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Genetically Engineered *Erwinia carotovora*: Survival, Intraspecific Competition, and Effects upon Selected Bacterial Genera

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Environmental use of genetically engineered microorganisms has raised concerns about potential ecological impact. This research evaluated the survival, competitiveness, and effects upon selected bacterial genera of wild-type and genetically engineered *Erwinia carotovora* subsp. *carotovora* to ascertain if differences between the wild-type and genetically engineered strains exist in soil microcosms. The engineered strain contained a chromosomally inserted gene for kanamycin resistance. No significant differences in survival in nonsterile soil over 2 months or in the competitiveness of either strain were observed when the strains were added concurrently to microcosms. For reasons that remain unclear, the engineered strain did survive longer in sterilized soil. The effects of both strains on total bacteria, *Pseudomonas* and *Staphylococcus* strains, and actinomycetes were observed. While some apparent differences were observed, they were not statistically significant. A better understanding of the microbial ecology of engineered bacteria, especially pathogens genetically altered for use as biological control agents, is essential before commercial applications can be accomplished.

Genetic engineering is being used to develop microorganisms that can perform a variety of commercial tasks. Application of genetically engineered microorganisms (GEMs) has been limited so far to small-scale trials because of a lack of knowledge about survival and impact on ecosystems and because of public concern over release. A need exists for development of risk assessment methodologies to predict environmental risk, if any, prior to release. Before this can be accomplished, however, several parameters relevant to potential environmental risk should be determined and their importance to risk assessment should be assessed (3, 4, 24).

Several factors must be considered before GEMs are released into the environment in large quantities. Survival of an organism under laboratory conditions does not ensure success in natural ecosystems. Conversely, the ability to contain and control species in the laboratory does not preclude their establishment, survival, and adverse effects in the field. Because of their potential to increase in numbers in the environment at the site of release or, following transport, at some unintended and unexpected location, GEMs present unique regulatory problems (3). GEMs have the potential to compete with and displace native populations, thereby affecting the normal processing of materials in soils and sediments (3, 16).

None of these potential adverse effects has been realized thus far (16). Seven authorized GEM releases (11) have produced no unanticipated effects; in fact, these organisms have all declined in concentration after release, often faster than investigators anticipated. However, all of these releases have involved the use of organisms that have not had substantial DNA sequences inserted into their genomes. There is also disagreement about the nature of GEMs used in ecological studies and about the environmental fate of the recombinant DNA they contain. Many researchers have used models that have plasmids containing recombinant DNA, even though plasmids are more promiscuous in regard to DNA exchange than are bacteria containing chromosomal alterations or nonconjugative plasmids (23).

Erwinia carotovora subsp. *carotovora* is a phytopathogenic enterobacterium that causes soft rot in tissues of many plants and is associated with plants in soil and aquatic habitats (6, 7, 13, 17). Although this bacterium is found in soil and water, it is likely not an indigenous inhabitant of those niches but rather a contaminant derived from plant tissue (22). In this study, *E. carotovora* was engineered to include an antibiotic resistance gene in its chromosome. This genetic alteration is useful as a marker for ecological and epidemiological studies and as a model for evaluating the relative importance of several parameters that may indicate the potential environmental risks of putative genetically engineered biological agents derived from *E. carotovora*. The objectives were (i) to determine *E. carotovora* survival in sterile and nonsterile soil, (ii) to ascertain intraspecific competitiveness between the engineered strain and its wild-type parent, and (iii) to determine any effects that engineered *E. carotovora* may have upon populations of selected bacterial genera.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. carotovora* subsp. *carotovora* EC14, engineered as previously described (2, 19), was used in this study. A spontaneous mutant of wild-type *E. carotovora* resistant to 150 μg of rifampin per ml (strain L-863) was used in this study to aid in isolation; L-833 is a strain engineered from wild-type *E. carotovora* by partially removing in vitro an extracellular pectate lyase gene on a plasmid (pDR40) by deletion mutation, inserting a DNA fragment from Tn*903* conferring kanamycin resistance, and chromosomally inserting, by gene replacement, part of the plasmid (pVS41) into the chromosome (19); and strain L-864 is a spontaneous rifampin (150 μg/ml)-resistant mutant of L-833. Chromosomal insertion was confirmed by demon-

