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Common Ancestry and Novel Genetic Traits of *Francisella novicida*-Like Isolates from North America and Australia as Revealed by Comparative Genomic Analyses▽†

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***Francisella novicida* is a close relative of *Francisella tularensis*, the causative agent of tularemia. The genomes of *F. novicida*-like clinical isolates 3523 (Australian strain) and Fx1 (Texas strain) were sequenced and compared to *F. novicida* strain U112 and *F. tularensis* strain Schu S4. The strain 3523 chromosome is 1,945,310 bp and contains 1,854 protein-coding genes. The strain Fx1 chromosome is 1,913,619 bp and contains 1,819 protein-coding genes. NUCmer analyses revealed that the genomes of strains Fx1 and U112 are mostly colinear, whereas the genome of strain 3523 has gaps, translocations, and/or inversions compared to genomes of strains Fx1 and U112. Using the genome sequence data and comparative analyses with other members of the genus *Francisella*, several strain-specific genes that encode putative proteins involved in RTX toxin production, polysaccharide biosynthesis/modification, thiamine biosynthesis, glucuronate utilization, and polyamine biosynthesis were identified. The RTX toxin synthesis and secretion operon of strain 3523 contains four open reading frames (ORFs) and was named *rtxCABD*. Based on the alignment of conserved sequences upstream of operons involved in thiamine biosynthesis from various bacteria, a putative THI box was identified in strain 3523. The glucuronate catabolism loci of strains 3523 and Fx1 contain a cluster of nine ORFs oriented in the same direction that appear to constitute an operon. Strains U112 and Schu S4 appeared to have lost the loci for RTX toxin production, thiamine biosynthesis, and glucuronate utilization as a consequence of host adaptation and reductive evolution. In conclusion, comparative analyses provided insights into the common ancestry and novel genetic traits of these strains.**

Francisella tularensis is an intracellular pathogen that causes tularemia in humans, and the public health importance of this bacterium has been well documented in recent history (81). The genome sequences of several *F. tularensis* isolates from disparate geographic origins have been sequenced to date (4, 6, 12, 13, 41). Comparative genome sequence analyses have provided insights into the taxonomy, physiology, and pathogenic evolution of different subspecies of *F. tularensis* (33, 91). *Francisella novicida* strain U112, which is β-galactosidase negative but citrulline ureidase and glycerol positive, originally was isolated from water collected in the Ogden Bay Bird Refuge in Utah (39). Early comparative studies indicated that *F. novicida* strain U112 is less fastidious than *F. tularensis*, and it differs from the latter in antigenic composition as well as virulence (64). The lipopolysaccharide (LPS) of *F. novicida* strain U112 also is structurally distinct and biologically more active than *F. tularensis* LPS (87, 92). Nevertheless, *F. novicida* strain U112 has been used as a surrogate of *F. tularensis* in scores of laboratory studies, primarily due to its pathogenicity for rodents and amenability to genetic manipulation (24).

F. novicida was formally included in the genus *Francisella* in 1959, and strain U112 was the sole member of the species until 1989 (62). Since then, there have been at least seven reports of *F. novicida* or *F. novicida*-like bacteria isolated from humans. These include the earliest human isolates described by the Centers for Disease Control and Prevention from Louisiana and California (30), two isolates from Texas (15), and an isolate each from Australia (95), Thailand (42), and Arizona (8).

Some of these reports were from immunocompromised patients who manifested a localized, relatively mild illness, which is consistent with *F. novicida* being an opportunistic pathogen. Despite these reports, *F. novicida* is thought to constitute an environmental lineage along with *Francisella philomiragia*, which also has been associated with human disease (30, 94).

The genome of *F. novicida* strain U112 has been sequenced and compared to the genome sequences of *F. tularensis* pathogenic to humans (75). The draft genome sequences of *Francisella* isolates from Louisiana and California (GA99-3548 and GA99-3549, respectively) also have been compared to the genome sequences of *F. tularensis* strains (12). The draft genome sequences of *F. novicida* strains FTE (a mouse passage of strain U112; GenBank project number 30717) and FTG (a human isolate; GenBank project number 55313) are available.

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TABLE 1. Characteristics of the genomes of strains U112, Fx1, and 3523

Strain	Chromosome size (bp)	No. of protein-coding genes[a]	No. of subsystems[a]	GC content (%)	No. of rRNA or tRNA genes[b]				GenBank accession no.
					5S	23S	16S	tRNA	
U112	1,910,031	1,821	250	32.48	3 + 1	3	3	38	CP000439
Fx1	1,913,619	1,819	253	32.54	3 + 1	3	3	38	CP002557
3523	1,945,310	1,854	253	32.32	3 + 1	3	3	37	CP002558

[a] Genes and subsystems predicted by RAST server.
[b] Each strain contains three rRNA operons (5S-23S-16S) and an orphan 5S rRNA fragment.

Whereas *F. tularensis* is a highly infectious zoonotic agent, the mechanism of transmission of *F. novicida* among vertebrate or invertebrate species is unknown. Despite the presence of ≥97.7% average nucleotide identities among the genome sequences of *F. novicida* and *F. tularensis* strains, they have been proposed to constitute separate species based on evolutionary analyses (40). However, amending the genus *Francisella* and the reclassification of *F. novicida* as a subspecies of *F. tularensis* have been formally proposed (31).

Francisella isolate Fx1, which is β-galactosidase, β-lactamase, and citrulline ureidase positive but glycerol negative, was cultured from the blood of a diabetic patient in the Galveston Bay area of Texas (15). The patient was thought to have disseminated infection due to isolate Fx1 manifesting with bacteremia, pneumonia, and brain abscesses. Virulence studies in laboratory animals performed by standard methods showed no difference between *F. tularensis* subsp. *tularensis* and isolate Fx1 (15). Subsequent studies have designated this isolate *F. tularensis* subsp. *novicida* strain Fx1 and *F. novicida* strain Fx1 (22, 66). *Francisella* isolate 3523, the first reported *Francisella* from the Southern Hemisphere, was cultured from a patient who had cut a toe in brackish water in the Northern Territory of Australia (95). The patient was afebrile, had no other clinical symptoms, and recovered after antibiotic treatment. Studies using Swiss-Webster mice showed that isolate 3523 was less virulent than *F. tularensis* subsp. *tularensis* (95). The true nature of this isolate, which is glycerol and β-galactosidase positive, is unknown and has been tentatively designated a *novicida*-like subspecies of *F. tularensis* (95). Since these isolates were from different continents/hemispheres, they were desirable candidates for genome sequencing and comparative analyses. The objectives of the present study were to decipher the genomes of *F. novicida*-like strains Fx1 and 3523 and to identify the genetic differences between these strains and *F. tularensis* subsp. *novicida* strain U112 by comparative genome analyses. Since genetic relationships and evolutionary contexts could be better understood by whole-genome analyses of conserved operons, it was envisaged to include the genomes of different *Francisella* species and strains in the comparisons when available.

MATERIALS AND METHODS

Bacterial cultivation and chromosomal DNA extraction were performed at the Centers for Disease Control and Prevention, Fort Collins, CO, using standard protocols (57, 66). Genomic library construction, sequencing, and finishing were performed at the Genome Science Facilities of Los Alamos National Laboratory, Los Alamos, New Mexico, as described previously (4, 6, 98). The prediction of the number of subsystems and pairwise BLAST comparisons of protein sets within strains Fx1 and 3523 were performed using Rapid Annotation using Subsystems Technology (RAST), which is a fully automated, prokaryotic genome annotation service (3). Proteins deemed to be specific to each strain were compared against the NCBI nonredundant protein database to determine whether they were hypothetical or conserved hypothetical. If there was no adequate alignment with any protein (less than 25% identity or the aligned region is less than 25% of the predicted protein length), the translated open reading frame (ORF) was designated a hypothetical protein.

Multiple genome comparisons were performed using the progressive alignment option available in the program MAUVE, version 2.3.0. Default scoring and parameters were used for generating the alignment. A synteny plot was generated using the program NUCmer. The program uses exact matching, clustering, and alignment extension strategies to create a dot plot based on the number of identical alignments between two genomes. Prophage regions (PRs) were identified using Prophinder (http://aclame.ulb.ac.be/Tools/Prophinder/), an algorithm that combines similarity searches, statistical detection of phage gene-enriched regions, and genomic context for prophage prediction. Insertion sequences (ISs) were identified by whole-genome BLAST analysis of strains Fx1, 3523, and U112 using the IS finder (http://www-is.biotoul.fr/). Gene acquisition and loss among the three strains were determined by comparing gene order, orientation of genes (forward/reverse), GC content of genes (the percentage above or below the whole-genome average), features of intergenic regions (e.g., remnants of IS elements, integration sites, etc.), and the similarity of proteins encoded by genes at a locus of interest (>90% identity at the predicted protein level).

DNA and protein sequences were aligned using the ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html) programs as described previously (80). Multiple-sequence alignments for phylogenetic analyses of strains 3523 and Fx1 were performed using the program MUSCLE, which is available at the website http://www.phylogeny.fr/ (20, 21). The alignment was followed by a bootstrapped (n = 100) neighbor-joining method for inferring the phylogenies (85). Only full-length 16S rRNA and succinate dehydrogenase (*sdhA*) gene sequences from high-quality, finished *Francisella* genomes were included in these comparisons.

