Symposium: Assessing the Effects of Agricultural Biotechnology

The Release and Tracking of Genetically Engineered Bacteria in the Environment

D. A. Kluepfel, E. L. Kline, H. D. Skipper, T. A. Hughes, D. T. Gooden,
D. J. Drahos, G. F. Barry, B. C. Hemming, and E. J. Brandt

First author, Department of Plant Pathology and Physiology; second and fourth authors, Department of Microbiology; third and fifth authors, Department of Agronomy and Soils, Clemson University, Clemson, SC 29634; remaining authors, Monsanto Co., St. Louis, MO 63198.

Technical contribution 3019 of the South Carolina Agricultural Experiment Station.
Accepted for publication 31 October 1990 (submitted for electronic processing).

The planned release of genetically engineered microorganisms into agroecosystems has been suggested as a means to control insects, plant pathogens, and as a possible vehicle to deliver plant growth promoting agents. These are just a few examples of the benefits potentially afforded by this application of agricultural biotechnology (29). However, before the release of any genetically engineered microorganism, we must first understand (and be able to predict with a reasonable degree of certainty) the behavior of the released organism in an agricultural environment (12,33). With this understanding we will be able to make important risk management decisions from a position of knowledge and experience rather than a fear of the unknown (27). We are attempting to answer some of the unknowns associated with the release of genetically engineered microorganisms using a genetically marked isolate derived from the soilborne fluorescent pseudomonad group.

Why should we study this group of microorganisms? Many natural isolates of root-colonizing fluorescent pseudomonads possess a variety of interesting natural biocontrol and plant growth promoting capabilities (6,8,10,11,15,16,23,35,36). In addition, these bacteria readily colonize root tissue and are amenable to genetic manipulation (4,7,30). These characteristics make members of this group a logical choice to exploit as a possible delivery vehicle for plant-beneficial agents, such as natural pesticides or plant hormones. We feel that many of these applications would not be practical or even possible without the precisely targeted input afforded by these bacteria growing in the rhizosphere (35).

It is crucial to be able to determine the root-colonizing ability

© 1991 The American Phytopathological Society

(location, population densities, and dynamics on crop roots), long-term persistence, relative competitiveness, and pesticidal effectiveness of engineered bacteria under actual field conditions. However, few tools exist to accurately monitor these parameters. The genetically modified strain described here provides a tool which, when coupled with existing techniques, provides a very powerful system to study the microbial ecology of the rhizosphere.

DEVELOPMENT AND UTILITY OF A GENETICALLY MARKED BACTERIAL STRAIN

Detecting and enumerating previously released microorganisms in the field always presents many challenges. Researchers typically rely on antibiotic resistance as the primary tool for monitoring movement and population dynamics of the introduced microorganism (32). By culturing field samples on media containing the appropriate antibiotics, the selection and enumeration of the introduced bacteria is often possible. This simple technique has been used successfully with both foliar and soilborne bacteria (26,30). However, bacteria marked in this fashion frequently fail to maintain their competitive ability, environmental persistence, or intended plant beneficial activity (9,18,31). In addition, antibiotic resistance characteristics of the indigenous microbial population often preclude the use of antibiotic resistance markers in many environments.

Here we describe the actual field use of a sensitive tracking method that reduces these problems and is particularly effective for the fluorescent pseudomonads. We have taken advantage of the inability of most, if not all, fluorescent pseudomonads to efficiently utilize lactose as a sole carbon source (13,20,21). By insertion of the *Escherichia coli* lac operon genes, *lac*Z and *lac*Y, into the chromosome of a root-colonizing strain of *Pseudomonas aureofaciens* (Ps3732RN), it was possible to select a lactose-utilizing derivative. Since this isolate also was resistant to rifampicin and nalidixic acid we could rely on three separate phenotypes for selection in addition to its natural ability to fluoresce under longwave ultraviolet light.

The *lac*ZY insertion into the chromosome was accomplished using a disarmed Tn7 vector derivative (2,3,4,17). Tn7 is a large transposon that contains antibiotic resistance genes, along with five transposition genes that mediate its ability to transpose (19). The advantage of Tn7 is that the transposition genes function in trans, and only a few hundred base pairs at each terminus are required for transposition. In addition, Tn7 apparently inserts itself into a single specific site in the bacterial chromosome (24). Therefore, by using the Tn7 based delivery system, we know precisely where the insertion occurs, which eliminates the need for screening through unwanted transposon mutants (28). Because the insertion does not cause any observable alteration in any of the phenotypes examined to date, such a gene delivery system offers many advantages.

