Ensifer, *Phyllobacterium* and *Rhizobium* species occupy nodules of *Medicago sativa* (alfalfa) and *Melilotus alba* (sweet clover) grown at a Canadian site without a history of cultivation

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Phage-resistant and -susceptible bacteria from nodules of alfalfa and sweet clover, grown at a site without a known history of cultivation, were identified as diverse genotypes of *Ensifer*, *Rhizobium* and *Phyllobacterium* species based on sequence analysis of ribosomal (16S and 23S rRNA) and protein-encoding (*atpD* and *recA*) genes, Southern hybridization/RFLP and a range of phenotypic characteristics. Among phage-resistant bacteria, one genotype of *Rhizobium* sp. predominated on alfalfa (frequency ~68 %) but was recovered infrequently (~1 %) from sweet clover. A second genotype was isolated infrequently only from alfalfa. These genotypes fixed nitrogen poorly in association with sweet clover and *Phaseolus vulgaris*, but were moderately effective with alfalfa. They produced a near-neutral reaction on mineral salts agar containing mannitol, which is atypical of the genus *Rhizobium*. A single isolate of *Ensifer* sp. and two of *Phyllobacterium* sp. were recovered only from sweet clover. All were highly resistant to multiple antibiotics. Phylogenetic analysis indicated that *Ensifer* sp. strain T173 is closely related to, but separate from, the non-symbiotic species '*Sinorhizobium morelense*'. Strain T173 is unique in that it possesses a 175 kb symbiotic plasmid and elicits ineffective nodules on alfalfa, sweet clover, *Medicago lupulina* and *Macroptilium atropurpureum*. The two *Phyllobacterium* spp. were non-symbiotic and probably represent bacterial opportunists. Three genotypes of *E. meliloti* that were symbiotically effective with alfalfa and sweet clover were encountered infrequently. Among phage-susceptible isolates, two genotypes of *E. medicae* were encountered infrequently and were highly effective with alfalfa, sweet clover and *Medicago polymorpha*. The ecological and practical implications of the findings are discussed.

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INTRODUCTION

Rhizobia are soil bacteria of considerable economic importance. In symbiotic association with legume crops they have the potential to fix nitrogen in amounts sufficient to reduce the dependency of agriculture on nitrogen fertilizers. Populations of rhizobia represent a valuable bioresource that may be exploited via selection of bacteria with desirable traits for use in inoculants intended to maximize legume crop productivity. Information on the diversity, dynamics and characteristics of populations of rhizobia residing in soil is therefore of considerable practical importance.

Studies on the diversity of rhizobia often depend on the analysis of 16S rRNA gene sequences for species-level identification. However, several studies have shown that 16S rRNA genes may undergo recombination and horizontal transfer resulting in sequence mosaicism (e.g. van Berkum *et al.*, 2003; Vinuesa *et al.*, 2005). Another disadvantage of bacterial identification based on the analysis of 16S rRNA genes is that closely related species cannot always be differentiated because of high levels of sequence conservation (Martens *et al.*, 2007). To surmount these difficulties, the use of other genes such as protein-encoding (housekeeping) genes with greater sequence divergence than 16S rRNA genes, but sufficiently conserved to be phylogenetically useful, are recommended as supplementary genetic markers for identification of the rhizobia (e.g. Martens *et al.*, 2007, 2008).

Abbreviations: PT, phage type; RE, relative effectiveness.

Supplementary material is available with the online version of this paper.

Medicago sativa (alfalfa) and *Melilotus alba* (sweet clover) are forage legumes that fix nitrogen in symbiotic association with the bacterial species *Ensifer meliloti*. Bromfield *et al.* (2001), characterized 1620 bacterial isolates from nodules of alfalfa and sweet clover) grown for two seasons at a Canadian site (latitude 45° 23' 6.28" N; longitude 75° 42' 15.65" W) without a known history of cultivation. Initially, bacteria able to nodulate alfalfa and sweet clover were not detected in soil, whereas in the second season their numbers increased markedly, presumably in response to the presence of legumes. Based on the use of 16 *E. meliloti* typing phages, a wide array of phage sensitivity patterns (phage types; PTs) were encountered among bacterial isolates sampled from both plant species. Phage-resistant isolates, designated PT14, from nodules of alfalfa were unusual in that they were predominant, whereas from sweet clover they were encountered infrequently. Based on *nod* gene hybridization patterns, the PT14 isolates from alfalfa were relatively homogeneous while those from sweet clover were heterogeneous.

Our aim was to obtain information on the species diversity and phenotypic characteristics of these unusual phage-resistant bacterial isolates from root nodules of alfalfa and sweet clover. This was accomplished by Southern hybrid-

ization using a variety of insertion sequence (IS) and symbiotic gene probes, by sequence analysis of ribosomal (16S and 23S rRNA) and protein-encoding (*atpD* and *recA*) genes, as well as a range of phenotypic determinations including plasmid profiles, resistance to antibiotics, symbiotic effectiveness and host range of nodulation. We also characterized phage-susceptible isolates belonging to PTs 13 and 24 that had unusual colony morphology relative to *E. meliloti* reference strains. A final objective was to estimate the frequency of genotypes and species among bacterial isolates comprising phage-resistant and -susceptible types.

METHODS

Bacteria. Single-colony isolates of bacteria from nodules of alfalfa and sweet clover were maintained at -80 °C in 20 % (v/v) glycerol. In the earlier work (Bromfield *et al.*, 2001), a subsample consisting of 180 of 561 phage-resistant isolates (PT14) from alfalfa and all 19 PT14 isolates from sweet clover were placed in a number of distinct groups based on *nodEFG* hybridization. Bacterial isolates representative of each of these groups as well as several isolates randomly selected from those belonging to each of PTs 13 and 24 (susceptible to eight and one of 16 typing phages, respectively) were used for further characterization in this study. Reference strains and representative examples of the bacterial isolates studied are shown in Table 1.

Table 1. Reference strains and representative bacteria used in this study

Superscript T designates type strains.

