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Phylogenetic Position of *Rhizobium* sp. Strain Or 191, a Symbiont of Both *Medicago sativa* and *Phaseolus vulgaris*, Based on Partial Sequences of the 16S rRNA and *nifH* Genes

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Phenotypic and DNA sequence comparisons are presented for eight *Rhizobium* isolates that were cultured from field-grown alfalfa (*Medicago sativa* L.) in Oregon. These isolates were previously shown to nodulate both alfalfa and common bean (*Phaseolus vulgaris* (L.) Savi.). The objective of the present study was to determine their phylogenetic relationships to the normal symbionts of these plants, *Rhizobium meliloti* and *Rhizobium leguminosarum* biovar phaseoli, respectively. Phenotypically, the Oregon isolates more nearly resemble strains from *P. vulgaris* than those from *M. sativa*. For example, even though nitrogen fixation levels were low with both host species, the symbiotic efficiency of a representative *Rhizobium* isolate (Or 191) with common bean was twice that observed with alfalfa. Comparative sequencing of a 260-bp segment of the 16S rRNA gene (directly sequenced after amplification by the polymerase chain reaction) demonstrated that Or 191 is not closely related to the type strain of *R. meliloti* (ATCC 9930), *R. leguminosarum* (ATCC 10004), or *Rhizobium tropici* (CIAT 899). Instead, sequence comparisons of the *16S* gene indicated that Or 191 belongs to a distinct and previously unrecognized taxonomic group that includes strains that have previously been called *R. leguminosarum* bv. phaseoli type I. Unlike type I strains, however, Or 191 has only a single copy of the *nifH* gene (type I strains have three), and the nucleotide sequence of this gene is substantially different from those of other rhizobial and nonrhizobial *nifH* genes examined thus far.

Alfalfa (*Medicago sativa* L.) grown on moderately acid soils in North America is often nitrogen deficient because its normal microsymbiont, *Rhizobium meliloti*, is intolerant of soil acidity (13, 22). A previous study of *Rhizobium* isolates cultured from alfalfa grown in moderately acid soil in Oregon indicated that they possess the unique ability to nodulate both alfalfa and common bean (*Phaseolus vulgaris* (L.) Savi.) (6). The objective of the present study was to determine the evolutionary relationships of these unusual isolates to the normal symbionts of alfalfa and common bean.

R. meliloti, which is native to Asia (7), is the only *Rhizobium* species that can fix nitrogen with the plants of the genus *Medicago* (31). Studies employing genomic DNA-DNA hybridization (3) and multilocus enzyme electrophoresis (7) have indicated that all strains of *R. meliloti* are closely related, although two divisions of the species can be distinguished by multilocus enzyme electrophoresis. In contrast, the symbionts of *Phaseolus vulgaris* are genotypically highly heterogeneous (10, 18, 20). Originally defined in reference to its host range, the species *Rhizobium phaseoli* was renamed *R. leguminosarum* biovar phaseoli and classified as a host-specific biovar of the same species as the symbionts of vetches (*R. leguminosarum* bv. viciae) and clovers (*R. leguminosarum* bv. trifolii) (15).

For some symbionts of *P. vulgaris*, this clearly is a realistic taxonomic treatment. For example, strain 8002 (which was isolated from *P. vulgaris* in England) has a chromosomal type that is also commonly found in English isolates recovered from pea (*Pisum sativum* L.) and clover (*Trifolium repens* L.) (34). Moreover, the nucleotide sequence of a diagnostic segment of the 16S rRNA gene in strain 8002 (33) is identical to the sequences of several other strains of *R. leguminosarum*, including the type strain for the species, ATCC 10004 (33a). However, the taxonomic placement of a large number of other *P. vulgaris* symbionts, such as the type I strains that represent extensive rhizobial populations in the Americas, is unclear because their chromosomal genotypes have not been directly compared with that of the type strain for the species.

It has long been recognized that the symbionts of *P. vulgaris* are genetically much more diverse than are strains of the other two biovars of *R. leguminosarum* (10, 14, 15). For example, a distinctive group of *Phaseolus*-nodulating strains, the type II strains, has recently been separated as the distinct species *Rhizobium tropici* (18). To avoid confusion, therefore, we will refer to all symbionts of *Phaseolus* species as *Rhizobium* sp. (*Phaseolus*) unless there are clear grounds for assigning particular strains to either *R. leguminosarum* or *R. tropici*.