By flanking the *lac*ZY genes with both the left and right Tn7 termini and cloning the transposition genes into an adjacent region of an unstable incompatible plasmid, it was possible to effect the transposition of Tn7::*lac*ZY into the chromosome of Ps3732RN (2,3,4,17). The result was a strain (designated Ps3732RNL11) that contains the *lac*ZY element in the chromosome and is devoid of the plasmid vector. The *lac*ZY genes also facilitate the cleavage of the chromogenic dye X-gal, which results in blue bacterial colonies on the selection medium, which greatly aids microbial enumeration. This marker, which aids selection on a defined lactose amended medium, allows us to track the microorganism, as well as the inserted genes themselves, in the environment and lessens our reliance on antibiotic resistance as the sole selective agent (13,14).

Not only does this insertion facilitate microbial enumeration, it provides a unique metabolic and physical chromosomal genetic marker. It is this latter feature that enables us to examine several basic questions concerning the microbial ecology of the rhizosphere. These include long-term genetic and microbial persistence, chromosomal gene transfer or rearrangements, genotypic verification, and monitoring of movement in the environment. Most of these would be difficult and in many cases impossible to document by depending solely on selected antibiotic resistance markers. For example, by using direct DNA isolation from the soil coupled with the polymerase chain reaction we will be able to follow persistence and gene transfer frequencies long after populations have fallen below dilution plating detection limits (typically 10^2 colony-forming units [cfu] per gram of soil).

FIELD RELEASE OBJECTIVES

On November 2, 1987, with approval from the Environmental Protection Agency, we released the genetically engineered bacterium Ps3732RNL11 and its nonengineered parent, Ps3732RN, at Clemson University's Edisto Research and Education Center near Blackville, SC (5). The objectives of this project were to: 1) evaluate the effectiveness of the *lac*ZY marking system in a field environment; 2) compare the ability of the engineered strain with its nonengineered parent to survive, multiply, and colonize the root system during three successive cropping cycles; 3) document the lateral and vertical movement of root colonizing bacteria in a field environment; and 4) determine the potential for chromosomal gene exchange in a soil environment.

MICROBIAL CONTAINMENT EFFORTS AT THE RELEASE SITE

Surrounding the inoculated plots was a 9.2-m wide border strip planted with winter wheat, which was in turn surrounded by a 4.6-m wide plant-free zone (Fig. 1). This was deemed necessary since we anticipated the majority of lateral movement of the bacteria to be through root to root contact (22). This layout was encompassed by a 3.1-m wide grass zone. To prevent run off, the plots and buffer zones were enclosed in a 30-cm high $\times$ 3.1-m wide containment terrace (Fig. 1). Tests plots and buffer strips were contained within a fenced-in 2.8 ha field. The fence, approximately 2.5 m high, was electrified to help keep the plot free of animals.

Adjacent to the test plots but within the fenced test area, two layers of thick plastic tarp were laid down to cover an area approximately 6 $\times$ 15 m. All bacterial loading, changing operations, and disinfestation of equipment used for planting were conducted on the tarp. This was done to ensure containment and easy disinfestation in the event of a spill during the loading operations. Both tractor and grain drill were disinfected with 2% Na-hypochlorite followed by a water rinse. The equipment was then sprayed with 95% ethanol to facilitate drying before loading of the subsequent bacterial strain. All appropriate tractor and planter surfaces were swabbed both presterilization and poststerilization to detect possible sources of cross-contamination.

INOCULATION AND SAMPLING PROCEDURES

Bacteria were inoculated onto winter wheat seeds (*Triticum aestivum* L. 'Pioneer 2555') at the time of planting. Both strains were introduced independently by pumping (20 psi) a water suspension of 5×10^8 cfu/ml (Ps3732RN) or 1×10^9 cfu/ml (PS3732RNL11) directly into the wheat seed furrow at 5 ml bacterial suspension per 30 cm of row using a peristaltic pump attached to the grain drill. The seeds and introduced bacteria were covered immediately with soil by the planting equipment. This method of application greatly reduced the potential for aerosol dissemination of the bacteria. The third treatment consisted of a water-inoculated control planting. Approximately 3 hr after inoculation, five seeds were collected from the field from each of the six replications in each of the three treatments. Each five-seed sample was placed in a microcentrifuge tube to which 1 ml of water was added and vortexed for 15 sec. The number of bacteria residing in the wash water and the number of bacteria adsorbed per seed were estimated.

