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Expression of *tfx* and Sensitivity to the Rhizobial Peptide Antibiotic Trifolitoxin in a Taxonomically Distinct Group of α-Proteobacteria Including the Animal Pathogen *Brucella abortus*

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Three phylogenetically distinct groups within the α-proteobacteria which differ in trifolitoxin sensitivity are described. Trifolitoxin sensitivity was found in strains of *Agrobacterium*, *Brucella*, *Mycoplana*, *Ochrobactrum*, *Phyllobacterium*, *Rhodobacter*, *Rhodopseudomonas*, *Rhodospirillum*, and *Rhizobium*. Strains of *Agrobacterium*, *Brucella*, *Phyllobacterium*, *Rhizobium*, and *Rhodospirillum* were capable of producing trifolitoxin upon conjugal transfer of *tfxABCDEFG*.

Triplett and Barta (24) found that trifolitoxin (TFX), a posttranslationally modified peptide antibiotic, inhibited only strains of *Rhizobium leguminosarum* and *Rhizobium fredii*. Bradyrhizobia, plant pathogens, and enteric bacteria were not inhibited by TFX. By comparison of the sequences of 16S rRNA genes and other analyses, a number of very interesting bacteria have been found to be close relatives of the genus *Rhizobium* (10, 16, 29–32). These include members of the leaf-nodulating genus *Phylobacterium* (14); intracellular animal pathogens such as *Bartonella*, *Brucella*, and *Rochalimaea* spp. (4, 6, 17, 18, 26); soilborne and aquatic diazotrophs such as *Beijerinckia*, *Blastobacter*, and *Mycoplana* spp.; *Ochrobactrum anthropi*, a symbiont of nematodes (1) which can cause bacteremia in humans (11); and photosynthetic diazotrophs such as *Rhodobacter*, *Rhodospirillum*, and *Rhodopseudomonas* spp.

In this work, the TFX sensitivities of species within the α-proteobacteria were determined. The construction, replicons, inserts, and references of the plasmids used in this work are listed in Table 1. DNA isolations and manipulations were primarily performed as described by Sambrook et al. (19). Bacterial strains and culture media used in this work are included in Table 2. Antibiotic concentrations used were described previously (8) except that the tetracycline concentration used was 1.5 μg/ml. *Brucella* strains were cultured on *Brucella* agar medium (0964-01-3; Difco) at 37°C with 5% CO_2 or at 28°C. All other α-proteobacteria were cultured at 28°C.

A modification of the triparental mating procedure described by Triplett et al. (25) was used here in the conjugation of all plasmids into α-proteobacteria. Cells were taken directly from stocks frozen at −70°C in 15% glycerol except *Escherichia coli* strains and *Brucella abortus* RB51 (20), which were

TABLE 1. Plasmids used in testing TFX sensitivity and in the transfer of *tfx* to various α-proteobacteria

Plasmid	Replicon (reference) and insertion site	Deletion or gene(s) added to replicon	Antibiotic resistance phenotype[a]	Reference
pTFX3[b]	pTFX2 (25); *Hpa*I deletion	Deletion of internal *Hpa*I fragment of Tn*5*	**Tc^r**	This work
pTFX23	pBluescript II KS$^+$ (2); *Hin*cII	7.1-kb *Mlu*I fragment of pTFX1 (22) containing *tfxABCDEFG*	Ap^r	8
pTFX24	pBluescript II KS$^+$ (2); *Hin*cII	Same as pTFX23 but in opposite orientation	Ap^r	8
pTFX24K	pTFX24 (8); *Xho*I	Insertion of 1.2-kb *Hin*cII fragment from pUX-BF5 (5) containing *npt*II	Ap^r Km^r	This work
pTX24K	pTFX24K; *Not*I deletion	Deletion of 1.5-kb *Not*I fragment containing *tfxAB'*	Ap^r Km^r	This work
pDTXC-12	pDSK519 (12); *Eco*RI, *Sst*I	5.8-kb *Not*I fragment of pTFX23 containing *tfxB'CDEFG*	**Km^r** TFX^r	This work
pDT42[b]	pDSK519 (12); *Eco*RI, *Sst*I	7.2-kb *Bss*HII fragment of pTFX24 containing *tfxABCDEFG*	**Km^r** TFX^r	This work
pT2TX3K	pTR102 (28); *Bam*HI	6.8-kb *Bss*HII fragment of pTX24K containing *tfxB'CDEFG*	Ap^r **Km^r Tc^r** TFX^r	This work
pT2TFXK[b]	pTR102 (28); *Bam*HI	8.3-kb *Bss*HII fragment of pTFX24K containing *tfxABCDEFG* and *npt*II	Ap^r **Km^r Tc^r** TFX^r	This work

[a] Antibiotic resistances in bold were used to select against recipients in triparental matings with α-proteobacteria.
[b] Plasmid confers TFX production in *Rhizobium* spp. upon transfer by conjugation.

