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# Survey of indigenous bacterial endophytes from cotton and sweet corn

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## Abstract

The genotypic diversity of indigenous bacterial endophytes within stems and roots of sweet corn (*Zea mays* L.) and cotton (*Gossypium hirsutum* L.) was determined in field trials throughout one growing season. Strains were isolated from surface-disinfested tissues and identified by fatty acid analysis. Gram-negative bacteria comprised 70.5% of the endophytic bacteria and 27 of the 36 genera identified. The most frequently isolated groups from sweet corn roots, were *Burkholderia pickettii* and *Enterobacter* spp.; from sweet corn stems, *Bacillus megaterium*. Bacterial genera present in sweet corn roots were also generally present in sweet corn stems. However, *Burkholderia gladioli*, *Burkholderia solanacearum* and *Enterobacter cloacae* were isolated much more frequently from sweet corn roots than stems, whereas *Methylobacterium* spp. were found more frequently in sweet corn stems than roots. *Agrobacterium radiobacter*, *Serratia* spp. and *Burkholderia solanacearum*, were the most frequently isolated groups from cotton roots; and *Bacillus megaterium* and *Bacillus pumilus* from cotton stems. *Acinetobacter baumannii* and *Arthrobacter* spp. were present in cotton stems but not in cotton roots. There were 14 taxonomic groups present in cotton roots that were not in cotton stems; all but one were Gram-negative. These included, *Agrobacterium radiobacter*, *Bacillus megaterium*, *Bacillus pumilus*, *Enterobacter asburiae*, *Pseudomonas chlororaphis*, *Serratia* spp. and *Staphylococcus* spp. *Rhizobium japonicum* and *Variovorax paradoxus* were isolated, almost exclusively, from the roots of both crops. Bacterial taxa present in both sweet corn and cotton early in the season were generally present late in the season. The diversity of bacteria was greater in roots than stems for each crop.

## Introduction

Bacterial endophytes have been reported in various plant tissues, including tubers (Hollis, 1951) fruit (Samish and Dimant, 1957; Samish et al., 1961), stems (Misaghi and Donndelinger, 1990) and seeds and ovules (Mundt and Hinkle, 1976). Occasionally, investigators have identified isolated endophytic bacteria, but in such cases identification consisted of a limited number of the predominant strains encountered. Hollis (1951) described 14 bacteria, representing different morphologies, from potato tubers. Mundt and Hinkle (1976) identified 395 bacterial endophytes of ovules and seeds of 27 plant species; the most commonly occurring genera included *Bacillus*, *Enterobacter*, *Erwinia*, *Flavobacterium* and *Pseudomonas*. Gardner et al. (1982), identified 556 endophytic bacteria from lemon-root xylem to thirteen different genera, with Gram-negative rod-shaped bacteria predominating.

There are indications that some rhizosphere bacteria colonize the internal tissues of roots. Patriquin et al. (1983) showed that *Azospirillum* spp. from the rhizosphere colonized plant roots internally, including the xylem, intercellular spaces and the inner cortex. *Pseudomonas* spp., *Erwinia*-like spp., and unidentified Gram-positive and Gram-negative bacteria were consistently isolated from the xylem of alfalfa roots at populations of $\log_{10}$ 3–4 cfu $g^{-1}$ (Gagné et al., 1989). Bacteria, once inside the plant, escape the competition of other rhizosphere microflora and may be better adapted to survival based on catabolism of plant metabolites.

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The aforementioned studies relied on classical biochemical tests for identifying bacteria. Advances in bacterial identification technologies have made bacterial identification of large numbers of strains more practical and relatively rapid. Microbial Identification, Inc. (MIDI) (Newark, DE), has developed a computer-driven system which can successfully identify approximately 900 different bacteria based on differences in total cellular fatty acids. This Microbial Identification System (MIS) (1990) involves extracting fatty acids from pure bacterial cultures and separating them using gas chromatography. The generated fatty acid profiles are then compared to a computer library of profile entries for bacterial identification. Experiments in the area of microbial ecology benefit from tools like MIS which can broaden experimental designs without increasing the time input. The objectives of this study were to identify and determine the diversity of bacterial endophytes and to compare diversity and frequency of endophytes in sweet corn and cotton, and in stems and roots, throughout a growing season. Results from this research will provide basic information on the microbial ecology of indigenous endophytes, information required for future efforts to use endophytes for enhancing crop development. A preliminary report on a portion of this project was presented in abstract form (McInroy and Kloepper, 1991).

