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Variable stability of antibiotic-resistance markers in *Bacillus cereus* UW85 in the soybean rhizosphere in the field

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Abstract

We compared the stability of antibiotic-resistance markers in strains derived from *Bacillus cereus* UW85 in culture media and in the soybean rhizosphere in a growth chamber and in the field. We studied two independent, spontaneous mutants resistant to neomycin, three independent, spontaneous mutants resistant to streptomycin, and strains carrying plasmid pBC16, which encodes tetracycline resistance. Antibiotic-resistance markers were maintained in populations of all UW85 derivatives in culture and in the rhizosphere of soybeans grown in soil in a growth chamber. In two field experiments, antibiotic resistance was substantially lost in rhizosphere populations of *B. cereus* as early as 14 or as late as 116 days after planting. To distinguish between death of the inoculated strain and loss of its marker, we tested populations of *B. cereus* for other phenotypes (orange pigmentation, plasmid-borne resistance to tetracycline, and biocontrol activity) that are typical of UW85-derivatives used as inoculum, but atypical of the indigenous populations of *B. cereus*, and these phenotypes were maintained in populations from which the marker was lost. In general, neomycin-resistance markers were maintained at a higher frequency than streptomycin-resistance markers, and maintenance of antibiotic-resistance markers varied with position on the root and with the year of the experiment. In a semi-defined medium, the UW85 derivatives grew at the same rate as the wild type at 28°C, but most grew more slowly than the wild type at 16°C, demonstrating that antibiotic resistance can affect fitness under some conditions. The results suggest that the stability of antibiotic-resistance markers should be assessed in the ecosystems in which they will be studied.

Keywords: *Bacillus thuringiensis*, ecology, genetic engineering, genetic selection, planned introduction

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Introduction

Concerns about risks associated with planned introductions of genetically engineered microorganisms into the environment are due, in part, to inadequate knowledge of microbial ecology (Levin & Harwell 1986; Tiedje *et al.* 1989). The debate has highlighted the lack of reliable methods for monitoring engineered and non-engineered microorganisms (McCormick 1986). DNA–DNA hybridization (Fredrickson *et al.* 1988; Holben *et al.* 1988), rDNA oligonucleotide probes (Stahl *et al.* 1988; Delong *et al.* 1989), immunofluorescence (Schmidt 1974; Tamplin *et al.* 1990), serology (Allan & Kelman 1977; Ohba & Aizawa 1978), β-galactosidase activity (Drahos *et al.* 1986; Höfte *et al.* 1990), and dilution plating on selective media (see Park & Lim 1985; Iswandi *et al.* 1987; Pettibone *et al.* 1987; Lindow *et al.* 1988) have been applied to the detection and enumeration of microorganisms in many types of ecosystems. Dilution plating on selective media is used extensively to study microbial populations, because plate counts can detect relatively small, culturable populations of a particular strain or species and numerous samples can be processed.

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Antibiotic resistance is one of the most common markers used to monitor the survival of genetically engineered and non-engineered bacteria introduced into soils (Temple *et al.* 1980; Liang *et al.* 1982; Mallory *et al.* 1982; West *et al.* 1985; Devanas *et al.* 1986; van Elsas *et al.* 1986; Acea *et al.* 1988; Compeau *et al.* 1988; Fredrickson *et al.* 1988; Trevors *et al.* 1990; Postma *et al.* 1991), aquatic habitats (Liang *et al.* 1982; Mallory *et al.* 1982; Pettibone *et al.* 1987; Scanferlato *et al.* 1989), rumens of animals (Flint *et al.* 1989), and onto plant leaf and root surfaces (Lai *et al.* 1977; Loper *et al.* 1984; Park & Lim 1985; Iswandi *et al.* 1987; Lindow *et al.* 1988; Scher *et al.* 1988; O'Brien & Lindow 1989; van Peer *et al.* 1990). Antibiotic-resistance markers can be obtained by mutations conferring resistance, acquisition of genes coding for resistance, or identification of intrinsic resistances.

Valid use of an antibiotic-resistance marker requires that it be stable. A marker can be expected to be stable in a population if its genetic transmission is faithful and if it does not affect fitness. Considerable data suggest that antibiotic-resistant mutants can be less fit than their wild-type parents in the environment (Pankhurst 1977; Jones & Bromfield 1978; Pettibone *et al.* 1987; Compeau *et al.* 1988). Antibiotic-resistance markers associated with reduced fitness will not be stably maintained in a bacterial population.

Stability of antibiotic-resistance markers is generally measured in culture (Liang *et al.* 1982; Mallory et al, 1982; Loper *et al.* 1984; Iswandi *et al.* 1987; Trevors *et al.* 1990; Postma *et al.* 1991) or in microcosms (Pankhurst 1977; Jones & Bromfield 1978; Pettibone *et al.* 1987; Compeau *et al.* 1988) before use of the marked strain in the field, yet few studies report measurements of stability in the field. The stability of spontaneous antibiotic-resistance mutations and intrinsic resistances in *Rhizobium* (which have been used to identify *Rhizobium* strains in nodules) has been examined in the field. Brockwell *et al.* (1977) reported that antigenic markers and spontaneous antibiotic-resistance markers in field isolates of *R. trifolii* were equally stable 4 years after inoculation, and Brunel *et al.* (1988) found that intrinsic resistances and antibiotic-resistance mutations in *Bradyrhizobium japonicum* were stable 8–13 years after inoculation. Lindström *et al.* (1990) have shown that after 5 years there was variability in intrinsic antibiotic-resistance profiles in two of 20 isolates of *R. galegae*. Although marker stability has been examined in various *Rhizobium* strains, only one (Brunel *et al.* 1988; Lindström *et al.* 1990) or two (Brockwell *et al.* 1977) antibiotic-resistant variants of each strain were examined. These studies emphasized marker stability in a few nodule isolates, which may not be representative of a population.

The objective of the study reported here was to measure the stability of antibiotic-resistance markers in populations of *Bacillus cereus* UW85 in the rhizosphere of field-grown soybeans. We examined the stability of five independent, spontaneous, antibiotic-resistance mutations (genomic markers) and the plasmid pBC16, which carries genes encoding tetracycline-resistance (plasmid marker) singly and in combination. The data suggest that antibiotic-resistance markers acquired by mutation or plasmids were lost from *B. cereus* UW85 populations in the field, that the loss was not predicted from laboratory experiments, and that loss of the markers was highly variable.

