Bacterial communities of the rhizosphere and endorhiza associated with field-grown cucumber plants inoculated with a plant growth-promoting rhizobacterium or its genetically modified derivative

W.F. Mahaffee and J.W. Kloepper

Abstract: The future use of genetically modified microorganisms in the environment will be dependent on the ability to assess potential or theoretical risks associated with their introduction into natural ecosystems. To assess potential risks, several ecological parameters must be examined, including the impact of the introduced genetically modified organism on the microbial communities associated with the environment into which the introduction will occur. A 2-year field study was established to examine whether the indigenous bacterial communities of the rhizosphere and endorhiza (internal root tissues) were affected differently by the introduction of an unaltered wild type and its genetically modified derivative. Treatments consisted of the wild-type strain *Pseudomonas fluorescens* 89B-27 and a bioluminescent derivative GEM-8 (89B-27::Tn*4431*). Cucumber root or seed samples were taken 0, 7, 14, 21, 35, and 70 days after planting (DAP) in 1994 and 0, 7, 14, 28, 42, and 70 DAP in 1995. Samples were processed to examine the bacterial communities of both the rhizosphere and endorhiza. Over 7200 bacterial colonies were isolated from the rhizosphere and endorhiza and identified using the Sherlock System (Microbial ID, Inc.) for fatty acid methyl ester analysis. Community structure at the genus level was assessed using genera richness and Hill's diversity numbers, $N1$ and $N2$. The aerobic–heterotrophic bacterial community structure at the genus level did not significantly vary between treatments but did differ temporally. The data indicate that the introduction of the genetically modified derivative of 89B-27 did not pose a greater environmental risk than its unaltered wild type with respect to aerobic–heterotrophic bacterial community structure.

Key words: diversity, ecology, PGPR, *Pseudomonas*, root colonizaton, GEM.

Résumé : L'emploi éventuel, dans l'environnement, de microorganismes modifiés génétiquement dépendra de la capacité d'évaluer les risques potentiels ou théoriques associés à leur introduction dans les écosystèmes. Pour évaluer les risques potentiels, plusieurs paramètres écologiques doivent être examinés, dont l'impact que de tels microorganismes modifiés génétiquement pourront avoir sur les communautés microbiennes associées à l'environnement où il se fera une introduction. Une étude au champ de 2 années a été poursuivie pour examiner si les communautés bactériennes indigènes des rhizosphères et les endorhizes (tissus racinaires internes) ont été affectées différemment par l'introduction d'un type indigène inaltéré et par celle de son dérivé modifié génétiquement. Les traitements ont comporté l'utilisation d'une souche de type indigène de *Pseudomonas fluorescens* 89B-27 et d'un dérivé bioluminescent GEM-8 (89B-27::Tn*4431*). En 1994, des échantillons de graines ou de racines de concombre ont été prélevés à 0, 7, 14, 21, 35 et 70 jours après l'ensemencement (JAE) et, en 1995, à 0, 7, 14, 28, 42 et 70 JAE. Les échantillons ont été traités de façon à examiner les communautés bactériennes tant des rhizosphères que des endorhizes. Plus de 7200 colonies bactériennes ont été isolées des rhizosphères et des endorhizes, lesquelles furent identifiées à l'aide du système Sherlock (Microbial ID, Inc.) pour l'analyse des esters méthylés d'acides gras. La structure communautaire au niveau du genre a été établie, utilisant la richesse des genres et les nombres de diversité de Hill, $N1$ et $N2$. La structure de la communauté bactérienne aérobie–hétérotrophe n'a pas varié de façon significative au niveau du genre entre les traitements, mais elle différé dans le temps. Les données indiquent que l'introduction de dérivés de 89B-27 modifiés génétiquement ne présente pas un risque environnemental plus graud que son type indigène inaltéré par rapport à la structure bactérienne aérobie–hétérotrophe communautaire.

Mots clés : diversité, écologie, PGPR, *Pseudomonas*, colonisation des racines, GEM.
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W.F. Mahaffee[1] and J.W. Kloepper. Department of Plant Pathology, Alabama Agriculture Experiment Station, Biological Control Institute, Auburn University, AL 36849, U.S.A.

[1] Author to whom all correspondence should be sent at the following address: Horticulture Crops Research Laboratory, Agriculture Research Station, U.S. Department of Agriculture, 3420 NW Orchard Avenue, Corvallis, OR 97330, U.S.A. (e-mail: mahaffew@bcc.orst.edu).

Introduction

Assessment of the environmental impact associated with the introduction of a genetically engineered microorganism into a habitat requires assessment of multiple ecological parameters (e.g., colonization patterns, survival, spread from target area, etc.) under field conditions (Tiedje et al. 1989). One component to the assessment of the risks of using genetically engineered bacteria is the effect on the indigenous fauna and flora (Seidler 1992; Tiedje et al. 1989). The bacterial communities associated with the plant roots are extremely important in plant development and would most likely be disrupted by the introduction of a genetically modified, plant growth-promoting rhizobacteria (PGPR) to plant roots. Thus bacterial rhizosphere and endorhiza (root tissues below the epidermis (Mahaffee and Kloepper 1997)) communities could serve as models for developing techniques and methods for assessing alterations in community dynamics of indigenous biota associated with the introduction of a genetically modified rhizobacterial strain.

