



Efficient bacterial colonizers of wheat roots  
by Monica Elliot Juhnke

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in  
Plant Pathology  
Montana State University  
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**Abstract:**

Native soil bacteria which efficiently colonize spring wheat roots were identified and characterized. Using standard techniques, these bacteria were isolated from the rhizosphere and rhizoplane of wheat and barley plants and include *Pseudomonas fluorescens* (Biotypes I and II), *Xanthomonas maltophilia*, *Bacillus subtilis*, *B. pumilus*, *Streptomyces* species and a coryneform type. At least one isolate from each taxonomic group can be considered an efficient root colonizer.

Fifty-six of the 60 isolates tested under field conditions were genetically marked for resistance to antibiotics via selection of spontaneous mutants. This allowed for simplified detection and monitoring in the field. These isolates were utilized as seed treatments and tested for spring wheat root colonization during the 1985 growing season in Bozeman, MT. Twenty were shown to persist on wheat roots and were recovered in appreciable numbers through harvest.

These twenty isolates were utilized again in 1986 as spring wheat seed treatments. At five weeks, the coryneform type had the highest root colonization values and composed an average of 27% of the total culturable rhizosphere bacterial flora. Mixtures of these isolates were also applied as seed treatments in 1986. Isolates of the same and different taxonomic groups were compatible on seeds and roots and could be differentiated on growth media via antibiotic resistance and gross morphology.

Five of the mutant isolates, one from each genus represented, could colonize the roots of plant species other than spring wheat. Winter wheat, barley, corn, soybean and safflower seed were bacterized with the mutant isolates resulting in root colonization of the plants. This suggests these bacterial root colonizers may not be host specific.

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MONTANA STATE UNIVERSITY  
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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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## ABSTRACT

Native soil bacteria which efficiently colonize spring wheat roots were identified and characterized. Using standard techniques, these bacteria were isolated from the rhizosphere and rhizoplane of wheat and barley plants and include Pseudomonas fluorescens (Biotypes I and II), Xanthomonas maltophilia, Bacillus subtilis, B. pumilus, Streptomyces species and a coryneform type. At least one isolate from each taxonomic group can be considered an efficient root colonizer.

Fifty-six of the 60 isolates tested under field conditions were genetically marked for resistance to antibiotics via selection of spontaneous mutants. This allowed for simplified detection and monitoring in the field. These isolates were utilized as seed treatments and tested for spring wheat root colonization during the 1985 growing season in Bozeman, MT. Twenty were shown to persist on wheat roots and were recovered in appreciable numbers through harvest.

These twenty isolates were utilized again in 1986 as spring wheat seed treatments. At five weeks, the coryneform type had the highest root colonization values and composed an average of 27% of the total culturable rhizosphere bacterial flora. Mixtures of these isolates were also applied as seed treatments in 1986. Isolates of the same and different taxonomic groups were compatible on seeds and roots and could be differentiated on growth media via antibiotic resistance and gross morphology.

Five of the mutant isolates, one from each genus represented, could colonize the roots of plant species other than spring wheat. Winter wheat, barley, corn, soybean and safflower seed were bacterized with the mutant isolates resulting in root colonization of the plants. This suggests these bacterial root colonizers may not be host specific.

## INTRODUCTION

One of the pitfalls of science and indeed all rational thought is the tendency to create words for concepts, words that take on a "reality" in their own right, independent of the concept. Words separated from the parent concepts tend to usurp the role of concepts and to create "ideas" that are actually meaningless.

This quote from Good (1986) very aptly applies to research of the rhizosphere. Although the term "rhizosphere" can be defined and does represent a concept, many of the other terms associated with rhizosphere research are just words which do not represent a fully mature concept and so can not be defined except by the researchers who coined the term originally. A recent review of rhizosphere research is provided by Curl and Truelove (1986).

The rhizosphere is the zone of soil that is influenced by living roots. Plant roots affect physical, chemical and biological properties of the soil. In turn, each of these soil properties affect plant roots. Thus, an immensely complex system lies below the soil - hidden from view.

The biology of the rhizosphere can be studied directly and without destruction but with great difficulty and only in those circumstances where the rhizotron (an underground container with transparent sides), or adaptations thereof, can provide the answers to the questions posed. After all, the rhizosphere is a dynamic system in flux. Once the roots are removed from the soil, they are no longer in their original environment and can not be placed back into the same

environment with the expectation that the replaced roots will return to their previous state. Thus, a rhizosphere researcher studies a particular root system once and only once. Conclusions are drawn from a population.

Another problem facing rhizosphere biologists is soil. All soils are different (physically, chemically and/or biologically) and even the same soil is subject to perpetual modifications. It is no coincidence that the rhizosphere literature is extensive and yet, so inconclusive. Therefore, how useful are the results from experiments conducted under field conditions? These results are quite serviceable since they provide a point of reference upon which to build a rhizosphere research data base. However, conclusions from this data base should be formulated cautiously lest they become dogma.

My primary interest in rhizosphere biology is rhizosphere bacteria (i.e. bacteria associated with plant roots) and the manipulation of these organisms to promote plant health. It is known that rhizosphere bacteria exist, but their isolation is difficult. We can observe these organisms microscopically. However, to work with them and manipulate them requires their isolation and subsequent growth on artificial media. Qualitative and quantitative reports concerning rhizosphere bacteria are common, but it is essential to realize these reports discuss total numbers of culturable rhizosphere bacteria and not the absolute total number of rhizosphere bacteria - a distinction which is often ignored. It is also not emphasized enough that the genera of bacteria isolated from the rhizosphere are a direct function of isolation techniques.

In general, the presently known rhizosphere bacteria are separated into two primary groups - deleterious and beneficial organisms. It is important to note that a particular organism can belong to both groups. Deleterious rhizosphere bacteria can be classified together as plant pathogens - minor or major. Beneficial rhizosphere bacteria, however, fall into four different groups.

The most frequently discussed beneficial group includes the symbiotic nitrogen-fixing genera Rhizobium and Frankia (Stacey and Brill,1982). A second group is the freeliving nitrogen-fixing bacterial genera of Azospirillum, Azotobacter, Bacillus, Clostridium and Klebsiella (Gaskins et al.,1985). The Azotobacter organisms were extensively utilized in the Soviet Union during the late 1950's and early 1960's as bacterial fertilizers to increase crop yields (Brown,1974). Azospirillum has been utilized most recently to promote plant growth of grasses, including cereal crops, via nitrogen-fixation (Stacey and Brill,1982; Gaskins et al.,1985; Bashan,1986b).

Biological control agents are a third group of beneficial rhizosphere bacteria and encompass all bacterial groups. The main criterion for inclusion in this group is that the organism suppress a soilborne plant pest - usually a pathogen, but insects and nematodes may be the suppressed pests.

The fourth group includes those organisms which promote plant growth. There are primarily four rhizosphere genera which are reported to produce plant growth-promoting compounds - Azospirillum, Azotobacter, Bacillus and Pseudomonas (Katznelson and Cole,1965; Brown and Burlingham,1968; Tien et al.,1979). However, some recent reviews

(Suslow,1982; Burr and Caesar,1984) imply that Pseudomonas species, specifically fluorescent pseudomonads, are the only organisms in this group which effectively establish on and colonize plant roots. These reviews refer to these fluorescent pseudomonads as root-colonizers or rhizobacteria or plant growth-promoting rhizobacteria. Interestingly, it is believed that these bacteria promote growth not by producing growth-stimulating compounds but rather by inhibiting and altering the normal root microflora (Suslow,1982).

The use of rhizosphere bacteria as seed treatments is referred to as seed bacterization (Brown,1974; Burr and Caesar,1984). Development of rhizosphere bacteria as seed treatments for the biological control of soilborne pests or for promoting plant growth has become a major emphasis in agricultural research (Schroth and Hancock,1982; Burr and Caesar,1984; Kloepper et al.,1985; Baker,1986). However, only one organism has thus far been commercialized for use as a seed inoculant with the potential of producing healthy plants and increasing yield due to disease control (Backman et al.,1984). That organism is a Bacillus subtilis strain currently being marketed by Gustafson, Inc. under the name Quantum-4000. The efficacy and commercial potential of genetically engineered bacterial organisms has, of course, not yet been tested under field conditions.

Perhaps, a major factor in the unsuccessful commercialization of rhizosphere bacteria has been the inconsistency of results obtained from field testing. There are numerous explanations to account for the variability reported including unsuccessful bacterial inoculation and survival on the seed, poor establishment by the bacteria on seed and

roots and inadequate survival of introduced bacteria in the natural soil environment (Mangenot and Diem,1979; Gaskins et al.,1985; Curl and Truelove,1986).

In general, the following seem to be true of the current status of the development of useful rhizosphere bacteria: First, the consistency between results obtained for similar experimental conditions is minimal (Kommedahl and Mew,1975; Suslow,1982; Liftshitz et al.,1986) as is the correlation between results of experiments conducted under controlled conditions (i.e. laboratory, growth chamber, greenhouse) and actual field conditions (Kommedahl et al.,1981; Suslow,1982; Weller et al.,1985). Schroth and Hancock (1981) indicated that a key finding in their work with beneficial rhizobacteria was that the most effective method for identifying these organisms was to initially test strains directly in the field without regard to lab results.

Second, results from experiments have often been reported in terms of pest control or plant growth-promotion efficacy and not the root colonization competence of organism(s) tested (Howell and Stipanovic,1979; Utkhede and Rahe,1980; Weller and Cook,1983). Quite often rhizosphere bacteria targeted for use as biological seed treatments are initially screened for inhibition of only one soilborne plant pathogen (Weller and Cook,1983) or for another specific characteristic (Suslow,1982).

Third, in the past ten years, a major emphasis has been placed on one group of bacteria, the fluorescent pseudomonads, in the development of rhizosphere bacteria for seed treatments. Previously, most

seed bacterization research had concentrated on Azotobacter, Bacillus and Streptomyces with only minor consideration given to Pseudomonas (Brown, 1974; Merriman et al., 1974). However, Rovira (1963) stated that in pure culture studies ". . . Azotobacter did not colonize the roots of lucerne, maize, tomato, or wheat to any great extent. Bacillus and Clostridium were moderate colonizers of plant roots reaching from 1 to 20 per cent the levels reached by Pseudomonas fluorescens on the same plants". Later, Cook and Rovira (1976) indicated that fluorescent pseudomonads may be the biological organisms responsible for a natural biological control phenomenon called take-all decline.

Additional research demonstrated that fluorescent pseudomonads were potential biological control agents (Howell and Stipanovic, 1979; Scher and Baker, 1980; Geels and Schippers, 1983; Weller and Cook, 1983). This research, plus the research conducted by the group working in the rhizobacteria program at the University of California, Berkeley (Suslow, 1982; Burr and Caesar, 1984), provided an impetus for concentrating on the fluorescent pseudomonads for seed bacterization purposes.

Although it is a Bacillus subtilis strain of unknown origin which is being utilized for the Quantum-4000 biological seed inoculant, there has not been the extensive effort to characterize Bacillus species as useful rhizosphere bacteria as there has been for the fluorescent pseudomonads. In those cases where Bacillus species have been studied, the organism has normally been evaluated in terms of biological control or growth promotion and not rhizosphere

colonization efficiency (Utkhede and Rahe, 1980). Notable exceptions would be the work by Merriman et al. (1974) and Kloepper et al. (1985).

Studies utilizing Streptomyces species as rhizosphere colonizers have been even less extensive than those with Bacillus. Some of the more comprehensive field studies were conducted in Australia (Broadbent et al., 1971; Merriman et al., 1974).

The primary problem in most field experiments utilizing Bacillus and Streptomyces isolates is that marked strains (i.e. antibiotic-resistant mutants) have not been utilized. Therefore, it can only be assumed that the organisms isolated from the rhizosphere/root complex were the same strains that were initially applied to the seed. This has not been true for the fluorescent pseudomonads where marked strains have been widely used in field studies.

Genes which determine rhizosphere colonization have not been characterized or cloned, even for the fluorescent pseudomonads. This task will be difficult as it is highly unlikely that colonization is controlled by a single gene or a single gene complex. However, genes which regulate production of toxins (Wabiko et al., 1986), antibiotics (Hopwood et al., 1985; Fayerman, 1986; Gutterson et al., 1986), enzymes (Fuchs et al., 1986) and plant growth hormones (Yomada et al., 1985) have been identified from naturally occurring soil bacteria. Some of these genes have already been transferred between organisms (Hopwood et al., 1985; Fuchs et al., 1986; Shivakumar et al., 1986).

With the rapid expansion of bacterial recombinant DNA (r-DNA) technology available, it would seem to be more productive to first

identify bacteria that colonize roots efficiently and effectively and then insert or amplify genes of interest to develop the biocontrol agent or growth-promoting agent of choice (Schroth and Hancock, 1981; Suslow, 1982; Curl and Truelove, 1986). This would be especially useful for intragenetic or even intraspecific r-DNA work.

Until the colonization genes are identified, it will be difficult to transform an inefficient or non-colonizing organism with agriculturally useful characteristics into an efficient root colonizer. However, it does seem feasible to transform an efficient root colonizing organism, with no biocontrol properties or at least not those of interest, into an organism which colonizes and achieves the desired biocontrol action.

A research group at Monsanto Chemical Co. may have used this approach when they transferred the insecticidal delta-endotoxin gene of Bacillus thuringiensis subsp. kurstaki into Pseudomonas fluorescens strains which colonize corn roots (Obukowicz et al., 1986). It is not reported, however, if the P. fluorescens strains were originally selected for their root colonization capability in the field or a characteristic identified in the laboratory. EPA approval for field tests with this intergeneric r-DNA organism has so far not been granted. Thus, the efficacy of this approach is still unknown.

The objective of the present research project was to identify and characterize naturally occurring rhizosphere bacteria of small grains which efficiently colonize spring wheat roots under field conditions. These bacteria would then be available for transformation and eventual

use as biocontrol agents or plant growth-promoting agents applied as seed treatments.

Spring wheat was initially selected as the model plant system due to the severe economic losses incurred by Montana irrigated spring wheat producers from take-all root rot disease caused by Gaeumannomyces graminis var. tritici (Ggt). There are presently no economic control methods, short of crop rotation, available for this disease, but current evidence would indicate this disease could be controlled via biological agents (Asher and Shipton,1981). Since wheat is an important food crop worldwide and there are a number of serious soilborne pests associated with this crop (Wiese,1977), results from this model system could also have practical applications outside of Montana.

## MATERIALS AND METHODS

Preliminary Screening

During the 1984 field season, rhizosphere and rhizoplane bacteria were isolated from spring wheat, winter wheat and barley roots. Winter wheat plant samples were obtained from a dryland commercial field in Teton Co., MT. Spring wheat and barley plant samples were from irrigated and dryland fields in Gallatin Co. and Broadwater Co., MT. After shaking the root systems hard and removing large clumps of soil attached to the roots by hand, only the tightly adhering rhizosphere soil remained. Plants were stored in paper bags at 2 C until the roots were sampled for bacteria 1-10 days after collecting.

For clarity, spermosphere refers to a soil zone influenced by the seed germination process (Lynch,1978); rhizoplane is the actual plant root surface plus closely adhering soil or debris; rhizosphere is the zone of soil which influences living roots; the laimosphere is the root-shoot transition zone or soil-shoot interface (Curl and Truelove,1986). For this work, the rhizoplane and rhizosphere were differentiated only in the initial isolation of root colonizing bacteria. Thereafter, the root samples are referred to as rhizosphere samples. The major part of the root system in the top 25 cm of soil, including the first 1-2 cm of the plant shoot, was routinely sampled

for introduced bacteria. In 1986, the seed, if present, was removed at sampling.

For each 1984 field sample, four root systems were mechanically shaken for 30 min in 100 ml of sterile distilled water (SDW). The roots were then transferred to a sterile flask. The soil suspension which remained was the rhizosphere subsample. The roots were macerated in 100 ml SDW in a sterile Waring blender for 60 sec to obtain the rhizoplane subsample.

Bacteria were isolated from each subsample via replicate dilution plating on the following media. BCBRVB, a modified King's medium B (Sands et al., 1980), was used to select for fluorescent pseudomonads while the actinomycete selective medium (Kuster and Williams, 1964; Williams and Davies, 1965) selected for actinomycetes, especially streptomycetes. Bacillus spp. were obtained primarily with a modified soil extract agar (Parkinson et al., 1971). The 1/10 strength tryptic soy broth agar (1/10 TSBA) was utilized as a general plate count medium. Lactobacillus MRS broth agar was used to select organisms capable of utilizing glucose aerobically. (See Appendix for media recipes.) Plates were incubated at 28 C for 5 days. For two locations, replicate plates were also incubated at 5 C for 10 days. Colonies representative of the morphological types present on the plates were selected and streaked for purity on tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI). A total of 537 bacterial isolates were selected.

Ten additional isolates were selected from dilution plates of a Quincy, WA. take-all suppressive soil. These isolates were obtained in February of 1985.

The antifungal activity of each isolate was tested utilizing Rhodotorula glutinis. Each isolate was inoculated onto the center of a potato dextrose agar plate (PDA) (Difco) and incubated at 28 C. Bacterial growth was terminated after 4 days by exposure to chloroform vapors. Cells from a 4 day old PDA culture of R. glutinis were suspended in SDW and then sprayed over the bacterial growth plates. After 36 hours zones of inhibition were measured. For isolates obtained from plates originally incubated at 5 C, antibiotic activity was tested at 5 C in addition to the normal 28 C incubation temperature.

Screening for Gaeumannomyces graminis var. tritici  
Inhibition and Lysis

Isolates which were selected for field experiments were also tested for their ability to inhibit Ggt in vitro at four pH levels and three temperatures. The pH levels were 5, 6, 7 and 8 and were obtained by adjusting the pH of PDA, after autoclaving, with either 0.1 N HCl or 0.1 N NaOH. Each isolate was inoculated onto the center of each of the four different PDA/pH level plates. Four 6-mm-diameter plugs of Ggt growing on PDA were then placed equidistant from the center of each plate. These plates were incubated at 28 C. Zones of inhibition were measured after 4 and 7 days of growth.

To determine the effect of temperature on inhibition, three replicate PDA plates (pH 7) were inoculated with each bacterial

isolate and Ggt as described previously. Plates were incubated at 2 C, 15 C or 28 C. Zones of inhibition were measured after 4, 7 and 10 days of growth.

The bacterial isolates were also screened for their ability to lyse hyaline or melanized Ggt hyphae. Single 6-mm-diameter plugs of Ggt growing on PDA were placed in the center of PDA or Czapek solution agar (CSA) (Difco) plates. CSA plates were incubated at room temperature for 14 days to obtain primarily hyaline hyphae. PDA plates were incubated for 11 days to obtain melanized hyphae.

After these incubation periods, each bacterial isolate was inoculated on one plate of each medium at four points equidistant from the center. The bacteria were inoculated by making a heavy bacterial suspension in SDW and placing a drop of the suspension directly on the Ggt hyphae growing on each medium. Plates were incubated at 28 C and examined after 4, 7 and 12 days for bacterial growth and lysis of the hyphae by the bacterial isolate.

#### Storage, Mutant Development and Identification

The 60 isolates selected for field testing were stored using three different systems for each isolate: a) on 1/10 TSBA slants at 2 C; b) in 30% glycerol at -15 C; c) in 35% glycerol at -70 C. Fresh 1/10 TSBA slants were made every six months. Colonies of each isolate were transferred to the fresh slants after determining that the isolate was still viable and, after mutants were developed, that they were still antibiotic resistant.

Glycerol storage solutions utilized tryptone yeast extract broth (TYE) for streptomycetes and Rhizobiaceae isolates or nutrient broth (NB) (BBL Microbiology Systems, Cockeysville, MD) for all other isolates. For glycerol storage, isolates were grown in small screw-cap tubes or vials of TYE and NB for 24-48 hr with shaking. An appropriate aliquot of 80% sterile glycerol was then added. Tubes and vials were shaken and placed in cold storage - tubes at -15 C and vials at -70 C.

Antibiotic-resistant mutants of the isolates selected for field testing were developed utilizing four antibiotics - streptomycin, rifampicin, nalidixic acid and erythromycin (Sigma Chemical Co., St. Louis, MO). All antibiotics were sterilized in 95% ethanol. Rifampicin and erythromycin were also solubilized in ethanol, but nalidixic acid and streptomycin were solubilized in SDW. Each isolate was spread on 1/10 TSBA plates, each plate containing one antibiotic at 25 or 100 µg/ml. Resistant colonies were selected and serially transferred twice on the appropriate antibiotic-supplemented 1/10 TSBA medium. From the last plate, resistant colonies were transferred to 1/10 TSBA without antibiotics to determine if these putative mutant isolates were resistant to the antibiotic or dependent on it for growth. To obtain double- or triple-marked mutants, the same procedures were used with the remaining antibiotics.