RESULTS AND DISCUSSION

General features of the chromosomes. The chromosome of strain 3523 was 31,692 bp larger than that of strain Fx1. Although the chromosomes of strains 3523, Fx1, and U112 differed in size, their average GC content and the percentage of sequence that encodes proteins were similar (Table 1). Genomic islands (GIs) are clusters of genes in prokaryotic genomes of probable horizontal origin (38). Comparative genomic analysis indicated that strain 3523 contained two GIs (GI1, 4,352 to 15,978 bp, 28.8% GC; GI2, 1,012,013 to 1,056,964 bp, 34.2% GC). Strain U112 also contained two genomic islands (GI1, 4,244 to 15,027 bp, 29.2% GC; GI2, 369,322 to 376,086 bp, 30.7% GC). However, strain Fx1 contained a single genomic island (GI1, 4,244 to 13,232 bp, 27.9% GC). Whereas GI2 of strain 3523 was unrelated to GI2 of strain U112, GI1 of strains 3523, Fx1, and U112 were related to each other, suggesting a common lateral origin. Interestingly, none of these GIs contained genes that have a role in pathogenicity.

Short sequence repeats, which include insertion sequences (IS), are the hallmark of *F. tularensis* genomes, and the IS ele-

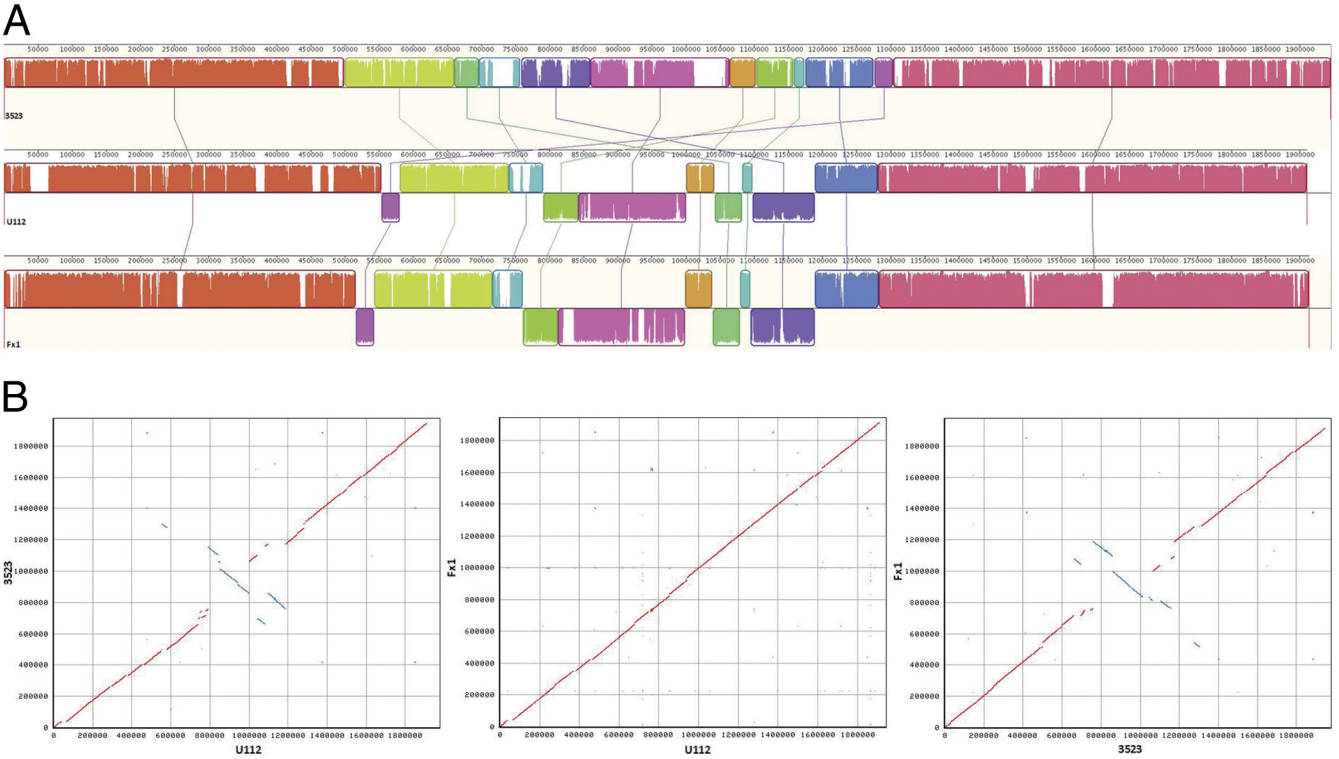


FIG. 1. (A) Alignment of the chromosomes of strains Fx1, U112, and 3523 using MAUVE 2. Identically colored boxes, known as locally colinear blocks (LCBs), depict homologous regions in the three chromosomes. The edges of LCBs indicate chromosome rearrangements due to recombination, insertions, and/or inversions. Sequences of strains Fx1 and U112 inverted in relation to those of strain 3523 are shown as blocks below the horizontal line. The vertical lines connecting the LCBs point to regions of homology among the three chromosomes. Numbers above the maps indicate nucleotide positions within the respective chromosomes. (B) Synteny plots of the chromosomes of strains Fx1, U112, and 3523 generated by NUCmer. Each plot shows regions of identity between the chromosomes based on pairwise alignments. Plus-strand matches are slanted from the bottom left to the upper right corner and are shown in red. Minus-strand matches are slanted from the upper left to the lower right and are shown in blue. The number of dots/lines shown in the plot is the same as the number of exact matches found by NUCmer. Numbers indicate nucleotide positions within the respective chromosomes.

ments are thought to be generally stable among different isolates despite their diverse geographical origins (75, 86). Whole-genome BLASTN and BLASTX analyses using IS finder showed that strain 3523 contained a single copy of an IS*481* family element (FN3523_0714, 348 amino acids [aa]) that had no homologs in strains Fx1 and U112. Strain Fx1 contained six copies of the IS*110* family element (FNFX1_0028, FNFX1_1255, FNFX1_1555, and FNFX1_1557, 315 aa each; FNFX1_0715 and FNFX1_0718, 290 aa each) that had no homologs in strains 3523 and U112. Strains 3523 and Fx1 contained IS*982* family elements (FN3523_1458, 258 aa; FNFX1_0226, 187 aa) that had no homologs in strain U112. Whereas strain 3523 lacked ISFtu1, ISFtu3, and ISFtu5 sequences, strain Fx1 lacked only ISFtu5 sequences. Nevertheless, both strains contained full-length or partial homologs of other ISFtu elements in various copy numbers (data not shown).

Whole-genome alignment using MAUVE showed the presence of extensive blocks of homologous regions among strains 3523, Fx1, and U112 (Fig. 1A). NUCmer analyses revealed that the genomes of strains Fx1 and U112 were mostly colinear, whereas the genome of strain 3523 had gaps, translocations, and/or inversions compared to the genomes of strains Fx1 and U112 (Fig. 1B). In-depth sequence examination indicated that some of these gaps and/or inversions were associated with integrative and conjugative elements, including the IS elements mentioned above. The occurrence of IS elements at genomic breakpoints also has been observed in comparisons of the genomes of different subspecies of *F. tularensis* (75).

BLAST comparison of protein sets. A three-way comparison of strains 3523, Fx1, and U112 revealed that they contained 1,583 orthologous protein-coding genes (bidirectional best hits). A similar comparison indicated that strain 3523 contained 149 protein-coding genes with no homologs in strains Fx1 and U112. Strain Fx1 contained 70 protein-coding genes with no homologs in strains 3523 and U112. Strain U112 contained 69 protein-coding genes with no homologs in strains 3523 and Fx1. A two-way comparison of protein-coding genes of strains 3523, Fx1, and U112 is shown in Table S1 in the supplemental material. In strain 3523, 494 genes could not be assigned a function based on BLAST analysis and therefore had been annotated as encoding hypothetical or conserved hypothetical proteins. In strain Fx1, 447 genes had been annotated as encoding hypothetical or conserved hypothetical proteins.