Recent work on the impact of genetically engineered microorganisms has focused on either changes of the metabolic functioning of microbial communities in vitro (Ellis et al. 1995; Vahjen et al. 1995) or differences in populations of specific bacterial genera (Orvos et al. 1990) in soil microcosms or greenhouse studies. The approach of examining metabolic functioning is based on the premise that microbial communities are very plastic (i.e., a large proportion of community members have similar metabolic functions in vitro). However, this approach does not account for possible differences in phenotypes expressed in vitro and those expressed in situ. While on the surface (i.e., in vitro), bacterial communities may appear to be plastic; they are probably far more rigid in situ because of the spatial scale of bacterial communities (Foster et al. 1988; Foster and Bowen 1982) and episodic activity of individual bacterial cells (Lee 1994). These properties of bacterial communities could allow for two species to utilize the same substrates without competition, since each strain may be either active under very different environmental conditions or be spatially separated, even though they colonize the same soil aggregate. The realized niche of bacterial species is very likely more restricted in situ than the theoretical niche observed in vitro. Thus, concentrating only on metabolic functioning in vitro could limit the understanding and conception of the potential environmental impacts of an introduced genetically modified microorganism. Therefore, in addition to developing and testing methodologies for examining community functioning, it would also be prudent to develop and test techniques for examining community structure based on taxonomic relationships.

One approach to assess community structure is the use of diversity indices and multivariate analyses. Richness and evenness indices have been used to describe plant and animal communities since their introduction almost 40 years ago (reviewed by Peet 1974) and are now being used to describe microbial communities (Banerjee and Anderson 1992; Boehm et al. 1993; Bolton et al. 1991; Mahaffee and Kloepper 1997; Workneh and van Bruggen 1994). Diversity indices and canonical discriminant analysis were used by Workneh and van Bruggen (1994) to show that rhizosphere communities of actinomycetes on tomato roots were more diverse in organically than conventionally managed rhizosphere soil. Species richness and evenness were used by Banerjee and Anderson (1992) to detect differences in rhizoplane microflora, which resulted from fumigation and organic nutrient addition. Differences in diversity of fungal communities lasted for at least 17 months, including two growing seasons. Boehm et al. (1993) used polar ordination to demonstrate that the decomposition level of organic matter selected for rhizosphere bacteria that were more suppressive to *Pythium* damping off. Mahaffee and Kloepper (1997) demonstrated that richness, Hill's diversity numbers ($N1$ and $N2$), and the coefficient of biotic similarity can be used to detect structural changes in the soil, rhizosphere, and endorhiza bacterial communities associated with cucumber plants. These studies indicate that changes in microbial communities can be detected using diversity indices and multivariate analyses.

A 2-year field study was established to examine whether aerobic–heterotrophic bacterial rhizosphere and endorhiza community structures were differentially affected by the introduction of a wild-type plant growth promoting rhizobacteria strain and its genetically modified derivative. This study was part of a larger 2-year field study on assessing methods for monitoring the environmental impact of introduced rhizobacteria in the field environment (Mahaffee 1996).

Materials and methods

Bacterial strains

The wild-type strain *Pseudomonas fluorescens* 89B-27 and its bioluminescent derivative GEM 8 (Mahaffee et al. 1997) were used. Strain 89B-27 has been shown to induce systemic resistance in cucumber (Liu et al. 1995*a*, 1995*b*, 1995*c*). GEM 8 is a bioluminescent derivative of 89B-27 with a chromosomal insertion of Tn*4431* (Shaw et al. 1987), which contains the promotorless operon of *Vibrio fischeri* and a gene for tetracycline (Tc) resistance. All bacteria were stored in tryptic soy broth (TSB; Difco) with 30% v/v glycerol at −80°C. Prior to each experiment, cultures were streaked for single colonies on 5% v/v TSB with 20 g agar/L (TSBA), incubated for 48 h at 28°C, and checked for purity. Seeds of cucumber cultivar Straight 8 were treated with a bacterial suspension in 2% methylcellulose (72 μL/g seed), which resulted in inoculum density of approximately 1×10^6 cfu/seed. Suspensions were prepared by growing cultures, taken from −80°C storage, on 5% TSBA at 28°C for 48 h. Bacteria were scraped from the surface of the agar after adding 10 mL of 0.1 M phosphate buffer (pH 7.0) (PPB). This suspension was centrifuged (8000 × *g*, 10 min) and the pellet was resuspended in 2% methylcellulose, yielding a concentration of approximately 1×10^8 cfu/mL.

Field experiment

Field plots were established in a sandy-loam field soil (pH 6.2, 0.7% organic matter, 98 kg P/ha, 202 kg K/ha, 684 kg Ca/ha, 104 kg Mg/ha) at the E.V. Smith substation of the Alabama Agricultural Experiment Station in 1994 and 1995 . Fertilization (6-0-0) was done every 7–10 days through drip irrigation at a rate of 9 kg/ha. Experiments were 2 × 6 factorials (bacterial strain × sampling time) designed as split plots with bacterial treatment as the main plot and sampling time as the subplot with six replications. Main plots consisted of two rows with 10 mounds per row spaced 90 cm apart in raised beds under plastic mulch with drip irrigation. Individual mounds were used as the subplots and randomly assigned for each sampling time. The field was maintained according to recommendations for commercial cucumber production by the Alabama Cooperative Extension Service. Treatments consisted of an unaltered wild-type strain 89B-27 and GEM 8 (89B-27::Tn*4431*). Five grams of seed were treated for each main plot and three seeds were planted

in each mound. Mounds were thinned to two plants per mound 7 days after planting (DAP) prior to sampling plots.

