Extensive Allelic Variation among *Francisella tularensis* Strains in a Short-Sequence Tandem Repeat Region

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Members of the genus *Francisella* and the species *F. tularensis* appear to be genetically very similar despite pronounced differences in virulence and geographic localization, and currently used typing methods do not allow discrimination of individual strains. Here we show that a number of short-sequence tandem repeat (SSTR) loci are present in *F. tularensis* genomes and that two of these loci, SSTR9 and SSTR16, are together highly discriminatory. Labeled PCR amplification products from the loci were identified by an automated DNA sequencer for size determination, and each allelic variant was sequenced. Simpson's index of diversity was 0.97 based on an analysis of 39 nonrelated *F. tularensis* isolates. The locus showing the highest discrimination, SSTR9, gave an index of diversity of 0.95. Thirty-two strains isolated from humans during five outbreaks of tularemia showed much less variation. For example, 11 of 12 strains isolated in the Ljusdal area, Sweden in 1995 and 1998 had identical allelic variants. Phenotypic variants of strains and extensively cultured replicates within strains did not differ, and, for example, the same allelic combination was present in 55 isolates of the live-vaccine strain of *F. tularensis* and another one was present in all 13 isolates of a strain passaged in animals. The analysis of short-sequence repeats of *F. tularensis* strains appears to be a powerful tool for discrimination of individual strains and may be useful for a detailed analysis of the epidemiology of this potent pathogen.

Francisella tularensis is the etiological agent of tularemia, a disease affecting many mammalian species including humans, rodents, and lagomorphs. Rodents and lagomorphs are highly susceptible to the disease and often die from the infection. These animals are believed to be a source of infection for other mammals, and the disease is transmitted to humans via vectors such as ticks or mosquitoes. *F. tularensis* isolates are highly infectious, and a bite by a vector may be sufficient to establish human infection (4).

Tularemia occurs over the whole Northern Hemisphere, and areas with a rather high incidence of the disease exist in Scandinavia, Russia, and United States. The most virulent *F. tularensis* isolates belong to the subspecies *tularensis*, and before the introduction of effective antibiotic treatment, human infections caused by such strains resulted in a mortality rate of up to 30% (5, 14). Although this subspecies was formerly believed confined to the United States, recent studies have reported its occurrence in Europe as well (9). Virtually all European isolates belong to the subspecies *holarctica*, and although such isolates may cause severe disease and are highly infectious, they rarely result in human mortality. *F. tularensis* subsp. *holarctica* strains exist in North America as well. Japanese *F. tularensis* strains belong to the subspecies *holarctica*, while those from Central Asia constitute a separate subspecies, *F. tularensis* subsp. *mediaasiatica* (25). There are only a few known isolates of the fourth subspecies, *F. tularensis* subsp. *novicida* (25). All have been linked to water and may cause disease in compromised individuals (3).

Although its subspecies differ dramatically in virulence, *F.*

tularensis appears to be a genetically homogenous species. Recent taxonomic work has aimed to develop molecular tools for discrimination of the four recognized subspecies and individual strains. This work has been successful insofar as it has allowed discrimination between the two most important subspecies from a clinical standpoint, subspecies *tularensis* and *holarctica*, but none of the developed methods allow high discrimination of individual strains (6, 11, 15). Thus, there is a need for better methods for the studies of the relationships of *F. tularensis* strains.

An ongoing project is aimed to sequence the genome of a prototypical isolate of *F. tularensis* subsp. *tularensis*, Schu S4. This work is more than 98% complete, and a thorough analysis of the genome is therefore possible. We used the sequence information to identify short-sequence repeats. Such repeats appear to exist in variable numbers in many prokaryotic genomes and, since they are rapidly evolving, have shown promise for discrimination of individual strains (29). We identified eight short-sequence tandem repeats in the *F. tularensis* genome. Two loci, each containing a unique repeat, showed high allelic variation. A typing method based on the allelic variation of the two loci is shown to be superior for discrimination of *F. tularensis* isolates compared to previously used methods.

