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Soybeans inoculated with root zone soils of Canadian native legumes harbour diverse and novel *Bradyrhizobium* spp. that possess agricultural potential

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ABSTRACT

An assessment was made of the evolutionary relationships of soybean nodulating bacteria associated with legumes native to eastern Canada to identify potential new sources of soybean inoculant strains.

Short season soybeans were used to selectively trap bacteria from root zone soils of four native legume species. Screening of more than 800 bacterial isolates from soybean root nodules by analysis of *rec*A gene sequences followed by analyses of selected genotypes using six core and two symbiosis (*nod*C and *nif*H) gene sequences permitted identification of diverse taxa that included eight novel and four named *Bradyrhizobium* species as well as lineages attributed to the genera *Afipia* and *Tardiphaga*.

Plant tests showed that symbionts related to four named species as well as a novel *Bradyrhizobium* lineage were highly efficient with regard to nitrogen fixation on soybeans relative to an inoculant strain.

A new symbiovar (sv. septentrionalis) is proposed based on a group of four novel *Bradyrhizobium* spp. that possess distinctive *nod*C and *nif*H gene sequences and symbiotic characteristics.

Evidence is provided for horizontal transfer of sv. septentrionalis symbiosis genes between novel *Bradyrhizobium* spp., a process that rendered recipient bacteria ineffective on soybeans.

Diverse lineages of non-symbiotic and symbiotic *Bradyrhizobium* spp. co-occured within monophyletic clusters in a phylogenetic tree of concatenated core genes, suggesting that loss and/or gain of symbiosis genes has occurred in the evolutionary history of the bacterial genus.

Our data suggest that symbiont populations associated with legumes native to eastern Canada harbour elite strains of *Bradyrhizobium* for soybean inoculation.

Introduction

The root nodule bacteria (collectively known as rhizobia) include economically important soil bacteria that fix atmospheric nitrogen in symbiotic association with crop legumes and thereby minimize the requirement for nitrogen fertilizer inputs in agriculture. Natural populations of rhizobia represent an important source of genetic diversity that may be exploited by selecting bacteria with desirable symbiotic characteristics for use in inoculants intended to optimize legume crop productivity.

Root nodule bacteria belonging to the genus *Bradyrhizobium* have the ability to form a nitrogen fixing symbiosis with soybean (*Glycine max*), an economically important grain legume and major source of oil and protein. Soybean cultivation in Canada is currently at its most northern limit. Although the development and use of early-maturing soybean cultivars has permitted expansion of the range of soybean cultivation, short seasons and sub-optimal growing conditions remain important constraints to symbiosis and crop productivity [21]. Most strains of *Bradyrhizobium* used in inoculants for soybeans originate from the soybean growing areas of Asia and the United States [8,23] and may not possess traits for optimal short-season symbiosis.

Diverse legumes native to east North America are associated with populations of symbiotic bradyrhizobia [10,12–14]. Several of these native legumes occur in eastern Canada and their bacterial symbionts are adapted to regional conditions including short-seasons.

In a previous study [23] we obtained 148 bacterial isolates from root zone (RZ) soils of two legume species native to eastern Canada using soybean cultivars as trap hosts. On the basis of preliminary analyses using two housekeeping (core) gene sequences, these bac-

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terial isolates were classified as either *B. japonicum* or a novel *Bradyrhizobium* lineage.

Our purpose was to extend this work by carrying out a detailed assessment of the diversity, evolutionary relationships and symbiotic characteristics of indigenous bacterial symbionts that elicit nodules on roots of short season soybeans with a view to identifying potential new sources of elite inoculant strains. This was achieved by using short season soybean cultivars to selectively trap root nodule bacteria from RZ soils of four native legume species (*Amphicarpaea bracteata*, *Desmodium canadense*, *Desmodium glutinosum* and *Apios americana*) growing at different sites in eastern Canada. Characterization of bacterial isolates from soybean nodules was achieved by using multiple locus sequence analysis (MLSA) of core (16SrRNA, *atp*D, *gln*II, *rec*A, *gyr*B and *rpo*B) and symbiosis (*nod*C and *nif* H) gene sequences. Genealogies were inferred using Bayesian [1] and maximum-likelihood [6] algorithms.

Materials and methods

Site description, soil sampling and bacterial isolation

Thirty root zone (RZ) soil samples were collected with aseptic precautions (10 cm depth) from each of four native legumes at five sites (S1 through S5) in the province of Québec during 2007: *A. bracteata* (S1), *D. canadense* (S2), *D. glutinosum* (S3), *A. bracteata* (S4) and *A. americana* (S5). These sites were separated by distances of up to 500 km and were located in, or adjacent to, natural woodland that had no known history of agriculture. Site descriptions including coordinates and soil characteristics are given in Supplementary Table S1.