Strain	Origin	Host plant	Source or reference
<i>E. meliloti</i>			
ATCC 9930 ^T	USA	<i>Med. sativa</i>	ATCC, USA
SU47	Australia	<i>Med. sativa</i>	Rothamsted, UK
T15	Canada	<i>Med. sativa</i>	This study
T1580	Canada	<i>Mel. alba</i>	This study
T1607	Canada	<i>Mel. alba</i>	This study
<i>E. medicae</i>			
M102	Syria	<i>Med. trunculata</i>	Eardly <i>et al.</i> (1990)
T2	Canada	<i>Mel. alba</i>	This study
T10	Canada	<i>Med. sativa</i>	This study
<i>Ensifer</i> sp.			
T173	Canada	<i>Mel. alba</i>	This study
'<i>Sinorhizobium morelense</i>'			
LC04 ^T	Mexico	<i>Leucaena leucocephala</i>	Wang <i>et al.</i> (2002)
<i>Rhizobium</i> sp.			
OR191	USA	<i>Med. sativa</i>	Eardly <i>et al.</i> (1992)
T1470	Canada	<i>Med. sativa</i>	This study
T1473	Canada	<i>Med. sativa</i>	This study
T136	Canada	<i>Mel. alba</i>	This study
T1155	Canada	<i>Med. sativa</i>	This study
<i>Phyllobacterium</i> sp.			
T1018	Canada	<i>Mel. alba</i>	This study
T1293	Canada	<i>Mel. alba</i>	This study
<i>R. etli</i>			
CFN42 ^T	Mexico	<i>Phaseolus vulgaris</i>	Segovia <i>et al.</i> (1993)
<i>R. radiobacter</i>			
PD67 (synthetic derivative of C58)			Bromfield <i>et al.</i> (1987)

Table 2. DNA probes

Insertion sequence or symbiotic gene	Plasmid	Probe characteristics	Reference
<i>ISRm1</i>	pRWRm13	0.9 kb <i>Bgl</i> II– <i>Eco</i> RV internal <i>ISRm1</i> fragment	Barran <i>et al.</i> (1991)
<i>ISRm3</i>	pRWRm31	1.3 kb <i>Eco</i> RI internal <i>ISRm3</i> fragment	Barran <i>et al.</i> (1994)
<i>ISRm5</i>	pRWRm61	0.8 kb <i>Bst</i> XI– <i>Xho</i> I internal <i>ISRm5</i> fragment	Barran <i>et al.</i> (1994)
<i>nodC</i>	pVR3	177 bp <i>Rsa</i> I internal <i>nodC</i> fragment	Rastogi <i>et al.</i> (1992)
<i>nodH</i>	pSW95	0.3 kb <i>Bam</i> HI– <i>Pst</i> I <i>nodH</i> fragment	Bromfield <i>et al.</i> (1994)
<i>nodEFG</i>	pEK10	7 kb <i>Eco</i> RI <i>nodEFG</i> fragment	Bromfield <i>et al.</i> (2001)
<i>nifH</i>	pVR6	1.23 kb <i>Hind</i> III– <i>Bgl</i> II <i>nifH</i> fragment	Rastogi <i>et al.</i> (1992)

RFLP and Southern hybridization. The DNA probes *IsRm1*, *IsRm3*, *IsRm5*, *nodEFG*, *nodH*, *nodC* and *nifH* were used for Southern hybridization (Table 2). Genomic DNAs were extracted and purified using the Wizard SV Genomic DNA Purification System (Promega) according to the manufacturer's protocol; DNA concentration was determined using a NanoDrop ND-1000 spectrophotometer. Genomic DNAs were digested to completion with *Eco*RI, *Bam*HI or *Sal*I. The resulting DNA fragments were separated by gel electro-

phoresis, blotted onto nitrocellulose and hybridized to the respective ³²P-labelled DNA probes (Barran *et al.*, 1994).

Nucleotide sequencing. Full-length 16S rRNA and partial 23S rRNA, *atpD* and *recA* genes were amplified by PCR for subsequent DNA sequence analysis. Primers used for amplification and sequencing as well as temperature and PCR cycling conditions are shown in Table 3. Primers were derived from the literature except for 23S3f and

Table 3. Oligonucleotide primers

Primers in bold were used for PCR amplification as well as sequencing; remaining primers were used only for sequencing.

Primer	Direction	Sequence (5'–3')	PCR cycling conditions	Reference
16Sa	Forward	CGCTGGCGGCAGGCTTAACA	1 min 95 °C, 25 × (30 s 95 °C, 3 min 68 °C), 8 min 72 °C	van Berkum & Fuhrmann (2000) (for both 16Sa and 16Sb)
16Sb	Reverse	CCAGCCGCAGGTTCCCCT		
16Sa2 (ssu336f)	Forward	GGCAGCAGTGGGGAATATTG	1 min 95 °C, 25 × (30 s 95 °C, 45 s 75 °C [70 °C]*), 8 min 72 °C	Mantelin <i>et al.</i> (2006)
16Sa3 (ssu843f)	Forward	CCTGGGGAGTACGGTCGCAAG		Mantelin <i>et al.</i> (2006)
16Sb2 (ssu1023r)	Reverse	GGGACTTAACCCAACATCT		Mantelin <i>et al.</i> (2006)
16Sb3 (ssu440r)	Reverse	GGCACGAAGTTAGCCGGGGC		Mantelin <i>et al.</i> (2006)
atpD294f	Forward	ATC GGC GAG CCG GTC GAC GA	1 min 95 °C, 25 × (30 s 95 °C, 45 s 75 °C [70 °C]*), 8 min 72 °C	Gaunt <i>et al.</i> (2001)
atpD771r	Reverse	GCC GAC ACT TCC GAA CCN GCC TG		Gaunt <i>et al.</i> (2001)
atpD255F†	Forward	GCT SGG CCG CAT CMT SAA CGT C	1 min 95 °C, 30 × (45 s 95 °C, 1 min 70 °C, 1.5 min 74 °C), 8 min 74 °C	Vinuesa <i>et al.</i> (2005)
atpD782R†	Reverse	GCC GAC ACT TCM GAA CCN GCC TG		Vinuesa <i>et al.</i> (2005)
recA6F	Forward	CGK CTS GTA GAG GAY AAA TCG GTG GA	3 min 95 °C, 35 × (45 s 95 °C, 45 s 60 °C, 1.5 min 72 °C), 8 min 72 °C	Gaunt <i>et al.</i> (2001)
recA555R	Reverse	CGR ATC TGG TTG ATG AAG ATC ACC AT		Gaunt <i>et al.</i> (2001)
23S129f	Forward	CYGAATGGGGVAACC	3 min 94 °C, 30 × (1 min 94 °C, 1 min 67 °C, 3 min 72 °C) 8 min 72 °C	Hunt <i>et al.</i> (2006)
23S2241r	Reverse	ACCGCCCCAGTHAAACT		Hunt <i>et al.</i> (2006)
23S3f	Forward	ATACCAAGGCGCTTGAGAGAACT	72 °C) 8 min 72 °C	This study
23S3r	Reverse	TTCGCAGAGCCCTGTGTTTGTG		This study
23S1447Mf‡	Forward	ATAGCTGGTTCTCCGC		van Berkum <i>et al.</i> (2003)
23S1447Mr‡	Reverse	GCGGAGAACCAGCTAT		van Berkum <i>et al.</i> (2003)

**S. morelense* LC04^T and *Rhizobium* sp. strains T136, T1470, T1155, T1473 and OR191 amplified only at 70 °C annealing temperature.