In the present study, the Oregon *Rhizobium* isolates were first screened for traits that have been shown to distinguish the symbionts of alfalfa from those of common bean, including environmental stress tolerance and symbiotic effectiveness. Next, multilocus enzyme profiles of the Oregon isolates were compared with those of symbionts of alfalfa and common bean; however, the results failed to place the Oregon isolates with either group. The results did show that all of the Oregon isolates have the same multilocus genotype (5a). Finally, DNA sequence comparisons were made among a typical Oregon isolate (Or 191) and several representative symbionts of alfalfa and common bean. The latter strains were selected to span the range of chromosomal genotypic diversity revealed in earlier surveys of enzyme polymorphism among isolates recovered from these host species (7,

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20). Segments of the chromosomal gene encoding 16S rRNA (designated as *16S*) (33) and the Sym plasmid-encoded nitrogenase reductase gene (*nifH*) were amplified by the polymerase chain reaction (19) and sequenced directly.

MATERIALS AND METHODS

Sources of strains. Eight Oregon isolates were originally cultured from ineffectively nodulated alfalfa seedlings growing in moderately acid silt loam soil at the Hyslop Crop Science Experiment Station of the Oregon State University in Corvallis (6). Legume crops grown previously at the site included pea, common bean, alfalfa, and clover, all of which were routinely inoculated with commercial rhizobial cultures.

The Oregon isolates were grown and maintained on yeast extract-mannitol (YEM) agar (31). Before use, stock cultures were authenticated by testing the infectivity of four single-colony isolates on common bean and alfalfa. The reciprocal infectivity of one of the isolates, Or 191, was confirmed by tests with both host species. Single colonies for subculture were obtained from spread plates inoculated with dilute YEM broth cultures that had been vortexed in 0.01% (vol/vol) Tween 80. A comparison of direct counts and plate counts from the broth cultures indicated that the spread plate colonies probably arose from single cells.

The sources of the *Rhizobium* laboratory strains used for comparative analysis were as follows. *R. meliloti* 102F34, 102F51, 102F82, 102F84, 102F85; CC 169, CC 2013; 3-3, 27-2, L5-30, 1021; and ATCC 9930 were provided by P. J. Bottomley (Oregon State University, Corvallis); J. Fourment (Institut National de la Recherche Agronomique, Castanet-Tolosan, France); B. Kneen (Boyce Thompson Institute at Cornell, Ithaca); and the American Type Culture Collection, respectively. *Rhizobium* sp. (*Phaseolus*) Tal 182; CIAT 144, CIAT 899 (type strain of *R. tropici* [18]); FL-27, Olivia-4 (20); CIAT 57, CIAT 134, CIAT 676, Viking 1, Olivia; and 127K12b were provided by H. J. Hoben (University of Hawaii NifTAL Project, Maui); J. Kipe-Nolt (Centro Internacionale de Agricultura Tropicale, Cali, Colombia); D. Pinero (UNAM, Mexico City, Mexico); E. L. Schmidt (University of Minnesota, St. Paul); and R. S. Smith (LiphaTech, Milwaukee), respectively.

Symbiotic effectiveness of Or 191 on alfalfa and bean. Because the nodules formed by Or 191 on both host species were normal in appearance, this particular isolate was selected for symbiotic characterization and comparison with the commercial inoculant strains *R. meliloti* 102F34 (on alfalfa) and *Rhizobium* sp. (*Phaseolus*) 127K12b (on common bean). Alfalfa (cultivar Apollo) and bean (cultivar Aurora) plants were grown axenically in sand in pots 7.5 and 15 cm in diameter, respectively. One week after planting, alfalfa seedlings were thinned to six plants per pot and bean seedlings were thinned to one plant per pot. Pots were initially watered with a one-third-strength nutrient solution (5) containing 3.6 mM N (as KNO_3) and subsequently watered with the same nutrient solution without KNO_3. Experiments were conducted in a room with a controlled environment under a combination of metal halide and mercury vapor lamps that provided a photon flux density of 750 $\mu mol\ m^{-2}\ s^{-1}$ over a 12-h photoperiod. Day and night temperatures were 26 and 22°C, respectively. With the exception of the uninoculated controls, each pot was inoculated at planting with 10^9 cells of an early-stationary-phase culture. After 35 days, plants were harvested to evaluate acetylene reduction activity (11), nodulation, and shoot dry matter production.