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TABLE 1. Soil characteristics of Hayter loam soil before inoculation and at day 46 after inoculation

Sample	pH	Concn (ppm) of:							% Organic matter
		P	K	Ca	Mg	Mn	Zn	NO_3-N	
PRE[a]	5.6	19	138	876	128	39	2.3	13	3.7
GEM[b]	5.4	17	119	852	128	22	3.4	30	3.0
MUT[c]	5.4	18	124	864	129	22	2.4	33	3.1

[a] Pretest soil sample.
[b] Postexperiment soil from microcosm inoculated with genetically engineered *E. carotovora*.
[c] Postexperiment soil from microcosm inoculated with wild-type bacteria.

strating that the constructed strain, L-833, no longer contained the plasmid (pVS41) and that the kanamycin resistance gene from Tn*903* was present: Southern hybridization of genomic DNA showed that the gene was contained in an *Eco*RI fragment not present in plasmid pVS41 (D. R. Orvos and G. H. Lacy, unpublished data). It is unlikely that the insertion is due to transposition by Tn*903*, since the inverted repeats flanking the kanamycin resistance gene required for transposition were removed prior to construction of pVS41 (V. K. Stromberg and G. H. Lacy, unpublished data). Both L-863 and L-864 displayed growth and biochemical characteristics similar to those of the wild-type strain.

Media and chemicals. Plate count agar (PCA; Difco Laboratories, Detroit, Mich.), diluted 1:10, was used to enumerate total bacteria. This dilution was chosen on the basis of preliminary experiments for maximum colonies recovered (data not shown). PCA amended with 40 μg of kanamycin monosulfate per ml and 150 μg of rifampin per ml (Sigma Chemical Co., St. Louis, Mo.) was used to detect the genetically engineered and wild-type *E. carotovora*, respectively. Actinomycete isolation agar (Difco) was used to estimate the density of actinomycetes; mannitol salt agar (Difco) was used to enumerate *Staphylococcus* strains and other salt-tolerant bacteria; and pseudomonas isolation agar (Difco) was used to isolate *Pseudomonas* spp.

Microcosms. Terrestrial microcosms consisted of 1-liter glass mason jars containing 200 g of soil. Humidified sterile air was circulated at 5 ml/min through the microcosm, minimizing evaporation of soil moisture while allowing for elimination of metabolic by-products. Both intake air and exhaust air were sterilized by membrane filtration with a Maxi Capsule (Gelman Sciences, Inc., Ann Arbor, Mich.). Soil in microcosms was gently mixed every other day to ensure a homogeneous distribution of microorganisms. This study utilized soil from the Virginia Polytechnic Institute and State University farm at Whitethorn, Va. Soil was a no-till Hayter loam type previously planted with corn, was dark grayish brown in color, and consisted of 39% sand, 41% silt, and 20% clay. After collection, soil was air dried for 48 h and passed through a 4.75-mm-pore-size sieve. Other soil characteristics are listed in Table 1.

Microcosm inoculation. *E. carotovora* was grown in 4 liters of nutrient broth to an optical density of approximately 1.0 at 550 nm. Bacterial cells were harvested by centrifugation at 10,000 × *g* for 20 min. The supernatant was discarded, and the pellet was washed with and suspended in sterile distilled water and centrifuged at 10,000 × *g*. This pellet was suspended in sterile distilled water, and 15 ml of inoculum was delivered to each microcosm (200 g of soil) to provide approximately 10^8 cells per g of soil. Inoculated soil was passed through a 2-mm-pore-size sieve, mixed, and randomly plated into microcosms. Microcosms were incubated at 20°C.

