Persistence & Diversity of Rhizobial Bacteria Nodulating Alfalfa

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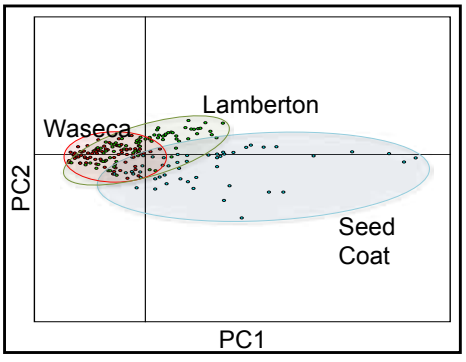
Most alfalfa seed is treated with an inoculant consisting of several strains of the nitrogen fixing bacterium *Sinorhizobium meliloti* to enhance nodulation of seedlings. However, little is known about the persistence of these strains later in the season. There is also little information on diversity of *S. meliloti* strains naturally occurring in the U.S., their capacity for nitrogen fixation, and competitive ability with strains introduced on seed. One strategy for increasing alfalfa forage yields, particularly in less fertile areas, is selection and use of highly competitive and efficient nitrogen fixing strains of rhizobia in the field. In other crops, inoculation of plant growth promoting bacteria (biologics) onto seeds has been shown to promote disease resistance and enhance growth. The objectives of this study are to characterize rhizobial strains nodulating alfalfa in two locations in Minnesota, identify bacteria associated with alfalfa nodules, and determine their capacity for promoting plant growth.

Alfalfa plants obtain nitrogen from the atmosphere by symbiotic nitrogen fixation and also obtain nitrogen from the soil. During the establishment year, plants obtain 40-60% of their nitrogen needs from soil nitrogen and the remainder from nitrogen fixation. Over the life of the stand, plants become more reliant on nitrogen fixation as soil nitrogen is depleted. The symbiotic bacteria do not rely on a host for survival but are found as free-living organisms in soil. However, in some soils the bacteria can decline to very low populations that are insufficient for adequate nodulation of the crop.

We evaluated diversity of rhizobial bacteria and nodule-associated bacteria in alfalfa plots established in 2013 at the University of Minnesota Long Term Agricultural Research Network sites in Waseca and Lamberton, MN. Alfalfa had not been grown at either site for over 30 years, although it was present in nearby fields. At both sites, the alfalfa cultivar DKA44-16RR pretreated with a commercial rhizobial seed coating was established in 2014 and 2015. At each site, eight soil cores were removed from each alfalfa plot and 48 nodules were collected from the combined eight cores (total of 768 nodules). Nodules were surface sterilized, crushed to release bacteria, and bacteria cultured on an agar nutrient medium. Bacteria were also isolated from nodules developing from the same seed grown in sterile vermiculite in a growth chamber. PCR-based assays and DNA sequencing confirmed the isolated bacteria were *Sinorhizobium*. A total of 562 pure strains were recovered from field-grown plants and 54 from inoculated seed. A subset of 158 field strains was used for DNA fingerprinting to assess genetic diversity of the bacteria.

Analysis of the DNA fingerprints showed bacteria from each location formed distinct populations (Figure 1). There were no differences in bacterial populations from 6-month-old and 18-month-old plants at either location but field isolates were significantly different from seed-derived strains. Populations at each site were highly diverse, with greater diversity at the Lamberton site. These results indicate indigenous populations of *S. meliloti* remained high in the soil at each site in the absence of alfalfa and out-competed strains introduced on seeds on mature plants.

Figure 1. Analysis of diversity of rhizobial strains isolated from field grown plants in Waseca and Lamberton compared to isolates from seed coat inoculant. Bacteria isolated from field grown plants are distinct from bacteria isolated from the seed coat inoculant.



Recent DNA sequencing of the genomes of 48 strains of *S. meliloti* by University of Minnesota scientists identified specific genes involved in bacterial-plant interactions may increase competitiveness for nodulating alfalfa. Approximately 33% of the field strains we isolated had these genes. We are currently evaluating whether they confer a competitive advantage in the field strains. Sequencing also revealed genetic diversity in *S. meliloti* occurs

in the chromosome as well as in small circular pieces of DNA called plasmids, not required for cell growth. The plasmids contain genes for a wide variety of functions useful for adapting to changes in environmental conditions, including genes able to increase nodulation. Not all bacteria contain the same plasmids, but these can be transferred among *S. meliloti* bacteria to spread useful plasmids when they are needed. This leads to the fascinating concept that the plasmids enable bacteria to access information for survival at the population level that otherwise could not be contained by a single bacterial cell. Research on bacterial genes may allow scientists to develop rhizobial strains with strong competitive ability and high rates of nodulation and nitrogen fixation.

In addition to the bacteria within nodules, we isolated the bacteria colonizing the outside of alfalfa root nodules. A total of 380 bacterial strains were cultured from the outer nodule surface and were identified to belong to seven classes of bacteria. Representative isolates from each location were tested for plant growth promoting activities in laboratory assays, including production of plant hormones, solubilization of phosphate, iron capture (chelation), and inhibition of root rot or crown rot pathogens (Figure 2). Almost all bacteria had at least one plant growth promoting activity with 40% having four. About 50% could solubilize phosphate and 65% could produce the hormone indole acetic acid, which is involved in root and shoot growth. Interestingly, ~70% had activity against one pathogen and 18% had activity against two. None of the isolates appeared to inhibit growth of *S. meliloti* in lab assays. Isolates with multiple growth promoting activities will be tested for enhancing nodulation, plant biomass, and disease resistance. Results suggest most of the bacteria associated with alfalfa root nodules are beneficial to plant growth.

Figure 2. Antifungal activity of nodule-associated bacteria in agar plate assays. The fungus was placed in the center of the plate with three dots of bacteria near the edge of the plate. The bacterial strain in panel A has no inhibitory activity while that in panel B shows moderate activity and the strain in panel C has strong activity.