Bacteroid ultrastructure. Transmission electron microscopy was used to compare thin sections of nodules formed by Or 191 with those of nodules formed by the symbiotically effective strains *R. meliloti* 102F34 and *Rhizobium* sp. (*Phaseolus*) 127K12b (on alfalfa and bean, respectively). Nodules were excised 35 days after planting and maintained in fixative (1% [wt/vol] paraformaldehyde and 2% [vol/vol] glutaraldehyde in 50 mM sodium cacodylate buffer at pH 6.5) for 4 h at 4°C. After buffer rinses, the tissue was fixed in 2% (wt/vol) osmium tetroxide, dehydrated in an acetone series, and embedded in Spurr epoxy resin. Thin sections were obtained from the late symbiotic zone (proximal zone III) of the alfalfa nodules, as defined by Vance et al. (29) and Vasse et al. (30). Comparable sections were also obtained from the central cores of 5-week-old bean nodules. The sections were mounted on copper grids, stained with lead citrate and uranyl acetate, and viewed with a Zeiss 10 transmission electron microscope.

pH and temperature tolerance. The eight Oregon isolates, nine strains of *R. meliloti* (102F34, 102F51, 102F82, 102F84, 102F85, 3-3, 27-2, L5-30, and 1021), and nine strains of *Rhizobium* sp. (*Phaseolus*) (CIAT 57, CIAT 134, CIAT 144, CIAT 676, CIAT 899, 127K12b, Olivia, Tal 182, and Viking 1) were screened for the ability to tolerate low pH by the modified YEM agar plate method of Cunningham and Munns (4). The agar pH was adjusted to seven levels (pH 4.4, 4.6, 4.8, 5.0, 5.2, 5.6, and 6.4) by the addition of sterile 1 N HCl to autoclaved media cooled to 55°C. Because dense inocula sometimes produced false-positive results, a drop-plate method was used to produce dilute inoculant suspensions (27). Growth was scored 10 days after inoculation. A water-jacketed incubator was used to screen strains for growth at 39°C.

Genomic DNA extraction and RFLP analysis. Genomic DNA was extracted from 7-ml stationary-phase broth cultures. The cells were pelleted, resuspended in 1 ml of Tris-EDTA (pH 8.0) (TE), and lysed in 2% (wt/vol) sodium dodecyl sulfate. After 40 μg of RNase A per ml was added, the suspension was incubated at 37°C for 10 min. The DNA was then precipitated by adding sodium acetate (to a final concentration of 250 mM) and isopropanol to 70% (vol/vol). The precipitate was collected on a hooked Pasteur pipette and resuspended in 400 μl of TE. Then 20 μg each of proteinase K and RNase A were added, and the solution was incubated for 3 h at 50°C. The preparation was extracted twice with equal volumes of phenol and three times with equal volumes of chloroform (16). The DNA in the supernatant was precipitated by adding 0.5 volume of 10.5 M ammonium acetate and then ethanol to 70% (vol/vol). The DNA was dried at room temperature, rinsed in 70% (vol/vol) ethanol, dried again, and resuspended to a final concentration of approximately 0.1 μg/μl in TE. The DNA in this preparation was used as template in polymerase chain reactions (PCRs) and for genomic digests in restriction fragment length polymorphism (RFLP) analyses.

DNA for the *nifH* RFLP analysis was digested with *Bam*HI and separated by electrophoresis overnight on a 0.8% agarose gel (17). Segments containing part or all of the *nifH* gene were identified by Southern hybridization (16). The *nifH* hybridization probe was an *Xho*I fragment excised from the plasmid p*Rm*R2:pACYC184 cloned in the *Escherichia coli* HB101 (23), which was provided by R. Wheatcroft, Agriculture Canada, Ottawa.