Fifty-six of the 60 isolates developed antibiotic resistance to one or more antibiotic. Two isolates became dependent on streptomycin for growth and two isolates developed neither resistance nor

dependence to any of the antibiotics. Mutants were stored using the three methods described previously.

In this report, mutant isolates are designated by the addition of letters to their identification numbers. Letters indicate which antibiotic(s) the isolate is resistant to or dependent upon for growth: E = erythromycin; N = nalidixic acid; R = rifampicin; S = streptomycin. Capital letters signify a resistance level of 100  $\mu\text{g}/\text{ml}$  and small letters signify a resistance level of 25  $\mu\text{g}/\text{ml}$ . If an isolate is dependent upon the antibiotic for growth, a "+" mark is inserted after the capital letter.

To determine which of the four antibiotics or combination of these antibiotics would be most useful as genetic markers for monitoring the bacterial isolates in the soil and on plant roots, an attempt was made to obtain 14 different mutants of each of four bacterial isolates. The isolates were 109 (Streptomyces sp.), D-39 (B. subtilis), D-56 (coryneform) and D-290 (P. fluorescens). The 14 different mutant types desired included resistance to each of the antibiotics separately, all double combinations of the antibiotics and all triple combinations of the antibiotics. The technique previously described was employed to obtain the mutants. These mutants were stored only on 1/10 TSBA slants at 2 C.

Mutant isolates were first categorized according to Gram reaction, morphology and oxygen requirements. Gram-negative rods were identified using procedures outlined in Bergey's Manual of Determinative Bacteriology (Krieg, 1984) and, since they were primarily Pseudomonas species, the taxonomic monograph of Stanier et

al. (1966) and the laboratory guidelines of Sands et al. (1980). Gram-positive rods which produced endospores aerobically (Bacillus species) were identified to species using the procedures of Gordon et al. (1973). The one aerobic Gram-positive rod which did not produce endospores was identified simply as a coryneform (Buchanan and Gibbons, 1974). The Gram-positive filamentous strains (actinomycetes) were identified as Streptomyces species based on morphological characteristics (Buchanan and Gibbons, 1974). Chemical analysis of the cell wall components are necessary to identify the coryneform isolate to genus and to confirm that the actinomycete isolates are members of the genus Streptomyces.

#### Mutant Isolate Stability

To determine the stability of antibiotic resistance and root colonization efficiency, six mutant isolates were transferred repeatedly on a rich non-selective medium. The mutant isolates utilized were 28Er (P. fluorescens), 88SE (X. maltophilia), D-39Sr (B. subtilis), D-56SR (coryneform), D-60R (B. subtilis) and D-185S (Streptomyces sp.). Isolates were transferred from storage slants to tryptic soy broth agar (TSBA).

Four randomly selected single colonies of each parental-mutant-isolate were subcultured on new TSBA plates - one plate per subisolate. Subsequently, each subisolate was transferred to new TSBA plates every 2-4 days until each one had been serially transferred ten times. Transfers were then made to 1/10 TSBA slants for storage at 2 C.

After all the subisolates were in storage, experiments were begun to compare antibiotic resistance and root colonization efficiency between the parental-mutant-isolates and their subisolates. Stability of antibiotic resistance was determined by replica plating the mutant isolates and mutant subisolates on the appropriate antibiotic media. If any mutant isolate or mutant subisolate failed to grow, it was transferred again to verify these results. Root colonization efficiency was determined via normal seed bacterization techniques and growth of the plants in an environmental growth chamber.

#### Bacterial Inoculation of Seed

Mutant isolates were transferred from storage slants to 1/10 TSBA plates with the appropriate antibiotic(s) and level(s) to insure stability of antibiotic resistance or dependence. These plates were then used as the inoculum source for the plates and flasks used to increase the bacterial isolates for seed treatment.

The Bacillus mutant isolates were grown on soil extract agar (SEA) for 4 days at 28 C. Depending on the mutant isolate, this provided a mixture of vegetative spores and endospores. Gram-negative mutant isolates, plus the one coryneform mutant isolate, were grown on TSBA for 2 days at 28 C. Plates were inoculated by smearing a single colony on each plate. Streptomycetes were grown from single colonies in TYE on a reciprocal shaker for 2 days.

Streptomycin-dependent mutant isolates were grown on TSBA supplemented with 100 µg/ml streptomycin. Rather than smearing a

single colony on each plate, a single colony was streaked on the plate via a series of closely spaced straight lines to facilitate growth.

For all experiments, the spring wheat cultivar Pondera was utilized. For root colonization studies of crops other than spring wheat, Winridge winter wheat, Pirolina barley, Pioneer Hybrid 3540 corn, Harosoy soybean, and Oker and MT 81B-3697 safflower were used. All seed was surface sterilized with 0.5% NaOCl for 5 min followed by three rinses in SDW. The seed was then spread out to dry overnight on paper towels in a clean air chamber under a sterile air stream.

Seed for single isolate treatments was coated with the following amounts of treatment components. The cells from one SEA or one TSBA plate or one 10 ml TYE flask were suspended in 6.25 ml sterile 1% carboxymethylcellulose (CMC) (medium viscosity) (Sigma). This suspension was used to coat 12.5 g seed.

The seed for isolate mixture treatments was inoculated using the same total mass of bacteria for the inoculum. Thus, for the treatments utilizing two mutant isolates, one-half the normal amount of each isolate was used - i.e. one-half plate or 5 ml of each isolate per 12.5 g seed. For the mixture treatment utilizing five different mutant isolates, one-fourth plate or 2.5 ml of each isolate was used to inoculate the seed.

For two of the 1986 field experiments, different inoculum rates of the same mutant isolates were utilized as seed treatments. Isolates 88SE (X. maltophilia) and 28Er (P. fluorescens) were applied individually to spring wheat seed at six different inoculum rates. The normal rate was described above. Three additional rates were 1/4

normal, 1/16 normal and 1/64 normal - designated as rates 2, 3 and 4 respectively. Rates 5 and 6 were not as precise. Rate 5 was one loopful (4-mm diameter) of bacteria, scraped off a TSBA plate, per 12.5 g seed. Rate 6 was 1/6 loop per 12.5 g seed or one loopful per 75 g seed - the total amount of seed inoculated per treatment. The methods utilized to obtain rates 5 and 6 were unorthodox but did achieve the desired effect of fewer bacteria per seed. Bacterial number per seed was determined as described later in this section.

For isolates grown on SEA or TSBA, bacteria were scraped from the plates, suspended in 1% CMC and then mixed with the seed. This technique was altered once for a 1986 field experiment where mutant isolates of isolate 199 (P. fluorescens) were compared. One mutant was streptomycin dependent and the other was streptomycin resistant. To insure that no streptomycin was carried over from the streptomycin supplemented TSBA growth plates, the mutant isolates were scraped off the plates, suspended in sterile phosphate buffer (SPB) (ph 6.8), shaken well, centrifuged at 3020 x g for 10 min and the pellet resuspended in 1% CMC.

Streptomycete isolates grown in TYE were collected via centrifugation at 3020 x g for 10 min. The pellet was then suspended in 1% CMC and mixed with the seed.

Seed was allowed to soak in the bacterial suspension for a minimum of 30 min before spreading out to dry overnight on butcher paper on clean bench tops in a room with minimal air movement. The seed was then packaged in clean paper envelopes for planting. Extra seed from each treatment was deposited in paper envelopes which were

placed in plastic boxes or bags and stored at 2 C. For all field experiments, seed was planted within 48 hr of inoculation. Seed for growth chamber experiments was planted within 24 hr of inoculation.

Within 24 hr after field planting or 6 hr after growth chamber planting, the extra seed was assayed to determine bacterial number per seed and seed germination. Five seeds per treatment were placed in SPB, sonicated (Mettler Ultrasonic Cleaner - Model ME 4.6) 30 sec, allowed to soak for 30 min and then plated by serial dilutions on the appropriate 1/10 TSBA antibiotic medium. Seed germination tests were also conducted at this time by placing 10 seeds per treatment in a sterile glass petri plate containing a sterile filter paper (Whatman #1) moistened with 5 ml SDW. For the mutant isolates field tested in 1985, the number of bacteria per seed was determined again after 3 months in cold storage.

Non-bacterized check treatments for field experiments included seed sterilized and coated only with 1% CMC, seed sterilized but not coated with CMC and seed not sterilized.

### Experimental Setup and Design

#### Field Experiments

Field experiments were established in 1985 and 1986 on the A. H. Post Agronomy Farm near Bozeman, MT. Supplemental sprinkler irrigation was provided in both years.

Experimental design for the three 1985 field experiments and two of the seven 1986 field experiments was a randomized complete block with four replications. Plots consisted of three rows if yield was

measured; otherwise, one-row plots were established. Plants sampled from yield plots for root colonization assays were obtained from the outside two rows since the middle row was evaluated for disease and/or harvested for grain yield. For the other five 1986 field experiments, each treatment within an experiment was planted as a block of 12 rows.

In 1985, the mutant isolates were used as single isolate treatments in each of the three field experiments. Since one of the initial intents of this project was to identify soil bacteria capable of controlling Ggt, two of the experiments were designed to evaluate this characteristic in addition to rhizosphere competence. The experiment evaluating root colonization only had no Ggt inoculum placed with the seed at planting. Instead, 5 g of chopped, autoclaved oat kernels were deposited in each row. This was intended to offset any effects of the oat kernel base of the Ggt inoculum on plants or bacterial isolates. Ggt inoculum consisted of autoclaved oat kernels infested with a virulent Ggt isolate. One gram of Ggt oat kernel inoculum was placed with the seed at planting in a second experiment to provide take-all symptoms at a severity typically observed in a commercial field. For the third experiment, 5 g of Ggt oat kernel inoculum was deposited with the seed to obtain severe take-all symptoms - i.e. plant death before maturity.

In 1986, one of the field experiments utilized 20 of the 60 field tested isolates of 1985 as single isolate treatments. These 20 mutant isolates were selected for further testing based on rhizosphere competence in the 1985 field experiments and stable

storage or antibiotic-resistant characteristics. In addition to the single isolate treatments, nine of these 20 mutant isolates were also utilized for ten treatments in which they were mixed together in various combinations and applied to the seed. Disease control was not evaluated in any of the 1986 field experiments so no Ggt inoculum or autoclaved oat kernels were added to the rows at planting.

#### Growth Chamber Experiments

Growth chamber experiments were established in plastic cone containers. The selected cone size depended on the plant growth medium with which they were filled. The tip of each cone was plugged with non-absorbent cotton. The experimental design for all experiments was a randomized complete block with four replications of each treatment.

Experiments with plants grown in non-sterile, horticultural grade vermiculite used 10-cm deep cones. These were filled with vermiculite to within 2.5 cm of the top. The vermiculite was then saturated with 20 ml of non-sterile, distilled water. Two bacterized seeds were planted in each cone. The cone was filled to the top with more vermiculite and wetted with 5 ml more of non-sterile, distilled water. After planting, all cones were covered with plastic wrap and placed in a growth chamber maintained at  $21\text{ C} \pm 2\text{ C}$  with fluorescent and incandescent lamps providing an average of  $4850\text{ lumens/m}^2$  for 12 hr each day. After plants emerged, the plastic wrap was removed and all plants watered with 10 ml non-sterile, distilled water. Plants were watered on a regular basis thereafter - usually 5 ml per application on alternating days.

Only Bozeman silt loam was utilized for experiments with plants grown in soil since this was the soil type at the A. H. Post Agronomy Farm where the field experiments had been conducted. Soil utilized was air-dried, stored dry at 15 C and ground to a 2-mm size before use. The 15-cm deep cones were filled with non-sterile soil to within 5 cm of the top and tapped down five times. The cones were covered with plastic wrap and placed for sub-irrigation into a container of non-sterile, distilled water at the same height as the soil in the cones. Cones were soaked for a minimum of 12 hr which saturated the soil in all cones. They were then drained for 48 hr - i.e. to field capacity. Two bacterized seeds were planted per cone and the cone filled with 2.5 cm more soil. Cones were covered with plastic wrap after planting and placed in the growth chamber under the conditions previously described. After plant emergence, the plastic wrap was removed and the plants watered as needed with non-sterile, distilled water.

#### Root Colonization Assays

The basal selective medium used to reisolate introduced bacteria from roots was 1/10 TSBA with 100 µg/ml cycloheximide (Sigma) and 25 µg/ml (a.i.) pimarinic acid (Gist-Brocades, Delft, The Netherlands) added to inhibit fungal growth. The four antibacterial antibiotics (nalidixic acid, rifampicin, streptomycin and erythromycin) and their levels added depended on which mutant bacterial isolate was being assayed. For the non-bacterized check treatments, the basal selective

medium was utilized to determine total culturable aerobic bacteria of rhizosphere samples.

#### 1985 Field Experiments

Two different types of assays were conducted to determine root colonization. One was a direct plate assay. Two plants from each replication of each experiment were pulled 25 days after planting. Plants were pulled from the soil rather than dug in 1985 due to the extremely dry conditions. Some roots were lost due to breakage but probably no more than would have been lost in the digging process. Plants had 4 to 5 leaves, 1 to 2 tillers (growth stage 2.1) (Zadoks et al., 1974) and the root system consisted primarily of seminal roots and the sub-crown internode with 1 or 2 crown roots beginning to develop. The stem was cut 2 cm above the crown and discarded. After shaking roots vigorously to remove excess soil and removing clumps by hand, roots were placed in clean paper envelopes which were placed in plastic bags and kept cool until used for the assay. Assays were completed within 6 hr of pulling the plants.

Roots were placed directly onto selective medium plates using sterile techniques and incubated at 28 C. Plates were evaluated at 3 and 6 days for growth of the inoculated isolate along roots. To confirm that bacteria on these plates were the inoculated mutant isolates, a rapid visual comparison was made by streaking a colony from the assay plate on half of a fresh selective medium plate and the known mutant isolate on the other half. Plates were examined after 2 and 4 days of growth.

This same assay was utilized 43 days (growth stage 4.5) after planting for the mutant isolates which tested positive in the first assay. At this time the root system consisted of both seminal roots and crown roots.

The second type of root colonization assay was a dilution plate assay and was first completed at the same time as the second direct plate assay (growth stage 4.5). The same techniques were used to obtain the root samples except only one plant from each replication was sampled. Roots were transferred from envelopes to tubes containing 10 ml SPB, sonicated 30 sec, allowed to soak 30-45 min and then plated from serial dilutions on the appropriate antibiotic-supplemented 1/10 TSBA.

To obtain the dry weight of the roots plus tightly adhering soil, the contents of each 10 ml SPB tube with root sample were poured onto preweighed filter paper (Whatman #1). Minimal soil passed through the filter paper. After filtration, the filter paper with root and soil sample was placed in a glass petri plate and allowed to dry for 3 days at 80 C. The paper was weighed again to obtain the weight of root-soil sample.

The dilution plate assay was also utilized to compare colonization of each portion of the root system. For the experiment evaluating root colonization only, one plant from each replication of the six treatments sampled plus the non-sterilized check treatment was obtained. Each root portion (seminal roots, sub-crown internode, crown roots) of each plant sampled was placed in a paper envelope. The rest of the assay was conducted as described above.

### 1986 Field Experiments

Dilution plate assays were conducted for evaluation of root colonization at various growth stages of each experiment. Only seminal roots and sub-crown internodes of the root system were evaluated.

Field sampling techniques were altered slightly from those in 1985. Four plants, rather than one plant, from each row sampled were dug from the soil and pooled together as one sample. Since the treatments of five of the seven experiments were planted as blocks of twelve rows, four rows were randomly selected at each sampling time to obtain four replications for the assay.

Stems were cut 2 cm above the crown and discarded. This time the remnant seed, if present, was also removed and discarded so only bacteria colonizing the roots and crown were assayed. After removing excess soil, the roots were placed directly into new plastic self-seal sandwich bags (Ziploc brand) and stored at 2 C until used for the assay. These bags were free of any bacteria not inhibited by the antibacterial antibiotics incorporated in the selective media.

Rather than transferring the roots to SPB in tubes, the SPB was added directly to the bag with the roots. The samples were then sonicated 30 sec in the bags, soaked 30-45 min and plated by serial dilution on the appropriate 1/10 TSBA antibiotic media. Dry weights were determined as previously described.

For 12 of the single isolate treatments, replica plates of the dilutions were made utilizing the basal selective medium (1/10 TSBA with cycloheximide and pimaricin). This was to determine what

percentage of the total aerobic bacteria isolated from the rhizosphere consisted of the treatment isolate.

Dilutions of mixture isolate treatments were also replica plated. The selective media used depended on the isolates applied to the seed.

For the field experiment examining the usefulness of antibiotic combinations as selection markers, border check rows planted with non-bacterized seed were sampled and replica plated on each of the 14 different antibiotic-supplemented selective media plus the basal selective medium. Nystatin (Sigma) at 25  $\mu\text{g}/\text{ml}$  was substituted for pimarinin in the selective media due to a temporary shortage of pimarinin.

#### Growth Chamber Experiments

Dilution plate assays were completed 4 weeks after planting for all growth chamber experiments. Plants were dislodged from the cone containers and excess vermiculite or soil removed by hand. The stems were cut 1 cm above the crown and discarded, as was the remnant seed. The roots were then placed directly into the Ziploc sandwich bags and stored at 2 C until used for the assay within 6 hr.

If more than one plant was present in the cone, only one plant's roots were assayed. For the mutant isolate stability experiment, the person working with the plant roots washed their hands with soap and water before extracting the next plant. The rest of the assay technique was the same as previously described.

## RESULTS

Preliminary Screening

During the 1984 field season, 537 bacterial isolates were selected from a large group of bacteria obtained via dilution plating of spring and winter wheat and barley roots on selective and general media. All gross morphological colony types present on the dilution plates were represented by the isolates selected. Since biological control of fungal soilborne plant diseases such as take-all was a primary research interest and it was logistically impossible to screen all 537 bacterial isolates in the field, the isolates were initially screened for in vitro antifungal activity. This was accomplished via an antibiotic plate assay utilizing the red yeast Rhodotorula glutinis.

Forty-nine isolates that demonstrated some degree of antifungal activity and were stable in storage were selected for field testing. Eleven additional isolates chosen for field testing were: a) five actinomycete isolates obtained from a Quincy, WA. take-all suppressive soil provided by D. Weller, b) a Xanthomonas maltophilia isolate previously shown to be a wheat root colonizer in Montana (R. Kuestner, personal communication) and c) five isolates from the original 537 isolates with potential plant protection characteristics other than production of antifungal antibiotics. One of these five isolates produced an antibacterial antibiotic, two produced a dark pigment in

culture and two produced acid from glucose aerobically. All 11 isolates were screened for inhibition of R. glutinis but only one, a Quincy soil actinomycete, was inhibitory. The extent of R. glutinis inhibition by the 60 isolates is reported in Table 1.

Gaeumannomyces graminis var. tritici Inhibition  
And Lysis In Vitro

Since my original goal was to identify bacteria capable of controlling take-all disease, the isolates selected for field testing were screened for their ability to inhibit Ggt in vitro. All the isolates were screened except the five Quincy soil actinomycetes since these had not yet been isolated. However, as determined in a later screening test, two of the Quincy soil actinomycetes (isolates 236 and 242) did inhibit Ggt in vitro.

The pH of the rhizosphere can differ from that of the surrounding bulk soil and will fluctuate during the growing season or can be manipulated to obtain the desired pH (Weinberger and Yee, 1984; Smiley, 1974). Therefore, the ability of the selected bacterial isolates to inhibit Ggt at four pH values was determined (Table 1). In general, for each isolate, there was little difference in Ggt inhibition at pH 6, 7 or 8. In contrast, at pH 5 distinct differences in inhibition by some of the isolates were observed. Twenty of the isolates, primarily Streptomyces and Bacillus spp., did not grow. Five isolates, all P. fluorescens, produced larger inhibition zones at pH 5 against Ggt - 7 mm or more - compared with those at the other three pH values.

Table 1. Antifungal activity of 60 bacterial isolates as affected by pH and temperature.