Materials and methods

Bacterial strains and isolation of antibiotic-resistant mutants

The strains used in this study and their relevant phenotypes are listed in Table 1. To isolate spontaneous antibiotic-resistant mutants, the wild-type parent *B. cereus* UW85 (ATCC 53522) was grown in Trypticase soy broth (TSB, BBL, Cockeysville, MD) to late exponential phase, and plated on media containing antibiotics (5-μg/ml neomycin, 20-μg/ml streptomycin). Mutants were considered to be independent if they were from cultures derived from separate single colonies. The orange-pigmented colony phenotype of strain UW85s1 arose from a spontaneous mutation independent of the mutation conferring streptomycin resistance. pBC16, a 4.2-kb plasmid that encodes tetracycline resistance (Battisti *et al.* 1985; Selinger *et al.* 1990), was transferred into the antibiotic-resistant mutants by the method of Battisti *et al.* (1985) from *B. cereus* strain 569 UM20-1 tr251-1. *B. cereus* 569 and 569 UM20-1 tr251-1 were a gift of C. B. Thorne (Department of Microbiology, University of Massachusetts, Amherst). Transconjugants were selected for tetracycline resistance (5 μg/ml), the appropriate genomic marker, and prototrophic growth on Min IC medium, a semi-defined medium (Green *et al.* 1985), containing 15-g/l noble agar (Difco Laboratories, Detroit, MI). We confirmed the presence of pBC16 in the antibiotic-resistant derivatives of UW85 by showing that each putative transconjugant contained plasmid DNA that co-migrated with, and had the same *Eco*R1 pattern in an agarose gel as pBC16.

Marker stability in culture

We used two methods to determine whether the antibiotic-resistance markers were maintained in populations of the UW85 derivatives in culture before initiating growth chamber or field experiments. The first method involved making 25 consecutive transfers of each UW85 derivative from solid media without antibiotics onto solid media with and without antibiotics. The second method involved growing the UW85 derivatives in TSB without antibiotics for approximately 100 generations and then plating onto media with and without antibiotics.

Determination of doubling times

Exponential growth rates of the wild-type UW85 and its antibiotic-resistant derivatives were measured by changes in the optical density of the culture at 600 nm. Fifty-millilitre Min IC cultures in 250-ml Erlenmeyer flasks were grown at 16 or 28°C on a rotary shaker at 170 r.p.m. The exponential doubling times presented are the means from three experiments.

Bacterial seed treatments

Seed treatments were prepared by coating soybean seeds (*Glycine max* L. Merr), cultivar AP-200 (Agripro Seeds, Ames, IA), with a paste of spores of the UW85 derivatives. This paste was obtained by inoculating a TSA plate with 1 ml of an overnight TSB culture, incubating the plate for 4 days at 28°C, and scraping the lawn of spores off the plates. Twenty-five seeds were mixed vigorously with the paste of spores obtained from one plate in a 50-ml conical centrifuge tube (VWR, Chicago, IL) and air-dried in a laminar-flow hood. *B. cereus* populations varied from 3.4×10^8 to 2.3×10^9 CFU per seed.

Growth-chamber experiments

Plants were grown in an unsterilized Joy silt-loam soil from Arlington, WI in 5-cm × 16-cm plastic cones (Cone-tainers, Canby, OR) in a growth chamber (24°C with a 12-h photoperiod and a light intensity of 244 μeinsteins/m/s). Plants were watered as needed. Three soybean seeds were placed in each cone and plants were thinned to one plant per cone 14 days after planting. The experimental design was a randomized complete block with two treatment factors: seed treatment and sampling time. Each combination of seed treatment and sampling time was assigned a single cone within a block, and each combination appeared once in each block. Seed treatments were UW85, the antibiotic-resistant derivatives, and an untreated control. Sampling times were 28 and 56 days after planting. The first and second growth chamber experiments consisted of 5 and 10 blocks, respectively.

Field experiments

Field experiments were performed at the University of Wisconsin Arlington Research Station, which is on a Joy silt-loam soil (fine-silty, mixed, mesic, aquic Hapludolls, pH 6.8). The 1987 and 1989 field experiments were planted on 19 and 24 May, respectively. The design for the 1987 field experiment was a randomized complete block with three seed treatments and three blocks. Seed treatments were UW85n1, UW85s1, and an untreated control. Seeds were spaced 15 cm apart in rows 7.6 m long and 76 cm apart. Seeds were planted at a depth of 2.5 to 4 cm. Within each block we sampled three plants from every treatment plot and all analyses were based on the average of the three plants. Sampling times were 0, 7, 14, 21, 28, 35, 49, 63, 91 and 126 days after planting.

The design for the 1989 field experiment was a randomized complete block with two treatment factors: seed treatment and sampling time. Each combination of seed treatment and sampling time was assigned a different plot within each of 8 blocks, and each plot consisted of 4 seeds spaced 15 cm apart and planted at a depth of 2.5–4 cm. Seed treatments were UW85n1, UW85n1-t, UW85n2, UW85n2-t, UW85s1, UW85s2, UW85s2-t, UW85s3, UW85s3-t, UW85-t, and an untreated control. Sampling times were 35, 83 and 116 days after planting. Within each block, we sampled one plant from every treatment plot. Samples were retrieved over two consecutive days, coinciding with groups of blocks. Rain interrupted the sampling 83 days after planting; for that sampling time, only four blocks were sampled.

Sample preparation and dilution-plating protocol

Population sizes of *B. cereus* were estimated by dilution plating sonicated samples of seeds, cotyledons, radicles, or 1- or 2-cm root segments taken from 0–1, 0–2, 2–3, 4–5 or 9–10 cm below the crown or from the last centimetre of the root or root tip (RT). The crown, which was marked on each plant as it was removed from the soil, is defined as the part of the plant at the soil–air interface. Most of the soil was shaken from the seed or root and the plant material was placed in either 5 ml or 10 ml of sterile distilled water; the aqueous suspension was sonicated for 15 s at 20% output with a 250-W Vibra-cell sonicator (Sonics and Materials, Danbury, CT) and then serially diluted in sterile distilled water. Aliquots (0.1-ml) of the dilutions were spread onto a semi-selective medium unamended or amended with one or more antibiotics.

Semi-selective media

The medium for the 1987 field experiment was half-strength TSA. For the 1989 field experiment, Min IC medium was used because fewer non-bacilli grew on it, thereby facilitating detection of *B. cereus*. Furthermore, when root samples were plated on both media, up to 20% more *B. cereus* grew on Min IC than on half-strength TSA plates. Both media contained 12.5-μg/ml polymyxin B-sulfate, 50-μg/ml ampicillin, and 100-μg/ml cycloheximide (Sigma Chemical Co., St Louis, MO). The antibiotics were included to reduce growth of other microorganisms; all *B. cereus* isolates that we screened from our collection from the field grew on the semi-selective media.

