 DAP in 1994 and 0, 7, 14, 28, 42, and 70 DAP in 1995. Prior to removal of the soil core, the above-ground portion of the plant was removed by cutting the stem 5 to 7 cm above the soil line. A 15-cm-diameter soil core, centered on the plant stem or furrow, was removed from each experimental plot to a depth of 25 cm and placed in a plastic bag. Seed or root systems were removed from the soil cores, gently shaken to remove any loosely adhering soil, and placed in sterile plastic bags.

For measuring rhizosphere and spermosphere colonization, stem tissue from each root sample was removed to 0.5 cm below the soil line prior to weighing. Seed or root samples obtained 0, 7, and 14 DAP were placed in test tubes with 10 ml of PPB; root samples from 21 and 28 DAP received 20 ml of PPB; root samples from 35 DAP received 50 ml of PPB; and root samples from 48 and 70 DAP were placed in bottles with 100 ml of PPB. All samples were sonicated in an FS28 bath (Fisher Scientific) for 6 min, and the sonicates were diluted serially and spiral plated onto 5% TSBA plus cycloheximide, 5% TSBA plus rifampin and cycloheximide, and 50% TSBA plus cycloheximide. After 48 h at 28°C, colonies were counted and bioluminescent colonies on 50 and 5% TSBA-plus-cycloheximide plates were counted in the dark after 1 μl of n-decyl aldehyde was placed on the lid of each plate. To confirm bioluminescence, a small portion of each bioluminescent colony or of colonies with a similar morphology was sampled with a sterile toothpick. The toothpick was placed in a cuvette with
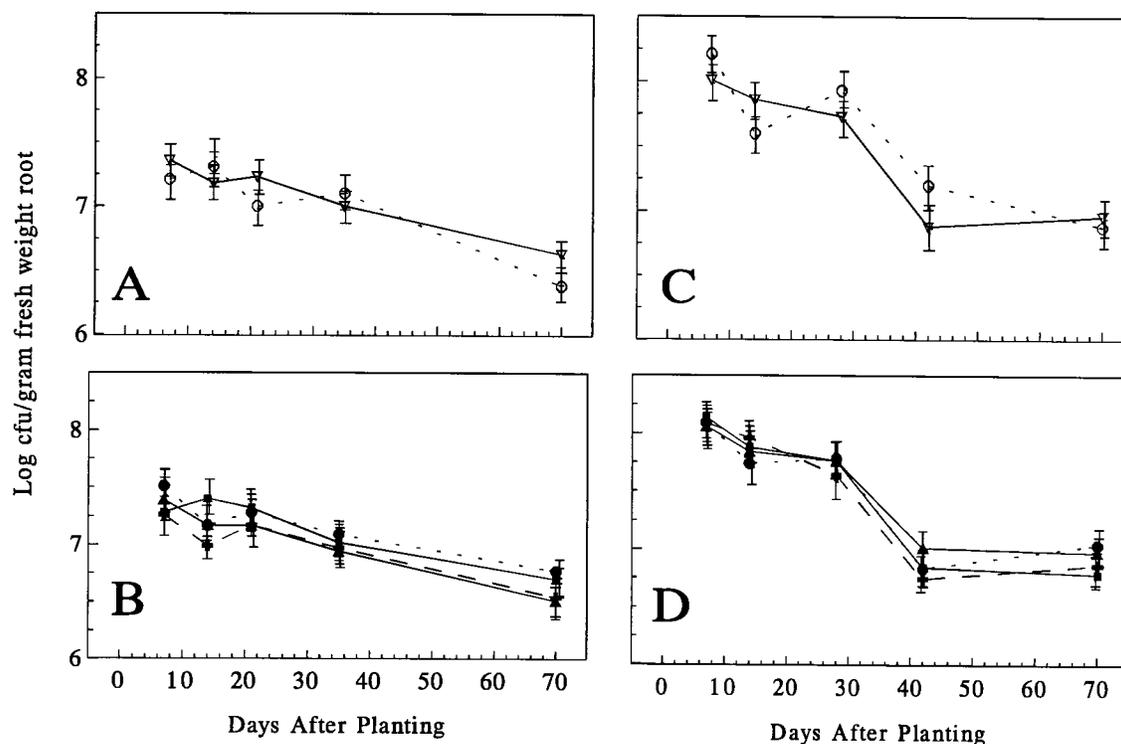
FIG. 1. Total bacterial rhizosphere populations from cucumber in 1994 (A and B) and 1995 (C and D). (A and C) Total populations as determined by total counts on 5% TSBA-plus-cycloheximide spiral plates ($\triangledown$) and total counts on 5% TSBA-plus-cycloheximide 24-well pour plates ($\bigcirc$). (B and D) Treatment-specific total populations from 24-well pour plates: methylcellulose control ($\blacktriangle$), 89B-27 ($\bullet$), GEM-8 ($+$), and R34 ($\blacksquare$). Error bars represent the standard deviation of each treatment at a given sampling time.

