Comparative Genomic Characterization of \textit{Francisella tularensis} Strains Belonging to Low and High Virulence Subspecies

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Abstract

Tularemia is a geographically widespread, severely debilitating, and occasionally lethal disease in humans. It is caused by infection by a gram-negative bacterium, \textit{Francisella tularensis}. In order to better understand its potency as an etiological agent as well as its potential as a biological weapon, we have completed draft assemblies and report the first complete genomic characterization of five strains belonging to the following different \textit{Francisella} subsp. species: the \textit{F. tularensis} subsp. \textit{tularensis} FSC033, \textit{F. tularensis} subsp. \textit{holarctica} FSC257 and FSC022, and \textit{F. tularensis} subsp. \textit{novicida} GA99-3548 and GA99-3549 strains. Here, we report the sequencing of these strains and comparative genomic analysis with recently available public \textit{Francisella} sequences, including the rare \textit{F. tularensis} subsp. \textit{mediasiatica} FSC147 strain isolate from the Central Asian Region. We report evidence for the occurrence of large-scale rearrangement events in strains of the \textit{holarctica} subsp. species, supporting previous proposals that further phylogenetic subdivisions of the Type B clade are likely. We also find a significant enrichment of disrupted or absent ORFs proximal to predicted breakpoints in the FSC022 strain, including a genetic component of the Type I restriction-modification defense system. Many of the pseudogenes identified are also disrupted in the closely related rarely human pathogenic \textit{F. tularensis} subsp. \textit{mediasiatica} FSC147 strain, including modulator of drug activity B (\textit{mdaB}) (FTT0961), which encodes a known NADPH quinone reductase involved in oxidative stress resistance. We have also identified genes exhibiting sequence similarity to effectors of the Type III (T3SS) and components of the Type IV secretion systems (T4SS). One of the genes, \textit{msrA2} (FTT1797c), is disrupted in \textit{F. tularensis} subsp. \textit{mediasiatica} and has recently been shown to mediate bacterial pathogen survival in host organisms. Our findings suggest that in addition to the duplication of the \textit{Francisella} Pathogenicity Island, and acquisition of individual loci, adaptation by gene loss in the more recently emerged \textit{tularensis}, \textit{holarctica}, and \textit{mediasiatica} subspecies occurred and was distinct from evolutionary events that differentiated these subspecies, and the \textit{novicida} subspecies, from a common ancestor. Our findings are applicable to future studies focused on variations in \textit{Francisella} subspecies pathogenesis, and of broader interest to studies of genomic pathoadaptation in bacteria.

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Introduction

\textit{Francisella tularensis} is a Gram-negative, facultative intracellular bacterium and its ability to survive and grow within macrophages is a trait that contributes to its virulence. Virulent isolates of the bacterium are the etiological cause of tularemia, a severely debilitating and occasionally fatal disease in humans. Transmission can occur by aerosolization when infected animal carcasses are disrupted, entry through skin abrasions or sites of bites from an arthropod vector, or by ingestion of contaminated food or water. As few as 10 cells have been found to cause respiratory tularemia, making \textit{F. tularensis} one of the most infectious pathogens known at...
Author Summary

Tularemia is a zoonotic disease that is widely disseminated throughout the Northern Hemisphere and is caused by different strain types of bacteria belonging to the genus Francisella. In general, Francisella tularensis subspecies are able to infect a wide range of mammals including humans and are often transmitted via insect vectors such as ticks. Depending on the strain and route of infection the disease may be fatal in humans. In order to better understand F. tularensis as an etiological agent as well as its potential as a biological weapon, we have completed draft sequence assemblies of five globally diverse strains. We have performed a comparative analysis of these sequences with other available public Francisella sequences of strains of differing virulence. Our analysis suggests that genome rearrangements and gene loss in specific Francisella subspecies may underlie the evolution of niche adaptation and virulence of this pathogen.

The effective dose of infection has contributed to past efforts to develop bioweapons containing the F. tularensis bacterium, and due to the particularly high mortality rate of respiratory tularemia, there is still concern that weapons of this nature still exist [3].