Identification and enumeration of B. cereus *in rhizosphere samples*

B. cereus colonies were identified by their distinctive colony morphology (large, flat, wrinkled, cream- or orange-coloured colonies) on half-strength TSA or Min IC. *B. anthracis* and *B. thuringiensis* have colony morphologies similar to *B. cereus* on the semi-selective media, which complicated detection of *B. cereus*. The haemolytic activity of *B. cereus* and *B. thuringiensis* (Parry *et al.* 1983; Claus & Berkeley 1986) was used to determine the proportion of the rhizosphere *B. cereus*-like population that was *B. cereus* and *B. thuringiensis*. *B. cereus*-like isolates obtained from treated and untreated roots (n=250 in 1987 and n=600 in 1989) were screened for haemolytic activity on blood-TSA plates. All isolates lysed red blood cells indicating that the total *B. cereus*-like population was comprised primarily of *B. cereus* and *B. thuringiensis*. Phenotypic and genetic analyses of *B. cereus* and *B. thuringiensis* (Baptist *et al.* 1978; Parry *et al.* 1983; Priest *et al.* 1988; Zahner *et al.* 1989) indicate that they should be placed in the same species, and therefore we did not attempt to distinguish between *B. cereus* and *B. thuringiensis*. In this paper, we will refer to the *B. cereus*-like population observed in the rhizosphere as *B. cereus*.

Total and antibiotic-resistant *B. cereus* colonies were counted after 2 and 3 days of incubation at 28°C, respectively. The CFU values of *B. cereus* per sample were $\log_{10}$-transformed, and expressed as mean $\log_{10}$ CFU. Prior to statistical analysis, for samples in which no *B. cereus* was detected, a value that was 10-fold less than the detection limit was assigned. The detection limit was 50 and 100 CFU per sample in the 1987 and 1989 field experiments, respectively.

Plating efficiency

To determine whether the indigenous bacterial community prevented growth of the mutants differentially on the semi-selective medium, we compared the CFUs from culture samples mixed with sonicated material from untreated roots from the 1989 experiment with the CFUs from samples of the same cultures mixed with sterile distilled water. Recovery of vegetative cells (500–5000 CFU) of UW85s1, UW85n1, or UW85n1-t was examined 35, 83 and 116 days after planting. Recovery of spores (275–6000 CFU) of UW85s1, UW85s3-t, or UW85-t was examined at 14 and 28 days after planting. The plating efficiency of vegetative cells and spores mixed with the sonicated material from roots was 83–100% of that obtained in sterile water, and did not differ between total and antibiotic-resistant populations of *B. cereus*. There were no detectable differences in plating efficiency among strains.

Statistical analysis

The SAS statistics computer package (SAS Institute Inc., 1985) was used for statistical analyses. In the 1987 experiment, for each sampling time and location on the root, population sizes of total and antibiotic-resistant *B. cereus* were each analysed as randomized complete blocks. To compare populations among treatments, a least significant difference (LSD; P=0.05) was calculated (Snedecor & Cochran 1980). We also performed t-tests for certain comparisons.

In the 1987 field experiment, a paired t-test was used to compare population sizes of total and antibiotic-resistant *B. cereus* for each sample. Because this experiment entailed multiple testing based on a repeatedly sampled plot, we used a Bonferroni correction (Snedecor & Cochran 1980; Miller 1981) whereby individual comparisons were judged significant at 0.0056, thus resulting in an overall error rate of P<0.05.

For the experiment in 1989, population sizes of total and antibiotic-resistant *B. cereus* were analysed as a split-plot experiment. In this analysis, seed treatments were whole-plot treatment factors, and selection on various semi-selective media was the sub-plot treatment factor. For each sampling time and location on the root, separate ANOVAs were performed to compare the population size of total *B. cereus* with the population size of *B. cereus* containing the genomic marker, the plasmid marker, and both the genomic and plasmid marker, and to compare populations with the plasmid or genomic marker with populations with both markers. Appropriate LSDs were calculated (Milliken & Johnson 1984) for comparisons among sub-plot treatment factors (e.g. comparisons among total and antibiotic-resistant populations) and whole-plot treatment factors (comparisons among treatments). An argument could be made for using a Bonferroni correction with the 1989 data; upon doing so the same results were observed with only four exceptions and the exceptions did not alter the conclusions. These results are not shown.

Modified alfalfa damping-off bioassay

Isolates of *B. cereus* obtained from treated and untreated roots were screened for biocontrol activity in an alfalfa damping-off bioassay, as described previously (Handelsman *et al.* 1990), with the following modifications. *B. cereus* was grown in 1.5 ml of half-strength TSB at 28°C for 2.5 days. Test-tubes, each containing three alfalfa seedlings in sterilized vermiculite, were inoculated with 3750–10 000 zoospores of *Phytophthora medicaginis* and 0.3 ml of a *B. cereus* culture. Plants were scored for survival 5–7 days after inoculation and a *B. cereus* isolate was

scored as positive for biocontrol activity if at least two of the three alfalfa seedlings inoculated with that isolate survived. Each isolate was screened in one test tube in each of two separate experiments. In the modified damping-off assay, 90% of the separate cultures of UW85 tested scored positive for biocontrol activity, whereas only 15 and 18% of the cultures of the non-protecting *B. cereus* strains 569 and UW30 scored positive for biocontrol, respectively. Biocontrol activity of the rhizosphere isolates of *B. cereus* was adjusted to the activity of the UW85 controls; thus, the biocontrol activity of UW85 is expressed as 100%.

Isolation and detection of plasmid DNA

Plasmid DNA was extracted by the alkaline digest method of Maniatis *et al.* (1982) with the following modifications. Cultures of *B. cereus* grown in 16-mm × 100-mm test-tubes containing 5 ml of Brain Heart Infusion Broth (Difco Laboratories, Detroit, MI) supplemented with 10-μg/ml tetracycline when appropriate, were incubated at 37°C on a rotary shaker at 170 r.p.m. After 12 h of growth, tunicamycin was added to obtain a final concentration of 5 ng/ml, and cultures were incubated for an additional 6 h. Cells were centrifuged and resuspended in the cell-lysis buffer containing 20-mg/ml lysozyme and incubated in a 37°C water bath for 30 min. Cells were lysed by adding a NaOH-SDS solution to the cell-lysis buffer suspension and incubating the mixture in a 65°C water bath for 20 min. Plasmid DNA was precipitated by adding 1.0 ml of ethanol and incubating at −20°C for 1 h. All reagents were obtained from Sigma Chemical Co. *Eco*R1 restriction digests of plasmid DNA were performed as described by Maniatis *et al.* (1982).

