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Analysis of 16S Ribosomal DNA Sequences of *Francisella* Strains and Utilization for Determination of the Phylogeny of the Genus and for Identification of Strains by PCR

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The 16S ribosomal DNAs (rDNAs) of two strains of *Francisella tularensis* and one strain of *Francisella philomiragia* were sequenced. On the basis of phylogenetic analysis data, the genus *Francisella* was placed in the γ subclass of the *Proteobacteria*. The most closely related organism was the intracellular bacterium *Wolbachia persica*. The sequenced 16S rDNA molecules of the *Francisella* species exhibited very high levels of similarity (98.5 to 99.9%). Two variable regions, comprising 390 to 450 nucleotides of the 16S rDNA molecules of 17 additional *Francisella* strains, including members of the species *F. tularensis* and *F. philomiragia*, were also sequenced. At most, six nucleotide differences were observed among the sequences of the *F. tularensis* strains. The sequence of *Francisella novicida* was virtually identical to the sequences of the *F. tularensis* strains, thereby supporting the hypothesis that these organisms are members of the same species. On the basis of the observed differences, primer pairs were designed to distinguish strains by using the PCR at the genus, species, and subspecies levels. This permitted sensitive identification of strains belonging to the genus *Francisella* and discrimination of the species *F. tularensis* and *F. philomiragia*.

Members of the genus *Francisella* are identified by biochemical characteristics and an unusual fatty acid composition (6). Two species, *Francisella tularensis* and *Francisella philomiragia*, have been recognized, and studies of *F. tularensis* have been responsible for virtually all of our knowledge concerning the genus. This organism is a facultatively intracellular bacterium and the causative agent of the zoonotic disease tularemia (25). There are two biovars of *F. tularensis* (20), and *F. tularensis* biovar tularensis, also designated type A, is the predominant biovar found in North America (20, 25); this organism is highly virulent in humans (20, 25). *F. tularensis* biovar palaearctica, also designated type B, occurs in Europe, Asia, and North America (20, 25); this biovar is less virulent in humans (5, 20, 25). The two biovars differ in only a limited number of biochemical characteristics, and serological studies have indicated that they are antigenically similar (15, 18, 20).

F. philomiragia is occasionally associated with disease in humans with compromised conditions (12). Its inclusion in the genus *Francisella* is based on similarities to *F. tularensis* in antigen and fatty acid compositions (12). Until recently, a third species, *Francisella novicida*, was recognized (6). However, because of phenotypic similarity of *F. tularensis*, the validity of this species has been questioned, and the designation *F. tularensis* biovar novicida has been suggested (12).

Currently used phenotypic criteria do not provide enough information to assess the relationship of *F. tularensis* to other bacterial genera, and the results of DNA hybridization experiments have not documented a close relationship between the genus *Francisella* and any of several other bacterial genera examined (6).

The lack of phylogenetic placement of the genus *Francisella* was the reason for undertaking this study. Our approach was to determine the 16S ribosomal DNA (rDNA) sequences of members of each of the two *Francisella* species. Phylogenetic analysis based on 16S rRNA and 16S rDNA sequences has been widely used (2–4, 10, 11, 17–19, 26).

Moreover, we wanted to determine whether a comprehensive analysis of the variable regions of the 16S rDNA molecule of *F. tularensis* would provide information that could clarify the relationship of strains within the species *F. tularensis*. We hoped not only to be able to define more precisely the validity of the species *F. novicida* (*F. tularensis* biovar novicida), but also to clarify the relationships of various *F. tularensis* strains.

MATERIALS AND METHODS

Bacteria, media, and growth conditions. The bacterial strains used in this study are listed in Table 1. These strains are part of our *Francisella* strain collection (FSC), which contains approximately 100 *Francisella* strains; strains have been generously donated to us by researchers from several countries. The available information about each strain was documented, and each strain was assigned a strain collection number (Table 1). We characterized all of the strains biochemically and by agglutination. With one exception, the strains used in this study were found to belong to the genus *Francisella*. The exception was strain FSC 059, which was identified as *Staphylococcus warneri* after it had been included in this study.

All *Francisella* strains were grown for 3 days on modified Thayer-Martin medium (22). Cell extracts of virulent *Francisella* strains (concentration, 10^9 bacteria per ml of saline) were prepared by heat treatment at 65°C for 2 h. All other bacterial strains were grown at 37°C in Luria-Bertani medium (1).

16S rDNA sequencing. Direct sequencing of amplified DNA was performed by using the method developed by Hultman et al. (13). The 16S rRNA gene to be sequenced was amplified by using prokaryotic 16S rDNA universal primers F1 and R13 (4). This set of primers was used in two separate pairs; in each pair, either F1 or R13 was biotiny-

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TABLE 1. Strains included in this study

