

ISOLATION AND IDENTIFICATION OF BACTERIA IN EASTERN OREGON AGRICULTURAL SOILS

Stephanie A. Boyle and Stephan L. Albrecht

Abstract

Because of their high occurrence and large diversity in the soil, the types of bacteria living in particular crop fields may have a significant impact on the quality of that soil and on the productivity of a crop. Isolating and identify soil bacteria is therefore important, but not always easy. Visual observations, under a microscope, of individual bacteria or their colony profiles are not always accurate or reliable. However, bacteria can be readily identified by their metabolic activity. A system, used in conjunction with a differential staining technique (Gram stain¹), has been developed by BIOLOG® Inc. that tests a bacterium's ability to use different carbon compounds and identifies them based on utilization patterns. This method was used on 42 strains isolated from the CBARC long-term plots in Pendleton, OR. Sixteen strains were positively identified. Many of the strains were Gram-positive bacteria suggesting this type of bacteria makes up the majority of the culturable populations residing in the plots investigated. This observation is in contrast to many agricultural soils that are inhabited by predominately Gram-negative bacteria. The bacterium *Bacillus subtilis* was one of the species identified. This microorganism

is reported to suppress disease-causing organisms in wheat crops.

Key Words

Long-term experiments, wheat, bacteria, gram stain, carbon substrate utilization

Introduction

Microbiologists currently estimate that only one percent of soil microorganisms have been observed (Sylvia et al. 1998). This small fraction points to the substantial diversity present in terrestrial habitats and the difficulties associated with identifying microorganisms, such as bacteria. Garland and Mills (1991) cite two major obstacles in the characterization of bacteria: small individual size and morphological similarity. Unlike plant and animal species that can typically be identified through careful visual observation, bacteria are not so easily distinguished. For example, two bacterial cells may look identical under a microscope, while further testing reveals the first capable of transforming nitrogen and the second of excreting a hormone that stimulates plant growth. Therefore, researchers must look beyond morphology to accurately differentiate bacterial species from one another.

Microbiologists have developed a variety of strategies to accurately identify bacterial species. Traditional identification requires extensive testing that includes staining and the inoculation of selective and determinant media (Benson 1998). Recently, a variety of

¹ Gram staining refers to bacterial smears treated with a series of solutions: crystal violet, Gram's iodine, isopropyl alcohol, and safranin. Because of the difference in cell wall compositions, these solutions react to stain the cells blue in the case of Gram-positive (GP) strains and red when the unknown is Gram-negative (GN). Gram staining allows bacteria to be quickly separated into groups, thus aiding in the identification (Benson 1998).

molecular identification procedures have been developed. These methods include the amplification of genetic material and—in some cases—the use of probes to detect targeted sequences. Whether traditional or cutting-edge, all of these techniques have unique problems associated with them. Some require large investments of time, while others depend on expensive tools. An alternative to these identification techniques, developed by BIOLOG[®] Inc. (Hayward, CA), uses microtiter plates² filled with a variety of sugars, each of which maybe a source of energy for microorganisms. When the added bacteria are able to use a particular carbon source, an oxidation-reduction dye changes the well from clear to purple. A reacted microtiter plate will contain a variety of clear and purple wells, and this pattern can then be compared against a known database. The BIOLOG[®] system was chosen for its relatively inexpensive cost and its ability to accurately identify organisms with minimal laboratory testing.

Testing an unknown strain in this system may yield several results. First, the bacterium can be positively identified if the utilization pattern matches a species included in the BIOLOG[®] software. If a resulting pattern does not match, a list of 10 similar patterns may indicate a species closely related to the unknown strain. At the very least, information on the metabolic potential of an unknown strain may limit the number of additional determinative tests that will be needed. The role of one bacterium in the soil community can sometimes be revealed without a species identification, simply by examining those carbon sources that the strain is able to use.

² Microtiter plate: 12.5 x 8.5 x 1.7 cm clear plastic tray containing 96 cylindrical wells. Each well is 1 cm in diameter, and wells are labeled 1-12 along the length of the tray and A-H along the width.

Biodiversity and microbial community structure are central to any agroecosystem. As management systems continue to evolve, they undoubtedly affect biodiversity, microbial community structure, and the important processes they control, such as soil quality and nutrient cycling. These processes in the dryland farming areas of the Pacific Northwest (PNW) are critical for agricultural stability and productivity. Because agroecosystems comprise a substantial amount of all ecosystems in the PNW, agriculture may be the most important ecosystem in preserving and maintaining biodiversity. Yet it is unclear how agricultural management practices affect biodiversity and ecosystem structure.