Sequencing DNA segments amplified by PCR. Two DNA

TABLE 1. Nucleotide sequences and positions of primers

Primer	Sequence	Position (bp)	Reference(s)
16S rRNA-1	5'-TGGCTCAGAACGAACGCTGGCGGC-3'	20	32, 33
16S rRNA-2	5'-CCCACTGCTGCCTCCCGTAGGAGT-3'	361	32, 33
nifH-1	5'-AAGTGCGTGGAGTCCGGTGG-3'	256	28
nifH-2	5'-GTTCGGCAAGCATCTGCTCG-3'	856	28
nifH-3	5'-GCCAACAACATCGCCAGGGGTAT-3'	484	28
nifH-4	5'-GCAGCCAGCGCTTCGGCGAG-3'	605	21
nifH-5	5'-GCGGCMAGTGCCTCGGCGAG-3'	605	28

segments, representing 300 bp of the 16S rRNA gene and 522 bp of the *nifH* gene, were separately amplified from each strain in standard 100-μl PCR mixtures under 50 μl of light mineral oil (24). The primers for PCR amplification (and subsequent nucleotide sequencing) are shown in Table 1. A DNA thermal cycler (Perkin-Elmer Cetus Instruments, Norwalk, Conn.) provided a 30-cycle amplification series; each cycle consisted of a 1-min denaturation step at 94°C, a 2-min primer-annealing step at 65°C, and a 2.5-min extension step at 72°C. Subsequently, the aqueous PCR mixture containing the amplified DNA was removed with a narrow pipette tip and concentrated (25) in a Centricon-30 microconcentrator (W. R. Grace Co., Danvers, Mass.).

Single-stranded templates for sequencing were prepared by the procedure of Higuchi and Ochman (12), and both strands were sequenced by the standard protocols in a Sequenase version 2.0 sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio).

RESULTS

Relative symbiotic effectiveness of Or 191. Although it was earlier reported that Or 191 was symbiotically ineffective (unable to fix dinitrogen) with alfalfa (6), our experiments showed that when the shoot of the host plant is not enclosed in the culture vessel, this strain is, in fact, capable of low levels of nitrogen fixation with both alfalfa and common bean (Table 2). However, the symbiotic potential of Or 191 with common bean was twice that with alfalfa. This symbiotic behavior was independent of the host cultivar tested (5a).

pH and temperature tolerance of the Oregon isolates. The stress tolerance characteristics of the eight Oregon field isolates resembled those of *Rhizobium* sp. (*Phaseolus*). Specifically, the Oregon isolates grew at pH 5.2 but not at 39°C. Similarly, most of the *Rhizobium* sp. (*Phaseolus*) strains grew at pH 5.2 (127K12b was the only exception) but not at 39°C (*R. tropici* CIAT 899 was the only exception). In contrast, the nine *R. meliloti* laboratory strains tested did not grow at pH 5.2 but did grow at 39°C.

Bacteroid ultrastructure of Or 191. The bacteroids produced by Or 191 did not strongly resemble those of either of the commercial inoculant strains (Fig. 1). This finding and the extensive cell lysis that was evident in the bacteroids produced by Or 191 (Fig. 1B and D) indicate that this strain is poorly adapted for nitrogen fixation with either of these host species (1, 29).

Nucleotide sequence analyses. The nucleotide sequence analysis involved comparisons of Or 191 with three alfalfa symbionts and three common bean symbionts. Pairwise comparisons were made between homologous 260-bp *16S* gene segments (sequences available under GenBank accession numbers M55233 through M55236 and M55241 through M55243) and homologous 492-bp *nifH* segments (sequences available under GenBank accession numbers M55225 through M55232).

There were 18 polymorphic nucleotide sites among the seven *16S* sequences. The number of nucleotide differences between each pair of sequences is given in Table 3, which also presents the results of a comparison of each sequence with the published sequence of *R. leguminosarum* bv. phaseoli 8002 (33). The *16S* sequence of strain 8002 is the same as that of ATCC 10004, which is the type strain of *R. leguminosarum* (15). We also observed this same *16S* sequence in other strains of *R. leguminosarum* bv. viciae and *R. leguminosarum* bv. trifolii (33a). It is noteworthy that the *16S* sequence of Or 191 is similar to those of *Rhizobium* sp. (*Phaseolus*) strains Olivia-4 and FL-27 (isolated in Minnesota and Mexico, respectively [20]) and that the *16S* sequences of these three strains are as different from that of *R. leguminosarum* as are those of the recognized species *R. meliloti* and *R. tropici* (Table 3).