Laboratory no.[a]	Species	Strain designation(s)[b]	Origin (trivial designation)[c]	Virulence for rabbits[d]	Nucleotide sequence accession no.[e]
1	*Francisella tularensis*	FSC 043	Human, Ohio (SCHU)	High	Z21932
2	*Francisella tularensis*	FSC 033	Squirrel, Georgia (SnMF)	High	Z22888, Z22889
3	*Francisella tularensis*	FSC 042	Hare, Canada (Utter)	High	Z22908, Z22909
4	*Francisella tularensis*	FSC 036	Beaver, Oregon (O-415)	NI[f]	
5	*Francisella tularensis*	FSC 041	Tick, Canada (Vavenby)	High	Z22898, Z22899
6	*Francisella tularensis*	FSC 058	Beaver, Montana (117)	NI	
7	*Francisella tularensis*	FSC 045	Squirrel monkey, United States	NI	
8	*Francisella tularensis*	FSC 046	Fox, Ohio	NI	Z22896, Z22897
9	*Francisella tularensis*	FSC 054	Tick, Nevada	High	Z22904, Z22905
10	*Francisella tularensis*	FSC 056	Eigelsbach strain, United States	Low	
11	*Francisella tularensis*	FSC 044	City water supply, United States (Helena)	Low	Z22902, Z22903
12	*Francisella tularensis*	ATCC 6223, FSC 138	Human, Utah	Low	Z22912, Z22913
13	*Francisella tularensis*	ATCC 29684, FSC 155	Live vaccine strain (LVS)	Low	Z21931
14	*Francisella tularensis*	FSC 108	Human, Sweden (SBL R45)	Low	Z22910, Z22911
15	*Francisella tularensis*	FSC 106	Human, Sweden (SBL R15)	Low	
16	*Francisella tularensis*	CCUG 17299, FSC 146	Human, Sweden	Low	Z22894, Z22895
17	*Francisella tularensis*	FSC 091	Human, Norway (9/15)	Low	
18	*Francisella tularensis*	FSC 025	Chatearoux, France	NI	
19	*Francisella tularensis*	FSC 026	Charney, France	NI	Z22921, Z22922
20	*Francisella tularensis*	FSC 151	Water, Russia (P-13863)	Low	Z22918, Z22919
21	*Francisella tularensis*	FSC 152	Common vole, Russia (P-13864)	Low	
22	*Francisella novicida*	ATCC 15482, FSC 040	Water, Utah	Low	Z22916, Z22917
23	*Francisella philomiragia*	ATCC 25017, FSC 153	Human, Utah		Z21933
24	*Francisella philomiragia*	CCUG 12603, FSC 145	Human, Sweden		
25	*Francisella philomiragia*	CDC E6588, FSC 154	Human, Switzerland		Z22890, Z22891
26	*Francisella philomiragia*	ATCC 25015, FSC 144	Muskrat, Utah		Z22900, Z22901
27	*Staphylococcus warneri*	CCUG 30782, FSC 059			
28	*Francisella tularensis*	FSC 090	Human, Japan (Jap4)	Low	Z22914, Z22915
29	*Francisella tularensis*	FSC 017	Human, Japan (S-2)	Low	
30	*Francisella tularensis*	FSC 022	Human, Japan (Ebina)	Low	Z22906, Z22907
31	*Francisella tularensis*	FSC 149	Gerbil, Middle Asia, CIS (120)[g]	Low	
32	*Francisella tularensis*	FSC 147	Hare, Middle Asia, CIS (543)	Low	Z22892, Z22893
33	*Francisella tularensis*	ATCC 29684, FSC 155	(LVS)	Low	
34	*Staphylococcus aureus*	ATCC 6538			
35	*Salmonella typhimurium*	ATCC 19585			
36	*Yersinia enterocolitica*	ATCC 9610			
37	*Bacillus subtilis*	ATCC 6633			
38	*Escherichia coli*	ATCC e23716			
39	*Coxiella burnetii*	ATCC VR 146			
40	*Legionella pneumophila*	ATCC 33152			
41	*Listeria monocytogenes*	ATCC 19111			
42	*Wolbachia persica*	ATCC VR 331			

[a] Numbers used to label lanes in Fig. 3 through 5.
[b] Culture collection designations.
[c] Trivial designations are designations other than culture collection designations.
[d] As defined in reference 22.
[e] EMBL data base and Genbank accession numbers.
[f] NI, no information.
[g] CIS, Commonwealth of Independent States.

lated. The starting materials used for DNA amplification were 1-μl portions of heat-treated *Francisella* cell extracts. PCRs were performed in a total volume of 50 μl, essentially as described previously (13). Primers F1 and R13 were used at a concentration of 0.5 μM, and 3 mM $MgCl_2$ was included. Samples were subjected to 30 cycles of amplification in a DNA thermal cycler (model 480; Perkin-Elmer Cetus, Norwalk, Conn.). Each amplification cycle consisted of denaturation for 30 s at 94°C, primer annealing to the template at 60°C for 1 min, and primer extension at 72°C for 1 min. After amplification, the PCR products were purified with a Centricon-3 cartridge (Amicon). Then, the double-stranded PCR products were immobilized on Dynabeads (type M-280 Streptavidin; Dynal A. S., Oslo, Norway) and made single stranded by adding NaOH (13). Sequence reactions were performed by the dideoxy termination method (23), using Sequenase (USB Corp., Cleveland, Ohio) according to the instructions of the manufacturer. The primers used for sequencing were either nonbiotinylated F1 or nonbiotinylated R13 primer and nine other primers synthesized on the basis of the previously sequenced regions of the *Francisella* 16S rRNA molecule (9), scattered around the whole *Francisella* 16S rRNA gene. Both strands were sequenced at least once in every position. Additional primers were also designed in order to sequence two variable regions of the 16S rRNA genes of 17 additional *Francisella* strains. These primers, designated FT9 (5′-CGGCCCAAACTCCTACG GGA-3′) and FT10 (5′-AAGTCCCGCAACGAGCGCAA-

TABLE 2. Positions of the primers used in the PCR

Primer	Position[a]	Sequence	Reference
F1	11-29	5'-GAGTTTGATCCTGGCTCAG-3'	4
R13	1544-1525	5'-AGAAAGGAGGTGATCCAGCC-3'	4
F5	1290-1272	5'-CCTTTTTGAGTTTCGCTCC-3'	This study
F11	149-168	5'-TACCAGTTGGAAACGACTGT-3'	This study
FP5	1290-1272	5'-CCTTTCTGAGTTTCGCTCC-3'	This study
FP8	457-478	5'-ATGAGTTAATAGCTTGTAGGAA-3'	This study
FTS8	457-478	5'-CAAGGTTAATAGCCTTGGGGA-3'	This study
FTS12	1171-1152	5'-CCTTGTCAGCGGCAGTCTCA-3'	This study
FTL8	457-478	5'-CAAGGTTAATAGCCTTGGGGGA-3'	This study
FTL12	1172-1152	5'-GCCTTGTCAGCGGCAGTCTTA-3'	This study

[a] *Escherichia coli* numbering.

3'), were located at positions corresponding to positions 329 to 348 and 1110 to 1091, respectively, on the *Escherichia coli* 16S rRNA gene.