Iron metabolism and FPI genes. A cluster of 17 to 19 genes has been proposed to constitute the *Francisella* pathogenicity island (FPI), and it is found in a single copy in *F. tularensis* subsp. *novicida* strain U112 (FTN_1309 to FTN_1325), but it is duplicated in the genomes of *F. tularensis* subsp. *tularensis* (75,

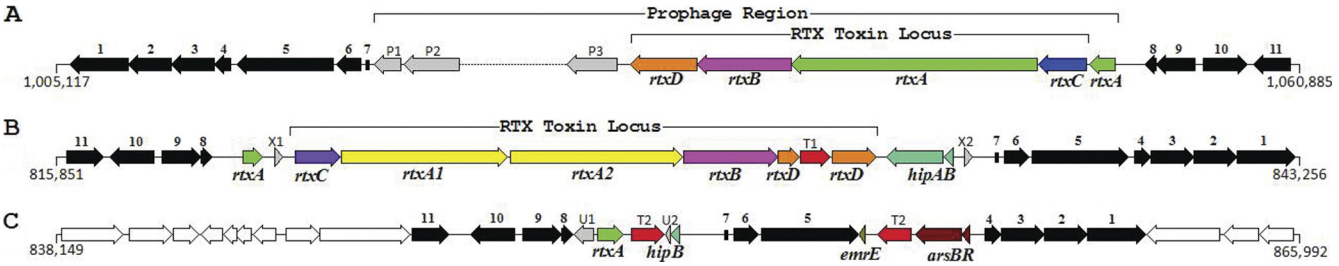


FIG. 2. (A) RTX toxin locus of strain 3523. Arrow 1, glutamate-1-semialdehyde aminotransferase; 2, glutathione synthetase; 3, methionyl-tRNA formyltransferase; 4, hypothetical protein; 5, methyltransferase; 6, probable fatty acid hydroxylase; 7, tRNA-Val-GAC; 8, hypothetical protein; 9, recombination associated protein; 10, Rhodanese-like domain protein; 11, hypothetical protein; P1, hypothetical protein; P2, phage-related tail protein; P3 (FN3523_1033), site-specific recombinase, phage integrase family; *rtxD* (FN3523_1034), membrane fusion protein (toxin secretion protein); *rtxB* (FN3523_1035), ABC-type toxin exporter; *rtxA* (FN3523_1036), RTX toxin; *rtxC* (FN3523_1037), RTX toxin activation protein; *rtxA* (FN3523_1038), vestigial RTX toxin gene. (B) RTX toxin locus of strain Fx1. Arrows 1 to 11 are as described for panel A. Arrow *rtxA* (FNFX1_0797), vestigial RTX toxin gene; *rtxC* (FNFX1_0799), RTX toxin activation protein; *rtxA1* and *rtxA2* (FNFX1_0800 and FNFX1_0801), RTX toxins; *rtxB* (FNFX1_0802), ABC-type toxin exporter; *rtxD* (FNFX1_0803 and FNFX1_0805), membrane fusion protein (toxin secretion protein, fragmented due to transposon insertion); *hipBA* (FNFX1_0806 and FNFX1_0807), persister cell locus; X1 and X2, hypothetical proteins; T1, IS*1016* family transposase. (C) Arsenic resistance locus of strain U112. Arrows 1 to 11 are as described for panel A. Arrow *rtxA* (FTN_0793), vestigial RTX toxin gene; *hipB* (FTN_0795), repressor of *hipA* (absent from this strain); U1 (FTN_0792) and U2, hypothetical proteins; T2, ISFtu2 transposase; *emrE* (FTN_0799), multidrug resistance antiporter; *arsRB* (FTN_0800 and FTN_0801), arsenite resistance proteins.

91). Genome comparisons revealed that strains 3523 and Fx1 also contained a single copy of the FPI (FN3523_1373 to FN3523_1389 and FNFX1_1347 to FNFX1_1363, respectively). The order and orientation of genes within the FPI of strains 3523, Fx1, and U112 were identical. The predicted proteins within the putative FPIs of strains 3523 and Fx1 had average identities of 87 and 97%, respectively, to those from strain U112. Furthermore, strains 3523, Fx1, and U112 contained the ferric uptake regulator gene (*fur*) as well as the *fslABCDEF* operon (FN3523_1749 to FN3523_1755, FNFX1_1722 to FNFX1_1728, and FTN_1681 to FTN_1687, respectively), which is implicated in the biosynthesis of a polycarboxylate siderophore in *F. tularensis* subsp. *tularensis* strain Schu S4 and *F. tularensis* subsp. *novicida* strain U112 (37, 71). An ortholog of strain Schu S4 *fupA* (FTT0918), whose product is required for the efficient utilization of siderophore-bound iron (47), also was found in strains 3523, Fx1, and U112 (FN3523_0408, FNFX1_0437, and FTN_0444, respectively). The presence of FPI, *fur*, *fslABCDEF*, and *fupA* in almost all *Francisella* genomes suggests that these functions are essential for their survival in the environment and/or host.

RTX toxin-related genes. Several pathogenic Gram-negative bacteria produce potent pore-forming cytotoxins that contain calcium-binding glycine and aspartate-rich repeat regions (93). The genetic determinants of RTX toxin synthesis and transport usually consist of a single operon (*rtxCABD*) and an unlinked gene (*tolC*) encoding an outer membrane channel (46). Although some researchers have alluded to the presence of toxins in cellular preparations of *F. tularensis*, there is no consensus opinion on the synthesis of toxins, and genes encoding toxins have not been found in this species (69, 90). However, homologs of *tolC* (FTT_1095c and FTT_1724c) have been characterized in *F. tularensis* (25) and also are found in strains U112 (FTN_0779 and FTN_1703), 3523 (FN3523_1096 and FN3523_1775), and Fx1 (FNFX1_0783 and FNFX1_1744).

The predicted prophage region of strain 3523 contained an operon (10,160 bp, 35.5% GC) related to those involved in RTX toxin synthesis and secretion (Fig. 2A). The first ORF (FN3523_1037, *rtxC*) had no homologs in the databases. Based on its location within the operon and predicted protein molecular mass (356 aa, 41.81 kDa), *rtxC* may encode a protein involved in fatty acylation during the activation of protoxin to cytotoxin. The second ORF (FN3523_1036, *rtxA*) encoded a putative protein (1,829 aa, 195 kDa) that had 30% identity to the RTX cytotoxin-related FrpC protein (1,829 aa, 197 kDa) of *Neisseria meningitidis* FAM20 (88) and α-hemolysin HlyA (1,024 aa, 110 kDa) of *Escherichia coli* (82). The third ORF (FN3523_1035, *rtxB*) encoded a putative ABC transporter (703 aa, 79.83 kDa) that had 50% identity to the α-hemolysin translocator ATP-binding protein HlyB (706 aa, 79.83 kDa) of *E. coli* (29). The fourth ORF (FN3523_1034, *rtxD*) encoded a putative membrane fusion protein (490 aa, 56.41 kDa) that had 32% identity to the type 1 translocator protein HlyD (478 aa, 54.48 kDa) of *E. coli* (67). The toxin-encoding ORF (FN3523_1036, 5,490 bp) was the largest among all protein-coding genes predicted in strain 3523. Several other *Francisella* genomes contained truncated genes related to *rtxA* of strain 3523 (e.g., FTM_1222, FTL_1124, FTT_1077c, FTH_1098, and FTA_1184; 76 to 94% identity at the predicted protein level). However, homologs of *rtxB*, *rtxC*, and *rtxD* were not found in any of these genomes.

The genomic analysis of strain Fx1 revealed a locus (12,973 bp, 32.6% GC) encoding proteins related to RTX toxins (Fig. 2B). The first ORF (FNFX1_0799, *rtxC*) had no homologs in the databases. Based on its location within the operon and predicted protein molecular mass (337 aa, 39 kDa), *rtxC* may encode a protein involved in fatty acylation during the activation of protoxin to cytotoxin. The second and third ORFs (FNFX1_0800 and FNFX1_0801, *rtxA1* and *rtxA2*, respectively) encoded putative proteins (1,234 and 1292 aa, 134.7 and 140 kDa, respectively) that are unrelated to α-hemolysin HlyA of *E. coli* but had 28 to 30% identity to the RTX cytotoxin-related proteins FrpA and FrpC of *Neisseria meningitidis* and the putative protein encoded by *rtxA* of strain 3523. Furthermore, the putative proteins encoded by *rtxA1* and *rtxA2* had 30% identity to each other. Interestingly, the *rtx* locus of strain Fx1 also contained *rtxB* and *rtxD* ORFs found in strain 3523. Whereas

the *rtxB* ORFs (FNFX1_0802 and FN3523_1035) were 96% identical among the two strains, the *rtxD* ORF of strain Fx1 was fragmented due to a transposition event (FNFX1_0803 and FNFX1_0805). Within this region, strain Fx1 contained a gene encoding a hypothetical protein (FNFX1_0797, 141 aa) that had 86% identity to the putative RTX toxin (1,829 aa, encoded by *rtxA*) of strain 3523. An operon (1,487 bp; 31% GC) related to the *hipBA* locus of multidrug-tolerant bacteria also was found in this region (Fig. 2B), and putative HipA (FNFX1_0806, 418 aa) of strain Fx1 was 30% identical to the HipA persistence factor (2WIU_C, 446 aa) of *E. coli*. Genomes of several other *F. tularensis* subsp. *tularensis* strains contained a similar *hipBA* operon, but strain U112 contained only a *hipB* ortholog (FTN_0795) and strain 3523 lacked *hipBA*.

Although the α-hemolysin of *E. coli* is the prototype of RTX toxins, genes encoding RTX toxins appear to be more common in pathogenic members of the *Pasteurellaceae* and are almost always associated with genes encoding a type I secretion system. The horizontal transfer of genes encoding RTX toxins across different bacterial families has been suggested based on phylogenetic analyses (23). The identification of a locus encoding putative RTX toxins in strains 3523 and Fx1 is especially intriguing, since such genes have not been reported thus far in any of the francisellae genomes. The bacteriocin ABC transporters of some bacteria have similarities to ABC transporters of type I secretion systems (99). Although bacteriocins of *Francisella* have been investigated previously and designated tularecins (1, 2, 5), very little is known about the genetic basis of their synthesis/secretion. The putative RTX toxin of strain 3523 is also related to the bacteriocins of *Rhizobium leguminosarum* and *Nitrococcus mobilis* Nb-231 (data not shown). Since the bacteriocin of *R. leguminosarum* has similarities to RTX toxins (63) and some cytolysins can function as bacteriocins (11), the putative RTX toxin of strain 3523 also may possess properties of bacteriocins and afford environmental fitness. The biochemical characterization of *rtxCABD* loci of strains 3523 and Fx1 is required to verify these hypotheses. Furthermore, the presence of a vestigial RTX toxin/bacteriocin-encoding ORF in several *Francisella* genomes, including strains 3523 (FN3523_1038), Fx1 (FNFX1_0797), and U112 (FTN_0793), indicates that the common ancestor of these bacteria was toxigenic/bacteriocinogenic. It is possible that the ancestral locus was truncated during habitat restriction and/or reductive evolution of these bacteria, and that it was reacquired by strains 3523 and Fx1 through horizontal transfer.