Isolate No.	Isolate Identification	Rhodotorula <sup>a</sup> Rating	Ggt <sup>b</sup> Lysis	Ggt Inhibition (mm) <sup>c</sup>				Ggt Inhibition (mm) <sup>c</sup>		
				pH 5 <sup>d</sup>	pH 6	pH 7	pH 8	2 C <sup>e</sup>	15 C	28 C
9	Streptomyces	2	0	NG	5	3	3	NG	3	3
22	Streptomyces	2	0	NG	4	5	5	NG	2	5
28	Pseudomonas	4	2	9	10	10	6	G	11	10
53	Pseudomonas	1	1	NG	0	0	0	NG	0	0
58	Bacillus	1	0	NG	2	0	0	NG	0	0
59	Xanthomonas	2	0	0	0	0	0	NG	0	0
74	Streptomyces	0	1	0	0	0	0	NG	0	0
80	Pseudomonas	1	1	NG	0	0	0	NG	0	0
88	Xanthomonas	0	1	0	0	0	0	NG	0	0
108	Streptomyces	1	1	0	3	4	3	NG	0	4
109	Streptomyces	1	0	NG	1	1	1	NG	2	1
116	Streptomyces	2	0	0	5	5	6	NG	3	5
118	Pseudomonas	2	1	14	5	4	1	G	7	4
121	Pseudomonas	1	1	12	9	5	5	G	12	5
125	Rhizobiaceae	1	1	NG	0	0	0	NG	0	0
131	Streptomyces	1	0	NG	4	5	5	NG	2	5
147	Bacillus	1	1	0	3	4	5	NG	NT	4
DI-152	Bacillus	3	1	4	3	7	6	NG	10	7
DI-155	Bacillus	2	0	NG	2	2	1	NG	0	2
157	Bacillus	4	1	NG	5	8	8	NG	8	8
162	Bacillus	2	0	NG	1	1	<1	NG	1	1
165	Pseudomonas	2	1	15	5	NT	1	G	6	NT
176	Pseudomonas	2	1	12	5	5	3	G	2	5
189	Pseudomonas	2	1	14	5	5	3	G	3	5
199	Pseudomonas	1	1	2	2	0	0	G	1	0
216	Pseudomonas	1	1	5	3	2	1	G	2	2
230	Bacillus	3	2	4	6	6	6	NG	7	6
231	Enterobacteriaceae	0	0	NG	I	3	I	NG	5	3
236	Streptomyces	1	0	NT <sup>f</sup>	NT	NT	NT	NT	NT	NT
238	Streptomyces	0	0	NT	NT	NT	NT	NT	NT	NT
239	Streptomyces	0	0	NT	NT	NT	NT	NT	NT	NT
241	Streptomyces	0	0	NT	NT	NT	NT	NT	NT	NT
242	Streptomyces	0	0	NT	NT	NT	NT	NT	NT	NT
1000	Xanthomonas	0	0	0	0	0	0	NG	0	0
1001	Bacillus	4	2	6	7	10	9	NG	10	10

Table 1--continued

Isolate No.	Isolate Identification	Rhodotorula <sup>a</sup> Rating	Ggt <sup>b</sup> Lysis	Ggt Inhibition (mm) <sup>c</sup>				Ggt Inhibition (mm) <sup>c</sup>		
				pH 5 <sup>d</sup>	pH 6	pH 7	pH 8	2 C <sup>e</sup>	15 C	28 C
D-4	Streptomyces	4	1	0	10	8	8	NG	5	8
D-39	Bacillus	3	1	NG	3	1	1	NG	3	1
D-56	Coryneform	3	1	NG	0	0	0	G	0	0
D-60	Bacillus	3	2	5	4	2	3	NG	10	2
D-82	Streptomyces	1	2	NG	3	4	4	NG	3	4
D-168	Bacillus	3	2	2	1	2	1	NG	8	2
D-173	Bacillus	3	2	5	7	7	8	NG	10	7
D-180	Streptomyces	2	1	NG	4	4	5	NG	5	4
D-181	Bacillus	3	1	4	I	I	I	NG	10	I
D-183	Bacillus	3	2	5	6	5	5	NG	7	5
D-185	Streptomyces	2	2	NG	2	3	4	NG	3	3
D-187	Bacillus	3	2	5	I	7	I	NG	10	7
D-188	Bacillus	3	2	6	5	5	3	NG	1	5
D-198	Bacillus	3	2	6	I	I	I	NG	10	I
D-199	Streptomyces	2	2	NG	5	3	4	NG	5	3
D-214	Bacillus	0	0	NG	0	0	0	NG	0	0
D-217	Enterobacteriaceae	0	1	0	0	0	0	G	0	0
D-220	Bacillus	3	2	NG	8	6	8	NG	8	6
D-221	Bacillus	3	2	5	5	8	7	NG	7	8
D-222	Bacillus	1	2	1	3	3	3	NG	9	3
D-226	Bacillus	3	2	2	7	7	6	NG	10	7
D-267	Pseudomonas	1	1	12	1	3	2	G	3	3
D-273	Pseudomonas	1	1	2	5	4	1	NG	1	4
D-288	Pseudomonas	2	1	12	4	3	2	G	4	3
D-290	Pseudomonas	3	2	4	11	NT	8	G	12	NT

<sup>a</sup>Rhodotorula glutinis inhibition rating: 0 = no inhibition; 1 = no distinct non-fungal zone, just less concentrated fungal growth; 2 = <5 mm zone of inhibition; 3 = 5 to 15 mm zone of inhibition; 4 = >15 mm zone of inhibition.

<sup>b</sup>Lysis of hyaline Gaeumannomyces graminis var. tritici (Ggt) hyphae: 0 = no bacterial growth and lysis; 1 = bacterial growth but no lysis; 2 = bacterial growth plus lysis.

<sup>c</sup>NT = Bacterial isolate not tested for this particular characteristic; I = Bacterial isolate inhibits Ggt but due to bacterium's spreading nature, unable to determine a distinct zone of inhibition.

<sup>d</sup>NG = No growth of bacterial isolate at this pH.

<sup>e</sup>G = Growth of bacterial isolate; NG = No growth of bacterial isolate.

<sup>f</sup>Isolates 236 through 242 were not tested for inhibitory activity of Ggt at this time.

Soil temperature fluctuations are also a natural occurrence during the growing season of spring wheat. To test the effect of temperature on bacterial inhibition of Ggt, the inhibition assay was conducted at three temperatures - 2 C, 15 C, and 28 C (Table 1). Ggt and 47 of the bacterial isolates did not grow at 2 C. When the size of Ggt inhibition zones were compared for temperatures 15 C and 28 C, only four of the isolates had inhibition zone differences of 5 mm or more.

By comparing R. glutinis and Ggt inhibition results for each isolate in Table 1, the isolates can be categorized into four groups: a) 46 isolates inhibited R. glutinis and Ggt; b) 5 isolates inhibited R. glutinis but not Ggt; c) 8 isolates did not inhibit either organism; d) 1 isolate (231) inhibited Ggt but not R. glutinis.

Three months later, after antibiotic-resistant mutants were developed, the mutant isolates and their parental isolates were again screened for the ability to inhibit Ggt in vitro. Twelve of the parental isolates, which originally had inhibited Ggt, were no longer inhibitory while a few isolates showed reduced inhibition. Two antibiotic-resistant mutants, derived from parental isolates which significantly inhibited Ggt (28 and D-226), were incapable of inhibition after developing antibiotic resistance.

One mechanism of control for take-all would be the actual lysis of melanized or hyaline Ggt hyphae (Tschudi and Kern, 1979; Campbell, 1983). An in vitro test was designed to determine if any of the bacterial isolates could lyse one or both types of hyphae

(Table 1). None of the isolates could even grow on melanized Ggt hyphae whereas 43 of the isolates grew on the hyaline hyphae. Only 18 of these could also lyse the hyaline hyphae.

#### Mutant Isolate Development and Identification

To monitor the presence or absence of the 60 selected isolates on plant roots under natural field conditions, antibiotic-resistant mutants of 56 of these 60 isolates were obtained. Two of the remaining isolates were antibiotic-dependent. The other two isolates developed neither resistance nor dependence on antibiotics. Four antibiotics were utilized as markers - streptomycin, erythromycin, rifampicin and nalidixic acid. Each antibiotic has a different site of action (Bryan,1982).

The streptomycetes and the one coryneform isolate were identified only to genus. The majority of gram-negative isolates and all the gram-positive, endospore producing isolates were also identified to genus, but only those isolates utilized for field studies in both 1985 and 1986 were identified to species. The 60 isolates included 14 Pseudomonas spp., 3 Xanthomonas spp., 17 Streptomyces spp., 22 Bacillus spp., 1 coryneform, 1 Rhizobiaceae and 2 Enterobacteriaceae family members.

#### Mutant Isolate Stability

As indicated in the materials and methods, bacterial growth from 1/10 TSBA storage slants was used as the initial source for increasing the antibiotic-resistant mutant isolates used as seed inoculum.

Mutant isolates were transferred from slants to 1/10 TSBA plates supplemented with the appropriate antibiotics for each isolate. This insured that the isolate used to inoculate seed was still viable and antibiotic resistant. Single colonies from these plates were then used to inoculate growth media for bacterial increase.

Fresh slants were made every 6 months. Each mutant isolate was transferred from the old slant to antibiotic-supplemented 1/10 TSBA plates. A single colony from these plates was then streaked on the fresh slant. Again, growth under these selective pressure conditions insured that the mutant isolate was still viable and resistant to the appropriate antibiotics.

The majority of the mutant isolates were stable in storage when handled as described above. If utilized for commercial seed bacterization, it is quite possible the mutant isolates would be serially transferred under non-selective growth conditions. Therefore, a study was conducted with six of the mutant isolates to determine the stability of antibiotic resistance and root colonization efficiency after repeated transfers of isolates on a rich, non-selective medium. The six mutant isolates utilized were 28Er (P. fluorescens), 88SE (X. maltophilia), D-39Sr (B. subtilis), D-56SR (coryneform), D-60R (B. subtilis) and D-185S (Streptomyces sp.). Four subisolates of each parental-mutant-strain were obtained for comparative purposes. Each subisolate had been transferred ten times.

After all the mutant subisolates were in storage, experiments were begun to compare antibiotic resistance and root colonization between the parental-mutant-isolates and their subisolates. All four

subisolates of 28Er, 88SE, D-60R and D-185S but only two subisolates of D-39Sr were still resistant to the appropriate antibiotics. None of the D-56SR (coryneform) subisolates remained resistant to streptomycin and rifampicin.

Those mutant subisolates which had maintained the parental phenotype for antibiotic resistance were increased along with the parental-mutant-isolate for inoculation on seed for use in a growth chamber experiment to test spring wheat root colonization. Four weeks after planting, dilution plate assays were conducted for root colonization. Results are summarized in Table 2.

Significant differences for root colonization between the parental-mutant-isolate and the subisolates were observed for mutant isolates 28Er and D-185S at the 5% significance level but not at the 1% significance level. Only one subisolate from each of these two groups was substantially different from the average root colonization values of each group. For those isolates which were stable for antibiotic resistance, it would appear that they were also stable for root colonization.

#### Antibiotic Markers for Monitoring Introduced Bacteria

The most important consideration in evaluating root colonization by introduced bacteria is the ability to reisolate the bacteria. One method of monitoring introduced microorganisms is to use antibiotic-resistance markers. For the field experiments of this research project, spontaneous antibiotic-resistant mutants of the bacterial

Table 2. Effect of serial transfers, using non-selective media, on the ability of antibiotic-resistant isolates to colonize Pondera spring wheat roots.

Isolate <sup>a</sup>	Log <sub>10</sub> CFU/Seed	Log <sub>10</sub> CFU/mg <sup>b</sup>
<u>Pseudomonas fluorescens</u>		
Parental 28Er	6.3	2.5 x
Subisolate 28Er-1	6.4	1.4 y
Subisolate 28Er-2	6.4	2.0 xy
Subisolate 28Er-3	6.5	2.3 x
Subisolate 28Er-4	6.3	2.3 x
		LSD = 0.6
<u>Xanthomonas maltophilis</u>		
Parental 88SE	7.0	2.7 x
Subisolate 88SE-1	7.0	3.0 x
Subisolate 88SE-2	6.5	2.7 x
Subisolate 88SE-3	7.2	2.6 x
Subisolate 88SE-4	7.2	3.0 x
		LSD = 0.4
<u>Bacillus subtilis</u>		
Parental D-39Sr	5.8	2.5 x
Subisolate D-39Sr-3	6.1	2.8 x
Subisolate D-39Sr-4	5.8	2.7 x
		LSD = 0.7
<u>Bacillus subtilis</u>		
Parental D-60R	4.6	2.6 x
Subisolate D-60R-1	3.9	2.6 x
Subisolate D-60R-2	5.2	2.6 x
Subisolate D-60R-3	5.8	2.8 x
Subisolate D-60R-4	5.9	2.8 x
		LSD = 0.4
<u>Streptomyces sp.</u>		
Parental D-185S	2.7	1.3 xy
Subisolate D-185S-1	2.8	0.7 y
Subisolate D-185S-2	2.7	1.0 xy
Subisolate D-185S-3	2.5	1.8 x
Subisolate D-185S-4	2.4	1.8 x
		LSD = 0.8

<sup>a</sup>The parental-mutant-isolate was not serially transferred. The subisolates were serially transferred ten times.

<sup>b</sup>Values represent average of four replications. Dry weight (mg) of root system sampled plus tightly adhering soil. Values followed by the same letter are not significantly different (P=0.05) according to Newman-Keuls multiple range test.

isolates were developed. These mutants were then field tested for root colonization capabilities.

In the 1985 field experiments, it was demonstrated for Bacillus species that streptomycin resistance was not an adequate marker since numerous other soil organisms were naturally resistant to streptomycin. Some P. fluorescens and Streptomyces isolates with nalidixic acid resistance were not stable in storage which seemed to preclude their ability to colonize.

Therefore, it would be beneficial to determine which antibiotics and combination of antibiotics would be most useful as genetic markers for monitoring introduced bacteria in the field. Four of the isolates were selected for this experiment - 109 (Streptomyces sp.), D-39 (B. subtilis), D-56 (coryneform) and D-290 (P. fluorescens).

An attempt was made to mark each of these isolates fourteen different ways using the antibiotics erythromycin, streptomycin, rifampicin and nalidixic acid at the level of 100 µg/ml. In other words, each isolate would be resistant to each of the four antibiotics separately, to two of the antibiotics in all combinations, and to three of them in all combinations. This was accomplished only for isolates D-56 (coryneform) and D-290 (P. fluorescens). Isolate 109 (Streptomyces) could only be marked four different ways and isolate D-39 (B. subtilis) marked only three ways (Table 3).

These 35 antibiotic-resistance mutant isolates were used as spring wheat seed treatments in a 1986 field experiment to evaluate their root colonization capability. This experiment also evaluated the efficacy of the antibiotic combinations to inhibit common

Table 3. Antibiotic-resistant mutants developed from four bacterial isolates for evaluation of bacterial monitoring with antibiotic markers.

Antibiotic-Resistance Marker <sup>a</sup>	Isolate <sup>b</sup>			
	109 <sup>c</sup>	D-39 <sup>d</sup>	D-56 <sup>e</sup>	D-290 <sup>f</sup>
S	+	+	+	+
R	+	+	+	+
N	+	-	+	+
E	-	-	+	+
SR	-	+	+	+
SN	+	-	+	+
SE	-	-	+	+
RN	-	-	+	+
RE	-	-	+	+
NE	-	-	+	+
SRN	-	-	+	+
SRE	-	-	+	+
SNE	-	-	+	+
RNE	-	-	+	+

<sup>a</sup>S = streptomycin; R = rifampicin; N = nalidixic acid; E = erythromycin; each at 100 µg/ml.

<sup>b</sup>+, - = resistant to antibiotic(s); - = susceptible to antibiotic(s).

<sup>c</sup>Streptomyces sp.

<sup>d</sup>Bacillus subtilis

<sup>e</sup>coryneform

<sup>f</sup>Pseudomonas fluorescens

rhizosphere microorganisms when incorporated into the basal selective medium, 1/10 TSBA with cycloheximide and pimaricin. Without efficient inhibition of the background bacterial growth on dilution plates, it is difficult to detect the marked isolate.

Dilution plate assays were completed 31 to 39 days and 74 to 78 days after planting. Mutant isolate colonies were counted as were the total number of bacterial colonies on each dilution plate. From these two counts, the percentage of bacteria on the dilution plate which were the mutant isolate was determined. In most cases, the mutant isolate colonies could be easily separated from background growth via colony morphology.

Also for the second sampling time, plants in the border rows were sampled. These rows were check rows which were planted with non-bacterized seed. Four samples were obtained and dilution replica plated on 15 media - 1/10 TSBA with each combination of antibacterial antibiotics plus 1/10 TSBA without these antibiotics. The average  $\log_{10}$ CFU for each medium is reported in Table 4 as is a percentage value which compares the number of bacteria on the antibiotic-supplemented media to the bacterial number on the non-supplemented medium. In general, rifampicin and antibiotic combinations with rifampicin were best at suppressing unmarked bacteria present in rhizosphere samples.

Isolate 109 (Streptomyces sp.) mutants were undetected or detected at very low numbers (109R) for the earliest sample time (Table 5). However, at the second sampling time the results for 109S and 109R were similar to those obtained for 109S in another 1986

Table 4. Effectiveness of four antibiotics at suppressing unmarked bacterial strains in the rhizosphere.

Antibiotics in Medium <sup>a</sup>	Log <sub>10</sub> CFU/mg <sup>bc</sup>	% of Bacteria on 1/10 TSBA <sup>bd</sup>
S	2.9	19.2
R	1.7	1.3
N	2.8	13.2
E	2.8	21.0
SR	0	0
SN	0.8	0.3
SE	1.8	2.6
RN	0	0
RE	0	0
NE	0.1	0.2
SRN	0	0
SRE	0	0
SNE	0	0
RNE	0	0
no antibiotics	3.7	----

<sup>a</sup>S = Streptomycin; R = Rifampicin; N = Nalixidic Acid; E = Erythromycin; each at 100 µg/ml.

<sup>b</sup>Values represent average of four replications.

<sup>c</sup>Dry weight (mg) of root system sampled and tightly adhering soil.

<sup>d</sup>% = (CFU per antibiotic medium ÷ CFU per non-antibiotic medium) \* 100.

Table 5. Colonization of Pondera spring wheat roots in 1986 by antibiotic--resistant mutants of isolate 109 (Streptomyces sp.) and isolate D-39 (Bacillus subtilis).

Mutant Isolate	Log <sub>10</sub> CFU/Seed	Growth Stage 1.3		Growth Stage 6.9	
		Log <sub>10</sub> CFU/mg <sup>a</sup>	% of Plate Bacteria <sup>bc</sup>	Log <sub>10</sub> CFU/mg <sup>a</sup>	% of Plate Bacteria <sup>bc</sup>
109S	2.9	0	0	0.9	2.6
109R	2.4	0.1	0	1.5	95.2
109N	3.7	0	0	0.2	0
109SN	4.3	0	0	0.1	28.0
D-39S	6.6	1.5	54.1	1.8	12.5
D-39R	6.2	1.1	100.0	2.0	97.8
D-39SR	6.4	1.0	99.4	1.6	99.4

<sup>a</sup>Dry weight (mg) of root system sampled plus tightly adhering soil. Values represent average of four replications.

<sup>b</sup>Plate bacteria include the mutant isolate and all natural antibiotic-resistant bacteria counted on the antibiotic-supplemented dilution plate.

<sup>c</sup>% = (Isolate CFU/mg ÷ Plate CFU/mg) \* 100; Average based on the number of replications with the mutant isolate present.

experiment using single isolate treatments (see Table 13). Mutant isolates 109N and 109SN were not very stable in culture. This could account for their inefficient root colonization. Mutant 109R colonies were not typical for isolate 109 growth as they no longer produced aerial spores.

All three D-39 (B. subtilis) mutant isolates colonized roots and had comparable bacterial counts for both growth stages sampled (Table 5).

Not all of the mutant isolates of D-56 (coryneform) colonized spring wheat roots (Table 6). Mutants D-56NE and D-56SNE were undetectable for both sampling times. The increase in the number of other colonizing mutant isolates from the first to second sampling time was due to an increased incubation period. Some of these mutant isolates were slower growing than the others and required 10 days rather than the normal 6 days to produce visible colonies with the typical D-56 colony morphology on the dilution plates.

As with isolate D-56, mutant isolates D-290NE (P. fluorescens) and D-290SNE were undetectable on roots for both growth stages sampled (Table 7). In addition, mutant D-290SNR was not detected in either sample. There was considerable variability in the amount of colonization by each of the D-290 mutant isolates.