After completion of the termination reaction, the reaction mixtures were heated at 65°C for 5 min. The supernatants containing the newly synthesized DNA strands were removed, and 2.5-μl portions of the samples were electrophoresed in a 6% polyacrylamide–7 M urea gel.

PCR identification of *Francisella* strains. One-microliter portions of heat-treated cell extracts of the virulent *Francisella* strains (corresponding to approximately 10^6 bacterial cells) were used in the PCR. *Wolbachia persica* ATCC VR331 was obtained lyophilized. The material was resuspended in 1 ml of saline, 0.1 ml of this preparation was treated by the alkaline lysis method as described previously (21) and the resulting material was resuspended to a volume of 10 μl; 1 μl of this preparation was used for each PCR.

The conditions used for the PCR were similar to those described above. A preheating cycle at 94°C for 7 min was included, and the optimum $MgCl_2$ concentration (1.5 mM) was used. Both *Taq* polymerase and primers were added during the preheating. The primers used are listed in Table 2. The amplification cycles used for denaturation, primer annealing, and primer extension were as follows: 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min for the F5-F11 primer pair; 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min for the FP5-FP8 primer pair; and 94°C for 1 min and 68°C for 1 min (only two temperatures) for the FTL8-FTL12 and FTS8-FTS12 primer pairs and combinations of these primer pairs. After 35 cycles of amplification, 5-μl portions of the PCR products were electrophoresed in a 1.0% agarose gel, stained with ethidium bromide, and photographed.

Data analysis. The 16S rDNA sequences of the species indicated in Table 3 were aligned and compared to determine similarity. Only positions that were identical in at least 50% of the species and positions that did not overlap with gaps in the alignment were included in the analysis. These constraints reduced the number of positions to 1,327. The levels of similarity between the sequences were converted to nucleotide substitution values (7, 14). These values were used to produce a phylogenetic tree by the method of Fitch and Margoliash (8). Statistical confidence in the topology was assessed by using SEQBOOT, DNADIST, FITCH, and CONSENSE in succession, as executed by the PHYLIP software package (version 3.5) (7).

The signature positions (Table 4) for the genus *Francisella* were identified by comparing the 16S rDNA sequences of *F. tularensis* SCHU and LVS, *F. philomiragia*, and *W. persica* with the 16S rRNA sequences of *Oceanospirillum linum*, *Ectothiorhodospira shaposhnikovi*, and the bacteria listed in Table 3, 21 16S rRNA sequences compiled by Dams et al. (2), and the sequences of 54 members of the family *Pasteurellaceae* (3).

RESULTS

Sequence determination. Virtually complete 16S rDNA sequences (1,518 to 1,521 nucleotides) for the following *Francisella* strains were determined: (i) a strain belonging to the species *F. philomiragia* (ATCC 25017), (ii) the live vaccine strain *F. tularensis* LVS (ATCC 29684), a type B strain from Russia, and (iii) the highly virulent strain SCHU, a typical type A strain (15). The sequences have been deposited in the EMBL and GenBank nucleotide sequence data bases.

F. tularensis SCHU exhibited 99.9% similarity to *F. tularensis* LVS and 99.2% similarity to *F. philomiragia*. Our analysis demonstrated that 14 of the 26 differences between

TABLE 3. Levels of sequence similarity and evolutionary distances based on alignment of 1,327 nucleotides of 16S rDNAs from *Francisella* species, *W. persica*, and some reference species belonging to the γ subclass of the *Proteobacteria*[a]

Species	% Sequence similarity or evolutionary distance[b]								
	Francisella tularensis	*Francisella philomiragia*	*Wolbachia persica*	*Legionella pneumophila*	*Pseudomonas aeruginosa*	*Coxiella burnetii*	*Proteus vulgaris*	*Escherichia coli*	*Agrobacterium tumefaciens*
Francisella tularensis		99.2	97.8	88.7	87.9	87.3	85.5	85.8	82.9
Francisella philomiragia	0.8		97.4	88.5	87.7	87.4	85.5	85.8	82.9
Wolbachia persica	2.2	2.6		88.5	88.1	87.0	85.1	85.3	83.3
Legionella pneumophila	12.6	12.9	12.9		90.3	90.5	87.5	87.4	83.7
Pseudomonas aeruginosa	13.4	13.7	13.3	10.7		90.3	87.6	88.8	83.6
Coxiella burnetii	14.2	14.1	14.8	10.4	10.6		86.5	87.8	84.0
Proteus vulgaris	16.6	16.6	17.2	14.1	13.8	15.2		95.0	82.6
Escherichia coli	16.3	16.3	17.0	14.3	12.4	13.7	5.2		82.2
Agrobacterium tumefaciens	20.1	20.1	19.7	19.1	19.2	18.7	20.7	21.3	

[a] Evolutionary distances were calculated as described in Materials and Methods. The EMBL accession numbers for sequences used in the alignment are as follows: *Agrobacterium tumefaciens*, M11223; *Coxiella burnetii*, M21291; *Escherichia coli*, J01859; *Legionella pneumophila*, M59157; *Proteus vulgaris*, X07652; *Pseudomonas aeruginosa*, M34133; and *Wolbachia persica*, M21292.

[b] The values on the upper right are levels of sequence similarity, and the values on the lower left are evolutionary distances.

TABLE 4. Sequence signatures for the genus *Francisella* and *W. persica*

Position[a]	Nucleotide in:		Exceptions[d]
	Francisella[b]	*Eubacteria*[c]	
40	U	C	
151	C	A, g	*Chloroflexus auranticus*, *Oceanospirillum linum*
294	U	C	
336	A	G	*Herpetosiphon aurantiacus*
401	A	G	
402	U	C	
849	C	U, g	*Streptomyces coelicolor*
889	C	G	*Herpetosiphon aurantiacus*
1280	A	U, c	*Ruminobacter amylophilus*, *Proteus vulgaris*
1290	G	C, u	*Chloroflexus aurantiacus*
1453	A	G	
1455	U	A, g	

[a] *Escherichia coli* numbering.
[b] Bases found in *Francisella* species, including *W. persica*.
[c] Bases found in other members of the *Eubacteria*. Lowercase letters indicate bases found in less than 25% of the strains of *Eubacteria* that have been examined.
[d] Bacterial species having the same base as *Francisella* strains.

the *F. philomiragia* and *F. tularensis* sequences were located within one variable region of the gene, nucleotides 375 to 526 (*E. coli* numbering). Moreover, a cluster of four differences was identified in another region, nucleotides 207 to 215.