1 µl of a 1:10 dilution of *n*-decyl aldehyde, and the number of light quanta/10 seconds was measured in a Monolight 2000 luminometer (Analytical Luminescence Laboratories, La Jolla, Calif.). Any sample measuring twice background or greater was considered positive for bioluminescence. In addition, all colonies were scraped from plates where no bioluminescent colonies were observed, placed in a cuvette, and monitored for light quanta as above. In 1995, the incubation time for 5 and 50% TSBA-plus-cycloheximide plates was reduced from 48 to 24 h after plating. In addition, after 1 µl of *n*-decyl aldehyde (24) was placed on the lid of each plate, the plates were incubated at 15°C for 2 h prior to counting in the dark.

All samples were processed for IFC in all media (5% TSBA plus cycloheximide, 5% TSBA plus rifampin and cyclohexi-mide, and 50% TSBA plus cycloheximide) by placing 100 µl of the first dilution in a well of a 24-well tissue culture plate and then serially diluting with a FinnPipet Diluter (Labsystems Oy, Helsinki, Finland). Next, 300 µl of agar (48°C) was added to each sample while the samples were being shaken at 150 rpm. The agar was allowed to harden, and then the plates were inverted and incubated for 48 h at 28°C. After incubation, the colonies were counted and the agar was air dried to a thin film with warm blowing air (45°C) for 4 to 6 h. The dried agar films were stained with 300 µl of a 1:75 dilution in 25% Ringer's saline–0.1% Tween 20 of FITC-conjugated polyclonal antibod-ies specific for 89B-27 under the described conditions (16). After staining for 36 h at 4°C, the films were rinsed three times for 1 h in 25% Ringer's saline–0.1% Tween 20. The stained preparations were viewed and counted with a light microscope equipped with incident blue light and an FITC filter system. A random sample of 20 IFC-positive colonies from each treat-

ment at each sampling date was isolated, purified, and stored at −80°C prior to cluster analysis of GC-FAME profiles (total *n* = 720 in both years). The specificity of the antiserum for 89B-27 was confirmed by comparing profiles of IFC-positive colonies to 10 profiles of 89B-27.

The initial populations of all three introduced bacteria on the seeds were 5.5 ± 0.5 log CFU/g of seed in 1994 and 6.8 ± 0.4 log CFU/g of seed in 1995 as measured by spiral plating and IFC. There were no significant differences between plating methods for each treatment. Initial populations on the seeds after planting on day 0 were not significantly different among bacterial treatments, but populations of all bacterial treat-ments were significantly greater than initial populations of the methylcellulose control (4.6 and 5.0 log CFU/g of seed in 1994 and 1995, respectively). Total bacterial rhizosphere popula-tions on 5% TSBA-plus-cycloheximide spiral plates were not significantly different from total bacterial populations in 5% TSBA-plus-cycloheximide plates (Fig. 1A and B). Total pop-ulations declined during the growing season from 7.3 to 6.5 log CFU/g (fresh weight) of root in 1994 and 8.0 to 7.0 log CFU/g (fresh weight) of root in 1995. Total rhizosphere bacterial populations did not significantly differ among treatments at any sampling time in either year (Fig. 1B and D), although rhizosphere populations were significantly greater in 1995 than 1994 at all comparable sampling dates. The detection limits ranged from 1.5 ± 0.3 to 2.8 ± 0.2 log CFU/g (fresh weight) and 1.2 ± 0.3 to 2.0 ± 0.1 log CFU/g (fresh weight) for spiral plating and IFC, respectively, depending on the sampling time.