Arsenic resistance genes. Arsenic is an environmental pollutant, and some microorganisms have evolved mechanisms of resistance to this cytotoxic agent. Arsenic exists in two oxidation states, arsenite [As(III)] and arsenate [As(V)], in biological systems. In most bacteria, the minimal arsenical resistance operon contains three ORFs (*arsRBC*), wherein the conversion of arsenate to arsenite is accomplished by a reductase (product of *arsC*), arsenite is transported out of the cell by a membrane-bound efflux pump (product of *arsB*), and *arsR* encodes an arsenic resistance regulatory protein. The plasmid- or transposon-mediated horizontal transfer of genes that confer arsenic resistance has been well documented (58). *F. tularensis* subsp. *novicida* strain U112 contained a two-gene operon (1,218 bp, 30.4% GC) that encoded a putative transcriptional regulator and an arsenite exporter (*arsRB*) (Fig. 2C). At the protein level, strain U112 ArsB (FTN_0800, 342 aa) was 61% identical to the arsenite efflux transporter of *Bacillus subtilis* (BSU25790, 346 aa), and ArsR (FTN_0801, 116 aa) was 38% identical to the ArsR repressor of *B. subtilis* (BSU25810, 105 aa). A gene encoding a putative IS*4* family transposase (247 aa) was found adjacent to the *arsRB* operon of strain U112. Within this region, strain U112 also contained a gene encoding a putative protein (FTN_0799, 109 aa) that was 44% identical to the small multidrug resistance antiporter EmrE of *E. coli*. The *arsRB* operon and homologs of *emrE* were present in *F. tularensis* subsp. *novicida* strain GA99-3548 and *F. philomiragia* strains ATCC 25015 and 25017, but not in strains 3523 and Fx1. In strain U112, the transposase gene associated with the *arsRB* operon was separated from an identical gene upstream by genes encoding a putative fatty acid hydroxylase (FTN_0797, 182 aa), an rRNA methyltransferase (FTN_0798, 718 aa), and a *hipB* gene. Adjacent to this transposase gene, strain U112 contained two ORFs encoding hypothetical proteins (FTN_0792 and FTN_0793, 140 and 189 aa, respectively) (Fig. 2C). Homologs of FTN_0792 were present in *F. tularensis* subsp. *novicida* strains GA99-3548 and GA99-3549 but not in strains 3523 and Fx1. Furthermore, FTN_0793 had 91% identity to the putative RTX toxin (1,829 aa, encoded by *rtxA*) of strain 3523. The occurrence of different loci (prophage region containing *rtxCABD* in strain 3523, transposon associated with *rtxCABD* in strain Fx1, and transposon associated with arsenite resistance genes in strain U112) flanked by conserved genes implies that this variable region is a genomic hot spot among different *Francisella* strains.

Polysaccharide biosynthesis gene clusters. The LPS of *Francisella* spp. has several unique features and has been demonstrated to undergo antigenic variation (28). In contrast to *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *novicida* strain U112 expresses a single chemotype of LPS which has been proposed to contribute to virulence in a mouse model of infection (34). Immunobiological studies have demonstrated the activation of complement by pathogenic strains of *F. tularensis* subsp. *tularensis* as well as *F. tularensis* subsp. *novicida* strain U112 and implicated their LPS O-antigen in mediating resistance to complement-mediated lysis (16). It also has been shown that mice immunized with LPS from strain U112 were protected against strain U112 but not against *F. tularensis* subsp. *holarctica*, and immunization with LPS from *F. tularensis* subsp. *tularensis* protected against *F. tularensis* subsp. *holarctica* but not against strain U112 (87). The *wbt* gene cluster of *F. tularensis* subsp. *tularensis* strain Schu S4 (17,378 bp, 31% GC) is involved in LPS biosynthesis and contained 15 ORFs (87). In contrast, a similar gene cluster of *F. tularensis* subsp. *novicida* strain U112 (13,880 bp, 30.6% GC) contained only 12 ORFs (87). The LPS O antigens of *F. tularensis* subsp. *novicida* and *F. tularensis* subsp. *tularensis* have been shown to be structurally and immunologically distinct, due in part to the differences in *wbt* genes involved in their biosynthesis (73, 87).

Comparative genomic analyses revealed that strains 3523 and Fx1 contained a cluster of 21 (23,452 bp, 31% GC) and 16 (15,841 bp, 30.6% GC) ORFs, respectively, that were related to the *wbt* gene cluster of *F. tularensis* subsp. *tularensis* strain Schu S4. In addition, these strains contained conserved ORFs encoding proteins putatively involved in mannose modification adjacent to the *wbt* gene cluster (FN3523_1475 to FN3523_1476 and

FNFX1_1451 to FNFX1_1452). Table S2 in the supplemental material contains a comprehensive list of the annotated ORFs found in the *wbt* gene clusters of these bacteria. The 10 strain-specific ORFs found in the *wbt* gene cluster of strain 3523 were organized into groups of five (FN3523_1480 to FN3523_1484, 4,575 bp, 29.6% GC), two (FN3523_1486 and FN3523_1487, 2,328 bp, 29.8% GC), and three (FN3523_1492 to FN3523_1494, 3,232 bp, 28.9% GC) contiguous ORFs. Of the six strain-specific ORFs found in the *wbt* gene cluster of strain Fx1, four were contiguous (FNFX1_1457 to FNFX1_1460, 3,888 bp, 28.6% GC). Of the five strain-specific ORFs found in the *wbt* gene cluster of strain Schu S4, four were contiguous (FTT_1452c to FTT_1455c, 4,150 bp, 30% GC). The *wbt* gene cluster of *F. tularensis* subsp. *novicida* strain U112 contained three noncontiguous ORFs that were strain specific (FTN_1422, FTN_1424, and FTN_1428). The *wbt* gene clusters of *F. novicida*-like strains 3523 and Fx1 contained four contiguous orthologous ORFs (FN3523_1488 to FN3523_1491, 4,175 bp, 30.7% GC, and FNFX1_1461 to FNFX1_1464, 4,204 bp, 30.6% GC, respectively) that had no homologs in strains U112 and Schu S4. The *wbt* gene clusters of strains U112 and Schu S4 contained three contiguous orthologous ORFs (FTN_1425 to FTN_1427, 3,380 bp, 32.5% GC, and FTT_1459c to FTT_1461c, 3,391 bp, 32.5% GC, respectively) that had no homologs in strains 3523 and Fx1.

The *wbt* gene clusters of strain Fx1 and *F. tularensis* subsp. *tularensis* Schu S4 contained two contiguous orthologous ORFs (FNFX1_1466 and FNFX1_1467, 1,225 bp, 32.7% GC, and FTT_1462c and FTT_1463c, 1,411 bp, 31% GC, respectively) that had no homologs in strains 3523 and U112. The *wbt* gene clusters of strains 3523 and U112 contained two contiguous orthologous ORFs (FN3523_1495 and FN3523_1496, 2,209 bp, 34% GC, and FTN_1429 and FTN_1430, 1,735 bp, 34.3% GC, respectively) that had no homologs in strains Fx1 and Schu S4. The strain-specific ORFs in these strains were flanked by highly conserved orthologous ORFs putatively encoding WbtM (FTT_1450c, FN3523_1477, and FNFX1_1453) and WbtA (FTT_1464c, FN3523_1497, FNFX1_1468, and FTN_1431). Furthermore, the *wbt* gene cluster of strain Schu S4 was flanked by genes encoding ISFtu1/IS*630* transposases (126 aa each), and the *wbt* gene cluster of strain U112 contained a copy of ISFtu3/IS*1016* (233 aa) that appears to have truncated the ORF encoding a putative dTDP-D-glucose 4,6-dehydratase (FTN_1420c; WbtM). However, the *wbt* gene clusters of strains 3523 and Fx1 lacked transposase genes (see Table S2 in the supplemental material).

Since the *wbt* gene clusters of strains U112, Fx1, 3523, and Schu S4 display a cassette/mosaic structure with an outer conserved region and an inner variable region, it can be hypothesized that genes in the outer region encode functions common to all strains, whereas genes in the inner region encode serogroup-specific functions. If the number of genes in the inner variable region is an indicator of the complexity of LPS, then the LPS of strains 3523 and Fx1 likely is more different from that of strains U112 and Schu S4. A similar chimeric arrangement has been observed in the gene clusters encoding polysaccharide antigens in *Salmonella enterica*, and it has been proposed that genes in the outer conserved region mediate the conspecific exchange of genes in the inner variable region (44).

