

Evolution of Subspecies of *Francisella tularensis*†

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Analysis of unidirectional genomic deletion events and single nucleotide variations suggested that the four subspecies of *Francisella tularensis* have evolved by vertical descent. The analysis indicated an evolutionary scenario where the highly virulent *F. tularensis* subsp. *tularensis* (type A) appeared before the less virulent *F. tularensis* subsp. *holarctica* (type B). Compared to their virulent progenitors, attenuated strains of *F. tularensis* exhibited specific unidirectional gene losses.

Francisella tularensis, the causative agent of tularemia, is considered a potential bioterrorist agent. Important premises are an extremely low infectious dose and a potential for airborne transmission (6). The two most clinically important entities of tularemia, type A and type B, correspond to the highly virulent subspecies *F. tularensis* subsp. *tularensis* and the moderately virulent *F. tularensis* subsp. *holarctica*, respectively (18, 24). Taxonomic work has identified two additional subspecies, *F. tularensis* subsp. *mediasiatica*, exhibiting a moderate virulence, and “*F. tularensis* subsp. *novicida*,” with a low virulence in animals and humans (24). The latter subspecies has less-fastidious extracellular growth requirements than the other subspecies and a distinct lipopolysaccharide O-antigen (10, 29).

Based on small subunit RNA sequences, *F. tularensis* is classified as a member of the γ -subgroup of proteobacteria (24). The two species *F. tularensis* and *F. philomiragia* and in addition a number of more recently identified tick endosymbionts are the only members of the genus *Francisella*, which diverges deeply among the γ -proteobacteria (22, 27).

Assuming that *F. tularensis* subspecies have evolved from a common ancestor, identification of genetic differences among subspecies might provide insights into species phylogeny and provides a basis for studies of more functional issues. Although exhibiting differences in virulence, geographical distribution, and a few biochemical tests, *F. tularensis* subspecies are highly similar in gene content (1). Mapping of genetic differences will allow future exploration of their relationships to functional correlates. In a recent DNA-based study using highly mutable variable-number tandem repeat (VNTR) sequences dispersed over the genome, *F. tularensis* subsp. *tularensis* (type A) showed more diversity than *F. tularensis* subsp. *holarctica* (type B), suggesting the former subspecies to be evolutionarily older (11). Significant linkage disequilibrium was detected among VNTR loci of *F. tularensis*, consistent with a predominantly clonal population structure.

The present work was based on findings from a whole-ge-

nome microarray study of multiple *F. tularensis* strains (1). The microarray study identified large size regions of difference (RDs) among *F. tularensis* strains. Notably, a pair of direct repeated sequences flanked seven of eight identified RDs. We hypothesized that the presence of direct repeats in a genomic region would represent a propensity for interstrain variation of that region. Therefore, direct repeated sequences were searched for in the genome sequence of strain SCHU S4, and when identified, the corresponding genomic regions were assayed for variability among various strains of *F. tularensis*. In parallel, single nucleotide variations (SNVs) were analyzed by sequencing of internal fragments of genes of *F. tularensis* and related bacteria of the γ -subgroup of proteobacteria.

Bacteria and identification of genomic regions with direct repeats. Criteria for including *F. tularensis* strains in this study were to ensure maximum spatial and temporal diversity. Forty-five isolates (Table 1) representing the four *F. tularensis* subspecies were selected from the *Francisella* Strain Collection (FSC) in Umeå, Sweden. Bacteria were grown on modified Thayer-Martin agar. Cells were suspended into phosphate-buffered saline and heat killed. DNA was prepared using silica and guanidine isothiocyanate buffer as described previously (23).

A Perl script (detailed in the supplemental text) was devised to search for direct repeats in the, at that time, unfinished genome sequence of *F. tularensis* strain SCHU S4 (FSC237). The completed genome sequence is assigned accession no. AJ749949 in GenBank (13). More than 70 genomic regions flanked by direct repeats were evaluated by PCR in a subset of five *F. tularensis* isolates that represented each of the four *F. tularensis* subspecies and the Japanese *F. tularensis* subsp. *holarctica* variant. Genomic regions that exhibited size polymorphism on agarose gels were further analyzed in 45 strains (Table 1). PCR amplicons were sequenced to identify the junctions of the deletion/insertion events. Primer sequences for PCR amplification were posted in the supplemental material in Table S1.