In 1994, very few bioluminescent colonies were detected visually on 50% TSBA-plus-cycloheximide spiral plates and none were detected visually on 5% TSBA-plus-cycloheximide
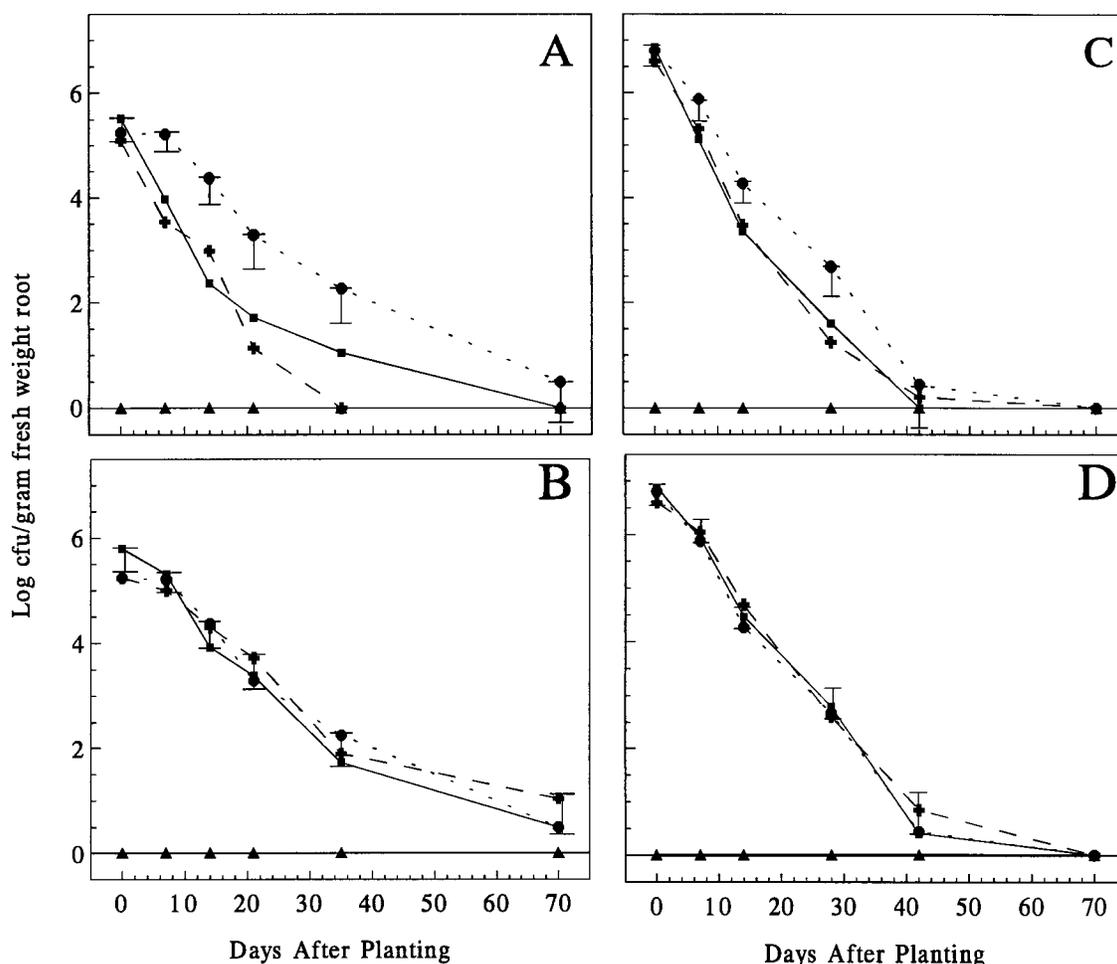
FIG. 2. Populations of introduced bacteria in the cucumber rhizosphere during 1994 (A and B) and 1995 (C and D): methylcellulose control (▲), 89B-27 (●), GEM-8 (✚), and R34 (■). (A and C) Populations of introduced bacteria determined by plating on 5% TSBA plus cycloheximide and rifampin for R34, plating on 50% TSBA plus cycloheximide and counting bioluminescent colonies for GEM-8, and IFC. (B and D) Populations of all introduced bacteria determined by IFC. Populations for the methylcellulose control were determined by using all of the above detection methods. The bars represent Fisher's protected least significant difference ($P = 0.05$) from the wild-type population measured by IFC.

plates. However, when colonies from 50 and 5% TSBA-plus-cycloheximide spiral plates with morphologies similar to the bioluminescent colonies were tested in the luminometer, light emission was detected. GEM-8 populations were determined by counting visibly bioluminescent colonies and by selecting individual colonies with morphologies similar to the wild type (89B-27) and testing for emission of light quanta. GEM-8 populations always were significantly greater on 50% TSBA than on 5% TSBA for spiral plating but not significantly different by the IFC plating technique. GEM-8 populations as determined by bioluminescence and tetracycline resistance were also significantly lower than those determined by IFC (data not shown).

The short incubation time and chilling of plates in 1995 appeared to improve the detection of bioluminescent colonies, since most colonies were detected visually as opposed to with the luminometer as in 1994. In both years, there were individual plates with no visually detectable bioluminescent colonies. However, when bacteria scraped from the whole plate were examined for light emission, bioluminescence was detected from some plates. All of these plates were from the 35-, 42-, or 70-DAP sampling times, when populations of introduced bac-

teria were near the detection limits of culturing methods. For statistical analyses, these plates were scored as having five bioluminescent colonies.