For all four isolates, the mutant isolate percentage of the total bacteria on antibiotic dilution plates was greater for the first sampling time than for the second sampling time. Overall, the three best combinations of antibiotics were rifampicin alone, rifampicin with streptomycin and a combination of rifampicin, streptomycin and

Table 6. Colonization of Pondera spring wheat roots in 1986 by antibiotic-resistant mutants of isolate D-56 (coryneform).

Mutant Isolate	Log <sub>10</sub> CFU/Seed	Growth Stage 1.3		Growth Stage 6.9 <sup>a</sup>	
		Log <sub>10</sub> CFU/mg <sup>b</sup>	% of Plate Bacteria <sup>cd</sup>	Log <sub>10</sub> CFU/mg <sup>b</sup>	% of Plate Bacteria <sup>cd</sup>
D-56S	7.8	1.6	58.2	2.2	15.6
D-56R	7.9	1.7	100.0	2.3	87.8
D-56N	7.6	0	0	1.6	17.1
D-56E	8.0	--- <sup>e</sup>	----	2.0	15.7
D-56SR	8.0	1.5	100.0	2.1	100.0
D-56SN	5.8	0	0	0.6	30.7
D-56SE	7.8	1.6	90.6	2.0	63.0
D-56RN	7.6	0	0	0.4	76.7
D-56RE	7.9	1.6	100.0	1.8	69.8
D-56NE	7.9	0	0	0	0
D-56SRN	8.0	0	0	1.6	100.0
D-56SRE	7.4	1.4	100.0	2.1	100.0
D-56SNE	7.4	0	0	0	0
D-56RNE	7.7	0	0	1.1	100.0

<sup>a</sup>Results reported are from dilution plate counts at 10 days growth rather than 6 days.

<sup>b</sup>Dry weight (mg) of root system sampled plus tightly adhering soil. Values represent average of four replications.

<sup>c</sup>Plate bacteria include the mutant isolate and all natural antibiotic-resistant bacteria counted on the antibiotic-supplemented dilution plate.

<sup>d</sup>% = (Isolate CFU/mg ÷ Plate CFU/mg) \* 100; Average based on the number of replications with the mutant isolate present.

<sup>e</sup>D-56E colonies could not be differentiated from background contaminants.

Table 7. Colonization of Pondera spring wheat roots in 1986 by antibiotic-resistant mutants of isolate D-290 (Pseudomonas fluorescens).

Mutant Isolate	Log <sub>10</sub> CFU/Seed	Growth Stage 1.3		Growth Stage 6.9	
		Log <sub>10</sub> CFU/mg <sup>a</sup>	% of Plate Bacteria <sup>bc</sup>	Log <sub>10</sub> CFU/mg <sup>a</sup>	% of Plate Bacteria <sup>bc</sup>
D-290S	7.5	3.4	96.1	2.1	13.8
D-290R	7.1	2.4	99.2	0.6	49.2
D-290N	6.6	---- <sup>d</sup>	----	0	0
D-290E	6.8	2.9	55.7	1.8	9.4
D-290SR	6.8	2.0	99.2	1.0	96.8
D-290SN	6.6	2.4	96.8	1.0	67.5
D-290SE	7.1	2.6	88.0	1.4	35.9
D-290RN	6.3	1.3	99.4	<0.1	100.0
D-290RE	6.8	1.6	100.0	0.6	38.6
D-290NE	4.0	0	0	0	0
D-290SRN	5.0	0	0	0	0
D-290SRE	6.2	2.0	100.0	0.7	100.0
D-290SNE	6.4	0	0	0	0
D-290RNE	5.9	1.4	100.0	0.2	100.0

<sup>a</sup>Dry weight (mg) of root system sampled plus tightly adhering soil. Values represent average of four replications.

<sup>b</sup>Plate bacteria include the mutant isolate and all natural antibiotic-resistant bacteria counted on the antibiotic-supplemented dilution plate.

<sup>c</sup>% = (Isolate CFU/mg ÷ Plate CFU/mg) \* 100; Average based on the number of replications with the mutant isolate present.

<sup>d</sup>D-290N colonies could not be differentiated from background contaminants.

erythromycin. These combinations were efficient in inhibiting background rhizosphere microorganisms on dilution plates and did not impair efficient colonization by the mutant isolate.

#### Storage of Bacterized Spring Wheat Seed

In 1985, the bacterized seed which remained after planting the field experiments was placed in cold storage (2 C). The number of bacteria per seed was determined for each test isolate at the time of planting and was determined again 3 months later for the seed which had been in storage. The results for both seed assays are reported in Table 8.

The majority of the mutant isolates differed between assays by  $\log_{10}$  1.0 CFU or less. After storage, 14 isolates had decreased levels of bacteria per seed of greater than  $\log_{10}$  1.0 CFU. Most of these isolates were Pseudomonas species. Two isolates had substantial increases in bacterial numbers after storage. One was a streptomycete and the other was a Bacillus sp.; both had not been detected in the previous seed assay at planting. The other nine mutant isolates which had not been detected at planting were also not detected after 3 months in cold storage.

#### 1985 Field Results

In 1985 the 60 test isolates selected, which included 58 antibiotic-resistant or -dependent mutants and 2 non-mutants, were utilized in three field experiments as single isolate seed treatments for spring wheat. All three experiments were planted from a single

Table 8. Effect of cold storage on bacterial number per seed.

Isolate	Isolate Identification	Log <sub>10</sub> CFU/Seed		
		Planting Time	3 Month Storage	Difference
9S	Streptomyces	1.8	0	-1.8
22N	Streptomyces	3.4	3.8	+0.4
28Er	Pseudomonas	7.5	4.8	-2.7
53Sr	Pseudomonas	5.9	0	-5.9
58R	Bacillus	7.0	6.9	-0.1
59SN	Xanthomonas	5.3	4.6	-0.7
74	Streptomyces	1.3	6.3	+5.0
80Sr	Pseudomonas	5.7	4.5	-1.2
88SE	Xanthomonas	6.7	6.3	-0.4
108S	Streptomyces	0	0	0
109S	Streptomyces	3.4	3.9	+0.5
116N	Streptomyces	4.1	4.1	0
118SN	Pseudomonas	5.0	4.0	-1.0
121Sr	Pseudomonas	6.1	3.6	-2.5
125SR	Rhizobiaceae	3.8	3.3	-0.5
131S	Streptomyces	5.2	5.9	+0.7
147R	Bacillus	7.3	6.3	-1.0
DI-152S	Bacillus	3.3	3.4	+0.1
DI-155s	Bacillus	5.7	6.2	+0.5
157R	Bacillus	5.1	4.4	-0.7
162s	Bacillus	6.1	3.9	-2.2
165SN	Pseudomonas	5.3	4.4	-0.9
176Sr	Pseudomonas	0	0	0
189SN	Pseudomonas	4.6	2.1	-2.5
199S+	Pseudomonas	5.6	4.8	-0.8
216SR	Pseudomonas	4.4	2.8	-1.6
230S	Bacillus	0	0	0
231S+	Enterobacteriaceae	2.1	2.7	+0.6
236N	Streptomyces	3.6	4.0	+0.4
238N	Streptomyces	1.2	1.1	-0.1
239N	Streptomyces	2.2	0	-2.2
241S	Streptomyces	0	0	0
242s	Streptomyces	0	0	0
1000SNR	Xanthomonas	5.1	5.1	0
1001S	Bacillus	0	0	0

Table 8--continued

Isolate	Isolate Identification	Log <sub>10</sub> CFU/Seed		
		Planting Time	3 Month Storage	Difference
D-4N	Streptomyces	1.6	1.6	0
D-39Sr	Bacillus	7.0	6.5	-0.5
D-56SR	Coryneform	7.8	6.3	-1.5
D-60R	Bacillus	7.2	6.8	-0.4
D-82N	Streptomyces	6.4	6.2	-0.2
D-168r	Bacillus	0	6.2	+6.2
D-173S	Bacillus	0	0	0
D-180N	Streptomyces	3.6	3.9	+0.3
D-181S	Bacillus	0	0	0
D-183S	Bacillus	5.4	4.5	-0.9
D-185S	Streptomyces	2.8	3.0	+0.2
D-187S	Bacillus	3.7	3.1	-0.6
D-188R	Bacillus	6.0	6.2	+0.2
D-198S	Bacillus	4.0	0	-4.0
D-199N	Streptomyces	5.6	5.5	-0.1
D-214Sr	Bacillus	2.1	3.0	+0.9
D-217Sr	Enterobacteriaceae	4.0	5.1	+1.1
D-220S	Bacillus	0	0	0
D-221Rs	Bacillus	4.7	3.1	-1.6
D-222	Bacillus	6.0	6.0	0
D-226Sr	Bacillus	1.7	2.1	+0.4
D-267SN	Pseudomonas	7.0	6.1	-0.9
D-273SN	Pseudomonas	5.8	5.1	-0.7
D-288SR	Pseudomonas	5.4	3.9	-1.5
D-290E	Pseudomonas	7.6	5.1	-2.5

batch of treated seed for each test isolate. The number of CFU per seed for the seed planted ranged from 0 to  $6.3 \times 10^6$  and was isolate dependent. It was observed in these experiments and subsequent experiments that for some of the Streptomyces mutant isolates, the dilution plates of the treated seeds would yield no colonies. However, when the Streptomyces treated seeds were germinated on sterile blotter paper in the lab, it was demonstrated that the seeds did carry viable inoculum of the mutant isolate since the germinating seed would be covered with Streptomyces colonies.

All three experiments were designed to assess spring wheat root colonization by the 60 bacterial isolates. In addition, two of the experiments also evaluated any effects each isolate may have had on take-all disease of spring wheat and/or the causal organism Ggt.

#### Root Colonization Assay - First Sampling Date

The first test assay, conducted 25 days after planting to determine if root colonization had occurred, was a direct plate assay of all three experiments. Of the 60 isolates tested, 38 were observed to be present on the root system in at least one of the experiments. Thirty-six isolates were positive in the experiment evaluating root colonization only. Those Bacillus isolates that were resistant only to streptomycin were indistinguishable from background contaminants. Therefore, it was not possible to confirm their presence or absence. The only exception was DI-155s (Bacillus sp.) which has a distinguishable colony morphology. The three isolates belonging to either the Rhizobiaceae or Enterobacteriaceae were not detected on the roots in

this first assay. Twenty of the 38 isolates were detected on the roots in all four replications of each experiment.

#### Root Colonization Assay - Second Sampling Date

For all three experiments a second assay using the direct plate method was executed 43 days after planting for the isolate treatments positive for the first sampling date. Only two of these isolates (118SN and 165SN - both P. fluorescens) were not observed on the roots. Another isolate (D-180N - Streptomyces sp.) was inadvertently not sampled.

At the same time this direct plate assay was completed, a dilution plate assay was conducted for the 36 positive isolates of the experiment evaluating root colonization only. Again, two isolates (D-180N - Streptomyces sp. and D-188R - Bacillus sp.) were inadvertently omitted. The average  $\log_{10}$  CFU of bacteria per mg dry weight of roots-plus-tightly-adhering soil for the isolates ranged from 0 to 3.4. Table 9 summarizes the data by genus. More detailed results for the 34 isolates are reported in Table 10.

#### Root Colonization Assay - Third Sampling Date

The root system of a spring wheat plant develops in the following manner. Seminal roots develop first and originate from the embryo when the seed germinates. The maximum number of seminal roots is six with three to five being most common. The sub-crown internode is the internode between the seed and the first leaf. Its length will vary according to the depth the seed is planted (Briggle, 1967; Kirby and Appleyard, 1981). Since the seminal roots and sub-crown internode

develop first, it can be inferred that these two regions will be colonized first by bacteria already present in the soil and by bacteria introduced on the seed.

Table 9. Average number of bacteria colonizing Pondera spring wheat roots assayed at plant growth stage 4.5 in 1985.

Genus	No. Isolates	Average Log <sub>10</sub> CFU/mg <sup>a</sup>	Range Log <sub>10</sub> CFU/mg <sup>a</sup>
<u>Pseudomonas</u>	9	1.4	0 - 3.4
<u>Xanthomonas</u>	3	1.4	0.6 - 2.5
<u>Bacillus</u>	7	1.7	0.6 - 2.3
<u>Streptomyces</u>	14	0.6	0 - 2.1
"coryneform"	1	2.8	-----

<sup>a</sup>Dry weight (mg) of root system sampled (sub-crown internode, seminal and crown roots) plus tightly adhering soil.

Crown roots originate from the lower node of the main stem and its tillers (Briggle, 1967; Kirby and Appleyard, 1981). Each new tiller gives rise to additional crown roots resulting in a massive, fibrous crown root system which is difficult to sample quantitatively for bacteria. For bacteria introduced on the seed to colonize crown roots, the bacteria must migrate through the soil or move upward via the sub-crown internode.

It was observed at the time of the second root sampling that the amount of soil adhering to the crown roots was considerably greater

Table 10. Colonization of Pondera spring wheat roots by bacterial isolates utilized as seed treatments in 1985.

Isolate	Log <sub>10</sub> CFU/Seed	Log <sub>10</sub> CFU/mg <sup>ab</sup>	
		Growth Stage	
		4.5	92
<u>Pseudomonas fluorescens</u>			
D-290E	7.6	3.4	— <sup>c</sup>
D-267SN	7.0	2.7	NT
28Er	7.5	2.4	1.6
216SR	4.4	1.4	NT
D-288SR	5.4	1.4	1.1
D-273SN	5.8	0.7	0
189SN	4.6	0	0
118SN	5.0	0	0
165SN	5.3	0	0
<u>Xanthomonas maltophilia</u>			
88SE	6.7	2.5	1.6
1000SNR	5.1	1.2	1.7
59SN	5.3	0.6	0.6
<u>Bacillus species</u>			
D-168r	0	2.3	NT
D-39Sr	7.0	2.1	1.8
58R	7.0	2.0	1.8
147R	7.3	1.8	3.3
D-60R	7.2	1.8	2.8
DI-155s	5.7	1.2	NT
157R	5.1	0.6	NT
D-188R	6.0	NT	3.5
<u>Streptomyces sp.</u>			
131S	5.2	2.1	2.4
D-185S	2.8	1.4	0.5
239N	2.2	0.8	1.8
22N	3.4	0.7	NT
109S	3.4	0.6	0.7
116N	4.1	0.6	NT
238N	1.2	0.6	NT
241S	0	0.6	NT
D-4N	1.6	0.5	NT
236N	3.6	0.5	NT
D-199N	5.6	0.3	NT
9S	1.8	0	NT
242s	0	0	NT
D-82N	0	0	NT
D-180N	3.6	NT	NT
<u>"Coryneform"</u>			
D-56SR	7.8	2.8	2.3

<sup>a</sup>Values represent average of four replications. NT = Bacterial isolate not sampled for this growth stage.

<sup>b</sup>Dry weight (mg) of root system sampled plus tightly adhering soil.

<sup>c</sup>D-290E colonies could not be separated visually from background contaminants on dilution plates.

than the amount adhering to seminal roots - even after shaking the root systems hard and removing large clumps of soil attached to the roots by hand. In contrast, the sub-crown internode had virtually no soil adhering to its surface.

Since the tightly adhering soil was considered to be rhizosphere soil that the bacterial isolates could be colonizing in addition to the roots, the increased amount of soil adhering to the crown roots could result in decreased CFU/mg dry weight values. In other words, the number of each bacterial isolate colonizing the crown roots could be the same or greater than the number colonizing the seminal roots, but the increased dry weight of the crown roots' rhizosphere soil, which may or may not be colonized by the isolate, decreases the reported value per unit weight.

I was, therefore, interested in determining the number of bacteria associated with each portion of the root system. For the experiment evaluating root colonization only, a dilution plate assay was conducted 61 days after planting for six of the mutant isolate treatments and a check treatment which had not been inoculated with bacteria. The results are reported in Table 11. The dry weight of the crown roots/rhizosphere soil was approximately twenty times the dry weight of either the sub-crown internode or seminal roots/rhizosphere soil. However, the number of bacteria per mg dry weight roots and soil was not proportionately less for the crown roots.

For each mutant isolate compared, the sub-crown internode or seminal roots had the greatest number of bacteria but the

Table 11. Comparison of Pondera spring wheat root portions for dry weight and bacterial number of six selected bacterial seed treatments in 1985.

Isolate	Isolate Identification	Root Portion	Dry WT (mg) Roots & Soil	Log <sub>10</sub> CFU/mg <sup>ab</sup>
28Er	<u>P. fluorescens</u>	seminal	32	1.1
		sub-crown internode	29	1.6
		crown	509	1.5
59SN	<u>X. maltophilia</u>	seminal	28	0.5
		sub-crown internode	35	1.1
		crown	543	0.6
147R	<u>B. pumilus</u>	seminal	31	2.2
		sub-crown internode	36	2.2
		crown	597	1.0
D-39Sr	<u>B. subtilis</u>	seminal	30	1.9
		sub-crown internode	30	1.9
		crown	726	1.2
D-56SR	"coryneform"	seminal	32	1.6
		sub-crown internode	32	2.3
		crown	520	2.1
D-185S	<u>Streptomyces</u> sp.	seminal	32	1.3
		sub-crown internode	31	1.2
		crown	665	0.9
Check <sup>c</sup>	----	seminal	27	4.3
		sub-crown internode	34	4.1
		crown	732	3.9

<sup>a</sup>Values represent average of four replications.

<sup>b</sup>Dry weight (mg) of root portions sampled plus tightly adhering soil.

<sup>c</sup>No bacterial isolates were applied to check seed. Values are for total aerobic bacterial counts on roots as determined via dilution plating on 1/10 TSBA without antibacterial antibiotics.

bacterial number associated with the crown roots was comparable with either of the other root portions. This was also true for the check treatment where total culturable aerobic bacterial counts were obtained. Based on these results, the decision was made that only the sub-crown internode and seminal roots would be sampled in future tests. This would be adequate in determining root colonization efficiency since these are the two root regions colonized first and the results for these two root regions are comparable with those for the crown roots. Crown roots would be sampled for only a few isolates of special interest.

#### Root Colonization Assay - Fourth Sampling Date

Another dilution plate assay was conducted for 20 of the bacterial isolate treatments 8 days after the grain was harvested. Only the sub-crown internode and seminal roots were sampled. Results are reported in Table 10. A majority of the colonization values ( $\log_{10}$ CFU) of the sampled mutant isolates decreased over time, but one X. maltophilia, two Bacillus spp. and three Streptomyces spp. mutant isolates increased in numbers. None of the P. fluorescens increased over time.

#### Disease Evaluation

In addition to evaluating rhizosphere competence by the bacterial isolates, two of the experiments also were designed to evaluate bacterial effect on take-all disease. One experiment simulated take-all disease symptoms commonly found in commercial spring wheat fields. For this experiment, the number of whiteheads in the middle row of

Table 12. The number of whiteheads observed and grain yield obtained in 1985 with bacterial isolate seed treatments of Pondera spring wheat inoculated with Gaeumannomyces graminis var. tritici (Ggt).