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TABLE 2. TFX sensitivities of selected α-proteobacteria

Organism	Growth inhibition (% of control)[a] by TFX from:	
	T24	KIM5s(pT2TFXK)
Agrobacterium radiobacter ATCC 19358[b]	0	0
C58[b]	0	0
Agrobacterium rhizogenes ATCC 11325[b]	75	109
Agrobacterium rubi TR3[b]	2	2
Agrobacterium vitis CG-48[b]	13	19
CG-64[b]	208	22
Azorhizobium caulinodans ATCC 43989[b]	0	0
Beijerinckia indica ATCC 9037[b]	0	0
Blastobacter denitrificans ATCC 43295[b]	0	0
Bradyrhizobium elkanii 61A76[b]	0	0
Bradyrhizobium japonicum 110Spc[b]	0	0
Mycoplana dimorpha ATCC 4279[b]	74	118
Ochrobactrum anthropi ATCC 49188[b]	30	52
ATCC 49188[c]	0	23
Phyllobacterium myrsinacearum ATCC 43590[b]	0	0
ATCC 43590[c]	5	23
Phyllobacterium rubiacearum ATCC 43591[b]	4	32
ATCC 43591[c]	16	58
Rhizobium etli USDA9032[b]	91	133
USDA9041[b]	167	158
USDA9043[b]	135	164
Rhizobium galegae USDA4128[b]	0	0
USDA4130[b]	13	9
USDA4136[b]	0	8
Rhizobium huakuii USDA4773[b]	9	39
USDA4776[b]	0	4
USDA4778[b]	0	0
Rhizobium leguminosarum 128C1[d]	100	100
Rhizobium meliloti 102F3[b]	3	15
1021[b]	0	0
Rhizobium tropici USDA9039[b]	0	0
USDA9030[b]	0	30
Rhizobium sp. strain ANU280[b]	45	64
Rhodobacter capsulatus B10[e]	0	0
Rhodobacter sphaeroides 2.4.1[e]	68	78
Rhodopseudomonas marina BN126[e]	18	35
Rhodopseudomonas palustris RPI[e]	0	0
Rhodospirillum rubrum UR2[e]	19	28

[a] Values are percentages of the area of the inhibition zone obtained with *R. leguminosarum* bv. viceae 128C1. Sources of TFX production were the producing strains T24 and KIM5s(pT2TFXK). No zones of inhibition were observed with the nonproducing strains T24::Tn*5*$_1$ and KIM5s(pT2TX3K) (data not shown).
[b] BSM (7) was used to culture test strains and the TFX-producing strains.
[c] YM (27) medium was used to culture test strains and the TFX-producing strains.
[d] 128C1 was cultured on the same medium as the test strains.
[e] ATCC medium 1139 was used to culture test strains and the TFX-producing strains.

TABLE 3. TFX production by transconjugants of *Agrobacterium*, *Brucella*, *Phyllobacterium*, *Rhizobium*, and *Rhodospirillum* spp.[a]

Species and transconjugant[b]	Area of net inhibition zone (mm^2)	
	128C1	102F3
Agrobacterium rhizogenes ATCC 11325(pDT42)	2,312	69
ATCC 11325(pDTXC-12)	0	0
Brucella abortus RB51(pDT42)	1,979	0
RB51(pDTXC-12)	0	0
Phyllobacterium rubiacearum ATCC 43591(pDT42)	1,477	59
ATCC 43591(pDTXC-12)	0	0
Rhizobium etli USDA9043(pDT42)	2,046	542
USDA9043(pDTXC-12)	0	0
Rhizobium galegae USDA4128(pDT42)	2,338	51
USDA4128(pDTXC-12)	0	0
USDA4130(pDT42)	2,604	623
USDA4130(pDTXC-12)	0	0
Rhizobium huakuii USDA4773(pDT42)	2,763	537
USDA4773(pDTXC-12)	0	0
Rhizobium leguminosarum bv. phaseoli KIM5s(pT2TFXK)	2,457	478
KIM5s(pT2TX3K)	0	0
Rhizobium leguminosarum bv. trifolii T24	1,913	41
T24::Tn*5*$_1$	0	0
Rhizobium tropici USDA9030(pDT42)	1,746	59
USDA9030(pDTXC-12)	0	34
USDA9039(pDT42)	2,425	453
USDA9039(pDTXC-12)	0	0
Rhodospirillum rubrum UR2(pTFX3)	528	0