Use of unidirectional deletions for phylogenetic analysis. The use of RDs for phylogenetic analysis relies on an assumption of unidirectional deletion events that eventually become fixed in bacterial populations (phylogenetic lineages) (2, 14).

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† Supplemental material for this article may be found at <http://jb.asm.org/>.

TABLE 1. Presence or absence of 17 regions of difference in 45 *F. tularensis* strains

Species and isolate information (no. of isolates)	FSC no.	Alternative strain designation	RD ^a																
			1a	1b	1c	2	3	4	5	6	7	11	16	17	18	19	20	21	22
<i>Francisella tularensis</i> subsp. <i>tularensis</i> (12)																			
Human ulcer, 1941, Ohio	237	SCHU S4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Human ulcer, 1941, Ohio	043	SCHU, avirulent	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Unknown, 1960, Eigelsbach	013	FAM standard	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Squirrel, Georgia	033	SnMF	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tick, 1935, B. C., Canada	041	Vavenby	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hare, Canada	042	Utter	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Human pleural fluid, 1940, Ohio	046	Fox Downs	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Rabbit, 1953, Nevada	054	Nevada 14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lab acquired when handling Nevada 14	053	F. tul AC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mite, 1988, Slovakia	198	SE-219/38	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mite, 1988, Slovakia	199	SE-221/38	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Human lymph node, 1920, Utah	230	ATCC6223	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Francisella tularensis</i> subsp. <i>mediasiatica</i> (4)																			
Experimental isolate, cap-	122	(TTC-R)6-4-1	+	+	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+
Miday gerbil, 1965, Kazakhstan	147	GIEM 543	+	+	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+
Ticks, 1982, Central Asia	148	240	+	+	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+
Hare, 1965, Central Asia	149	120	+	+	-	+	+	+	+	+	+	+	-	+	+	-	+	+	+
<i>Francisella tularensis</i> subsp. <i>holarctica</i> (from Eurasia and North America) (24)																			
Tick, 1941, Montana	012	425F4G	+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Beaver, 1976, Hamilton, Montana	035	B423A	+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Human ulcer, 1999, Karlstad, Sweden	236		+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Human, Vosges, France	247	T 20	+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Tick, 1949, Moscow area, Russia	257	GIEM 503/840	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Human, 2000, Pyhäjärvi, Finland	286		+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Unknown, 1960, Eigelsbach	014	FAM SR	+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Human, 1994, Norway	158	CCUG 33391	+	-	+	-	-	-	-	-	-	-	-	±	-	+	+	+	+
Hare, 1974, Näs (W), Sweden	074	SVA T7	+	-	+	-	-	-	-	-	-	-	-	±	+	+	+	+	+
Passage in small mammals of FSC074	069	SVA T7K	+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Live vaccine strain, Russia	155	LVS	+	-	+	-	-	-	-	-	-	-	-	-	+	+	+	+	D
Vaccine strain from Russia	338	Strain 015	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	D
Water, 1991, Elista, Kalmykiya, Russia	118	14687	+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	D
Water, 1985, Rostov region, Russia	121	12267	+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	D
Norwegian rat, 1988, Russia	150	250	+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	D
Water, 1988, Rostov, Russia	151	25, A13863	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	D
Human, 1995, Ockelbo, Sweden	178	R39/95	+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	D
Tick, 1995, Lanzhot, Czech Republic	180	T-17	+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	D
Mite, 1988, Slovakia	196	SE-210/37	+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	D
Human, 1998, Ljusdal, Sweden	200		+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	D
Human, 1995, Äänekoski, Finland	249		+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	D
Water, 1991, Odessa region, Ukraine	116	14670	+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	T
Water, 1990, Odessa region, Ukraine	124	14588	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	T
Human blood, 1994, Örebro, Sweden	157	CCUG 33270	+	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	T
<i>Francisella tularensis</i> subsp. <i>holarctica</i> (from Japan) (4)																			
Human, 1958, Japan	021	Tsuchiya	-	+	+	-	-	+	-	-	+	-	N	-	+	+	+	+	+
Human, 1950, Japan	022	Ebina	-	+	+	-	-	+	-	-	+	-	N	-	+	+	+	-	+
Human lymph node, 1926, Japan	017	S-2	-	+	+	-	-	+	-	-	+	-	N	-	+	+	+	-	+
Tick, 1957, Japan	075	Jama	-	+	+	-	-	+	-	-	+	-	N	-	+	+	+	-	D
<i>Francisella tularensis</i> subsp. <i>novicida</i> (1)																			
Water, 1950, Utah	040	ATCC 15482	I	I	I	+	+	+	N	+	N	+	N	+	+	+	N	+	+