The goal of this research was to isolate several bacterial strains from soil collected in the Columbia Basin Agricultural Research Center (CBARC) long-term plots, identify these strains, and develop a database of bacteria present in eastern Oregon agricultural soils. After bacteria were successfully identified, further literature searches were conducted to uncover their possible functions. The identification of local bacteria could provide important insights into the transformation of carbon, nitrogen, sulfur, and other nutrients. Additional long-term benefits of this research could include, discovering a biological control for soil-borne pathogens or detecting a beneficial strain that encourages plant growth.

Material and Methods

Soil was collected during summer 2000 and 2001, from the CBARC long-term plots (Rasmussen and Smiley 1994). Five long-term plots were sampled, including a grass pasture (GP), conventionally-tilled annual winter wheat (AWCT), no-till winter wheat (NT), wheat/pea (WP), and two treatments

in a residue management experiment (CR). Both residue management treatments were in a wheat-fallow rotation, one with manure (CRM) and the other with no fertilizer and burned in September (CRFB). Using sterile collection techniques, cylindrical cores 2 cm in diameter were collected to a depth of 10 cm from four random locations.

Using dilution/plating methods, bacterial colonies were grown from composite soil samples. According to the procedure outlined in Holt (1984), sterile water was used to make dilutions of 1:10,000, 1:100,000 and 1:1,000,000. These dilutions were spread on nutrient agar plates and incubated at 30° C for 48 hours. Bacteria from well-defined colonies were then transferred to fresh plates using a sterile needle. An isolate was considered to be a pure strain after three consecutive transfers without evidence of other microorganisms, and purity was verified by Gram staining and examination of cells at 1000x magnification. Cellular morphology was also examined at this time; most isolates could be characterized as either rod or coccus. The term rod denotes cells appearing as ovals either individually or in chains of five to ten. Coccus shape cells appear round and usually occur unconnected. If an isolate was Gram positive, then an additional stain, performed after 72 hours, tested for the presence of endospores (Benson 1998).

After collection of preliminary data, each isolate was tested in the BIOLOG® system. Isolates were streaked on Biolog Universal Growth (BUG™) agar (57 g/L) and grown for 24 hours at 30° C. A cell suspension was then created by transferring growth from the plates into tubes of inoculating fluid. To ensure an even distribution of cells throughout the suspension, tubes were gently shaken. Prior to the addition of cells,

100 microliters of 7 percent sodium thioglycolate solution was added to inoculating fluid, decreasing clumping. In some cases, clumping occurred in spite of thioglycolate addition. These problematic strains were grown on 25 percent BUG agar (13 g/ L), before creating a cell suspension. Microtiter plates were inoculated with uniform suspensions.

The pattern of carbon substrate utilization was observed at 4 to 6 hours and 24 hours. Any reading from the top left well (A-1) was subtracted from values on the other 95 wells, because it did not contain a substrate. Individual wells were characterized as positive, negative, or borderline, and patterns were compared against those of known bacteria. Statistical tests, built into the software, established the validity of any identification. A microtiter plate inoculated and incubated for 24 hours is pictured (Fig. 1).

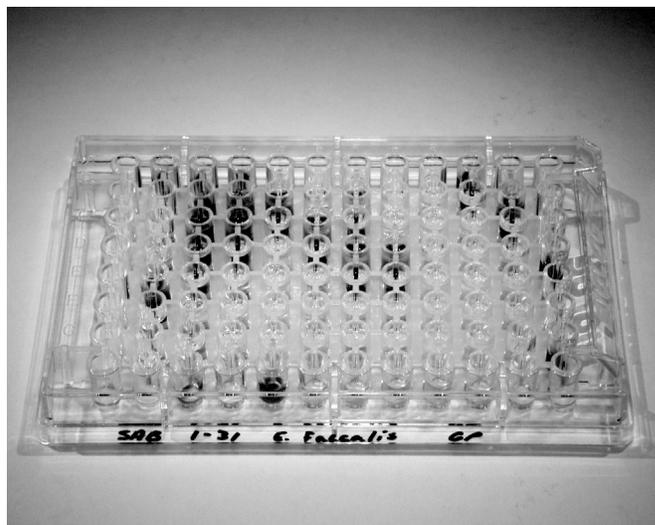


Figure 1. BIOLOG® microtiter plate inoculated with a bacterial strain and incubated to produce a carbon substrate utilization pattern, Columbia Basin Agricultural Research Center, 2000, 2001.

Results

A total of 42 strains were isolated, purified, and assigned lab identifications. Nine of these isolates were GN and the remaining 33 were GP. Seven GN and nine GP strains were positively identified (Table 1).