Plant samples were taken from field plots 0, 7, 14, 21, 35, and 70 DAP in 1994 and 0, 7, 14, 28, 42, and 70 DAP in 1995. A 15-cm-diameter soil core, centered on the plant stem or furrow, to a depth of 25 cm was removed from each sampled mound. The soil core was placed in a plastic bag to facilitate the dislodging of seed and root material. Any nonrhizosphere soil was removed by gently shaking seed or root systems after removal from the soil.

Enumeration and sampling of rhizosphere bacteria

Seed and root samples were weighed and processed for enumeration. For time 0, all seeds were placed in a test tube with 10 mL of 0.2 M potassium phosphate buffer (pH 7.0) (PPB) and processed as below. Root samples from 7 and 14 DAP samples were placed in test tubes with 10 mL PPB, root samples from 21 and 28 DAP were placed in test tubes with 20 mL PPB, samples from 35 DAP were placed in test tubes with 50 mL PPB, and root samples from 48 and 70 DAP were placed in bottles with 100 mL PPB. All samples were sonicated in a FS28 sonicator bath (Fisher Scientific) for 6 min to remove rhizosphere and rhizoplane microorganisms. Sonication treatment was optimized to yield the highest colony-forming units of bacteria on 5% TSBA from cucumber roots of varying ages (W.F. Mahaffee, unpublished). Sonicate was serially diluted and plated with a spiral plater (Spiral Systems, Bethesda, Md.) on 5% TSBA amended with 200 μL cycloheximide (Cm)/mL. Plates were incubated for 48 h at 28°C prior to hand counting.

Root samples were removed from sonicate and rinsed under running tap water, prior to surface disinfestation in 40 mL of 1.05% NaClO with 0.1% Tween 20. During disinfestation, each sample was shaken at 350 rpm for 3 min and then rinsed three times in sterile PPB (McInroy and Kloepper 1995). Surface-disinfested root samples were triturated for 1 min in a Kleco tissue pulverizer (Kinetic Laboratory Equipment Company, Visalia, Calif.) with 1 mL PPB, and then serially diluted and spiral plated as above. Efficiency of surface disinfestation was checked by plating 1.0 mL of the final rinse buffer onto 5% TSBA and incubating at 28°C for 72 h (McInroy and Kloepper 1995). Samples with observable growth after 72 h of incubation were removed from data analysis.

Community structure

Random samples of 35 or 25 bacterial colonies were picked from 5% $TSBA^{Cm}$ plates of the same dilution and streaked for purity on TSBA (Mahaffee and Kloepper 1997). A single colony of each isolate was then transferred to 96-well plates with 50 μL TSBA and incubated for 48 h prior to overlaying with 75 μL of TSB with 30% v/v glycerol. All 96-well plates were stored at −80°C until extraction. Fatty acid methyl esters (FAMEs) were extracted from each isolate using standard and recommended procedures for gas chromatographic (GC) FAME analysis (Sasser 1990). After extraction, samples were stored at −20°C until analysis with a Hewlett–Packard gas chromatograph and the Sherlock Microbial Identification System software using the aerobe method and TSBA library version 3.9 (MIDI, Newark, Del.). Identification at the genus level was used and isolates with a similarity index below 0.200 were considered as "no match" and grouped together.

After the isolates were identified, richness, diversity, and similarity at the genus level were used to compare bacterial rhizosphere communities among treatments over time. Richness was determined by direct counts of the numbers of genera identified in each sample. The total number of genera, as well as number of isolates of each genus, were used to determine genera diversity (Hill 1973; Ludwig and Reynolds 1988). Hill's first and second diversity numbers, $N1$ and $N2$, were used to estimate genera diversity. $N1$ ($e^{H'}$) is a modification of Shannon's index $H' = -\Sigma[(n_i/n)\ln(n_i/n)]$ and $N2$ ($1/\lambda$) is a modification of Simpson's index $\lambda = -\Sigma[n_i(n_i - n)]/[n(n - 1)]$. For both equations, n_i is the number of individuals of the i^{th} genus and n is the total number of individuals in the sample (Hill 1973). $N1$ is an estimate of the abnndant genera and $N2$ is a measure of the very abundant genera.

Statistical analysis

Population data were converted to log(cfu/g fresh weight tissue), with populations below the detection limits registered as 0 for calculation of means (Kloepper and Beauchamp 1992). Analysis of variance for population and diversity estimates was done using the MANOVA procedure in PC-SAS (SAS Institute, Cary, N.C.). Significant differences ($P = 0.05$) were determined using single degree of freedom contrasts.

Results

Enumeration and sampling of rhizosphere bacteria

The aerobic–heterotrophic bacterial populations of the indigenous rhizosphere and endorhiza microflora did not significantly differ between the unaltered wild-type strain and its genetically modified derivative in either 1994 or 1995 field seasons (Fig. 1). Bacterial populations of the rhizosphere were significantly greater than the endorhiza in both years and did not significantly vary at any sampling time except in 1994 for 0 DAP. The seed populations 0 DAP were significantly lower than subsequent rhizosphere populations. Rhizosphere populations did not significantly vary between the unaltered wild type and GEM-8. Endorhiza populations were significantly greater at the end of the season than early in the season. Populations of the wild type and GEM-8 have been reported previously (Mahaffee et al. 1997) and did not significantly vary in either 1994 or 1995.