^a The symbol + indicates presence and the symbol - indicates absence of a particular region of difference relative SCHU S4. The symbol ± indicates that the strain is heterogeneous at the particular RD. The letters D or T indicate a duplication or triplication, respectively. N, not detected; I, insertion of a unique sequence (1). Regions used in deletion-based phylogenetic analysis were 1c, 2, 3, 4, 5, 6, 7, 11, and 16. Boldface letters indicate strains subjected to SNV analysis.

An RD was used for depicting evolution only when it was found to represent a deletion and not an insertion. Three criteria were used for excluding RDs from use in phylogenetic analysis. (i) The first criterion was whether the corresponding region in the SCHU S4 genome exhibited three or more closely localized direct repeats. We reasoned that the presence of more than two repeats might indicate an evolutionary history

of genome amplification. Therefore, the SCHU S4 genome was scrutinized for repeats present in the vicinity of RDs ($\pm 10,000$ bp). For a review of direct repeat mediated excision or amplification in bacteria, see references 19 and 20. (ii) The second criterion was whether the RD was flanked by multicopy insertion sequence elements. This criterion was based on an assumption that the genomic region is potentially prone to re-

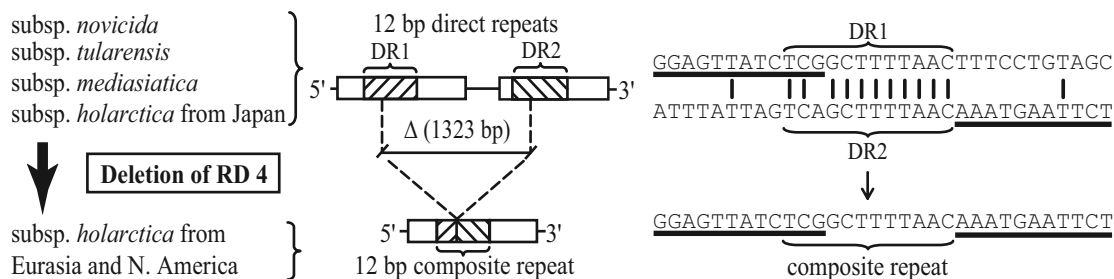


FIG. 1. Unidirectional repeat-mediated deletion mechanism exemplified by RD4. The symbol Δ represents deleted sequence. Flanking direct repeats (DR1 and DR2) and the resulting composite repeat are depicted as hatched bars. Open bars represent open reading frames that were truncated by the deletion. Partial nucleotide sequences are shown before (DR1 and DR2) and after (composite repeat) the deletion event and allowed analysis of evolutionary direction.

combination events within and among taxa in phylogenetic lineages. (iii) The third criterion was whether the RD showed polymorphism within a subspecies.

SNVs and calculation of phylogenetic trees. A subset of 15 *F. tularensis* strains was selected for analysis of SNVs (Table 1). Additionally, DNA was isolated from a freeze-dried ampoule of the type strain of the tick endosymbiont *Wolbachia persica* (ATCC VR-331) as described previously (23). A set of seven genes was selected, previously used in multiple locus sequence typing of various bacteria: *aroA*, *parC*, *pgm*, *tpiA*, *trpE*, *atpA*, and *uup* (28). Internal fragments of the genes were PCR amplified. Primer sequences for PCR amplification were posted in supplemental material in Table S1. Amplification was successful for seven gene fragments from each *F. tularensis* strain and four gene fragments from *W. persica* (*aroA*, *parC*, *tpiA*, and *atpA*). PCR amplicons were sequenced on both strands using the amplification primers and standard protocol on an ABI 377 platform (PE Applied Biosystems). Sequences of gene orthologs in the genomes of *Yersinia pestis* (CO92) and *Agrobacterium tumefaciens* (C58) were retrieved from GenBank. Phylogenetic analyses were performed using concatenated nucleotide sequences, individual gene fragments, and concatenated in-frame-translated amino acid sequences. Maximum likelihood trees were reconstructed from DNA sequences by using PAUP v. 4.0b10 (25) as described previously (7). The extent of pairwise congruence between individual gene trees was estimated by a method comparing the likelihood values calculated for single gene trees to the values for other gene trees and to 200 randomly generated trees of the same size (7). Congruence of gene trees indicates a common evolutionary history of investigated genes. Phylogenetic reconstruction from amino acid sequences was performed using PHYLIP (v. 3.6b). Phylogenetic methods are further detailed in the supplemental text.

