Genomic Deletion Marking an Emerging Subclone of *Francisella tularensis* subsp. *holarctica* in France and the Iberian Peninsula[▽][†]

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Francisella tularensis **subsp.** *holarctica* **is widely disseminated in North America and the boreal and temperate regions of the Eurasian continent. Comparative genomic analyses identified a 1.59-kb genomic deletion specific to** *F. tularensis* **subsp.** *holarctica* **isolates from Spain and France. Phylogenetic analysis of strains carrying this deletion by multiple-locus variable-number tandem repeat analysis showed that the strains comprise a highly related set of genotypes, implying that these strains were recently introduced or recently emerged by clonal expansion in France and the Iberian Peninsula.**

The species *Francisella tularensis* is a gram-negative intracellular pathogen comprising four different subspecies: subsp. *tularensis*, subsp. *holarctica*, subsp. *mediasiatica*, and subsp. *novicida*. Of these four, the *tularensis* and *holarctica* subspecies are clinically significant for humans, with the *tularensis* subspecies causing a more serious disease in humans and with animal models (7, 25). In addition to virulence characteristics, *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* also display unique geographic distributions. *F. tularensis* subsp. *holarctica* is found in the Eastern and Western hemispheres, whereas *F. tularensis* subsp. *tularensis* appears to be limited to North America (7).

The basis for these apparent differences in ecological and virulence characteristics is not well understood. Comparative genomics studies show that genomic organization among the subspecies is distinct, with multiple rearrangements, translocations, inversions, and recombination events that have occurred during divergence of the subspecies (1, 6, 15, 20, 23). Nonetheless, the composition of the genomes is remarkably similar among the subspecies.

Though *F. tularensis* subsp. *tularensis* populations display a more limited geographic range, at least two different sublineages (AI and AII) have been detected in the Eastern and Western portions of the United States by at least two different genotyping methods (8, 13, 18, 24). In contrast, *F. tularensis* subsp. *holarctica* populations found throughout North America and the Palearctic ecozone of Eurasia show surprisingly little genetic diversity, leading many to speculate that these populations are derived from a relatively recent bottleneck or clonal expansion event (6, 18, 20–22, 25).

The dearth of genetic diversity in *F. tularensis* poses substantial epidemiological problems, particularly with *F. tularensis* subsp. *holarctica*, constraining molecular epidemiological analyses to the most rapidly evolving genetic markers, such as tandem repeats. Because these markers can be susceptible to homoplasy, additional genomic markers or events can greatly enhance both epidemiological and phylogenetic inference. With this problem in mind, we used our previously described shotgun DNA microarray (23) to search *F. tularensis* subsp. *holarctica* strains for genetic diversity by comparative genome hybridization (CGH). Our strain collection included a set of 42 *F. tularensis* subsp. *holarctica* strains derived from an epidemic of tularemia in animals and humans in Spain and Southern France in 1997 to 1999 (5, 10), as well as three strains from Czech Republic (Tu-28, Tu-29, and Tu-35) and one from the Russian Federation (Tu-42) (see Table S1 in the supplemental material). A set of 16 *F. tularensis* subsp. *tularensis* strains, an *F. tularensis* subsp. *novicida* strain, and a *Francisella philomiragia* strain that had previously been tested with this same array were also included as controls (23). DNA isolation, labeling, hybridization, washing, and data analyses were performed as we have previously described, with binary conversion of the ratios based on >2 standard deviations from the mean of each array (23, 26).