Genetic and spatial diversity patterns among a variety of Francisella strain isolates have been previously reported and together with phylogenetic analyses, have provided much insight into the evolutionary divergence of the Francisella genus [4–6]. Francisella is the only genus of the family Francisiellaceae, and has no close pathogenic relatives [7]. The divergent nature of the F. tularensis lineage is evident from phylogenetic studies examining a subset of homologous genes and proteins present in Francisella and 15 other genomes from species also belonging to the γ subclass of proteobacteria [8,9]. The variation of previously characterized genetic attributes between different F. tularensis subspecies (subsp.) is generally minor, despite the more distinct variations in virulence and geographical origin. Previous phylogenetic studies have examined the relationships between the subspecies of Francisella and have recently demonstrated that there are distinct clades of the F. tularensis subsp. tularensis (Type A) lineage, Type A.I and Type A.II [4,10,11]. Divergence of the Type A strains predated the F. holarctica FSC022 japonica strain, which is distinct from the main F. tularensis subsp. holarctica (Type B) radiation lineage [5,6,12].

Studies of strain dispersion and divergence have provided insight into likely migration histories of different Francisella lineages. It has been proposed that the A.I strains originated in the Midwestern North American region prior to the emergence of the A.II strains [11]. The subsequent divergence of the F. tularensis subsp. holarctica biovar japonica strain likely occurred prior to the other Type B strains (reviewed in [12]). Although F. tularensis subsp. novicida has been isolated in Thailand and Australia, the geographical distribution of F. tularensis generally spans the Northern Hemisphere and the most virulent subspecies, F. tularensis subsp. tularensis (Type A) is found exclusively in North America. Cluster analysis of microarray hybridization data has shown overall genomic similarities between F. tularensis subsp. tularensis and F. tularensis subsp. mediasiatica strains, even though strains of the latter subspecies are geographically distinct and are distinguishable by their moderate virulence for mammals [13]. F. tularensis subsp. mediasiatica strains have only been isolated from Kazakhstan and Turkmenistan in Central Asia. This subspecies is virulent in mice [14] and is thought to be more closely related to the highly virulent Francisella tularensis subspecies tularensis. However, F. tularensis subsp. mediasiatica is believed to be of relatively low virulence in humans, and only rare cases of human disease caused by this subspecies are known. F. tularensis subsp. mediasiatica virulence, therefore, more closely resembles that of F. tularensis subsp. holarctica strains (reviewed in [12]). The subspecies F. tularensis subsp. holarctica (Type B) is generally more benign than F. tularensis subsp. tularensis (Type A) strains and has been used to develop the potential vaccine strain, LVS [11,12,13,16].

Strain divergence in Francisella subspecies is likely due to smaller scale genetic differences including those previously characterized in pathogenicity gene clusters and individual gene families, although the biological significance of these variations in regulating virulence remains to be deciphered [9,13,17,18]. It has, however, been well established from evolutionary studies of bacterial pathogens that both gene gain and loss can contribute to virulence as well as pathoadaptation to specific hosts [19]. Virulence and host-range can be influenced by the acquisition of genes that are either structurally organized into pathogenicity islands, or distributed throughout in the genome [20]. And the acquisition of virulence gene clusters often marks the evolutionary differentiation of bacterial pathogens from nonpathogenic ancestors (reviewed in [19]). A duplication of a cluster of genes characterized in Francisella as the Francisella Pathogenicity Island (FPI), for example, could have contributed to the differentiation of the more pathogenic Francisella Type A and B strains from the human non-pathogenic, or rarely pathogenic F. tularensis subsp. novicida and F. philomiragia strains [18].

Although pathoadaptation by gene loss is usually thought of as an opposing evolutionary force to gene acquisition, the presence of both mechanisms may be advantageous for dynamic host niche colonization by bacteria. There is evidence that loss of gene function, as evident by a higher abundance of pseudogenes in the genome, can promote either increased virulence or attenuation [19,21]. An evolutionary fluctuation of niche adaptation is likely due to a lack of selective pressures for genes encoding functions specialized to certain host environments [22–24]. In the case of Francisella, previous studies have suggested that the genomes of more recently diverged subspecies may have adapted to intramacrophage growth via disruptions in many genes (pseudogenes) that include protein products involved in DNA metabolism, amino acid biosynthesis and transport [17,25]. The increased presence of pseudogenes has also been correlated with a high frequency of Insertion Sequence (IS) elements and genome rearrangements in more virulent strains of numerous bacterial pathogens, including strains of Francisella [17]. Specifically, comparison of the more ancestral F. tularensis subsp. novicida U112 strain with F. tularensis subsp. tularensis Schu S4 and the F. tularensis subsp. holarctica LVS identified multiple IS elements associated genomic rearrangements and a collection of genes specific to the human pathogenic strains [17]. Extensive rearrangements have been characterized in Francisella and are known to have occurred from analysis across different subspecies (e.g., OSU18 vs SCHU S4) [26], as well as from comparisons across different clades of the Type A subspecies (WY96-3418 vs SCHU S4) [10]; reviewed in [23]. However, to date, there has been no reported evidence for rearrangements from comparisons among whole genome sequences of the Type B lineage. Previous examination of gene acquisition and loss occurring prior to the divergence of the human pathogenic tularensis (Schu S4) and holarctica (LVS) strains by comparison to a nonpathogenic novicida relative (U112), identified numerous factors potentially involved in human infection [16]. Since these strains are quite phylogenetically distant from one another and are all isolates from...
Francisella subspecies-rich geographical locales, this comparison is of limited value in identifying potential factors required for lethal human infection by the Type A subtype strains. Now, the recently available sequences of the geographically distinct and moderately human pathogenic FSC022 Type B strain from Japan as well as the closely related rarely human pathogenic *F. tularensis* subsp. *mediasiatica* FSC147 strain from the Central Asian Region has enabled a more comprehensive comparison between highly virulent, human pathogenic and human-non-(or rarely) pathogenic strains. Our studies, therefore, provide new insights into how structural genomic rearrangements has contributed to the acquisition and loss of factors regulating virulence and pathoadaptation of different Francisella subspecies, and how shared polymorphisms between the more recently emerged *mediasiatica* and *holarctica* subspecies might be signatures of attenuation.