Southern hybridization of *Bam*HI genomic digests of the Oregon isolates with a *R. meliloti nifH* probe indicated that these strains contain only a single copy of the *nifH* gene (Fig. 2). The DNA sequence of this gene was not determined in its entirety, but a central segment 492 bp in length was determined for Or 191 and for three representative symbionts of both common bean and alfalfa. This segment corresponds to bp 313 through 804 of the published *nifH* sequence of *R.*

TABLE 2. Symbiotic characteristics of isolate Or 191 and commercial inoculant strains for alfalfa[a] and common bean[b]

Host	Treatment	No. of nodules per plant	Nodule dry wt (mg/plant)	Acetylene reduction sp act (μmol/h/g of nodule dry wt)	Shoot dry wt (mg/plant)	Relative symbiotic effectiveness[c] (%)
Alfalfa	Control[d]	0		0	23 ± 4	0
	Or 191	15 ± 2	5 ± 1	67 ± 8	32 ± 3	5
	102F34	11 ± 1	6 ± 1	437 ± 5	191 ± 21	100
	Fisher PLSD[e]	NS	NS	24	41	
Bean	Control[d]	0		0	168 ± 5	0
	Or 191	347 ± 15	702 ± 31	71 ± 5	296 ± 58	13
	127K12b	617 ± 54	805 ± 11	112 ± 22	1,177 ± 52	100
	Fisher PLSD[e]	137	80	NS	144	

[a] Values are means (± standard errors) of four replicate samples (containing six plants each) per treatment.
[b] Values are means (± standard errors) of four plants per treatment.
[c] $[(x - x_u)/(x_e - x_u)]100$; where x, x_u, and x_e are mean shoot dry weights of plants receiving a specified *Rhizobium* strain, uninoculated control plants, and effectively nodulated plants, respectively.
[d] Uninoculated control plant.
[e] Fisher (protected) least significant difference ($P \leq 0.05$). NS, nonsignificant differences.