Isolate <sup>a</sup>	Isolate Identification	3.3-m Row	
		Whiteheads <sup>b</sup>	Yield (g) <sup>b</sup>
9S	Streptomyces	16 xy	194 xy
22N	Streptomyces	14 xy	249 xy
28Er	Pseudomonas	12 xy	205 xy
53Sr	Pseudomonas	17 xy	217 xy
58R	Bacillus	27 xy	156 y
59SN	Xanthomonas	29 y	184 xy
74	Streptomyces	11 xy	222 xy
80Sr	Pseudomonas	17 xy	203 xy
88SE	Xanthomonas	11 xy	224 xy
108S	Streptomyces	22 xy	204 xy
109S	Streptomyces	17 xy	232 xy
116N	Streptomyces	18 xy	174 xy
118SN	Pseudomonas	15 xy	197 xy
121Sr	Pseudomonas	21 xy	189 xy
125SR	Rhizobiaceae	23 xy	179 xy
131S	Streptomyces	24 xy	202 xy
147R	Bacillus	9 xy	226 xy
DI-152S	Bacillus	9 xy	238 xy
DI-155s	Bacillus	25 xy	177 xy
157R	Bacillus	18 xy	204 xy
162s	Bacillus	9 xy	234 xy
165SN	Pseudomonas	16 xy	228 xy
176Sr	Pseudomonas	20 xy	200 xy
189SN	Pseudomonas	19 xy	202 xy
199S+	Pseudomonas	18 xy	186 xy
216SR	Pseudomonas	18 xy	179 xy
230S	Bacillus	16 xy	242 xy
231S+	Enterobacteriaceae	14 xy	208 xy
236N	Streptomyces	11 xy	178 xy
238N	Streptomyces	12 xy	226 xy
239N	Streptomyces	23 xy	203 xy
241S	Streptomyces	31 y	148 y
242s	Streptomyces	17 xy	203 xy
1000SNR	Xanthomonas	22 xy	196 xy
1001S	Bacillus	33 y	156 y

Table 12--continued

Isolate <sup>a</sup>	Isolate Identification	3.3-m Row	
		Whiteheads <sup>b</sup>	Yield (g) <sup>b</sup>
D-4N	Streptomyces	22 xy	190 xy
D-39Sr	Bacillus	28 xy	155 y
D-56SR	Coryneform	9 xy	239 xy
D-60R	Bacillus	13 xy	260 xy
D-82N	Streptomyces	18 xy	203 xy
D-168r	Bacillus	13 xy	199 xy
D-173S	Bacillus	15 xy	202 xy
D-180N	Streptomyces	17 xy	234 xy
D-181S	Bacillus	22 xy	196 xy
D-183S	Bacillus	16 xy	213 xy
D-185S	Streptomyces	15 xy	228 xy
D-187S	Bacillus	18 xy	218 xy
D-188R	Bacillus	23 xy	171 xy
D-198S	Bacillus	27 xy	178 xy
D-199N	Streptomyces	17 xy	206 xy
D-214Sr	Bacillus	20 xy	184 xy
D-217Sr	Enterobacteriaceae	17 xy	186 xy
D-220S	Bacillus	24 xy	156 y
D-221Rs	Bacillus	24 xy	178 xy
D-222	Bacillus	14 xy	231 xy
D-226Sr	Bacillus	27 xy	191 xy
D-267SN	Pseudomonas	20 xy	201 xy
D-273SN	Pseudomonas	21 xy	205 xy
D-288SR	Pseudomonas	12 xy	194 xy
D-290E	Pseudomonas	19 xy	179 xy
Check <sup>c</sup>	----	22 xy	173 xy
Check/No <u>Ggt</u> <sup>c</sup>	----	1 x	287 x

<sup>a</sup>1.0 g Ggt inoculum added to each row of the experiment except for the Check/No Ggt treatment which had no Ggt inoculum added.

<sup>b</sup>Values in the same column followed by the same letter are not significantly different (P=0.05) according to Newman-Keuls multiple range test. Values represent average of four replications.

<sup>c</sup>No bacterial isolates were applied to Check seed treatments.

each three-row plot was determined at growth stage 77 (Table 12). The check treatment (non-bacterized seed) without Ggt inoculum applied with the seed developed no disease symptoms. No significant decreases in disease severity were observed, but three bacterial isolates significantly increased disease severity.

A second experiment simulated severe take-all disease conditions - i.e. plant death before maturity. It was observed in this experiment that none of the bacterial seed treatments decreased severe take-all disease symptoms when compared to the check treatment with Ggt inoculum. Again, the check treatment without Ggt inoculum developed no disease symptoms.

#### Grain Yield

For the experiment simulating take-all disease conditions in a commercial spring wheat field, the middle row of each three-row plot was harvested 101 days after planting. Significant decreases in yield were observed for five of the bacterial seed treatments when compared with the check treatment with no take-all disease (Table 12). Most of the treatments yielded more than the check treatment with Ggt inoculum but none yielded more than the check treatment without Ggt inoculum added to the row.

#### 1986 Field Results - Single and Mixture Isolate Treatments

The 20 mutant isolates which colonized the spring wheat root system most efficiently in 1985 were selected for further field testing as single isolate treatments for the 1986 growing season. Some of these mutant isolates were also tested as combination

treatments in which mixtures of more than one isolate were applied together as a single treatment to determine which genera and/or species were compatible as co-colonizers of spring wheat roots. Colonization efficacy and grain yield under non-disease conditions were evaluated.

#### Single Isolate Treatments

Dilution plate assays were conducted on all single isolate treatments 32 days and 83 days after planting, growth stages 1.2-1.3 and 6.9 respectively. Results of sub-crown internode and seminal root assays for single isolate treatments are summarized in Table 13. As demonstrated in 1985, mutant isolates within each genus varied considerably in their ability to colonize spring wheat roots. Grain yields for each treatment are reported in Table 13 also. No plant diseases were prevalent in the field.

In general, the number of bacteria reported in 1985 at growth stage 4.5 was quite similar to the number reported in 1986 at growth stage 1.2-1.3. However, the number of bacteria reported in 1986 at growth stage 6.9 decreased considerably from the previous counts for most treatments. The general exceptions were the Bacillus spp. seed treatments.

For 12 of the mutant isolates, I determined what proportion of the total culturable aerobic bacteria isolated from the spring wheat rhizosphere samples were composed of the specific introduced isolate. This was accomplished by replica plating of rhizosphere samples on antibiotic-supplemented and non-supplemented 1/10 TSBA (Table 14). Substantial differences between the 12 isolates were

Table 13. Colonization of Pondera spring wheat roots by bacterial isolates utilized as single isolate seed treatments in 1986.

Isolate	Log <sub>10</sub> CFU/Seed	Log <sub>10</sub> CFU/mg <sup>ab</sup>		Yield <sup>c</sup> (grams)
		Growth Stage		
		1.2-1.3	6.9	
<u>Pseudomonas fluorescens</u>				
D-290E	7.0	3.2	- <sup>d</sup>	304
28Er	7.0	2.8	1.1	306
216SR	6.6	1.7	0.1	328
D-288SR	6.4	1.5	0	330
D-273SN	6.4	0	0	332
<u>Xanthomonas maltophilia</u>				
88SE	7.6	2.8	1.1	357
59SN	6.5	1.7	1.6	321
1000SNR	7.3	0.9	0.9	315
<u>Bacillus subtilis</u>				
D-39Sr	6.9	2.0	1.6	295
D-60R	5.7	1.8	1.4	314
157R	0	0	0	316
<u>Bacillus pumilus</u>				
147R	7.0	2.2	2.1	322
<u>B. subtilis/pumilus group</u>				
D-168r	6.8	2.3	1.2	309
58R	6.4	2.2	1.4	333
<u>Streptomyces sp.</u>				
109S	3.4	2.2	0.6	345
D-185S	2.9	1.9	1.2	337
131S	3.5	1.7	0	274
239N	0 <sup>e</sup>	0	0	304
D-4N	0	0	0	307
<u>"Coryneform"</u>				
D-56SR	7.8	3.2	1.5	324
Check <sup>f</sup>	---	---	---	311

<sup>a</sup>Values represent average of four replications.

<sup>b</sup>Dry weight (mg) of root system sampled plus tightly adhering soil.

<sup>c</sup>No significant differences between yield values (P=0.05) according to Newman-Keuls multiple range test. Values represent average of four replications. Row length was 3.3-m.

<sup>d</sup>D-290E colonies could not be separated visually from background contaminants on dilution plates for growth stage 6.9.

<sup>e</sup>Present - observed on germinating seed.

<sup>f</sup>No bacterial isolates were applied to check seed.

Table 14. Colonization of Pondera spring wheat roots in 1986 by bacterial isolates introduced as seed treatments and by native culturable aerobic bacteria.

Isolate	Growth Stage 1.2-1.3 <sup>a</sup>		Growth Stage 6.9 <sup>a</sup>	
	Isolate % of Total Aerobic Bacteria <sup>b</sup>	Total Aerobic Bacteria Log <sub>10</sub> CFU/mg <sup>c</sup>	Isolate % of Total Aerobic Bacteria <sup>b</sup>	Total Aerobic Bacteria Log <sub>10</sub> CFU/mg <sup>c</sup>
<u>Pseudomonas fluorescens</u>				
28Er	13.4	3.7	0.6	3.5
D-288SR	1.4	3.5	0	3.1
D-290E	13.6	4.2	--- <sup>d</sup>	3.1
<u>Xanthomonas maltophilia</u>				
88SE	11.0	3.9	0.4	3.6
1000SNR	3.7	3.4	0.5	3.3
<u>Bacillus subtilis</u>				
D-39Sr	1.9	3.9	2.0	3.3
D-60R	1.8	3.8	1.4	3.4
<u>Bacillus pumilus</u>				
147R	5.6	3.6	2.8	3.7
<u>Streptomyces sp.</u>				
109S	1.2	4.3	0.3	3.4
239N	0	3.8	0	3.2
D-185S	1.5	3.8	0.8	3.5
<u>"Coryneform"</u>				
D-56SR	27.8	3.8	3.0	3.4
Check <sup>e</sup>	---	3.6	---	3.4

<sup>a</sup>Values represent average of four replications.

<sup>b</sup>X = (Isolate CFU/mg ÷ Total Aerobic Bacteria CFU/mg) \* 100.

<sup>c</sup>Dry weight (mg) of root system sampled and tightly adhering soil.

<sup>d</sup>D-290E colonies could not be separated visually from background contaminants on dilution plates for sampling at growth stage 6.9.

<sup>e</sup>No bacterial isolates were applied to check seed.

observed when the plants were primarily seedlings with only seminal roots (growth stage 1.2-1.3). Mutant isolate D-56SR (coryneform) had the highest percentage with 27.8%. These differences did not exist at growth stage 6.9 where the highest percentage was 3.0%. For most of the 12 mutant isolates, the percentage values for both growth stages were less than 6% and they did not differ extensively between sampling periods.

Interestingly, the average for the total number of culturable aerobic bacteria ( $\log_{10}$ CFU) did not vary greatly between isolate treatments or between the growth stages sampled. The range for the first sampling period was 3.7 to 4.7, and for the second sampling period it was 3.4 to 4.3. The average number of culturable aerobic bacteria for the check treatment, where no bacterial isolates were applied to the seed, was 3.7 for the first sampling period and 3.4 for the second sampling period.

Based on colony morphology, an antibiotic-resistant mutant isolate was rarely observed on the non-supplemented 1/10 TSBA used for determining total culturable aerobic bacteria. Even mutant D-56SR (coryneform), which was easily isolated on 1/10 TSBA with streptomycin and rifampicin and had a distinguishable colony morphology, was not observed on the replica plates of non-supplemented 1/10 TSBA. For growth stage 1.2-1.3, mutant D-56SR should have composed at least 25% of the bacterial microflora on the non-supplemented 1/10 TSBA based on the counts from the antibiotic-supplemented 1/10 TSBA. It was also noted that the non-supplemented 1/10 TSBA plates from all treatments, including the check treatment, usually had very similar morphological

colony types growing on them. These plates were normally dominated by Bacillus-like organisms.

These observations suggest that the total culturable aerobic bacterial counts and the introduced isolate percentages determined from these counts are erroneous. If the introduced mutant isolates are a part of the total aerobic bacterial counts, why are they normally not observed on the non-supplemented 1/10 TSBA? These introduced mutants are probably not as competitive as some of the fast-growing native rhizosphere bacteria on media. It would seem that there are certain bacteria which are capable of quickly establishing growth on a plate to the exclusion of all other organisms - introduced and native bacteria. This means that total rhizosphere aerobic bacterial counts are not being determined. Therefore, while a researcher can ascertain the numbers of an introduced isolate colonizing a plant root, one can not determine, conclusively, the percentage of the total aerobic bacteria colonizing the root which is composed of that introduced isolate.

#### Mixture Isolate Treatments

When isolates were mixed together and applied as a single treatment, the primary problem to be solved was how to separate and identify each mutant isolate of the mixture on dilution plates. If the isolates belonged to different genera, there was usually little difficulty in separating them based on colony morphology. However, isolates of the same genus were a problem. This was solved by the use of mutant isolates resistant to different antibiotics and/or

combinations of antibiotics; by the selection of isolates with different morphological colony types; or a combination of these two methods. For example, 28Er and D-288SR had very similar colony morphology types and were both identified as Pseudomonas fluorescens but could be separated based on their antibiotic resistance. Results for mixture treatments are summarized in Table 15.

The bacterial counts for each mutant isolate in the mixture are directly comparable with the bacterial counts for the single isolate treatments since all seed was treated and planted as part of the same field experiment in 1986. Comparison of the values obtained for the mutant isolates as single isolate treatments and mixture treatments is given in Table 16. For seven of the nine isolates, there were no significant differences in the  $\log_{10}$ CFU values obtained for each isolate between their use as single isolate treatments or in mixture treatments with one or more of the other isolates.

The B. subtilis (D-39Sr) and Streptomyces (D-185S) mutants did have significantly different  $\log_{10}$ CFU values for single versus mixture treatments. For both of these isolates at both growth stages, the single isolate treatments had higher colony counts than the same isolate in a mixture treatment.

There were fewer numbers of each mutant isolate applied to the seed with mixture treatments than with single isolate treatments. One-half the amount of each isolate normally used for single treatments was used for mixture treatments with two bacterial isolates. Only one-fourth the normal amount was used for mixture F which utilized five isolates. Despite these lower amounts for mixture

Table 15. Effect of using mixtures of bacterial isolates as seed treatments on Pondera spring wheat root colonization in 1986.

Mixture	Isolates in Mixture	Isolate Identification	Log <sub>10</sub> CFU/Seed	Log <sub>10</sub> CFU/mg <sup>ab</sup>	
				Growth Stage	
				1.2-1.3	6.9
A	28Er	<u>P. fluorescens</u>	7.4	2.1	1.0
	D-288sr	<u>P. fluorescens</u>	6.8	1.8	0
B	88SE	<u>X. maltophilia</u>	7.7	2.3	1.6
	1000SNR	<u>X. maltophilia</u>	7.2	0.7	1.0
C	D-39Sr	<u>B. subtilis</u>	7.1	1.5	1.2
	D-60R	<u>B. subtilis</u>	6.3	1.6	1.0
D	D-185S	<u>Streptomyces</u> sp.	2.3	0.8	0.3
	239N	<u>Streptomyces</u> sp.	0 <sup>c</sup>	0	0
E	28Er	<u>P. fluorescens</u>	7.2	2.4	1.1
	88SE	<u>X. maltophilia</u>	7.7	2.6	1.8
F	28Er	<u>P. fluorescens</u>	6.5	2.4	1.4
	88SE	<u>X. maltophilia</u>	6.9	2.3	1.1
	D-39Sr	<u>B. subtilis</u>	6.2	1.2	0.7
	D-56SR	"coryneform"	7.4	2.9	1.8
	D-185S	<u>Streptomyces</u> sp.	0	0	0
G	D-185S	<u>Streptomyces</u> sp.	- <sup>d</sup>	0.1	0.3
	D-60R	<u>B. subtilis</u>	- <sup>d</sup>	1.8	1.1
H	88SE	<u>X. maltophilia</u>	7.3	2.4	1.2
	D-39Sr	<u>B. subtilis</u>	6.2	1.4	1.4
I	88SE	<u>X. maltophilia</u>	7.2	2.4	1.8
	D-56SR	"coryneform"	6.2	2.7	1.9
J	D-56SR	"coryneform"	7.4	3.1	1.8
	D-39Sr	<u>B. subtilis</u>	6.2	1.2	0.8

<sup>a</sup>Values represent average of four replications.

<sup>b</sup>Dry weight (mg) of root system sampled plus tightly adhering soil.

<sup>c</sup>Present - observed on germinating seed.

<sup>d</sup>No counts available.

Table 16. Colonization of Pondera spring wheat roots in 1986 by nine bacterial isolates as single isolate seed treatments and components of isolate mixture seed treatments.

Isolate	Isolate Identification	Treatment <sup>a</sup>	Log <sub>10</sub> CFU/Seed	Log <sub>10</sub> CFU/mg <sup>bc</sup>	
				Growth Stage	
				1.2-1.3	6.9
28Er	<u>P. fluorescens</u>	single	7.0	2.8	1.1
		mixture A	7.4	2.1	1.0
		mixture E	7.2	2.4	1.1
		mixture F	6.5	2.4	1.4
88SE	<u>X. maltophilia</u>	single	7.6	2.8	0.6
		mixture B	7.7	2.3	1.6
		mixture E	7.7	2.6	1.8
		mixture F	6.9	2.3	1.1
		mixture H	7.3	2.4	1.2
		mixture I	7.2	2.4	1.8
239N	<u>Streptomyces</u> sp.	single	0	0	0
		mixture D	0	0	0
1000SNR	<u>X. maltophilia</u>	single	7.3	0.9	0.9
		mixture B	7.2	0.7	1.0
D-39Sr	<u>B. subtilis</u>	single	6.9	2.0 x	1.6 x
		mixture C	7.1	1.5 y	1.2 xy
		mixture F	6.2	1.2 y	0.7 y
		mixture H	6.2	1.4 y	1.4 xy
		mixture J	6.2	1.2 y	0.8 y
D-60R	<u>B. subtilis</u>	single	5.7	1.8	1.4
		mixture C	6.3	1.6	1.0
		mixture G	<sub>-d</sub>	1.8	1.1
D-56SR	coryneform	single	7.8	3.2	1.5
		mixture F	7.4	2.9	1.8
		mixture I	6.2	2.7	1.9
		mixture J	7.4	3.1	1.8
D-185S	<u>Streptomyces</u> sp.	single	2.9	1.9 x	1.2 x
		mixture D	2.3	0.8 y	0.3 x
		mixture F	0	0 z	0 x
		mixture G	<sub>-d</sub>	0.1 z	0.3 x
D-288SR	<u>P. fluorescens</u>	single	6.4	1.5	0
		mixture A	6.8	1.8	0

<sup>a</sup>Refers to single isolate treatments listed in Table 13 and mixture treatments listed in Table 14.

<sup>b</sup>Values represent average of four replications. For isolates D-39Sr and D-185S, values in the same column followed by the same letter are not significantly different (P=0.05) according to Newman-Keuls multiple range test. There were no significant differences between treatments for the other mutant isolates.

<sup>c</sup>Dry weight (mg) of root system sampled plus tightly adhering soil.

<sup>d</sup>No counts available.

treatments, the seed bacterial counts for each isolate were not substantially different and neither were most of the root colonization counts, as demonstrated previously. This would seem to indicate that lower numbers of bacteria could be applied while still obtaining the same root colonization results. Thus, two field experiments utilizing variable inoculum rate treatments were established to examine this observation more closely.

#### 1986 Field Results - Variable Rate Treatments

High numbers of bacteria were normally applied to the seed - an average of  $10^6$  or  $10^7$  CFU per seed. For comparative purposes, root colonization was evaluated on a dry weight basis resulting in bacterial counts that were lower than on the seed. Also, bacterial counts per mg of roots and soil sampled decreased over time for most isolates. Therefore, there was some concern that the bacterial isolates were not actually increasing in numbers on the roots but were simply being transferred from seed to roots and surviving in a non-replicative state.

The early results from the single and mixture isolate seed treatments in 1986 indicated that a lower number of each mutant bacterial isolate could be applied to the seed without decreasing the bacterial numbers colonizing roots. This suggested that the bacterial isolates were increasing numerically on the roots. Thus, field experiments were designed to determine if bacterial isolates replicate in the rhizosphere and if lower inoculum rates were adequate for bacterial establishment on spring wheat roots.

Kloepper et al. (1985) used a simple technique to determine soybean spermosphere establishment in non-sterile soil. They used decreasing amounts of bacteria to inoculate soybean seed and then "planted" the seed in a large petri plate containing soil. Their results demonstrated that the bacteria did replicate in the spermosphere. This technique was utilized in a natural field environment with two of my antibiotic-resistant mutant isolates - 88SE (X. maltophilia) and 28Er (P. fluorescens). These isolates were applied individually to spring wheat seed at six different inoculum rates.

One experiment was planted in the spring of 1986 and utilized mutant isolate 88SE (X. maltophilia) only. Dilution plate assays were conducted on all treatments at 14, 20, 26, and 54 days after planting. The seed was removed from the root system before the sample was placed in the plastic bag. Therefore, any bacteria remaining on the seed were not included in the determination of the number of bacteria colonizing the roots.

Results for these sub-crown internode and seminal root assays are reported two different ways. First, as bacterial counts per mg dry weight of the sample (Table 17); and second as the bacterial count for the entire root system sampled - i.e. total number of the mutant isolate on the roots (Table 18). There were no significant differences between the six inoculum rates for any of the tests despite the fact that there were differences in the amount of bacteria originally applied to the seed. The results for the two lowest inoculum rates reported in Table 18 indicate that isolate 88SE (X. maltophilia) did increase on the roots since the number of bacteria

Table 17. Effect of Pondera spring wheat seed inoculum rates of isolate 88SE (Xanthomonas maltophilia) on bacterial number per seed and bacterial number per mg root.