To validate performance of this microarray data set, the

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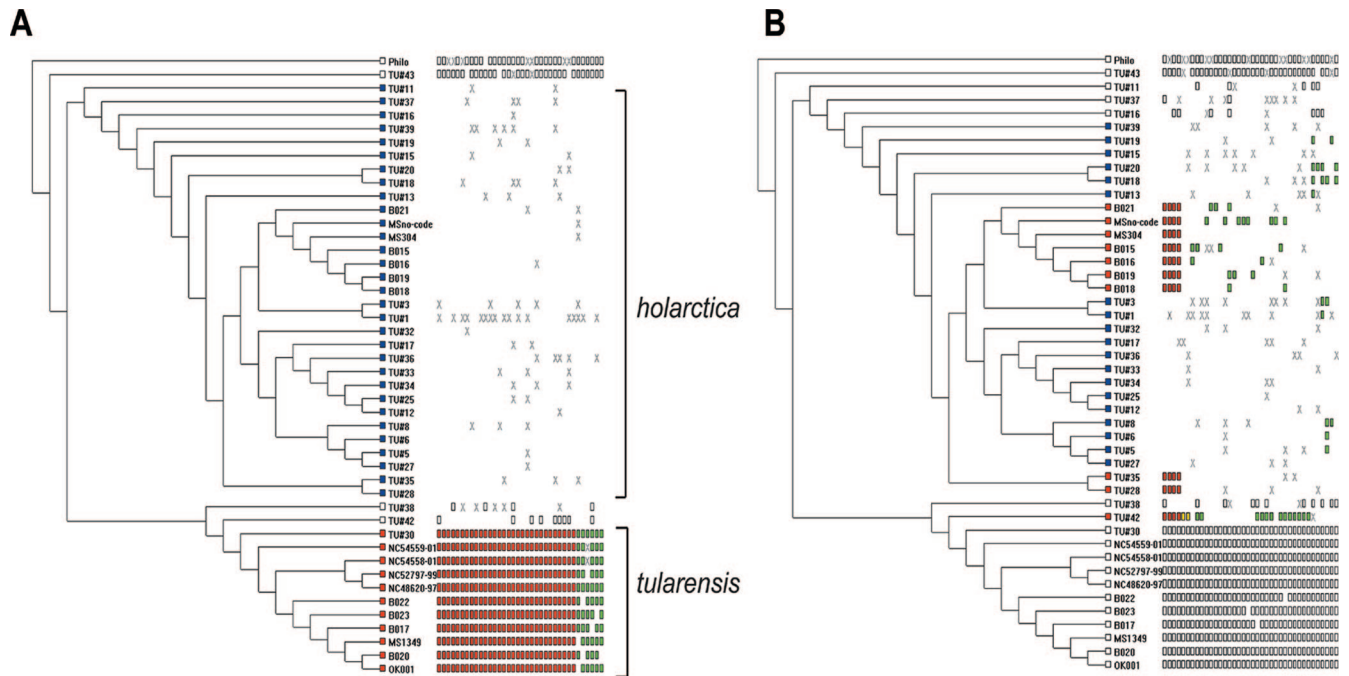▽ Published ahead of print on 21 September 2007.

FIG. 1. MARKFIND output identifying microarray addresses corresponding to RD$_{23}$. The dendrograms were generated using the MARKFIND program (26). Dendrograms were generated using the unweighted-pair group method using average linkages algorithm of the binary converted comparative genome hybridization data. The data sets were converted to binary 1 if the ratio of test to reference was <2 standard deviations from the mean (segment present) and converted to 0 if the ratio of test to reference was >2 standard deviations from the mean (significant deviation in hybridization signal). Strain names are indicated to the right of the leaves of the tree. In panel A, the binary data were sorted in the MARKFIND program to identify segments unique to *F. tularensis* subsp. *tularensis* strains (red squares at leaves of branches) and absent in *F. tularensis* subsp. *holarctica* (blue squares at leaves of branches). The white or colored rectangles to the right of the branches illustrate individual array features that score binary 1 (segment present), whereas white space indicates binary 0 (significant deviation in hybridization signal). An X indicates missing data or instances where the standard deviation was <2 but >1. In panel B, the same UPGMA dendrogram is depicted, except the binary data were sorted to identify array features that score binary 0 for all *F. tularensis* subsp. *holarctica* strains derived from Spain and France (marked by blue squares at the leaves) but binary 1 for all other *F. tularensis* subsp. *holarctica* stains (red squares at leaves) and binary 1 for all *F. tularensis* subsp. *tularensis* strains and *F. philomiragia* strains (white rectangles at leaves).