Biosynthesis of polysaccharides requires several glycosyltransferases (GTs), which catalyze the transfer of sugars from an activated donor to an acceptor molecule and are usually specific for the glycosidic linkages created (48). The genomes of strains U112, Fx1, 3523, and Schu S4 contained yet another cluster of 10 to 17 ORFs oriented in the same direction that encoded putative GTs and other proteins related to enzymes involved in polysaccharide biosynthesis or cell wall/membrane biogenesis. This cluster has been tentatively designated *psl* (*p*olysaccharide *s*ynthesis *l*ocus). Table S3 in the supplemental material contains a list of the annotated ORFs found within this gene cluster. Strain 3523 had 11 contiguous strain-specific ORFs (FN3523_1278 to FN3523_1288, 10,580 bp, 30.4% GC) within the *psl* cluster. Strain Fx1 had two contiguous strain-specific ORFs (FNFX1_1260 and FNFX1_1261, 1,897 bp, 26.8% GC) within the *psl* cluster. Strain Schu S4 had three contiguous strain-specific ORFs (FTT_0794 to FTT_0796, 2,675 bp, 27% GC) within the *psl* cluster. The *psl* clusters of strains Schu S4 and Fx1 contained two orthologous ORFs that were not found in strain 3523 (FTT_0797/FNFX1_1259 and FTT_0793/FNFX1_1262). These strain-specific ORFs were flanked by three contiguous orthologous ORFs at both ends. Furthermore, strain Fx1 contained a copy of the IS*110* family transposase (FNFX1_1255; 315 aa) adjacent to the ORF that encoded a putative HAD family hydrolase (FNFX1_1256; see Table S3 in the supplemental material). Based on gene content and organization, it may be surmised that the *psl* gene cluster was involved in LPS and/or exopolysaccharide (EPS) biosynthesis. Since the *psl* gene cluster of strain 3523 contained more genes than strains Fx1, U112, and Schu S4, it is possible that the LPS/EPS of this strain is more complex.

Thiamine biosynthesis genes. Vitamin B_1 (thiamine pyrophosphate) is involved in several microbial metabolic functions (74). Prokaryotes have evolved elaborate mechanisms to either synthesize this important cofactor *de novo* or acquire it from their niche (7). Thiamine biosynthesis in most bacteria is accomplished by two major pathways; one involves the formation of hydroxymethylpyrimidine pyrophospate (HMP-PP) from aminoimidazole ribotide using ThiC and ThiD, and the other involves the formation of hydroxyethylthiazole phosphate (HET-P) using ThiS, ThiF, ThiG, and ThiO. The enzyme thiamine phosphate synthase (ThiE) combines HMP-PP and HET-P to produce thiamine phosphate, which is phosphorylated by thiamine monophosphate kinase (ThiL) to produce thiamine pyrophosphate (7). The *in vitro* growth of most *Francisella* species is achieved by supplementing culture media with thiamine hydrochloride or thiamine pyrophosphate, indicating the absence of the thiamine biosynthesis (TBS) pathway in these bacteria (59).

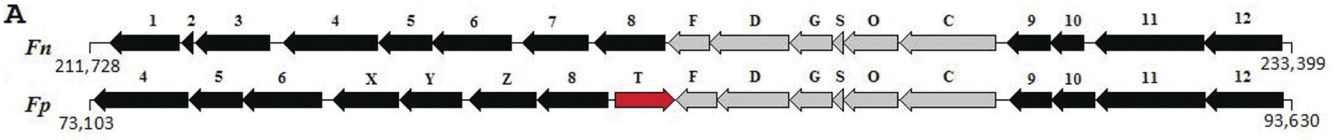
Strain 3523 and *F. philomiragia* strain ATCC 25017 contained an operon with six ORFs (*thiCOSGDF*; FN3523_1212 to FN3523_1217, 6,035 bp, 32% GC) encoding proteins related to enzymes involved in thiamine biosynthesis in several prokaryotes (Table 2). This gene cluster was not found in other *Francisella* genomes and was associated with a transposable element in *F. philomiragia* strain ATCC 25017 (Fig. 3A). The genetic organization of the strain 3523 *thiCOSGDF* locus was similar to that of the plasmid-encoded *thiCOGE* locus involved in thiamine biosynthesis in *Rhizobium etli* (56). An analogous cluster (*thiOGF*) is found within plasmid pEA29 of the plant pathogen *Erwinia amylovora* strain Ea88 (50). The chromosome of lithoautotrophic bacterium *Ralstonia eutropha* H16

TABLE 2. Strain 3523 genes involved in thiamine biosynthesis, glucuronate metabolism, and spermidine biosynthesis

Locus tag and function	Protein size (aa)	Annotation	Closest homolog outside *Francisella*			
			Locus tag	Protein size (aa)	% Identity	E value
Thiamine biosynthesis						
FN3523_1212	251	Thiazole biosynthesis adenylyltransferase (ThiF)	Mrub_1727	266	37	3e−39
FN3523_1213	494	Fused protein phosphomethylpyrimidine kinase (ThiD)/thiamine-phosphate pyrophosphorylase (ThiE)	lpg1568	495	36	9e−76
FN3523_1214	259	Thiazole synthase (ThiG)	lpg1567	263	59	2e−87
FN3523_1215	66	Thiamine biosynthesis protein (ThiS)	IL-0768	66	34	5e−05
FN3523_1216	339	Thiamine biosynthesis oxidoreductase (ThiO)	Kkor_0127	351	33	6e−44
FN3523_1217	592	Thiamine biosynthesis protein (ThiC)	CV_0235	632	72	0.0
Glucuronate metabolism						
FN3523_0892	325	Inositol oxygenase	56727 Miox	285	38	7e−48
FN3523_0893	462	D-Xylose-proton symporter (XylT)	CBUD_1731	463	43	2e−91
FN3523_0894	473	Glucuronate isomerase (UxaC)	Sde_1272	471	51	2e−140
FN3523_0895	182	KDPG aldolase (KdgA)	BC1003_2949	213	37	5e−36
FN3523_0896	306	2-Keto-3-deoxygluconate kinase (KdgK)	Sde_1269	296	43	1e−59
FN3523_0897	396	Mannonate dehydratase (UxuA)	PROSTU_04181	396	58	2e−134
FN3523_0898	490	D-Mannonate oxidoreductase (UxuB)	CJA_0180	492	43	2e−106
FN3523_0899	774	Alpha-glucosidase	BL00280	802	53	0.0
FN3523_0900	486	Rhamnogalacturonide transporter (RhiT)	KPK_1307	502	45	8e−116
Spermidine biosynthesis						
FN3523_0489	163	*S*-Adenosylmethionine decarboxylase (SpeD)	Tcr_0272	167	66	3e−47
FN3523_0490	289	Spermidine synthase (SpeE)	Pfl01_1732	291	57	7e−97
FN3523_0491	550	Arginine decarboxylase (SpeA)	Avin_07050	636	31	8e−64
FN3523_0492	328	Agmatine deiminase (AguA)	Kkor_1127	327	51	5e−90
FN3523_0493	286	*N*-Carbamoylputrescine amidase (AguaB)	VP1774	288	57	4e−92

also contains a *thiCOSGE* locus that is proposed to be involved in the *de novo* synthesis of thiamine (68). At the protein level, strain 3523 ThiC was 68% identical to ThiC of *R. etli* (AAC45972) and *R. eutropha* (H16_A0235), whereas strain 3523 ThiF was 35% identical to ThiF of *E. amylovora* (NP_981993; E value, 3e−24). Furthermore, strain 3523 ThiO and ThiS were 27 and 31% identical to ThiO (H16_A0236) and ThiS (H16_A0237) of *R. eutropha*, respectively (E values, 9e−27 to 0.001). Putative thiazole synthase ThiG of strain 3523 was ~50% identical to ThiG of *R. etli* (AAC45974; E value, 4e−66) and *R. eutropha* (H16_A0238; E value, 8e−77).

In strain 3523, FN3523_1213 appeared to encode a putative fused protein containing hydroxy-phosphomethylpyrimidine kinase and thiamine-phosphate pyrophosphorylase domains. In some bacteria, these functions are encoded by two different ORFs (*thiD* and *thiE*, respectively). Homologs of FN3523_1213 were found in several bacteria (e.g., *Legionella pneumophila*, *Coxiella burnetii*, *Geobacter sulfurreducens*, and *Colwellia psychreryth-*



B

THI-box sequence upstream of *thiC*	bp to *ATG*	*Species* (Locus tag; protein)
ACCCTTTGAACCTGATCTAGTTAGCACTAGTGTAGG	105	*F. novicida*-like 3523 (FN3523_1217; 592 aa)
ACCCTTTGAACCTGATCTAGTTAGCACTAGTGTAGG	106	*F. philomiragia* ATCC 25017 (Fphi_0090; 592 aa)
ACCCTTTGAACCTGATCTAGTTAGCACTAGCGTAGG	201	*Staphylococcus carnosus* TM300 (Sca_0594; 467 aa)
ACCCTTTGAACCTGATCTGGGTCATGCCAGCGTAGG	190	*Geobacillus sp.* Y412MC61 (GYMC61_1249; 562 aa)
ACCCTTTGAACCTGATCTGGATAATGCCAGCGCAGG	142	*Burkholderia pseudomallei* MSHR346 (GBP346_A1419; 643 aa)
ACCCTTTGAACCTGATCCAGTTCATACTGGCGTAGG	125	*Mesorhizobium sp.* BNC1 (Meso_2561; 608 aa)
ACCCTTTGAACCTGATCCGGGTAATGCCGGCGCAGG	103	*Rhodothermus marinus* DSM 4252 (Rmar_0078; 627 aa)
ACCCTTTGAACCTGATCCGGGTCATACCGGCGAAGG	152	*Azospirillum sp.* B510 (AZL_005290; 575 aa)