Sequence similarity analysis. The sequences of *Francisella* strains were compared with previously determined 16S rRNA sequences of prokaryotes included in the EMBL data base (version 32). The signatures (determined by the method of Stackebrandt et al. [24]) of the sequences beginning at positions 168, 310, 506, and 1405 were almost identical to the consensus signatures of the γ subclass of *Proteobacteria*. Only one deviation was found; the C at position 168 in *Francisella* strains was replaced with a U. Signature positions for the genus *Francisella* were determined (Table 4).

To determine where to place the genus *Francisella* within the γ subclass, levels of similarity to other genera were calculated. Interestingly, the sequence of *W. persica* exhibited 97.4% similarity to the *Francisella* sequences (Table 3), which was the highest level of similarity observed with any of the sequences included in the data base. A phylogenetic tree (Fig. 1) was constructed. *Proteus vulgaris* and *E. coli* were included as reference strains of the γ subclass, and *Agrobacterium tumefaciens* was included as an outgroup. The other strains included were those that exhibited the highest levels of similarity to *Francisella* strains.

Determination of the sequences of the variable regions of *Francisella* strains. To assess the relationships among vari-

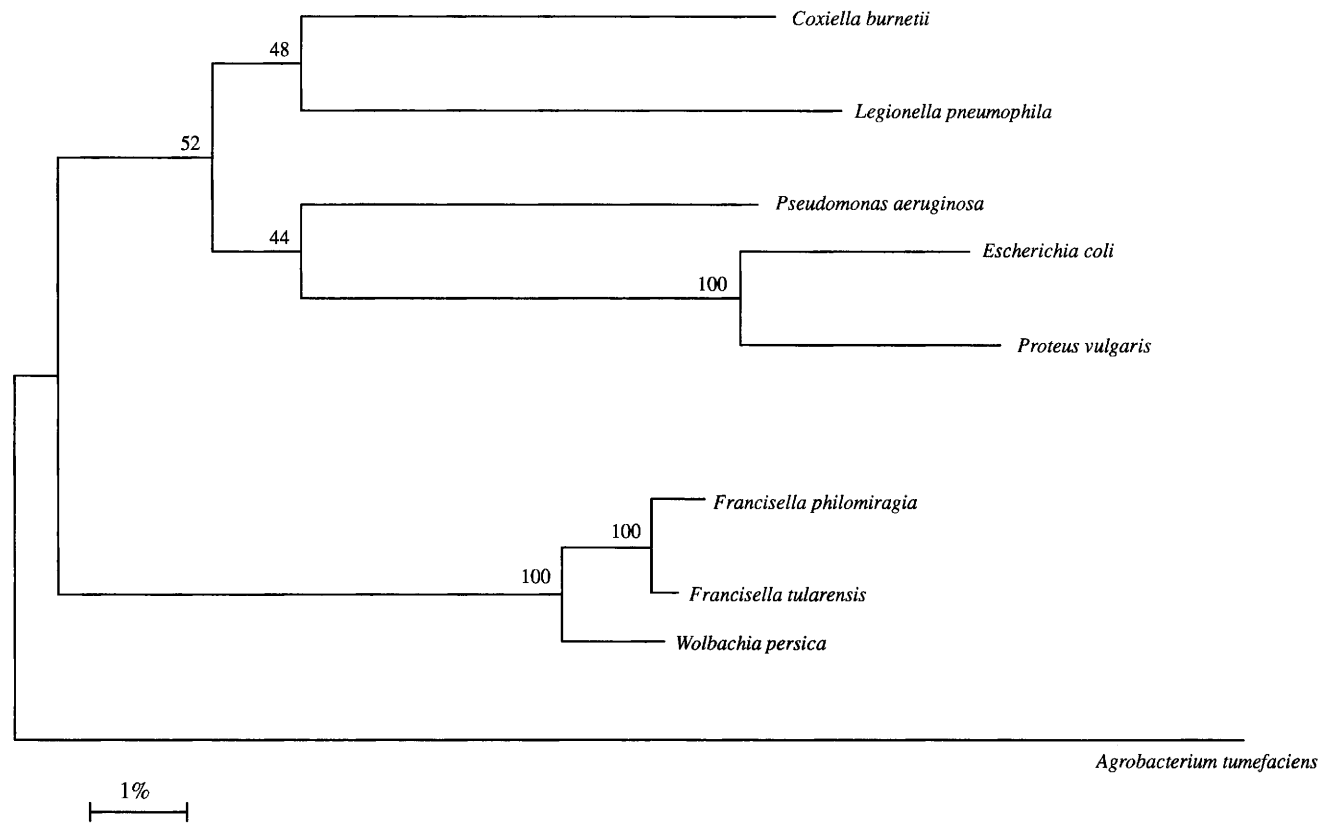


FIG. 1. Evolutionary distance tree showing relationships among the organisms used in this study and members of the γ subclass of the *Proteobacteria* that exhibit the highest levels of similarity to members of the genus *Francisella*. *Agrobacterium tumefaciens* was included as an outgroup. The unrooted tree was constructed by the algorithm of Fitch and Margoliash after evolutionary distances had been calculated from nucleotides substitution values. The numbers at the nodes of branches are the confidence values (expressed as percentages) obtained from 100 replications. Bar = sequence similarity difference of 1.0%.

