Molecular Ecology of Rhizosphere Microorganisms

Biotechnology and the Release of GMOs

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This book helps evaluate the state of the art of rhizosphere microbial ecology and biotechnology. Experts in the field review methods and strategies applied to the detection, identification and monitoring of microorganisms in the rhizosphere. Major topics treated included:

- construction of genetically marked rhizosphere bacteria
- detection of marked wildtype and genetically modified organisms (GMOs)
- identification of wildtype and GMOs by DNA probes and PCR amplification
- rapid typing of non-modified and GMOs by PCR-based techniques
- assessment of the role of gene transfer
- EU regulations for the use and release of GMOs
- biosafety results from field testing of GMOs

In addition, technologies for the modifying gene expression and gene products for specific traits of agronomic interest in genetically engineered rhizosphere bacteria are covered.

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2 Studies on Indigenous Endophytic Bacteria of Sweet Corn and Cotton

John A. McInroy and Joseph W. Kloepper

2.1 Introduction

Several reports since 1948 have demonstrated that bacteria naturally inhabit healthy plant tissues, including fruits (1), vegetables (2, 3), stems (4) and roots (5). Mundt and Hinkle (6) found endophytic bacteria within seeds and ovules of 25 of 27 plant species sampled, establishing the presence of endophytes prior to germination. Endophytic bacteria were found throughout cotton plants, radicles, roots, stems, unopened flowers, and bolls (7). Since bacterial endophytes have a natural association with plants and can colonize plant tissues without inciting disease, they are potenial candidates for use as agricultural inoculants which provide plant growth-promotion or biological control of plant diseases.

To date, there has been little research aimed at determining possible benefits of endophytic bacteria on crops. Van Peer et al. (8) reported that 30% of *Pseudomonas* endophytes reduced plant growth after seed bacterization and 2% actually stimulated plant growth. Researchers at Crop Genetics International have modified a xylem-inhabiting endophyte of bermudagrass (*Cynodon dactylon*), *Clavibacter xyli* subsp. *cynodontis*, using recombinant DNA techniques to produce an endotoxin from *Bacillus thuringiensis*, which combats the European corn borer (*Ostrinia nubilalis*) in corn (9). These two reports demonstrate that select endophytic bacterial strains may benefit plants.

It is possible that some endophytes can systematically colonize plants and overcome the limitations of phyllioplane or rhizosphere bacteria. The internal tissues of plants provide a more uniform and protective environment for introduced biological control agents compared to the phylloplane, where exposure to ultraviolet radiation, rainfall and temperature fluctuations negatively affects introduced microorganisms, or compared to the rhizosphere where introduced microorganisms compete for nutrients with other microbes. Reports on the extent and density of tissue colonization by endophytic bacteria are limited, especially for above-ground tissues. It is therefore useful to have a quantitative understanding of the indigenous endophytic bacterial community to help assess endophytes as potential sources of effective strains for plant growth-promotion or biological control of plant disease.

The objectives of this study were to determine the population dynamics of bacterial endophytes in stems and roots during the growing season; to identify the major

taxa of the endophytic community; and to compare populations in a model monocotyledonous plant, sweet corn (*Zea mays L.*) and a dicotyledonous plant, cotton (*Gossypium hirsutum L.*).

2.2 Materials and Methods

2.2.1 Media

Bacteria were isolated on three different media; tryptic soy agar (TSA) (Difco Laboratories, Detroit, MI) was used to support the growth of a broad range of microorganisms; medium R2A (Difco Laboratories, Detroit, MI) was used for bacteria requiring a low level of nutrients (oligotrophs); medium SC (10) was included to support the growth of some fastidious organisms, e.g. *Clavibacter xyli.*

2.2.2 Field Experiments

Cotton ("DES 119") and sweet corn ("Silver Queen") were planted in 1990 in the field in a fine-loamy, siliceous, thermic, Typic Hapludult soil at Tallassee, AL. Ten blocks of four 25-ft rows were planted for both crops. Plants were sampled at emergence and 2, 7, 14, 21, 28, 42, 56, 70 and 112 days after emergence. The experiment was repeated the following year under the same conditions and sampled once prior to emergence, at emergence and 7, 14, 28, 42, 56 and 70 days after emergence. At each sampling date, one randomly selected cotton plant from each of the ten replicate blocks was manually uprooted and transported at 10 °C to the laboratory. Sweet corn was sampled similarly.