Results/Discussion

**General Features of Five Sequenced Francisella Genomes**

We have gleaned further insight into subspecies specific differences in gene content with a comprehensive comparative analysis of 20 *Francisella* strains. Included in our analysis are new genome sequences for five *Francisella tularensis* strains. The draft genomic sequences of *F. tularemia* subsp. *tularensis* strain FSC033, *F. tularemia* subsp. *holarctica* strains: FSC022, and FSC257, and *F. tularemia* subsp. *novicida* strains: GA99-3548 and GA99-3549 have been annotated and deposited in GenBank (Materials and Methods). Genome and assembly statistics for each strain are summarized in Table 1. All of the genomes consist of a single circular chromosome and are approximately 2 Mb in size. Although the five strains represent different subspecies of *Francisella*, the overall features of the genomes are quite similar (Table 1). The average GC content and distribution is consistent with previous studies reporting the lower G+C content in *Francisella* [9]. The average number of genes is 1,734, with a mean of 1,374 total protein-coding genes. The genomes of the *novicida* subspecies carry the highest percentage of intact ORFs (97%) and conversely, the *F. tularensis* subsp. *holarctica* FSC257 strain sequence carries the lowest percentage (84%) in comparison to the other five genomes. Pairwise alignment using blastn (1e-5, 95%) of the draft genomes with the *F. tularemia* subsp. *holarctica* OSU18 reference genome shows the high level of overall similarity between genomes across subspecies (95%) (Figure 1). The average gene length does vary across the different strains of *Francisella* and is correlated with the abundance of pseudogenes (Table 1). We also report a significantly larger number of total transposable elements present in the Type A and Type B strains in comparison to the *novicida* strains, which is consistent with previous studies [17]. It is worth noting that the highest numbers are present in Type B strains, even though rearrangement events have not been characterized by previous comparisons between these genomes.

| Table 1. Gene and Assembly Statistics Summary. |
|---------------------------------------------|
| **Human virulence** | low | low | high | rarely | rarely |
| **Strains** | *F. tularensis* subsp. *holarctica* FSC033 | *F. tularensis* subsp. *holarctica* FSC022 | *F. tularensis* subsp. *tularensis* FSC033 | *F. tularensis* subsp. *novicida* GA99-3548 | *F. tularensis* subsp. *novicida* GA99-3549 |
| Length (Mb) | 1.89 | 1.87 | 1.85 | 1.86 | 1.90 |
| GC Content (%) | 32.10 | 32.07 | 32.17 | 32.34 | 32.23 |
| Total ORFs | 1,764 | 1,745 | 1,715 | 1,705 | 1,720 |
| Average ORF Length (nt) | 916 | 953 | 964 | 986 | 1000 |
| Protein Coding ORFs | 1,487 | 1,510 | 1,514 | 1,649 | 1,661 |
| Disrupted ORFs | 277 | 235 | 201 | 56 | 59 |
| Percent intact ORFs (%) | 84 | 87 | 88 | 97 | 97 |
| Proteins of Unknown Function | 427 | 604 | 598 | 521 | 537 |
| Pathogenicity Islands | 2 | 2 | 2 | 1 | 1 |
| IS elements | 113 | 110 | 74 | 9 | 24 |
| tRNA | 27 | 32 | 27 | 36 | 30 |
| rRNA | 5 | 5 | 4 | 7 | 6 |
| ncRNA | 4 | 4 | 4 | 4 | 4 |
| Coverage | 10.01× | 10.31× | 10.48× | 9.88× | 9.88× |
| Assembly Size (Mb) | 1.89 | 1.87 | 1.85 | 1.86 | 1.90 |
| Total Contig Length (Mb) | 1.89 | 1.86 | 1.84 | 1.85 | 1.90 |
| Scaffolds | 21 | 9 | 8 | 5 | 9 |
| Scaffold N50 (Kb) | 245.19 | 488.1 | 387.07 | 554.42 | 258.79 |
| Contigs | 31 | 19 | 15 | 18 | 15 |
| Contig N50 (Kb) | 116.80 | 293.90 | 295.52 | 238.33 | 209.54 |
| %Q40 | 97.53 | 98.76 | 98.76 | 98.78 | 98.93 |
| EndSequenced Fosmids | 27,832 | 24,312 | 24,672 | 26,882 | 23,927 |