[a] Rifampin (50 μg/ml) and streptomycin were used to select against the *E. coli* donor in the *Brucella* and *Rhodospirillum* matings, respectively. In all other matings, the donor was selected against by using a minimal medium (7) in noble agar, which supports only poor growth of *E. coli* and good growth of the α-proteobacteria.
[b] For each strain number, the first and second transconjugant are TFX producing and non-TFX producing, respectively.

stored in Luria-Bertani broth and *Brucella* broth, respectively, each in 15% glycerol. The medium used to interrupt the matings was that used to culture the recipient (Table 2).

The TFX sensitivity assay used was as described previously (8) with minor modifications. The sources of TFX production were *R. leguminosarum* bv. trifolii T24 and *R. leguminosarum* bv. phaseoli KIM5s(pT2TFXK). The latter overproduces TFX compared with T24. This is presumably the result of multiple copies of pT2TFXK in the cell. As controls for TFX production in these experiments, we included T24::Tn*5*$_1$ (24) and KIM5s(pT2TX3K). Turbid suspensions (5 μl) at an optical density at 600 nm of 1 from the four strains were spotted in the center of agar plates containing the appropriate medium. After 2 days at 28°C, the plates were sprayed with a slightly turbid suspension (optical density at 600 nm of 0.1) of the strain to be tested for TFX sensitivity. No medium on which both *Brucella* and *Rhizobium* strains would grow well was found. Thus, TFX sensitivity of the *Brucella* strains was determined by using *Brucella* agar as an overlay on YM (27) plates which had been spotted with rhizobia 2 days earlier. After 2 days at 28°C, the area of the center colony was subtracted from the area of the inhibition zone to determine the net area of inhibition. In most assays, *R. leguminosarum* bv. viceae 128C1 was assayed along