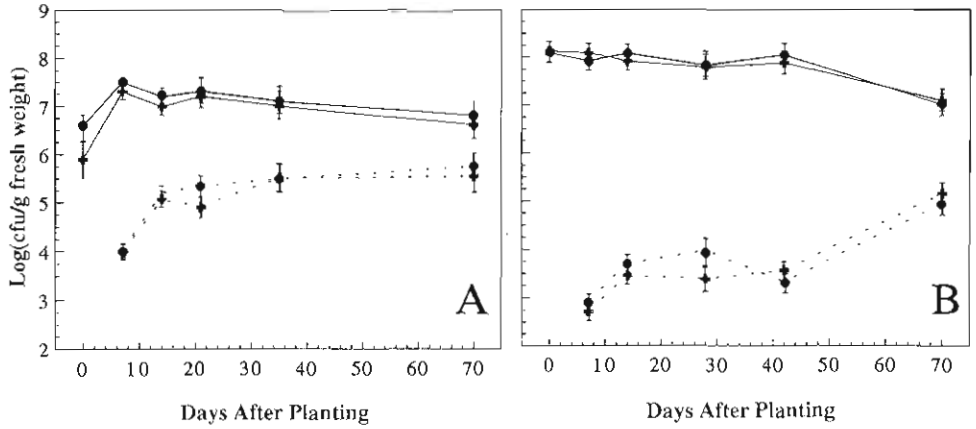
Community structure

In total, 7200 individual bacterial isolates were identified to the genus level, 4200 for the rhizosphere and 3000 for the endorhiza (Tables 1 and 2). For both 1994 and 1995, analysis of the factorial for the rhizosphere community resulted in no bacteria × sampling time interactions for richness, $N1$, and $N2$. Only the main factor of sampling time was significantly varied for all three indices. Single degree of freedom contrasts were still used to compare the wild type and GEM-8 at each sampling time for both years. Genera richness, $N1$, and $N2$ for the rhizosphere communities were not significantly different ($P = 0.05$) between the wild-type and GEM-8 treated plants (Fig. 2) in either 1994 or 1995. However, there was a trend to significance ($P > 0.1$) at 14 and 21 DAP in 1994 for $N2$ (Fig. 2C) but not for genera richness or $N1$. There was more variation associated with the estimates of bacterial community structure in 1995 than in 1994, because of a large number of rare genera isolated (less than three times during the course of the season) in 1995 (Tables 1 and 2).

Analysis of the main factor of sampling time showed that there were similar temporal trends in rhizosphere community structure, even though there were differences in the timing of community shifts between the 2 years. In both years, genera richness, $N1$, and $N2$ significantly increased, and then decreased and increased until the last sampling time.

Analysis of the endorhiza data was similar to that of the rhizosphere in that no significant bacteria × sampling time interaction was observed. Only the main factor of sampling time was significantly varied in both years (Fig. 3). However, as above, single degree contrasts were still used to

Fig. 1. Total bacterial populations of the rhizosphere (—), and endorhiza (- - -) habitats over time from plants inoculated with wild-type 89B-27 (●) and its genetically modified derivative GEM-8 (+). (A) 1994; (B) 1995. Error bars represent ± SD.



compare between the wild type and GEM-8. No significant differenees were observed in either year for genera richness, $N1$, or $N2$.

Analysis of the main factor of sampling time showed that the diversity of the endorhiza was significantly greater later in the growing season than early in the season. In 1994 genera richness, $N1$, and $N2$ were significantly greater at 21 and 35 DAP (but not 70 DAP) than at 7 and 14 DAP, while in 1995 genera richness, $N1$, and $N2$ for all sampling times were significantly greater than 7 DAP. In addition, 28 and 70 DAP were significantly greater than 14 and 42 DAP.

Discussion

There were no differences at the genus level in the aerobic–heterotrophic bacterial community structures of the rhizosphere and endorhiza from field-grown cucumber plants treated with either the wild-type 89B-27 or its genetically modified derivative GEM-8. These results suggest that the introduction of the luciferase operon and a gene for tetracycline resistance into 89B-27 did not cause the genetically modified derivative GEM-8 to alter the community structure of the rhizosphere and endorhiza differently from the wild type. This does not mean, however, that the introduction of less innocuous genetic material (e.g., genes for antibiotic or siderophore production) may not cause changes in the microbial structure, nor does it iudicate that other bacterial strains, modified in the same manner, wonld not have an effect on the indigenous microflora. Since significant differences were observed in the temporal changes of the rhizosphere and endorhiza bacterial communities from field-grown cucumber roots, these results support reports (Boehm et al. 1993; Mahaffee and Kloepper 1997; White et al. 1994; Workneh and van Bruggen 1994) tbat indices for measuring community structure can be used to detect differences in bacterial communities.