Inoculum <sup>c</sup> Rate	Log <sub>10</sub> CFU/Seed <sup>d</sup>	Log <sub>10</sub> CFU/mg <sup>ab</sup>			
		Growth Stage			
		1.2-1.3	2.1	2.2	2.3
Normal	7.4	2.6	3.1	1.6	1.8
1/4 Normal	7.1	2.6	2.9	1.8	1.5
1/16 Normal	6.5	3.0	3.2	1.6	1.6
1/64 Normal	5.9	3.0	3.2	1.8	1.5
1 Loop	4.8	3.0	2.8	1.5	1.0
1/6 Loop	3.6	2.3	2.7	1.6	--- <sup>e</sup>

<sup>a</sup>Dry weight (mg) of root system sampled plus tightly adhering soil.

<sup>b</sup>Values represent average of four replications. There were no significant differences between values (P=0.05) according to Newman-Keuls multiple range test.

<sup>c</sup>Normal inoculum rate is one TSBA plate of bacterial growth per 12.5 g seed. "1 Loop" rate is one loopful of bacteria per 12.5 g seed. "1/6 Loop" rate is one loopful of bacteria per 75 g seed - the total amount of seed inoculated per treatment.

<sup>d</sup>Experiment established in the spring of 1986.

<sup>e</sup>88SE colonies could not be separated visually from background contaminants on dilution plates.

Table 18. Effect of Pondera spring wheat seed inoculum rates of isolate 88SE (*Xanthomonas maltophilia*) on bacterial number per seed and bacterial number per root system.

Inoculum <sup>b</sup> Rate	Log <sub>10</sub> CFU/Seed <sup>c</sup>	Log <sub>10</sub> CFU (Total) per Root System <sup>a</sup>			
		Growth Stage			
		1.2-1.3	2.1	2.2	2.3
Normal	7.4	4.7	4.7	4.3	4.3
1/4 Normal	7.1	5.1	4.6	4.5	4.1
1/16 Normal	6.5	4.9	4.9	4.4	4.3
1/64 Normal <sup>d</sup>	5.9	5.2 x	5.0 xy	4.2 y	4.1 y
1 Loop <sup>d</sup>	4.8	4.7 x	4.6 xy	4.3 xy	3.8 y
1/6 Loop	3.6	4.3	4.2	4.3	--- <sup>e</sup>

<sup>a</sup>Values represent average of four replications. There were no significant differences between values in the same column (P=0.05) according to Newman-Keuls multiple range test.

<sup>b</sup>Normal inoculum rate is one TSBA plate of bacterial growth per 12.5 g seed. "1 Loop" rate is one loopful of bacteria per 12.5 g seed. "1/6 Loop" rate is one loopful of bacteria per 75 g seed - the total amount of seed inoculated per treatment.

<sup>c</sup>Experiment established in the spring of 1986.

<sup>d</sup>Values in the same row followed by the same letter were not significantly different (P=0.05) according to Newman-Keuls multiple range test. Significant differences were observed for only two inoculum rates - 1/64 and 1 loop.

<sup>e</sup>88SE colonies could not be separated visually from background contaminants on dilution plates.

on the root system sampled was equal to or greater than the number of bacteria on the seed at planting. The total number of bacteria on the root system sampled did not differ between inoculum rates for each growth stage sampled and did not differ extensively between assays for each inoculum rate - even though the number of bacteria on the seed for each rate varied considerably. These results also suggest that a lower number of bacteria applied to the seed achieves the same goal as the normal rate now used.

A second experiment was planted in the fall of 1986 and utilized both mutant isolates (88SE - X. maltophilia and 28Er - P. fluorescens) applied to spring wheat seed at the six different inoculum rates. A fall experiment with spring wheat is unconventional, but this allowed for an immediate replication of the spring planted experiment, plus the opportunity to include another mutant isolate for comparison with mutant isolate 88SE.

The fall weather was mild and very similar to the spring and early summer weather of 1986. Soil temperatures were similar also (NOAA, 1986). Three dilution plate assays were completed 16, 27, and 40 days after planting. Adverse weather prevented a fourth root sampling. The same techniques for sampling were used as described earlier except that plants for each isolate were sampled in order beginning with the lowest rate and ending with the highest rate.

The results for mutant isolate 88SE (X. maltophilia) are reported in Tables 19 and 20 and those for mutant isolate 28Er (P. fluorescens) are summarized in Tables 21 and 22. The same trends as observed in the spring planted experiment were evident. The seed assay showed a

Table 19. Effect of Pondera spring wheat seed inoculum rates of isolate 88SE (*Xanthomonas maltophilia*) on bacterial number per seed and bacterial number per mg root.

Inoculum <sup>c</sup> Rate	Log <sub>10</sub> CFU/Seed <sup>d</sup>	Log <sub>10</sub> CFU/mg <sup>ab</sup>		
		Growth Stage		
		1.1-1.2	1.2-1.3	2.1
Normal	7.7	4.1 x	3.2	3.1
1/4 Normal	7.0	3.8 x	3.1	2.9
1/16 Normal	6.8	3.5 xy	2.8	2.5
1/64 Normal	5.7	3.6 xy	2.6	2.8
1 Loop	4.6	3.1 y	2.7	2.8
1/6 Loop	4.4	3.1 y	2.5	2.8

<sup>a</sup>Values represent average of four replications. Values in the same column followed by the same letter were not significantly different (P=0.05) according to Newman-Keuls multiple range test. Significant differences were observed only at growth stage 1.1-1.2.

<sup>b</sup>Dry weight (mg) of root system sampled plus tightly adhering soil.

<sup>c</sup>Normal inoculum rate is one TSBA plate of bacterial growth per 12.5 g seed. "1 Loop" rate is one loopful of bacteria per 12.5 g seed. "1/6 Loop" rate is one loopful of bacteria per 75 g seed - the total amount of seed inoculated per treatment.

<sup>d</sup>Experiment established in the fall of 1986.

Table 20. Effect of Pondera spring wheat seed inoculum rates of isolate 88SE (*Xanthomonas maltophilia*) on bacterial number per seed and bacterial number per root system.

Inoculum <sup>b</sup> Rate	Log <sub>10</sub> CFU/Seed <sup>c</sup>	Log <sub>10</sub> CFU (Total) per Root System <sup>a</sup>		
		Growth Stage		
		1.1-1.2	1.2-1.3	2.1
Normal	7.7	5.9 x	5.6	5.2
1/4 Normal	7.0	5.6 xy	5.4	5.3
1/16 Normal	6.8	5.0 y	5.0	4.8
1/64 Normal	5.7	5.4 xy	4.9	5.2
1 Loop	4.6	5.1 y	4.9	5.2
1/6 Loop	4.4	5.2 y	4.8	5.0

<sup>a</sup>Values represent average of four replications. Values followed by the same letter were not significantly different (P=0.05) according to Newman-Keuls multiple range test. Significant differences were observed only at growth stage 1.1-1.2.

<sup>b</sup>Normal inoculum rate is one TSBA plate of bacterial growth per 12.5 g seed. "1 Loop" rate is one loopful of bacteria per 12.5 g seed. "1/6 Loop" rate is one loopful of bacteria per 75 g seed - the total amount of seed inoculated per treatment.

<sup>c</sup>Experiment established in the fall of 1986.

Table 21. Effect of Pondera spring wheat seed inoculum rates of isolate 28Er (Pseudomonas fluorescens) on bacterial number per seed and bacterial number per mg root.

Inoculum <sup>c</sup> Rate	Log <sub>10</sub> CFU/Seed <sup>d</sup>	Log <sub>10</sub> CFU/mg <sup>ab</sup>		
		Growth Stage		
		1.1-1.2	1.2-1.3	2.1
Normal	7.3	4.0 x	3.4	2.2
1/4 Normal	6.4	3.6 xy	3.0	2.7
1/16 Normal	5.3	3.3 xy	2.7	2.8
1/64 Normal	4.0	3.4 xy	3.1	2.4
1 Loop	4.0	3.3 xy	2.8	2.4
1/6 Loop	4.1	2.8 xy	3.0	2.4

<sup>a</sup>Values represent average of four replications. Values in the same column followed by the same letter were not significantly different (P=0.05) according to Newman-Keuls multiple range test. Significant differences observed only at growth stage 1.1-1.2.

<sup>b</sup>Dry weight (mg) of root system sampled plus tightly adhering soil.

<sup>c</sup>Normal inoculum rate is one TSBA plate of bacterial growth per 12.5 g seed. "1 Loop" rate is one loopful of bacteria per 12.5 g seed. "1/6 Loop" rate is one loopful of bacteria per 75 g seed - the total amount of seed inoculated per treatment.

<sup>d</sup>Experiment established in the fall of 1986.

Table 22. Effect of Pondera spring wheat seed inoculum rates of isolate 28Er (*Pseudomonas fluorescens*) on bacterial number per seed and bacterial number per root system.

Inoculum <sup>b</sup> Rate	Log <sub>10</sub> CFU/Seed <sup>c</sup>	Log <sub>10</sub> CFU (Total) per Root System <sup>a</sup>		
		Growth Stage		
		1.1-1.2	1.2-1.3	2.1
Normal	7.3	5.8	5.6	4.7
1/4 Normal	6.4	5.3	5.2	5.0
1/16 Normal	5.3	5.4	5.0	5.0
1/64 Normal	4.0	5.5	5.3	4.6
1 Loop	4.0	5.4	5.0	4.8
1/6 Loop	4.1	5.0	5.1	4.6

<sup>a</sup>Values represent average of four replications. No significant differences (P=0.05) between treatments according to Newman-Keuls multiple range test.

<sup>b</sup>Normal inoculum rate is one TSBA plate of bacterial growth per 12.5 g seed. "1 Loop" rate is one loopful of bacteria per 12.5 g seed. "1/6 Loop" rate is one loopful of bacteria per 75 g seed - the total amount of seed inoculated per treatment.

<sup>c</sup>Experiment established in the fall of 1986.

difference of  $\log_{10}$  3.3 CFU between the normal inoculum rate and the lowest inoculum rate for isolate 88SE (X. maltophilia). However, for each of the three growth stages sampled, the root colonization assay values differed by only 1.0, 0.7 and 0.6 ( $\log_{10}$ CFU) between the normal and lowest inoculum rates (Table 19). The seed assay for isolate 28Er (P. fluorescens) showed a difference of  $\log_{10}$  3.2 CFU between the normal and lowest inoculum rates with only a maximum difference of 1.2, 0.7 and 0.6 between these inoculum rates for the root colonization assays (Table 21). Only for the earliest sampling date of both isolates was there a significant difference between inoculum rates.

The total number of the mutant isolate per root system sampled did differ significantly between inoculum rates for the first sampling date of isolate 88SE (X. maltophilia) but not for the other root colonization sampling dates or for any of the sampling dates of isolate 28Er (P. fluorescens) (Table 20 and Table 22). Again, the results for the two lowest inoculum rates for both of these mutant isolates indicated that they did increase on the roots since the total number of bacteria on the root system was greater than the number of bacteria per seed at planting. This was also true for the "1/64 Normal" treatment of both isolates in this experiment.

#### 1986 Field Results - Pseudomonas fluorescens (199) Mutants

Three additional field experiments were established in 1986 to help determine if the mutant bacterial isolates were establishing and increasing in numbers on the roots. These experiments utilized antibiotic-resistant and antibiotic-dependent spontaneous mutants of

isolate 199 (P. fluorescens). Mutant isolate 199ER was resistant to 100 µg/ml of both erythromycin and rifampicin and required neither antibiotic for growth. Mutant isolate 199S<sup>+</sup>NR was resistant to 100 µg/ml of nalidixic acid and rifampicin but also required a minimum of 5 µg/ml of streptomycin for growth. As long as this mutant isolate does not replicate in the absence of streptomycin, it will remain viable. However, as soon as it attempts to multiply in the absence of streptomycin, death will result due to impairment of protein synthesis (Gale et al., 1981).

Two experiments were established in 1986, one in the spring and one in the fall, utilizing mutant isolates 199ER and 199S<sup>+</sup>NR as single isolate treatments and as a mixture treatment of the two isolates. Dilution plate assays in the spring were conducted 25, 45, 63, and 85 days after planting. Fall assays were completed 16, 27, and 40 days after planting.

The results for these assays were very similar and are summarized in Table 23. In both experiments, mutant isolate 199ER does appear to be colonizing spring wheat roots and has values comparable to 28Er, also a fluorescent pseudomonad, in the single isolate seed treatment experiment of 1986 (Table 13). Mutant isolate 199S<sup>+</sup>NR was present on roots at substantially lower levels than mutant 199ER for all sampling times of both experiments. Colonization values of 199 S<sup>+</sup>NR declined to less than log<sub>10</sub> 0.5 CFU for both treatments of both experiments. Reisolated rhizosphere colonies of 199 S<sup>+</sup>NR had the same growth requirement for streptomycin as the mutant isolate applied to the seed.

Table 23. Colonization of Pondera spring wheat roots by two mutants of a *Pseudomonas fluorescens* isolate (199) utilized as single isolate and mixture treatments in 1986.

"Spring Planted Experiment"						
Isolate	Treatment <sup>a</sup>	Log <sub>10</sub> CFU/Seed	Log <sub>10</sub> CFU/mg <sup>b</sup>			
			Growth Stage			
			1.2-1.3 <sup>c</sup>	2.2 <sup>c</sup>	5.9 <sup>c</sup>	7.7 <sup>c</sup>
199ER	Single	7.3	2.3	1.0	1.8	1.0
	Mixture	6.9	2.3	0.5	1.5	0.9
199S <sup>+</sup> NR	Single	6.4	0.6	0	0	0
	Mixture	6.1	0.2	0	0	0

  

"Fall Planted Experiment"						
Isolate	Treatment <sup>a</sup>	Log <sub>10</sub> CFU/Seed	Log <sub>10</sub> CFU/mg <sup>b</sup>			
			Growth Stage			
			1.1 <sup>c</sup>	1.2-1.3 <sup>c</sup>	2.1 <sup>c</sup>	
199ER	Single	6.2	2.2	2.2	2.0	
	Mixture	5.4	2.2	2.1	1.8	
199S <sup>+</sup> NR	Single	5.6	1.2	0.7	0.4	
	Mixture	4.6	0.9	0.2	0	

<sup>a</sup>Single treatment consisted of 199ER or 199S<sup>+</sup>NR applied to the seed individually. Mixture treatment consisted of the two isolates applied to the seed together as one treatment.

<sup>b</sup>Dry weight (mg) of root system sampled plus tightly adhering soil.

<sup>c</sup>Values represent average of four replications.

One way to provide streptomycin in the rhizosphere for the growth of 199 S<sup>+</sup>NR would be to utilize a seed treatment mixture which included a streptomycin producing organism. Streptomyces griseus (NRRL B-1965) is a known producer of streptomycin in culture (D. P. Labeda, personal communication), but it was not known whether this organism produced streptomycin in the soil. Since it was a laboratory strain, there was also no evidence that it was capable of colonizing spring wheat roots.

A field experiment was established in the spring of 1986 utilizing mutant isolate 199S<sup>+</sup>NR and S. griseus (NRRL B-1965) as single isolate and mixture treatments. Dilution plate assays were conducted for this experiment along with those of the previously described spring experiment utilizing mutant isolates 199ER and 199S<sup>+</sup>NR. The results for 199S<sup>+</sup>NR were quite similar for both experiments. There were log<sub>10</sub>CFU 0.5 and 0.4 per mg roots and soil obtained at growth stage 1.1 for single and mixture treatments, respectively. Subsequent test values declined to 0 indicating that this mutant isolate was not surviving and/or colonizing spring wheat roots. Since S. griseus was not marked as antibiotic resistant, there was no way to confirm that the streptomycete colonies that did appear on the dilution plates were this particular isolate.

Root Colonization of Crops Other Than Spring Wheat

From the field experiments conducted in 1985 and 1986, it was evident that some mutant isolates could be considered efficient colonizers of spring wheat roots. Could these same mutant isolates colonize other important field crops? A series of four experiments was conducted under growth chamber conditions to answer this question.

Five mutant isolates and six field crops were evaluated. For the first two experiments, plants were grown in vermiculite. Only four of the mutant isolates were tested. The final two experiments were conducted using Bozeman silt loam soil and involved all five mutant isolates.

The root colonization values for plants grown in vermiculite were considerably higher than for those grown in soil (Table 24). This would be expected since the soil was not sterile and the introduced bacteria would have to compete with the indigenous microflora.

Few significant differences for root colonization were observed between crops for the same mutant isolate, either in vermiculite or soil. However, this in itself is significant because it indicates the mutant isolates are capable of colonizing crops other than spring wheat even though selection of these bacterial root colonizers was exclusively on spring wheat. If one closely examines Table 24 for the results of 28Er (P. fluorescens), 88SE (X. maltophilia), D-39Sr (B. subtilis) and D-56SR (coryneform) in all four experiments, one observes that in all cases the highest root colonization values for

Table 24. Root colonization by five bacterial isolates on six field crops grown in vermiculite or soil in a growth chamber.

Isolate	Crop	Vermiculite		Soil	
		Log <sub>10</sub> CFU/mg <sup>a</sup>			
		Expt. 1	Expt. 2	Expt. 3	Expt. 4
28Er ( <i>P. fluorescens</i> )	spring wheat	1.8 x	---	0.8 x	0.8 x
	winter wheat	1.8 x	2.5 x	0.4 x	1.0 x
	barley	2.4 x	3.0 x	0.8 x	0.6 x
	corn	1.8 x	1.6 y	1.0 x	1.6 x
	soybean	2.2 x	3.2 x	1.1 x	0.6 x
	safflower	--- <sup>b</sup>	3.0 x	---	1.2 x
88SE ( <i>X. maltophilia</i> )	spring wheat	NT <sup>c</sup>	NT	0.2 y	1.6 x
	winter wheat	NT	NT	0.8 xy	1.7 x
	barley	NT	NT	1.3 x	1.2 x
	corn	NT	NT	1.1 xy	1.8 x
	soybean	NT	NT	1.8 x	2.1 x
	safflower	NT	NT	---	---
D-39Sr ( <i>B. subtilis</i> )	spring wheat	2.6 x	2.8 x	1.6 x	1.6 x
	winter wheat	2.5 x	3.0 x	1.8 x	1.8 x
	barley	2.8 x	2.9 x	1.6 x	1.8 x
	corn	2.8 x	3.1 x	2.0 x	1.8 x
	soybean	2.7 x	2.9 x	1.7 x	1.6 x
	safflower	1.6 y	1.8 y	---	---
D-56SR ( <i>coryneform</i> )	spring wheat	2.6 x	3.4 x	1.2 x	0.7 x
	winter wheat	3.1 x	3.6 x	1.0 x	0.5 x
	barley	2.8 x	3.8 x	1.4 x	0.6 x
	corn	2.8 x	3.4 x	1.1 x	1.0 x
	soybean	3.5 x	3.7 x	1.6 x	1.2 x
	safflower	3.1 x	3.4 x	---	---
D-185S ( <i>Streptomyces</i> sp.)	spring wheat	2.6 x	2.6 x	0.6 x	0.6 x
	winter wheat	1.4 y	2.8 x	0.8 x	0.2 x
	barley	1.4 y	2.7 x	0.8 x	0.6 x
	corn	0 z	1.2 y	0.1 x	0.1 x
	soybean	2.0 xy	2.5 x	---	---
	safflower	0 z	1.2 y	---	---

<sup>a</sup>Dry weight (mg) of root system sampled plus tightly adhering soil. Values represent average of three or four replications. Values in the same column followed by the same letter are not significantly different (P=0.05) according to Newman-Keuls multiple range test.

<sup>b</sup>Data not presented because values were average of two replications or less; not included in statistical analysis either.

<sup>c</sup>Isolate 88SE not tested in vermiculite.

each isolate are not for spring wheat. In 12 of the 14 cases, the field crop with the highest value is soybean or corn. For isolate D-185S (Streptomyces sp.), spring wheat does have the highest root colonization value in two cases with corn consistently being the field crop with the lowest values. For the experiments established in soil (Experiments 3 and 4), the mutant isolate which had the most consistent results between trials was D-39Sr (B. subtilis).

Table 25 contains the same data as Table 24 but presented differently so as to emphasize root colonization by the isolates on a crop by crop basis. In the first experiment, D-56SR (coryneform) and D-39Sr (B. subtilis) were consistently the best root colonizers for all field crops. Isolate D-56SR continued as one of the best colonizers in the second experiment. Both of these experiments were established in vermiculite. In the soil experiments (Experiments 3 and 4), D-39Sr was one of the best colonizers followed by 88SE (X. maltophilia).