MATERIALS AND METHODS

Bacteria, media, and growth conditions. The bacterial strains (Table 1) all belong to the *Francisella* strain collection of approximately 250 *Francisella* strains and have been tested for specific agglutination and examined by PCR specific for a gene encoding an *Francisella*-specific 17-kDa lipoprotein (24). In addition, they have been biochemically characterized, with the exception of a few recently isolated Swedish strains. The strains included in the study encompassed 39 nonrelated *F. tularensis* isolates representing each of the four subspecies, 32 isolates from five human tularemia epidemics, 55 replicates of a strain serially passaged on solid media, and 13 replicates of a strain isolated after passages in mice, guinea pigs, or alpine hare (21) (Table 1). All strains were characterized

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TABLE 1. *F. tularensis* strains used in this study

Species and origin (no. of strains)	FSC no.	Alternative strain designation	Size (bp) of:	
			9-bp SSTR	16-bp SSTR
<i>F. tularensis</i> subsp. <i>holarctica</i>				
Human, Aänekoski, Finland	249	1468	288	361
Tick, 1949, Moscow area, Russia	257	503/840	360	361
Human outbreak, 1997, Oulu, Finland (6)			396–414	361
Human ulcer, 1984, Norway	097	83933/84	288	361
Hare, 1984, Finland	080	SVA T13	288	361
Tick, 1953, Norway	032	Norway	288	361
Human, 1996, Piteå, Sweden	188		288	361
Human outbreak 1998, Ljusdal, Sweden (6)			297–306	361
Hare, 1952, France	025	Chateauroux	297	361
Human, 1993, Vosges, France	247	SVA T20	297	361
Human outbreak, 1995, Ljusdal, Sweden			297	361
Ticks, 1995, Czech Republic	180	T-17	306	361
Human outbreak, 1981, Ljusdal, Sweden (7)			297–351	361
Norway rat, 1988, Rostov, Russia	150	250	333	361
Live vaccine strain, Russia	155	ATCC 29684	351	361
Water, 1990, Odessa region, Ukraine	124	14588	360	361
Human blood, 1989, Norway	089	45F2	360	361
Tick, 1941, Montana	012	425F4G	369	361
Beaver, 1976, Hamilton, Mont.	035	B423A	378	361
Hare, 1974, Västerdalälven, Sweden	074	SVAT7	315	361
Human blood, 1994, Örebro, Sweden	157	CCUG 33270	387	361
Human, 1999, Karlstad, Sweden	236		396	361
Human epidemic, 1995, Västerdal River, Sweden (6)			315–414	361
Human lymph node, 1926, Japan	017	Jap-S2	234	393
Human, 1950, Japan	022	Ebina	234	393
Tick, 1957, Japan	075	Jama	234	393
Human, 1958, Japan	021	Tsuchiya	234	457
<i>F. tularensis</i> subsp. <i>mediaasiatica</i>				
Tick, 1982, Central Asia, former USSR	148	240	432	361
Midway gerbil (<i>Meriones meridianus</i>), 1965, Kazakhstan	147	543	459	361
Hare, 1965, Central Asia, former USSR	149	120	450	361
<i>F. tularensis</i> subsp. <i>tularensis</i>				
Squirrel, Georgia "CDC standard"	033	SnMF	360	425
Tick, 1935, British Columbia, Canada	041	Vavenby	378	489
Human pleural fluid, 1940, Ohio	046	Fox Downs	459	409
Mite, 1988, Slovakia	198	SE-219/38	369	505
Mite, 1988, Slovakia	199	SE-221-38	468	505
Human ulcer, 1941, Ohio (2 variants)	043, 237	Schu, Schu S4	432	617
Canada	042	Utter	279	345
Human lymph node, 1920, Utah	230	ATCC 6223	279	345
Hare, 1953, Nevada	054	Nevada 14	351	345
Human ulcer, laboratory acquired when handling Nevada 14	053	F. tul AC	351	345
<i>F. tularensis</i> subsp. <i>novicida</i>				
Water, 1950, Utah	040	ATCC 15482	486	NA ^a
Human blood, 1991, Texas	156	Fx1	585	NA
<i>F. philomiragia</i> Muskrat, 1959, Utah	144	ATCC 25015	NA	NA

^a NA, no amplicons were generated under the conditions used.

also by use of a PCR assay based on primers C1 and C4, which identifies *F. tularensis* subsp. *holarctica* strains (15). Bacteria were grown for 2 days on modified Thayer-Martin agar plates (23) at 37°C under 5% CO₂, harvested by scraping, suspended in saline at a concentration of 10⁹/ml, and heat killed at 65°C for 2 h. The cell suspensions were stored at –20°C until used.