The results presented in this study appear to contradict those of White et al. (1994), where the soil bacterial community was initially altered after the introductiou of a bioluminescent strain of *P. fluorescens* compared with the wild type in soil microcosms. The contradiction may be a result of basing the calculation of diversity on colony morphology, which could have biased the estimation of bacterial diversity. Although there are several reports that colony morphology is sufficient for examining diversity (Frederickson et al. 1991; Haldeman and Amy 1993; Herman et al. 1994), they are based on limited isolations (18–198 isolates). Our experience in identifying over 20 000 bacterial isolates to species using FAME analysis (Mahaffee and Kloepper 1997; Mahaffee et al. 1996; McInroy and Kloepper 1995; Press et al. 1995, 1996) indicates that colony morphology is not reliable for determining taxonomic relationships. We have observed that several bacterial genera and species have indistinguishable colony morphologies, particularly Gram-positive bacteria, and that colony morphologies of a particular species vary greatly in regards to the colony density on the isolation medium. Thus, using colony morphology to distinguish bacterial groups would most likely cause cousiderable variation in accuracy of diversity estimates.

The field data presented here on the effects of a genetically engineered bacterium introduced into the soil environment agree with reports from soil microcosms or greenhouse-grown plants (Ellis et al. 1995; Orvos et al. 1990; Vahjen et al. 1995). Ellis et al. (1995) reported that the metabolic profiles of microbial communities from sugar beet rhizosphere samples were not affected hy the introduction of a fluorescent pseudomonad strain (SBW25) genetically modified with two gene cassettes encodiug β-galactosidase, kanamycin resistance, and catechol-2,3-dioxygenase. Vahjen et al. (1995) reported that the introduction of *Corynebacterium glutamicum* ATCC 13032, genetically modified with plasmid pUN1 encoding production of aprotinin, into soil microcosms of two different soils had no effect on the microbial community in one soil and only a transient effect in the other soil. The transient effects on microbial communities were only observed when the introduced genetically modified bacterium occurred at popnlations of 10^5 cfu/g soil. The introduction of kanamycin resistance in *Erwinia carotovora* into soil microcosms did not affect populations of actinomycetes, pseudomonads, or salt-tolerant bacteria (Orvos et al. 1990). These reports indicate that genetic modification of soil and rhizosphere bacteria with genctic material encoding antibiotic resistance or novel marker phenotypes do not cause long-term effects on microbial communities. There is still the possibility that the introduction of less innocuous genes (i.e., antibiotic production, toxin production, substrate utilization)

Table 1. The number of baeterial isolates per genus from the rhizosphere of cucnmber plants inoculated with cither 89B-27 or GEM-8 at different days after planting.

	No. of isolates[a]																			
	1994										1995									
	89B-27					GEM-8					89B-27					GEM-8				
Genus[b]	7[c]	14	21	35	70	7	14	21	35	70	7	14	21	42	70	7	14	21	35	70
Acidovorax		2	1	6	13			1	3	18	2		1	2	5	3	6	3	2	2
Acinetobacter		3	8	1	1	1		7	1	2		5	4	3	2	3	3	2	2	
Agrobacterium	1	11	7	2	1	5	5	1	1	5	4	15	6	30	20	5	12	13	39	28
Alcaligenes	2	4	1	8	10	4	2	4	4	7	4	4	5	1	4	4	2	1	5	1
Aquaspirillium																				
Arthrobacter	2	4	4	2	4	3	3	6	4	1	11	10	6	2	9	6	10	2	1	10
Aureobacterium		1	3	1	1	1	2	1	3	1	1	2	3	1	3		6	6	2	2
Azospirillium																				1
Azotobacter																2				
Bacillus	31	46	12	14	19	28	54	19	20	3	61	57	80	15	16	62	59	93	19	9
Bergeyella	1			1																1
Brevibacter															2					
Brochothrix						1														
Burkholderia	2	4	3	15	25	2	4	2	3	33	26	20	1	22	2	13	3	2	25	5
Cellulomonas	1	4	2	2	5		3	4	1	1	3	3			11		5	5	2	5
Chryseobacterium	31	14	11	10	25	24	25	16	8	26	11	17	18	7	8	7	14	5	3	6
Chryseomonas			1					1												
Clavibacter				1	1			1		2			10		15			2	1	17
Comamonas	3		4	1	3	1	1	2	5	4	1	5	2	6	10		8	1	2	2
Corynebacterium	2	2	2	1	3			1		2	1	1	5		2					1
Curtobacterium			5	2	1				1	1		2	6		10		2		1	7
Cytophaga			3	2	8	2	2		3	7	4	4	1	5	1		3	3	2	2
Enterobacter	57	49	51	8	4	52	55	77	7	1		7	2	6		1	5		7	
Erwinia				1		1		1		2				1						
Escherichia					1									5						
Exiguobacterium			1						1	1										
Flavimonas		1	5											3						
Flavobacterium				1	3	1	1			6		1		4			1			1
Gluconobacter									1	1							1			1
Hydrogenophaga		8		1	10			1		13							1			
Janthinobacterium		3															1			
Klebsiella		1		1	1		2		3	1										
Kurthia	1		1			1	2			1						1	1			
Leuconostoc								1												
Listeria																	1			
Methylobacterium		1						1							2	1				
Microbacterium												3								
Micrococcus	1	2	4	4	4	2	4	3	2	1	2	6	9	1	10	5	3	8	2	13
Nocardia																1				
Ochrobactrum												1			1		1			
Oerskovia		1					1				4	4		5	4		1	1		5
Paenibacillus	1	8	2	1		2	1	2	3							2	14		1	10
Pantoea	1	1	13	1	4			4	4	3	8	7	1	2	1	5			1	
Phyllobacterium												2		1	1					
Provdencia											3					2				
Pseudomonas	39	19	41	86	35	29	19	31	80	50	45	10	14	56	19	71	21	15	65	28
Rathyibacter													1		1					
Serratia				3	2			1		1			1				10			
Sphingobacterium					2						7	9	1		2	2	3	8	1	1
Sphingomonas		1					1		1			1		1	1				1	
Staphylococcus		1									1		3		1					5
Stenotrophomonas	22	11	12	28	5	41	7	29	35	5	3	4	14	12	13	9	3	18	14	11
Variovorax										2	3	1	3	4		3	2			
Weeksella				2						3		1		3						
Xanthobacter			1															2	2	
Xanthomonas		1	2		2		2		1			3		3	2		1	4	3	3
No match[d]	12	8	10	4	15	11	13	5	10	14	4	5	13	9	32	2	6	16	6	33
Total No. of isolates	210	210	210	210	210	210	210	210	210	210	210	210	210	210	210	210	210	210	210	210