Sampling of microcosms. Samples were withdrawn from microcosms at various intervals, depending on the parameters under study. For survival and structural effects studies, samples were taken on days 0, 1, 3, 6, 12, 24, 42, and 46 after inoculation. Competition study samples were withdrawn from microcosms as follows: on days 0, 2, 4, 6, 10, 15, 20, and 25 for 1:1 competition experiments; on days 0, 2, 5, 10, and 15 for other competition studies; and on days 0, 2, 5, 10, 16, 20, 40, and 55 for sterile-soil experiments.

To allow for diffusion of inocula, day 0 samples were withdrawn approximately 2 h after inoculation. After the soil in the microcosms was mixed, samples (1 g) were randomly removed and placed in milk dilution bottles containing 99 ml of sterile distilled water. These were mixed, first manually for a brief period and then for 20 min on an orbital shaker at 150 rpm, and subsequent dilutions were prepared. Dilutions were plated onto appropriate media by using the spread plate technique. Inoculated plates were incubated at 25°C (PCA, actinomycete isolation agar) or 30°C (PCA with antibiotics, pseudomonas isolation agar, mannitol salt agar) for 48 to 96 h, and plates with appropriate numbers of colonies were counted. Soil for determination of water content was removed at the time of sampling, and results were expressed as CFU per gram of dry weight.

Two selection strategies were used for isolation. In survival studies, strain L-864 (Kan^r, Rif^r) was selected on media containing both antibiotics to increase the sensitivity of detection (700 CFU/g of soil). In competition studies, strains L-833 (Kan^r) and L-863 (Rif^r) were selected separately on media containing the appropriate antibiotic.

Soil sterilization. Soil was sterilized by exposing it to 1.5 Mrad of gamma radiation from a ^{60}Co source over a 15-h period at the University of Virginia Reactor Facility, Charlottesville. Soil was checked for sterility by plating onto 1:10 PCA and 1:10 nutrient agar; no growth was observed after incubation for 7 days.

Experimental design and statistical analysis. For survival and effects studies, three microcosms were inoculated with the genetically engineered (L-864) strain and three were inoculated with the wild-type (L-863) strain of *E. carotovora*. On each sampling day, one sample from each of the six microcosms was withdrawn and diluted. Three plates were prepared from each dilution, and plates with approximately 30 to 300 colonies were counted. Statistical significance between groups at each sampling day was evaluated by using Student's *t* test, and statistical significance between sampling intervals was evaluated by using a repeated-measures analysis (20). Statistical analysis was accomplished with SAS (18).

Competition experiments were conducted in soil containing (i) equal concentrations (1:1) of engineered (L-833) and wild-type (L-863) bacteria, (ii) 10 times as many engineered as wild-type bacteria, and (iii) 10 times as many wild-type as engineered bacteria. Survival in sterile soil was evaluated in radiation-sterilized soil inoculated with either the engineered or the wild-type strain of *E. carotovora*. For each competition study, six microcosms were inoculated with bacteria. Experiments with sterile soil used three microcosms with the genetically engineered strain and three with the wild-type strain.

RESULTS

Survival. Populations of genetically engineered and wild-type *E. carotovora* declined at significantly ($P \leq 0.05$) different rates over the duration of the experiment; at days 1,