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IS Element-Based Genome Rearrangement Events in Type B Strains

Extensive rearrangements have been characterized in Francisella and are known to have occurred from comparing different subspecies (e.g., OSU18 vs SCHU S4) [26], as well as from comparing different clades of the tularensis subspecies Type A (WY96-3418 vs SCHU S4) [10]; (reviewed in [25]). Here, we provide evidence for the occurrence of large-scale genome rearrangements from whole-genome comparisons between the more ancestral FSC022 japonica strain and other Type B strains (Figure 2A) as well as from comparisons within the Type B radiation lineage (Figure 2B).

In order to gain further insight into the possible mechanisms underlying rearrangements in the Type B strains, we have analyzed IS element content. In general, our analysis of the different classes of IS elements in the five new Francisella sequences agrees with what is known of the different Francisella subspecies (Table 2) and the conservation profile and genomic context of the IS elements in Francisella subspecies are also consistent with present models of phylogenetic relationships of the Francisella genus [25] (Table 2). In agreement with earlier reports, we also find noticeable copy number differences between ISFtu elements when comparing the F. tularensis subsp. novicida and F. philomiragia subsp. philomiragia species with the F. tularensis subsp. tularensis (Type A) or F. tularensis subsp. holarctica (Type B) subspecies. Specifically, the F. tularensis subsp. novicida and F. philomiragia subsp. philomiragia genomes contain predominantly ISFtu2 and ISFtu3 elements, respectively. However, the genomes of Type A and B strains contain significantly higher numbers of ISFtu1 and ISFtu2 elements in comparison to the other ISFtu types 3–6. The abundance of ISFtu1 and ISFtu2 elements in the F. tularensis subsp. holarctica genomes suggests that the mechanism mediating genome rearrangements in this subspecies may in part be similar to the rearrangements characterized from comparisons between the Type A and B subspecies [26]. However, ISFtu2 copy number and
genetic structural differences between Type A and Type B strains may determine rearrangement potential between different Type B strains [25].

Previous studies have characterized distinct biochemical characteristics, as well as differences in erythromycin sensitivity, among strains of the Type B clade [12]. The *F. tularensis* subsp. *holarctica* is comprised of strains isolated from different geographical regions in the Northern Hemisphere. *F. tularensis* subsp. *holarctica* is, therefore, endemic to different continents with diverse ecologies. As a result, many variations occur in local transmission cycles, environments, and hosts; And give rise to distinctive biochemical and epidemiological traits between certain isolates of the subspecies. For example, the FSC022 *japonica* isolate is recognized as a biovar variant [27] separable from the other Type B strains. In addition, Lagomorphs (rabbits, hares) have been reported to be the predominant natural reservoir for *F. tularensis* in North America, Europe and Japan. Whereas isolates from the former Soviet Union, Sweden, and Norway also inhabit lemmings in addition to other small rodents as a natural reservoir [28].

Furthermore, the finding that Type B strains are more resistant to ingestion by water borne ciliates is consistent with the suggestion that Type B strains are able to survive in aquatic environments [29,30]. Although further subdivisions of the subspecies *holarctica* have been proposed, very few phenotypic attributes have been demonstrated to formally support this and in the absence of comparative genomic evidence, phylogenetic subdivisions of the *F. tularensis* subsp. *holarctica* has not been formally established or recognized [reviewed in [12]].