At each subsequent sample date five random subsamples of roots from each plot were pooled into a single sample (six samples

per treatment). In addition, five random root subsamples were taken from each of the two plant rows immediately adjacent to the left and right of the treated rows. Rows were planted 18 cm apart. During the first 6 wk after planting, subsamples were placed in 50-ml conical disposable tubes, to which 40 ml of sterile distilled water was added. Tubes were shaken at 250 rpm for 15 min on a rotary shaker at room temperature. After shaking, the water was decanted and the washing procedure repeated twice. Bacteria in the first wash were considered to be weakly associated with the roots, whereas bacterial populations present in the third washing were considered more tightly associated with the root and yielded more consistent population estimates. After the third washing, the roots were blotted dry and weighed. Eight weeks after inoculation, 500-ml centrifuge bottles and larger volumes of water were used to wash roots because of increased root mass. At each sample time the entire root system for each of the five plants was harvested and processed through the washing procedure described above (five subsamples per tube). During the first 4 wk after inoculation, root weights increased from an average of 0.365 ± 0.057 g to 0.810 ± 0.18 g. At the termination of the first wheat crop (31 wk) roots weighed an average of 24.6 ± 5.7 g.

Five weeks after inoculation, we tested for the presence of the bacteria at various points below the soil surface. This was done by digging a 1.5-m long $\times$ 1.0-m deep trench adjacent to a Ps3732RNL11 treated row. Before taking samples, approximately 18 cm of soil was gently removed from the wall of the trench, starting from the lowest depth to be sampled. Five subsamples were extracted at each depth, with a tube 20 mm in diameter, and pooled. Samples were taken at 61, 46, 30, 15, and 5 cm below the soil surface. The vertical movement test was performed in four of the six Ps3732RNL11 replications.

Five random subsamples of soil from each of the four plant-free zones that surrounded the plot were also sampled and tested for the presence of either Ps3732RN or Ps3732RNL11 at each sample time. In addition to the plant-free zone, we sampled the irrigation pond, which was 923 m from the test site. This was done for two reasons. First, we felt that it was important to screen for possible movement of the released organisms into the irrigation pond. However, after analyzing more than 90 water samples over a 31-wk period, we did not detect either of the released strains. The second reason for pond sampling was to ensure that we were not applying a large population of rifampicin-resistant or nalidixic-acid-resistant microorganisms to the test plots during irrigation, which could confound our enumeration procedures.

During the entire inoculation and sampling process great care was taken not to transfer soil or plant debris to adjacent treatment plots. Before entering and after leaving each treatment plot, boots and equipment were washed with 2% Na-hypochlorite, followed by a water rinse. At no time during the test did we detect the occurrence of cross-contamination.

Seed washes, root washes, and soil suspensions from plant-free zones and vertical dissemination samples were serially diluted in water and plated on the appropriate selection medium. All selection plates were incubated in the dark at room temperature (24–28 C), and the colonies were tallied 2–3 days after inoculation.

MICROBIAL MOVEMENT, PERSISTENCE, AND ROOT COLONIZATION

Three hours after inoculation/planting, we were able to detect approximately 2×10^3 cfu per seed. Seven to 10 days after planting, populations of both strains reached a maximum of approximately 3×10^6 cfu/g root and remained at that level for the next several weeks. The fourth week after inoculation, populations of both strains began a long steady decline until levels of 2.3×10^2 and 4.6×10^2 cfu/g root, for Ps3732RN and Ps3732RNL11 respectively, were reached at the first wheat crop harvest 31 wk after planting. These population values were at or near the limits of detection for this system.

In laboratory experiments we were able to detect as few as 10^2 cfu/g soil when soil samples were analyzed within one hour after inoculation (*unpublished*). Frequently we were able to detect the introduced strains at even lower levels, however, the probability of detection at these low levels was significantly lower than 100%. Although direct extrapolation from soil detection to detection limits of bacteria in the rhizosphere may be tenuous, it does provide a useful point of reference.