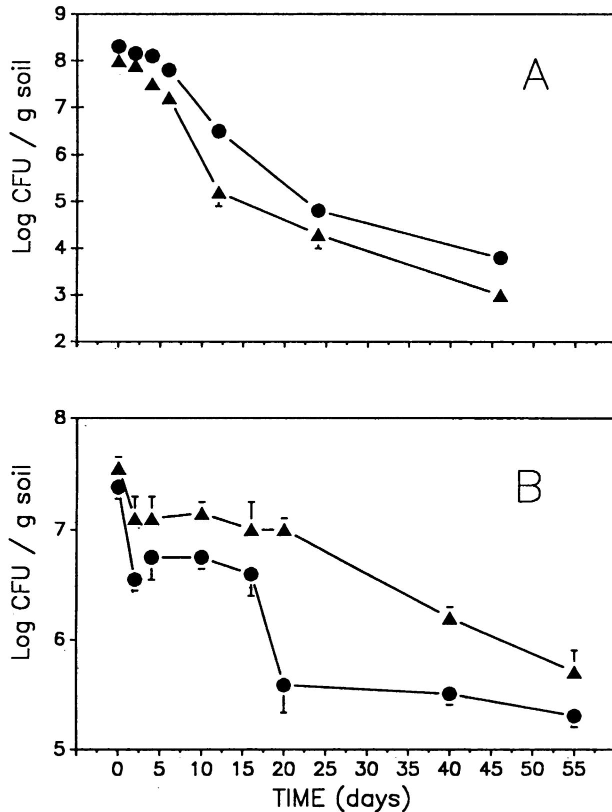

FIG. 1. (A) Survival of genetically engineered (L-864) (●) and wild-type (L-863) (▲) *E. carotovora* in soil microcosms. (B) Survival of genetically engineered (L-864) (▲) and wild-type (L-863) (●) *E. carotovora* in radiation-sterilized soil. Error bars represent 1 standard deviation unit and are included in the symbol when not shown.

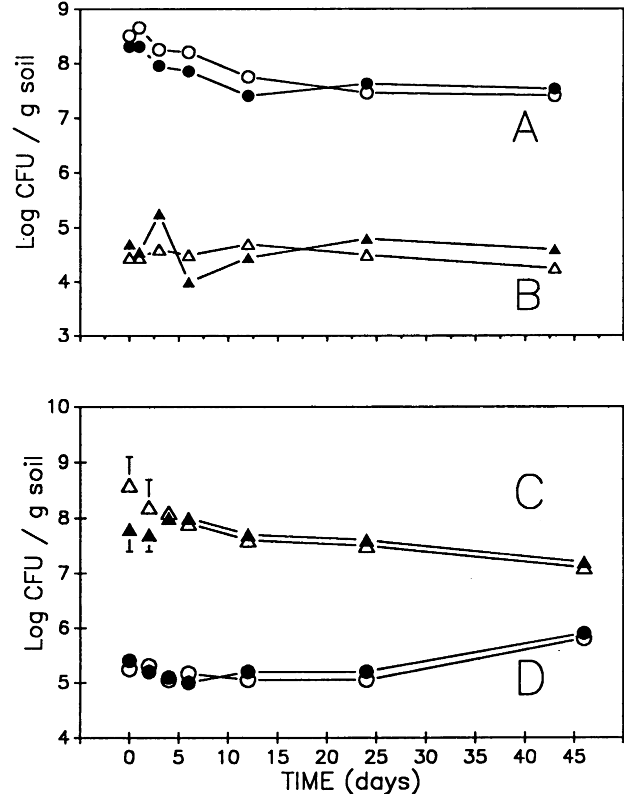

FIG. 2. Populations of total bacteria (A), pseudomonads (B), actinomycetes (C), and salt-tolerant bacteria, including *Staphylococci* spp. (D), in microcosms inoculated with genetically engineered (L-864) (solid symbols) and wild-type (L-863) (open symbols) *E. carotovora* in soil microcosms. Error bars represent 1 standard deviation unit and are included in the symbol when not shown.

3, 6, 12, and 46, genetically engineered *E. carotovora* (L-864) was present at a significantly lower concentration ($P \leq 0.05$) than L-863 (wild type) (Fig. 1A), although initial concentrations were not statistically different. For both strains, the decline was fastest early in the experiment and then it leveled off. The threshold of detection for both the engineered and the wild-type strain was approximately 700 CFU/g of soil.

Structural effects upon selected genera. Populations of total bacteria remained constant in both treatment groups and declined only 1 order of magnitude during the study (Fig. 2A). Significant decreases in populations were observed between days 1 and 3 and between days 6 and 12. There were no significant differences in initial densities, and no difference due to group effect ($P > 0.05$) was observed.