RZ soil samples from each site were pooled to form a composite and maintained at 4 °C before use. Isolation of bacteria from composite soil samples was done within 10 days of sampling using short season soybeans AC Glengarry and AC Orford as trap plants. Soil suspensions (fivefold dilutions in water) representing each native legume and site combination were thoroughly mixed and inoculated onto soybean seedlings from surface sterilized seed planted in Leonard jars [26] containing vermiculite and supplied with nitrogen free nutrient solution [3]; negative controls consisted of uninoculated plants. Soybeans were grown for 35 days in a controlled environment cabinet at 25 °C (16 h day), 16 °C (8 h night).

Bacteria were isolated from surface sterilized nodules taken at random from roots of each soybean cultivar and inoculation treatment in seven replicate Leonard jars (two plants per jar). Bacteria were grown at 28 °C on modified yeast-extract mannitol (YEM) agar medium [23] and purified by repeated streaking and single colony picking. Pure bacterial cultures were maintained at −80 °C in 20% w/v glycerol.

Further details of the procedures used for plant growth, bacterial isolation and culture are given in our previous study [23].

Bacteria

The 819 bacterial isolates used in this study are listed in Supplementary Table S2. All isolates were slow growing (colony diameter ≤1 mm after 7–21 days at 28 °C) on modified YEM agar medium [23]. Reference taxa included type strains of named *Bradyrhizobium* species as well as species of closely related bacterial genera (Supplementary Table S3).

Nucleotide sequencing

Nucleotide sequences of six core genes (16S rRNA, *atp*D, *gln*II, *rec*A, *gyr*B and *rpo*B) were generated. These genes were chosen based on their use in phylogenetic studies of the genus *Bradyrhizobium* [11,18,23] and availability of nucleotide sequences in Public databases. To assess evolutionary relationships based on symbiosis genes, partial sequences of nodulation (*nod*C) and nitrogen fixation (*nif*H) genes were generated. Preparation of genomic DNA, primers and conditions for amplification, nucleotide sequencing and sequence editing was as described by Tang et al. [23] (*atp*D, *gln*II, *gyr*B, *rec*A, *rpo*B, *nod*C and *nif*H genes) and Yu et al. [29] (16S rRNA gene). Genbank accession numbers of the nucleotide sequences used in this study are shown in Supplementary Tables S2–S4.

Phylogenetic analysis

Best fit substitution models were selected using the Bayesian information criterion implemented in jModelTest version 2 [4]. Bayesian phylogenetic analyses were carried out using MrBayes version 3.2.1 with default priors [1] as previously described [29].

Maximum Likelihood (ML) phylogenetic analyses [6] were done as described by Tang et al. [23] using 1000 non parametric bootstrap replications to assess support.

Throughout this work the topologies of phylogenetic trees from Bayesian and ML analyses were similar and for the sake of brevity only Bayesian trees are shown.

Plant tests

Host range of nodulation and relative symbiotic nitrogen fixing effectiveness (RE) of bacterial isolates was assessed using plants grown in Leonard jars for 35 days [26] using methods and conditions detailed by Bromfield et al. [3] and Tang et al. [23]. RE values were calculated as $([x - x^o]/[x^e - x^o]) \times 100$, where x, x^o, and x^e are the mean shoot dry weights of, respectively, plants inoculated with a given bacterial isolate, uninoculated plants, and plants inoculated with effective reference strain, *Bradyrhizobium diazoefficiens* USDA 110^T. Unless otherwise stated, RE values were derived from means of five replicates (two plants/replicate) for each bacterial isolate.

Results

Bacterial isolation from RZ soils of native legumes

Using soybean cultivars AC Glengarry and AC Orford as trap hosts, a total of 819 bacterial isolates were obtained from RZ soils of native legumes at sites S1 through S5: *A. bracteata* (302 isolates), *D. canadense* (440 isolates), *D. glutinosum* (36 isolates) and *A. americana* (41 isolates). Data for the origins of these bacterial isolates (RZ soil, native legume, site and soybean cultivar used as trap host) are given in Supplementary Table S2.

Relatively few nodules (average number per plant between 0.5 and 27) were elicited on roots of trap plants with considerable variation in numbers between soybean cultivars and inoculation treatments consisting of RZ soils from different native legumes and sites. Populations of symbiotic bacteria in the different RZ soils varied with regard to nitrogen-fixing effectiveness on soybeans as indicated by low to intermediate overall relative effectiveness (RE) values of between −0.5 and 51.4 (Supplementary Table S5). It is noteworthy that symbionts in RZ soil from *D. canadense* were the most effective (RE = 32.9–51.4).

Sequence analysis using core genes

Based on a review of the literature, the protein encoding core gene, *rec*A, was selected for sequence analysis of all 819 isolates as a first step in bacterial characterization. The Bayesian *rec*A gene tree (Fig. 1) shows that these isolates were divided into 32 distinct *rec*A genotypes placed in multiple lineages. In some cases bacterial isolates sharing a particular *rec*A genotype originated from different