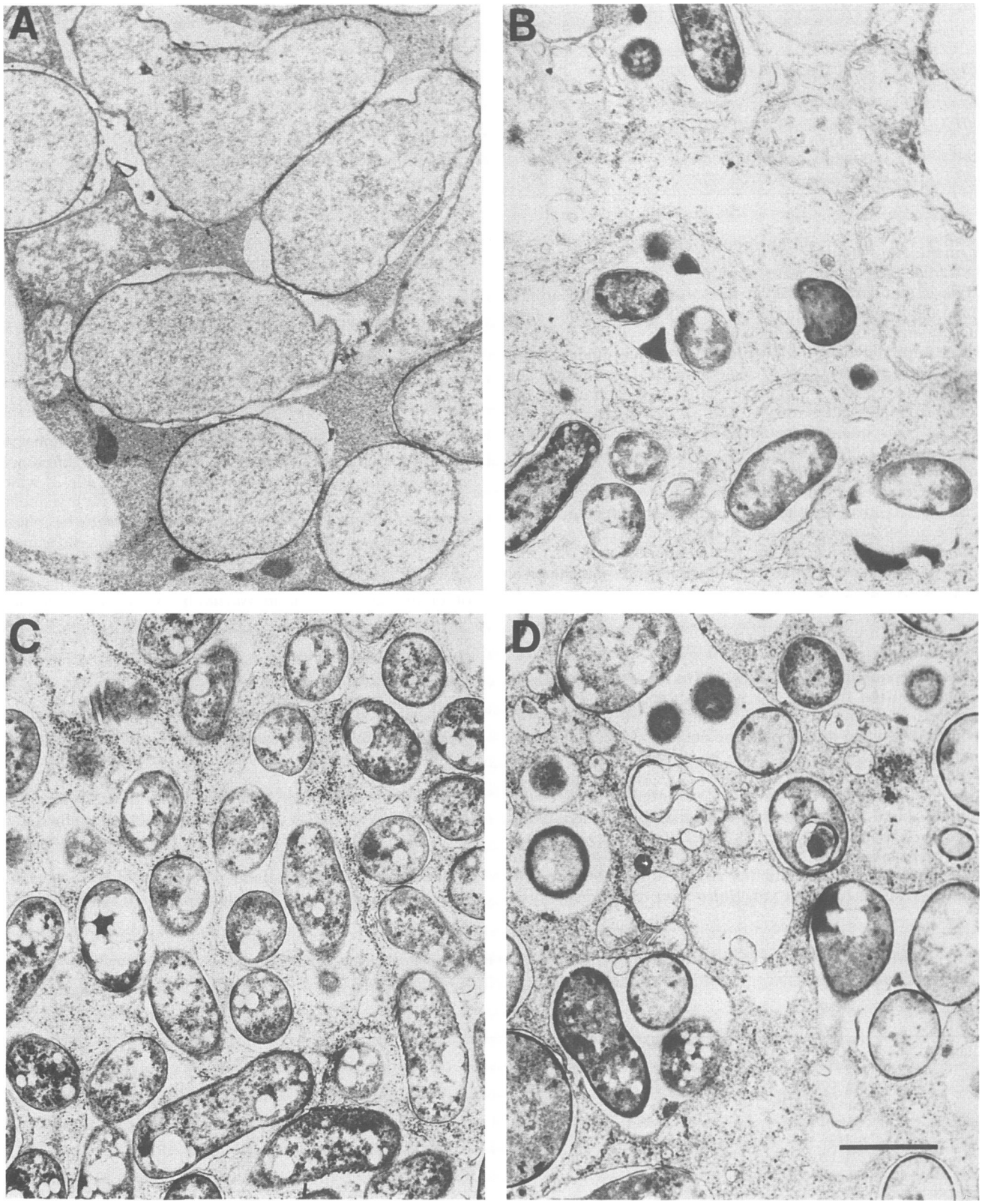

FIG. 1. Electron micrographs of nodule thin sections for comparison of bacteroids in alfalfa and common bean. (A) Late symbiotic zone (or proximal zone III) of an alfalfa nodule formed by *R. meliloti* 102F34. (B) Late symbiotic zone of an alfalfa nodule formed by Or 191. (C) Central core section of a bean nodule formed by *R. leguminosarum* bv. phaseoli 127K12b. (D) Central core section of a bean nodule formed by Or 191. Bar, 1.0 μm.

TABLE 3. Pairwise nucleotide sequence distance matrix[a]

Rhizobium species	Strain	No. of sequence differences[b]						
		1	2	3	4	5	6	7
1 *Rhizobium* sp. (*Medicago*)	Or 191		**69**	**61**	**64**	**61**	**63**	**75**
2 *Rhizobium* sp. (*Phaseolus*) type I	Olivia-4	2		**21**	**75**	**69**	**66**	**52**
3 *Rhizobium* sp. (*Phaseolus*)	FL-27	4	2		**70**	**64**	**62**	**47**
4 *R. meliloti* division A	ATCC 9930	10	8	8		**15**	**20**	**74**
5 *R. meliloti* division A	CC 2013	10	8	8	0		**21**	**66**
6 *R. meliloti* division B	CC 169	11	9	9	1	1		**66**
7 *R. tropici*	CIAT 899	14	12	10	8	8	9	
8 *R. leguminosarum* bv. phaseoli[c]	8002	11	9	9	7	7	8	7

[a] The data for a 492-bp segment of the *nifH* gene are shown in boldface type, and those for a 260-bp segment of the 16S rRNA gene are shown in lightface type.

[b] Values are the numbers of nucleotide sequence differences between the corresponding (numbered row and column) sequences.

[c] The sequence of the 16S rRNA gene of *R. leguminosarum* bv. phaseoli 8002 was previously published (33).

meliloti (28). Among these seven sequences, 128 of the 492 nucleotide sites were polymorphic; at most (84%) of these sites, the polymorphism involved synonymous substitution.

Pairwise differences among the *nifH* nucleotide sequences are shown in Table 3. The number of *nifH* sequence differences between Or 191 and the other strains ranged from 61 to 75 (Table 3), which is similar to the level of *nifH* sequence divergence observed among *R. meliloti*, *Rhizobium* sp. (*Phaseolus*), and *R. tropici*. In contrast, there were only 15 to 21 *nifH* sequence differences among the three strains of *R. meliloti* and only 21 differences between the two strains of *Rhizobium* sp. (*Phaseolus*). The partial *nifH* sequence determined for the type I *Rhizobium* sp. (*Phaseolus*) strain Olivia-4 was identical to the published sequence for strain CFN 42 (21), which is the best-studied representative of the type I *Rhizobium* sp. (*Phaseolus*) strains (8, 17, 18, 20). The sequence of ATCC 9930, the type strain of *R. meliloti*, differed at only four sites from the published *nifH* sequence for *R. meliloti* 41 (28).

