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Population Structure of *Francisella tularensis*†

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We have sequenced fragments of five metabolic housekeeping genes and two genes encoding outer membrane proteins from 81 isolates of *Francisella tularensis*, representing all four subspecies. Phylogenetic clustering of gene sequences from *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* aligned well with subspecies affiliations. In contrast, *F. tularensis* subsp. *novicida* and *F. tularensis* subsp. *mediasiatica* were indicated to be phylogenetically incoherent taxa. Incongruent gene trees and mosaic structures of housekeeping genes provided evidence for genetic recombination in *F. tularensis*.

Francisella tularensis is a facultative intracellular bacterium and the causative agent of tularemia, a zoonosis affecting a great variety of vertebrate species. Due to its highly infectious nature and its ability to cause severe disease in humans, *F. tularensis* in the past was explored as a potential biological warfare agent in military research programs in the United States, Japan, and the former Soviet Union (5). Recently, *F. tularensis* has been listed among the top six potential bioterrorism agents (22).

Four subspecies of *F. tularensis* are currently recognized (23). *F. tularensis* subsp. *tularensis* (also termed "type A") encompasses those isolates most virulent to humans. *F. tularensis* subsp. *holarctica* ("type B") is also highly infectious but rarely fatal in cases of human infection. Several strains of *F. tularensis* subsp. *mediasiatica* have been collected in central Asia, but little is known about their characteristics. *F. tularensis* subsp. *novicida* rarely causes disease in humans. It is the only subspecies of *F. tularensis* evidently not confined to the Northern Hemisphere, as it was recently reported in Australia (29).

Despite distinct variations in virulence and different geographic origins, isolates of *F. tularensis* display little genetic variation. Subspecies are closely related phylogenetically. The most efficient strain discrimination reported to date was achieved by PCR analyses of size polymorphisms of short-sequence tandem repeats in the genome of *F. tularensis* (variable-number tandem repeat analysis [VNTR]) (6, 7, 11, 12). A recent study revealed two genetically distinct clusters among isolates of *F. tularensis* subsp. *tularensis* and several clusters within *F. tularensis* subsp. *holarctica* (11). Analysis of the most-variable locus, a 9-bp repeat, provided 31 length variants (alleles) among 192 strains (11). In addition, pronounced linkage among alleles at different VNTR loci suggested a clonal population structure (11).

However, while highly variable short sequence repeats permit the currently most discriminatory typing procedure for *Francisella tularensis*, sequences from genes encoding proteins may be less prone to repeated mutations, in the absence of selection, and hence provide a more reliable phylogenetic signal (9, 14). In addition, typing results based on DNA sequencing are more portable between laboratories (24). In this study, we have investigated the genetic population structure of *F. tularensis* on the basis of nucleotide sequences from five housekeeping genes (*tpiA*, *dnaA*, *mutS*, *prfB*, and *putA*) and two genes encoding membrane proteins (*tul4* and *fopA*) that had been exploited for PCR-based species identification in the past.

Sequence analyses. Bacterial isolates investigated in this study are listed in Table 1. Oligonucleotide primers for PCR were designed on the basis of genome sequences available from *F. tularensis* strains Schu S4 (subspecies *tularensis*; sequence accession no. AJ749949) and LVS (subspecies *holarctica*; preliminary sequence data available at http://bbrp.llnl.gov). Primer sequences are posted in Table S1 in the supplemental material. The seven loci investigated are located at physically distant positions on the bacterial chromosome as judged from the genome sequence from strain Schu S4 (Table 2). PCR and sequence analyses of both strands of the PCR products were performed by standard procedures. Sequences were deposited in the EMBL sequence database. Sequence divergence within *F. tularensis* (Table 2) was based on single-nucleotide polymorphisms rather than insertions or deletions. The ratio of mean nonsynonymous to synonymous substitutions per site (d_N/d_S), calculated with the software START (13), varied from 0.066 (*dnaA*) to 0.242 (*tul4*), indicating strong purifying selection (Table 2) acting on all loci investigated, including the genes encoding membrane proteins (*tul4* and *fopA*). Sequences from locus *fopA*, however, were unusually uniform (Table 2). Different sequences at the five housekeeping loci resulted in 13 different allelic profiles or sequence types (ST1 to ST13) (Table 3). When the two loci encoding membrane proteins (*tul4* and *fopA*) were included in the analysis, three additional sequence types were detected (ST14 to ST16).