Analysis of RDs among *F. tularensis* strains. PCR analysis of the genomes of 45 *F. tularensis* isolates identified 17 RDs in *F. tularensis* (Table 1). Generally, PCR amplification showed the presence of one of two alternate fragment sizes for each RD and isolate. The pattern of amplification allowed an immediate subspecies identification. With very few exceptions, all isolates of a subspecies showed the same fragment size for each RD. A polymorphism was present at RD21-22 among isolates of a subspecies and at RD18-19 among variants of a strain.

F. tularensis genomes showing alternate PCR fragment sizes were sequenced across each RD. Alignment of sequences for

each RD showed the smaller-sized PCR fragments to contain only one direct repeat and to be missing a large genomic segment compared to the SCHU S4 sequence. The boundary of the missing sequence was defined by the two direct repeats (Fig. 1). Nucleotide degeneracy of flanking repeats allowed further analysis. We found each single repeat present in a smaller PCR fragment to be a composite repeat derived from fusion of a left and right flanking repeat (Fig. 1; also Fig. S1 in the supplemental material). The finding is consistent with a deletion mechanism mediated by recombination of flanking repeats followed by excision of the intervening sequence. The deletion will be irreversible if two direct repeats were originally present and a single composite repeat is left after excision. As an alternative, two direct repeats may mediate genome amplification of intervening sequence as demonstrated for RD22 (Table 1). At this locus, sequence analysis showed duplication (12 isolates) or triplication (3 isolates) of the region flanked by repeats (sequence data not shown).

Genes were truncated in isolates with smaller-sized PCR fragments at 16/17 RDs (Table 2; also Table S2 in the supplemental material). Only RD1c represents an intergenic sequence. RD11 and RD16-22 were identified and characterized de novo in this study and found to affect 12 open reading frames in the *F. tularensis* SCHU S4 genome (Table 2). Gene content of RD1-7 has been previously described (1) and was further characterized in this study (Table S2 in the supplemental material). Among deleted genes, some require discussion with regard to virulence properties of various isolates. The gene *pdpD* of RD6 was recently suggested to play a role in *F. tularensis* virulence (15). In concordance to previous analysis of a smaller number of isolates, we confirmed the gene to be deleted in all type B isolates including the live vaccine strain (1, 15). Genes in RD18 and RD19 discriminated among isolates of individual subspecies. Genes in RD19 represent type IV pili building block proteins that were absent in, e.g., the live vaccine strain of *F. tularensis*. Homologous genes have been implied as virulence determinants in other bacteria (5). Finally, genes in RD18 represent genes of a novel protein family unique to *F. tularensis* (13). The latter genes were found to be deleted in an avirulent phenotype of strain SCHU (FSC043) and in the live vaccine strain of *F. tularensis*.

A deletion-based phylogeny of *F. tularensis*. Nine RDs fulfilled our strict selection criteria for a unidirectional genetic event (see above) and were included in the deletion-based phylogenetic analysis (Table 1 and Fig. 2). An evolutionary

TABLE 2. Gene content of RDs of *F. tularensis*^a

RD	Genomic position of deletion ^b (size in bp)	Gene comprised by deletion	Genome position of flanking direct repeats ^b
RD11 ^c	551874–552722 (849)	FTT0531, ABC transporter, ATP-binding protein, pseudogene	551856–551879 552705–552728
RD16	1664669–1665013 (345)	FTT1598, hypothetical membrane protein	1664591–1664721 1664936–1665066
RD17	1535418–1535717 (300)	<i>aceF</i> , FTT1484, pyruvate dehydrogenase, E2 component	1535696–1535923 1535396–1535623
RD18 ^c	928556–930035 (1,480)	FTT0918, hypothetical protein FTT0919, hypothetical protein	928215–928753 929698–930236
RD19 ^c	898418–898956 (539)	FTT0890, type IV pili fiber building block protein FTT0889, type IV pili fiber building block protein	898901–899020 898363–898482
RD20	546921–547446 (526)	FTT0524, conserved hypothetical protein	546916–546939 547442–547465
RD21	124728–125948 (1,221)	<i>nupC1</i> , FTT0116, nucleoside permease NUP family protein <i>nupC2</i> , FTT0115, nucleoside permease NUP family protein	123791–125005 125009–126233
RD22	992350–992819 (470)	FTT0980, aminotransferase, class II FTT0981, hypothetical protein	992250–992349 992720–992819