2.2.3 Sample Preparation and Surface Sterilization

Individual plant samples were washed in running tap water to remove adherent soil. Sections, 2–3 cm in length, were excised with a flamed scalpel. Root sections were taken just below the soil line in younger plants (14 days or less after emergence) and from 5–10 cm below the soil line in older plants (21 days or more after emergence). Stem sections were taken 1–2 cm above the soil line in younger plants and 10 cm above the soil line in older plants.

All sections were blotted dry with a paper-towel and weighed before processing. Stem samples were surface-disinfested in 20% hydrogen peroxide for 10 min and

rinsed four times with sterile 0.02 M potassium phosphate buffer, pH 7.0. Surface-disinfestation parameters for all tissues were optimized prior to experimentation. Root samples were surface-disinfested with 1.05% sodium hypochlorite for 10 min and rinsed four times as previously described. A 0.1 ml aliquot was taken from the final buffer wash of each sample and transferred to a tube of tryptic soy broth to serve as a sterility check. Samples were discarded if growth from the sterility check occurred within 48 hr. Each sample was triturated with a sterile mortar and pestle in 9.9 ml of the final buffer wash. Serial dilutions were made using phosphate buffer, as previously described, and plated with a spiral plater (Spiral Systems, Inc., Bethesda, MD). Each dilution of every sample was plated on 1 plate each of TSA, R2A and SC.

2.2.4 Growth Conditions, Bacterial Counts and Data Analysis

Agar plates were incubated at 28 °C for 48–72 hr except where noted. Colonies were counted with a laser colony counter (Spiral Systems, Inc., Bethesda, MD) and populations were determined by Bacterial Enumeration software (Spiral Systems, Inc., Bethesda, MD) in colony forming units per ml. Populations were transformed to log 10 colony forming units per gram fresh weight (cfu/g-fw) prior to calculating mean population densities.

2.2.5 Isolation and Preservation of Endophytes

At each sampling date, and for each treatment, one representative of each bacterial colony morphology was transferred to a fresh TSA plate to establish pure cultures. Individual strains were shaker-cultured at room temperature for 18–24 hr in tryptic soy broth. Cultures were then centrifuged at 5000 x g for 7 min at 4 °C. The resulting pellet was resuspended in 2.0 ml TSB amended with 20.0% glycerol and maintained at −80 °C in Nalgene cryovials for later identification by MIS as outlined below.

2.2.6 Strain Identification

Each strain was identified by membrane fatty acid analysis using the Microbial Identification System (11). Strains that could not be identified with a similarity index above 0.100 were considered unidentified.

2.3 Results

2.3.1 Population Dynamics

Bacteria were recovered from surface-disinfested stems and roots of cotton and sweet corn during both growing seasons on all media. Populations from medium R2A and medium SC were significantly greater than populations on TSA ($P=0.0001$). Populations from medium R2A were not significantly different from medium SC ($P=0.0001$). Plate counts from medium R2A were more accurately determined because of less colony overlap and smaller colony size which was due to the low nutritional status of the medium. For these reasons, data are presented from medium R2A.

Total endophytic bacterial (TEB) populations of sweet corn roots and stems (Fig. 1) from the field showed that endophytic bacteria were present at emergence at 10^4 cfu/g-fw for both seasons. TEB populations in corn stems and roots in 1990 remained between $10^4 - 10^6$ cfu/g-fw for most of the growing season. These populations increased to $10^8 - 10^{10}$ cfu/g-fw post-harvest. TEB populations in 1991 were from $10^4 - 10^7$ cfu/g-fw for the entire growing season. Although not significant, there was no similar population increase in cotton roots or stems at the end of the 1991 growing season.