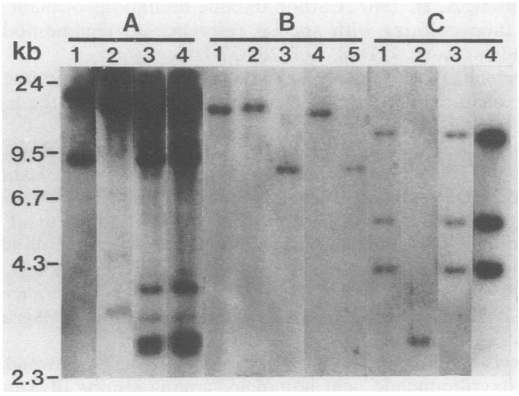

FIG. 2. Southern hybridization of a *R. meliloti nifH* gene segment with *Bam*HI-digested genomic DNA from various strains. (A) *R. meliloti* strains. Lanes: 1, 102F85; 2, 102F84; 3, 27-2; 4, 3-3. (B) Oregon field isolates. Lanes: 1, Or 191; 2, Or 188; 3, Or 172; 4, Or 084; 5, Or 082. (C) *P. vulgaris* symbionts. Lanes: 1, 127K12b; 2, CIAT 899 (type strain of *R. tropici*); 3, CIAT 144; 4, CIAT 57.

DISCUSSION

In our experiments, strain Or 191 was able to form nitrogen-fixing nodules on both alfalfa and common bean. This is the first report of a rhizobial strain that is able to fix nitrogen with both of these hosts, and this finding contradicts the original description of this strain, which indicated that it formed only ineffective nodules on alfalfa (6). The explanation for this discrepancy appears to lie in the different plant culture conditions that were employed in the two studies. In the original study, alfalfa was grown under conditions (on agar slants in glass tubes) that were probably carbon dioxide limited for the plants (9), whereas in the present study plants were grown in open pots. Shoot dry weights were several-fold greater with the open system, and the improved culture conditions resulted in measurable levels of symbiotic nitrogen fixation by Or 191 with both hosts (Table 2). It is apparent, however, that this strain is not well adapted for nitrogen fixation with either host species.

The primary objective of the present study was to determine the evolutionary relationships of Or 191 and the other Oregon isolates to a representative range of alfalfa and common bean symbionts. The assumption prevalent at the initiation of our work was that all the symbionts of alfalfa belong to one species, *R. meliloti*, and those of common bean belong to another species, *R. leguminosarum*. Hence, at the time, the most plausible interpretation seemed to be that the Oregon isolates represented either a host-range variant of one of these species or a hybrid between them. But the results of our study revealed a different picture.

In the introduction, we cited several studies indicating that the symbionts of *M. sativa* are a coherent group in terms of genetic relatedness, whereas those of *P. vulgaris* are markedly heterogeneous. The nucleotide sequence comparisons presented in Table 3 are consistent with this conclusion, inasmuch as the three strains of *R. meliloti* are similar in both their *16S* and *nifH* sequences, whereas certain *P. vulgaris* symbionts differ markedly from each other in their sequences for these same genes. Because only a single variable nucleotide site was found among the three *R. meliloti 16S* sequences, and because no *16S* sequence variation has thus far been detected in *R. leguminosarum*, our analysis suggests that strains differing in more than a few nucleotides are probably too distant phylogenetically to be placed in the same species.

Martinez-Romero et al. (18) recently proposed a new species, *R. tropici*, for those strains isolated from *P. vulgaris* that had previously been called type II strains. They showed that these strains differ from the common type I *Phaseolus*-nodulating strains in many ways, including in a nucleotide sequence of part of the 16S rRNA gene. (The segment sequenced by these workers differs from that which we studied.) Their conclusion is clearly supported by our results: as shown in Table 3, the *16S* and *nifH* sequences of CIAT 899 (the type strain of *R. tropici*) are markedly divergent from those of Olivia-4 (a type I strain) and another *P. vulgaris* symbiont, FL-27. Indeed, the *16S* sequence of CIAT 899 differs more from those of the other two *P. vulgaris* symbionts than it does from the *16S* sequences of the *R. meliloti* strains. Martinez-Romero et al. (18) refer to the type I strains as *R. leguminosarum* bv. phaseoli, according to current convention, but the novel finding of our study is that neither the type I strains nor the Oregon isolates belong to *R. leguminosarum*.