†Primers used only for strains T173 (*Ensifer* sp.) and T1018 (*Phyllobacterium* sp.).

‡Primers modified at the locations shown in bold.

23S3r, which were determined by analysis of partial sequences using the software Primer Select (DNASTAR Lasergene 7). Primer locations for 23S3f and 23S3r were 1633–1655 and 1784–1763, respectively, relative to the 23S rRNA sequence in the complete genome of *E. meliloti* 1021 (GenBank accession no. NC003047).

The volumes of PCRs were 50 µl, containing 50–75 ng DNA, 0.15 mM each dNTP, 1.0 µM each primer, 1 × Titanium *Taq* DNA polymerase (Clontech Laboratories) and 1 × buffer that was supplied with the enzyme. An Eppendorf Mastercycler Gradient PCR was used to carry out amplifications. Subsequently, 1 µl of the PCR products was examined by electrophoresis in 1% agarose gels containing ethidium bromide, using a marker ladder as reference. PCR products with a clean single band of the correct molecular size were purified by using Montage PCR Centrifugal Filter Devices (Millipore). For products with multiple bands, the bands with the expected size were excised from the gel and purified using QIAEX II DNA gel extraction kit (Qiagen). Purified PCR products were sequenced using ABI BigDye Terminator chemistry v3.0 (Applied Biosystems) as described by Tambong *et al.* (2006) and analysed on an ABI 3100-Avant automated sequencer (Applied Biosystems/Hitachi).

Phylogenetic analysis. Nucleotide sequences of 16S rRNA, 23S rRNA, *atpD* and *recA* genes were edited using SEQMAN (DNASTAR, v.7.0) and multiple alignments for each set of sequences were done using CLUSTAL W as implemented in MEGALIGN. Nucleotide sequences for type strains of relevant taxa were obtained from GenBank and included in the analyses. For reference purposes we sequenced the 16S rRNA, 23S rRNA, *atpD* and *recA* genes of '*Sinorhizobium morelense*' strain Lc04^T (obtained from E. Martinez-Romero, UNAM, Mexico) as well as the non-type strains *Ensifer medicae* M102 (Eardly *et al.*, 1990) and *Rhizobium* sp. OR191 (Eardly *et al.*, 1992).

Molecular phylogenies were estimated using MEGA4 (Tamura *et al.*, 2007). The model for each set of sequences was selected on the basis of the Akaike information criterion implemented in Model Test 3.7 (Posada, 2006). Evolutionary histories were inferred using the neighbour-joining method (Saitou & Nei, 1987) and bootstrap consensus trees inferred from 1000 permutations of the datasets (Felsenstein, 1985). Evolutionary distances were computed using the Tamura–Nei method (Tamura & Nei, 1993) as the number of base substitutions per site. The rate variation among sites was modelled with a gamma distribution. All positions containing gaps and missing data were eliminated from each dataset (complete deletion option).

Nucleotide sequences generated in this study were deposited in GenBank and their accession numbers are shown in the phylogenetic trees.

The name *Ensifer* was used to designate bacterial species comprising the former genus *Sinorhizobium* with the exception of '*S. morelense*', which retains its nomenclature (Young, 2003; Martens *et al.*, 2007, 2008).

Frequency. Frequency estimates for bacterial genotypes were based on the phage type data of Bromfield *et al.* (2001), where 810 bacterial isolates is the total sample size from alfalfa and sweet clover, respectively. Frequencies of bacterial genotypes among phage-resistant isolates (PT14) were estimated by combining the *nodEFG* hybridization data of Bromfield *et al.* (2001) with those obtained by RFLP/Southern hybridization in this study. The frequencies of genotypes among PT14 isolates from alfalfa (A) were based on a subsample of 180 from 561 isolates and estimated from $[(xA14/180) \times (561/810)] \times 100$, where *xA14* is the number of PT14 isolates in the subsample of 180 belonging to a particular *nodEFG* type and 561/810 represents the proportion of isolates belonging to PT14 in the total sample from alfalfa.

Frequency estimates of genotypes for phage-susceptible isolates (PTs 13 and 24) were based on the assumption that phage type and

genotype (defined by Southern hybridization) are correlated as demonstrated by Barran *et al.* (1994).

Plasmids. Analysis of plasmid content was done by horizontal agarose gel electrophoresis as described by Bromfield *et al.* (1987). Blotting, labelling (³²P) and hybridization with DNA probes for *nifH* and *nodC* genes was as described by Barran *et al.* (1994). Estimates of the molecular size of bacterial plasmids were made by reference to standard plasmids of 410, 270, 180 and 55 kb in *Rhizobium radiobacter* PD67 (Bromfield *et al.*, 1987). The megaplasmid band of *E. meliloti* SU47, consisting of pSymA (~1300 kb) and pSymB (~1700 kb) (Barnett *et al.*, 2001), was used as a standard to detect plasmids of equivalent mobility.

Antibiotic resistance and acid production. Assays of intrinsic antibiotic resistance were done on tryptone yeast-extract (TY) agar medium (Bromfield *et al.*, 1994) amended with filter-sterilized solutions of the following antibiotics (Sigma Aldrich) at the following final concentrations (µg ml⁻¹): carbenicillin, 5, 50, 100, 1000; kanamycin, 5, 50, 100, 500; erythromycin and neomycin sulphate, 5, 50, 100, 300; gentamicin sulphate and tetracycline hydrochloride, 5, 50; nalidixic acid, 5, 20. Inoculation was by a multiple inoculator that simultaneously delivered 16 samples (~2 µl) to each plate. The inoculum for each bacterial strain, suspended in water, was standardized at ~10⁸ cells ml⁻¹. Plates were incubated for 4 days at 28 °C and bacterial growth recorded from duplicate plates of each antibiotic concentration.

Acid-production (final pH) assays were carried out using slopes of modified yeast-extract mannitol (YEM) agar medium (Bromfield *et al.*, 1994), inoculated with 0.1 ml washed bacterial cell suspension (~10⁸ cells ml⁻¹). Bromothymol blue (0.0025%) was added to the growth medium as pH indicator. The pH of the agar medium (five replicates per bacterial culture) was recorded after 21 days at 28 °C.