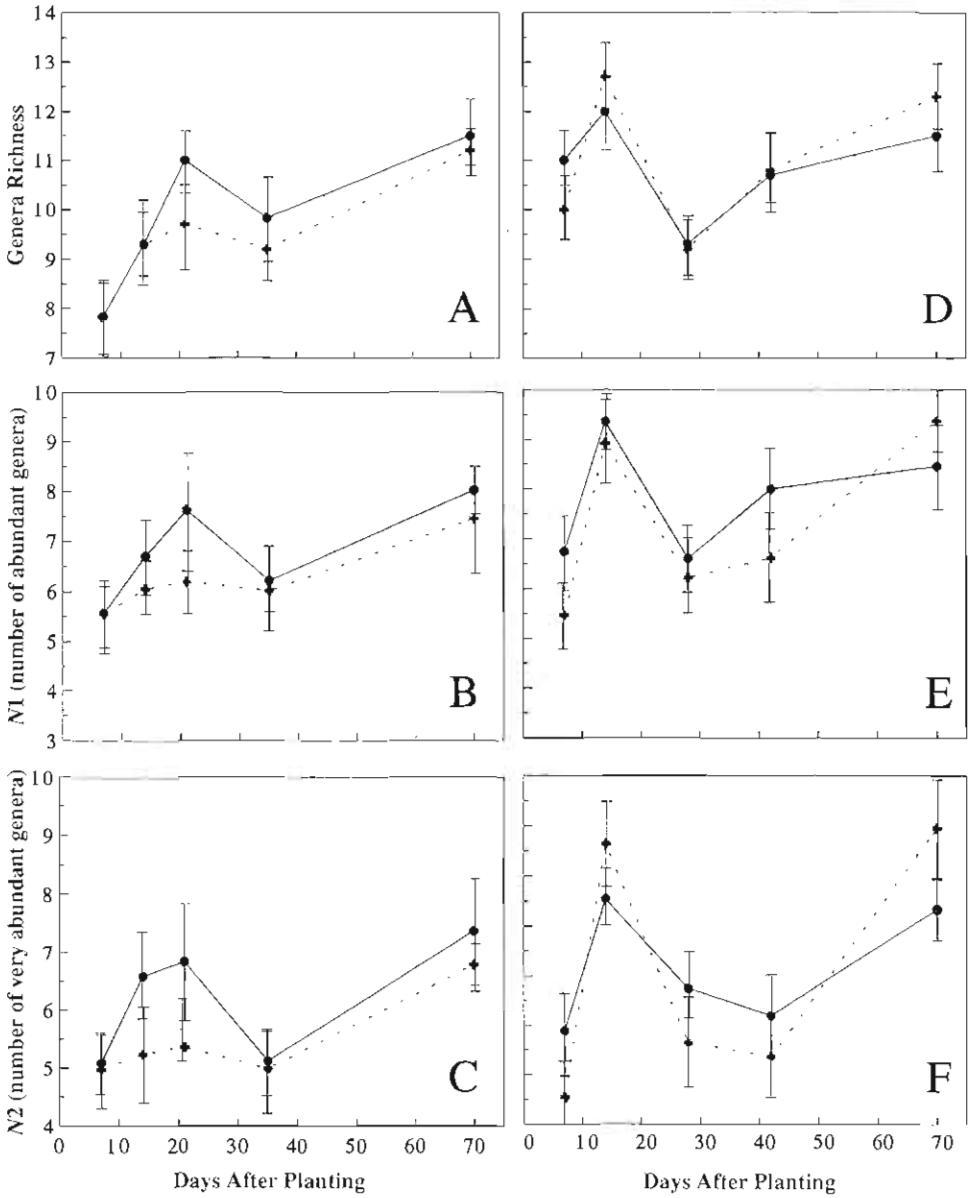
[a]The number of isolates identified per genus for each sampling time pooled over six replications.
[b]Bacterial isolates identified to genus based on FAME analysis and Microbial ID software and TSBA library 3.9.
[c]Days after planting.
[d]Bacterial isolates not identifiable using TSBA library 3.9.