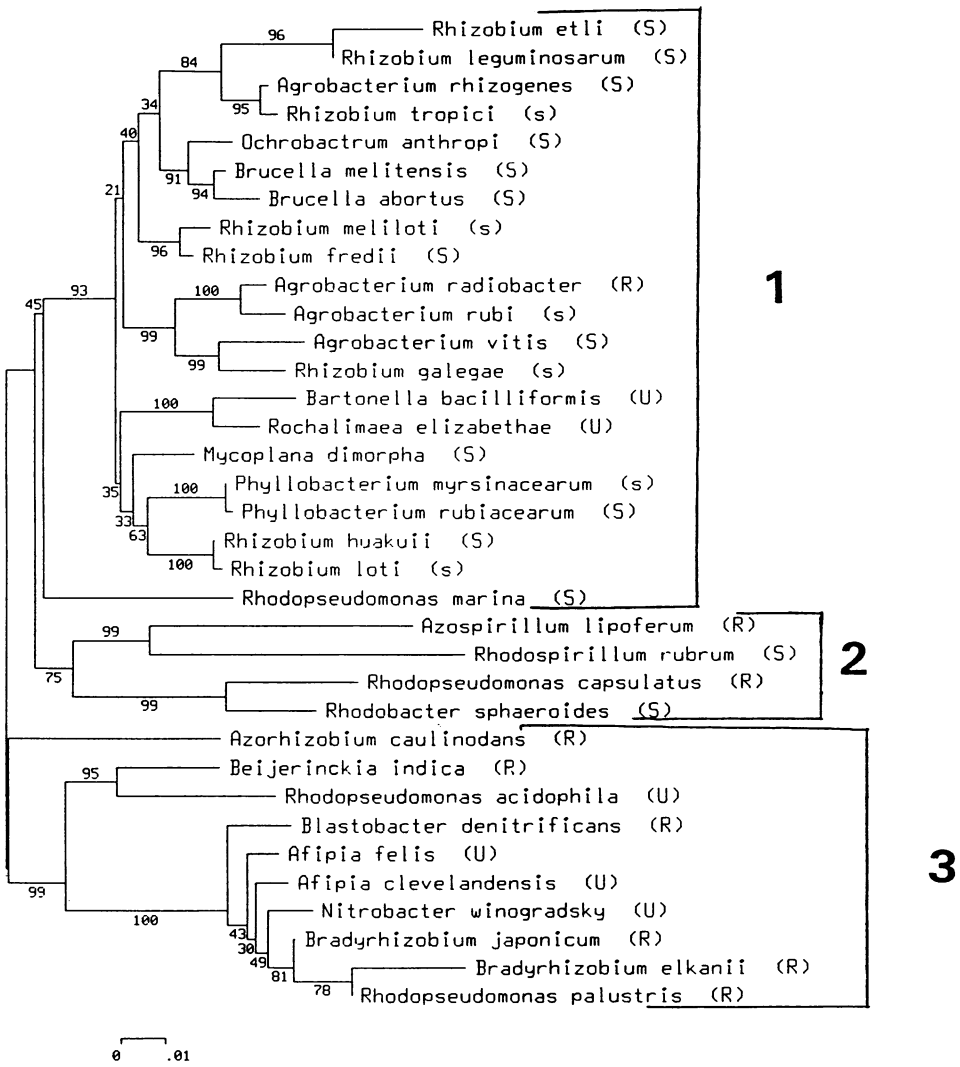

FIG. 1. Phylogenetic tree of full-length 16S rRNA sequences of selected α-proteobacteria prepared from sequences in the GenBank database by using the PILEUP program of the Genetics Computer Group package (3). The pairwise distance matrix and phylogenetic tree were constructed by using the Jukes-Cantor algorithm and the Neighbor-Joining method, respectively, in the MEGA package (13). Species names are flanked by a letter indicating sensitivity to TFX at levels produced by T24 (S) or an overproducing strain(s), resistance to TFX at either level of production (R), or unknown TFX sensitivity (U). Groups 1, 2, and 3 specify clusters of species which differ in TFX sensitivity. Group 1 and 3 species are nearly always TFX-sensitive and -resistant, respectively. Group 2 species are either sensitive or resistant to TFX. Confidence levels (percentages) shown above each node were generated from 500 bootstrap trees. The numbers of changes per sequence position are represented on the distance scale assuming a median rate of change. GenBank accession numbers for the 16S rRNA sequences are D12782, D12784, D12786, D12788, D12789, D12790, D12791, D12792, D12793, D12794, D12795, D12797, D12798, L01260, L11661, L11664, L20762, L26166, M27534, M32020, M34218, M34129, M55490, M59060, M59061, M65248, M65249, M69186, S46917, X13695, X53853, X66024, X67221, X67228, and X67228.

with other strains to provide an internal standard for our quantitative assay for TFX sensitivity.

The TFX sensitivities of several genera within the α-proteobacteria were determined (Table 2). Since our last survey of TFX inhibition of *Rhizobium* strains in 1987 (24), four new species of *Rhizobium* have been described. Strains of all known species of *Rhizobium* are inhibited by TFX at levels produced by either T24 or overproducing strains (Table 2). Several *Agrobacterium* strains were previously shown to be resistant to T24 levels of TFX (24). In this work, strains of *Agrobacterium rhizogenes* and *Agrobacterium vitis* were very sensitive to T24 levels of TFX and a strain of *Agrobacterium rubi* was sensitive to the overproducing strain (Table 2). Other α-proteobacterium species which are sensitive to T24 levels of TFX were found, including *Brucella abortus*, *Brucella melitensis*, *Mycoplana dimorpha*, *O. anthropi*, *Phyllobacterium rubiacearum*, *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, and *Rhodopseudomonas marina* (Tables 2 and 3). *Phyllobacterium myrsinacearum* is sensitive only to overproduction of TFX (Table 2). *Brucella abortus* RB51 was sensitive to T24 and KIM5s, with net inhibition zone areas of 537 and 844 mm^2, respectively. These data are not included in Table 2 because it was not possible to measure 128C1 and RB51 inhibition on the same medium.

Upon transfer of *tfx* to several *Rhizobium* strains by conjugation, TFX was produced by members of the four new species of *Rhizobium*: *Rhizobium etli*, *Rhizobium galegae*, *Rhizobium huakuii*, and *Rhizobium tropici* (Table 3). TFX production by *Brucella abortus*, *A. rhizogenes*, *P. rubiacearum*, and *Rhodospi-*

rillum rubrum following transfer of either pDT42 or pTFX3 was also observed (Table 3).