Most of the GP strains were characterized as rods, including 17 strains capable of forming

endospores. Taxonomically, only three genera contain GP spore-forming rods: *Bacillus*, *Clostridium*, and *Sporolactobacillus*. The possible identity of unknown strains could be further narrowed to only *Bacillus* strains, given that members of the genus *Clostridium* are anaerobic and the genus *Sporolactobacillus* contains only one species. The six positively identified isolates are listed in Table 1.

Table 1. Bacteria identified using BIOLOG[®] system, Columbia Basin Agricultural Research Center, 2000, 2001.

Bacterial type ¹	Sample location ²	Genus	Species
GP, SF	AWCT	<i>Bacillus</i> †	<i>amyloliquefaciens</i>
GP, SF	CRFB	<i>Bacillus</i> †	<i>halodurans</i>
GP, SF	CRFB	<i>Bacillus</i>	<i>subtilis</i>
GP, SF	CRFB	<i>Bacillus</i>	<i>circulans</i>
GP, SF	CRM	<i>Bacillus</i>	<i>maroccanus</i>
GP, SF	WPA	<i>Bacillus</i>	<i>subtilis</i>
GP	GP	<i>Aureobacterium</i>	<i>testaceum</i>
GP	CRM	<i>Staphylococcus</i>	<i>lentus</i>
GP	WP	<i>Arthrobacter</i>	<i>ilicis</i>
GN	GP	<i>Acinetobacter</i>	<i>genospecies 13</i>
GN	GP	<i>Chryseobacterium</i>	<i>scophthalmum</i>
GN	NT	<i>Alcaligenes</i>	<i>denitrificans</i>
GN	AWCT	<i>Pseudomonas</i>	<i>fluorescens</i> <i>biotype G</i>
GN	AWCT	<i>Listonella</i>	<i>pelagia</i>
GN	CRFB	<i>Burkholderia</i>	<i>glumae</i>
GN	CRM	<i>Stentrophomonas</i>	<i>maltophilia</i>

¹Identifications were made using standard techniques, except if marked (†). Bacterial types include Gram positive spore-formers (GP, SF), Gram positive non-spore-formers (GP), and Gram negative (GN). ²Sample locations are as follows: conventional-till annual wheat (AWCT), crop residue fall burned (CRFB), crop residue manure (CRM), wheat/pea (WP), grass pasture (GP), and no-tilled wheat (NT).

Two of the remaining GP strains were cocci and 14 were rods that did not form spores. One coccus was identified as *Curtobacterium*; however, the species could not be determined at this time. The other coccus produced a clear utilization pattern, but did not match any known organism. Because of clumping and false positives in the control wells, only three rods were positively identified (Table 1). Four of the non-spore-forming GP rods were particularly difficult to evaluate. They formed long chains of cells, and when observed at 1000x magnification, these chains appeared intertwined. These strains also had a tendency to be Gram-variable in cultures older than 48 hours. All clumped when added to inoculating fluid, never yielding a uniform suspension or positive identification.

Nine isolates were determined to be GN. All of these isolates were grown on BUG agar with added maltose (2.5 g/L); when added to inoculating fluid, they all formed uniform cell suspensions. Additionally, these isolates yielded clear patterns of utilization after 24 hours. Table 1 lists seven strains that produced patterns closely matching those of known species.

Discussion

The majority of bacteria isolated from CBARC long-term plots were GP, and approximately half of the GP strains had spore-forming capabilities. If these results are representative, it may mean that local soils are composed primarily of GP bacteria, some of which form endospores. Strains capable of forming spores may remain inactive in the soil during stressful times of year, including periods of drought or cold weather, and return to an active state during periods with more favorable conditions. The isolation techniques employed in this

particular study may have inadvertently selected for spore-forming organisms, however, meaning that the ratio of GP to GN bacteria in the soil is still unknown. The isolated spore-formers may be culturable, while other types of bacteria, perhaps present in larger numbers, do not grow on nutrient agar. These spore-forming strains may also overwhelm other culturable strains when placed on nutrient-rich media.

Although this study did not conclusively determine the relative population size of *Bacillus* species, the identified strains indicated the presence of potentially beneficial bacteria in eastern Oregon soils. *Bacillus subtilis*, a relatively common soil bacterium, was isolated and identified from two different long-term treatments. One greenhouse study suggested a decrease in the wheat pathogen, take-all, and an increase in seedling growth when *B. subtilis* was added to soil (Ryder et al. 1999). *B. subtilis* is present in local soils, although the population size and contribution is not known. The confirmed presence of *Bacillus circulans* may also be of interest, because some strains are capable of weakly degrading cellulose (Holt 1984) and may play a role in the breakdown of residue.