The introduced strains exhibited limited lateral migration through the soil. In the loosely associated rhizosphere soil (wash 1), Ps3732RNL11 was found in the 36-cm border row (2nd adjacent row) 12 and 16 wk postinoculation at levels of 1×10^3 and 6.3×10^1 cfu/g root respectively. In the same rhizosphere fraction (wash 1) Ps3732RNL11 levels in the 18-cm border rows (1st adjacent row) at weeks 12 and 16 were 1.32

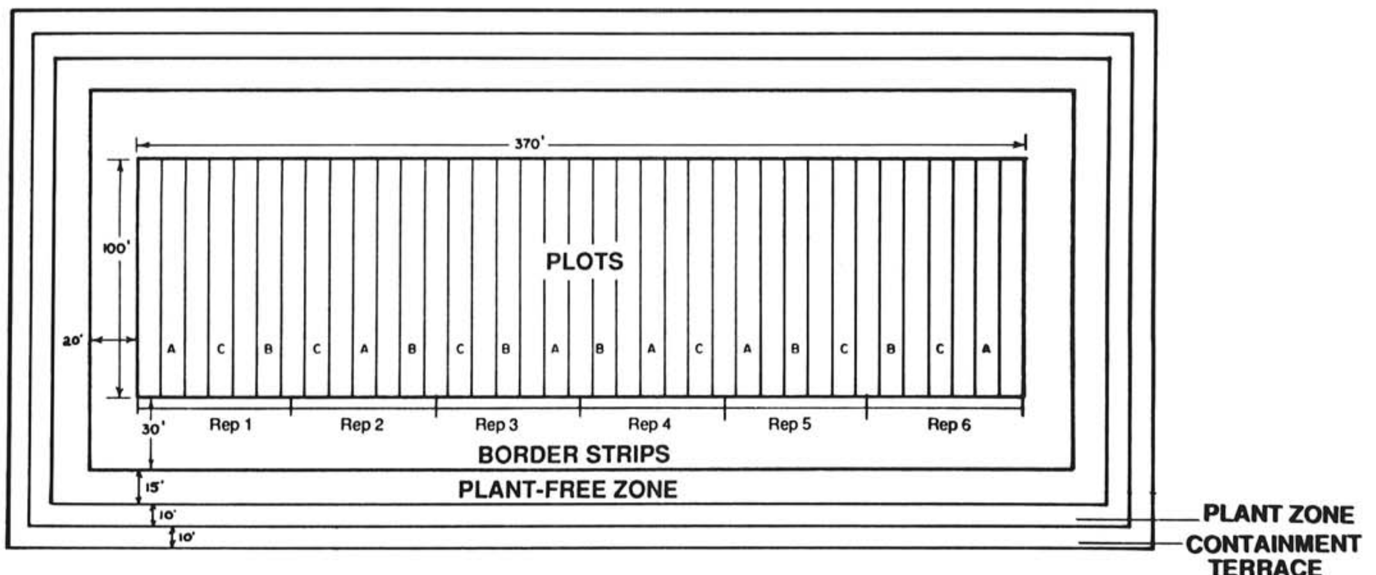

Fig. 1. Field plot design. The field plot is a randomized complete block design. The treatment plots are 30.8 m long and 3 m wide. There were three treatments: treatment A, water (control); treatment B, inoculated with the nonengineered parent (Ps3732RN); and treatment C, inoculated with the *lac*ZY engineered strain (Ps3732RNLll); which were replicated six times. Surrounding the inoculated plots is a border strip 9.2 m wide, planted with wheat, followed by a plant-free zone 4.6 m wide, and a grass zone (plant zone) 3.1 m wide. The entire release area is surrounded by a containment terrace 30 cm high $\times$ 3.1 m wide.

$\times 10^4$ and 3.9×10^3 cfu/g root, respectively. Ps3732RNL11 was found only at one sample time in the tightly associated fraction (wash 3) in any of the adjacent border rows. At week 16 it was found at a population of 5.3×10^1 cfu/g root 18 cm from the point of application. The lateral movement data for Ps3732RN was similar to that described for Ps3732RNL11.