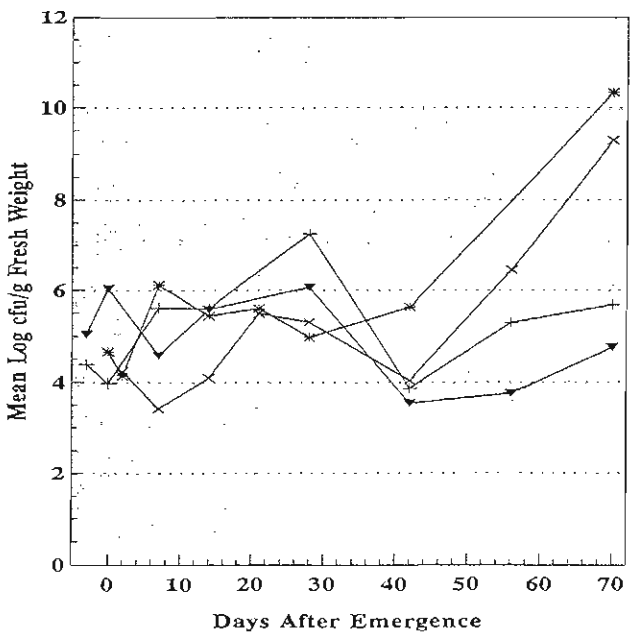

Fig. 1. Population densities of endophytic bacteria from roots (*) and stems (x) of field-grown sweet corn, 1990; and roots (+) and stems (▼) of field-grown sweet corn, 1991.

Endophytic bacteria were present at emergence in cotton roots in 1990 and 1991. In 1990, TEB populations from field-grown cotton roots (Fig. 2) were 10^4 cfu/g-fw for the first week and from 10^5 – 10^8 cfu/g-fw for the rest of the season. In 1991, TEB populations from cotton roots were 10^7 cfu/g-fw during the first week and 10^4 – 10^6 cfu/g-fw for the rest of the season. No cotton stem populations in 1990 were detected at emergence, but bacteria were present 2 days after emergence at 10^3 cfu/g-fw. Cotton stem populations in 1990 remained between 10^4 – 10^6 cfu/g-fw for the rest of the season. In 1991, TEB populations in cotton stems were 10^7 cfu/g-fw at emergence and 10^6 – 10^7 cfu/g-fw for the first week. For the remainder of the season cotton stem populations in 1991 ranged from 10^3 – 10^6 cfu/g-fw.

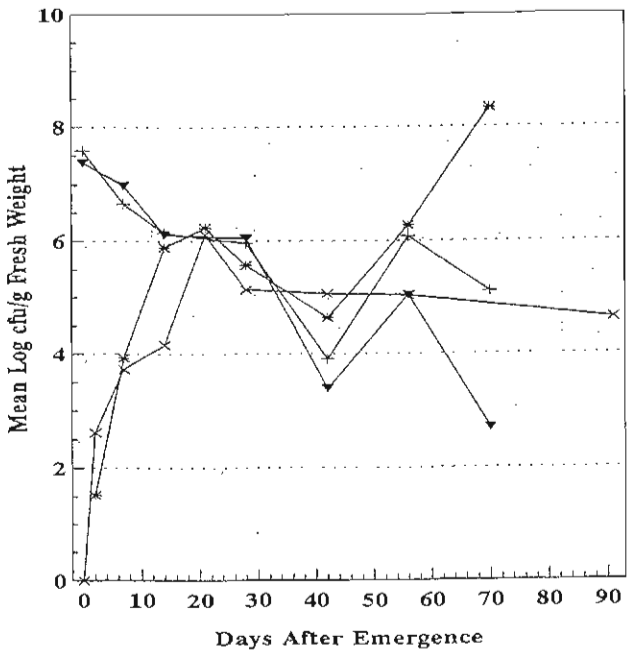

Fig. 2. Population densities of endophytic bacteria from roots (*) and stems (x) of field-grown cotton, 1990; and roots (+) and stems (▼) of field-grown cotton, 1991.