binary-converted data were sorted to identify probes hybridizing specifically to *F. tularensis* subsp. *tularensis* strains (Fig. 1A) but not the *F. tularensis* subsp. *holarctica*, *F. tularensis* subsp. *novicida*, or *F. philomiragia* strains. This yielded 36 probes, which DNA sequence and contig analysis subsequently showed to all be derived from the previously identified RD$_{tularensis}$1 to RD$_{tularensis}$13, validating that the array performance was similar to that in our previous study (23). To screen the data for diversity within *F. tularensis* subsp. *holarctica*, the data were then sorted among the *F. tularensis* subsp. *holarctica* strains according to geography. As shown in Fig. 1B, four of the microarray probes hybridized to *F. tularensis* subsp. *holarctica* strains isolated from the United States, the Czech Republic, and Russia but not to the large set of *F. tularensis* subsp. *holarctica* isolated from Spain. DNA sequence analysis revealed that all four probes formed a 1.8-kb contig (Fig. 2), corresponding to the FTL_1083 to FTL_1080 coding regions of the *F. tularensis* subsp. *holarctica* LVS genome sequence and the corresponding region encoding FTT1006 to FTT1009 in the *F. tularensis* subsp. *tularensis* SCHU S4 genome sequence (15, 21). The CGH pattern, the fact that these probes formed a contig, and the fact that the Spanish strains are believed to comprise an epidemic (19) suggested that this genome alteration could represent a marker of a clonal population. This

segment is referred to herein as the region of genomic difference 23 (RD$_{23}$) in keeping with the previously used RD numbering system (1, 23, 25).

To precisely map the endpoints of the RD$_{23}$ deletion, PCR primers were designed within the FTT1005c and FTT1009 coding regions abutting the 1.8-kb RD$_{23}$ contig (RD$_{23}$Forward, 5′-GTCTTGTTGAGCAAATGCCC-3′; RD$_{23}$Reverse, 5′-CGGAGCAGGCTTAAATAGTGA-3′) and PCR was performed
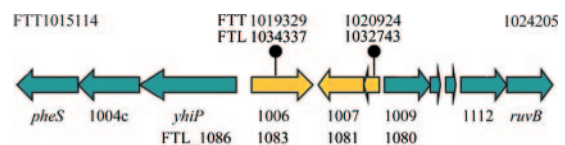

FIG. 2. Map of the RD$_{23}$ genomic region from the *F. tularensis* subsp. *tularensis* SCHU S4 genome. The arrows depict individual coding regions. Coordinates of the 3′ end of *pheS* and the 5′ end of *ruvB* (relative to the *F. tularensis* subsp. *tularensis* strain SCHU S4 genome sequence) are indicated above the respective arrows. Coordinates of the deletion endpoints are indicated above the ball and sticks for the corresponding *F. tularensis* subsp. *tularensis* SCHU S4 genome sequence (FTT) and the corresponding *F. tularensis* subsp. *holarctica* LVS genome sequence (FTL). Coding regions from the FTT and FTL genome sequences are indicated below the relevant portion of the map.
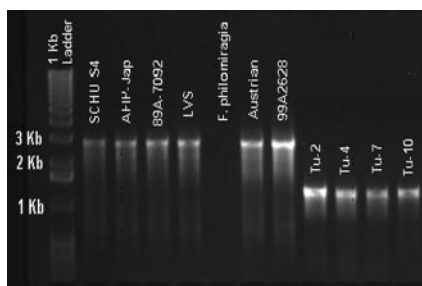
FIG. 3. PCR analysis of RD$_{23}$. Primers for the RD$_{23}$ PCR assays were positioned an additional 1 to 2 kb from the left and right ends of the respective RD to ensure inclusion of the junctions in the amplicons. PCRs were performed with 25-μl volumes containing 5 mM MgCl$_2$ and 160 μM of each deoxynucleoside triphosphate (Idaho Technology, Salt Lake, UT), 500 nM (each) of forward and reverse primer, and 2.5 U of Platinum *Taq* (Invitrogen). Thermocycling conditions were optimized and performed using both T-Gradient (Biometra, Göttingen, Germany) and Dyad (MJ Research, Reno, Nevada) thermocyclers according to the following cycling parameters: initial hold at 95°C for 2 min, 30 s; 30 cycles of 95°C for 30 s, 64°C for 1 min, and 72°C for 1 min; final extension at 72°C; and a final indefinite hold at 4°C. PCR products were electrophoresed on an agarose gel and stained with ethidium bromide. Strain designations are indicated above the respective lanes. The marker lane on the left contains the 1-kb ladder with sizes of the 1- to 3-kb fragments indicated to the left. (For more information on strain designations, see Table S1 in the supplemental material.)

