ITM 6002-609

IDENTIFICATION AND METABOLIC DIVERSITY OF RHIZOBACTERIA FROM BENTGRASS AND BERMUDAGRASS GREENS.

Sponsored by:

United States Golf Association, Green Section Research.

Submitted to:

Dr. Michael P. Kenna
Director, Green Section Research
United States Golf Association
P.O. Box 2227
Stillwater, OK 74076-2227

November 1, 2000

Principal Investigators:

Horace D. Skipper
Crop and Soil Environmental Science

Voice: 864-656-3525 Fax: 864-656-3443
E-Mail: Skipper@Clemson.Edu

John A. McInroy, Department of Plant Pathology, Auburn University, Auburn, AL 36849-5409

Daniel A. Kluepfel, Department of Plant Pathology & Physiology, Clemson University, Clemson, SC 29634-0377

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ANNUAL REPORT: NOVEMBER 3, 2000

The Sherlock System[1] software for bacterial identification comes with a few additional software tools to help get the most information out of a single analysis. One of these tools is the 2-D plot program[2]. The 2-D plot program is especially useful for finding relationships among large numbers of organisms. This is done by employing the statistical procedure of principal component analysis to plot results of individual analyses in a three-dimensional array (x, y, and z axes). These 3-dimensional relationships are graphically represented in 2 dimensions by printing on paper. Each bacterial strain analyzed with the Sherlock System software can be used in a 2-D plot. Each analysis of a strain or different strains is represented by a single point in the 2-D plot graph. The unit of distance between two points is Euclidean Distance (ED). In general, a cluster of points within 110 ED^2 (approximately 10.5 on the x-axis and 10.5 on the y-axis) constitutes representatives within the same species. Similarly, data points within 60 ED^2 constitute members of a single subspecies.

Approximately 900 strains, which were unidentifiable with the Sherlock System software, were analyzed with the 2-D plot software program. Although these strains remained without a taxonomic category, the analysis on the Sherlock System could still prove useful if the strains could be put into species groups and representatives of each group could be subjected to further tests to determine their identity. After analyzing these strains with the 2-D plot software, it was determined that 15 predominant clusters were present. Most of these clusters exceeded the limits for a species cluster, indicating that each cluster represented a genus or at least multiple species of a single genus. When each cluster was analyzed further, it was determined that subclusters existed within each main cluster. These subclusters are about the size of a 'species' cluster and there are approximately 38 in total. Of the 15 main clusters, 4 appear to be most similar to Gram positive bacteria and 8 appear to be most similar to Gram negative bacteria, with 3 clusters being inconclusive. Representative isolates from these clusters will be subjected to MicroLog™ System and DNA techniques for identification.

Sasser, M. 1990. Identification of bacteria by gas chromatography of cellular fatty acids. MIDI Technical Note 101. MIDI, 115 Barksdale Prof. Center, Newark, DE 19711.

Sasser, M. 1990. "Tracking" a strain using the Microbial Identification System. MIDI Technical Note 102. MIDI, 115 Barksdale Prof. Center, Newark, DE 19711.

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GOAL:

The goal is to fill a void in the identification of turf rhizobacteria generated by the FAME analysis used in our current USGA project. It is essential to determine if the approximately 500 unidentified isolates represent many species of a few genera, many genera of a few species, or perhaps entirely new, unidentified and unnamed, turf rhizobacteria.

OBJECTIVES:

1. Complete the identification of approximately 500 turf rhizobacteria by a combination of biochemical profiling and DNA techniques.

2. Determine the metabolic diversity of turf rhizobacteria by carbon substrate utilization patterns and potential for denitrification.

Research Methodology:

Over the past three years, rhizobacteria have been isolated quarterly from the roots of bentgrass or bermudagrass at newly constructed or reconstructed greens in a USGA sponsored project entitled "Bacterial Populations and Diversity within New USGA Putting Greens". The goal of this project was to identify these bacterial isolates using fatty acid methyl ester (FAME) analysis (Sherlock System™ from Microbial ID, Inc., Newark, DE; Sasser, 1990a).