FIG. 3. (A) Thiamine biosynthesis locus. *Fn*, *F. novicida*-like strain 3523; *Fp*, *F. philomiragia* ATCC 25017. Arrow 1, metabolite:H^+ symporter (MHS) family protein; 2, hypothetical protein; 3, exodeoxyribonuclease VII large subunit; 4, transglutaminase-like superfamily protein; 5, conserved hypothetical protein; 6, conserved hypothetical protein; 7, hypothetical protein; 8, major facilitator superfamily transporter; 9, glutamate racemase; 10, hypothetical protein; 11, excinuclease B subunit; 12, exodeoxyribonuclease; COSGDF (FN3523_1212 to FN3523_1217), thiamine biosynthesis proteins; T, transposase; X, hypothetical protein; Y, hypothetical protein; Z, major facilitator transporter. (B) Putative thiamine regulatory element. A conserved 36-bp sequence upstream of *thiC* from eight species is boxed. Nucleotides identical in all eight sequences are shown in boldface. The length of sequence between the THI box and the start codon of *thiC* in each species is indicated (bp to *ATG*). The locus tag and the number of amino acids encoded by *thiC* in each species are shown in parentheses.

raea; ~30% protein identity) and plants (e.g., *Arabidopsis thaliana*, *Zea mays*, and *Brassica napus*; ~29% protein identity). It has been proposed that these bifunctional enzymes are involved in the synthesis of HMP-PP as well as the condensation of HMP-PP and HET-P to produce thiamine monophosphate (36, 72, 74). The 5′ untranslated regions of operons involved in thiamine biosynthesis and transport have been shown to contain a regulatory element called the THI box sequence (55). Based on the alignment of conserved sequences upstream of operons involved in thiamine biosynthesis from various bacteria, a putative THI box sequence was identified upstream of *thiC* in strain 3523 and *F. philomiragia* strain ATCC 25017 (Fig. 3B). The identification of a putative THI box upstream of *thiC* in strain 3523 suggests a thiamine-dependent regulation of this gene, similarly to other bacteria that have TBS genes (74).

In bacteria that lack a TBS pathway, thiamine kinases may facilitate the salvage of dephosphorylated thiamine intermediates from the environment or growth medium (32). A gene that encodes a putative thiamine pyrophosphokinase (TPK) was found in most members of *Francisella*, including strains 3523, Fx1, and U112 (FN3523_0611, FNFX1_0669, and FTN_0662, respectively). *F. novicida* strain U112 TPK was 27% identical to *Bacillus subtilis* TPK (THIN_BACSU; E value, 3e−10), which catalyzes the direct conversion of thiamine to thiamine pyrophosphate (52). In *Listeria monocytogenes*, which has an incomplete thiamine biosynthesis pathway, it has been shown that proliferation within host cells is reduced upon the deletion of *thiD* and *thiT*, encoding an HMP salvage protein and a thiamine transporter, respectively (77). In addition, strains of *E. amylovora* that lack the *thiOSGF* operon-containing plasmid pEA29 have been shown to be altered in exopolysaccharide biosynthesis and less virulent (51). *Aspergillus nidulans*, the filamentous ascomycete which induces a fatal systemic mycosis in mice, has been shown to be less pathogenic when its thiamine biosynthetic pathway was blocked by mutation (70). It also has been suggested that *Brucella abortus* and *R. etli*, which have similar intracellular lifestyles, require increased thiamine in the stationary phase (56, 76). In view of these observations, the TBS gene cluster of strain 3523 could serve to enhance its survival in the environment/host and also may have an indirect role in pathogenesis. Furthermore, the TBS genes of strain 3523 could be useful in accelerating the growth of thiamine auxotrophs of *F. tularensis* in the laboratory.

Glucuronate metabolism genes. Some bacteria have evolved mechanisms for the metabolism of uronic acids and uronates using the Entner-Doudoroff pathway (65). In this pathway, α-D-glucuronic acid (GlcUA) is converted into 2-keto-3-deoxygluconate (KDG) by a three-step process. The subsequent phosphorylation of KDG yields 2-keto-3-deoxy-6-phosphogluconate (KDPG), which is finally cleaved to produce glyceraldehyde 3-phosphate (G3P) and pyruvate. Comparative genomic analyses indicated that strains 3523 and Fx1 contained a cluster of nine ORFs that appear to constitute a polycistronic operon (FN3523_0892 to FN3523_0900 and FNFX1_0904 to FNFX1_0912, respectively, 11,784 bp, 31.7% GC). These ORFs encoded putative proteins related to enzymes involved in GlcUA catabolism (Table 2). Furthermore, genes encoding putative transposases of IS*1106* and IS*1016* families were found near the GlcUA utilization gene cluster of strain Fx1 but not in strain 3523 (Fig. 4A). This gene cluster was not found in other *Francisella* genomes, with the exception of *F. philomiragia* strain ATCC 25015, which appeared to contain the entire cluster (79 to 89% identity at the protein level).

The predicted mannonate dehydratase, 2-keto-3-deoxygluconokinase, KDPG aldolase, and glucuronate isomerase proteins from strain 3523 had 57, 37, 37, and 28% identities to *E. coli* UxuA (ECs5281), KdgK (ECs4406), KdgA (ECs2560), and UxaC (ECs3974), respectively (E values, 5e−132 to 9e−26). These enzymes catalyze the dehydration of D-mannonate to KDG, phosphorylation of KDG, cleavage of KDPG to pyruvate and G3P, and the conversion of D-glucuronate to D-fructuronate, respectively (65). The GlcUA utilization gene clusters of strains 3523 and Fx1 had some similarities to that of *Bacillus stearothermophilus* T-6, which has been predicted to metabolize GlcUA akin to *E. coli* and *Bacillus subtilis* (79). Furthermore, one end of the GlcUA utilization gene cluster of *B. stearothermophilus* T-6 also contains an ORF encoding a protein that has similarities to transposases of the IS*481* family (49), indicating the possible horizontal transfer of this locus.

The ORFs encoding a putative inositol oxygenase in strains 3523, Fx1, and ATCC 25015 had no bacterial homologs in the public databases outside the genus *Francisella*. However, strain 3523 inositol oxygenase had 38% identity to *Mus musculus myo*-inositol oxygenase (56727 Miox; E value, 7e−48), which catalyzes the conversion of *myo*-inositol to GlcUA (9). *myo*-Inositol and its derivatives are ubiquitous among eukaryotes and archaea, but their synthesis and metabolism is believed to be less common among bacteria (54). Although none of the francisellae genomes sequenced to date contained ORFs encoding proteins putatively involved in the transport and/or metabolism of *myo*-inositol, most of them, including strains 3523 and Fx1, had a *suhB* homolog (FN3523_1410 and FNFX1_1384, respectively). This evolutionarily conserved gene encoded inositol-1-monophosphatase, which hydrolyzes *myo*-inositol-1-phosphate to yield free *myo*-inositol (60, 61). Thus, it appears that most members of *Francisella* can convert *myo*-inositol-1-phosphate to free *myo*-inositol. However, only strains 3523 and Fx1 as well as *F. philomiragia* strain ATCC 25015 are able to utilize *myo*-inositol to synthesize GlcUA, which then is metabolized using the Entner-Doudoroff pathway.

Polyamine biosynthesis and transport genes. Polycations such as putrescine and spermidine are precursors for several cellular components, and pathways for the biosynthesis of these polyamines have been described in a number of species (97). Polyamines also have been implicated in bacterial oxidative stress response and virulence (78). The ubiquitous presence of genes encoding putrescine transport systems (*potFGHI*) in bacterial genomes suggests that these small molecules play crucial roles in cellular physiology. Strains 3523, Fx1, and U112 as well as *F. tularensis* subsp. *tularensis* strains Schu S4, WY96-3418, and OSU18 contained a *cadA* gene (FN3523_0462, FNFX1_0489, FTN_0504, FTT_0406, FTW_1667, and FTH_0474, respectively). This gene encoded a putative protein that had 55% identity to *E. coli* lysine decarboxylase (NP_418555; E value, 0), which is involved in cadaverine biosynthesis (53). The strains mentioned above also contained a *potGHI* operon (e.g., FN3523_1141 to FN3523_1143) and an unlinked *potF* gene (e.g., FN3523_0514).