Tularemia epidemics. Strains from epidemics occurring in areas along the rivers Ljusnan and Västerdalälven in central Sweden in 1981, 1995, and 1998 and from an area surrounding the city of Oulu in northern Finland in 1997 were received from laboratories which had performed the primary isolation. The isolation had been performed by (i) the Swedish Defense Research Agency; (ii) the Swedish Institute for Infectious Disease Control, Stockholm, Sweden; or (iii) the Clinical Microbiology Laboratory, Oulu University Hospital, Oulu, Finland.

All strains were further characterized by our laboratory to ascertain the species to which they belonged. For each epidemic, the suspected locations where infection had been contracted were at most 50 km apart. Cases associated with the same epidemic occurred within a period of at most 2 months, with the exception of the Oulu outbreak, where isolation dates encompassed 1 year. The area of Västerdalälven is located at latitude 60.5° and longitude 14.5°, Ljusdal is located at latitude 61.8° and longitude 16.1°, and Oulu is located at latitude 65.2° and longitude 25.5°. The distance from Ljusdal to Västerdalälven is 180 km, and the distance from Ljusdal to Oulu is 650 km.

Identification of repetitive elements. At present, the genome of the Schu S4 strain of *F. tularensis* subsp. *tularensis* is being sequenced. Our analysis was based on a total of 1.9 million bases, estimated to represent >98% of the total genome.

TABLE 2. Sequence heterogeneity of the 9-bp SSTR does not alter the encoded amino acids^a

Variants of 9-bp tandem repeats encoding amino acids Asn-Lys-Asp	Designation ^b	No. of tandem repeats found in the 9-bp locus	Presence in all strains of subspecies:
AACAAAGAC	SSTR9A	1–6	<i>tularensis</i> , <i>holarctica</i> , and <i>novicida</i>
AATAAAGAC	SSTR9B	1–3	<i>tularensis</i> and <i>novicida</i>
AACAAAGAT	SSTR9C	1	<i>mediaasiatica</i>
AATAAAGAT	SSTR9D	1–24	<i>mediaasiatica</i> and <i>novicida</i>
AATAAGGAT	SSTR9E	2–26	All

^a The alleles were sequenced in strains belonging to subspecies *tularensis* (*n* = 6), *holarctica* (*n* = 19), *mediaasiatica* (*n* = 2), and *novicida* (*n* = 2).

^b Designations SSTR9A to SSTR9E refer to the sequence variants of the 9-bp repeat. The variants were each arranged in tandem, and these tandem regions always appeared in the order A, B, C, D, and E.

there were correlations between repeat variants and subspecies; i.e., some repeats were found only in some subspecies but in all sequenced alleles of the subspecies. Moreover, the sequencing revealed that each of the variants of the 9-bp repeats were arranged in tandem and that the tandem regions were always arranged in the order A, B, C, D, and E (designations as indicated in Table 2).

Analysis of the region containing the SSTR16 element. A 16-bp SSTR was identified in multiple loci of the genome sequence, and each 16-bp repeat was part of a larger repetitive element resembling an IS element. In the current assembly of the Schu S4 genome, 35 identical IS elements containing 2 to 18 copies of the 16-bp repeat were found. After an initial evaluation of 10 loci (data not shown), a locus containing 18 copies in tandem, designated SSTR16, was found to be the most discriminatory and was selected for further analysis (Fig. 2). The 1,208-bp IS element of this locus contained two open reading frames encoding putative proteins of 126 (ORF1) and 118 (ORF2) amino acids. A GenBank Blastx search identified homology of ORF2 to a transposase of *Aspergillus niger* (GenBank accession no. AAB50684; E value, 1×10^{-15} ; 48 of 120 identical amino acids).