2.3.2 Bacterial Identification

A total of 947 bacterial endophytes were isolated; 313 were from sweet corn roots, 230 from sweet corn stems, 250 from cotton roots and 154 from cotton stems. The endophytic bacteria isolated comprised 34 genera; 31 of these were present in sweet

corn and 31 were present in cotton. Twenty five of the 34 genera were Gram-negative taxa. Of the total isolates, 71.4% were Gram-negative, and 25.9% were Gram-positive. Bacteria which were unidentifiable by MIS represented 2.6% of the total. Results of bacterial identification by fatty acid analysis (Table 1) indicated that the diversity of bacteria did not vary between sweet corn and cotton; however, the frequency of occurrence did. The most frequently isolated groups were *Pseudomonas pickettii* and *Pseudomonas solanacearum* from sweet corn roots; *Serratia* spp. from sweet corn stems; *Agrobacterium radiobacter, Serratia* spp. and *Staphylococcus* spp. from cotton roots; and *Bacillus megaterium* and *Bacillus pumilus* from cotton stems. *Acinetobacter baumannii, Comamonas testosteroni,* and *Cellulomonas* spp. were only isolated from cotton, and *Pantoea agglomerans, Flavimonas oryzihabitans,* and *Xanthomonas campestris* pathovars were only isolated from sweet corn. Several taxonomic groups were isolated much more frequently from sweet corn than they were from cotton; these included *Pantoea agglomerans, Enterobacter cloacae, Pseudomonas cepacia, Pseudomonas gladioli, Pseudomonas putida, Clavibacter* spp., *Klebsiella* spp., and *Kluyvera* spp. There were no taxonomic groups that were isolated much more frequently from cotton than from sweet corn.

In general, bacteria isolated from sweet corn stems were also isolated from sweet corn roots, and vice versa. This was not so in cotton. *Acinetobacter baumannii, Bacillus subtilis, Arthrobacter* spp., and *Citrobacter* spp. were present in cotton stems but not in cotton roots. There were 13 taxonomic groups present in cotton roots but not in cotton stems, all of which were Gram-negative except for *Microbacterium* spp. isolated at only one sampling date.

Agrobacterium radiobacter was isolated from roots of both crop plants more frequently than from stems of both plants. The group of strains that was unidentifiable came, almost exclusively, from the roots of both crops. All taxonomic groups frequently isolated from stems of both crops were also present in roots of both crops.

2.4 Discussion

Healthy monocotyledonous and dicotyledonous plants were naturally infested with endophytic bacteria at average populations of $10^3 - 10^7$ cfu/g-fw throughout two growing seasons. Endophytes colonized plants early in the season, beginning prior to emergence, based on recovery from seedlings. TEB populations of sweet corn roots and stems, even through germination, generally remained between $10^4 - 10^6$ cfu/g-fw (Fig. 1). Endophytic bacterial populations tended to decrease acropetally, although they do seem to colonize most plant tissues. Root populations were generally slightly greater than stem populations.

The internal tissues of sweet corn and cotton, host a diverse microflora that is similar to common soil bacteria, rhizosphere bacteria, and previously reported endophytic bacteria. Previously reported endophytes have been isolated predominantly from fruits, vegetables, and storage organs of other plant systems and include species of *Bacillus, Agrobacterium, Enterobacter, Erwinia, Flavobacterium, Micrococcus,*