Table 2. The number of bacterial isolates per genus from the endorhiza of cucumber plants inoculated with either 89B-27 or GEM-8 at different days after planting.

	No. of isolates[a]																			
	1994										1995									
	89B-27					GEM-8					89B-27					GEM-8				
Genus[b]	7[c]	14	21	35	70	7	14	21	35	70	7	14	21	42	70	7	14	21	35	70
Acidovorax							2		2	8			1							
Acinetobacter		3		1	7			1	1	3		1	2	1			3	5		2
Agrobacterium		6	7	23	4	9	2	10	15	24		24	23	45	22	5	26	26	45	20
Alcaligenes										1		1	6	7	2			5	15	
Ancyclobacter			1																	
Arthrobacter			1					1			5	1	1	1	1		1	1		
Aureobacterium								1	1				1	3	4		1	3	1	
Bacillus	55	3	37		11	57	2	28	1	10	98	44	45	12	14	90	40	52	17	18
Bergeyella							1		5											
Burkholderia				2	20		11	3		6	8	17		5	1		13		3	
Cellulomonas				2	1	2			11	7		1		1	9	3	1		1	6
Chryseobacterium	15	1	8	62	31	20	5	25	52	27	5	5	12	37	5	7	5	16	35	4
Chryseomonas			1																	
Clavibacter			1		4			2			4	1	7	1	5					
Comamonas		1								3		3								
Corynebacterium									1	2	2	1	3	4		2	1	9		3
Curtobacterium			1										5	1	2		1	3		1
Cytophaga			1	1	20				5	17										
Enterobacter	62	70	61	5	1	46	60	58		9		15	10	4	7		6			5
Erwinia															7		1			
Flavimonas				1																5
Flavobacterium			3																	
Hydrogenophaga									6											
Janthinobacterium									5											2
Kurthia						1			1		2									1
Methylobacterium															1					
Microbacterium											1	1			1			2		5
Micrococcus	9					4		1	4		1	1	1	4	5		2	7	2	1
Nocardia												1						2		
Ochrobactrum		1				1			1		3	2	6	1	1			1		2
Paenibacillus	6		1		1	6	1	3	1		4	8	3	5	4	2	14	1	5	17
Pantoea			2	1	2			4					3	2	1				5	
Phyllobacterium				1									5	5	2			2	1	
Pseudomonas		49	20	16	36	1	55	12	4	21	15	5	1		19	34	13	2		24
Rathyibacter														1						1
Serratia																		1		2
Sphingobacterium									6				1	1	1	2	2			
Staphylococcus														5	2	2				1
Stenotrophomonas	1	12	3	31	6			6	22	5		12	10		25		12	6	11	21
Variovorax										1						1		1		4
Weeksella																			3	5
Xanthobacter									1			1	4		2		2		5	
Xanthomonas				3	1				2		1	2			1	2	1	4	1	
No match[d]	4	2	2	2	4	5	3	1	3	6	1	3	1	4	6		4	2		
Total No. of isolates	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150	150

[a]The number of isolates identified per genus for each sampling time pooled over six replications.
[b]Bacterial isolates identified to genus based on FAME analysis and Microbial ID software and TSBA library 3.9.
[c]Days after planting.
[d]Bacterial isolates not identifiable using TSBA library 3.9.

Fig. 2. Temporal changes in three measures of bacterial rhizosphere community structure from cucumber plants treated with 89B-27 (●) and GEM-8 (+). (A–C) 1994 field trial; (D–F) 1995 field trial; (A, D) genera richness; (B, E) *N*1: Hill's modified Shannon's diversity index; (C, F) *N*2: Hill's modified Simpson's diversity index. See Materials and methods for details. Error bars represent ± SD.