Microbial identification using FAME analysis (Sherlock System™) requires pure bacterial strains to be cultured on tryptic soy agar for 24 hours at 28°C. Approximately 40 mg of bacterial cells are harvested and subjected to heat and sodium hydroxide treatment to disrupt the cell membranes. The fatty acids are saponified then methylated prior to extraction with methyl tert-butyl ether and hexane. After the fatty acid methyl esters have been collected, they are analyzed on an Hewlett-Packard 5890 gas chromatograph fitted with an ultra 2 capillary column and a flame-ionizing detector. Profiles of retention time and relative percentage of the fatty acids are then generated and compared to a database of known species to identify each strain. The resulting identification data has been used to monitor microbial shifts over the duration of the study in SC, NC, FL, and

AL. With three years of data nearly complete, approximately 5,000 strains have been analyzed. However, approximately 500 of these isolates remain unidentified using the Microbial ID/Sherlock System™.

The presence of unidentified strains skews the data analysis since it is not known whether these unidentified strains represent one or more taxonomic groups. Alternatively, removing the unidentified strains would result in unbalanced treatments. Ideally, these unidentified strains should be subjected to alternative identification schemes to help clarify their taxonomic position. If these 500 unidentified isolates represent 1 to 3 species, they could represent the dominant rhizobacteria rather than the *Pseudomonas* and *Bacillus* species as identified in the current quarterly profiles. We therefore propose to use two alternative means to identify the problem strains that are not catalogued in the Sherlock System™.

It would be expensive and time consuming to consider identifying each of the 500 unidentified strains using a traditional biochemical or genetic approach. Instead, we propose to reduce the number of unknown isolates to a more manageable number through the use of cluster analysis of the fatty acid retention time profiles already generated (Romesburg, 1990). Cluster analysis is a statistical procedure that takes raw data, such as fatty acid retention time profiles, and categorizes the data based on similarity (or dissimilarity). Through the use of cluster analysis of our unidentified bacteria, we can determine which isolates in the group of 500 are most related to each other. Based on preliminary indications using cluster analysis on the existing data, the 500 unknown isolates appear to cluster into 20 to 30 different groups (Sherlock System™; Sasser, 1990b). Since members of each resulting cluster are closely related, and expected to represent a single species, we propose to take two representative strains from each cluster and subject them to the more detailed identification schemes presented here.

Currently there exists three powerful approaches/tools to speciate bacterial strains isolated from the environment: a) extraction and analysis of fatty acids, b) carbon source utilization patterns, and finally, c) the cloning and sequencing of 16S rRNA genes. We propose to use the MicroLog™ System and the MicroSeq System for rRNA sequence analysis.

The MicroLog™ System (Biolog, Hayward, CA) is one of the most versatile bacterial identification systems due to its extensive database. The MicroLog™ System relies on the principles of classical taxonomy in its use of various carbon sources as indicators of which enzymes are present in the strain tested. To analyze a pure bacterial culture with the MicroLog™ System, an individual strain is cultured on solid growth medium, harvested and suspended in a saline solution. This inoculum is used to fill the wells of a MicroPlate™ - commercially available 96-well ELISA plates. One well of the MicroPlate™ is a negative control while the others contain either a carbohydrate or an amino acid in powdered form in

addition to a tetrazolium dye indicator. As the wells are hydrated with the bacterial suspension, enzyme activity is registered by the breakdown of the carbon source, resulting in color variation from clear to dark purple. These color variations are read with an ELISA-plate reader and used to generate a 'fingerprint' for the sample. The sole carbon-source utilization 'fingerprints' are then compared against a database of 'known' profiles for identification.

The number of profiles in the MicroLog™ System database currently exceeds the number in the Sherlock System™ database which allows for a greater chance of identification. If two representative isolates from each of the anticipated 20 to 30 clusters are chosen for further characterization using the MicroLog™ System, then 40 to 60 strains would make up our focus group. Although it is likely that many of the unknown clusters will be identified with carbohydrate utilization profiling, it is proposed that 16S rRNA gene sequence analysis be used for confirmation and identification of the remaining unknowns. The use of rRNA sequence data to determine the phylogeny of microbial life has been well documented and will be used in this project to complement both fatty acid and MicroLog™ System analysis (Amann et al., 1995; Pace, et al., 1996; Van Damme, et al., 1996).

