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

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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Monica Elliott Juhnke

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ABSTRACT

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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 intrageneric 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. Lactobacillis 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 μg/ml and small letters signify a resistance level of 25 μg/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 coryneforym 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, Piroline 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 C $\pm$ 2 C with fluorescent and incandescent lamps providing an average of 4850 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.) pimaricin (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 µg/ml was substituted for pimaricin in the selective media due to a temporary shortage of pimaricin.

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 of 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*[a] Rating	*Ggt*[b] Lysis	*Ggt* Inhibition (mm)[c]				*Ggt* Inhibition (mm)[c]		
				pH 5[d]	pH 6	pH 7	pH 8	2 C[e]	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[f]	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