Densities of *Pseudomonas* spp. (Fig. 2B), actinomycetes (Fig. 2C), and *Staphylococcus* spp. (Fig. 2D) were not significantly different in microcosms inoculated with either strain ($P > 0.05$). While *Pseudomonas* spp. and actinomycetes experienced significant declines of 0.5 and 1 order of magnitude, respectively, a significant increase in densities of *Staphylococcus* spp. in both test groups was observed between days 24 and 46; these concentrations probably include other salt-tolerant species in addition to *Staphylococcus* spp. Initial and final populations of *Staphylococcus* spp. (Fig. 2D), *Pseudomonas* spp. (Fig. 2B), and actinomycetes (Fig. 2C) were not significantly different ($P > 0.05$). Populations of these bacteria in control microcosms inoculated with sterile water showed no significant changes over time (data not shown).

Survival of *Erwinia* strains in sterile soil. Both strains of *E. carotovora* survived longer in radiation-sterilized soil (Fig. 1B) than they did in nonsterile soil. In the absence of competitors, numbers of *E. carotovora* declined only 2 orders of magnitude over 55 days. Longer survival in sterile soil was expected, since less competition for resources probably occurs.

Intraspecific competition. Numbers of both engineered and wild-type *E. carotovora* declined at similar rates when added in equal (Fig. 3A) or unequal (Fig. 3B) proportions to the same microcosms. No significant difference between strain densities was observed on sampling days ($P > 0.05$), but overall decline over time was significant ($P \leq 0.05$). The observed decline curve differed from that of either the wild-type or the engineered strain (Fig. 1A) when they were added separately to microcosms, in that the characteristically large initial decrease followed by a smaller rate of decrease was not observed. Instead, decline was linear, with density diminishing about 2 orders of magnitude every 10 days.

DISCUSSION

Survival and competition are interrelated and often used interchangeably in microbial ecology. However, while survival is an important parameter in predicting the risk of

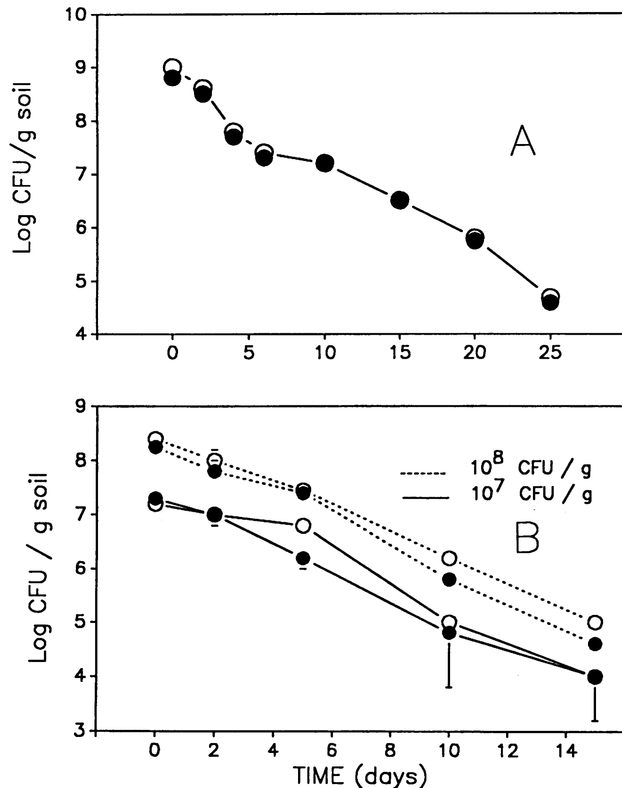

FIG. 3. (A) Intraspecific competition of genetically engineered (L-864) (○) and wild-type (L-863) (●) *E. carotovora* in nonsterile soil when added concurrently to soil microcosms in even amounts (1:1). (B) Intraspecific competition of genetically engineered (L-864) and wild-type (L-863) *E. carotovora* in nonsterile soil when added concurrently to soil microcosms in varied amounts. Symbols: (●), 10:1 ratio of engineered strain to wild-type strain; (○), 10:1 ratio of wild-type strain to engineered strain. Error bars represent 1 standard deviation unit and are included in the symbol when not shown.

releasing GEMs, competition of the introduced bacterium with its parental strain is another way to ascertain if the GEM possesses an enhanced ability to survive. In addition, removing competitors by substrate sterilization can also provide conditions that favor GEM survival (16). This observation raises the possibility that this GEM will survive longer in perturbed habitats than the wild type.