The 16-bp tandem repeats were located in a noncoding region adjacent to a 14-bp inverted repeat at one end of the putative *F. tularensis* transposon. PCR amplification of the SSTR16 region from different *F. tularensis* strains showed that its size ranged from 345 to 617 bp. No sequence heterogeneity of the 16-bp direct repeat was observed by sequencing of PCR products from 12 strains.

Variability of the SSTR9 and SSTR16 loci among *F. tularensis* strains. PCR amplification was performed on samples from each strain by using labeled primers, and amplicons were

FIG. 2. Sequence of the SSTR16 region in the Schu S4 strain. The 16-bp direct repeats are marked by arrows and are present in 18 copies. Primer positions are shaded. The direct repeats are located inside an IS element; terminal inverted repeats of the IS element are shown in boldface. Thirty-five copies of the IS elements have been identified in the current assembly of the Schu S4 genome, each containing 2 to 18 copies of the 16-bp tandem repeat.

FIG. 3. Representative electrophoretic analysis of PCR fragments of the SSTR9 loci from different isolates of *F. tularensis*. Fragments were detected by fluorescence on an ABI 377XL DNA sequencer. Strains of each of the four subspecies are represented; *F. tularensis* subsp. *tularensis* in lanes 7 (FSC198), 9 (FSC199), and 17 (FSC054); *F. tularensis* subsp. *holarctica* nonrelated European and North American isolates in lanes 1 (FSC176), 3 (FSC180), 4 (FSC247), 5 (FSC188), 6 (FSC012), 14 (FSC025), 16 (FSC032), 20 (FSC150), 21 (FSC076), 22 (FSC155), 23 (FSC080), 24 (FSC157), 25 (FSC089), 26 (FSC161), and 27 (FSC097), isolates from Ljusdal, Sweden, 1995 and 1998 in lanes 2 (FSC245), 11 (FSC200), 13 (FSC201), 15 (FSC202), and 29 (FSC102), and nonrelated Japanese isolates in lanes 8 (FSC017), 10 (FSC021), 12 (FSC022), and 19 (FSC075); *F. tularensis* subsp. *mediasiatica* in lane 18 (FSC149); and *F. tularensis* subsp. *novicida* in lane 28 (FSC040). Allele sizes (in base pairs) are indicated to the left.

analyzed for size by electrophoresis on nondenaturing polyacrylamide gels. A representative electrophoretic analysis of the SSTR9 PCR amplicons is shown in Fig. 3. The size range and sequencing of the SSTR9 locus showed that it contained a minimum of 3 and a maximum of 43 copies whereas the SSTR16 locus contained a minimum of 1 and a maximum of 18 copies of the respective SSTR. The amplicon sizes are summarized in Table 1. The SSTR9 locus showed a high allelic variation, and if the strains listed in Table 3 are excluded from the calculation because of their epidemiological relationship, Simpson's index of diversity was 0.95 based on results from 39 unrelated strains.

The SSTR16 region was present in only two allelic variants in the *F. tularensis* subsp. *holarctica* and *mediaasiatica* strains, whereas it was highly discriminatory among *F. tularensis* subsp. *tularensis* strains. Simpson's index of diversity for this locus was

0.42. If the discriminatory power of the two loci was combined, the index of diversity was 0.97.

No allelic variation was observed in extensively cultured replicates within strains (Table 3). Of 55 replicates of the live-vaccine strain of *F. tularensis* subjected to repeated passages on agar medium over a period of 3 months, all had identical allelic variants of SSTR9 and SSTR16 (Table 3). Analysis of 13 isolates from 7 passages of strain FSC074 in mice, guinea pigs, and hares showed no allelic variation and no sequence heterogeneity. The strain was originally isolated in Sweden from a hare. Similarly, the prototypical *F. tularensis* subsp. *tularensis* strain, Schu, and the laboratory-derived smooth variant, Schu S4, contained the same allelic combination (Table 3). Moreover, a rabbit isolate of *F. tularensis* subsp. *tularensis* and an isolate subsequently cultured from a laboratory worker infected with the same strain (2) had an identical