Furthermore, strain 3523 contained a cluster of five ORFs (FN3523_0489 to _0493, 4,873 bp, 32.6% GC) encoding putative proteins related to enzymes involved in the biosynthesis of

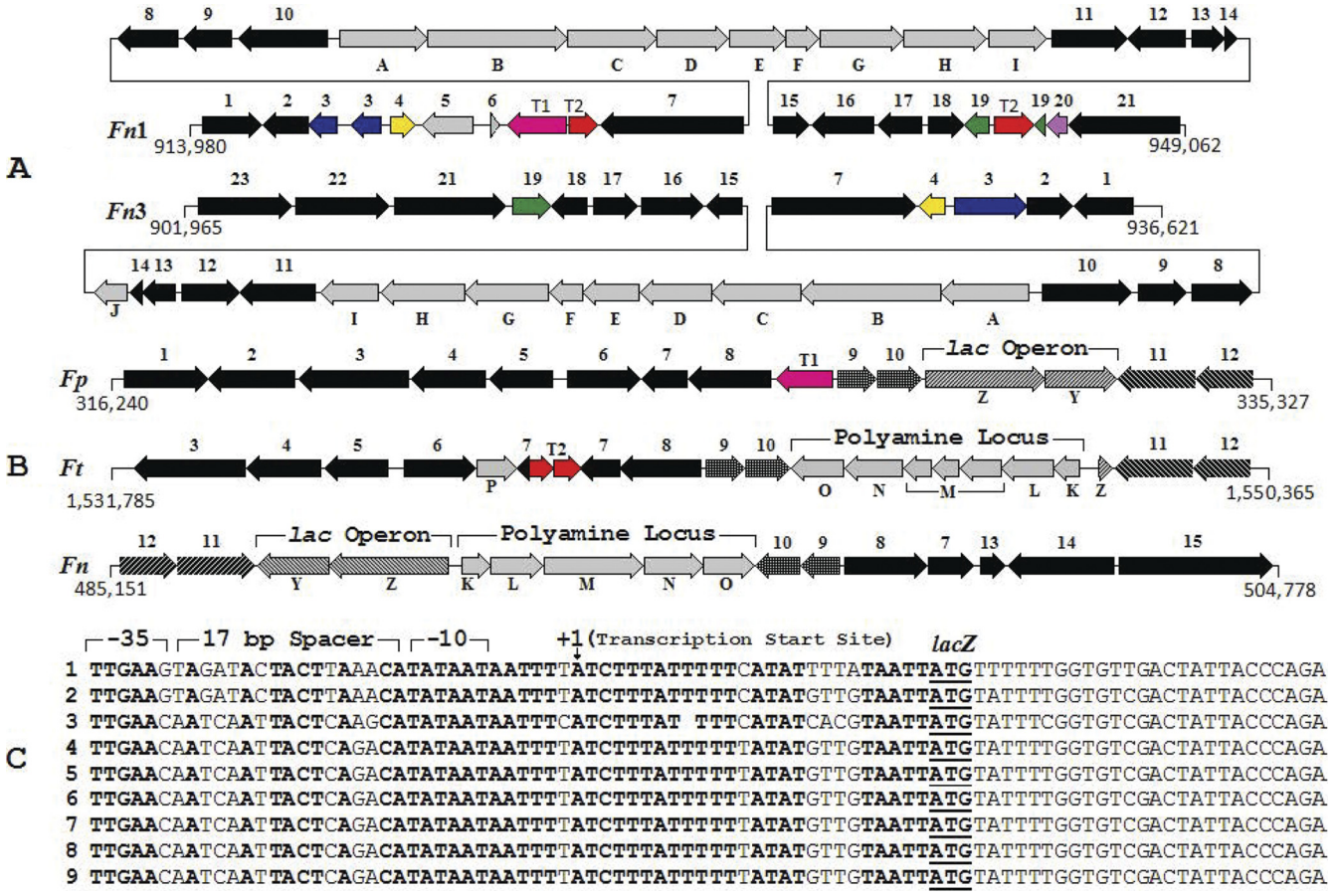


FIG. 4. (A) Glucuronate metabolism locus. *Fn*1, *F. novicida*-like strain Fx1; *Fn*3, *F. novicida*-like strain 3523. Arrow 1, hypothetical protein; 2, zinc-iron permease family protein; 3, cobalamin synthesis protein (putative GTPase); 4, zinc-iron uptake regulatory protein; 5, RNase T2 family protein; 6, hypothetical protein; 7, phenylalanyl-tRNA synthetase beta chain; 8, phenylalanyl-tRNA synthetase alpha chain; 9, drug/metabolite transporter superfamily protein; 10, putative transporter; 11, sugar transporter; 12, conserved hypothetical protein; 13, hypothetical protein; 14, transcriptional regulator; 15, hypothetical protein; 16, Holliday junction DNA helicase RuvB; 17, short-chain dehydrogenase family protein FabG; 18, CBS domain pair protein; 19, GDSL-like lipolytic enzyme; 20, hypothetical protein; 21, 1-deoxy-D-xylulose-5-phosphate synthase; 22, GMP synthase; 23, amino acid permease; ABCDEFGHI (FNFX1_0904 to FNFX1_0912, FN3523_0892 to FN3523_0900), glucuronate metabolism genes; J, hypothetical protein; T1, IS*1106* transposase; T2, IS*1016* transposase. (B) Lactose and polyamine metabolism loci. *Fp*, *F. philomiragia* ATCC 25017; *Ft*, *Francisella tularensis* subsp. *tularensis* WY96-3418; *Fn*, *F. novicida*-like strain 3523. Arrow 1, glutaminyl-tRNA synthetase; 2, peptide transporter; 3, ABC transporter ATP-binding protein; 4, tetracycline efflux protein TetA; 5, conserved domain protein; 6, drug:H^+ antiporter-1 (DHA1) family protein; 7, 23S rRNA (guanosine-2′-*O*)-methyltransferase (RlmB); 8, UDP-*N*-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase; 9, orotate phosphoribosyltransferase; 10, UDP-2;3-diacylglucosamine hydrolase; 11, threonine synthase; 12, homoserine kinase; 13, hypothetical protein; 14, arginyl-tRNA synthetase; 15, organic solvent tolerance protein; Y (FN3523_0487), sugar permease; Z (FN3523_0488), beta-galactosidase; K (FN3523_0489), *S*-adenosylmethionine decarboxylase; L (FN3523_0490), spermidine synthase; M (FN3523_0491), biosynthetic arginine decarboxylase; N (FN3523_0492), agmatine deiminase; O (FN3523_0493), *N*-carbamoylputrescine amidase; P, hypothetical protein; T1, transposase/integrase-like protein; T2, ISFtu1 transposase fragments. (C) Presumptive *lac* promoter of *Francisella*. A conserved 61-bp sequence upstream of the *lacZ* ORF from nine strains of *Francisella* is depicted. Nucleotides identical in all nine sequences are shown in boldface. The −10 (TATAAT), −35 (TTGAAC or TTGAAG), and 17-bp spacer regions are indicated. An arrow points to the putative transcription start site (+1). The predicted start codon of *lacZ* is underlined. Sequences 4 through 9 are identical. Row 1, *F. philomiragia* ATCC 25017; 2, *F. novicida*-like strain Fx1; 3, *F. novicida*-like strain 3523; 4, *F. tularensis* FSC198; 5, *F. tularensis* WY96-3418; 6, *F. tularensis* FSC147; 7, *F. tularensis* OSU18; 8, *F. tularensis* FTNF002-00; 9, *F. tularensis* LVS.

spermidine and putrescine (Table 2, Fig. 4B). This gene cluster was not found in strains Fx1 and U112 but was present in other *F. tularensis* subsp. *tularensis* genomes (e.g., strains Schu S4, OSU18, and FSC147). In *F. tularensis* subsp. *tularensis* strain WY96-3418, fragmented ISFtu1 elements were found near this gene cluster (Fig. 4B). Strain 3523 *S*-adenosylmethionine decarboxylase had 45% identity to SpeD (MJ_0315; E value, 9e−27) of *Methanocaldococcus jannaschii*, but it is unrelated to SpeD of *E. coli*. Strain 3523 spermidine synthase and arginine decarboxylase had 55 and 28% identities to *E. coli* SpeE (3O4F_A; E value, 1e−92) and SpeA (3NZQ_B; E value, 3e−60), respectively. Strain 3523 agmatine deiminase and N-carbamoylputrescine amidohydrolase had 37 and 57% identities to *Pseudomonas aeruginosa* PAO1 AguA (PA0292; E value, 1e−58) and AguB (PA0293; E value, 8e−94), respectively. These proteins are key components in the biosynthesis of polyamines in *M. jannaschii*, *E. coli*, and *P. aeruginosa* (14, 35, 45). The acquisition of genes encoding polyamine biosyn-

thesis/metabolism functions may enhance the adaptive capabilities of a bacterium (78, 97). The presence of spermidine biosynthesis genes in strain 3523 as well as *F. tularensis* subsp. *tularensis* strains, but their absence in strains Fx1, U112, and *F. philomiragia* strain ATCC 25017, is a notable feature from a pathogenic and evolutionary standpoint.