The phylogeny of TFX sensitivity correlates well with the phylogeny of the α-proteobacteria on the basis of 16S rRNA sequences (Fig. 1). There are three groups within the α-proteobacteria which differ in TFX sensitivity (Fig. 1). Group 1 species, with rare exceptions, are sensitive to TFX. Group 2 includes species that differ widely in TFX sensitivity. Group 3 includes only strains which are resistant to TFX. Strains within this third group are often resistant to many antibiotics (9, 15). Thus, the taxonomic limits of TFX sensitivity are confined to two groups within α-proteobacteria which are closely related genetically but inhabit very different environments. This allows us to predict whether a species within the α-proteobacteria is sensitive to TFX. For example, we predict that group 1 bacteria such as the animal pathogens *Rochalimaea* and *Bartonella* spp. are inhibited by TFX while group 3 genera such as *Afipia* and *Nitrobacter* are resistant to TFX.

An understanding of the taxonomic relationships of antibiotic-producing organisms can lead to entirely new applications for antibiotics. A potential agricultural application of the TFX system as a means to limit nodulation of legume roots by indigenous, TFX-sensitive rhizobia has been described (21–24). The ability of TFX to inhibit *Agrobacterium*, *Brucella*, and *Ochrobactrum* spp. may lead to new treatments for infections with these animal and plant pathogens. The ability of *Brucella abortus* RB51(pDT42) to produce TFX shows that genes from *Rhizobium* spp. can be expressed in *Brucella* spp., permitting extensive genetic comparisons between these two genera. Also, the use of TFX-producing strains under agricultural conditions may have broad ecological implications because a far wider spectrum of bacteria are inhibited than previously thought.