Five weeks after inoculation, bacteria were not detected deeper than 30 cm. Since root tissue was also found at the same depth, it appears that the bacteria recovered from this depth were associated with the roots of the inoculated plants.

PRERELEASE GROWTH CHAMBER TESTING OF Ps3732RN AND Ps3732RNL11

Before the actual field release of the genetically engineered microorganisms, we conducted contained growth chamber experiments with both Ps3732RN and Ps3732RNL11. Nonsterilized field soil from the planned field release site was used in a randomized complete block design growth chamber study with four replications. Both Ps3732RN and Ps3732RNL11 were introduced at a rate of 5 ml of a 2×10^8 cfu/ml suspension every 30 cm of row on the wheat seeds at the time of planting. Populations of both strains reached approximately 3×10^6 cfu/g root within 2 wk after planting and then began a long steady decline over the next several months until the populations fell below detection limits.

MOVEMENT OF THE ENGINEERED GENES INTO OTHER ORGANISMS

Gene transfer experiments were also conducted. Since Tn7 is primarily found in microbes associated with humans and animals treated with trimethoprim and is not found in soilborne microbes, we chose to screen for gene transfer with a small portion of Tn7 (3). Using approximately 501 bp of the right terminus of Tn7 as a probe, DNA hybridizations were performed to detect the transfer and expression of the *lac*ZY::Tn7 moiety in other members of the root-colonizing microflora. Because of the tight genetic linkage of the Tn7 border regions with the *lac*Z and *lac*Y genes, simultaneous transfer of both would be expected (4).

Over a 2-wk period, beginning 8 wk after inoculation, a total of 10,058 bacterial rhizosphere isolates were collected from minimal salts M9 medium plates amended with 2% lactose, X-Gal, and cycloheximide and were screened for the presence of the Tn7::*lac*ZY moiety. Total genomic extracts were made from each of these isolates. Five μg of DNA from each extract was blotted independently onto nitrocellulose paper and probed with the ^{32}P-labeled right terminus of Tn7. All positive hybridizations were confirmed to be Ps3732RNL11 by a thorough phenotyping (chromosomal DNA fingerprint analysis, fatty acid profiles, fluorescence, and antibiotic markers). The transfer of the Tn7::*lac*ZY chromosomal insert out of Ps3732RNL11 into another microbial member of the rhizosphere was not detected in the population sampled, suggesting that the chromosomal insertion remains stable under nonselective conditions for long periods of time. This was judged to be a rigorous examination of the potential for gene transfer, since others have shown that gene-transfer frequency decreases as the microbial mating partners move away from the root surface (34). It should be noted that these experiments were designed to detect the genetic transfer, which provided for expression of the transferred *lac*ZY genes.

SUMMARY

Agronomically important microorganisms present in the natural environment are a tremendous resource that we have just begun to understand. Selecting natural isolates that enhance the health of specific plant systems or increase plant yields is a very time consuming and laborious task. Through the use of genetic engineering we may be able to decrease the time required to obtain microbial isolates with the desired biological traits while at the same time enhancing their effectiveness in the environment. However, use of these genetically engineered plant beneficial organisms will require their release into the environment, a situation that must be preceded by small-scale field testing of such organisms. The field test of a *lac*ZY engineered root colonizing fluorescent pseudomonad reported here is a first step in our attempts to more thoroughly understand the behavior and life history of a genetically engineered microorganism in an agroecosystem.

Bacterial populations of both the engineered and nonengineered strains increased rapidly on wheat roots during the first 2 wk after inoculation and reached a maximum of approximately 2×10^6 cfu/g root. Both strains were at or near detection limits 31 wk later.

Lateral dissemination of the bacteria through the soil has been negligible and appears largely limited to the first 18 cm from the point of application. The limited movement and failure to colonize may be due to the inability of a small number of migrating bacteria to compete on a root system occupied by the indigenous microflora. Though well studied in foliar environments, the notion of preemptive exclusion is not nearly as well understood for microbes interacting in the rhizosphere (25).

Vertical dissemination also was limited, extending to a maximum of 30 cm below the surface. Significantly, the low bacterial populations detected appeared to be in association with roots of the plant originally inoculated at the time of planting. Similar patterns of downward dispersal of root-colonizing fluorescent pseudomonads have been reported by others (1).