TABLE 5. 16S rRNA signature nucleotides of the different clusters of *F. tularensis*

F. tularensis strain(s)	Nucleotide at position:			
	477	1153	1174/385	1292
SCHU, FSC 033, FSC 041, FSC 046	Δ[a]	G	G/C	A
FSC 042, FSC 054, FSC 138, FSC 022	G	G	G/C	A
LVS, FSC 026, FSC 044, FSC 108, FSC 146, FSC 151	G	A	G/C	A
FSC 040[b], FSC 090	G	G	Δ/Δ	A
FSC 147	G	G	G/C	G

[a] Δ, deletion at that position.
[b] Strain FSC 040 has also been identified as *F. novicida*.

ous *Francisella* strains, 16S rDNAs of 17 additional strains were sequenced; these strains included strains having different phenotypic traits and strains obtained from different geographical areas. The sequences of two regions of the 16S rDNAs from positions 375 and 1139 were determined; each of these regions was 170 to 250 nucleotides long. The first region exhibited the highest level of diversity in the alignment of the three complete sequences, whereas the second region was selected because it encompassed nucleotide 1153, which has previously been demonstrated by 16S rRNA sequencing and hybridization to vary among *Francisella* strains (9, 22). Of the 26 differences between the complete sequence of *F. philomiragia* (ATCC 25017) and the *F. tularensis* sequences, 16 were located within these two regions. The sequences of the *F. philomiragia* strains differed at six nucleotides. The *F. tularensis* strains exhibited variations at five positions, yielding clusters of strains (Table 5).

The first cluster, distinguished by a deletion at position 477, contained strains isolated in North America. Three of these strains were highly virulent when they were isolated (Table 1). The second cluster also comprised strains from North America, two of which were highly virulent when they were isolated. Also, avirulent strain B38, which was highly virulent when it was originally isolated (15), was a member of this cluster. The third cluster, which was characterized by an A at position 1153, contained strains isolated in Europe. The fourth cluster contained *F. tularensis* 090 (a strain isolated in Japan) and *F. novicida*. This may indicate that within the genus *Francisella*, the phenotypic markers that distinguish *F. tularensis* and *F. novicida* (12) are not necessarily reflected as differences in the 16S rRNA sequences. Actually, the sequence of *F. novicida* was very closely related to all of the *F. tularensis* sequences; within the variable region at positions 375 to 546 at most four differences were found (Fig. 2). Altogether, 743 bp (positions 375 to 642, 819 to 929, and 1036 to 1402) of the 16S rRNA gene of *F. novicida* was sequenced. No differences were found except those in the region from position 375 to position 546. The fifth cluster contained a strain isolated in the central Asian portion of the former Commonwealth of Independent States.

Besides the differences indicated in Table 5, only one additional deviation was found in the 16S rDNA sequences; *F. tularensis* FSC 146, which originally was isolated from a Swedish patient with meningitis, had a U at position 490 (Fig. 2).

Detection of the genus *Francisella*. Oligonucleotides were designed for specifically identifying strains belonging to the genus *Francisella*. On the basis of the sequences determined, two primers, designated F5 and F11, located at positions 1290 to 1272 and 149 to 168, were selected (Fig. 3A). All 31 *Francisella* strains but none of eight strains belonging to other genera yielded the appropriate amplified PCR product. In addition to *F. tularensis* 16S rDNA, the 16S rDNA of *W. persica* was amplified, a finding that was anticipated as the sequences of the two species are identical in the primer-complementary regions. We found no primers that distinguished all of the strains belonging to the genus *Francisella* from *W. persica*. We also confirmed that all preparations contained DNA that could be amplified, since the F1-R13 primer pair, which was specific to regions conserved in all 16S rDNAs, yielded a fragment of the expected size (Fig. 3B).

Identification of *Francisella* species. Species-specific regions in the two variable regions were identified, and a number of different primer pairs were assessed for usefulness for discriminating these variable sequences. The FTS8-FTS12 and FTL8-FTL12 primer pairs, used in equal amounts, specifically amplified the 16S rDNAs of all *F. tularensis* strains, including *F. novicida* (Fig. 4A). The FP5-FP8 primer pair was found to react with the four strains of *F. philomiragia* analyzed, but not with *F. tularensis* or any other bacterial species investigated (Fig. 4B).

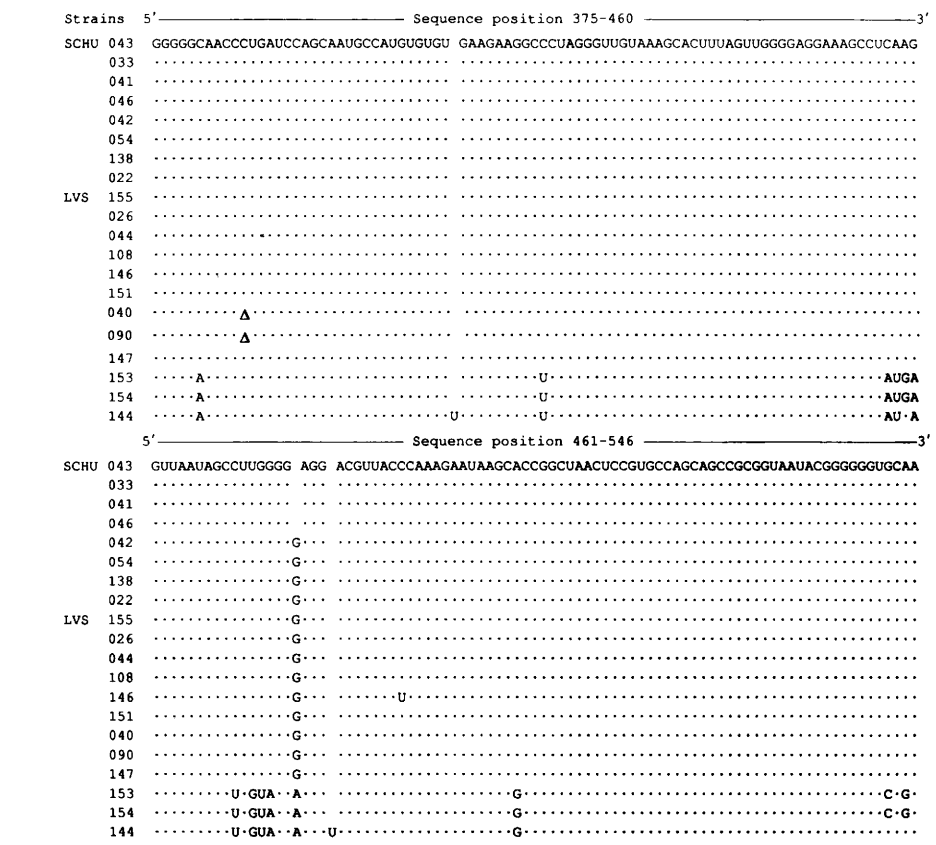
Differentiation of strains within the species *F. tularensis*. The usefulness of the PCR for identifying different clusters of *F. tularensis* was also assessed. To do this, primers that amplified a fragment encompassing positions 457 to 1172 of the 16S rDNA sequence were used. The FTS8-FTS12 primer pair specifically amplified the 16S rDNA of four *Francisella* strains that lacked a G at position 477 (Fig. 5), three of which were highly virulent when they were isolated. Conversely, the FTL8-FTL12 primer pair identified all of the strains that were not detected by the FTS8-FTS12 primer pair (data not shown).