Screen of rhizosphere isolates of B. cereus for antibiotic resistance

We tested the possibility that the introduced populations of *B. cereus* were unable to express antibiotic resistance immediately after removal from the rhizosphere or that they were unable to compete with the indigenous bacterial community on the medium selecting for antibiotic-resistant populations and were therefore unable to produce visible colonies. *B. cereus* isolates, obtained from the medium selecting for total *B. cereus* populations, were screened for antibiotic resistance by transferring them onto a semi-selective medium unamended or amended with one or more antibiotics.

Results

Isolation and characterization of mutants

We constructed strains that were singly or doubly marked with antibiotic resistance. To study stability of each marker, each strain had at least one other characteristic (orange pigmentation, pBC16, biocontrol activity) that distinguished it from indigenous *B. cereus* (Table 1).

Marker stability in culture and in the growth chamber

We examined marker stability of UW85 derivatives in culture and in the rhizosphere of soybeans. After 100 generations in broth or 25 consecutive transfers on solid medium at 28°C, the markers were stable in populations of all UW85 derivatives. In two growth chamber experiments, at all sampling times and locations on the root, there were no significant differences ($P>0.1$) between population sizes of

Table 1 Strains of *B. cereus* used in this study. All antibiotic-resistant mutants of UW85 were isolated independently from each other and appeared at frequencies of 10^{-7} to 10^{-8} per cell. Antibiotic-resistant mutants reverted to the wild-type antibiotic-sensitive phenotype at frequencies of 10^{-7} to 10^{-8} per cell. Transconjugants appeared at frequencies of 10^{-3} to 10^{-4} per donor. Abbreviations: Bic, biocontrol activity in an alfalfa bioassay (Handelsman *et al.* 1990); Neo^r, neomycin resistant (5 μg/ml); Str^r, streptomycin resistant (20 μg/ml); Tet^r, tetracycline resistant (5 μg/ml); Ant, anthranilic acid; Ora, orange-pigmented colony

Strain	Parent	pBC16	Relevant phenotype	Source or reference
B. cereus UW85				
UW85		-	Wild type, Bic$^+$	Handelsman *et al.* (1990)
UW85n1	UW85	-	Neo^r Bic$^+$	This work
UW85n2	UW85	-	Neo^r Bic$^+$	This work
UW85s1	UW85	-	Str^r Ora$^+$ Bic$^+$	This work
UW85s2	UW85	-	Str^r Bic$^+$	This work
UW85s3	UW85	-	Str^r Bic$^+$	This work
UW85n1-t	UW85n1	+	Neo^r Tet^r Bic$^+$	This work
UW85n2-t	UW85n2	+	Neo^r Tet^r Bic$^+$	This work
UW85s2-t	UW85s2	+	Str^r Tet^r Bic$^+$	This work
UW85s3-t	UW85s3	+	Str^r Tet^r Bic$^+$	This work
UW85-t	UW85	+	Tet^r Bic$^+$	This work
UW030	UW85n1-t	+	Neo^r Tet^r Bic$^-$	S. Raffel
B. cereus 569				
569		-	Wild type, Bic$^-$	Battisti *et al.* (1985)
569 UM20-1 tr251-1		+	Ant$^-$ Str^r Tet^r Bic$^-$	Battisti *et al.* (1985)

Table 2 Population sizes of total and antibiotic-resistant *B. cereus* 2–3 cm below the crown 56 days after planting in a growth chamber experiment. The data are from one sampling period from one experiment and are representative of a second similar experiment. Population sizes of the total and antibiotic-resistant *B. cereus* on treated roots were significantly larger (P=0.05) than those on untreated roots for all treatments and sampling times. Values in parentheses are percentages of the total population of *B. cereus* that were antibiotic resistant.

	Mean±SEM $\log_{10}$ CFU (%)					
Treatment	Total *B. cereus*	Neo^r *B. cereus*	Str^r *B. cereus*	Tet^r *B. cereus*	Neo^r & Tet^r *B. cereus*	Str^r & Tet^r *B. cereus*
Untreated	3.20±0.10	1.83±0.27	1.65±0.17	2.00±0.28	ND	ND
UW85n1	4.17±0.26	4.30±0.20 (134)				
UW85n1-t	4.51±0.28	4.61±0.22 (126)		4.52±0.23 (102)	4.56±0.25 (112)	
UW85n2	4.32±0.15	4.36±0.18 (110)				
UW85n2-t	4.22±0.28	4.38±0.23 (144)		4.40±0.22 (150)	4.30±0.23 (120)	
UW85s1	4.44±0.10		4.46±0.26 (105)			
UW85s2	4.42±0.31		4.53±0.20 (128)			
UW85s2-t	4.29±0.14		4.41±0.18 (131)	4.39±0.18 (126)		4.34±0.20 (115)
UW85s3	4.69±0.10		4.66±0.17 (91)			
UW85s3-t	4.56±0.20		4.45±0.16 (78)	4.49±0.13 (85)		4.50±0.16 (87)
UW85-t	4.39±0.22			4.39±0.21 (100)		

ND = none detected at our detection limit of 50 CFU/root section.

total and antibiotic-resistant *B. cereus* from treated roots (Table 2). The results indicated that the markers were stable under these conditions.

Marker stability in the 1987 field experiment

To measure marker stability, we compared the population sizes of total and antibiotic-resistant *B. cereus* on treated roots with those on untreated roots, and the populations sizes of total and antibiotic-resistant *B. cereus* on treated roots, and we measured maintenance of a second marker in populations of *B. cereus* from treated roots. The population sizes of total and neomycin- and streptomycin-resistant *B. cereus* from UW85n1- and UW85s1-treated roots were significantly larger (P<0.05) than those from untreated roots (Tables 3–5), with three exceptions. The population sizes of indigenous neomycin- and streptomycin-resistant *B. cereus* on untreated roots were generally near or below our detection limits of 100 CFU per sample (Table 3).

There were no significant differences between the population sizes of neomycin-resistant and total *B. cereus* on UW85n1-treated seeds, seedlings, and roots the first 21 days after planting (Table 4). By 35 days after planting and until seed harvest, however, the population sizes of total *B. cereus* were significantly larger than the population sizes of neomycin-resistant *B. cereus* (Table 4).