Plant tests. Seedlings from surface-sterilized seed were inoculated with washed bacterial culture (~10⁸ cells) and grown axenically in plant growth pouches (Mega International), in glass tubes (30 × 200 mm) containing vermiculite or in Leonard jar assemblies containing sand/grit mixture supplied with nitrogen-free nutrient solution as described by Bromfield *et al.* (1987). Plants were grown in controlled-environment cabinets for 35 days at ~250 µE m⁻² s⁻¹ (16 h light, 25 °C; 8 h dark, 16 °C). Significance in variation of symbiotic characteristics was determined by analysis of variance (GENSTAT, Release 8.1).

The relative effectiveness (RE) with regard to nitrogen fixation of bacterial strains was calculated as $[(x - x^0)/(x^c - x^0)] \times 100$ where *x*, *x*⁰ and *x*^c designate the mean shoot dry weights of, respectively, plants inoculated with a given bacterial strain, uninoculated plants and plants inoculated with a symbiotically effective standard strain.

RE values were derived from means of five (*Medicago sativa*, *Melilotus alba* and *Phaseolus vulgaris*) and nine (*Medicago polymorpha*) replicates (two plants per replicate) for each inoculation treatment and host plant combination.

RESULTS

RFLP and Southern hybridization

Data for Southern hybridization patterns of genomic DNAs from bacterial strains representative of phage-resistant and -susceptible types and *nodEFG* hybridization groups (Bromfield *et al.*, 2001) are shown in Table 4. Hybridization profiles generated using DNA probes for

Table 4. Estimated frequency of bacteria from nodules of *Med. sativa* and *Mel. alba* and characterization by insertion sequence (IS) and *nod* gene hybridization

Genomic DNAs were digested with *EcoRI* (*ISRm1*), *BamHI* (*ISRm3*, *ISRm5*, *nodH*) or *Sall* (*nodEFG*). Values in parentheses (PTs 13 and 24) represent the number of isolates characterized by Southern hybridization in this study.

Representative bacterial strain	No. of hybridizing bands with DNA probe*					IS/ <i>nod</i> genotype	Identification†	Phage type‡	<i>Med. sativa</i> ‡		<i>Mel. alba</i> ‡	
	<i>nodEFG</i>	<i>nodH</i>	<i>ISRm1</i>	<i>ISRm3</i>	<i>ISRm5</i>				No. isolates	Frequency	No. isolates	Frequency
Phage-resistant strains												
T15	4	1 a	1	8	5	F	<i>E. meliloti</i>	14	1	0.38	0	
T1580	2	1 a	0	3	6	G	<i>E. meliloti</i>	14	0		6	0.74
T1607	6	1 b	2	4	7	H	<i>E. meliloti</i>	14	0		2	0.25
T173	7	1 b	0	1	0	C	<i>Ensifer</i> sp.	14	0		1	0.12
T1470, T1473	3	1 c	0	1	0	A	<i>Rhizobium</i> sp.	14	3	1.15	0	
T136, T1155	2 a	1 c	0	0	0	B	<i>Rhizobium</i> sp.	14	176	67.72	8	0.99
T1018	0	0	0	0	0	I	<i>Phyllobacterium</i> sp.	14	0		1	0.12
T1293	0	0	0	0	0	I	<i>Phyllobacterium</i> sp.	14	0		1	0.12
Phage-susceptible strains												
T2	9	1 d	2	5	5	D	<i>E. medicae</i>	24	10 (2)	1.23	57 (4)	7.04
T10	9	1	2	6	2	E	<i>E. medicae</i>	13	3 (2)	0.37	9 (3)	1.11
Reference strains												
<i>E. meliloti</i> ATCC 9930 ^T	6	1 b	1	5	5	J						
<i>Rhizobium</i> sp. OR191	2 a	1 c	0	0	0	B						
<i>E. medicae</i> M102	9	1 d	1	3	0	K						
No. of hybridization profiles	11	6	7	10	7							

*Identical hybridization profiles for a particular DNA probe are indicated by a common letter.

†Identification based on analyses of 16S rRNA, 23S rRNA, *atpD* and *recA* gene sequences.

‡Phage type and frequency data based on Bromfield *et al.* (2001); PT14 isolates from *Med. sativa* represent a subsample of 180 from 561 isolates.

Fig. 1 (continues on following pages). Neighbour-joining bootstrap consensus trees based on sequences of 16S rRNA (A), 23S rRNA (B), *recA* (C) and *atpD* (D) genes. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Evolutionary distances were computed using the Tamura–Nei method and are in the units of the number of base substitutions per site. Rate variation among sites was modelled with a gamma distribution (shape parameter 0.71955, 1.54552, 1.67945 and 0.74334, respectively). All positions containing gaps and missing data were eliminated from each dataset. Strain designations and accession numbers in bold type are from this study.

IS*Rm1*, IS*Rm3*, IS*Rm5*, *nodH* and *nodEFG* divided the bacterial strains into nine distinct IS/*nod* genotypes, designated A through I. All reference strains had separate genotypes except for *Rhizobium* sp. OR191, which shared genotype B with strains T136 and T1155. Genomic DNAs from strains T1018 and T1293 (genotype I) did not hybridize with any of the IS or *nod* gene probes.

Hybridization profiles for *nodH* and *nodEFG* gene probes divided the test strains into six and nine symbiotic (*nod*)

genotypes, respectively, whereas profiles for the three insertion sequence probes divided the strains into eight IS genotypes. Hybridization profiles are shown in Supplementary Fig. S1 (available with the online version of this paper).

Analysis of nucleotide sequences

Phylogenetic trees reconstructed from sequences of ribosomal (16S and 23S rRNA) and protein-encoding (*atpD*)

and *recA* genes (Fig. 1), grouped representative bacterial strains with *E. meliloti*, *E. medicae*, '*S. morelense*' and *Ensifer adhaerans* and with the genera *Phyllobacterium* and *Rhizobium*.

Based on sequences of 16S rRNA (Fig. 1A), phage-resistant strain T173, the sole representative of genotype C, grouped with '*S. morelense*' (99% sequence identity) with strong bootstrap support of 95%.

Phage-resistant strains T1470 and T1473 (genotype A) and T136 and T1150 (genotype B) had identical 16S rRNA sequences to *Rhizobium* sp. OR191. The cluster formed by strains of *Rhizobium* sp. was separate from the closest relative (*Rhizobium etli* CFN42^T).

BLASTN searches indicated that several bacterial strains isolated from different regions of the world have high 16S rRNA sequence similarity to reference strain OR191 and related strains. Notable examples include strain HB8c1 (GenBank accession no. DQ280489) isolated from a root nodule of *Phaseolus vulgaris* in France, strain UMR 7370 isolated from *Dalea purpurea* in the USA (GenBank accession no. EF107507), and strains SEMIA 6436, SEMIA 6437 and SEMIA 6438 (GenBank accession nos FJ025119, FJ 025118 and FJ025120, respectively), which are used in inoculants for tropical legumes such as *Adesmia latifolia* and *Acaciella angustissima* in Brazil. As expected, these strains grouped closely with *Rhizobium* sp. OR191

and with strains, T1470, T1473, T136 and T1150 when they were included in the 16S rRNA gene tree (not shown).