DISCUSSION

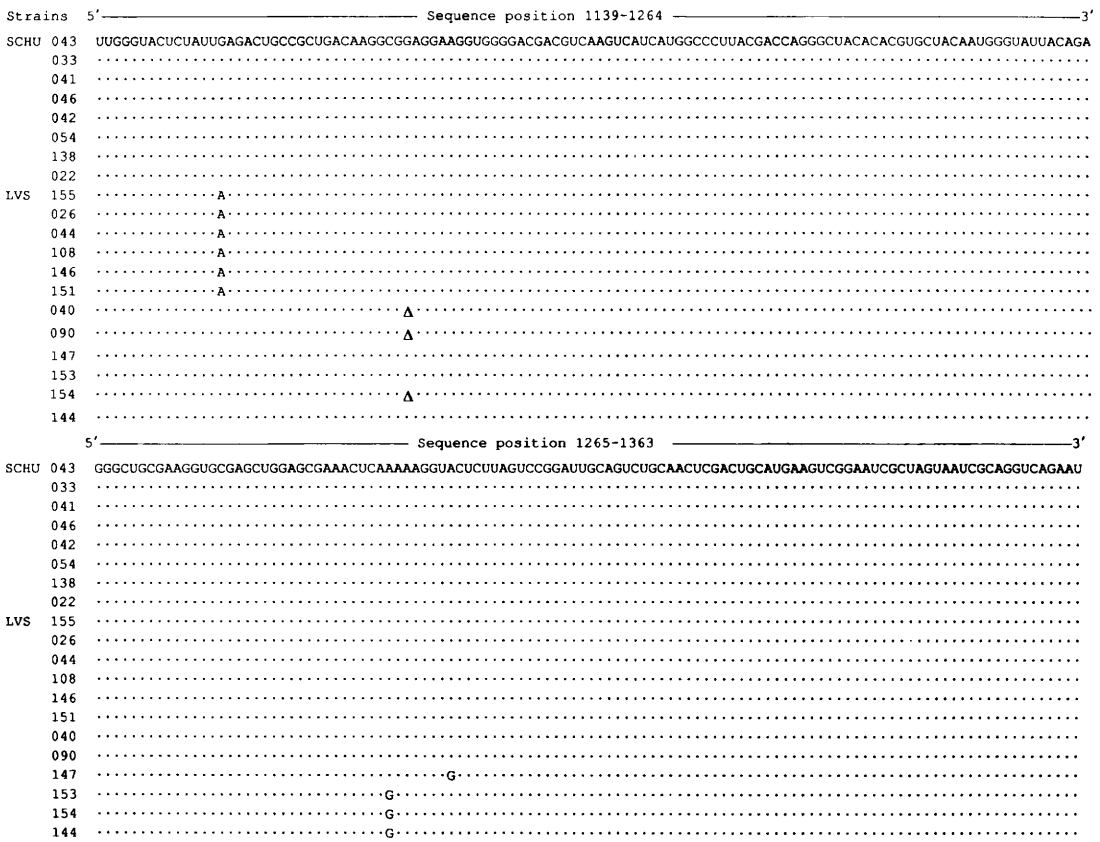
DNA-DNA hybridization is the standard method used to define bacterial species and, together with phenotypic characteristics, effectively delineates bacterial taxa. Phylogenetic parameters alone do not provide sufficient information for taxonomic evaluation, since evolutionary rates differ among various groups of bacteria. Nonetheless, rRNA analysis is thought to be the only valid method for assessing the present taxonomy. rRNA sequences are considered to be reliable phylogenetic indicators as their variability reflects evolutionary mutations that occur at a relatively regular pace. The variable regions of the molecule include a sufficient number of bases to provide adequate information for analysis of close as well as distant relationships among

FIG. 2. Sequences of the variable regions of the 16S rDNA molecules of *Francisella* strains. The sequences of two regions, positions 375 to 546 (A) and positions 1139 to 1363 (B), of the 16S rDNA molecules were determined. The sequence of *F. tularensis* SCHU FSC 043 is shown, and the nucleotides of the other sequences that differ from the nucleotides in the strain SCHU sequence are indicated. See Table 1 for strain designations. Δ, deletion at that position.