on *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* strains. As shown in Fig. 3, *F. tularensis* subsp. *holarctica* strains showing the RD$_{23}$ CGH pattern (Tu-2, Tu-4, Tu-7, and Tu-10) gave rise to PCR products of the same size, 1.38 kb, whereas all other strains produced the expected 2.97-kb amplicon, in common with the SCHU S4 strain.

DNA sequence analysis of the shorter, 1.38-kb fragment from strain Tu-19 showed that the RD$_{23}$ deletion extends from the FTT1006 coding region to that of FTT1008, as predicted from the microarray data. The deletion begins in the central region of the FTT1006 (403 amino acids; hypothetical membrane protein) coding region at the Leu160 codon (SCHU S4 coordinate 1019329) and extends into the FTT1008c (54 amino acids; hypothetical protein) coding region, ending at codon Leu23 (SCHU S4 coordinate 1020924). The protein encoded by FTT1006 shares similarity with several different proteins, including transporter proteins and *flgJ* product-like amidases. FTT1007 and FTT1008 encode hypothetical proteins.

RD$_{23}$ is limited to strains from Spain and Southern France. To test whether the RD$_{23}$ deletion is a clonal marker for an epidemic population, a real-time PCR assay was developed to specifically identify the RD$_{23}$ deletion and subsequently used to test a large set of *F. tularensis* strains. The design of the assay was based on use of TaqMan fluorophore probes, which can be detected on the ABI 7900HT Fast real-time PCR system (Applied Biosystems, Foster City, CA). Primers were designed to generate amplicons approximately 200 bp in size using Primer Express v. 2.0 software (Applied Biosystems). The primers and probe for the leftward flank of the deletion were RD$_{23}$Left Forward (5′-TTTGTTAGGATTTAGTTTTTGTTTACTTATAGGT-3′), RD$_{23}$Left Reverse (5′-ACTGACTCCCTTAGAACCAGAGT CA-3′), and RD$_{23}$Left Probe (VIC-5′-AGTCGATACTAATCAA **K**AATTTGTTGCACC-3′-6-carboxytetramethylrhodamine)

(where K = T or G). The oligonucleotide sequences for the rightward flank of the deletion were RD$_{23}$Right Forward (5′-G AAATATTCCATCTCCATCAAAATGC-3′), RD$_{23}$Right Reverse (5′-ATGGTTTAAAGATGACAATAGTAAGTCGA-3′), and RD$_{23}$Right Probe (6-carboxyfluorescein-5′-ACTACTTTGAT TA**R**GCATAAAAGCAAG-3′-6-carboxytetramethylrhodamine) (where R = A or G). Because naturally occurring single nucleotide polymorphisms were discovered in the SCHU S4 and LVS genome sequences in the probe segments, the probes were constructed with degenerate bases at the "K" and "R" positions (shown in boldface).