Underestimation of GEM-8 populations could be due to competition from other bacteria for nutrients. Light production by luciferase is dependent on two main components: availability of reduced flavin mononucleotide ($FNMH_2$) and the amount of aldehyde available to act as an electron receptor for reducing $FNMH_2$ (25). Both of these components would be affected by the nutritional status of individual cells. Competition between colonies on 5% TSBA could have reduced the amounts of available nutrients, causing a decrease in the metabolic activity of the cells and reduction in the amount of available $FNMH_2$, thus decreasing light production. However, even if $FNMH_2$ is readily available, light production is still dependent on the amount of aldehyde present in the system. In this study, limiting amounts of aldehyde were overcome by the addition of n-decyl aldehyde, but competition for nutrients could still be a factor. This conclusion is supported by observations that bioluminescent colonies were not visually detected on 5% TSBA plus cycloheximide but were detected on the more nutrient-rich medium 50% TSBA plus cycloheximide,

that the use of shorter incubation times in 1995 increased the number of bioluminescent colonies detected visually while decreasing colony crowding on plates, and that bioluminescent bacteria were detected from 5% TSBA-plus-cycloheximide plates by using a luminometer but were not detected visually. All of these observations suggest that nutrient availability probably affected the detection of bioluminescent colonies, resulting in the observed underestimation of GEM-8 by counting bioluminescent colonies.

No IFC-positive colonies were observed from the 10^{-1} to 10^{-8} dilutions of methylcellulose-treated controls, representing millions of colonies for each experimental unit. In addition, all isolates of IFC-positive colonies ($n = 720$) removed from samples treated with strain 89B-27 or its variants were grouped with 89B-27 below a Euclidean distance of 2 by cluster analysis of GC-FAME profiles, indicating that they were identical to 89B-27 (22). All IFC-positive colonies isolated from R34 and GEM-8 treatments also expressed their respective phenotypes.

In 1994, populations of R34, as measured by growth on 5% TSBA plus rifampin, were significantly lower than populations of the wild type, as measured by IFC, at 7, 14, 21, and 35 DAP (Fig. 2A). This trend also was observed for GEM-8 (Fig. 2A). Differences between the unaltered wild type and the genetically modified variants generally were greater than 1 log CFU/g (fresh weight) of root for all sampling times except 0 and 70 DAP. However, populations of GEM-8 and R34 were not significantly different from those of the wild type when populations of the genetically modified variants were determined by the IFC technique (Fig. 2B). This phenomenon also was observed in 1995 (Fig. 2C and D), when the populations of R34 and GEM-8, as measured by rifampin resistance and bioluminescence, respectively, were significantly lower than the populations of the unaltered wild type at 7, 14, and 28 DAP. However, population differences usually were less than 1 log CFU/g (fresh weight) of root. Thus, differences in populations from 5% TSBA plus rifampin and those determined by IFC appear to be related to the presence of rifampin in the medium.

The population data, as measured by IFC, indicate that there was no loss of ecological fitness when *P. fluorescens* 89B-27 was genetically modified either by selecting for spontaneous resistance to rifampin or by introduction of Tn*4431*. However, bacterial populations of the two genetically modified variants estimated from phenotypic markers (resistance to rifampin or bioluminescence) were significantly lower than populations estimated by IFC (Fig. 2), indicating that the survival and distribution of 89B-27 were underestimated in population determinations based on rifampin resistance or bioluminescence.

Similar phenomena have been observed in other systems (6, 15, 26, 30), where introduced bacteria have been shown to be nonculturable on media amended with antibiotics or other selective agents. Some viable bacterial cells from environmental samples are thought to be nonculturable because environmental stresses have resulted in altered cellular physiology or injury (5, 18) so that the bacteria can no longer tolerate the stresses resulting from culture on selective media. This phenomenon has been observed in both soil and plant systems (17, 19, 20, 30), where large differences between direct viable cell counts and those determined by plating on selective media were seen. None of these studies, however, directly examined whether the supposedly viable but nonculturable cells could be cultured on media without selective agents.

These data indicate that with careful characterization, genetic variants that are similar in ecological fitness to the unaltered wild-type parent can be selected. It has previously been reported that genetically modified variants are as ecologically

fit as the parent strain (9, 13, 14), but this hypothesis has not specifically been tested before by comparing variants to the unaltered wild-type strain under field conditions. Although the marked strains in this study did not appear to be less ecologically fit, the methods for recovering them significantly underestimated their population size in the environment. Thus, the use of such markers can result in an underestimation of population sizes, potentially influencing conclusions drawn by using them in ecological studies.

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