A


Strains 5′ Sequence position 375-460 3′
SCHU 043 GGGGGCAACCCUGAUCCAGCAAUGCCAUGUGUGU GAAGAAGGCCCUAGGGUUGUAAAGCACUUUAGUUGGGGAGGAAAGCCUCAAG
033
041
046
042
054
138
022
LVS 155
026
044
108
146
151
040 Δ
090 Δ
147
153 AUGA
154 AUGA
144 AU·A
5′ Sequence position 461-546 3′
SCHU 043 GUUAAUAGCCUUGGGG AGG ACGUUACCCAAAGAAUAAGCACCGGCUAACUCCGUGCCAGCAGCCGCGGUAAUACGGGGGGUGCAA
033
041
046
042
054
138
022
LVS 155
026
044
108
146
151
040
090
147
153 C·G·
154 C·G·
144

B


Strains 5′ Sequence position 1139-1264 3′
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033
041
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138
022
LVS 155
026
044
108
146
151
040 Δ
090 Δ
147
153
154 Δ
144
5′ Sequence position 1265-1363 3′
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033
041
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LVS 155
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147 G
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144 G

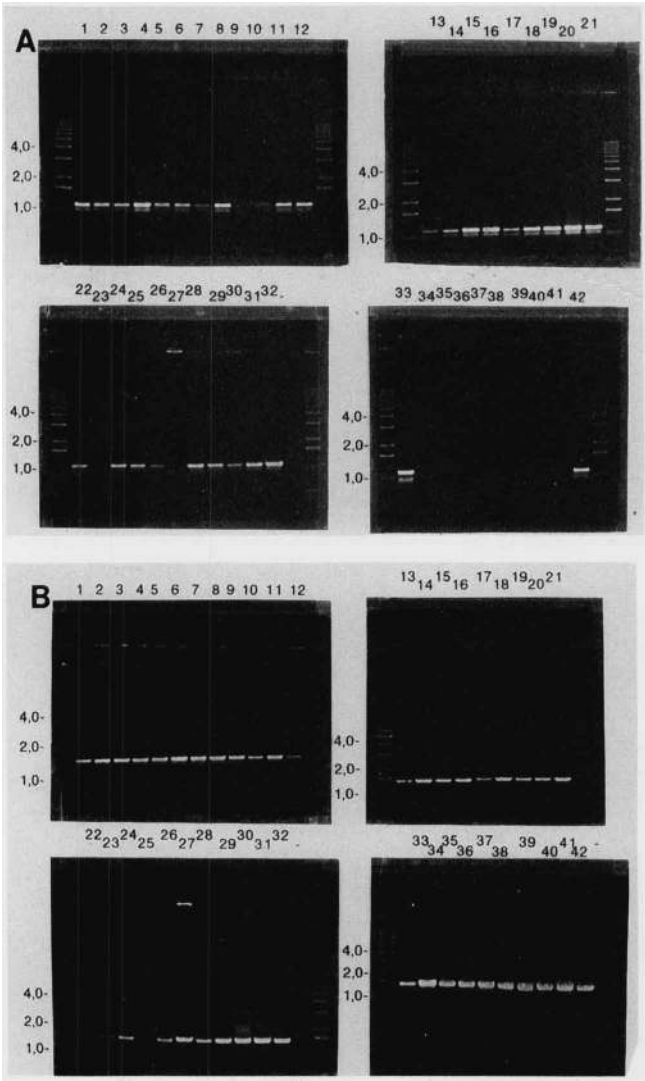

FIG. 3. Results of PCR-assisted amplification of whole cells by the *Francisella*-specific F5-F11 primer pair (A) and the F1-R13 primer pair complementary to conserved regions in all eubacterial 16S rRNA sequences (B). See Table 1 for an explanation of the bacterial strain numbers above the lanes. The chromosomal DNA from approximately 10^6 bacteria was subjected to 35 cycles of amplification. The resulting PCR product was then electrophoresed in an agarose gel, stained with ethidium bromide, and photographed.

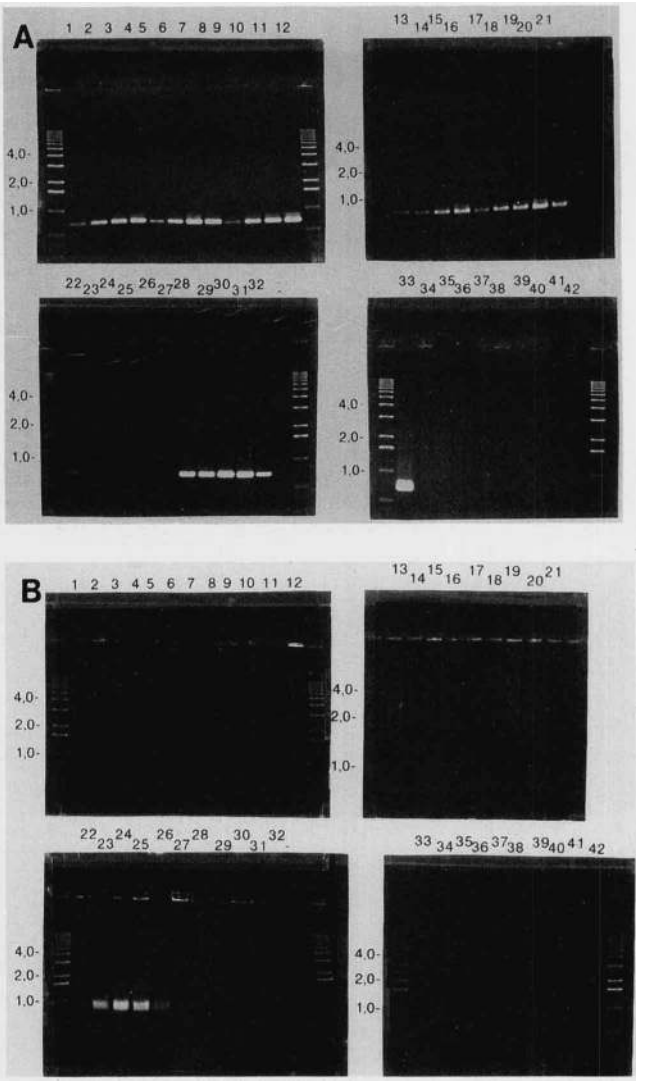

FIG. 4. Results of PCR-assisted amplification of whole cells by the *F. tularensis*-specific FTS8-FTS12 and FTL8-FTL12 primer pairs (A) and the *F. philomiragia*-specific FP5-FP8 primer pair. See Table 1 for an explanation of the bacterial strain numbers above the lanes. The chromosomal DNA from approximately 10^6 bacteria was subjected to 35 cycles of amplification. The resulting PCR product was then electrophoresed in an agarose gel, stained with ethidium bromide, and photographed.

species. Generally, good agreement has been found between previously existing taxonomic groups and phylogenetic data, inasmuch as 95% of all genera examined exhibit perfect concordance (19). Generally, the majority of recognized species that have been examined differ in their 16S rRNA sequences from related species in at least 1.5% or more of the positions (10).