Lactose metabolism genes. The *lac* operon, which contains the structural genes encoding proteins that facilitate lactose metabolism, is found in a variety of bacteria. Lactose is imported into the cell as a free sugar by means of a permease, and the enzyme β-galactosidase hydrolyzes this disaccharide into galactose and glucose (96). Genome comparisons revealed that strain 3523 contained a cluster of two ORFs (3,165 bp, 32% GC) adjacent to the spermidine biosynthesis locus that appear to constitute an operon (FN3523_0487 to FN3523_0488) (Fig. 4B). The predicted LacZ of strain 3523 had 29% identity to the β-galactosidase (AAF16519, BgaB; E value, 2e−79) of *Carnobacterium maltaromaticum* (17) and 26% identity to the β-galactosidase (O07012.2, GanA; E value, 8e−76) of *Bacillus subtilis* (19), but it is unrelated to the β-galactosidase of *E. coli*. Strain 3523 LacY had 25% identity to the putative oligogalacturonide transporter (NP_752266; E value, 3e−07) of *E. coli* CFT073 and 22% identity to the putative sugar transporter (YP_081973; E value, 1e−08) of *Bacillus cereus*. A similar *lac* operon was found in strain Fx1 and *F. philomiragia* strains ATCC 25015 and 25017. However, genes encoding the galactoside O-acetyltransferase (*lacA*) and the regulatory protein (*lacI*) were not found in any of these bacteria. In *F. philomiragia* strain ATCC 25017, an ORF encoding a putative transposase/integrase-like protein was found near the *lac* operon (Fig. 4B). Furthermore, other *F. tularensis* subsp. *tularensis* genomes (e.g., strains Schu S4, WY96-3418, OSU18, and FSC147) contained a truncated *lacZ*. Based on the alignment of conserved sequences upstream of *lacZ* from nine different *Francisella* genomes, a putative *lac* promoter also was identified (Fig. 4C). This promoter had high AT content (78 to 86%) and was similar to the *lac* promoters of several Gram-negative bacteria.

Although the classic *lac* operon of *E. coli* consists of three ORFs (*lacZYA*), which are regulated by the product of the adjacent *lacI* repressor gene, bacteria containing only *lacZY* or *lacZ* have been identified (27, 43, 83). While the evolutionary origin of the *E. coli lac* operon is uncertain (83), the occurrence of *lac* genes near integrative and conjugative elements and the identification of *E. coli*-like *lac* operons in some Gram-positive bacteria suggest their lateral mobility (18, 26, 89). From genome comparisons, it appeared that the last common lactose-utilizing ancestor of strains Fx1 and 3523, *F. philomiragia* strain ATCC 25017, and *F. tularensis* subsp. *tularensis* strains have acquired the *lacZY* operon by transposon-mediated horizontal transfer. The loss of this operon in strain U112 and most *F. tularensis* subsp. *tularensis* strains may be due to niche selection or through genetic drift, and a similar mechanism for some members of *Enterobacteriaceae* has been proposed (83). The ability to metabolize lactose probably affords strains Fx1 and 3523 a growth advantage in environments where the sugar is present, and these may represent a subset of *Francisella* species that have retained an ancestral copy of the *lac* operon. Although detailed phylogenetic analyses are required to establish the evolutionary origin of the *Francisella lac* operon, the *lacZ* gene and its promoter identified in this study should be useful in genetic studies requiring a reporter/marker within this group of bacteria.

Summary of important genetic traits and phylogenetic analyses. Based on whole-genome comparisons of different *Francisella* species and strains, the following genetic acquisitions and losses are evident. While strain Fx1 contained the *lac* operon in addition to genes for GlcUA utilization, strain 3523 contained the *lac* operon along with GlcUA utilization, thiamine biosynthesis, and spermidine biosynthesis genes. Whereas most *F. tularensis* subsp. *tularensis* strains had lost loci for GlcUA utilization and thiamine biosynthesis, they contained a vestigial *lacZ* gene and a complete *speDE* system. Furthermore, strains 3523 and Fx1 were the only ones containing the loci for RTX toxin production. Notably, strain U112 lacked all of these loci but contained an *arsRB* operon, which was absent from strains 3523 and Fx1 as well as *F. tularensis* subsp. *tularensis* strains. A summary of the presence/absence of these loci is presented in Fig. 5A. It is possible that strain U112, an environmental isolate of *F. tularensis* subsp. *novicida*, had retained an *arsRB* operon but had lost loci for lactose and GlcUA utilization as well as genes for thiamine biosynthesis because of niche selection. Strains 3523 and Fx1 appeared to be in transition from being environmental to pathogenic, since they have lost the *arsRB* operon but contain loci for lactose and GlcUA utilization, in addition to genes for RTX toxin production. Extant strains of *F. tularensis* subsp. *tularensis* appeared to have lost the *arsRB* operon and loci for lactose and GlcUA utilization, in addition to genes for thiamine biosynthesis, as a consequence of host adaptation and reductive evolution. Since strain 3523 and most *F. tularensis* subsp. *tularensis* strains contain a complete *speDE* system, the role of spermidine in the pathogenesis of tularemia needs to be carefully studied. This is especially important in light of recent reports implicating spermine and spermidine in the stimulation of host cells by *F. tularensis* subsp. *tularensis* (10).

Based on these observations, it may be surmised that the genes/loci for arsenite resistance, GlcUA utilization, lactose utilization, and thiamine biosynthesis are more ancient. It is possible that genes for RTX toxin production and spermidine biosynthesis were acquired later by an *F. tularensis*/*novicida*-like clade. Although strains 3523 and Fx1 were isolated in different parts of the globe, both were associated with marine environments, and their recent common ancestry cannot be ruled out. Deletion-based phylogenetic analyses have indicated *F. tularensis* subsp. *novicida* to be the oldest and that both the acquisition and loss of genes have occurred during the evolution of *F. tularensis* (84). Phylogenetic analyses based on full-length 16S rRNA and *sdhA* genes support the proposition that *F. tularensis* subsp. *novicida* is very closely related to *F. tularensis* subsp. *tularensis* and indicate that strain 3523 represents a new lineage parental to the other *F. tularensis* subsp. *novicida* and *F. tularensis* subsp. *tularensis* strains (Fig. 5B and C).

In conclusion, previous genome comparisons have indicated that subspecies of *F. tularensis* have independently and intermittently acquired and lost genes, and that the net loss in *F. tularensis* subsp. *tularensis* is more than the net loss in *F. tularensis* subsp. *novicida* (4, 75). Based on the analyses of unidirectional genomic deletion events and single-nucleotide variations, it has been suggested that the different subspecies of *F.*

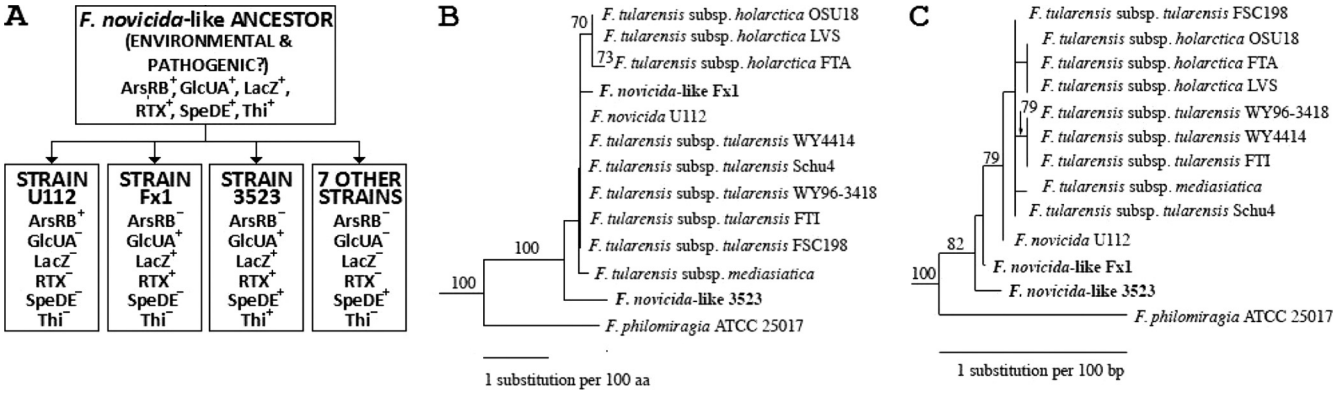


FIG. 5. (A) Summary of important genetic traits among strains U112, Fx1, 3523, and seven other *Francisella* isolates (strains FSC198, Schu S4, WY96-3418, LVS, FTA, OSU18, and FSC147). ArsRB, arsenic resistance locus; GlcUA, glucuronate metabolism locus; LacZ, lactose metabolism locus; RTX, RTX toxin locus; SpeDE, spermidine biosynthetic locus; Thi, thiamine biosynthesis locus. A plus indicates the presence and a minus indicates the absence of a particular locus in each strain. (B) Neighbor-joining tree using succinate dehydrogenase (*sdhA*) genes showing phylogenetic relationships among strains U112, Fx1, 3523, and 11 other *Francisella* isolates for which complete genomes are available. Nodes with bootstrap support greater than 70% are indicated. (C) A neighbor-joining tree like that in panel B is shown, but full-length 16S rRNA genes were used.

tularensis have evolved by vertical descent (84). Analyses of the genomes of strains 3523 and Fx1 imply that these strains represent new links in the chain of evolution from the *F. novicida*-like ancestor to the extant strains of *F. tularensis*. The presence of genes encoding novel biochemical properties appeared to have contributed to the metabolic enrichment and niche expansion of *F. novicida*-like strains. The inability to acquire new genes coupled with the loss of ancestral traits and the consequent reductive evolution may be a cause for, and an effect of, niche restriction of *F. tularensis* subsp. *tularensis*. Although numerous previous studies have discovered the genetic basis for some of the biochemical and antigenic dissimilarities between *F. tularensis* subsp. *novicida* and *F. tularensis* subsp. *tularensis* strains, comparative genome sequence analyses have provided a comprehensive account of innate and acquired genetic traits in this important group of bacteria.

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