## Materials and methods

### *Field experiments and sample preparation*

One field trial each of cotton *Gossypium hirsutum* (L.) cv. 'DES 119' and sweet corn *Zea mays* (L. ) cv. 'Silver Queen' was planted in 1990 in a fine-loamy, siliceous, thermic, Typic Hapludult soil at the E. V. Smith Station of the Alabama Agricultural Experiment Station near Tallassee, AL. Each trial contained nontreated seed and included 10 replications, each consisting of 25-ft long 2-row plots. Plants were sampled at emergence and 2, 7, 14, 21, 28, 42, 56, and 70 days after emergence. At each sampling date, one randomly selected cotton or sweet corn plant from each replication was manually uprooted and transported at 10 °C to the laboratory. Stem and root samples were taken from the same plants.

Sections 2 – 3 cm in length were excised with a flamed scalpel. Root sections were taken just below the soil line in younger plants ($\leq$ 14 days) and from 5 – 10 cm below the soil line in older plants ($\geq$ 21 days). Stem sections were taken 1 – 2 cm above the soil line in younger plants and 10 cm above the soil line in older plants.

Stem samples were weighed, surface-disinfested in 20% hydrogen peroxide for 10 min, and rinsed four times with sterile 0.02 *M* potassium phosphate buffer, pH 7.0 (PB). Root samples were surface-disinfested with 1.05 % sodium hypochlorite and rinsed four times in PB. (Surface-disinfestation parameters for all tissues were optimized prior to experimentation. Optimization included; selection of disinfestant, strength of disinfestant, duration of immersion in disinfestant, epiphyte detection after disinfesting and confirming absence of viable bacteria in cut-end of sample.) A 0.1 mL aliquot was taken from the final buffer wash and transferred to 9.9 mL tryptic soy broth (TSB) to serve as a sterility check. This method was previously determined (McInroy, unpublished) to detect surface contamination of stems, roots and seeds with the same accuracy as agitating samples in tryptic soy broth or imprinting samples on TSA. Samples were discarded if growth was detected in the sterility check within 48 hr. Each sample was triturated with a sterile mortar and pestle in 9.9 mL of the final buffer wash.

### *Isolation and preservation of endophytes*

Serial dilutions of the triturate were made in PB and plated with a spiral plater (Spiral Systems, Inc., Bethesda, MD). Each dilution of every sample was plated on 1 plate each of three different media; medium R2A (Difco Laboratories, Detroit, MI) for oligotrophic bacteria, TSA (Difco Laboratories, Detroit, MI) for culturable heterotrophic bacteria, and medium SC (Davis et al., 1990) was included to support the growth of fastidious organisms. Agar plates were incubated at 28 °C for 48 – 72 hr.

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 $\times$ *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 fatty acid analysis as outlined below.

*Strain identification*

Each strain was identified by analysis of fatty acid methyl-esters (FAMEs) of total cellular fatty acids (Sasser, 1990). Extraction of fatty acids required 24 h growth of bacterial strains at 28 °C on tryptic soy agar (Becton Dickinson Microbiology Systems, Cockeysville, Maryland). A 40 mg mass of colonial growth was transferred to individual glass tubes. Samples were first saponified with 1.0 mL saponification reagent (45 g sodium hydroxide, 150 mL methanol, 150 mL deionized water), vortexed for 10 sec, heated to 100 °C for 5 min in a boiling water bath, vortexed again and reheated to 100 °C for 25 min. Methylation of cellular fatty acids was accomplished with the addition of 2 mL methylating reagent (325 mL 6.0 *N* HCl, 275 mL methanol), vortexing and heating to 80 °C for 10 min. FAMEs were separated from the aqueous phase with the addition of 1.25 mL extraction reagent (100 mL hexane, 100 mL methyl-tert butyl ether) and tumbling for 10 min. Samples were then washed by removing the aqueous phase with a pasteur pipet and adding 3 ml base wash (10.8 g sodium hydroxide, 900 mL distilled water) and tumbling for 5 minutes. The organic phase, containing FAMEs, was then transferred to glass vials for chromatographic analysis.