On UW85s1-treated roots 7 days after planting there were no significant differences between the population sizes of total, streptomycin-resistant, or the orange-pigmented *B. cereus*. By 14 days after planting, at 0–1 cm

Table 3 Population sizes of total, neomycin- and streptomycin-resistant *B. cereus* on untreated seeds and roots (1987). Populations were compared on the cotyledon, radicle, or on 1-cm root segments 0–1, 2–3, 4–5 or 9–10 cm below the crown, or on the last cm of root (RT).

		Mean±SEM $\log_{10}$ CFU		
Days after planting	Sample	Total *B. cereus*	Neo^r *B. cereus*	Str^r *B. cereus*
0	Seed	-*	-	-
7	Cotyledon	4.21±0.15	1.74±0.29	1.32±0.44
	Radicle	3.88±0.18	1.99±0.10	ND
14	0–1	4.19±0.26	2.09±0.31	1.48±0.38
	RT	4.33±0.32	2.17±0.13	ND
21	0–1	3.14±0.32	1.98±0.35	ND
	2–3	3.32±0.16	2.10±0.37	ND
	RT	3.25±0.21	ND	ND
28	0–1	3.35±0.29	2.04±0.26	ND
	2–3	3.00±0.37	1.96±0.41	2.02±0.18
	RT	3.18±0.36	ND	1.78
35	0–1	3.34±0.10	2.38±0.24	1.57±0.23
	2–3	3.43±0.32	1.59±0.29	ND
	RT	2.65±0.41	1.74±0.12	1.65±0.13
49	0–1	3.95±0.06	2.28±0.52	-
	2–3	4.38±0.27	2.37±0.48	-
	4–5	3.79±0.18	1.37±0.37	-
	RT	3.24±0.32	ND	-
63	0–1	3.58±0.12	2.07±0.56	-
	2–3	3.70±0.11	2.50±0.50	-
	4–5	3.38±0.31	2.52±0.42	-
	9–10	3.40±0.31	1.43±0.12	-

*Not determined.
ND = none detected at detection limits (100 CFU per sample).

Table 4 Population sizes of total and Neo^r *B. cereus* on UW85n1-treated roots (1987). Populations were compared on the seed, cotyledon, radicle or on 1-cm root segments 0–1, 4–5, and 9–10 cm below the crown, or the last cm of the root (RT). %Neo is the proportion of the total population of *B. cereus* that was neomycin resistant

		Mean±SEM Log_{10} CFU		
Days after planting	Sample	Total *B. cereus*	Neo^r *B. cereus*	% Neo^r
0	Seed	8.13±0.24	8.40±0.35	186
7	Cotyledon	8.12±0.16†	7.11±0.41	10
	Radicle	6.42±0.26†	5.84±0.46	26
14	0–1	6.02±0.26†	6.81±0.18	616
	RT	5.41±0.62†	5.12±0.65	51
21	0–1	5.64±0.25†	5.50±0.39	72
	2–3	5.33±0.40†	4.24±0.47	8
	RT	3.85±0.57	3.19±0.33	22
28	0–1	5.16±0.39†	4.75±0.60	39
	2-3	5.19±0.39†	3.91±0.10*	5
	RT	4.40±0.10†	3.39±0.72	10
35	0–1	5.53±0.14†	4.66±0.36	14
	2-3	5.14±0.18†	4.40±0.20*	18
	RT	3.66±0.06†	2.73±0.10*	12
49	0–1	6.72±0.15†	5.47±0.22*	6
	2–3	6.98±0.16†	6.18±0.27*	16
	4–5	6.49±0.12†	5.60±0.17*	13
	RT	6.70±0.33†	4.17±0.13*	0.3
63	0–1	6.13±0.21†	4.56±0.25*	3
	2–3	6.23±0.45†	5.19±0.34*	9
	4–5	6.58±0.45†	4.35±0.13*	0.6
	9–10	4.20±0.62	3.55±0.31*	22

†$P<0.05$ (significantly greater than the untreated control as determined by Fisher's LSD).

*$P<0.05$ (population size of Neo^r *B. cereus* was significantly smaller than the total *B. cereus* as determined by a paired *t*-test with a Bonferroni correction).

below the crown and at the RT, 58% and 0.1% of the population was streptomycin resistant, whereas 62% and 69% were orange pigmented, respectively (Table 5). By 21 days after planting, no streptomycin-resistant *B. cereus* could be detected, although 78% and 35% of the population of total *B. cereus* was orange pigmented at 0–1 cm below the crown and at the RT, respectively. Orange-pigmented *B. cereus*-like colonies were not detected in the rhizosphere of untreated roots.

Loss of antibiotic resistance from populations of *B. cereus* could result from loss of the marker or death of the introduced strain. Because population sizes of total *B. cereus* were significantly larger on UW85n1- and UW85s1-treated roots than they were on untreated roots, death of the inoculum strain would have to be coupled with selection for *B. cereus* strains on treated roots. To determine the origin of the antibiotic-sensitive *B. cereus* population on treated-roots, we examined other traits characteristic of the UW85 derivatives and not of the indigenous *B. cereus*. Because populations of *B. cereus* from UW85s1-treated roots retained the orange pigment and biocontrol activity when there were no detectable streptomycin-resistant *B. cereus* (Table 5), the results suggested that the streptomycin-resistance marker was lost from the introduced population and not that the streptomycin-resistant mutant died. In the modified damping-off assay, 98% of the isolates from UW85s1- and UW85n1-treated roots (n=225) had biocontrol activity, but only 18% of the isolates from untreated roots (n=125) had biocontrol activity.

Marker stability in the 1989 field experiment

In 1989, we examined maintenance of antibiotic-resistance markers in UW85n1, UW85s1, and other independently isolated neomycin- and streptomycin-resistant mutants of UW85, and we examined the stability of pBC16, which carries tetracycline-resistance, in the presence and absence of each genomic marker. At planting, we observed no significant differences ($P>0.1$) between the population sizes of total and antibiotic-resistant *B. cereus* on seeds coated with spores of the UW85 derivatives (data not shown). At all subsequent sampling times and locations on the root, population sizes of total *B. cereus* on treated roots were significantly larger ($P<0.01$) than they were on untreated roots, and population sizes of antibiotic-resistant *B. cereus* on treated roots were significantly larger ($P<0.001$) than they were on untreated roots (Tables 6 and 7). On untreated roots, at all sampling times and locations on the root, population sizes of neomycin-, streptomycin- and tetracycline-resistant *B. cereus* were significantly smaller ($P<0.001$) than total *B. cereus* (Tables 6 and 7).