Phage-resistant strains T1018 and T1293 (genotype I), were placed in the genus *Phyllobacterium* (Fig. 1A). Strain T1018 represented a distinct lineage within the *Phyllobacterium* group with bootstrap support of 74 %. Strain T1293 was distinct from, but clustered with, *P. brassicacearum* and *P. myrsinacearum* with relatively low (<70 %) bootstrap support.

Phage-resistant strains, T15, T1580 and T1607 (genotypes F, G and H, respectively) clustered with *E. meliloti* whereas

phage-susceptible strains T2 (PT24; genotype D) and T10 (PT13; genotype E), clustered with *E. medicae* A321^T and reference strain M102.

The grouping of our bacterial strains in the tree inferred from sequences of 16S rRNA (Fig. 1A) was generally consistent with the grouping in trees based on sequences of 23S rRNA, *recA* and *atpD* genes (Figs 1B, C and D, respectively).

An exception was the relative placement of strain T173 in the tree inferred from *atpD* sequences; bootstrap support for the placement of T173 as a neighbour of *E. adhaerans*

genomovar (gv.) C was low ($\leq 50\%$). Strain T173 was also differentiated from 'S. *morelense*' in the gene tree inferred from *recA* sequences (bootstrap support, 98 %).

In trees inferred from 23S rRNA, *recA* and *atpD* sequences, bacteria comprising *Rhizobium* sp. were placed in two subgroups: one consisting of T136, T1155 and reference strain OR191 and the other consisting of strains T1470 and T1473. These two subgroups correspond to *Rhizobium* sp. genotypes A and B defined by Southern hybridization.

E. medicae strain T2 (genotype D) and T10 (genotype E) were differentiated only in the *recA* gene tree topology (Fig. 1C).

BLASTN searches revealed that *Ensifer* sp. strain T173 is highly similar to non-symbiotic *Ensifer* sp. strain LMG 20571 isolated from Belgian soil in that maximum sequence identity is 100 % for 16S rRNA, 23S rRNA, *atpD* and *recA* gene sequences (GenBank accession nos AJ420776, AM418718, AM418772 and AM182151, respectively). *Phyllobacterium* sp. strain T1018 is similar to non-symbiotic strain CCBAU 13011 isolated in China, with a maximum sequence identity of 100 % for 16S rRNA, 99 % for *recA* and 97 % for *atpD* (GenBank accession nos EU170548, EU255214 and EU170569, respectively). Phylogenetic trees that included the respective nucleotide sequences for *Ensifer* sp. LMG 20571 and *Phyllobacterium* sp. CCBAU 13011 showed that the former strain grouped closely with *Ensifer* sp. T173 and that the latter strain grouped with *Phyllobacterium* sp. T1018 (not shown).

Frequency

Table 4 shows estimates of the frequency of occurrence of bacterial genotypes (defined by RFLP and Southern hybridization). Phage-resistant bacteria belonging to *Rhizobium* sp. genotype B predominated on alfalfa (frequency about 68 %), whereas from sweet clover, this genotype was recovered infrequently ($\sim 1\%$). In contrast, *Rhizobium* sp. genotype A was recovered only from alfalfa at low frequency ($\sim 1\%$).

Phage-resistant isolates of *E. meliloti* (genotypes F, G, H), *Ensifer* sp. (genotype C) and the two *Phyllobacterium* spp. (genotype I) were recovered from alfalfa and/or sweet clover at frequencies of 1 % or less.

Phage-susceptible *E. medicae* isolates consisting of genotypes D and E were recovered from sweet clover and alfalfa at low frequency ($\sim 1\text{--}7\%$).

Symbiotic characteristics

Additional Southern analyses of genomic DNAs from *Phyllobacterium* sp. strains T1018 and T1293 (digested with *EcoRI*) revealed no hybridization with *nifH* and *nodC* gene probes, suggesting that these strains are non-symbiotic. Plant tests showed that neither strain elicited nodules on alfalfa, sweet clover or *Medicago lupulina* (black medic).

All strains comprising the genera *Ensifer* and *Rhizobium* nodulated alfalfa and sweet clover (Table 5a). *E. meliloti*

strains T15, T1580 and T1607 were of moderate to high relative effectiveness (RE) with regard to nitrogen fixation in association with these two hosts.

E. medicae M102 was poorly effective on alfalfa (RE=13 %) whereas strains T2 and T10 were highly effective on this host (RE=159 % and 155 %, respectively). In association with sweet clover, strains T2, T10 and M102 were more effective than the standard strain of *E. meliloti*.

Ensifer sp. strain T173 elicited large numbers of small white nodules on roots of alfalfa and sweet clover but did not fix nitrogen (RE=−2.8 %).

Strains representing *Rhizobium* sp. were moderately effective in association with alfalfa, with RE values of between 64 % and 77 %. In contrast, reference strain OR191 was poorly effective. On sweet clover, all *Rhizobium* sp. isolates showed low levels of effectiveness.

Bacteria and reference strains representing *E. meliloti*, *E. medicae* and *Ensifer* sp. elicited a few small white nodule-like structures on roots of *Phaseolus vulgaris* (beans) that did not fix nitrogen (Table 5b). In contrast, bean plants inoculated with strains of *Rhizobium* sp. were well nodulated. However, these strains were of low to moderate effectiveness on this host, with RE values varying between 19 % (strain T1470) and 78.1 % (strain OR191).

E. medicae strains T2 and T10 nodulated the annual legume *Medicago polymorpha* and were effective relative to standard strain M102 and uninoculated control plants; RE values were 101 % (T2) and 109 % (T10). *E. meliloti* ATCC 9930^T did not elicit nodules on *Med. polymorpha* (data not shown).

The host range of *Ensifer* sp. strain T173 was further examined to determine whether this bacterium is capable of fixing nitrogen with a legume host. The following additional plant hosts were tested: *Medicago lupulina*, *Medicago laciniata*, *Trigonella foenum-graecum*, *Trifolium repens*, *Lotus corniculatus*, *Lotus pedunculatus*, *Vicia sativa*, *Vicia cracca*, *Onobrychis vicifolia*, *Macroptilium atropurpureum* 'Siratro', *Lupinus nootkatensis*, *Leucaena leucocephala*, *Lathyrus sativus*, *Pisum sativum*, *Glycine max* and *Vigna unguiculata*. Of these plant hosts, strain T173 elicited small white nodules on roots of all 10 replicate plants of *Med. lupulina* and *Macroptilium atropurpureum* 'Siratro', but did not fix nitrogen.