Evidence for homologous genetic recombination. Phylogenetic trees based on different genes are incongruent (Fig. 1).

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TABLE 1. Bacterial strains[a]

Species and origin	Strain	Alternative designation	Length (bp) of indicated fragment			Sequence type
			Ft-M10	Ft-M3	RD1	
F. tularensis subsp. *holarctica*						
Live vaccine strain, Russia	ATCC 29684		361	351	924	ST2
Wild rabbit, Bulgaria, 1998	F80	RKI 03-1289, L1	361	351	924	ST2
Water animal, Bulgaria, 1962	F81	RKI 03-1292, Srebarna 19	361	351	924	ST2
United States	F82	RKI 03-1293, Kodar R	361	351	924	ST2
Russia, 1965	F76	RKI 03-1294, Kosho	361	306	924	ST6
United States	F83	RKI 03-1295, Tun Fac	361	396	924	ST16
Russia	F84	RKI 03-1297, Gaiski 15	361	351	924	ST2
United States, 1959	F85	RKI 03-1298, O-284	361	297	924	ST6
United States	F86	RKI 03-1299, O-363	361	378	924	ST15
United States, 1958	F77	RKI 03-1302, KF479	361	306	924	ST6
Hare, France, 1952	F78	RKI 03-1303, Chateauroux	361	423	924	ST2
Origin unknown	F1	BGA	361	297	924	ST6
Human, Austria (Mistelbach), 1997	F2		361	297	924	ST2
Human, Austria (Wien), 1997	F11		361	306	924	ST2
Human, Austria (Horn), 1997	F36		361	306	924	ST2
Hare, Austria, 1994 (6 isolates)	F6, F7, F53–F56		361	297–324	924	ST2
Hare, Austria, 1995 (2 isolates)	F8, F57		361	306	924	ST2
Hare, Austria, 1996 (2 isolates)	F9, F10		361	306	924	ST2
Hare, Austria, 1997 (31 isolates)	F3–F41		361	297–324	924	ST2
Hare, Austria, 1997 (1 isolate)	F28		361	306	924	ST14
Hare, Austria, 1997 (1 isolate)	F30		361	342	924	ST7
Hare, Austria, 1998 (5 isolates)	F42–F47		361	297–351	924	ST2
F. tularensis subsp. *tularensis*						
Human, Ohio, 1941	Schu S4		617	432	1,522	ST1
Human, Utah, 1920	F66	ATCC 6223	345	279	1,522	ST4
United States, 1959	F87	RKI 03-1300, 8859	345	306	1,522	ST5
Squirrel, Georgia	FSC033	SnMF, "CDC standard"	425	360	1,522	ST1
Tick, British Columbia, 1935	FSC041	Vavenby	489	378	1,522	ST1
Canada	FSC042	Utter	345	279	1,522	ST5
Human, Ohio, 1941	FSC043	Schu	617	432	1,522	ST1
Hare, Nevada, 1953	FSC054	Nevada 14	351	345	1,522	ST5
F. tularensis subsp. *mediasiatica*						
Miday gerbil, Kazakhstan, 1965	F63	FSC147, 543	361	450	1,453	ST10
Tick, central Asia, 1982	F64	FSC148, 240	361	432	1,453	ST11
Hare, central Asia, 1965	F65	FSC149, 120	361	450	1,453	ST12
F. tularensis subsp. *novicida*						
Water, Utah, 1950	ATCC 15482		NA	486	3,300	ST3
Water, United States, 1950	F79	RKI 03-1304	NA	486	3,300	ST3
Human, Texas, 1991	F59	FSC156, Fx1	NA	585	NA	ST8
Human, Texas, 1995	F60	FSC159, Fx2	NA	585	3,000	ST9
Water, Utah, 1950	F61	FSC040	NA	486	3,300	ST3
Spain	F62	FSC454	NA	NA	NA	ST13
F. philomiragia[b]						
Muskrat, Utah, 1959	DSM 7535		NA	NA	NA	NA
Water, Utah	F50	ATCC 25016	NA	NA	NA	NA
Muskrat, Utah, 1959	F51	ATCC 25015	NA	NA	NA	NA
Water, Utah	F93	ATCC 25017	NA	NA	NA	NA
Water, Utah	F94	ATCC 25018	NA	NA	NA	NA

[a] NA, no amplification.
[b] From *F. philomiragia*, only *tpiA*, *prfB*, and *fopA* could be amplified and sequenced.