In samples from roots treated with either UW85n1-t or its parent UW85n1, the population sizes of neomycin-resistant *B. cereus* were not significantly different from the total *B. cereus* until 116 days after planting, whereas populations of UW85n2 were significantly smaller than the total *B. cereus* population by 83 days after planting (Tables 6 and 7). No differences were observed between UW85n1 and UW85n1-t, suggesting that the acquisition of pBC16 by UW85n1 did not affect the stability of the neomycin-resistance marker. In samples from UW85s1-treated roots, the population sizes of streptomycin-resistant *B. cereus* were significantly smaller than the total *B. cereus* with one ex-

Table 5 Population sizes of total, Ora+, and Str^r *B. cereus* from UW85s1-treated plots (1987). Populations were compared on the seed, cotyledon, radicle or on 1-cm root segments 0–1, 2–3, 4–5 and 9–10 cm below the crown, or on the last cm of root (RT). %Ora or %Str^r is the percentage of the total population of *B. cereus* that was orange or streptomycin resistant

		Mean±SEM $\log_{10}$ CFU				
Days after planting	Sample	Total *B. cereus*	Ora+ *B. cereus*	Str^r *B. cereus*	% Ora	% Str^r
0	Seed	8.20±0.24†	8.04±0.19	7.90±0.44	69	50
7	Cotyledon	7.88±0.25†	7.39±0.18	4.56±0.61	32	0.05
	Radicle	6.74±0.32†	6.56±0.23	5.90±0.35	66	14
14	0–1	6.10±0.56†	5.89±0.30	5.86±0.19	62	58
	RT	5.08±0.33†	4.92±0.30	2.22±0.23*	69	0.1
21	0–1	5.34±0.12†	5.23±0.11	ND	78	0
	2–3	5.37±0.19†	5.21±0.16	ND	69	0
	RT	3.47±0.59	3.01±0.26	ND	35	0
28	0–1	5.39±0.10†	4.85±0.36	ND	29	0
	2–3	4.66±0.12†	3.97±0.14	ND	20	0
	RT	3.64±0.17†	3.34±0.41	ND	50	0

†$P<0.05$ (significantly greater than the untreated control as determined by Fisher's LSD).
*$P<0.05$ (population size of Str^r *B. cereus* was significantly smaller than the total *B. cereus* as determined by a paired *t*-test with a Bonferroni correction).
ND = none detected at our detection limit of 100 CFU/sample.

ception. The apparent loss of the streptomycin- and neomycin-resistance markers in populations of *B. cereus* from UW85s1- or UW85n1-treated roots generally was not as great in 1989 (Tables 6 and 7) as in 1987 (Tables 4 and 5).

The proportion of the total population of *B. cereus* that was neomycin- or streptomycin-resistant varied between sampling times, location on the root, and between strains (Tables 6 and 7). The results suggest that marker loss was greater further from the site and time of inoculation.

On UW85s3-t treated roots, 35 days after planting 2–3 cm below the crown and 83 days after planting 0–2 and 2–3 cm below the crown, the population sizes of streptomycin-resistant *B. cereus* were smaller than the total *B. cereus* (Tables 6 and 7). However, on roots treated with its parent, UW85s3, there were no significant differences between the population sizes of streptomycin-resistant and total *B. cereus* until 116 days after planting (Tables 6 and 7), suggesting that the acquisition of pBC16 by UW85s3 was associated with reduced maintenance of the streptomycin-resistance marker in the population.

At 35 days after planting, 0–2 cm below the crown, there were no significant differences between the population sizes of the total and tetracycline-resistant *B. cereus* on roots treated with strains carrying pBC16 (Table 6), although there were significant differences 2–3 cm below the crown (Table 7). By 83 days after planting, tetracycline resistance was not maintained for some mutants in populations of *B. cereus* 0–2 cm below the crown (Table 6) and with one exception, the proportion of tetracycline-resistant cells in populations of *B. cereus* 2–3 cm below the crown was significantly greater than in the sampling 35 days after planting (Table 7).

All tetracycline-resistant isolates of *B. cereus* tested from treated roots (n=20 from each treatment) and none of the tetracycline-resistant isolates from untreated roots had plasmid DNA that co-migrated with pBC16 in agarose gels (data not shown). None of the streptomycin-resistant, tetracycline-sensitive isolates from UW85s3-t treated roots screened (n=10) had plasmid DNA that co-migrated with pBC16 in agarose gels, demonstrating that tetracycline sensitivity was a good indicator of plasmid loss.

On roots treated with strains containing genomic and plasmid markers, the population size of *B. cereus* resistant to both antibiotics should be the same as the population size observed for the marker (genomic or plasmid) that is least stable, unless there was selection for cells that have neither marker. In most cases, population sizes of *B. cereus* resistant to both antibiotics were the same as the population sizes resistant to either the genomic or plasmid marker (Tables 6 and 7). On UW85s3-t-treated roots 83 days after planting, however, the population size of *B. cereus* resistant to both markers was significantly smaller (P=0.05) than the size of the population of *B. cereus* resistant to tetracycline (Tables 6 and 7).

Isolates from populations of the total *B. cereus* were screened for biocontrol activity. Of the isolates from treated roots (n=323), 90% had biocontrol activity whereas of the isolates from untreated roots (n=174), only 12% had biocontrol activity, suggesting that the isolates from treated roots were derived from the inoculum.

Table 6 Population sizes of total and antibiotic-resistant *B. cereus* 0–2 cm below the crown 35, 83 and 116 days after planting (1989). Population sizes of the total and antibiotic-resistant *B. cereus* on treated roots were significantly larger ($P<0.01$) than those on untreated roots. Values in parentheses are percentages of the total population of *B. cereus* that were antibiotic resistant.