TABLE 3. Variation of the SSTR9 types in epidemic or passages of *F. tularensis* strains^a

Origin	Yr of isolation	No. of isolates	Source	SSTR9 allele sizes (bp)	No. of allelic variants of SSTR9
Human epidemic, Oulu area, Finland	1996–1997	7	Ulcer, pleural fluid, blood	396–414	3
Human epidemic, Ljusdal area, Sweden	1981	7	Ulcer	297–351	3
Epizootic in hares, Ljusdal area, Sweden	1981	1	Autopsy	351	1
Human epidemic, Ljusdal area, Sweden	1995	6	Ulcer	297	1
Human epidemic, Ljusdal area, Sweden	1998	6	Ulcer	297–306	2
Human epidemic along the river Västerdalälven, Sweden	1995	6	Ulcer	315–414	3
Phenotypic variants of Schu strain (Schu and Schu S4), originally a human isolate	1941	2	Ulcer	432	1
Rabbit isolate (Nevada 14) and laboratory-acquired infection (F. tul. AC) from handling the isolate	1953 and 1954	2	Autopsy of hare and human ulcer	351	1
Passages of LVS (ATCC 29684) on agar medium		55		351	1
Passages of hare isolate (FSC074) in small mammals	1974	13	Autopsy	315	1

^a Schu strain, Nevada 14, and F. tul. AC belong to *F. tularensis* subsp. *tularensis*; all other isolates belong to *F. tularensis* subsp. *holarctica*.

TABLE 4. Allelic variation of SSTR9 of *F. tularensis* strains from Ljusdal, Sweden, isolated at different times

Allele size (bp)	Allelic variation for strains isolated in ^a :		
	1981	1995	1998
297	●	●●●●●●	●●●●●●
306	●●●●●		●
351	●○		

^a Each human isolates is shown as a solid circle, and an isolate from a hare is shown as an open circle.

allelic combination that was not present in any other analyzed strain (Table 3).

The analysis of strains from outbreaks that occurred in the Ljusdal area of Sweden in 1981, 1995, and 1998 showed that only three allelic variants were represented among the 18 strains and at most two variants were present in each outbreak (Tables 3 and 4). The presumed location where infection had been contracted was within an area of 10 km along the river Ljusnan. A detailed summary of the allelic variants is shown in Table 4. All except one strain showed an identical allelic combination from the outbreaks in 1995 and 1998. The only aberrant strain contained an allelic variant identical to the one found in most strains from the 1981 outbreak. During the latter outbreak, one strain was isolated with an allelic variant different from that in all other human isolates from Ljusdal but identical to that in an isolate from a dead hare found in the Ljusdal area the same year.

Isolates from Ljusdal showed a closer relationship than did isolates from an area along the river Västerdalälven in Sweden. In the latter case, the allele sizes were 315, 315, 405, 414, 414, and 414 bp (Table 3). The cases occurred in locations up to 50 km apart. The six isolates from the Oulu area in Finland showed three allelic variants (Table 3). In this case, the times of isolation varied over a period of more than 1 year. The size of the allelic variants varied from 396 to 414 bp. The cases occurred in locations to 50 km apart.

DISCUSSION

Our previous work showed that *F. tularensis* strains display a very limited genetic diversity, despite their varied geographical origin and wide variation in virulence, and this has hampered the development of useful tools for studies of the epidemiology of the pathogen. Restriction enzyme cleavage or sequence analysis of 16S rRNA genes demonstrated that the four subspecies could be discriminated, but individual strains could not be (6, 11). A recent study evaluated the application of PCR based on the use of various arbitrary primers or primers specific to repetitive extragenic palindromic or enterobacterial repetitive intragenic consensus sequences (15). It was concluded that the methods were useful for rapid analysis and may be a technically simple strategy for discrimination of subspecies but not of individual strains. Pulsed-field gel electrophoresis has become the standard for typing of many bacterial species (27). The method has, however, not allowed much discrimination of *F. tularensis* strains within the same subspecies (A. Johansson and A. Sjöstedt, unpublished data). Thus, there is a need for methods that allow discrimination of individual strains.