Those GP strains that proved hard to culture may be actinomycetes. The order *Actinomycetales*, composed of bacteria capable of producing long branching hyphae similar to fungi, has been previously studied in soils and is known to be Gram variable. Some laboratory strains resembled actinomycetes in cellular configuration and even relatively young cultures were Gram variable. It may be possible to identify these strains in a future study by using a microtiter plate designed specifically for actinomycetes and filamentous fungi.

The identification of *Arthrobacter ilicis* demonstrated both the wide variety of genera that could be present in eastern Oregon soils and problems associated with accurately identifying bacterial strains. Members of the genus *Arthrobacter* have previously been found in soils and may compose a significant fraction of the aerobic bacterial population, but *A. ilicis* is described as a blight-causing organism in American holly (Holt 1984). Although the unknown strain had a utilization pattern similar to this known species and registered a positive identification, it is possible that the unknown strain was not *A. ilicis*. The unknown strain may resemble *A. ilicis*, but was not included in the current BIOLOG[®] database. Further testing is required to confirm identification.

Gram-negative strains proved easier to identify than the GP isolates, and results showed a potentially diverse soil population. Most of the GN strains could be identified because of low levels of clumping and clear utilization patterns. All genus level identifications were unequivocal, given current information on the habitats and morphological characteristics of known species. Various members of the genera *Acinetobacter*, *Chryseobacterium*, *Alcaligenes*, *Pseudomonas*, *Listonella*, *Burkholderia*, and *Stentrophomonas* have previously been found and studied in agricultural soils. *A. denitrificans* has previously been identified as a soil bacteria capable of reducing nitrate and nitrite in terrestrial ecosystems (Holt 1984).

The initial soil bacteria database suggested eastern Oregon agricultural soils support a diverse population of bacteria. It remains to be discovered, however, if Pacific Northwest cropping systems encourage the growth of certain bacterial populations. Given the abundance of bacteria in agricultural soils and their vital functions, it

is important to determine what impact particular crops and cropping systems have on the structure of local communities. Identifying a small fraction of these bacteria proved to be a useful starting point, but more extensive research should be conducted before a clear bacteriological profile of our local soils emerges.

Summary

The isolation and identification of isolates indicated a wide variety of soil bacteria present in the CBARC long-term experiments. The BIOLOG[®] system proved useful in the identification of Gram-negative isolates, but less successful in the determination of Gram-positive strains. Some minor modifications to the identification procedure yielded limited success, but did not positively identify strains that continually clumped in suspension. Thirty-three of the 42 isolated strains were Gram-positive, suggesting the possible dominance of Gram-positive bacteria in local soils. Sixteen of the 42 were positively identified, including *Bacillus subtilis*, a common soil bacterium with potential benefits to Pacific Northwest wheat.

Acknowledgements

We thank Katherine Skirvin for technical assistance and helpful discussions, and Karl Rhinhart, Richard Smiley, and Steve Petrie for operational support of the long-term plots.

References

Alexander, D.B. 1998. Bacteria and archaea. Pages 44-71 in D.M. Sylvia, J.J. Fuhrmann, P.G. Hartel, and D.A. Zuberer (editors). Principles and Applications of Soil Microbiology. Prentice-Hall, Upper Saddle River, NJ.

Benson, H.J. 1998. Microbiological Applications: Laboratory Manual in General Microbiology. WCB McGraw-Hill, Boston.

Garland, J.L., and A. Mills. 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole carbon-source utilization. *Applied and Environmental Microbiology* 57:2351-2359.

Holt, J.G, editor. 1984. Bergey's Manual of Systematic Bacteriology. Vol. 1. Williams and Wilkins, Baltimore.

Rasmussen, P.E., and R.W. Smiley. 1994. Long-term experiments at the Pendleton

Agricultural Research Center. 1994 Columbia Basin Agricultural Research Annual Report 933:14-20.

Ryder, M.H., Z. Yan, T. Terrace, A. Rovira, W. Tang, and R. Correll. 1999. Use of strains of *Bacillus* isolated in China to suppress take-all and rhizoctonia root rot, and promote seedling growth of glasshouse-grown wheat in Australian soils. *Soil Biology and Biochemistry* 31:19-29.

Sylvia, D.M., J.J. Fuhrmann, P.G. Hartel, and D.A. Zuberer (editors). Principles and Applications of Soil Microbiology. Prentice-Hall, Upper Saddle River, NJ.