The real-time (RT)-PCR assay was developed as a duplex assay, consisting of the leftward forward primer as the common forward primer, the leftward probe just inside the deletion and flanked by its reverse primer, and the rightward probe outside the right junction and flanked by its reverse primer. This scheme allows detection of the RD$_{23}$Left Probe in instances of an intact region and detection of the RD$_{23}$Right Probe in strains carrying the RD$_{23}$ deletion. The RT-PCRs were performed in 25-μl reaction volumes containing 1× Universal Master Mix (Applied Biosystems), 400 nM of each primer, 600 nM of each probe, and 0.55 U of Platinum *Taq* (Invitrogen). The thermocycling conditions included 50°C for 2 min; a hold for 10 min at 95°C; 45 cycles of 95°C for 15 s, 56°C for 15 s, and 61°C for 1 min; and a final indefinite hold at 4°C. The RT-PCR was validated by first comparing a set of 90 strains, using the conventional PCR and the RT-PCR, where perfect correlation was observed (data not shown). We subsequently used the RT-PCR to screen a large strain collection comprising isolates of *F. tularensis* subsp. *tularensis* (*n* = 88), *F. tularensis* subsp. *holarctica* (*n* = 300), *F. tularensis* subsp. *novicida* (*n* = 7), *F. tularensis* subsp. *mediasiatica* (*n* = 4), and *F. philomiragia* (*n* = 6) strains.

Only 51 of 405 strains tested carried the RD$_{23}$ deletion (see Table S1 in the supplemental material). All 51 of the RD$_{23}$ deletion strains were *F. tularensis* subsp. *holarctica* strains, and 50 of the 51 RD$_{23}$-positive strains were isolated from Spain or France. The six strains isolated from France were from Chateneaux (AFIP3 and F0295), St. Germaine (AFIP4), Lorraine (FR-LauR), Langres (FR-Syl-S), and Vosges (F0020), implying that RD$_{23}$-positive strains may be widespread in France. The FR-LauR and FR-Syl-S strains were isolated in 1993 and 2000, respectively, with the FR-Syl-S strain being isolated from one of the first cases of human *F. tularensis* bacteremia reported in Southern Europe (12). However, the F0020 strain was isolated in 1952, implying that the RD$_{23}$-positive strains may have been in France for some time.

The larger set of RD$_{23}$-positive strains from Spain comprised isolates from the Valladolid, Palencia, Zamora, and Leon regions, representing a wide geographic distribution in this country. These strains were all isolated during a narrow time frame of 1997 to 1999, coinciding with an epidemic of tularemia found in humans and animals during that time (11). The only exception of a strain carrying RD$_{23}$ that was isolated outside of Spain or France was F0228, which was isolated in Uppsala, Sweden. Thus, with the exception of the F0228 isolate, the RD$_{23}$ allele appears to be concentrated among strains isolated from the Iberian Peninsula and France.