Plasmid analysis

Data for plasmids and Southern hybridization with *nifH* and *nodC* gene probes are given in Table 6; examples are shown in Fig. 2. Bacterial strains possessed between two and four plasmids and had distinct plasmid profiles, except for reference strain OR191, which shared an identical profile with strains of *Rhizobium* sp. belonging to genotype B.

Table 5. Symbiotic characteristics

Effective standard strains were *E. meliloti* ATCC 9930^T for *Med. sativa* and *Mel. alba*, and *R. etli* CFN42^T for *P. vulgaris*. Values are means of five replicates (two plants per replicate) for each inoculation treatment and host species combination; uninoculated plants were without nodules. RE, relative effectiveness; SED, standard error of difference between means. Letters in parentheses represent IS/*nod* genotypes (see Table 4).

Inoculation treatment	(a) Test hosts: <i>Med. sativa</i> and <i>Mel. alba</i>						(b) Test host: <i>Phaseolus vulgaris</i>		
	<i>Med. sativa</i>			<i>Mel. alba</i>			Shoot dry wt [log ₁₀ (mg per plant)]	Nodule dry wt [log ₁₀ (mg per plant)]	RE
	Shoot dry wt [log ₁₀ (mg per plant)]	Nodule no. per plant (log ₁₀)	RE	Shoot dry wt [log ₁₀ (mg per plant)]	Nodule no. per plant (log ₁₀)	RE			
<i>R. etli</i> CFN42 ^T							3.45	2.41	100.0
<i>E. meliloti</i>									
ATCC 9930 ^T (J)	1.24	0.88	100.0	1.02	0.85	100.0	2.20	1.00	−0.9
T15 (F)	1.39	0.84	156.8	1.03	0.68	101.9	2.23	0.67	−0.6
T1580 (G)	1.37	0.91	149.3	0.92	0.75	88.8	2.37	0.32	1.4
T1607 (H)	1.37	0.92	146.1	1.26	1.00	228.3	2.17	0.70	−1.2
<i>E. medicae</i>									
M102 (I)	0.77	1.50	13.0	1.22	0.97	200.3	2.20	1.29	−1.1
T2 (D)	1.37	0.95	159.1	1.09	0.81	130.6	2.23	1.08	−0.5
T10 (E)	1.38	1.09	155.0	1.22	1.20	201.0	2.19	1.15	−1.2
<i>Ensifer</i> sp.									
T173 (C)	0.72	1.57	6.2	0.61	1.21	−2.8	2.19	0.79	−1.2
<i>Rhizobium</i> sp.									
OR191 (B)	0.91	1.30	31.4	0.69	1.08	9.9	3.27	2.12	78.1
T1470 (A)	1.15	1.13	77.3	0.69	1.03	11.0	2.85	2.16	18.9
T1473 (A)	1.10	1.08	65.4	0.66	0.89	6.5	2.90	2.24	21.1
T136 (B)	1.09	1.18	62.9	0.73	1.19	19.3	2.95	2.01	24.8
T1155 (B)	1.09	1.29	64.3	0.75	0.94	22.5	3.09	1.94	43.2
Uninoculated	0.64		0	0.62		0	2.28		0
SED (<i>P</i> <0.01)	0.083	0.123		0.083	0.123		0.107	0.264	
	(d.f.=112)	(d.f.=104)		(d.f.=112)	(d.f.=104)		(d.f.=60)	(d.f.=56)	

*Effective standard strains.

Strains of *E. meliloti*, *E. medicae*, *Ensifer* sp. and *Phyllobacterium* sp. possessed plasmid bands with mobilities in agarose gels less than or equivalent to the megaplasmid band of the reference strain, *E. meliloti* SU47.

Only strains representing *E. meliloti* and *E. medicae* had megaplasmid bands in agarose gels that hybridized to *nifH* and *nodC* gene probes (i.e. symbiotic megaplasmids or pSymA). *E. medicae* strain T10 possessed a pSymA as well as a 170 kb plasmid that hybridized to *nifH*, but not to *nodC* gene sequences.

Ensifer sp. strain T173 was unusual in that it had a non-symbiotic megaplasmid as well as a 175 kb symbiotic plasmid.

All *Rhizobium* sp. strains possessed a relatively large symbiotic plasmid that was only slightly smaller in size (i.e. had greater mobility in agarose gels) than the reference megaplasmid band of *E. meliloti* SU47 (Fig. 2).

Acid production and antibiotic resistance

All strains produced an acidic reaction in mineral salts agar medium containing mannitol (Table 6). *Phyllobacterium* sp. produced the most acid (pH range 4.5–4.7), *E. meliloti* and *E. medicae* were intermediate (pH range 5.4–5.7), whereas *Rhizobium* sp. produced a slightly acid to near-neutral reaction (pH range 6.4–6.7).

Ensifer sp. T173 and ‘*S. morelense*’ Lc04^T differed from strains of *E. meliloti* and *E. medicae* in that they showed relatively high levels of resistance to carbenicillin (>1000 µg ml^{−1}), kanamycin (100–500 µg ml^{−1}) and neomycin (100 µg ml^{−1}) (Table 6). *Phyllobacterium* sp. strains T1018 and T1293 also showed relatively high levels of resistance to carbenicillin and kanamycin. Strain T1293 was unusual in that it showed a high level of resistance (>50 µg ml^{−1}) to tetracycline. *E. medicae* T2, T10 and reference strain M102 differed in their levels of resistance to carbenicillin.