Limited bacterial dissemination and no detectable genetic exchange, coupled with the fact that Ps3732RN and Ps3732RNL11 populations decreased to their detection limit within 31 wk, indicate to us that a minimal amount of risk is associated with this type of release. At the present time we are monitoring the ability of both the engineered and nonengineered bacteria to colonize two subsequent crops (soybeans and winter wheat) without reinoculation in the same test plots.

It is also interesting to note that root-colonization data obtained from the growth chamber studies followed a pattern similar to that obtained in the field. This was true for both the engineered and nonengineered strains. These data suggest that studies in controlled environments may have some predictive value for the fate of soilborne root-colonizing bacteria in an agroecosystem.

The initial results of this planned release of a genetically engineered, soilborne root-colonizing bacterium have shown that such a release can be conducted in a safe and responsible manner. Though we should guard against generalizations, we and others have not observed any inherent danger in the use of genetically engineered bacteria in the environment simply because the organism has been modified genetically (26,33). This, however, does not exclude the necessity of a case-by-case evaluation of the microorganism and its new genetic construct before its release into the environment. By continuing to build our data base on the microbial ecology of both native and genetically engineered microorganisms we will move toward the establishment of scientifically based risk assessment procedures that will guide their future release.

LITERATURE CITED

1. Bahme, J. B., and Schroth, M. N. 1987. Spatial-temporal colonization patterns of a rhizobacterium on underground organs of potato. Phytopathology 77:1093-1100.
2. Barry, G. F. 1986. Permanent insertion of foreign genes into the chromosomes of soil bacteria. Bio/Technology 4:446-449.
3. Barry, G. F. 1988. Construction of reporter strains. Pages 211-220 in: The Release of Genetically Engineered Microorganisms. M. Sussman, C. H. Collins, F. A. Skinner, and D. E. Stewart-Tull, eds. Academic Press, San Diego. 306 pp.
4. Barry, G. F. 1988. A broad-host range shuttle system for gene insertion into the chromosomes of Gram-negative bacteria. Gene 71:75-84.
5. Biotechnology premanufacturing notification, P-87-1292. 1987. Proposal for field testing of host *Pseudomonas fluorescens* (Ps3732RN) and genetically engineered *P. fluorescens* (Ps3732RNLll). Office of Toxic Substances Ts-793, U.S. Environ. Prot. Agency.