A recent development in molecular typing is based on the analysis of short-sequence repeats in prokaryotic genomes. The repeats may be a result of replication slippage and, although they are more infrequent in prokaryotic than in eukaryotic genomes, appear to be present in variable and often relatively large numbers (31). In many cases their role is obsolete, but recent studies show that they may regulate antigenic variation of, for example, virulence-associated genes and that their presence within genes may contribute to the coding potential of the messenger or the level of translation efficiency (29). Amplification of regions containing variable numbers of repeat elements has formed a basis for successful typing of individual strains from a variety of pathogens such as *Escherichia coli* (20), *Haemophilus influenzae* (30), *Staphylococcus aureus* (28), *Mycobacterium tuberculosis* (7, 17), *Mycobacterium africanum* (8), *Helicobacter pylori* (19), *Yersinia pestis* (1), and *Bacillus anthracis* (12, 16, 22).

Our findings show that typing of *Francisella* strains based on the analysis of SSTR is the method with the highest resolution tested so far. The repeats differ between strains of the same subspecies, and when the combined variability of the repeats was assessed, Simpson's index of diversity was 0.97. Therefore, it is the first method used for typing of *F. tularensis* strains that fulfills the recommended criterion, ≥ 0.95 , for routine typing of individual isolates (26). The relevance of the index requires that the test population reflect the diversity of the investigated species. We consider that our strain collection fulfills this criterion since the isolates originated from all relevant regions of the Northern Hemisphere and all four subspecies of *F. tularensis* are represented. The usefulness of the markers for epidemiological analyses of strains is supported by the finding that the allelic variants were not affected by extensive culturing in vitro or by passages in laboratory animals.

Despite the high discriminatory power of the method, we observed that epidemiologically related Scandinavian strains, i.e., strains isolated from a relatively confined area during a period of 2 months, contained the same or a few alleles of SSTR9. Moreover, the size variation of the SSTR9 allele was small in the related strains, only 9 bp in a majority of the strains from Ljusdal and at most 18 bp in the strains from Oulu. This may indicate that the strains from each epidemic, although containing distinct SSTR9 alleles, were genetically closely related. Moreover, it indicates that the same or similar clones of *F. tularensis* may persist in a geographically confined area, as supported by the finding that 18 of 20 strains from Ljusdal isolated during a period of 17 years differed in allele sizes by at most 9 bp. In Scandinavia, transmission to humans occurs predominantly in late summer or early autumn. If there is a persistence of clones of bacteria for years in various regions, this indicates that the seasonal outbreaks of tularemia occurring in Scandinavia are due to specific climatic conditions.

The copy number polymorphism of the SSTR9 locus, in particular, indicates that repeated insertions and deletions of repeats occur among *F. tularensis* isolates and is suggestive of a slipped-strand mispairing that create and maintain directly repeated sequences (31). A functional role of the repeat is implied by the conservation present at the first and second codon positions in all of the sequenced strains, whereas the point mutations that obviously occur in the third codon posi-

tion may be allowed due to their minor effect on protein structure.

Despite a limited diversity within the genomes of various bacterial species, several recent studies on, for example, *Y. pestis* (1) and *B. anthracis* (13, 16) show that even in such genomes, variable numbers of repetitive elements exist that evolve rapidly and thus show great discriminatory power. In this respect, the results of the studies are analogous to our findings with *Francisella*. The discrimination of *F. tularensis* strains was, however, considerably higher than that for *Y. pestis* or *B. anthracis* strains. With regard to *B. anthracis* isolates, Keim et al. suggested that strains of different geographical origins may show identical allelic variants since clones have been dispersed worldwide due to human activities (16). In the case of *Y. pestis*, typing based on a tetranucleotide repetitive element appear to yield a more limited discrimination since the same allelic variant was present in strains of different biovars.

In conclusion, our findings demonstrate that the analysis of short-sequence repeats is a powerful tool to understand the relationship of *F. tularensis* strains and may become an important tool for a detailed analysis of the epidemiology of the pathogen. The role of the *Francisella* short-sequence repeats is not understood, and it will be important to determine their biological significance.

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