^a Genes comprised by deletion at RD1–RD7 have been described by Broekhuijsen et al. (1). Details of RD1–RD7 have been posted as supplemental material (Table S2).

^b Position and gene name refer to the genome sequence of *F. tularensis* subsp. *tularensis*, strain SCHU S4 (13). Boundaries of repeats were defined by use of a threshold value set at >65% nucleotide identity.

^c Region of difference independently identified by Samrakandi et al. (21). RD18 and RD19 correspond to L3 and L2, respectively.

scenario is suggested where successive losses of genetic material at RDs parallel the taxonomic units of *F. tularensis* at the subspecies level. The analysis proposes an evolution of *F. tularensis* where the highly virulent *F. tularensis* subsp. *tularensis* (type A) preceded the appearance of the less virulent *F. tularensis* subsp. *holarctica* (type B). Our findings partially contrast to the evolution of *Y. pestis* that apparently became more virulent while losing genetic material in the transition from its ancestor, *Yersinia pseudotuberculosis* (3). The evolutionary transitions of *F. tularensis* subspecies (*F. tularensis* subsp. *novicida*, subsp. *tularensis*, and subsp. *holarctica*) are more complex and apparently involve both acquisition and loss of virulence since *F. tularensis* subsp. *novicida* exhibits the lowest virulence and was found to be evolutionarily oldest. Work on other pathogens has shown that the evolution of virulence, in particular the adaptation to living within eukaryotic hosts, may involve both acquisition and loss of genes (16), a fact that may explain the observations.

All nine RDs were present in all *F. tularensis* subsp. *tularensis* isolates. The deletion data show isolates of *F. tularensis* subsp. *mediasiatica* to be very similar to isolates of *F. tularensis*

subsp. *tularensis*. Only RD1c showed a deletion event unique for *F. tularensis* subsp. *mediasiatica* isolates. Therefore, the branching order of *F. tularensis* subsp. *mediasiatica* and *F. tularensis* subsp. *tularensis* remains unclear. Isolates from Japan of *F. tularensis* subsp. *holarctica* showed deletion at six RDs. Two additional deletions at RDs were found in Eurasian and North American *F. tularensis* subsp. *holarctica* isolates. This suggests that Japanese isolates are evolutionary intermediates between the highly virulent type A (*F. tularensis* subsp. *tularensis*) isolates and the less virulent Eurasian/North American type B isolates (*F. tularensis* subsp. *holarctica*). The single isolate of *F. tularensis* subsp. *novicida* was chosen for rooting, based on the most extensive number of SNVs in seven gene fragments (see below). The deletion analysis per se could not place *F. tularensis* subsp. *novicida* due to failure of PCR amplification of three RDs (RD5, RD7, and RD16). Notably, none of 17 RDs showed evidence of deletion events in *F. tularensis* subsp. *novicida*. The evolutionary direction might also be inferred from analysis of the gene content of RDs. The finding that 16/17 deletions truncate conserved genes (genes with homologs in other bacteria) implies deletions but not