There are several reasons for relying on rRNA gene sequence data for identification and phylogenetic relatedness. First, rRNA is the key element of the protein synthesizing machinery of all organisms and as a result, is extremely conserved in overall structure. This structural conservation extends to the DNA sequence of the rRNA genes where the typical 16S rRNA genes contain regions of highly conserved sequence and regions that have been termed, hypervariable regions. Both of these regions are very useful in the identification and phylogenetic placement of microorganisms in the living world. The rRNA also constitutes a significant component of the cellular biomass, are easily recovered, and are free from artifacts of lateral transfer between phylogenetically distant organisms. Combined, all these features make rRNA genes uniquely suited for establishing phylogenetic relationships in the microbial world.

In addition to the use of the previously mentioned GC-FAME and MicroLog™ System analysis, we will incorporate the use of 16S rRNA DNA sequence data to identify and speciate the rhizosphere isolates that remain unidentified after fatty acid and MicroLog™ System analysis. Initially we will isolate and sequence approximately 500 base pairs (bp) of the more conserved region of the 16S rRNA gene. Sequence data from this region of the gene has been shown to be effective in speciating more than 70% of the unknown bacterial isolates. The entire 16S rRNA gene (approx. 1,600 bp) will be sequenced for those isolates for which 500 bp of sequence data is insufficient for identification. We

anticipate that sequencing of the entire 16S rRNA gene will only be required for three to four of the unknown isolates.

The 20 to 25 bacterial isolates selected for 16S rRNA gene analysis will be grown in trypticase soy broth agar. Using previously published procedures, genomic DNA will be isolated and purified from each strain (Amann et al., 1995; Pace, et al., 1996; Van Damme, et al., 1996). Using primers specific for eubacteria the entire 16S rRNA gene (1,600 base pairs) will be amplified from the genomic DNA isolated above. The amplified product will be purified from excess primers and dNTPs using Microcon 100 (Amicon) membrance filtration. DNA sequencing of the 16S rRNA gene will be carried out using AmpliTaq FS DNA polymerase and cRhodamine dye terminators. The sample will be analyzed on an ABI Prism 377 DNA or Nicor sequencer in the Clemson University central sequencing facility. DNA sequence analysis will be performed using PE/Applied Biosystems MicroSeq microbial identification/analysis software and databases. The initial sequence analysis will cover both strands of the first 500 base pairs of the 16S rRNA gene. If warranted, the entire gene, approximately 1,600 base pairs) will be sequenced and analyzed as described above.

The 16S rRNA gene sequence from the unknown isolated will be compared to the PE Applied Biosystem MicroSeq database. The top 10 alignment matches will be determined by calculating the percent difference between two aligned sequences taking into account any mismatched base pairs, gaps, and IUB ambiguity codes. Using these top ten alignment matches, a nearest neighbor and/or UPGMA plylogenetic tree will be generated for each of the unknown isolates examined. In addition, we will perform the same statistical analysis (nearest neighbor.UPGA) on the 20 to 25 unknown isolates to obtain a quantitative estimate of relatedness of the unknown isolates.

Once the sequencing and initial identification has been completed, a cluster analysis will be performed to examine the relatedness of the isolates for which we have DNA sequence data. This will reveal, not only the relationship or association that may be present between specific isolates, but it will shed light on the bacterial isolate relationship to such ecological factors as: the plant species it was isolated from, disease suppressiveness of the soil site, plant growth promoting abilities, and drought tolerance.

In addition to identification of isolates, the MicroLog™ System analysis will help to define the metabolic diversity of rhizobacteria from bentgrass and bermudagrass. This data is essential with respect to biodegradation of thatch, root exudates, and pesticides. To further characterize the turf rhizobacteria, we will select 200 isolates from the major clusters and evaluate them for their denitrification potential. Denitrifiers are key microorganisms in the fate of nitrogen in the turfgrass ecosystem.

Isolates will be evaluated for denitrification as described by Tiedje (1994). Aliquots of rhizobacteria grown in nutrient broth to approximately 10^6 CFU/mL will be added to tubes containing nutrient or nitrate broth and incubated at 28^0C for 14 days. Nitrate powder and zinc dust will be used to test for NO_3 and NO_2 and tubes inspected for gas bubbles as presumptive test for denitrification. A confirmatory test via GC analysis for N_2O is not planned for the 200 isolates due to cost. As the numbers are reduced to 25 to 30 via identification protocols described above, a confirmatory test will be pursued.

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