Several isolates of *F. tularensis* subsp. *novicida* (F59, F60, and F62) show different phylogenetic affiliations depending on the gene considered (Fig. 1). For example, and most obviously, based on four of the seven genes investigated, isolate F62 branches off deeply from the *F. tularensis* clade, with strong bootstrap support and pairwise sequence differences of up to 9.2%, while it clusters together significantly with isolate F59 in the *dnaA* tree and has *fopA* and *mutS* sequences that are identical or nearly so (one nucleotide difference) to those from isolates ATCC 15482 and *F. tularensis* subsp. *mediasiatica* F63 and F64 (Fig. 1). Discordances between sequence data from different loci were evaluated by utilizing the incongruence length difference test implemented in PAUP 4b10. Results are indicated in Table S2 in the supplemental material. For four gene combinations (*dnaA-mutS*, *dnaA-tul4*, *dnaA-fopA*, and *tpiA-fopA*), significant conflict between data sets was indicated

TABLE 2. Genomic positions of the seven loci and sequence variation within *F. tularensis*

Gene	Genomic position[a]	Sequence length (bp)	No. of alleles	Maximum sequence divergence (%)	d_N/d_S
dnaA	370	471	12	7.0	0.066
tpiA	83715	417	9	6.0	0.155
prfB	207710	304	7	9.9	0.083
fopA	599187	467	8	1.4	0.099
tul4	909891	314	10	9.5	0.242
putA	1165439	371	5	7.8	0.070
mutS	1553295	411	9	5.6	0.093

[a] Within the genome of strain Schu S4 (sequence accession number AJ749949).

by *P* values below the recommended threshold of 0.05 (4, 8, 15). When strains F59, F60, and F62 were excluded from these analyses, all *P* values were above 0.05 (not shown).

To identify mosaic genes generated through recombination, we utilized both phylogenetic and nucleotide substitution distribution methods (20). The likelihood method of Grassly and Holmes (10), which is implemented in the software PLATO, indicated the presence of anomalously evolving regions that do not fit with a respective single phylogenetic topology in sequences from *tpiA*, *dnaA*, *prfB*, and *mutS* (Bonferroni-corrected significance level [α], 0.05). Reinvestigations allowing gamma-distributed evolution rate heterogeneity (four rate categories; shape parameter, 0.1) and altered transition/transversion ratios (range, 0.5 to 2.05) did not change these results for *dnaA*, *prfB*, and *mutS*, affirming recombination to be the underlying evolutionary cause for phylogenetic discordance rather than spatial variation of the evolution rate or selective pressure along the sequences (10). Predicted recombinational breakpoints in these sequences are indicated in Table 4. For *tpiA*, in contrast, a higher transition/transversion ratio suggested a locally increased negative, conservative, selective pressure (since transitions are more likely to be synonymous changes [10]).

Application of the maximum chi-square method (17), which is implemented in RDP, version 2.0 (16), confirmed the inferred mosaic structures of the genes *dnaA*, *prfB*, and *mutS* in *F. tularensis* subsp. *novicida* isolates F59 and F62 ($P < 0.001$) and predicted recombinational breakpoints in these sequences similar to those predicted by PLATO (Table 4).

The homoplasy test (implemented in the START software package [13]) was used to relate the frequency of observed synonymous homoplasies (that is, the same nucleotide changes in different branches of a maximum-parsimony tree) in the concatenated sequences from *F. tularensis* to the frequency expected under free recombination (18). Depending on the conservative consideration of potential very high or medium-high codon usage bias (18), respectively, this homoplasy ratio was calculated to be 0.09 to 0.10, which is low in comparison to several other bacterial species (26), suggesting that the overall contribution of recombination to the observed sequence variation is limited. However, the probability that the observed homoplasies could not be explained by mutations alone (and hence required recombination) was indicated to be 95% to 99% (18).