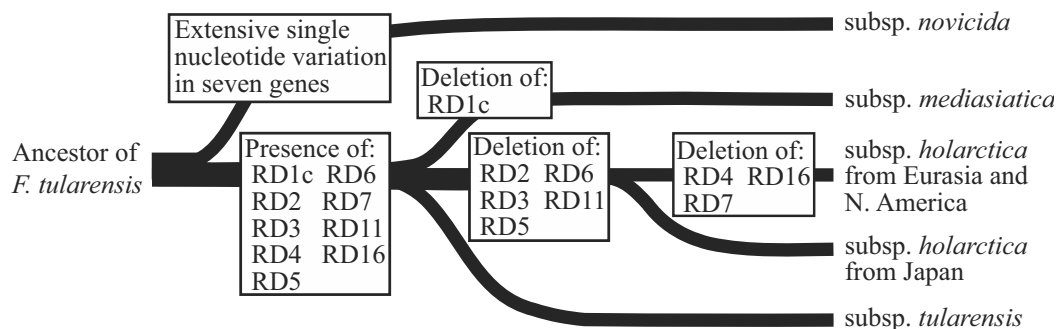


FIG. 2. Evolutionary scenario of *F. tularensis* based on deletion characters assuming a successive loss of genetic material. The scheme reads from left (*F. tularensis* ancestor) to right. *F. tularensis* subspecies are indicated. The horizontal distance may not correspond to actual phylogenetic distances calculated by other methods.

insertions have occurred during evolution. (Table 2; also Table S2 in the supplemental material). Because deletions are expected to represent unidirectional genetic events, RDs of *F. tularensis* are suggested to be valuable evolutionary markers. In fact, if all RDs with a uniform pattern within a subspecies were used in phylogenetic analysis the depicted evolutionary scenario would not change (data not shown).

A phylogeny inferred from analysis of SNVs. Compared to other genomic characters, SNVs exhibit slow mutation rates, making them valuable for phylogenetic analysis (9, 17). We analyzed seven housekeeping gene fragments (3,135 bp) in 15 *F. tularensis* isolates and four gene fragments (1,830 bp) of the tick endosymbiont *W. persica*. A total of 78 SNVs was detected among 15 *F. tularensis* isolates (see Fig. S2 in the supplemental material). The single isolate of *F. tularensis* subsp. *novicida* was the most divergent, showing 41 unique SNVs. Overall, the sample of worldwide *F. tularensis* isolates exhibited a low level of average pairwise sequence diversity (π) with values in the range of 0.0026 to 0.0101 for individual genes. The pattern of SNVs allowed a straightforward subspecies recognition, each *F. tularensis* subspecies being identified by several fixed polymorphisms. There was an extensive sequence similarity among isolates of a *F. tularensis* subspecies.

Four-gene phylogenetic trees were computed to describe the phylogenetic position of *F. tularensis* and its four subspecies among the gamma proteobacteria (Fig. 3). To root the phylogeny, we used *A. tumefaciens* as outgroup, as the α -subgroup is considered ancient among gamma proteobacteria (8). *Y. pestis* of the γ -subgroup of proteobacteria was included as a second outgroup. In the phylogeny obtained, the tick endosymbiont *W. persica* is the first and deepest branch, thus representing an early divergence from a common *Francisella* ancestor. Among the four *F. tularensis* subspecies, *F. tularensis* subsp. *novicida* appeared to represent the first branching. The result is in agreement to findings of a greater amount of genetic divergence among tick endosymbionts or isolates of *F. tularensis* subspecies (4, 12, 22). A greater diversity among isolates likely represents a longer evolutionary history. The four-gene trees demonstrated the split between *F. tularensis* subsp. *tularensis*/*mediasiatica* on one hand and *F. tularensis* subsp. *holartctica* isolates from Japan/Eurasia/North America on the other hand. Seven-gene trees illustrated the position of Japanese *F. tularensis* subsp. *holartctica* isolates close to but distinct from Eurasian/North American *F. tularensis* subsp. *holartctica* isolates (Fig. 3). The five *F. tularensis* subsp. *tularensis* isolates were grouped into two distinct clades. Overall, the present SNV and deletion-based phylogenies support the genetic relationships of *F. tularensis* subspecies calculated from VNTR data, including a genetic division of type A isolates into two distinct clades recently designated A.I and A.II, respectively (Fig. 2 and Fig. 3) (11).

The pairwise statistical comparisons of congruence between gene trees indicated that recombination across *F. tularensis* subspecies occurs rarely or not at all and justifies the use of concatenated gene sequences for tree reconstruction (data not shown). Based on the pattern of deletions and SNVs among isolates, we conclude that the distribution of fixed characters unique to *F. tularensis* subspecies reflects a genetic separation