		Mean±SEM $\log_{10}$ CFU (%)					
Treatment	Days after planting	Total *B. cereus*	Neo^r *B. cereus*	Str^r *B. cereus*	Tet^r *B. cereus*	Neo^r & Tet^r *B. cereus*	Str^r & Tet^r *B. cereus*
Untreated	35	3.56±0.29	2.50±0.20	2.57±0.23	1.29±0.59	ND	ND
	83	3.27±0.13	1.70±0.56	ND	1.36±0.37	ND	ND
	116	3.84±0.14	2.34±0.24	1.49±0.11	2.11±0.16	ND	ND
UW85n1	35	5.29±0.28	5.09±0.14 (63)				
	83	5.00±0.22	4.66±0.10 (46)				
	116	4.67±0.24	4.10±0.19* (27)				
UW85n1-t	35	5.02±0.56	5.56±0.51 (347)		5.20±0.46 (151)	4.55±0.13 (33)	
	83	4.35±0.12	4.45±0.04 (126)		3.62±0.26* (19)	3.66±0.30* (20)	
	116	5.16±0.19	4.36±0.24* (16)		4.33±0.25* (15)	4.35±0.21* (15)	
UW85n2	35	5.08±0.09	5.03±0.16 (89)				
	83	5.71±0.31	5.16±0.09* (28)				
	116	4.66±0.18	4.27±0.12 (41)				
UW85n2-t	35	5.23±0.19	5.28±0.31 (112)		5.21±0.06 (95)	4.90±0.18 (47)	
	83	4.64±0.20	4.63±0.11 (98)		4.48±0.16 (69)	4.43±0.22 (62)	
	116	4.59±0.11	4.21±0.15 (42)		4.27±0.09 (48)	4.09±0.07 (32)	
UW85s1	35	5.15±0.28		4.15±0.17* (10)			
	83	4.69±0.11		4.57±0.24 (76)			
	116	4.88±0.19		4.13±0.25* (18)			
UW85s2	35	5.34±0.33		5.08±0.39 (55)			
	83	5.51±0.43		5.21±0.29 (50)			
	116	4.50±0.31		4.02±0.28 (33)			
UW85s2-t	35	5.59±0.22		5.39±0.23 (63)	5.27±0.11 (48)		5.47±0.12 (76)
	83	4.71±0.16		4.57±0.08 (61)	4.29±0.04 (38)		4.57±0.05 (72)
	116	4.75±0.21		4.18±0.18* (27)	4.35±0.15 (40)		4.41±0.18 (46)
UW85s3	35	5.13±0.14		5.08±0.12 (89)			
	83	4.63±0.40		4.32±0.21 (49)			
	116	4.58±0.09		3.77±0.18* (15)			
UW85s3-t	35	5.01±0.15		4.65±0.19 (44)	4.85±0.42 (69)		4.90±0.32 (78)
	83	4.49±0.09		3.83±0.08* (22)	3.29±0.33* (6)		2.76±0.20* (2)
	116	4.72±0.21		4.25±0.20* (34)	4.11±0.37* (25)		4.26±0.43 (35)
UW85-t	35	5.27±0.11			5.40±0.19 (135)		
	83	5.09±0.37			4.98±0.39 (78)		
	116	5.22±0.21			4.63±0.36* (26)		

*$P<0.05$ (population size of antibiotic-resistant *B. cereus* was significantly smaller than the total *B. cereus* using LSD, as described in Materials and Methods).

ND = none detected at our detection limit of 50 CFU/root section.

Table 7 Population sizes of total and antibiotic-resistant *B. cereus* 2–3 cm below the crown 35, 83 and 116 days after planting (1989). Population sizes of the total and antibiotic-resistant *B. cereus* on treated roots were significantly larger ($P<0.01$) than those on untreated roots. Values in parentheses are percentages of the total population of *B. cereus* that were antibiotic resistant.

		Mean±SEM $\log_{10}$ CFU (%)					
Treatment	Days after planting	Total *B. cereus*	Neo^r *B. cereus*	Str^r *B. cereus*	Tet^r *B. cereus*	Neo^r & Tet^r *B. cereus*	Str^r & Tet^r *B. cereus*
Untreated	35	3.75±0.26	2.22±0.17	2.13±0.12	1.20±0.33	ND	ND
	83	3.64±0.31	1.98±0.03	ND	1.13±0.28	ND	ND
	116	3.94±0.16	1.87±0.23	1.62±0.21	1.70±0.38	ND	ND
UW85n1	35	5.12±0.32	4.87±0.50 (56)				
	83	4.98±0.17	5.00±0.15 (105)				
	116	4.75±0.14	4.26±0.21* (32)				
UW85n1-t	35	4.73±0.45	4.24±0.18 (35)		3.93±0.21* (17)	3.88±0.24* (15)	
	83	4.31±0.20	4.32±0.09 (102)		4.32±0.17 (102)	4.24±0.17 (85)	
	116	5.20±0.32	4.23±0.34* (11)		4.67±0.17* (30)	4.27±0.28* (12)	
UW85n2	35	5.61±0.50	5.15±0.27 (35)				
	83	5.12±0.17	5.22±0.11 (126)				
	116	4.76±0.18	4.45±0.17 (49)				
UW85n2-t	35	4.91±0.43	4.87±0.36 (91)		4.41±0.30* (32)	4.4±0.33* (31)	
	83	4.65±0.35	4.49±0.34 (69)		4.69±0.36 (110)	4.43±0.36 (60)	
	116	4.94±0.12	4.64±0.05 (50)		4.58±0.06 (44)	4.48±0.05 (35)	
UW85s1	35	5.27±0.36		4.37±0.26* (13)			
	83	4.85±0.07		4.22±0.18* (23)			
	116	4.92±0.33		4.12±0.26* (16)			
UW85s2	35	5.27±0.55		5.19±0.57 (83)			
	83	6.04±0.39		5.73±0.30 (49)			
	116	5.30±0.25		4.41±0.29* (13)			
UW85s2-t	35	5.81±0.27		5.27±0.17* (36)	5.07±0.43* (18)		5.03±0.43* (17)
	83	5.41±0.28		5.22±0.31 (65)	5.29±0.35 (76)		5.23±0.36 (66)
	116	4.78±0.09		4.61±0.08 (68)	4.61±0.07 (68)		4.56±0.14 (60)
UW85s3	35	5.31±0.35		5.24±0.30 (85)			
	83	4.57±0.13		4.44±0.22 (74)			
	116	5.02±0.26		4.29±0.22* (19)			
UW85s3-t	35	6.00±0.19		5.37±0.29* (23)	5.31±0.14* (20)		5.32±0.14* (21)
	83	4.57±0.20		4.11±0.21* (35)	3.43±0.13* (7)		2.25±0.23* (0.5)
	116	4.74±0.18		4.35±0.16 (41)	4.52±0.25 (60)		4.02±0.36* (19)
UW85-t	35	5.94±0.64			5.18±0.39* (17)		
	83	5.08±0.27			5.04±0.24 (91)		
	116	5.37±0.29			4.12±0.41* (6)		

*$P<0.05$ (population size of antibiotic-resistant *B. cereus* was significantly smaller than the total *B. cereus* using LSD, as described in Materials and Methods).