In conclusion, we have found several lines of evidence for

TABLE 3. Allelic profiles

Sequence type	No. of isolates	Representative strain	Allele no.						
			Housekeeping genes					Membrane protein genes	
			tpiA	*dnaA*	*prfB*	*mutS*	*putA*	*tul4*	*fopA*
ST1	4	Schu S4	1	1	1	1	1	1	1
ST2	35	ATCC 29684	2	2	2	2	2	2	2
ST3	3	ATCC 15482	3	3	3	3	3	3	3
ST4	1	F66	4	4	1	4	1	4	4
ST5	3	F87	5	4	1	5	1	4	4
ST6	4	F1	6	5	2	2	2	2	2
ST7	1	F30	6	6	2	2	2	2	2
ST8	1	F59	4	7	4	6	4	5	6
ST9	1	F60	7	8	1	7	3	6	6
ST10	1	F63	7	9	5	9	3	3	5
ST11	1	F64	7	10	5	9	3	3	5
ST12	1	F65	8	11	6	5	1	7	7
ST13	1	F62	9	12	7	8	5	8	5
ST14	1	F28	2	2	2	2	2	9	2
ST15	1	F83	6	5	2	2	2	10	8
ST16	1	F86	6	5	2	2	2	2	8

limited horizontal genetic transfer and recombination in *F. tularensis*. No evidence was found, however, for recombination events in *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica*. Rather, mosaic genes were discovered in *F. tularensis* subsp. *novicida* exclusively, and, in fact, omission of isolates F59, F60, and F62 from phylogenetic analyses eliminated the discrepancies among gene trees detected by incongruence length difference testing. These results are consistent with an underlying population in which recombination may be common and from which more-virulent clones that dominate medical interest and culture collections today have emerged and become widespread. Such a population structure previously has been termed "epidemic" (19). Consistently, based on gene content, *F. tularensis* subsp. *novicida* was recently concluded to be evolutionarily the oldest subspecies (27). Alternatively, recombination may be more frequent in, or restricted to, *F. tularensis* subsp. *novicida*. Our findings do not contradict the recently reported linkage disequilibrium among VNTR loci of this species, since only 2% of the isolates investigated in that study were affiliated with *F. tularensis* subsp. *novicida* (11). Currently, the genomes from several *F. tularensis* isolates are being sequenced (see www.genomesonline.org); those projects will provide abundant data for investigating the extent of recombination in these bacteria.

Phylogenetic diversity. Despite the detected signatures of recombinational events, the gene trees are far more similar than would be expected by chance alone (Fig. 1; also see Table S2 in the supplemental material). Hence, the extent of recombination in *F. tularensis* is not sufficient to completely obliterate the phylogenetic signal in the gene sequences. We therefore prefer to calculate phylogenetic relationships among the isolates on the basis of concatenated nucleotide sequences rather than allelic profiles, because the latter do not consider the extent of sequence difference (21, 24).

For *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *tularensis*, results of phylogenetic analyses based on concate-