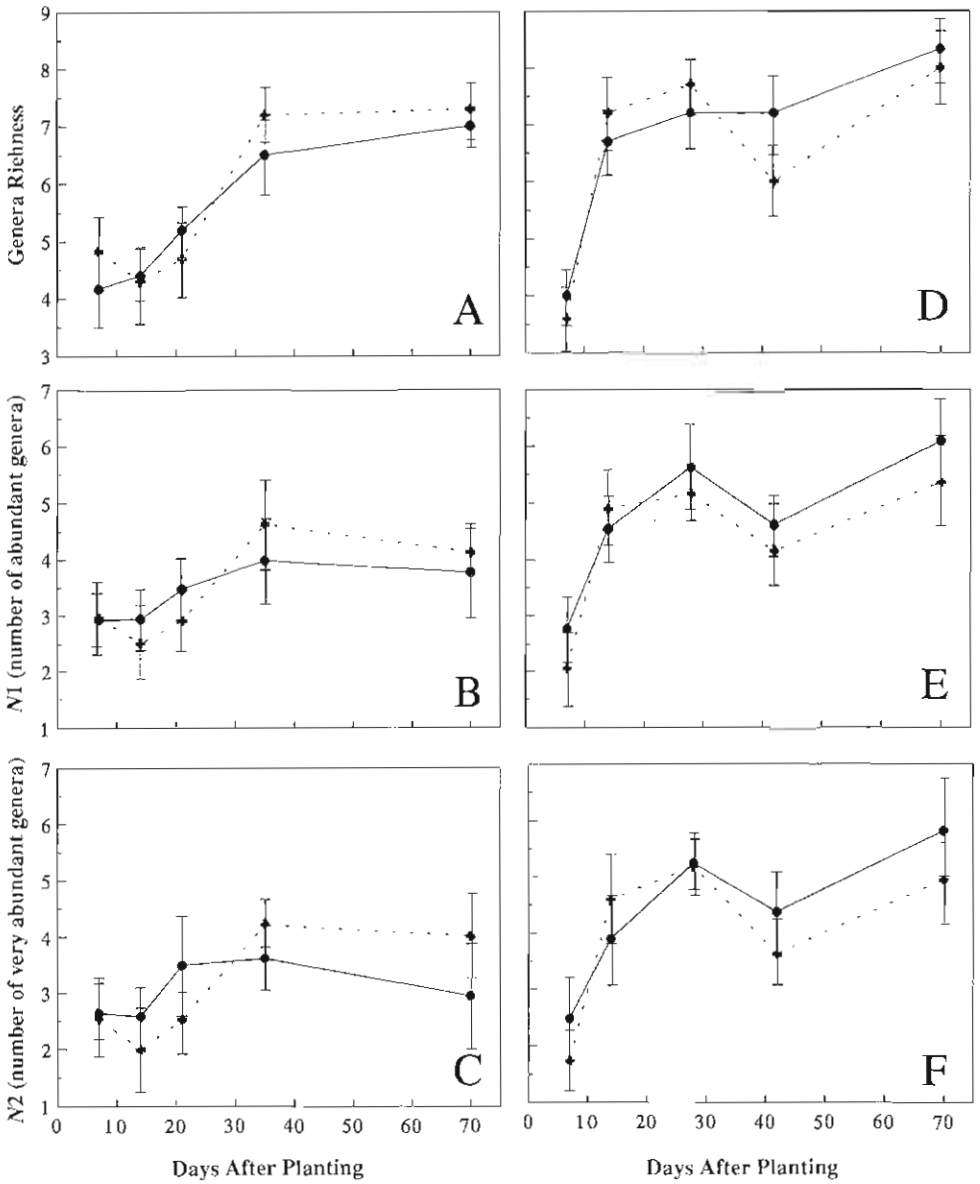
could have substantial effects on the microbial communities. However, it should be pointed out that the purpose behind introducing and genetically modifying biological control agents is the alteration of microbial communities through alteration of the survival, growth, or proliferation of at least one member of that community.

There were a large number of rare genera isolated from the endorhiza and rhizosphere in both 1994 and 1995 field samples (Tables 1 and 2). In several samples of the rhizosphere and endorhiza, there were several genera with only one isolate, and there were a large number of genera that were only isolated once or twice in each year. The occurrence of these rare genera appeared to cause the estimates of community structure to be larger than expected (Hill 1973; Ludwigg and Reynolds 1988). This sensitivity to rare genera is most likely related to the relatively small sample sizes employed in this study.

The increase in rare genera also could be related to the disruption of the natural community structure that may have developed in the absence of an introduced rhizobacterial strain. Ecological theory states that when a disruptive force is placed on a community, the diversity of that community initially increases and its membership becomes highly variable before reaching a new equilibrium (Petratis et al. 1989). This phenomenon has been observed in plant, insect, protozoa, and algae communities (Brown 1993; Muotka and Virtanen 1995; Palmer et al. 1995; Peterson and Stevenson 1992) and could account for some of the variability observed in this study.

A possible shortcoming of this work is that the bacterial

Fig. 3. Temporal changes in three measures of bacterial endorhiza community structure from cucumber plants treated with 89B-27 (●) and GEM-8 (+). (A–C) 1994 field trial; (D–F) 1995 field trial; (A, D) genera richness; (B, E) *N*1: Hill's modified Shannon's diversity index; *N*2: Hill's modified Simpson's diversity index. See Materials and methods for details. Error bars represent ± SD.



community structures are based on the heterotrophic bacteria that are culturable on 5% $TSBA^{Cm}$ in an aerobic environment. There are numerous reports indicating that culturing on artificial media underestimates the bacterial populations (0.01–1% of direct cell counts) in soil and other environments (Torsvik et al. 1990; Wilson and Lindow 1997), but the relationship of this underestimation of populations to representation of total number of species is unknown. It is very probable that a number of species are not isolated on artificial media, just as it is possible that a large number of bacterial species are not detected using molecular techniques that were developed for extracting DNA from Gram-negative bacteria. Based on observations that colony-forming units are usually aggregates of one to three bacterial cells (Olsen and Bakken 1987) and that only a portion (0.02–60%) of the population of a bacterial species or introduced isolate is recoverable on artificial media (Wilson and Lindow 1997), the percentage of bacterial species represented on artificial media could be 0.017–150% of the species present in the soil. Obviously, the real value is somewhere between these two extremes. Nevertheless, this subset of bacteria is ecologically significant in that there are numerous plant beneficial and detrimental bacteria that are culturable under the conditions used in this study (Balows et al. 1992).

The data reported indicate that the introduction of genes for bioluminescence and tetracycline resistance into strain 89B-27 did not result in the genetically modified derivative GEM-8 differentially affecting the aerobic–heterotrophic bacterial community structures at the genus level of field-grown cucumber roots. They also indicate that indices for

estimating community structure can be used to detect differences in the aerobic–heterotrophic bacterial communities of the rhizosphere and endorhiza habitats of cucumber roots under field conditions.

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