The population of the genetically engineered strain of *E. carotovora* declined significantly faster than that of the wild-type mutant over the duration of the experiment, even though both had identical growth curves in nutrient broth, tryptic soy broth, and minimal media. The reason for this is not clear, but it may reflect inherent variability from the sampling technique rather than an actual difference in rate of decline, since no difference was seen at day 24. It is possible, however, that insertion of the kanamycin resistance gene into the GEM conferred a competitive disadvantage not observed in growth curves or competition experiments (Fig. 3). Engineered organisms will probably be less fit than the parent strains (16, 24), as has been observed in previous studies (16); this is especially true for those with transposon introduction (F. J. Brockman, M.S. thesis, Washington State University, Pullman, 1988). Regardless, it is important to note that survival of the genetically engineered strain was not enhanced compared with that of the wild-type strain; this was expected on the bases of the phenotypes and genotypes of both introduced strains. Even at high initial concentrations characteristic of those associated with rotting plant tissue (5) and potential commercial application, the added bacteria, regardless of origin, were not able to compete with indigenous oligotrophic bacteria. This has been observed with wild-type *E. carotovora* (6) as well as with other species of genetically engineered (9, 12) and nonengineered (8) bacteria released into soil systems. The lower detection limits for both genetically engineered and wild-type strains were approximately 10^3 CFU/g; this correlates well with results of other studies (19).

Proliferation of nonresistant indigenous microbes on antibiotic-fortified media was noted when high inoculum levels were used; detection limits may indeed be even lower if these problems are overcome. Indigenous microbes did not grow on plates containing rifampin at dilutions less than 10^{-3}; however, kanamycin by itself was not found to be a satisfactory marker antibiotic, since some indigenous soil bacteria, particularly *Streptomyces* spp., were already resistant to it.

Total populations of bacteria experienced a significant increase between days 0 and 1 in both microcosm groups (Fig. 2A), probably because of added water and organic matter, in the form of bacteria, from the inoculum. The small decrease could be a reflection of cropping by nematodes and protozoa, though no conclusive evidence has been obtained (6). Although no one medium will allow growth of all indigenous bacteria (15), a 1:10 dilution of PCA allowed accurate sampling of a subset of total bacteria. The number sampled remained consistent throughout the experiment and therefore allowed determination of the effect of *E. carotovora* release on this subset.

Numbers of actinomycetes in both microcosm groups declined significantly ($P \leq 0.05$) over time, although the decline was slower in microcosms containing engineered *E. carotovora* (Fig. 2C). The initial populations in both treatment groups differed significantly (by an order of magnitude), which may partially explain that finding. Actinomycetes may have increased to high levels while soil was air dried and then declined to equilibrium in moist soil.

Pseudomonas spp. declined by only 0.5 order of magnitude during the study. No significant treatment effects were observed, but some group-time interactions were noted (Fig. 2B). Increased variability was also noted among pseudomonads compared with the other bacteria sampled, possibly because of fluctuations in moisture or nutrients in the microcosms.

Staphylococcus spp. and other salt-tolerant species were found to have stable populations over time but experienced a significant increase in both treatment groups between days 24 and 46 (Fig. 2D). No similar increase was seen in any of the other selected genera, and it is believed that this increase may have been due to the increased moisture content of the soil or to an increase in nutrients because of dead *E. carotovora*.

The slow declines in numbers of total bacteria, *Pseudomonas* spp., and actinomycetes, as well as the small increase in *Staphylococcus* spp. and other salt-tolerant species, reflect a microcosm that permitted indigenous populations of bacteria to remain viable during the course of the experiment. Lack of other confounding factors, such as plant and environmental influences, permitted as to directly determine if the genetically engineered bacterium affected these important groups of soil microorganisms in a manner different from that of the wild type. Each of these groups, as well as the total populations, is important within its own ecological

niche, is easy to monitor, and may be indicative of organisms that are affected by the addition of an exogenous bacterium.