One problem with the experiment concerned germination of soybean and safflower seeds. The germination rate for untreated soybean seed was 60%. Even though two soybean seeds per cone were planted, usually only three replications would have at least one emerged plant. For seeds treated with isolate D-185S and planted in soil, even fewer plants emerged. For the third experiment, only one replication had an emerged plant and there were only two replications with plants in the fourth experiment. The seeds usually germinated but did not emerge.

Table 25. Root colonization of six field crops inoculated with five bacterial isolates and grown in vermiculite or soil in a growth chamber.

Crop	Isolate	Vermiculite		Soil	
		Log <sub>10</sub> CFU/mg <sup>a</sup>			
		Expt. 1	Expt. 2	Expt. 3	Expt. 4
Spring Wheat	28Er	1.8 y	--- <sup>b</sup>	0.8 x	0.8 x
	88SE	NT <sup>c</sup>	NT	0.2 x	1.6 x
	D-39Sr	2.6 x	2.8 y	1.6 x	1.6 x
	D-56SR	2.6 x	3.4 x	1.2 x	0.7 x
	D-185S	2.6 x	2.6 y	0.6 x	0.6 x
Winter Wheat	28Er	1.8 z	2.5 y	0.4 y	1.0 x
	88SE	NT	NT	0.8 y	1.7 x
	D-39Sr	2.5 y	3.0 y	1.8 x	1.8 x
	D-56SR	3.1 x	3.6 x	1.0 y	0.5 x
	D-185S	1.4 z	2.8 y	0.8 y	0.2 x
Barley	28Er	2.4 xy	3.0 y	0.8 x	0.6 x
	88SE	NT	NT	1.3 x	1.2 x
	D-39Sr	2.8 x	3.1 x	2.0 x	1.8 x
	D-56SR	2.8 x	3.4 x	1.1 y	1.0 xy
	D-185S	0 z	1.2 y	0.1 z	0.1 y
Corn	28Er	1.8 y	1.6 y	1.0 y	1.6 x
	88SE	NT	NT	1.1 y	1.8 x
	D-39Sr	2.8 x	3.1 x	2.0 x	1.8 x
	D-56SR	2.8 x	3.4 x	1.1 y	1.0 xy
	D-185S	0 z	1.2 y	0.1 z	0.1 y
Soybean	28Er	2.7 xy	3.2 y	1.1 y	0.6 x
	88SE	NT	NT	1.8 x	2.1 x
	D-39Sr	2.7 xy	2.9 y	1.7 x	1.6 x
	D-56SR	3.5 x	3.7 x	1.6 x	1.2 x
	D-185S	2.0 y	2.5 z	---	---
Safflower	28Er	---	3.0 x	---	1.2
	88SE	NT	NT	---	---
	D-39Sr	1.6 y	1.8 y	---	---
	D-56SR	3.1 x	3.4 x	---	---
	D-185S	0 z	1.2 y	---	---

<sup>a</sup>Dry weight (mg) of root system sampled plus tightly adhering soil. Values represent average of three or four replications. Values in the same column followed by the same letter are not significantly different (P=0.05) according to Newman-Keuls multiple range test.

<sup>b</sup>Data not presented because values were average of two replications or less; not included in statistical analysis either.

<sup>c</sup>Isolate 88SE not tested in vermiculite.

The main reason the safflower did not germinate was due to seed contamination by fungi. The clorox solution used to sterilize the other crops' seeds seemed to have no effect on safflower seed contaminants or the number of contaminants was so much greater that the technique utilized was ineffective. The problem was accentuated when the seeds were planted in soil. The bacterial isolates were present on the safflower seed at planting in all four trials as determined by dilution plating of the bacterized seed. Due to the large number of replications missing in the third and fourth experiments, it is difficult to say conclusively that the isolates colonized safflower in soil. The exception is isolate 28Er (P. fluorescens) which did colonize safflower roots in all four replications of the fourth experiment.

Since the soil utilized in Experiments 3 and 4 was not sterile, weeds also grew along with the crop plants in the cones. Most of the weeds were pulled from the soil so they would not interfere with water use by the crops but a few escaped the weeding process.

The weeds were one species, the redroot pigweed (Amaranthus retroflexus L.). Four of the pigweed plants which were present at sampling were pulled from the soil and plated directly on the appropriate antibiotic medium. There were pigweed plants in cones planted with seeds inoculated with D-56SR (coryneform), 28Er (P. fluorescens), 88SE (X. maltophilia) and D-185S (Streptomyces sp.). Three of the four isolates were present on the pigweed roots; D-185S was not. This would suggest that some of the bacteria transferred from the intended colonization target to closely associated non-target

roots or moved through the soil to colonize roots of plants whose seed was not inoculated with bacteria.

## DISCUSSION

The primary objective of this research project was to identify and characterize rhizosphere bacteria capable of colonizing spring wheat roots effectively under natural field conditions - i.e. evaluate rhizosphere competence (Schmidt, 1979). This objective was achieved. At least one bacterial isolate from each group represented was determined to be rhizosphere competent. The colonizers included Bacillus pumilus, B. subtilis, Pseudomonas fluorescens, Streptomyces spp., Xanthomonas maltophilia and a coryneform. In addition to achieving this objective, basic concepts central to root colonization and development of commercial seed inoculant were evaluated.

Screening was conducted at four different stages in this research to arrive at the primary objective: first, initial selection of bacterial isolates from small grain roots; second, production of antifungal compounds in vitro; third, field screening of antibiotic-resistant mutants for rhizosphere competence; fourth, a second field screening for rhizosphere competence using the mutant isolates determined to be the most rhizosphere competent in stage three.

Although a diverse group of bacteria were isolated from small grain roots, the majority of these organisms represented only three genera - Bacillus, Pseudomonas and Streptomyces. This was not necessarily because they were the dominant rhizosphere or rhizoplane colonizing organisms. Rather, their selection was due to the type of

media utilized for isolating bacteria from roots. Perhaps more coryneform and X. maltophilia colonies would have been selected if media which select specifically for these organisms had been available. This is an inherent problem when isolating bacteria from a source such as soil. Basically, the selection process is only as good as the selective media used. Even if a general medium such as 1/10 TSBA allows one to isolate "all" culturable bacteria from the sample, this medium is still selective since the fastest-growing organisms may dominate and prevent others from growing.

The highest bacterial counts for the early plant growth stages in 1986 were associated with Gram negative mutant isolates (28Er - P. fluorescens, 88SE - X. maltophilia, D-290E - P. fluorescens) and the coryneform mutant isolate (D-56SR). However, the counts for these mutant isolates decreased by 1.7 log units when sampled at a later growth stage. Possibly by the time anthesis is complete for spring wheat, the amount of root exudates has decreased such that the nutrient status of the rhizosphere is unfavorable for these zymogenous organisms (Rivière,1960; Sands and Rovira,1971).

Although the Bacillus mutant isolates did not result in the highest bacterial counts in either year, as a group they were the most consistent colonizers. Their numbers on the roots varied the least from year to year and from plant growth stage to plant growth stage. Perhaps the Bacillus spp. have been previously overlooked because their numbers are usually lower than those of the fluorescent pseudomonads in the rhizosphere (Rovira,1963). Nonetheless, an organism which maintains a steady state in the natural soil

environment would be more desirable under certain circumstances - such as the need for long term plant protection beyond seedling stages.

The Bacillus spp. produce endospores which are more resistant than vegetative cells to destructive forces, such as desiccation, in the soil (Gordon et al.,1973). These spores may allow these organisms to colonize wheat roots during the entire growing season. This should be true for Streptomyces spp. since they also produce a protective resting spore, but only one of the mutant isolates (D-185S) followed a consistent colonization pattern. Interestingly, two of the three X. maltophilia mutant isolates (59SN, 1000SNR) were consistent colonizers. Other Streptomyces and Xanthomonas isolates had colonization patterns similar to the Gram negative organisms described earlier.

Until recently, X. maltophilia was classified as P. maltophilia. By transferring this organism from Pseudomonas to Xanthomonas (Swings et al.,1983), the genus Xanthomonas is no longer restricted to phytopathogenic bacteria. X. maltophilia is also distinguished from other xanthomonads because it is a saprophyte found in the soil. Phytopathogenic xanthomonads are not known to survive in the soil (Starr,1981).

Although X. maltophilia has been reported in association with plant roots (Debette and Blondeau,1980; Kwok et al.,1986), this is the first report of X. maltophilia colonizing small grain roots. This organism has recently been suggested as a possible biological control agent for pathogens of live oaks (Gregory et al.,1986) and cucumber (Kwok et al., 1986). In this study, the highest yield in 1986 (i.e.

non-disease conditions) was obtained from a X. maltophilia treatment (88SE). This mutant did not inhibit the saprophyte R. glutinis or the plant pathogen Ggt in vitro. It had been selected for field screening because it produced a dark pigment on TSA. Iverson et al. (1984) have demonstrated that X. maltophilia produces chitinase in vitro in amounts equivalent to that of Serratia liquefaciens.

The inconsistencies between laboratory and field results point out one of the major problems in the development of biological control agents of soilborne pests - initial selection of candidate isolates. Quite often selection is based on bacterial antagonism toward selected plant pathogens in vitro. However, in vitro results do not always correlate with field results (Kommedahl et al., 1981; Suslow, 1982; Weller et al., 1985).

The same problem exists for selecting organisms which can efficiently colonize roots. Since we do not know exactly what factors influence root colonization in the soil, it is difficult to design methods which will predict this characteristic in vitro with randomly selected bacteria.

Since my primary interest was the selection of rhizosphere competent bacteria, I did not screen the 537 isolates obtained from small grain roots against one particular soilborne pathogen. Instead, the isolates were initially screened for inhibition of R. glutinis, a non-plant pathogen. In retrospect it probably would have been better to screen bacterial isolates for their ability to inhibit other common rhizosphere bacteria since these are the organisms which the colonizer needs to displace. However, this approach might eliminate bacterial

colonizers which are compatible on roots with other bacterial colonizers and prevent development of seed treatments using more than one organism. Thus, screening methods must be designed so both characteristics (inhibition and compatibility) can be evaluated.

In this study, the 60 isolates selected for field testing, based primarily on results from the R. glutinis inhibition screen, were also examined for their ability to inhibit Ggt in vitro. However, these results were not utilized as a selection criterion at any time in the study. As stated previously, the bacteria could be divided into four groups - inhibitors of R. glutinis and Ggt; inhibitors of R. glutinis but not Ggt; inhibitors of Ggt but not R. glutinis; non-inhibitors of both R. glutinis and Ggt. Most isolates that were inhibitory to R. glutinis were also inhibitory to Ggt. Only one of the nine isolates not inhibitory to R. glutinis was inhibitory to Ggt. This isolate was not rhizosphere competent either; but, one isolate is hardly a representative sample. However, successful colonizers did come from the other three groups.

It would be a logistical nightmare to screen hundreds of organisms in the field for rhizosphere competence only. This is the primary reason why an initial screening of the organisms for antifungal activity was necessary. R. glutinis was selected as the test organism because it is not a plant pathogen. If a plant pathogenic organism had been used, it is possible a bias would have been introduced into the selection process. In addition, R. glutinis is a fast growing organism (assay plates could be read in 36 hours), and it is red in color which makes growth easily detectable. It would

seem that R. glutinis was a good choice for selecting Ggt inhibitors since 90% of the isolates which inhibited R. glutinis also inhibited Ggt and only 2% of the isolates that inhibited Ggt did not inhibit R. glutinis.

Presently, the primary method for confirming colonization is dilution plating of roots using selective media and antibiotic-resistant mutant isolates. It is necessary to mark introduced isolates in order to separate and identify them from the rest of the microbial community naturally occurring on roots. While it would be very difficult to develop hundreds of antibiotic-resistant mutant isolates for a preliminary field screen, it would be realistic to develop 100 or less.

Another reason to field screen only marked isolates concerns the following three statements made by W. Brill recently in Bio/Technology (McCormick, 1986): First, culturing a bacterium may weaken it if the cell wall is lost. Antibiotic resistance may decrease the ability of the organism to survive in the soil. Even if it does survive, it could lose the markers and so be present but undetectable. In the current study, by only screening marked isolates and restricting the definition of a rhizosphere competent organism to an organism which is readily detectable and identifiable, one selects for organisms which do survive in the soil as stably marked isolates.

Another reason to develop antibiotic-resistant mutants is to enhance rhizosphere colonization. Hossain and Alexander (1984) and Bashan (1986b) utilized this concept with soybean/Rhizobium and wheat/Azospirillum inoculation systems. Antibiotic-resistant and

fungicide-resistant mutants were developed in both cases. These mutants were applied to the seed and the seed planted in soil containing the appropriate antibiotics and fungicides; or the latter compounds were applied to the seed before bacterization with the mutant bacterial isolates. In both cases, root colonization was increased due to inhibition of the normal rhizosphere population.

Problems were encountered in developing antibiotic-resistant mutants from the 60 field isolates of this study. In general, the pseudomonads and xanthomonads developed spontaneous mutations for resistance to each of the four antibiotics. For most of these isolates, this allowed for development of different antibiotic-resistant marker combinations enabling differentiation of specific mutant isolates under natural field conditions. As observed previously (Drahos et al., 1986), phenotypic characteristics of Gram-negative isolates with nalidixic acid resistance were usually not stably maintained. For a majority of the Gram negative, nalidixic acid-resistant mutants, storage capabilities were lost and perhaps also their ability to colonize roots or survive in soil since they were not reisolated from roots.

This was not true for the Streptomyces which did develop stable, spontaneous mutants resistant to either streptomycin or nalidixic acid. However, mutants which were resistant to both streptomycin and nalidixic acid were not stably maintained in storage. Previous work with the streptomycetes has indicated that simultaneous loss of resistance to more than one antibiotic occurs more frequently than loss of resistance to one antibiotic (Danilenko, in Chater and

Hopwood, 1984). Resistance to either rifampicin or erythromycin was not developed by any of the streptomycete isolates.

Although the Bacillus isolates could be easily marked as streptomycin resistant, resistance to rifampicin was inconsistently achieved and resistance to erythromycin and nalidixic acid was never obtained. Only one Bacillus isolate developed a spontaneous mutant resistant to both streptomycin and a low level of rifampicin. Kloepper et al. (1985) and Hall et al. (1986) utilized rifampicin-resistant Bacillus mutants in their work with spermosphere competent organisms and biological control agents, respectively. In both cases, no mention is made of having difficulty in developing these spontaneous mutants using standard techniques.

It was amply demonstrated that streptomycin alone was not a sufficient marker for detecting bacterial isolates in a field situation. As Barry (1986) has also noted, a large number of soil bacteria are naturally resistant to streptomycin. The streptomycetes were perhaps one of the few groups which were susceptible to streptomycin. This, plus their unique colony morphology, permitted me to separate streptomycin-resistant Streptomyces mutant isolates from background contamination on 1/10 TSBA supplemented with 100 µg/ml streptomycin. Bacillus-like colonies were the most common background contaminants found on this medium making it impossible to confirm the presence of Bacillus mutants resistant only to streptomycin.

Since detection is necessary for evaluating rhizosphere competence, one experiment was designed to specifically determine which of the four antibiotics or combination of these antibiotics

would be most useful as genetic markers for monitoring introduced bacteria in the field. The pseudomonad and coryneform isolates easily developed resistance to all combinations of the antibiotics but the streptomycete and Bacillus isolates did not and so did not contribute extensively to the experimental results.

Media supplemented with antibiotic combinations which included rifampicin did not permit the normal rhizosphere microflora to grow (Table 4). Overall, rifampicin alone and all double and triple combinations of the antibiotics allowed the growth of less than 3% of the culturable bacterial flora normally found on non-supplemented 1/10 TSBA. The use of streptomycin, erythromycin or nalidixic acid alone as antibiotic supplements in 1/10 TSBA permitted 13% to 21% of background microflora to grow. This suggests that rifampicin alone and all combinations of the four antibiotics would be useful markers for introduced bacteria since 97% of the background rhizosphere microflora was inhibited. However, the data presented in Tables 6 and 7 show that this is not the case. The introduced mutant isolate percentage of the total rhizosphere microflora present on the antibiotic supplemented media is often less than 70%.

Nalidixic acid was usually not a stable marker - alone or in combination with other antibiotics - which resulted in decreased counts for those introduced mutants. Isolates with nalidixic acid resistance were difficult to maintain in storage and growth was often impaired. Mutants resistant to combinations of streptomycin and erythromycin or rifampicin and erythromycin were easily detected during the first 3-4 weeks after planting. This selectivity

diminished as the season progressed. This was also observed in the other field experiments.

Rifampicin alone, and a combination of rifampicin and streptomycin, were the best antibiotic marker systems for the Bacillus, Pseudomonas and coryneform isolates. These markers resulted in the mutant isolate composing 90%-100% of the total culturable microflora on a dilution plate. However, for one of the samplings, a large number of very small, non-sporulating actinomycete colonies were present.

Combinations of streptomycin/rifampicin/erythromycin and rifampicin/nalidixic acid/erythromycin were very selective also; but, if a double antibiotic marker system is sufficient for selectivity, it is probably redundant to add a third antibiotic marker - especially if it is possible the third marker might reduce rhizosphere competence. The rifampicin and rifampicin/ streptomycin combination markers are suitable marker systems since they efficiently inhibit the natural rhizosphere microflora and do not seem to impair root colonization.

One exercise that should be done with double and triple marked isolates is to determine if there would be differences in bacterial counts on different antibiotic media. In other words, replicate dilution plate a root sample with an introduced triple marked isolate on all combinations of the antibiotics to which it is resistant. If antibiotic resistance does not affect root colonization, there should be no differences in the plate counts.

With the Streptomyces isolate, the streptomycin-resistant mutant was the only mutant that was stable in storage, colonized roots and

had phenotypic characteristics similar to the parental isolate (i.e. aerial spore chains). Therefore, streptomycin was the best marker even though the background microflora dominated the dilution plates. The background microflora that was resistant to streptomycin did not include streptomycetes. This allowed for the detection of streptomycin-resistant streptomycetes. It is important to note that familiarization with the colony morphology of the isolate and the common background microflora can play a key role in detecting isolates - even on a selective medium.

One way to improve selectivity in the future might be by the use of genus- or species-specific selective media. For example, the use of a modified King's B medium (Sands et al., 1980) to improve detection of P. fluorescens isolates might not be an improvement over 1/10 TSBA, but it should at least be evaluated after an isolate has been designated an efficient root colonizer. The primary difficulty in using selective media is logistical. By using 1/10 TSBA as the basal medium in all experiments, the only difference in the selection process for each isolate was the antibiotic(s) added. This simplified media preparation and also eliminated a bias against organisms for which there are no selective media.

The importance of efficient antibiotic-resistant markers can not be over emphasized. This was the main reason why the Bacillus mutants with only streptomycin resistance were not selected for further field testing in 1986. Table 26 is a summary of the root colonization and yield results obtained for the 20 mutant isolates which were

Table 26. Summary of root colonization and yield results for 20 bacterial isolates utilized for spring wheat bacterization field studies in 1985 and 1986.

Isolate	Log <sub>10</sub> CFU/mg <sup>ab</sup>				Yield <sup>bc</sup> grams/3.3-m	
	1985 Growth Stage		1986 Growth Stage		1985	1986
	4.5 <sup>c</sup>	92	1.2-1.3 <sup>c</sup>	6.9 <sup>c</sup>		
<u>P. fluorescens</u>						
D-290E	3.4 y	--- <sup>d</sup>	3.2 v	--- <sup>d</sup>	179	304
28Er	2.4 yz	1.6	2.8 vw	1.1 xyz	205	306
216SR	1.4 yz	NT	1.7 xy	0.1 z	179	328
D-288SR	1.4 yz	1.1	1.5 xy	0 z	194	330
D-273SN	0.7 z	0	0 z	0 z	205	332
<u>X. maltophilia</u>						
88SE	2.5 yz	1.6	2.8 vw	1.1 xyz	224	357
59SN	0.6 z	0.6	1.7 xy	1.6 xy	184	321
1000SNR	1.2 z	1.7	0.9 y	0.9 yz	196	315
<u>B. subtilis</u>						
D-39Sr	2.1 yz	1.8	2.0 wx	1.6 xy	155	295
D-60R	1.8 yz	2.8	1.8 xy	1.4 xy	260	314
157R	0.6 z	NT	0 z	0 z	204	316
<u>B. pumilus</u>						
147R	1.8 yz	3.3	2.2 wx	2.1 x	226	322
<u>B. subtilis/pumilus</u>						
D-168r	2.3 yz	NT	2.3 wx	1.2 xy	199	309
58R	2.0 yz	1.8	2.2 wx	1.4 xy	156	333
<u>Streptomyces</u>						
109S	0.6 z	0.7	2.2 wx	0.6 yz	232	345
D-185S	1.4 yz	0.5	1.9 wx	1.2 xyz	228	337
131S	2.1 yz	2.4	1.7 xy	0 z	202	274
239N	0.8 z	1.8	0 z	0 z	203	304
D-4N	0.5 z	NT	0 z	0 z	190	307
<u>"Coryneform"</u>						
D-56SR	2.8 yz	2.3	3.2 v	1.5 xy	239	324
Check - with <u>Ggt</u>					173	---
Check - without <u>Ggt</u>					287	311

<sup>a</sup>Dry weight (mg) of root system sampled plus tightly adhering soil.