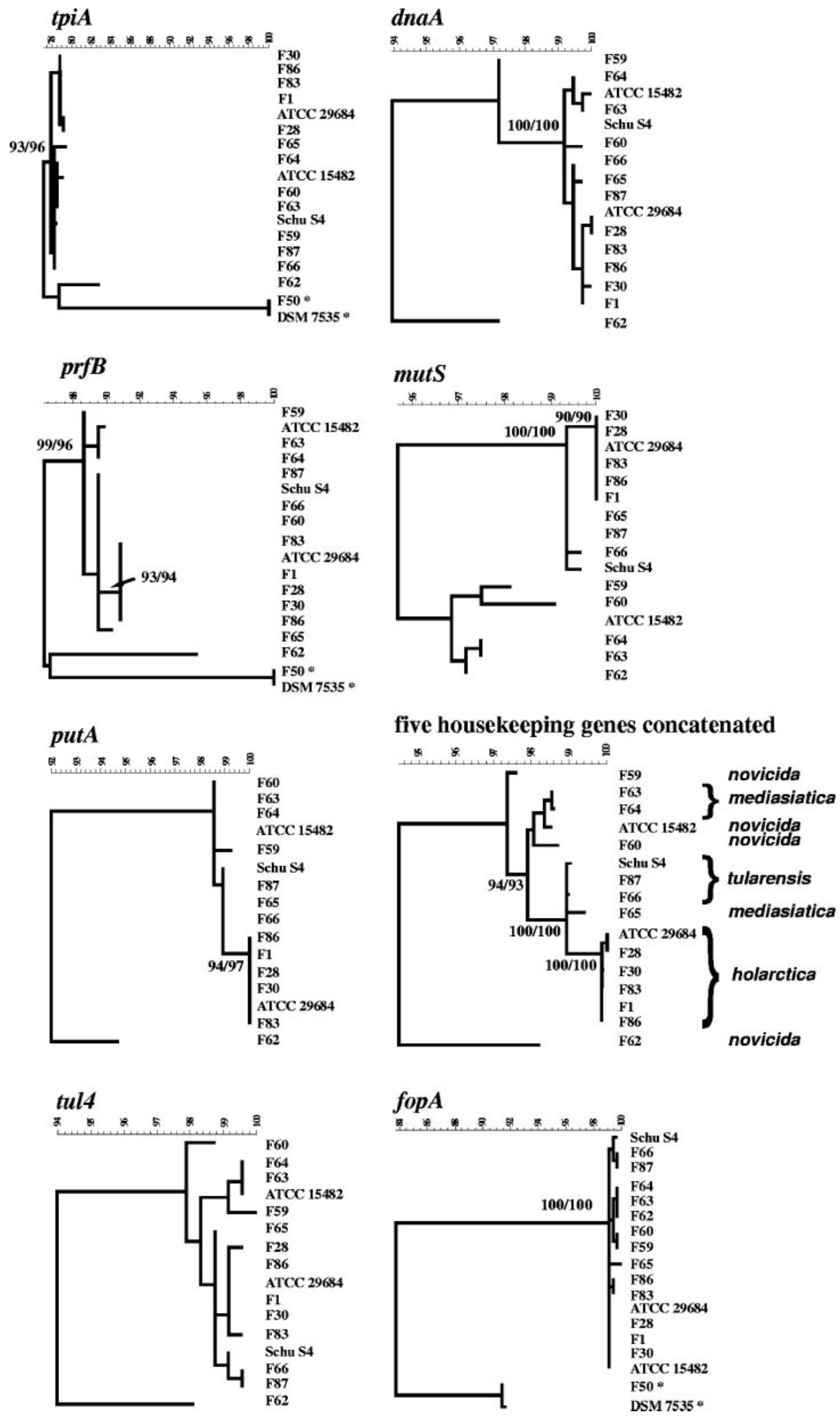

FIG. 1. Phylogenetic trees based on gene sequences as indicated and calculated by using the maximum likelihood algorithm in the BIONUMERICS software package, version 4.0 (Applied Maths). One representative isolate from each sequence type (Table 3) was included in the computations. When available, gene sequences from *Francisella philomiragia* isolates (indicated by asterisks) were included as outgroups to enable bootstrap analyses of the deepest nodes within the *F. tularensis* clade. Bootstrap values from 100 resamplings are indicated when they are at least 90%. They are based on both the maximum-parsimony and neighbor-joining algorithms and were computed by using PHYLIP 3.65. Subspecies affiliations are indicated in the tree based on concatenated gene sequences. Scale bars indicate percent sequence similarity.