Strains carrying RD$_{23}$ comprise a diverging clonal complex. Many of the RD$_{23}$-positive isolates in our collection from Spain have previously been shown to share a close genetic
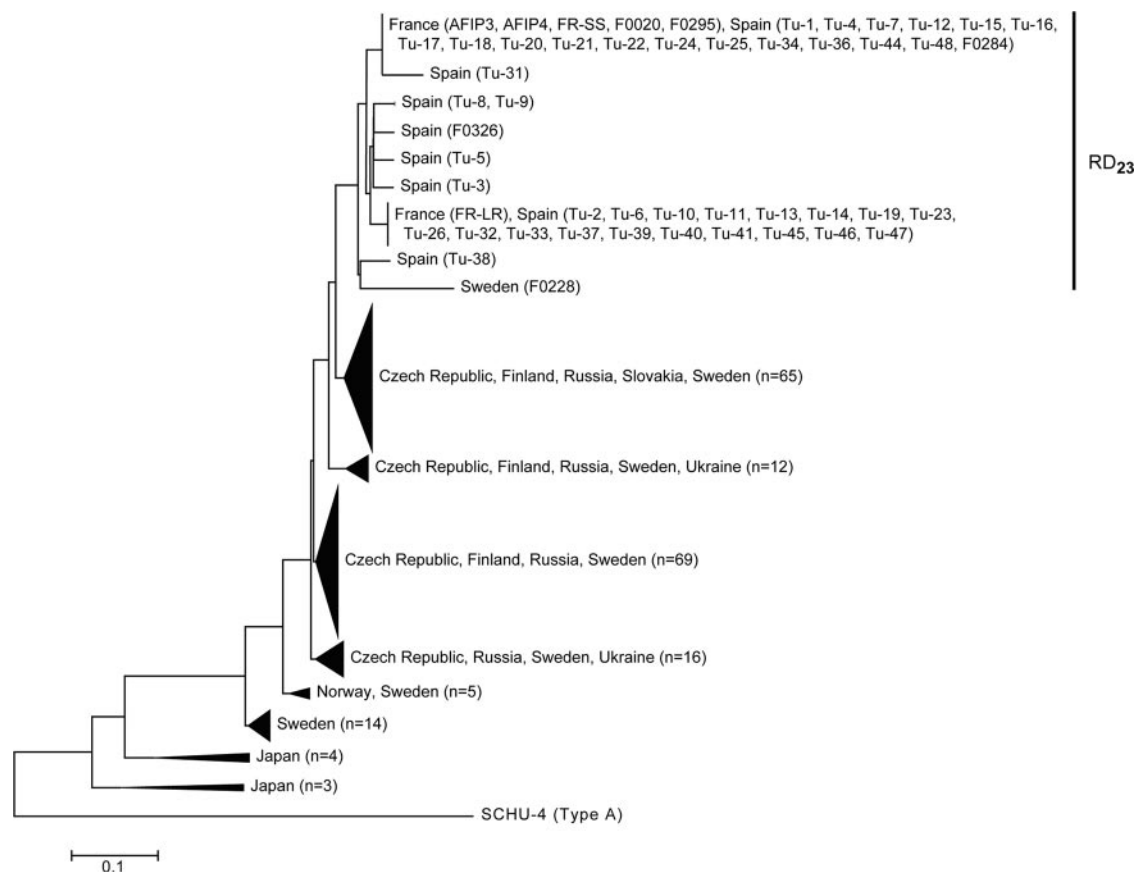
FIG. 4. Cluster analysis of MLVA data from *F. tularensis* subsp. *holarctica* strains with or without the RD_{23} deletion. The dendrogram was generated by neighbor joining using a midpoint root with MEGA 3.1 software. Multiple strains in the same cluster are indicated by filled triangles at the tips of the branches. SCHU S4, an *F. tularensis* subsp. *tularensis* strain, was included as an outgroup.

relationship by amplified fragment length polymorphism and other genotyping methods (5, 9). If the RD_{23} deletion is indeed a clonal marker of an epidemic population, then we would expect that the strains from France carrying RD_{23} should also be genetically related. The RD_{23}-positive strains were therefore genotyped by multiple-locus variable-number tandem repeat analysis (MLVA) and normalized to MLVA data generated previously (8, 13). MLVA was performed as described previously (13) using primers amplifying regions Ft-M1 through Ft-M25. All reverse primers were labeled with IRD800 (Li-Cor, Lincoln, NE), and the reactions were resolved on 41-cm gels using a Li-Cor 4000L automated DNA sequencer. Control reactions were run using the SCHU S4 and LVS strains. Sizes of MLVA products were predicted from a combination of known SCHU S4 and LVS sizes, the 50- to 350-bp and 50- to 700-bp IRD800 size standards (Li-Cor), and a 1-bp sequencing ladder. The data from each strain were tabulated with the previously published MLVA data (13) run on an ABI 377 system (Applied Biosystems). Data from the two data sets were converted to numeric base pair values and normalized for allele size by the NAU Laboratory. An unweighted, midpoint-rooted, neighbor-joining tree was created using MEGA 3.1 software (14).

As shown in Fig. 4, all strains carrying the RD_{23} deletion comprised a single cluster based on MLVA genotypes. All strains in this cluster shared a unique 466-bp allele at M24 that was not observed in any other strain, supporting the conclusion that they are clonally related. Divergence within the cluster was due to multiple alleles at the M3, M6, M8, and M10 loci. Polymorphism at the M3 and M6 loci alone accounts for six of the nine different MLVA genotypes in the RD_{23} cluster. These loci are among the most highly polymorphic of the MLVA loci examined to date and would be expected to show some differentiation even within a clonal complex (13). Polymorphic alleles at the M8 and M10 loci within the RD_{23} cluster were detected only in strain F0228 (unique allele at M8) and strain Tu-31 (unique allele at M10). Thus, strains carrying the RD_{23} deletion appear to form a clone complex with divergence largely marked by events at the most rapidly evolving loci. The most divergent member of the RD_{23} clade was strain F0228, which carries unique alleles at M3, M6, and M8. The F0228 strain is an interesting case because it is the only isolate carrying RD_{23} which was not originally isolated from the Iberian Peninsula or France. It was isolated from a skin lesion of a human with tularemia in Uppsala, Sweden, in the year 2000 during a large epidemic in that country but was the only isolate among 28 tested from that epidemic that carries RD_{23}. Further surveillance using a combination of the RD_{23} deletion and the MLVA genotypes identified in our study will now provide a