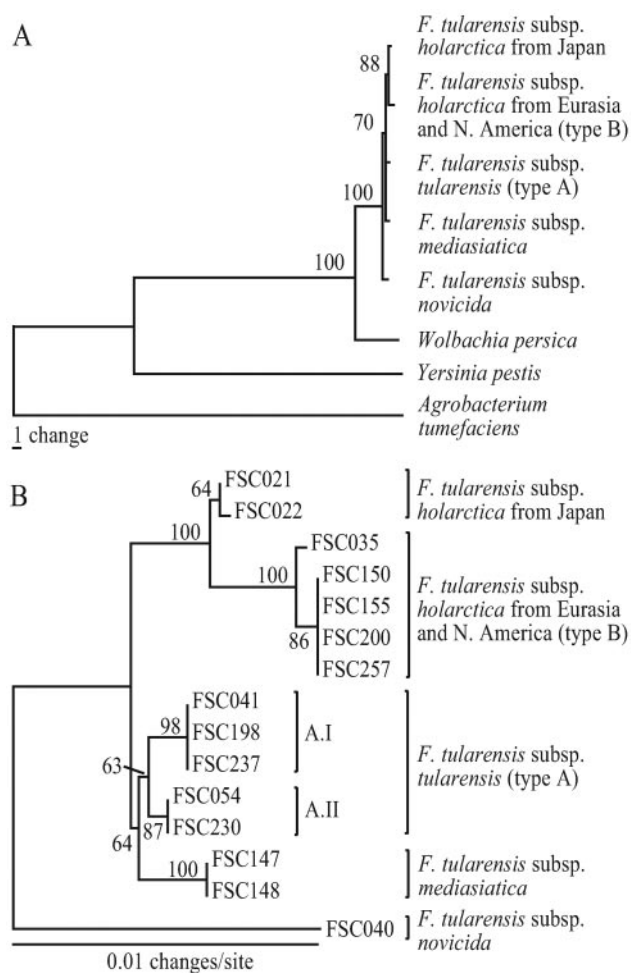


FIG. 3. Phylogenetic analysis of *F. tularensis* using a maximum-likelihood algorithm and based on concatenated sequences of gene fragments. A. Four-gene tree constructed using amino acid sequences (631 amino acids). The tree was rooted using *A. tumefaciens* and *Y. pestis* as outgroups. B. Seven-gene analysis using nucleotide sequences (3,135 bp). The tree was rooted using *F. tularensis* subsp. *novicida* as outgroup. *F. tularensis* subspecies and the division of type A isolates into two clades (A.I, A.II) are indicated. Bootstrap values from 1,000 replications are indicated at interior branch nodes (values of <50% not shown).

of subspecies and also that the subspecies have descended clonally.

Deletions that are specific to laboratory strains. Cell populations within a single *F. tularensis* strain might carry unique unidirectional deletions, i.e., at RD18-19 (Table 1). The finding of unique deletion characters among bacterial colonies derived from a single bacterial seed stock suggests that regions flanked by direct repeats are prone to mutation in *F. tularensis* genomes. Under natural evolution, it appears that direct repeat-mediated deletions only rarely became fixed, as illustrated in our analysis across *F. tularensis* subspecies. According to our results, the majority of observed deletions might be regarded as evolutionary footprints in *F. tularensis* genomes spanning over wide evolutionary time scales. In contrast, it seems that laboratory culture on artificial media facilitates the fixation of deletion variants. We suggest that deletion at RD18-19 might

represent genetic events that occurred at the laboratory during passage or storage of *F. tularensis* strains. This is supported by the finding of a mix of two distinct cell populations that exhibit these RDs in laboratory stocks of strains FSC074 (RD19) and FSC158 (RD18) (Table 1). Based on sequence analysis, direct repeat-mediated excisions at RD18 and RD19 have occurred also in the genome of the live vaccine strain of *F. tularensis*. The live vaccine strain was derived during repeated passages on artificial media (26). Thus, it seems plausible that the fixation of a deletion variant (the live vaccine strain) was facilitated by a repeated selection of single colonies during laboratory passages. It remains to be determined if genes located in RD18 and/or RD19 have a role in the attenuation of the live vaccine strain, the Russian vaccine strain 015 (FSC338), and the avirulent phenotype of the SCHU strain (FSC043). All virulence-attenuated strains included in this study showed deletion at RD18 and/or RD19.

Nucleotide sequence accession numbers. All sequences reported in this paper have been deposited in the GenBank database with accession no. AY794406 to AY794434 (RD2 to RD7, RD11, RD16–22) and AY794435 to AY794543 (SNVs in seven gene fragments). Sequences of RD1 are assigned accession no. AF469614 to AF469619 (1).

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