Pseudomonas, Xanthomonas, Citrobacter, and coryneforms including *Curtobacterium, Cellulomonas, Arthrobacter,* and *Clavibacter* (5, 7, 12, 13, 14, 15, 16). The endophytes identified in this study reflect this commonality. However, there are taxonomic groups that have only been previously isolated on one occasion. Lu and Chen (17), among other endophytes already mentioned, identified *Chromobacterium* spp. from cotton infested with *Fusarium*. Gardner et al. (18) identified *Enterobacter sakazakii, Pseudomonas aeruginosa, Serratia liquefaciens, Acinetobacter lwoffii, Yersinia* spp., *Shigella* spp, *Achromobacter* spp., *Providencia* spp., and *Vibrio* spp. from lemon roots. Mundt and Hinkle (6) identified 395 endophytic bacteria from seeds and ovules of 27 different plants and reported several species that had not previously been shown to colonize internal plant tissues. These bacteria, and some of the endophytes that were isolated on only one or two sampling dates in this study, represent what can be called casual opportunistic colonizers of internal plant tissues, e.g., *Acinetobacter, Comamonas, Alcaligenes, Flavimonas,* and *Microbacterium* (Table 1). Since these groups of bacteria are not common soil inhabitants, they probably exist in the environment in association with plants, either with decomposing organic matter, in the rhizosphere, or in the rhizoplane and phylloplane. They may colonize internal plant tissues through natural avenues but are not competitive with other endophytes.

Table 1. Identification and isolation frequency of bacterial endophytes from sweet corn and cotton.

Taxa[1]	Tissue Source Yielding Endophytic Bacteria			
	Sweet Corn		Cotton	
	Root	Stem	Root	Stem
Acinetobacter baumannii				+
Agrobacterium radiobacter	+	+	+	+
Alcaligenes piechaudii		+	+	
Arthrobacter spp.	+	+	+	
Aureobacterium spp.		+	+	+
Bacillus megaterium	+	+	+	+
Bacillus pumilus	+	+	+	+
Bacillus subtilis	+	+	+	
Bacillus thuringiensis	+	+	+	+
Bacillus spp.	+	+	+	+
Cellulomonas spp.		+	+	
Citrobacter spp.	+	+	+	
Clavibacter spp.	+	+	+	+
Comamonas testosteroni			+	
Curtobacterium spp.	+	+	+	+
Enterobacter asburiae	+	+	+	+
Enterobacter cloacae	+	+	+	
Enterobacter taylorae	+	+	+	
Erwinia spp.	+	+	+	+
Escherichia spp.	+	+	+	
Flavimonas oryzihabitans	+			
Flavobacterium spp.	+	+	+	
Hydrogenophaga pseudoflava	+	+	+	+
Klebsiella spp.	+	+	+	+
Kluyvera spp.	+	+	+	

Table 1. (continued).

Taxa[1]	Tissue Source Yielding Endophytic Bacteria			
	Sweet Corn		Cotton	
	Root	Stem	Root	Stem
Methylobacterium spp.	+	+	+	+
Microbacterium spp.	+	+	+	
Micrococcus spp.	+	+	+	+
Ochrobactrum anthropi		+	+	
Pantoea agglomerans	+	+		
Phyllobacterium spp.	+	+	+	+
Pseudomonas cepacia	+	+	+	+
Pseudomonas chlororaphis	+	+	+	+
Pseudomonas gladioli	+	+	+	
Pseudomonas pickettii	+	+	+	+
Pseudomonas putida	+	+	+	
Pseudomonas saccharophila	+	+	+	+
Pseudomonas solanacearum	+	+	+	+
Pseudomonas fluor. spp.	+	+	+	+
Pseudomonas nonfluor. spp.	+	+	+	+
Rhizobium japonicum	+		+	+
Salmonella spp.	+	+	+	+
Serratia spp.	+	+	+	+
Sphingomonas paucimobilis	+	+	+	
Staphylococcus spp.	+	+	+	+
Variovorax paradoxus	+	+	+	
Xanthomonas campestris	+	+	+	+
Xanthomonas maltophilia	+			
Unknown[2]	+	+	+	