Table 6. Plasmids, acid production and antibiotic resistance

Bacterial strain*	IS/ <i>nod</i> genotype	Plasmid configuration†	Acid production (pH)‡	Antibiotic resistance (µg ml ⁻¹)§						
				Car	Kan	Ery	Neo	Gen	Tet	Nal
<i>E. meliloti</i>										
ATCC 9930 ^T	J	M, 180	5.44	ND						
T15	F	M, 220, 190, 45	5.60	50	5	50	<5	5	<5	>20
T1580	G	M, 100	5.61	>50	>5	100	>5	>5	<5	>20
T1607	H	M, 200, 170,150	NT	100	>5	100	>5	>5	<5	>20
<i>E. medicae</i>										
M102	K	M, 300	5.63	50	<5	100	<5	>5	>5	>20
T2	D	M, 300, 160	5.67	>100	<5	100	5	>5	<5	>20
T10	E	M, 170#	5.41	<5	<5	100	<5	>5	<5	>20
<i>‘S. morelense’</i>										
Lc04 ^T	ND	>1000, 670, 540	ND	>1000	500	100	100	5	<5	20
<i>Ensifer</i> sp.										
T173	C	M, >400, >400, 175	5.75	>1000	>100	100	100	<5	<5	20
<i>Rhizobium</i> sp.										
OR191	B	C, <M, 140	6.74	>50	5	5	<5	<5	<5	>20
T136	B	C, <M, 140	6.60	100	5	5	<5	<5	<5	>20
T1155	B	C, <M, 140	6.77	100	5	5	<5	<5	<5	>20
T1470	A	C, <M, 280, 120	6.44	100	5	5	<5	<5	<5	>20
T1473	A	C, <M, 280, 140, 120	6.48	100	5	5	<5	<5	<5	>20
<i>Phyllobacterium</i> sp.										
T1018	I	≥M, 220, 180	4.65	>1000	100	100	5	<5	<5	>20
T1293	I	≥M, 280, 260, 180	4.45	>1000	>100	100	50	5	>50	>20

**E. meliloti* ATCC 9930^T, *E. medicae* M102, 'S. morelense' Lc04^T and *Rhizobium* sp. OR191 are reference strains.

†Plasmid sizes (kb) estimated from comparisons to reference plasmids (410, 270, 180 and 55 kb) in *R. radiobacter* PD67. C denotes band with mobility corresponding to the chromosomal band of PD67 or with mobility less than the reference megaplasmid band of *E. meliloti* SU47. M denotes band with mobility corresponding to the megaplasmid band of SU47. Plasmids in bold hybridized to *nodC* and *nifH* gene probes, with the exception of the 170 kb plasmid (designated #), which hybridized only to *nifH*.

‡Values are means of five replicates. Mean value for uninoculated control=pH 7.1. SED=0.044 (d.f.=56).

§Car, carbenicillin; Kan, kanamycin; Ery, erythromycin; Neo, neomycin; Gen, gentamicin; Tet, tetracycline; Nal, nalidixic acid. Values (based on duplicates) represent the highest antibiotic concentration at which bacterial growth was inhibited relative to plates without antibiotic. ND, Not determined.

||Plasmid data from Wang *et al.* (1999).

Strains of *Rhizobium* sp. showed a similar spectrum of resistance to reference strain OR191.

DISCUSSION

Bacteria related to *Rhizobium* sp. strain OR191 represent an anomalous group that were first isolated from nodules of alfalfa grown in acid soil in the United States and were reported to nodulate and fix nitrogen poorly in association with *Phaseolus vulgaris* (beans) and alfalfa (Eardly *et al.*, 1992).

Our data indicate that *Rhizobium* sp. OR191 and related strains are unusual in that they produce a slightly acid to near neutral reaction in mineral salts agar medium containing mannitol, a characteristic that is more typical of the genus *Bradyrhizobium* (Bergey's *Manual of Systematic Bacteriology*, 2005). They are also unusual in that they

possess a large symbiotic plasmid that is only slightly smaller than the pSymA and pSymB megaplasmids (~1300–1700 kb) characteristic of *E. meliloti* (Barnett *et al.*, 2001).

In the previous work (Bromfield *et al.*, 2001), alfalfa and sweet clover were grown for two seasons in slightly acid field soil (pH 6.1). Here, we show that the phage-resistant bacteria that were recovered at high frequency (69%) from alfalfa consisted almost entirely of a single genotype (genotype B) that was indistinguishable from *Rhizobium* sp. strain OR191 by any of the genetic markers used or by plasmid profile analysis. The fact that isolates of genotype B were predominant on alfalfa but were recovered infrequently (~1%) from sweet clover probably reflects differential host plant selection of specific bacteria for symbiosis.

Del Papa *et al.* (1999) reported that infrequently occurring acid-tolerant bacteria isolated from alfalfa grown in acid

Fig. 2. Examples of variation in size and number of plasmids that hybridize to DNA probes for *nodC* and *nifH* among bacteria from *Medicago sativa* and *Melilotus alba*. Isolate designations are given below each respective agarose gel (left) and autoradiogram (right). *E. meliloti* SU47, *E. medicae* M102 and *Rhizobium* sp. OR191 are reference strains. Arrows indicate plasmids of mobility equivalent to that of the reference megaplasmid band in *E. meliloti* SU47. (A) Plasmids in *E. meliloti* SU47 and *Rhizobium* sp. OR191, T1155 and T1473 that hybridize to *nodC*. Identical hybridization profiles were obtained for the *nifH* gene probe (not shown). (B, C) Plasmids in *E. medicae* M102, T10 and *Ensifer* sp. T173 that hybridize to *nodC* and *nifH*, respectively. See Table 6 for estimated plasmid sizes.

soils in South America, were genetically homogeneous and closely related to *Rhizobium* sp. OR191. Our identification of a second bacterial genotype (genotype A) that occurred at low frequency (~1 %) only from alfalfa, suggests that populations of OR191-related bacteria need not necessarily be genetically homogeneous.

The isolation of OR191 related bacteria from nodules of *Phaseolus* grown in acid, copper-contaminated soil in France (Laguerre *et al.*, 2006) indicates that their distribution is not restricted to acid soils in the Americas. Moreover, phylogenetic analysis of 16S rRNA sequences similar to strain OR191 revealed that related bacteria are associated with legumes such as *Dalea purpurea* in Minnesota (Mártir *et al.*, 2007) and with *Adesmia latifolia* and *Acaciella angustissima* in Brazil (Binde *et al.*, 2009), suggesting that these bacteria may be promiscuous with regard to their host range for symbiosis.

Our observation that OR191-related strains were more effective with regard to nitrogen fixation with alfalfa than with sweet clover might be indicative of their adaptation to symbiosis with alfalfa. Interestingly, our strains were less effective than OR191 in symbiotic association with *Phaseolus vulgaris*, perhaps reflecting the fact that OR191 originated in the United States, where beans have long been cultivated, whereas in eastern Canada this legume is rarely cultivated.

Competition for nodulation of the host legume by populations of rhizobia resident in soil represents a serious problem affecting the establishment of effective nitrogen-fixing inoculant strains in crop ecosystems (e.g. Barran & Bromfield, 1997). From a practical viewpoint, the extent to which soil populations of suboptimally effective *Rhizobium* sp. related to OR191 compete with effective introduced

inoculant strains of *E. meliloti* for nodulation of alfalfa remains to be investigated.

Bacteria of the species '*S. morelense*' are closely related to the predatory bacterial species, *E. adhaerens*. They are described as non-symbiotic (i.e. *Nod*⁻ *Nif*⁻), highly resistant to various antibiotics and were first isolated from nodules of *Leucaena leucocephala* (Wang *et al.*, 2002).