FAMEs were analyzed with a Hewlett-Packard series II gas chromatograph model 5890 equipped with 25-m × 0.2 mm × 0.33 $\mu$ phenyl methyl silicone capillary column. Samples were processed with the MIS which calibrated the gas chromatograph with a commercial FAME mixture (MIDI) at the beginning of each analysis and after every ten samples. FAME peaks were named by the MIS software, and bacterial isolates were identified using the MIS 'Aerobe Library' (Version 3.7). Strains that could not be identified with a similarity index above 0.100 were considered unidentified.

## Results

Endophytic bacteria were isolated from healthy sweet corn and cotton root and stem tissues at each sampling date. Bacterial isolates were identified from each tissue source at each of these sampling dates, except for sweet corn roots and stems and cotton roots at emergence when identification was not attempted. A total of 1078 bacterial endophytes were isolated; 311 from sweet corn roots, 232 from sweet corn stems, 276 from cotton roots and 259 from cotton stems. The endophytic bacteria isolated comprised 36 genera; 31 of these were present in sweet corn and 32 were present in cotton (Table 1). Of the total isolates, 23.4% were Gram-positive and 72.2% were Gram-negative, with the Gram-negative bacteria comprising 27 of the 36 genera identified. Bacteria which were unidentifiable by the MIS software represented 4.3% of the total.

Results of bacterial identification by fatty acid analysis (Table 1) indicated that several endophytic species isolated from sweet corn and cotton were different. *Acinetobacter baumannii*, *Alcaligenes* spp., *Cellulomonas* spp., *Comamonas testosteroni* (basonym, *Pseudomonas testosteroni*), and *Erwinia carotovora* were isolated from cotton but not from sweet corn. Alternatively, *Citrobacter koseri*, *Flavimonas oryzihabitans* (basonym, *Pseudomonas oryzihabitans*), *Microbacterium* spp. and *Stenotrophomonas maltophilia* (basonym, *Xanthomonas maltophilia*) were isolated only from sweet corn. Several taxonomic groups were isolated more frequently (more than twice as frequently) from sweet corn than from cotton; these included *Burkholderia cepacia* (basonym, *Pseudomonas cepacia*), *Burkholderia gladioli* (basonym, *Pseudomonas gladioli*), *Clavibacter michiganensis*, *Curtobacterium* spp., *Enterobacter cloacae*, *Klebsiella* spp., *Kluyvera* spp. and *Pseudomonas putida*. Of the frequently isolated groups, there were none that were isolated more frequently from cotton than from sweet corn.

There also were differences in frequently isolated bacterial taxa based on tissue source. With sweet corn, the commonly isolated groups, i.e. from 5 or more sample dates, were *Burkholderia pickettii* (basonym, *Pseudomonas pickettii*) and *Enterobacter* spp. from roots, but only *Bacillus megaterium* from stems. With cotton, *Agrobacterium radiobacter*, *Burkholderia solanacearum* (basonym, *Pseudomonas solanacearum*) and *Serratia* spp. were commonly isolated from roots, while stems yielded *Bacillus megaterium* and *Bacillus pumilus* as common isolates. In general, bacteria isolated from sweet corn stems were also isolated from sweet corn roots, and vice versa, with occasional exceptions; *Aureobacterium* spp., *Ochrobactrum anthropi* and *Yersinia frederiksenii* were isolated from sweet corn stems but not roots and *Rhizobium japonicum*, *Flavimonas oryzihabitans* and *Stenotrophomonas maltophilia* were isolated from sweet corn roots but not stems. This was not so in cotton. *Acinetobacter baumannii* and *Arthrobacter* spp. were present in cotton stems but not in cotton roots. There were 14 taxonomic groups present in cotton