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REFERENCES

1. **Aguillera, M. M., N. C. Hodge, R. E. Stall, and G. C. Smart, Jr.** 1993. Bacterial symbionts of *Steinernema scapterisci*. J. Invertebr. Pathol. **62:**68–72.
2. **Alting-Mees, M. A., and J. M. Short.** 1989. pBluescript II: gene mapping vectors. Nucleic Acids Res. **17:**9494.
3. **Anonymous.** 1994. Program manual for the Wisconsin Package, version 8, August 1994. Genetics Computer Group, Madison, Wis.
4. **Ariasstella, J., R. A. Erlandson, and P. H. Lieberman.** 1986. Histology, immunohistochemistry, and ultrastructure of the verruga in Carrion's disease. Am. J. Surg. Pathol. **10:**595–610.
5. **Bao, Y., D. P. Lies, H. Fu, and G. P. Roberts.** 1991. An improved Tn7-based system for the single-copy insertion of cloned genes into chromosomes of Gram-negative bacteria. Gene **109:**167–168.
6. **Benson, L. A., G. M. Ihler, S. Kar, and G. McLaughlin.** 1986. Entry of *Bartonella bacilliformis* into erythrocytes. Infect. Immun. **54:**347–353.
7. **Bergersen, F. J.** 1961. The growth of *Rhizobium* in synthetic media. Aust. J. Biol. Sci. **14:**349–360.
8. **Breil, B. T., P. W. Ludden, and E. W. Triplett.** 1993. DNA sequence and mutational analysis of genes involved in the production and resistance of the antibiotic peptide trifolitoxin. J. Bacteriol. **175:**3693–3702.
9. **Cole, M. A., and G. H. Elkan.** 1979. Multiple antibiotic resistance in *Rhizobium japonicum*. Appl. Environ. Microbiol. **37:**867–870.
10. **De Lay, J., W. Mannheim, P. Segers, A. Lievens, M. Denijn, M. Vanhoucke, and M. Gillis.** 1987. Ribosomal ribonucleic acid cistron similarities and taxonomic neighborhood of *Brucella* and CDC group Vd. Int. J. Syst. Bacteriol. **37:**35–42.
11. **Gransden, W. R.** 1992. Seven cases of bacteremia due to *Ochrobactrum anthropi*. Clin. Infect. Dis. **15:**1068–1069.
12. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene **70:**191–197.
13. **Kumar, S., K. Tamura, and M. Nei.** 1993. MEGA: molecular evolutionary genetics analysis, version 1.0. Pennsylvania State University, University Park, Pa.
14. **Lersten, N. R., and H. T. Horner, Jr.** 1976. Bacterial leaf nodule symbiosis in angiosperms with emphasis on *Rubiaceae* and *Myrsinaceae*. Bot. Rev. **42:**145–214.
15. **Maurin, M., H. Lepocher, D. Mallet, and D. Raoult.** 1993. Antibiotic susceptibilities of *Afipia felis* in axenic medium and in cells. Antimicrob. Agents Chemother. **37:**1410–1413.
16. **O'Connor, S. P., M. Dorsch, A. G. Steigerwalt, D. J. Brenner, and E. Stackebrandt.** 1991. 16S rRNA sequences of *Bartonella bacilliformis* and cat scratch disease bacillus reveral phylogenetic relationships with the alpha-2 subgroup of the class *Proteobacteria*. J. Clin. Microbiol. **29:**2144–2150.
17. **Regnery, R. L., J. G. Olson, B. A. Perkins, and W. Bibb.** 1992. Serological response to *Rochalimaea henselae* antigen in suspected cat-scratch disease. Lancet **339:**8807.
18. **Relman, D. A., S. Falkow, P. E. LeBoit, L. A. Perkocha, K. W. Min, D. F. Welch, and L. N. Slater.** 1991. The organism causing bacillary angiomatosis, peliosis hepatitis, and fever and bacteremia in immunocompromised patients. N. Engl. J. Med. **324:**1514.
19. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Schurig, G. G., R. M. Roop II, T. Bagchi, S. Boyle, D. Buhrman, and N. Sriranganathan.** 1991. Biological properties of RB51; a stable rough strain of *Brucella abortus*. Vet. Microbiol. **28:**171–188.
21. **Schwinghamer, E. A., and R. P. Belkengren.** 1968. Inhibition of rhizobia by a strain of *Rhizobium trifolii*: some properties of the antibiotic and of the strain. Arch. Mikrobiol. **64:**130–145.
22. **Triplett, E. W.** 1988. Isolation of genes involved in nodulation competitiveness from *Rhizobium leguminosarum* bv. trifolii T24. Proc. Natl. Acad. Sci. USA **85:**3810–3814.
23. **Triplett, E. W.** 1990. Construction of a symbiotically effective strain of *Rhizobium leguminosarum* bv. trifolii with increased nodulation competitiveness. Appl. Environ. Microbiol. **56:**98–103.
24. **Triplett, E. W., and T. M. Barta.** 1987. Trifolitoxin production and nodulation are necessary for the expression of superior nodulation competitiveness by *Rhizobium leguminosarum* bv. trifolii T24. Plant Physiol. **85:**335–342.
25. **Triplett, E. W., M. J. Schink, and K. L. Noeldner.** 1989. Mapping and subcloning of the trifolitoxin production and resistance genes from *Rhizobium leguminosarum* bv. trifolii T24. Mol. Plant-Microbe Interact. **2:**202–208.
26. **Trujillo, I. Z., A. N. Zavala, J. G. Caceres, and C. Q. Miranda.** 1994. Brucellosis. Infect. Dis. Clin. N. Am. **8:**225–241.
27. **Vincent, J. M.** 1970. A manual for the practical study of root-nodule bacteria. International programme handbook no. 15. Blackwell Scientific Publications, Oxford.
28. **Weinstein, M., R. C. Roberts, and D. R. Helinski.** 1992. A region of the broad-host-range plasmid RK2 causes stable in planta inheritance of plasmids in *Rhizobium meliloti* cells isolated from alfalfa root nodules. J. Bacteriol. **174:**7486–7489.
29. **Willems, A., and M. D. Collins.** 1993. Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA gene sequences. Int. J. Syst. Bacteriol. **43:**305–313.
30. **Yanagi, M., and K. Yamasato.** 1993. Phylogenetic analysis of the family Rhizobiaceae and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. FEMS Microbiol. Lett. **107:**115–120.
31. **Young, J. P. W.** 1992. Phylogenetic classification of nitrogen-fixing organisms, p. 43–86. *In* G. Stacey, R. H. Burris, and H. J. Evans (ed.), Biological nitrogen fixation. Chapman and Hall, New York.
32. **Young, J. P. W.** 1993. Molecular phylogeny of rhizobia and their relatives, p. 587–592. *In* R. Palacios, J. Mora, and W. E. Newton (ed.), New horizons in nitrogen fixation. Kluwer Academic, Dordrecht, The Netherlands.