basis on which to further track the distribution and spread of this apparent clone complex.

Within the *F. tularensis holarctica* subsp., genetic variation is scarce, even among strains from unlinked geographies. This lack of diversity is perplexing given that the *F. tularensis* subsp. *holarctica* genome has undergone substantial rearrangements with respect to the *F. tularensis* subsp. *tularensis* genome (6, 20, 21). MLVA studies also bear this out, showing little allelic diversity in *F. tularensis* subsp. *holarctica* populations compared to *F. tularensis* subsp. *tularensis* populations (13). These findings have been taken to indicate that the *F. tularensis* subsp. *holarctica* population may have recently been through a global evolutionary bottleneck or, more likely, that a clone has emerged and spread globally within a relatively recent time frame.

Our study has now shown that a distinct but highly related clone complex of *F. tularensis* subsp. *holarctica*, carrying the distinct RD_{23} genomic signature, has emerged within France and the Iberian Peninsula. Because tularemia has not commonly been reported in Spain and Southern France, we believe the isolation of highly related strains carrying RD_{23} is a consequence of recent epidemic spread of a single founding population. Further differentiation of the clone during its spread across the peninsula likely resulted in the eight distinct genotypes that are observed within the RD_{23} clade of the MLVA tree (Fig. 4). The RD_{23} strain F0228 appears unusual among the other RD_{23} strains. It was isolated outside Spain or France, and it bears a relatively distantly related MLVA genotype. It is unclear whether F0228 share ancestry with isolates from France and Spain or if RD_{23} occurred independently in a separate *F. tularensis* subsp. *holarctica* subclone.

Among isolates from France and Spain, phylogenetic inference from the clustering shown in Fig. 4 implies that two primary subpopulations carrying the RD_{23} signature are circulating. The F0020 strain, isolated from France in 1993, and the F0295 strain, isolated from France in 1952, are within one of these two large subpopulations. If a founder effect can explain the relative abundance of this subpopulation, then the F0295 strain implies that the RD_{23} clone or its immediate ancestor has been present in the region for >50 years. Although the exact origin of the clone is unknown, its emergence in Northern Spain is now well documented. The RD_{23} marker will be highly useful in tracking epidemiological and ecological characteristics of this clone complex as it spreads and in understanding how its genome is evolving over time.

It has generally been believed that *F. tularensis* subsp. *holarctica* strains are indeed virulent but less so than the *F. tularensis* subsp. *tularensis* strains (7). The outbreak of tularemia in northwest Spain, which occurred between 1997 and 1998, is the first recorded outbreak in that region, resulting in over 500 human cases (11). It is not yet clear whether the RD_{23} clone complex is a hypervirulent clone or whether other factors may explain its epidemic spread. Although a gain of virulence characteristics is usually associated with allelic diversity or gene acquisition, there are well-documented instances of specific deletions enhancing virulence of other species (2, 4, 16, 17) and of genome decay resulting in emergence of highly virulent species (3). The RD_{23} deletion itself does not include any obvious genes associated with virulence functions, but the adjacent genomic region contains several pseudogenes and

genes that have been inactivated by multiple stop codons. One of these degraded coding regions encodes a member of the Mip family (macrophage infectivity potentiator), suggesting some type of association of the region with virulence. Clearly more study will be necessary to determine if the RD_{23} deletion itself influences any of the apparent virulence characteristics of the clone complex.

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