An increase in nitrogen by-products and a decrease in soil organic matter (Table 1) may have contributed to the large reduction in numbers of exogenous bacteria as well as to the slow decline of indigenous species. Even with the small reduction in organic matter and possible accumulation of nitrogen metabolites, this microcosm design appeared to be satisfactory for preliminary studies regarding risk assessment of GEMs released into the environment. By removing extraneous confounding factors, direct determination of GEM effects upon microbial communities is possible. In addition, GEMs and their wild-type parents survived for over 40 days after being inoculated into microcosms at high concentrations. Perturbation of structural parameters (such as effects on selected bacterial genera or changes in diversity indices) as well as potential genetic exchange would be expected to occur within this period. Finally, since the addition of the *E. carotovora* itself represents a nutrient source (up to 1 mg of bacteria per g of soil at 10^9 CFU; 10^7 CFU of *E. carotovora* per ml is equal to approximately 2.2 μg of ash-free dry weight [V. S. Scanferlato, unpublished data]), bacteria may have effects upon various functional parameters of an ecosystem. These functional effects should also manifest themselves within this period, and they are being evaluated in our laboratory since they may have stimulated the increase in nitrogen by-products and the reduction in organic nitrogen.

Inoculating microcosms with concurrent, varied doses of engineered and wild-type *E. carotovora* did not affect survival. Both strains declined in concentration at similar rates regardless of whether the concentration of the engineered strain was 10 times higher or 10 times lower (Fig. 3B) than that of the wild type. However, the rate of decline was higher in microcosms receiving mixed concentrations of bacteria (Fig. 3B) than in microcosms receiving the same concentration (1:1) of each strain (Fig. 3A). This could be due to the higher number of bacteria added to microcosms in 1:10 and 10:1 competition studies as opposed to the 1:1 competition study. The higher-density bacteria would utilize nutrients at a higher rate. Microbial competition in nature is difficult to understand because of its complexity. However, intraspecific competition is easily determined in the laboratory and in field test situations. It is an important parameter in risk assessment, since it may indicate if the engineered strain will survive longer than its parental wild type. However, field validation of results (3) must be executed, since laboratory observations may not correlate with field data. Meade et al. (14) found that indigenous strains of *Rhizobium leguminosarum* competed successfully against inoculant strains in the field but demonstrated no difference in competitiveness in the laboratory.

An engineered strain must compete successfully with oligotrophic microbes in an oligotrophic environment if its desired commercial effect is to be achieved. To do this, however, introduced microbes must displace, at least temporarily, indigenous organisms already occupying a niche (1); otherwise, they are of no commercial value. This displacement may be transient. Introduction of *Rhizobium* spp. has provided some data for interspecific competition (21). In addition, Lindow (9) studied intraspecific competition between engineered (Ice^-) and wild-type (Ice^+) strains of *Pseudomonas syringae*. Lindow found that ice^- strains inoculated on leaf surfaces effectively inhibited ice^+ strains by competitive exclusion (10). However, it appears that the vast majority of mutations will impart a competitive disadvantage rather than an advantage (16), especially transposon insertions (Brockman, M.S. thesis). In this study, gene insertion did not affect the ability of *Erwinia* spp. to compete.

Environments that lack potential predators and perturbed systems may be more susceptible to GEM invasion than was first thought (1, 3, 16). In this study, engineered and wild-type *E. carotovora* survived longer in sterile soil because of a lack of competition for available nutrients and a lack of predators. However, the concentration of *E. carotovora* did not increase during the experiments. Using protozoan models, other researchers have determined that the ability to resist invasion appears to be related to species richness and to the particular successional state of the ecosystem (3). Additional studies are warranted to determine if this also applies to GEMs.

In summary, although a simple microcosm design was employed, it yielded data regarding the survival and competitiveness of a GEM as well as effects upon selected bacterial populations without the presence of confounding factors. Future research will examine the effect of these confounding factors, such as vegetation and temperature fluctuations, on survival and structural effects as well as potential functional effects of different GEMs released into the environment. By studying the microbial ecology of this microbe as it is developed for commercial use, it is hoped that predictive models that forecast survival and effects under a variety of conditions may be developed.

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