The type strain of *R. leguminosarum* was not studied by Martinez-Romero et al. (18) and was not included in an

earlier survey of genetic variation in *Rhizobium* sp. (*Phaseolus*) by multilocus enzyme electrophoresis (20). Consequently, although Martinez-Romero et al. (18) clearly showed that there are major phylogenetic divisions among *Phaseolus*-nodulating symbionts, they had no means of determining which, if any, of the groups that they identified correspond to *R. leguminosarum*. We sequenced a segment of the 16S rRNA gene of strain 8002, which was recovered from *P. vulgaris* in England (33). We are confident that this is an authentic example of *R. leguminosarum* bv. phaseoli, because ATCC 10004, which is the type strain of *R. leguminosarum* (and also represents *R. leguminosarum* bv. viciae), has precisely the same sequence in the corresponding segment of its 16S rRNA gene (32a). Moreover, strain 8002 has a chromosomal genotype, MFF/B (as defined by multilocus enzyme electrophoresis and RFLP analysis), that is common in British isolates of all three biovars of *R. leguminosarum* (34). However, the sequence of the 16S rRNA gene in strain 8002 is quite distinct from those of the *16S* genes of the type I strain (Olivia-4), the type II strain (CIAT 899), and unassigned (FL-27) *P. vulgaris* symbionts that we have examined. We conclude, therefore, not only that *R. tropici* is a justifiable new taxon for the type II strains but also that the type I *P. vulgaris* symbionts need to be reclassified as well, for they are at least as distant phylogenetically from authentic *R. leguminosarum* as are the type II strains. Formal proposal of a new name for these strains should await a comparison of additional chromosomal loci in more strains; however, recognition of the fact that these strains are members of a distinct phylogenetic group is important for our understanding of the genetic nature and the evolutionary relationships among *P. vulgaris* symbionts.

Although the four taxa represented in Table 3, *R. meliloti*, *Rhizobium* sp. (*Phaseolus*), *R. tropici*, and *R. leguminosarum*, are all distinct and equally differentiated in *16S* sequence, they are much more closely related to each other than to species of the related genera *Agrobacterium*, *Brucella*, and *Rochalimaea* (33). It is therefore justifiable, in terms of phylogenetic coherence, to retain them all in the genus *Rhizobium*.

In the sequence of its 16S rRNA gene, strain Or 191 is quite similar to the type I *Rhizobium* sp. (*Phaseolus*) strain Olivia-4. This similarity is reinforced by the observation that, like most type I strains, it can grow at pH 5.2 but not at 39°C. However, in contrast to the similarity we observed in phenotypic characteristics and *16S* gene sequences between these strains, the sequence of the *nifH* gene of Or 191 is substantially different from other published *nifH* sequences and also from rhizobial *nifH* sequences that we have determined thus far. Although the *nifH* sequence of Or 191 falls within the range of variation found within the genus *Rhizobium*, it is no more similar to the *nifH* genes of *R. meliloti*, *R. tropici*, or the *Rhizobium* sp. (*Phaseolus*) than the *nifH* genes in these strains are to each other. It should also be noted that type I strains of *Rhizobium* sp. (*Phaseolus*) usually carry three copies of the *nifH* gene (17, 21), whereas our evidence indicates that Or 191 carries only one copy (Fig. 2). Thus, although Or 191 may be related to the type I *Rhizobium* sp. (*Phaseolus*) strains in terms of its chromosomal genotype (as represented by the 16S rRNA gene), it has a distinctive Sym plasmid genotype (as represented by its *nifH* gene).

These observations indicate that phylogenies based on Sym plasmid genes cannot be expected to correspond to phylogenies based on chromosomal genes. Presumably this is due, at least in part, to the fact that Sym plasmid genes can move across chromosomal backgrounds by horizontal transfer between strains (2, 26, 34). For this reason, it is important to distinguish between the evolutionary histories of plasmids and the evolutionary histories of chromosomes in phylogenetic studies of bacteria, and it is also important to base conclusions on taxonomic relatedness on characteristics known to be chromosomally encoded.

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