ND = none detected at our detection limit of 50 CFU/root section.

Screening isolates of B. cereus *for antibiotic resistance*

When we screened isolates from the the total population of *B. cereus* for antibiotic resistance, the percentage of the total population of *B. cereus* resistant to streptomycin, neomycin, or tetracycline did not differ significantly from the estimates obtained by direct plating on antibiotics in either field experiment (data not shown).

Doubling times

When the antibiotic-resistant derivatives were grown in Min IC at 28°C, the doubling times of the antibiotic-resistant derivatives did not differ significantly from that of the wild-type UW85 (72–84 min). At 16°C, the doubling times of the antibiotic-resistant derivatives (148–456 min) were 12–32% longer than that of the wild-type UW85 (132 min, $P<0.05$ or $P<0.1$).

Discussion

The results suggest that antibiotic-resistance markers were not maintained in populations of *B. cereus* in the rhizosphere of soybeans grown in the field and that marker maintenance varied with strain, location on the root, time of sampling, and year of experiment. Loss of the marker from the rhizosphere populations could have been due to death of the inoculum or to loss of the marker from the inoculum strains. Because the population sizes of total *B. cereus* were significantly greater on treated roots than they were on untreated roots, if death of the inoculum strain were the basis for marker loss, death would have to be coupled with selection on treated roots for *B. cereus* that were sensitive to the antibiotics. However, a number of lines of evidence indicated that most of the total *B. cereus* populations on treated roots were derived from the inoculum, because they also had other phenotypes characteristic of UW85 derivatives, such as orange pigmentation, tetracycline resistance, or biocontrol activity. We do not know whether indigenous *B. cereus* could have acquired these phenotypes from UW85 by some means of genetic exchange (Graham & Istock 1979) or whether application of UW85 could select for these characteristics in the indigenous population, although these possibilities seem unlikely.

Differences in marker stability at different locations on the root over time and between treatments were not caused by sample-to-sample variability. The effect of inherent sample-to-sample variability was reduced by replication and was minimized by a conservative statistical analysis.

Between certain time points, there was an increase in the proportion of the *B. cereus* population with either the genomic or plasmid marker. Generally, the increases (Tables 6 and 7) were associated with a decrease in the population size of total *B. cereus* and a concurrent increase in the population size of *B. cereus* with either the genomic or plasmid marker compared with the previous sampling period at the same location on the root. Because we have not observed transfer of pBC16 from UW85 to other cells in culture, the increase in the population size of tetracycline-resistant *B. cereus* (Table 7) probably resulted from selection for pBC16-bearing cells and not from transfer of pBC16 to plasmid-less cells. The results may be caused by changes in environmental conditions that favour cells with the antibiotic-resistant phenotype.

The ability of the wild-type UW85 to grow significantly faster than some of the mutants and transconjugants at 16°C suggests that cooler soil temperatures could select for revertants with the antibiotic-sensitive phenotype. Although not all UW85 derivatives grew significantly more slowly than the wild type at 16°C, a small reduction in growth rates over a long period of time could result in loss of the antibiotic-resistance marker in the population (Stewart & Levin 1977). During the 1989 experiment, for example, surface soil temperatures fluctuated between 4 and 41°C, and at 5 cm below the soil surface temperatures fluctuated between 12 and 31°C (S. Baker, University of Wisconsin-Madison, pers. comm.). Because selection for cells with the antibiotic-sensitive phenotype could vary with changes in soil temperature, temporal and spatial differences in soil temperatures could explain differences in marker stability between sampling periods and location on the root. Alternatively, slower growth rates could assist in maintaining the marker in the populations by reducing the rate at which antibiotic-sensitive revertants appear in the population or the rate of plasmid loss (Stewart & Levin 1977). Soil temperature may not be the only factor contributing to marker instability, because nutrient availability has been demonstrated to affect plasmid stability in culture (Helling *et al.* 1981; Caulcott *et al.* 1987), soil (Devanas *et al.* 1986), and agricultural drainage water (Trevors *et al.* 1989).

The biological cost of spontaneous antibiotic-resistance mutations could be substantial if the mutations conferring antibiotic resistance reduce fitness. Compeau *et al.* (1988) reported that several spontaneous, rifampicin-resistant mutants of *Pseudomonas fluorescens* that grew slower and had altered membrane protein compositions were less able to compete with wild-type parents in sterile-soil microcosms. Spontaneous, antibiotic-resistant mutants of *Rhizobium* spp. were less competitive for nodulation and less effective in the symbiosis than their wild-type parents (Pankhurst 1977; Jones & Bromfield 1978). Pettibone *et al.* (1987) demonstrated that survival of antibiotic-resistant mutants of fecal indicator organisms was diminished compared to the wild-type parent in estuarine waters. These results demonstrate that before initiating ecological studies with antibiotic-resistant mutants, it is important to de-

termine whether the mutations have effects on fitness. Our results suggest that reduced fitness could affect the maintenance of antibiotic-resistance markers in a population, since revertants that are more fit than the introduced strain could be selected. If antibiotic-resistant mutants are less fit under stressful conditions in the environment, then a death rate under stressful conditions in the laboratory may be more predictive of behaviour in the field than would be a growth rate under conditions that do not restrict growth. Fidelity of genetic transmission of a marker also contributes to its stability, and the frequency of reversion to the antibiotic-sensitive phenotype may be greater in the environment than in laboratory cultures (Cairns *et al.* 1988; Miller 1988; Hall 1990). Our methods do not distinguish between loss of viability and loss of the ability to grow on culture medium. Additional studies will be required to determine the fate of the antibiotic-resistant cells that were not recovered.

Monitoring genetically engineered and non-engineered microorganisms introduced into the environment requires reliable methods for determining the survival and dispersal of the released microorganism. Our results suggest that the use of antibiotic-resistance markers for tracking microbes in the environment should be approached cautiously because the markers may not be maintained. Although other markers may not be as unstable as those we examined, stability should be established before markers are used to study microbial population biology in the environment.

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This work was part of L. J. Halverson's thesis 'Population biology of *Bacillus cereus* UW85: a case study for biotechnology policy' for the PhD degree at the Institute for Environmental Studies. Halverson's project in J. Handelsman's lab was to study the root-colonization ability of the disease-suppressive bacterium *Bacillus cereus* UW85. An experiment was designed in collaboration with M. K. Clayton to test the reliability of antibiotic-resistance markers to track the bacterium. Halverson noticed substantial loss of the markers in the field, leading to the development of the experiments reported in this paper.