<sup>b</sup>Values represent average of four replications.

<sup>c</sup>Values followed by the same letter were not significantly different (P=0.05) according to Newman-Keuls multiple range test. There were no significant differences between yield values (P=0.05)

<sup>d</sup>D-290E colonies could not be separated visually from background contaminants on dilution plates.

screened under field conditions for rhizosphere competence in both 1985 and 1986.

Only one of the mutant P. fluorescens isolates (28Er) can be considered rhizosphere competent. This mutant had one of the highest colonization values in 1985 and 1986 at the earlier plant growth stages. Although its  $\log_{10}$ CFU values declined over time, they did not decline below 1.0. In the first root assay of 1986 (growth stage 1.2-1.3), 13.4% of the total aerobic bacteria isolated from the rhizosphere was mutant 28Er. Parental isolate 28 was one of the best inhibitors of R. glutinis and Ggt in vitro, but these characteristics were lost after the development of antibiotic resistance. Grain yields in both years were very close to those of the checks. Loss of antifungal activity may have affected the ability of 28Er to increase yield but it certainly did not affect root colonization.

The fluorescent pseudomonad mutant D-290E may also be a good root colonizer but the single antibiotic resistance marker of erythromycin does not permit D-290E to be detected after the spring wheat plants flower due to an increase in the number of natural erythromycin-resistant rhizosphere organisms. The addition of another antibiotic marker, such as rifampicin, would help to solve this problem. When D-290E could be detected, the root colonization values were the highest of the 20 bacterial mutants. For the first root assay (growth stage 1.2-1.3) in 1986, 13.6% of the total aerobic bacteria isolated were D-290E. The other three P. fluorescens mutant isolates simply were not consistent in their colonization of roots.

All three of the X. maltophilia mutants should be considered rhizosphere competent with mutant 88SE the best of the three in terms of higher colonization values. This isolate comprised 11% of the total culturable rhizosphere bacterial population when the plants were seedlings in 1986. Mutants 59SN and 1000SRN, however, seem to be more stable colonizers over the entire season. For each year, their root colonization values did not differ greatly between root assays, unlike 88SE and the P. fluorescens isolates. These three xanthomonad mutants were among the least inhibitory isolates of R. glutinis and Ggt in vitro. However, the 88SE seed treatment produced the highest yield in 1986 and one of the higher yields in 1985. This is a good example of non-correlation between in vitro studies and field studies.

Although there were three Bacillus groups represented, they will be considered as one group. Only one of the six Bacillus mutants (157R) did not appear to be rhizosphere competent. The other mutants all had very similar root colonization values which should be adequate in the development of biotic agents. Based on grain yield results, it is possible that mutant D-39Sr is a deleterious rhizosphere organism since seed treated with this isolate produced one of the lowest yields in both years.

The reason mutant 157R did not colonize as well as other Bacillus mutants may be due to the dissimilar type of endospore 157R produced on SEA. After endospore staining, a typical Bacillus endospore was green and vegetative cells were pink. All of 157R endospores were green with distinct pink borders. While these may be the typical

cells of 157R, they may not be conducive to cell survival in soil or on roots.

Only one of the Streptomyces mutant isolates appeared to be rhizosphere competent (D-185S), although two more (109S and 131S) may belong in this category. Mutant 131S had consistently high root colonization values until the last root assay of 1986. It was unexplainably not detected at all in this assay. Mutant 109S does colonize spring wheat roots but not at very high levels. Since there is so little information available on Streptomyces root colonization, it is quite possible that these low values could be adequate for biocontrol or growth-promoting purposes. Root colonization might be improved by increasing the amount of inoculum applied to the seed. The  $\log_{10}$  CFU per seed for Streptomyces mutants was usually about half the amount for all other mutants.

The coryneform mutant (D-56SR) had some of the highest root colonization values in every root assay test of both years. In addition, it comprised 27.8% of the total aerobic bacteria isolated from the rhizosphere in 1986. Therefore, mutant D-56SR should be considered rhizosphere competent.

Since D-56SR was readily reisolated from the rhizosphere of inoculated plants, D-56SR must be stable in the soil with streptomycin and rifampicin as its antibiotic markers even though there is no selective pressure for maintenance of the antibiotic resistance. The problem with this isolate is the instability of the mutant in storage and in culture without selective pressure for the markers. This was demonstrated best by the mutant isolate stability experiment. After

four subisolates of D-56SR were serially transferred ten times, they were no longer resistant to streptomycin and rifampicin. A "temporary" loss of resistance was also observed during the preparation of inoculum for seed treatments for other field experiments. Several times when D-56SR was transferred from storage 1/10 TSBA slants to 1/10 TSBA supplemented with streptomycin and rifampicin, the mutant did not grow. However, if the mutant was transferred to unamended 1/10 TSBA first and then this growth transferred to the antibiotic medium, the isolate would grow. This also occurred with the glycerol storage solutions at -15 C and -70 C. Perhaps maintenance of antibiotic resistance is also a problem in the soil. If this is true, then root colonization values for D-56SR have been underestimated.

Although research with small grain rhizosphere bacteria has been extensive, utilization of marked bacterial isolates under natural field conditions has been limited. Sugar beets and potatoes have been the crops studied most often with marked strains (Suslow, 1982). Wong and Baker (1984) and Fredrickson and Elliott (1985) have used marked bacterial isolates with wheat, but these experiments were conducted only under laboratory and greenhouse conditions. There appears to be only one published report (Weller, 1983) which presents wheat root colonization values for a marked bacterial isolate introduced into the soil under field conditions.

Weller (1983) established a seed bacterization experiment with spring wheat using a P. fluorescens isolate resistant to rifampicin and nalidixic acid (2-79RN<sub>10</sub>). The average bacterial plate count

values for 2-79RN<sub>10</sub> were log<sub>10</sub> 1.1 CFU/mg roots (wet weight) at 55 days after planting and log<sub>10</sub> 0.7 CFU/mg roots at 83 days after planting. The growth stage was not reported. Note that the root weight is for wet root tissue and not dried root tissue as in my study. So, Weller's values would be greater on a dry weight basis. The introduced isolate composed 0.003% (55 days) and 0.008% (85 days) of the total aerobic bacterial population after planting. These values would be independent of wet or dry weight calculations.

The colonization values for the best P. fluorescens in my study (28Er) were (per mg root dry weight) log<sub>10</sub> 2.4 CFU and log<sub>10</sub> 1.6 CFU at 43 and 109 days after planting in 1985 and log<sub>10</sub> 2.8 CFU and log<sub>10</sub> 1.1 CFU at 32 and 83 days post-plant in 1986. In addition, this mutant comprised 13.4% and 0.6% of the total aerobic rhizosphere bacteria isolated at 32 and 83 days after planting in 1986. Some of the non-fluorescent pseudomonads had colonization and percentage values which were better than mutant 28Er. Therefore, I have selected bacterial colonizers which would seem to be more efficient colonizers of spring wheat than the P. fluorescens in Weller's study.

In his report (Weller,1983), results were also presented for winter wheat experiments. The fall root colonization values for one experiment were an improvement over the spring wheat values. Isolate 2-79RN<sub>10</sub> was originally developed for seed bacterization of winter wheat and most work with this isolate has been with winter wheat (Weller,1983; Weller and Cook,1983; Weller,1984). This may explain the low values obtained with spring wheat. Root colonization values for unmarked Bacillus subtilis and Streptomyces griseus

isolates were reported by Merriman et al. (1974). These organisms were used to bacterize wheat, oat and barley seed which was subsequently planted in field plots. Since the isolates were not marked, the bacterial counts were for the "number of actinomycetes resembling S. griseus" or "number of bacteria resembling B. subtilis". To eliminate non-spore forming bacteria and actinomycetes from the root sample, it was heated at 52 C for 20 min before dilution plating was completed. For wheat, there were  $\log_{10}$  2.5 CFU/mg root for S. griseus and  $\log_{10}$  3.3 CFU/mg root for B. subtilis. Root weights were for dry roots. Samples were obtained at growth stage 1.2. These values are all greater than colonization values obtained in my study for this growth stage, but this would be expected if they were using unmarked isolates.

As indicated previously, at least one bacterial isolate from each genus in this study should be considered rhizosphere competent. Mixing the different bacterial isolates together to achieve co-colonization of spring wheat roots also proved to be successful. Isolate mixing may enhance the development and increase the utilization of biotic agents. Cook and Baker (1983) discuss the potential usefulness of mixtures in terms of commercial production. Bacterial isolates with different pest control properties could be mixed together for a "super" seed treatment.

Another approach would be to mix an efficient root colonizer with an inefficient root colonizer that has an excellent pest control property. The efficient root colonizer could create a niche for the inefficient colonizer. For example, one could mix a streptomycin

producing S. griseus strain with a biocontrol agent that is resistant to streptomycin. The streptomycin producer would colonize and eliminate some of the native root microflora because of the streptomycin produced (Hossain and Alexander, 1984; Bashan, 1986b). This would provide an empty niche for the streptomycin-resistant biocontrol agent to colonize. Also, mixing of isolates with the same biocontrol property but with different niche requirements could produce a more stable and, so more consistent, biological seed treatment (Cook and Baker, 1983).

Published reports concerning the mixing of bacterial isolates together for utilization as a single seed treatment are few and often these mixtures have been composed of the same species (Schroth and Hancock, 1981; Suslow and Schroth, 1982; Weller and Cook, 1983). These reports do indicate that a mixture of isolates is more effective than a single isolate.

Bennett and Lynch (1981) mixed together isolates from three different bacterial genera for direct application to roots of wheat, barley and maize grown under gnotobiotic conditions. The bacteria included Curtobacterium sp. (a coryneform), Mycoplana sp. (a Gram negative rod) and a Pseudomonas sp. (fluorescent type). When the Pseudomonas sp. was part of the mixture on wheat roots, the Curtobacterium was not reisolated from the roots. There had been no evidence of antibiotic production by the Pseudomonas sp. in culture but that does not eliminate this as a means of inhibition. Microscopic studies did show that the Pseudomonas sp. was "more intimately associated" with the roots than the Curtobacterium or

Mycoplasma. Perhaps the pseudomonad was competing with Curtobacterium for space and nutrients on the roots.

Despite the fact that only half or less than half the amount of bacteria used for single isolate treatments was used for mixture treatments in my study, only two of the nine isolates used for mixture treatments had bacterial counts which differed significantly from their counts as single isolate treatments. This would imply that the majority of the mutant isolates were compatible with each other. The only mutant isolate having colonization values that dropped to unacceptable colonization levels in the mixture treatments was the Streptomyces mutant D-185S. Without further tests, it is difficult to understand why this Streptomyces mutant did not colonize as well in a mixture of organisms as it did alone. One or all of the other bacterial mutants could have been inhibiting its growth in the rhizosphere. However, I think it is more likely that its colonization values dropped due to the decrease (one-half less) in the amount of D-185S inoculum applied to the seed. One of the mixtures contained D-185S and another streptomycete mutant (239N). Mutant 239N was not reisolated from the seed or from the rhizosphere in either root assay test. Therefore, if 239N was not present, it is unlikely it could have been inhibiting D-185S which was reisolated from seed and roots. Since the root colonization values for D-185S in this mixture were similar to those in the other mixtures it comprised, it is probable the low values were due to inoculum amount and not inhibition.

Only half or less than half of the normal inoculum amount of each mutant isolate was used in the mixture treatments. The reduced

inoculum levels did not affect the colonization values for the majority of the isolates. This implies that one could inoculate with lower numbers of bacteria than used in this experiment and still maintain efficient rhizosphere colonization. This could have an important impact on commercialization of seed inoculants.

The results from the field experiments utilizing different bacterial inoculum rates for seed treatments did demonstrate that a very low amount of inoculum resulted in colonization values equivalent to those obtained with the normal amount of inoculum applied to the seed. My results are different from those of Kloepper et al. (1985). They determined that inoculant level on soybean did affect spermosphere populations. The different conclusions could be due to experimental procedure. Kloepper's group was working with soil under lab conditions and the spermosphere was sampled after only 48 hours. Using a hydroponic system, Bashan (1986a) determined that high levels of Azospirillum could decrease plant growth response and low levels were simply ineffective. He also stated that unpublished research results demonstrated that "over 70 different rhizosphere bacteria isolates showed similar trends". Obviously, there is an optimum inoculum level, but it may differ for each organism and each target crop.

From many studies there are reports which have suggested that survival and colonization of bacterial isolates or the disease suppressiveness exhibited by bacterial isolates was related to crop specificity and/or soil type (Millet et al., 1985; Yuen et al., 1985; Suslow and Schroth, 1982; Bennet and Lynch, 1981). Therefore, some of

the most interesting and unexpected results from this study were those obtained from growth chamber experiments which tested the capacity of five bacterial isolates to colonize crops other than spring wheat in vermiculite and in non-sterile soil. These isolates were capable of colonizing other crops and actually colonized corn and soybean roots better than spring wheat roots even though these isolates were selected based on spring wheat colonization results. It is important to remember that similar results may not be obtained in an actual field study. However, bacterial isolates with a broad host range would certainly be useful in developing commercially acceptable biological seed treatments.

## SUMMARY

Efficient bacterial root colonizers were identified and characterized. A next step could be the development of biotic agents to control soilborne pests or to promote plant growth. This could be done by inserting useful genes into these root colonizing bacteria, amplifying genes already present or modifying genes and gene products.

For example, mutant isolate 88SE (X. maltophilia), a doubly marked rhizosphere competent xanthomonad, appears to produce compounds which promote plant growth. If the genes which direct the production of these compounds can be isolated, it is possible that the gene sequence could be amplified or put under the control of a stronger promoter to increase production of the compounds.

If antibiotics are produced by Streptomyces in the soil (Rothrock and Gottlieb, 1984), inserting gene sequences for antibiotic production into Streptomyces root colonizers could produce an effective biocontrol agent. This same idea could apply to enzyme or toxin production, such as chitinase or B. thuringiensis endotoxin, in other bacterial root colonizers. In theory, the number of different agriculturally useful biotic agents which could be developed appears limitless. The only way one determines the boundary limits is to expand as far as possible!

Once the biotic agents have been developed, they can be mixed together to develop seed bacterization treatments with expanded

biological control capabilities. Presently, a biological seed treatment usually controls one soilborne pest or promotes growth for unknown reasons. With each bacterial colonizer in a mixture possessing specific useful genes, seed treatments can be designed to fit the targeted agricultural market.

My preliminary results which indicate that some of the bacterial isolates are capable of colonizing roots of field crops other than spring wheat in non-sterile soil are especially intriguing. If an organism could colonize several economically important crops in a number of different soil environments and be genetically manipulated easily, its value as a biotic agent for agriculture would be greatly enhanced. The ability to use significantly smaller amounts of bacterial isolates than used presently to inoculate seeds would also be extremely useful commercially.

Rhizosphere competent bacteria have been identified which have the potential for commercial development - but primarily via the use of recombinant DNA technology. Presently, the formidable problem is to gain EPA approval for release of genetically engineered microorganisms into the soil environment. Perhaps the best use of the organisms in this study is not in commercial development as seed inoculants but rather in microbial ecology studies to help answer the questions EPA and the general public are posing concerning the release of genetically engineered microorganisms.

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## APPENDIX

## MEDIA

All antibiotics and Sigma products were obtained from Sigma Chemical Co., St. Louis, MO. Bacto products were procured from Difco Laboratories, Detroit, MI. BBL products were obtained from BBL Microbiology Systems, Cockeysville, MD.

Modified King's Medium B (Sands et al., 1980)

Proteose Peptone # 3, Difco	20.0 g
K <sub>2</sub> HPO <sub>4</sub> · 3 H <sub>2</sub> O	1.5 g
MgSO <sub>4</sub> (anhydrous)	1.5 g
Glycerol, Sigma	15.0 ml
Bacto Agar, Difco	15.0 g
Double Distilled H <sub>2</sub> O	1.0 L

Autoclave 20 min at 121 C; cool to 45 C and add following:

Bacitracin	10.0 mg
Vancomycin	6.0 mg
Rifampicin	0.5 mg
Cycloheximide	100.0 mg
Benomyl	250.0 mg

Actinomycete Selective Medium (Kuster and Williams, 1964; Williams and Davies, 1965)

Soluble Starch, Sigma	10.0 g
Casein - Vitamin Free, Sigma	0.3 g
KNO <sub>3</sub>	2.0 g
NaCl	2.0 g
K <sub>2</sub> HPO <sub>4</sub> · 3 H <sub>2</sub> O	2.0 g
MgSO <sub>4</sub> (anhydrous)	0.05 g
CaCO <sub>3</sub>	0.02 g
FeSO <sub>4</sub> · 7 H <sub>2</sub> O	0.01 g
Bacto Agar, Difco	20.0 g
Distilled H <sub>2</sub> O	1.0 L

Autoclave 20 min at 121 C; cool to 45 C and add following:

Polymyxin B Sulfate	5.0 mg
Sodium Penicillin	1.0 mg
Cycloheximide	50.0 mg
Nystatin	50.0 mg

Soil Extract Agar (Norris et al., 1981)

Nutrient Broth, BBL	8.0 g
Soil Extract*	250.0 ml
Bacto Agar, Difco	15.0 g
Distilled H <sub>2</sub> O	750.0 ml

Autoclave 20 min at 121 C.

\*Soil Extract obtained by autoclaving 1 kg soil in 1 L distilled H<sub>2</sub>O for 60 min, filtering and restoring to 1 L volume with distilled H<sub>2</sub>O.

Modified Soil Extract Agar (Parkinson et al., 1971)

K <sub>2</sub> HPO <sub>4</sub> · 3 H <sub>2</sub> O	0.2 g
Glucose	1.0 g
Bacto Gelatin, Difco	30.0 g
Bacto Agar, Difco	15.0 g
Soil Extract*	1.0 L

Autoclave 20 min at 121 C; cool to 45 C and add following:

Cycloheximide	100.0 mg
Polymyxin B Sulfate	5.0 mg

\*Soil Extract obtained by autoclaving 1 kg soil and 1 L distilled H<sub>2</sub>O for 20 min, filtering and restoring to 1 L volume with distilled H<sub>2</sub>O.

Tryptic Soy Broth Agar

Bacto Tryptic Soy Broth, Difco	30.0 g
Bacto Agar, Difco	15.0 g
Distilled H <sub>2</sub> O	1.0 L

Autoclave 20 min at 121 C.

Note: This is not the same as the commercially available Tryptic Soy Agar.

1/10 Tryptic Soy Broth Agar

Bacto Tryptic Soy Broth, Difco	3.0 g
Bacto Agar, Difco	15.0 g
Distilled H <sub>2</sub> O	1.0 L

Autoclave 20 min at 121 C.

MRS Agar

Bacto Lactobacilli Broth, Difco	55.0 g
CaCO <sub>3</sub>	3.5 g
Bacto Agar, Difco	15.0 g
Distilled H <sub>2</sub> O	1.0 L

Autoclave 20 min at 121 C.

Tryptone Yeast Extract Broth (Kutzner, 1981)

Bacto Tryptone, Difco	5.0 g
Bacto Yeast Extract, Difco	3.0 g
Distilled H <sub>2</sub> O	1.0 L

Autoclave 20 min at 121 C.

Phosphate Buffer (ph 6.8)

NaCl	8.5 g
K <sub>2</sub> HPO <sub>4</sub> · 3 H <sub>2</sub> O	11.4 g
KH <sub>2</sub> PO <sub>4</sub>	6.8 g
Distilled H <sub>2</sub> O	1.0 L

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