[1] Grouped taxa consist of the following species; *Arthrobacter crystallopoietes, A. globiformis, A. mysorens, A. pascens; Aureobacterium barkeri, A. saperdae, A. testaceum; Bacillus amyloliquefaciens, B. cereus, B. coagulans, B. laterosporus, B. lentus, B. licheniformis, B. macerans, B. mycoides, B. pabuli, B. pasteurii, B. polymyxa, B. psychrophilus, B. sphaericus; Cellulomonas cartae, C. cellulans; Clavibacter michiganense* subsp. *insidiosum, C. michiganense* subsp. *nebraskense; Citrobacter diversus, C. freundii; Curtobacterium flaccumfaciens* subsp. *flaccumfaciens, C. flaccumfaciens* subsp. *oortii, C. flaccumfaciens* subsp. *poinsettiae, C. pusillum; Erwinia carnegieana, E. carotovora* subsp. *carotovora, E. herbicola, E. uredovora; Escherichia coli, E. hermannii; Flavobacterium indologenes, F. meningosepticum; Klebsiella planticola, K. pneumoniae* subsp. *ozaenae, K. terrigena; Kluyvera ascorbata, K. cryocrescens; Methylobacterium fujisawaense, M. mesophilicum, M. radiotolerans, M. rhodesianum; Microbacterium imperiale, M. laevaniformans; Micrococcus agilis, M. kristinae, M. luteus, M. lylae. M. roseus, M. varians; Pantoea agglomerans, P. ananas; Phyllobacterium myrsinacearum, P. rubiacearum; Pseudomonas* (fluorescent species) *P. coronafaciens, P. cichorii, P. fluorescens, P. syringae; Pseudomonas* (nonfluorescent species) *P. diminuta, P. marginalis, P. rubrisubalbicans, P. vesicularis; Salmonella bongori, S. choleraesuis* subsp. *arizonae, S. choleraesuis* subsp. *diarizonae, S. choleraesuis* subsp. *houtenae, S. choleraesuis* subsp. *salamae; Serratia marcescens, S. plymuthica. S. proteamaculans* subsp. *proteamaculans; Staphylococcus capitis* subsp. *capitis, S. capitis* subsp. *ureolyticus, S. cohnii, S. epidermidis, S. hominis, S. warneri.*

[2] Bacteria unable to be identified by MIS, 25 total.

Root tissues of both crops generally harbored the same endophytes found in stem tissue, with a few exceptions. The unidentified strains and *A. radiobacter* came almost exclusively from root tissue, suggesting that these microbes are strict colonizers of internal root tissue as opposed to stem tissue. There also were taxa which, al-

though isolated from both stem and root, were more frequently isolated from one over the other, e.g., *B. megaterium* and *B. subtilis* in cotton stems, and *P. cepacia, P. gladioli* and *P. solanacearum* in sweet corn roots. This suggests that strains can adapt to specific plant tissues. The total number of bacteria isolated from each tissue is an indirect measure of tissue diversity, since the bacteria were selected based on unique colonial morphology per treatment and per replicate. The number of endophytes isolated from roots of both crops is greater than that of stems, and the number of endophytes isolated from sweet corn tissues surpasses that isolated from the respective tissues in cotton. These data indicate that internal sweet corn tissues support a more diverse microbial flora than cotton. They also support the hypothesis that bacterial endophytes originate in the rhizosphere and from there proceed into stem tissue.

In order to make valid comparisons of endophytic bacteria with rhizosphere or soil bacteria, surveys of rhizobacteria and soil microbes of the past will have to be re-evaluated. This is due, in part, to the fact that most identification studies were conducted to the genus level only. But re-evaluation is also necessary to compensate for the changing bacterial nomenclature that has taken place over the past 15 years. The *Pseudomonas* genus alone has been fragmented into *Acidovorax, Comamonas, Flavimonas, Hydrogenophaga, Methylobacterium*, and *Sphingomonas*. Plant-associated members of the genus *Corynebacterium* are now in *Aureobacterium, Clavibacter, Curtobacterium*, and *Rathayibacter*.

Endophyte colonization of sweet corn and cotton tissues shown in this study suggests that internal plant habitats are exploited by a wide variety of bacteria. Screening of endophytic bacteria as potential plant growth-promoters and biological control agents can now include representatives from more diverse bacterial taxa, and the list may lengthen as more crops are studied.

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