An interesting observation in this analysis was the unusually high levels of homology between the 16S rRNA sequences of *W. persica* and *Francisella* strains. *W. persica*, an obligately intracellular microorganism, has previously been referred to the tribe *Rickettsieae*, but it has been noted that the phenotype of this organism does not closely resemble the phenotypes of other members of the genus *Wolbachia* (27). Recently, when its 16S rRNA sequence was determined by Weisburg et al., *W. persica* was found to be most similar to members of the *Coxiella-Legionella* cluster (30) and not related to other *Wolbachia* species or any other species of *Rickettsieae*. The most notable difference in topology between this study and that of Weisburg et al. is the branching of *W. persica* (Fig. 1). This may have resulted from the different methods used for phylogenetic analysis, the distant relationships of the bacteria included, the low confidence values for the positioning of the *Coxiella-Legionella* cluster, or the outgroups included.

Our results show that *W. persica* is much more similar to *Francisella* species than to any other species (Table 3). This finding is also supported by the high confidence values (100%) obtained (Fig. 2). It should be remarked that the degree of similarity (97.4 to 97.8%) does not mean that *W. persica* should be placed in the genus *Francisella*, but rather

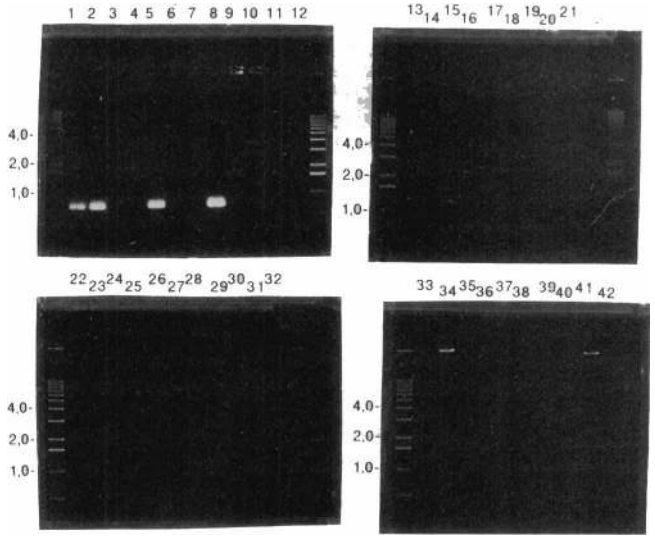

FIG. 5. Results of PCR-assisted amplification of whole cells by the FTS8-FTS12 primer pair. See Table 1 for an explanation of the bacterial strain numbers above the lanes. The chromosomal DNA from approximately 10^6 bacteria was subjected to 35 cycles of amplification. The resulting PCR product was then electrophoresed in an agarose gel, stained with ethidium bromide, and photographed.

does indicate that the species should be further characterized phenotypically to determine its relationship to *Francisella* species. The high level of similarity of the sequences complicated the search for a *Francisella*-specific primer pair. Indeed, all primer pairs that could be used to distinguish members of the genus *Francisella* from other bacteria also reacted with *W. persica*.

The data obtained for the 16S rDNA sequences of the strains belonging to the genus *Francisella* seem to corroborate the present differentiation of the genus into the species *F. tularensis* and *F. philomiragia*. Another observation is that, despite the clear distinction between the two species, the level of sequence homology (99.2%) between *F. philomiragia* and *F. tularensis* is actually higher than the levels of sequence homology found between many species belonging to the same genus (17). Moreover, our results also strengthen the previous suggestion, based on phenotypic data, that *F. novicida* should be classified as a subspecies of *F. tularensis* (12), as the degree of similarity is 99.6%.

Little is known about other intraspecies variations of 16S rRNA sequences. Like our results for *Francisella* strains a study of the 16S rRNA sequences of *Legionella pneumophila* strains revealed very little diversity (11). In contrast, up to 1.0% divergence was demonstrated in a study of *Borrelia burgdorferi* strains (16). It would be interesting to consider whether the few differences that exist in the 16S rRNA sequences of *Francisella* strains are reflected by differences in geographical distribution or in phenotypic traits, such as virulence.

In accordance with previous findings, *F. tularensis* strains could be divided into two groups depending on the nucleotide present at position 1153 (9, 22). All European strains had an A in this position, whereas strains isolated outside Europe, with one exception, *F. tularensis* FSC 044, had a G (Fig. 2). However, in the non-European group the strains were phenotypically heterogeneous, as they had different fermentation patterns and exhibited different degrees of virulence (22). At least in this respect, the nucleotide at position 1153 did not correlate with a phenotypic trait.

Identification of *Francisella* strains by the PCR has advantages compared with methods used previously to identify these bacteria (e.g., biochemical property and fatty acid composition analyses, which are laborious and time consuming). An additional advantage of using the PCR is that the limited diversity found in the 16S rRNA sequences of *Francisella* strains should permit rapid screening of the genotypes of new isolates. If a strict correlation between genotype and phenotype is found in future studies, the PCR would be a suitable method for classifying new isolates.

In conclusion, our study took advantage of the technique of sequencing 16S rRNA (rDNA) as a powerful method for establishing bacterial phylogeny and validating previously described taxa. Our phylogenetic analysis confirmed the validity of the present taxonomy of the genus *Francisella*. In addition, the signature positions of the 16S rDNA sequences at the genus, species, and subspecies levels can be used for rapid and specific identification of *Francisella* strains by the PCR.

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