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

| Taxa[b] | Source and isolation date[a] | | | | Number of Isolates |
|---|---|---|---|---|---|
| | Sweet corn | | Cotton | | |
| | Root | Stem | Root | Stem | |
| *Acinetobacter baumannii* | | | | 56 | 1 |
| *Agrobacterium radiobacter* | 14 42 70 | 7 70 | 2 7 21 28 70 | 2 | 36 |
| *Alcaligenes* spp. | | | 2 7 | | 2 |
| *Arthrobacter* spp. | 42 | 7 | | 21 | 6 |
| *Aureobacterium* spp. | | 2 70 | 70 | 2 | 6 |
| *Bacillus megaterium* | 0 14 28 42 70 | 7 14 21 28 42 | 2 7 | 2 7 14 28 42 56 70 | 35 |
| *Bacillus pumilus* | 0 28 42 | 2 7 14 28 | 7 28 | 2 14 21 28 42 56 70 | 23 |
| *Bacillus subtilis* | 0 28 | 2 7 | 7 | 2 | 9 |
| *Bacillus thuringiensis* | 70 | 21 28 | 2 7 21 28 | | 11 |
| *Bacillus* spp. | 0 21 28 42 70 | 2 7 28 56 70 | 2 7 28 42 70 | 2 7 28 42 56 | 52 |
| *Burkholderia cepacia* | 0 2 7 70 | 2 7 21 | 7 | 2 7 | 50 |
| *Burkholderia gladioli* | 0 2 7 42 70 | 2 7 | 7 | | 29 |
| *Burkholderia pickettii* | 0 2 7 14 21 70 | 7 21 70 | 7 42 70 | 21 42 70 | 95 |
| *Burkholderia solanacearum* | 0 2 14 21 70 | 21 70 | 7 21 28 42 56 70 | 21 28 42 56 | 28 |
| *Cellulomonas* spp. | | | 42 56 | 28 | 4 |
| *Citrobacter koseri* | 7 | 2 | | | 6 |
| *Clavibacter michiganensis* | 0 7 70 | 2 7 21 28 70 | 2 21 | 2 | 29 |
| *Comamonas testosteroni* | | | 7 28 | | 2 |
| *Curtobacterium* spp. | 7 21 42 70 | 21 42 70 | 28 70 | 42 | 16 |
| *Enterobacter asburiae* | 0 7 14 28 42 | 7 14 28 | 7 14 28 42 | 7 | 43 |
| *Enterobacter cloacae* | 2 7 14 28 70 | 7 | 56 | | 14 |
| *Enterobacter* spp. | 0 2 7 14 28 42 70 | 0 7 14 28 | 7 14 56 | 7 14 28 | 72 |
| *Erwinia carotovora* | | | 14 | 14 | 5 |
| *Escherichia* spp. | 7 56 70 | 70 | 56 | 14 | 5 |
| *Flavimonas oryzihabitans* | 42 | | | | 1 |
| *Flavobacterium* spp. | 7 | 2 70 | 42 | | 5 |
| *Hydrogenophaga* spp | 0 28 | 7 | 28 | 28 | 3 |
| *Klebsiella* spp. | 7 28 42 | 2 14 21 56 | 7 14 | | 15 |
| *Kluyvera* spp. | 7 | 2 7 14 | 14 | | 21 |
| *Methylobacterium* spp. | 0 | 21 28 42 70 | 2 7 28 | 7 14 28 | 13 |
| *Microbacterium* spp. | 70 | 70 | | | 3 |
| *Micrococcus* spp. | 7 14 21 | 7 21 70 | 2 7 21 42 56 | 7 21 56 70 | 20 |
| *Ochrobactrum anthropi* | | 7 28 | 2 7 | | 5 |
| *Pantoea* spp. | 7 70 | 2 7 70 | 7 14 | 2 14 | 52 |
| *Phyllobacterium* spp. | 0 7 14 | 7 14 70 | 7 21 28 70 | 7 70 | 90 |
| *Pseudomonas chlororaphis* | 42 56 | 28 56 | 7 21 28 42 | 14 | 12 |
| *Pseudomonas putida* | 56 | 2 14 56 | 7 | | 10 |
| *Pseudomonas saccharophila* | 0 28 70 | 7 28 42 70 | 7 28 | 21 28 42 56 | 35 |
| *Pseudomonas* fluor. spp. | 70 | 14 28 56 | 21 70 | 2 | 10 |
| *Pseudomonas* nofluor. spp | 0 42 | 2 7 42 70 | 7 70 | 21 70 | 12 |
| *Rhizobium japonicum* | 70 | | 7 | | 3 |
| *Serratia* spp. | 7 14 28 56 | 7 14 28 56 70 | 7 21 28 42 56 70 | 21 56 | 53 |

*Table 1.* Continued

| | | | | | |
|---|---|---|---|---|---|
| *Sphingomonas paucimobilis* | 42 | 28 42 | 14 28 56 | | 6 |
| *Staphylococcus* spp. | 0 2 14 | 7 14 28 42 | 2 7 14 21 42 | 56 | 39 |
| *Stenotrophomonas maltophilia* | 56 70 | | | | 5 |
| *Variovorax paradoxus* | 14 | 28 | 7 21 28 | | 13 |
| *Xanthomonas campestris* | 42 70 | 14 | 7 | 2 | 18 |
| *Yersinia frederiksenii* | | 14 | 7 | 14 | 7 |
| Unknown[c] | 0 7 28 42 70 | 2 7 56 | 2 21 28 42 70 | 28 56 70 | 46 |

[a]Dates refer to isolation date in days after emergence, sampling occurred at 0, 2, 7, 14, 21, 28, 42, 56 and 70 days after emergence.