In this study, evidence was obtained that *Ensifer* sp. strain T173 (from sweet clover), is closely related to, but separate from, '*S. morelense*' LC04^T. Moreover, the data suggest that T173 belongs to the same genospecies as non-symbiotic *Ensifer* sp. strain LMG 20571 (Martens *et al.*, 2007, 2008). However, strain T173 differs from both '*S. morelense*' and *Ensifer* sp. LMG20571 by its ability to elicit ineffective nodules on alfalfa, sweet clover, black medic and *Macroptilium atropurpureum* and by the presence of a 175 kb symbiotic plasmid.

It is possible that strain T173 acquired its symbiotic plasmid via horizontal transfer (Broughton, 2003). In this connection, it is of interest to note that the *in vitro* introduction of a functional symbiotic plasmid to a strain of the non-symbiotic species *E. adhaerans* conferred on it the ability to nodulate certain legume hosts (Rogel *et al.*, 2001).

While strain T173 did not fix nitrogen with any of a wide array of test legumes, it remains to be ascertained whether its symbiotic plasmid carries functional *nif* genes and is capable of conferring on this bacterium the ability to fix nitrogen in association with a legume host.

The fact that strain T173 was encountered only once among numerous bacterial isolates suggests either that it

occurred infrequently in soil or that it is poorly competitive for nodulation of the plant host. Alternatively, root nodules occupied by T173 may have escaped sampling due to their small size. However, the fact that the similar, but non-symbiotic, strain LMG 20571 was isolated from soil in Belgium (Willems *et al.*, 2003; Martens *et al.*, 2007) suggests that T173-related bacteria may have a wide geographical distribution.

The finding that *Phyllobacterium* sp. strains T1018 and T1293 are non-symbiotic and were each recovered only once from nodules suggests that they may be opportunistic bacteria that occasionally occupy root nodules. Our data suggest that these strains may represent distinct genospecies within the genus *Phyllobacterium*. The fact that strain T1018 is related to the non-symbiotic strain CCBAU 13011, isolated from a root nodule of *Vicia* sp. in China (Lei *et al.*, 2008), suggests that these bacteria may have a worldwide distribution.

Strains T1018 and T1293 were highly resistant to multiple antibiotics, in common with *Ensifer* sp. T173 in this study, and with 'S. morelense' described by Wang *et al.* (2002). In particular, strain T1293 was unusual in that it was highly resistant to the broad-spectrum antibiotic tetracycline. These *Ensifer* sp. and *Phyllobacterium* sp. strains originated from a site with no known history of antibiotic use (Bromfield *et al.*, 2001). The ecological and biological significance of multiple antibiotic resistance in these naturally occurring bacteria remains to be ascertained.

The species *E. medicae* is closely related to *E. meliloti*, and represents a group of bacteria that nodulate and fix nitrogen in association with annual *Medicago* species (e.g. *Med. polymorpha*) that are native to Africa, Asia and the Mediterranean region (Rome *et al.*, 1996).

Silva *et al.* (2007) obtained several isolates of *E. medicae* from naturalized *Med. polymorpha*, *Med. lupulina* and cultivated alfalfa in Mexico. These isolates may have originated from a single clone because they had identical plasmid profiles and were genetically homogeneous for all genetic markers analysed.

In contrast, we identified two distinct genotypes of *E. medicae* among phage-susceptible isolates from alfalfa and sweet clover. These two genotypes, represented by strains T2 and T10 respectively, were also differentiated on the basis of distinctive plasmid profiles. While these genotypes were minority components of the bacterial population inhabiting nodules, both were recovered at significantly higher frequency from sweet clover than from alfalfa, perhaps reflecting differential selection by the plant host.

It is of interest to note that strain T10 not only possessed a symbiotic megaplasmid (pSymA), but also a 170 kb plasmid carrying reiterated sequences of the *nifH* gene. It is possible that the 170 kb plasmid was acquired from another rhizobial species (Broughton, 2003). Alternatively, the reiterated *nifH* sequences may have originated from the pSymA of *E. medicae* via recombination (Rastogi *et al.*, 1992).

The annual legume *Med. lupulina* (black medic) was observed to occur sporadically in the vicinity of the experimental site (Bromfield *et al.*, 2001). It is possible that this legume served as a source of the *E. medicae* that were recovered from nodules of alfalfa and sweet clover plants grown in the field plots.

Our *E. medicae* strains were highly effective with regard to nitrogen fixation on alfalfa, sweet clover and *Med. polymorpha*, whereas strain M102 isolated from *Medicago trunculata* in Syria (Eardly *et al.*, 1990) was poorly effective on alfalfa but effective on the other plant hosts. Although more comparisons are needed, it is tempting to suggest that the high level of effectiveness of our strains on alfalfa and sweet clover might reflect adaptation to symbiosis with naturalized species of *Medicago* (*Med. sativa*, *Med. lupulina*) and *Melilotus* (*Mel. alba*, *Mel. officinalis*) that are widespread in eastern Canada. In this regard, further study of the agricultural potential of Canadian *E. medicae* for alfalfa inoculation may be warranted.

The bacteria that were identified as *E. meliloti* constituted a minority of phage-resistant bacteria from alfalfa and sweet clover. Their low frequency was expected a priori because the typing phages were developed using *E. meliloti* strains as hosts (Bromfield *et al.*, 2001) and because earlier studies showed that an overwhelming majority of bacterial isolates from *Medicago* and *Melilotus* species grown in Canadian soils are susceptible to these phages (e.g. Thurman & Bromfield, 1988). The isolates identified as *E. meliloti* were divided into three genotypes. This heterogeneity is in line with earlier reports of moderate levels of genetic diversity in naturalized populations of *E. meliloti* in eastern Canada (e.g. Bromfield *et al.*, 1998).

This study has provided new insights into the species diversity and characteristics of bacteria that occupy nodules of alfalfa and sweet clover. Diverse bacterial genotypes representing *Ensifer* sp., *Phyllobacterium* sp., *Rhizobium* sp., *E. meliloti* and *E. medicae* were identified among phage-resistant and -susceptible bacterial isolates from these two legume species grown at a site with no known history of cultivation. It is remarkable that this wide bacterial diversity was encountered at a site, where initially bacteria able to nodulate alfalfa and sweet clover were not detected in soil (Bromfield *et al.*, 2001).

We conclude that these diverse bacterial species were originally present in soil, although the possibility cannot be ruled out that they were introduced via transcontinental airborne dust, or on seed. Further study is needed to elucidate their origin and dispersal.

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