6. Brisbane, P. G., and Rovira, A. D. 1988. Mechanisms of inhibition of *Gaeumannomyces graminis* var. *tritici* by fluorescent pseudomonads. Plant Pathol. 37:104-111.
7. Burr, T. J., and Caesar, A. 1984. Beneficial plant bacteria. Crit. Rev. Plant Sci. 2:1-20.
8. Chet, I. 1987. Innovative Approaches to Plant Disease Control. Wiley, New York. 372 pp.
9. Compeau, G., Al-Achi, B. J., Platsouka, E., and Levy, S. B. 1988. Survival of rifampin-resistant mutants of *Pseudomonas fluorescens* and *Pseudomonas putida* in soil systems. Appl. Environ. Microbiol. 54:2432-2438.
10. Cook, R. J., and Rovira, A. B. 1976. The role of bacteria in the biological control of *Gaeumannomyces*. Biochemistry 8:269-273.
11. Davison, J. 1988. Plant beneficial bacteria. Biotechnology 6:282-286.
12. Domsch, K. H., Driesel, A. J., Goebel, W., Andersch, W., Lindenmaier, W., Lotz, W., Reber, H., and Schmidt, F. 1988. Considerations on release of gene-technologically engineered microorganisms into the environment. FEMS Microbiol. Ecol. 53:261-272.
13. Drahos, D. J., Hemming, B. C., and McPherson, S. 1986. Tracking recombinant organisms in the environment: β-galactosidase as a selectable non-antibiotic marker for fluorescent pseudomonads. Bio/Technology 4:439-444.
14. Drahos, D. J., Barry, B. C., Hemming, B. C., Brandt, E. J., Skipper, H. D., Kline, E. L., Kluepfel, D. A., Hughes, T. A., and Gooden, D. T. 1988. Pre-release testing procedures: US field test of a *lac*ZY-engineered soil bacterium. Pages 181-191 in: The Release of Genetically-Engineered Micro-Organisms. M. Sussman, C. Collins, F. Skinner, and D. Stewart-Tull, eds. Academic Press, San Diego. 306 pp.
15. Fravel, D. R. 1988. Role of antibiosis in the biocontrol of plant diseases. Ann. Rev. Phytopathol. 26:75-91.
16. Gaskins, M. H., Albrecht, S. L., and Hubbell, D. H. 1985. Rhizosphere bacteria and their use to increase plant productivity: A review. Agric. Ecosyst. Environ. 12:99-116.
17. Grinter, N. J. 1983. A broad-host-range cloning vector transposable to various replicons. Gene 21:133-143.
18. Godwin, D., and Slater, J. H. 1979. The influence of the growth environment on the stability of a drug resistance plasmid in *Escherichia coli* K-12. J. Gen. Microbiol. 111:201-210.
19. Hauer, B., and Shapiro, J. A. 1984. Control of Tn7 transposition. Mol. Gen. Genet. 194:149-158.
20. Hemming, B. C., and Drahos, D. J. 1984. β-Galactosidase, a selectable non-antibiotic marker for fluorescent pseudomonads. J. Cell. Biochem. Supp. 8b:252.
21. Hugh, R., and Gilardi, G. L. 1980. Pseudomonads. Pages 288-317 in: Manual of Clinical Microbiology, 3rd Ed. E. H. Lennette, ed. American Society of Microbiology, Washington, D.C.
22. Kelman, A., and Sequeira, L. 1965. Root-to-root spread of *Pseudomonas solanacearum*. Phytopathology 55:304-309.
23. Kloepper, J. W., Leong, J., Teintze, M., and Schroth, M. N. 1980. *Pseudomonas siderophores*: A mechanism explaining disease-suppressive soils. Current Microbiol. 4:317-320.
24. Lichtenstein, C., and Brenner, S. 1982. Unique insertion site of Tn7 in the *E. coli* chromosome. Nature 297:601-603.
25. Lindow, S. E. 1987. Competitive exclusion of epiphytic bacteria by ice$^-$ mutants of *Pseudomonas syringae*. Appl. Environ. Microbiol. 53:2520-2527.
26. Lindow, S. E., Knudsen, G. R., Seidler, R. J., Walter, M. V., Lambou, V. W., Amy, P. S., Schmedding, D., Prince, V., and Hern, S. 1988. Aerial dispersal and epiphytic survival of *Pseudomonas syringae* during a pretest for the release of genetically engineered strains into the environment. Appl. Environ. Microbiol. 54:1557-1563.
27. MacKenzie, D. R. 1991. The national biological impact assessment program. Phytopathology 81:361.
28. McKown, R., Orle, K. A., Chen, T., and Craig, N. L. 1988. Sequence requirements of *Escherichia coli att*Tn7, a specific site of transposon Tn7 insertion. J. Bacteriol. 170:352-358.
29. Milewski, E. 1985. Field testing of microorganisms modified by recombinant DNA techniques. Applications, issues, and development of "points to consider" document. Recomb. DNA Tech. Bull. 8:102-108.
30. Schippers, B., Bakker, A. W., and Bakker, P. A. H. M. 1987. Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. Annu. Rev. Phytopathol. 25:339-358.
31. Stotzky, G., and Babich, H. 1984. Fate of genetically-engineered microbes in natural environments. Recomb. DNA Tech. Bull. 7:163-188.
32. Sussman, C. H., Collins, C. H., Skinner, F. A., and Stewart-Tull, D. E., eds. 1988. The release of genetically-engineered microorganisms. Academic Press, San Diego. 306 pp.
33. Tiedje, J. M., Colwell, R. K., Grossman, Y. L., Hodson, R. E., Lenski, R. E., Mack, R. N., and Regal, P. J. 1989. The planned introduction of genetically engineered organisms: Ecological considerations and recommendations. Ecology 70:298-315.
34. Van Elsas, J. D., Nikkel, M., and Van Overbeek, L. S. 1989. Detection of plasmid RP4 transfer in soil and rhizosphere, and the occurrence of homology to RP4 in soil bacteria. Current Microbiol. 19:375-381.
35. Weller, D. M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. 26:379-47.
36. Windels, C. E., and Lindow, S. E., eds. 1985. Biological control on the phylloplane. The American Phytopathological Society, St. Paul, MN. 169 pp.