TABLE 4. Mosaic gene structures

Recombinant sequence		Breakpoint(s) predicted by:	
Gene	Isolate[a]	MAXCHI[b]	PLATO[c]
prfB	F62	152	152
dnaA	F62	40	48
mutS	F59	65, 143, 330	58, 136, 323

[a] Isolate in which the respective recombinant sequence was detected by MAXCHI.
[b] MAXCHI is an implementation of the maximum chi-square method in RDP, version 2.0. Sequence pairs were scanned, and the window size was set at 30 variable sites. With multiple-comparison correction turned off, the highest acceptable probability that potentially recombinant regions shared identity by mere chance was set at 0.001 ($P < 0.001$).
[c] Input maximum likelihood phylogenetic trees for PLATO were computed by using DNAML implemented in PHYLIP 3.65. Bonferroni-corrected significance level (α), 0.05.

nated nucleotide sequences correlate very well with RD1 amplification fragment lengths and VNTR data, notably the amplification fragment sizes from the 16-bp repeat locus termed Ft-M10 (Fig. 1, Table 1); analyses of RD1 fragments and the short sequence repeats Ft-M3 and Ft-M10 were performed as described previously (3, 12). The sizes of both amplification fragments, RD1 and Ft-M10 (also termed SSTR16 or Ft-V2 [6, 11, 12]), have previously been reported to be conserved in each of these two subspecies (3, 6, 11, 12). Hence, we conclude that our sequence data confirm these two subspecies each to be monophyletic entities.

Isolates of *F. tularensis* subsp. *holarctica* display little genetic diversity, with the majority assigned to two genotypes (ST2 and ST6) and a few single-locus and double-locus variants of these. Generally, Ft-M3 fragment lengths from *F. tularensis* subsp. *holarctica* were found to be more variable than our allelic profiles. However, in several cases, isolates affiliated with different sequence types had Ft-M3 fragments of identical lengths. Remarkably, sequence types ST2 and ST6 differ at two housekeeping loci (*tpiA* and *dnaA*) and, on this basis, would not be considered particularly closely related (9) (Table 3). Even so, several isolates of both sequence types have Ft-M3 fragment lengths of 297 bp (for example, isolates F1 and F2) and 306 bp (for example, isolates F77 and F8 [Table 1]). We conclude that these identical Ft-M3 fragment lengths are homoplastic and provide misleading information about the isolates' phylogenetic relationships.

Isolates of *F. tularensis* subsp. *tularensis* form two separate phylogenetic clusters which likely correspond to the two distinct groups A.I (includes Schu S4) and A.II (includes ATCC 6223/F66) that were recently discovered on the basis of VNTR analyses (11). Definitive correlation with VNTR groups A.I and A.II cannot be confirmed at present, however, because the corresponding VNTR fragment lengths were not published in that previous report (11). One of the *F. tularensis* subsp. *tularensis* isolates, F87, has an Ft-M3 fragment of 306 bp which is not homologous to the same fragment in several sequence types (ST2, ST6, and ST14) among *F. tularensis* subsp. *holarctica* isolates. Nucleotide sequences of Ft-M3 fragments from the two subspecies differ by 5.5%.

Previously, restriction fragment length polymorphism analyses and genome comparisons through DNA microarray hybridization led to the conclusion that the subspecies *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *tularensis* are highly homogeneous genetically (3, 28). While our data do not contradict these results, it should be considered that, even based on our limited data set, each of these subspecies appears to be more diverse than several highly uniform pathogens, including *Mycobacterium tuberculosis*, *Bacillus anthracis*, and *Yersinia pestis* (2, 21, 25). Hence, both subspecies of *F. tularensis* are likely to be significantly older than any of these recently emerged species (1).

Francisella tularensis subsp. *novicida* was indicated to be paraphyletic, as it does not include *F. tularensis* subsp. *mediasiatica* isolates F63 and F64, which descend from the same common ancestor (Fig. 1). Moreover, the three isolates assigned to *F. tularensis* subsp. *mediasiatica* did not form a single cluster, and F65 did not appear to be particularly related to F63 and F64. Instead, some affinity of F65 to *F. tularensis* subsp. *tularensis* may exist, which previously had been suggested for the entire subspecies (3, 11). Obviously, *F. tularensis* subsp. *novicida* and *F. tularensis* subsp. *mediasiatica* do not form phylogenetically coherent taxonomic entities and possibly should be reclassified. However, these taxonomic problems can be solved only when more isolates of these subspecies become available. Obviously, much of the diversity of *F. tularensis* extant in nature remains to be discovered.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been submitted to the EMBL database under accession no. AM 261086 to AM 261205.

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