[b]Grouped taxa consist of the following species: *Alcaligenes piechaudii, A. xylosoxidans* subsp. *xylosoxidans, Arthrobacter crystallopoietes, A. globiformis, A. mysorens, A. nicotianae, Aureobacterium barkeri, A. saperdae, A. testaceum*; *Bacillus alvei, B. amyloliquefaciens, B. brevis, B. cereus, B. coagulans, B. laterosporus, B. lentus, B. macerans, B. pabuli, B. pasteurii, B. polymyxa, B. sphaericus*; *Cellulomonas cellulans, C. turbata*; *Clavibacter michiganensis* subsp. *insidiosus, C. michiganensis* subsp. *nebraskensis*; *Curtobacterium flaccumfaciens* subsp. *flaccumfaciens, C. flaccumfaciens* subsp. *oortii, C. flaccumfaciens* subsp. *poinsettiae, C. pusillum*; *Enterobacter cancerogenus, E. taylorae*; *Erwinia carotovora* subsp. *carotovora*; *Escherichia coli, E. vulneris*; *Flavobacterium indologenes, F. meningosepticum*; *Hydrogenophaga flava, H. pseudoflava*; *Klebsiella planticola, K. pneumoniae* subsp. *ozaenae, K. terrigena*; *Kluyvera ascorbata, K. cryocrescens*; *Methylobacterium fujisawaense, M. mesophilicum, M. radiotolerans, M. Rhodesianum, M. zatmanii*; *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. cichorii, P. coronafaciens, P. fluorescens, P. savastanoi, P. syringae*; *Pseudomonas* (nonfluorescent species) *P. marginalis, P. mendocina, P. rubrisubalbicans, P. vesicularis*; *Serratia liquefaciens, S. marcescens, S. plymuthica*; *Staphylococcus capitis* subsp. *capitis, S. capitis* subsp. *ureolyticus, S. cohnii, S. epidermidis, S. hominis, S. warneri.*

[c]Bacteria unable to be identified by MIS, 46 total.

roots but not in cotton stems, all of which were Gram-negative except for *Bacillus thuringiensis.*

## Discussion

The results demonstrate that stems and roots of healthy, field-grown sweet corn and cotton serve as a microbial habitat for a diverse bacterial endophytic microflora. In this study, 36 genera were isolated as endophytes from either sweet corn or cotton, which expands the list of known endophytic bacteria from previous studies. The crop system which has previously been investigated most for endophytic bacteria is potato where DeBoer and Copeman (1974), Hollis (1951) and Sturdy and Cole (1974) in separate studies identified 11 bacterial genera. Cotton endophytes have previously been studied by Misaghi and Donndelinger (1990). The diversity of isolated endophytes in their paper was limited to six bacterial species from a relatively small collection of sixty identified strains. Data presented from our study indicate that species diversity is greater when a larger number of isolates is identified.

Genera identified in this study which have not previously been reported as endophytes, include; *Aureobacterium barkeri* (basonym *Corynebacterium barkeri*), *A. saperdae, A. testaceum, Flavimonas oryzihabitans, Hydrogenophaga flava* (basonym *Pseudomonas flava*), *H. pseudoflava* (basonym *Pseudomonas pseudoflava*), *Microbacterium imperiale* and *Ochrobactrum anthropi.* Differences in the identity of bacterial endophytes from previous studies and this study are most likely explained by recent taxonomical restructuring (i.e. *Aureobacterium barkeri, Hydrogenophaga flava*), and by the increased level of sophistication of the identification technique used in this study. Another possible explanation is that sweet corn and cotton may support a greater diversity of endophytes than other crops.

Endophyte colonization of sweet corn and cotton tissues shown in this study suggests that internal plant niches 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 will likely lengthen as more crops are studied.

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