The occurrence of rearrangement events in the Type B lineage emphasizes the phylogenetically distinct nature of the FSC022 strain and an appreciation of the impact that genetic decay likely has on the differentiation of *F. tularensis* subsp. *holarctica*. In this regard, it is important to emphasize the more recent emergence of the *F. tularensis* subsp. *holarctica* subspecies in comparison to other *Francisella* clades. FSC022 represents a more distinct lineage that predates strains presently experiencing the highest levels of genome decay in comparison to other subspecies, as evident by the abundance of pseudogenes (Table 1, and Tables 3–6).

Comparative Analysis of Human Pathogenic and Non-Pathogenic Strains of *Francisella tularensis* Subspecies

**Phylogeny of 20 *Francisella* strains by analysis of genome-wide SNP sequences.** Phylogenetic studies of genomewide SNP sequences from 20 *Francisella* strains show the population structure and subspecies divergence of *Francisella* and
A recent study using this same transposon library to perform a negative-selection screen in a mouse model identified 396 candidate essential genes required for growth in vitro [32]. Previous analysis of a mutant library identified 164 genes important for F. tularensis subsp. novicida virulence in mice [34]. The F. tularensis subsp. novicida strain is commonly used as a model organism to assay general Francisella subspecies virulence due to its pathogenicity in vivo and in small animal models. However, the very rare occurrence of human infection from F. tularensis subsp. novicida provides a need for studies that identify genes involved in regulating growth and virulence by comparative approaches across subspecies [35]. Other, more comparative, studies have identified genes that are either absent or disrupted (pseudogenes) in certain Francisella subspecies [9,17,36]. A recent comparative study of the Type A, Schu S4, Type B LVS, and F. tularensis subsp. novicida U112 strains reported 41 genes unique to the Type A and B strains, with most of these encoding proteins of unknown functions [17].

Our analysis of 20 Francisella genomes has identified a subset of ~500 coding sequences that are disrupted in different subspecies and included are genes known to encode protein products involved in metabolic pathways, intercellular transport, secretion, the Type I restriction-modification defense system, transcription, signalling (ie. two component systems), and many hypothetical proteins with unknown functions (listed in Tables 3–6 and Table S1a–h). Only 112 genes identified in our comparative analysis have also been previously reported (Figure 3) [4,11,31]. In agreement with these studies, it is evident from our phylogenetic analysis that the differentiation of F. tularensis subsp. novicida preceded differentiation of the more pathogenic F. tularensis subsp. tularensis and F. tularensis subsp. holarctica subspecies from a common ancestor (Figure 3). We also find that the branch length leading to F. tularensis subsp. tularensis Type A.II strains is shorter than to the F. tularensis subsp. tularensis Type A.I strains, suggesting differential rates of evolution along these two lineages. The F. tularensis subsp. holarctica FSC022 strain diverged basally from the main holarctica lineage prior to the radiation of the main holarctica group and also has a greatly reduced branch length consistent with a much slower rate of evolution. The F. tularensis subsp. mediasiatica FSC147 strain is phylogenetically more closely related to the Type A.II subspecies (Figure 3, and Larsson et al., submitted), however, cases of human infection are very rare. Interestingly, findings that human mortality results from infection with strains of the Type A subtype is indicative of host adaptation mechanisms possibly leading to an attenuation of virulence in both the F. tularensis subsp. mediasiatica and the F. tularensis subsp. holarctica (Type B) subspecies (reviewed in [12]).

Phylogenetic differentiation associated with genetic acquisition and decay. Due to the similarity of their overall genomic sequences (Figure 1), it has been proposed that subspecies and strain-to-strain pathogenicity differences in Francisella are most likely the result of smaller-scale polymorphisms found proximal to predicted breakpoints of genomic rearrangement events [17]. Previous analysis of a transposon generated F. tularensis subsp. novicida mutant library identified 396 candidate essential genes required for growth in vitro [32]. A recent study using this same transposon library to perform a negative-selection screen in a mouse model identified 125 candidate virulence genes required for infection of the lung, liver and spleen [33]. A different in vivo screen, identified 164 genes important for F. tularensis subsp. novicida virulence in mice [34]. The F. tularensis subsp. novicida strain is commonly used as a model organism to assay general Francisella subspecies virulence due to its pathogenicity in vitro and in small animal models. However, the very rare occurrence of human infection from F. tularensis subsp. novicida provides a need for studies that identify genes involved in regulating growth and virulence by comparative approaches across subspecies [35]. Other, more comparative, studies have identified genes that are either absent or disrupted (pseudogenes) in certain Francisella subspecies [9,17,36]. A recent comparative study of the Type A, Schu S4, Type B LVS, and F. tularensis subsp. novicida U112 strains reported 41 genes unique to the Type A and B strains, with most of these encoding proteins of unknown functions [17].

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## Table 3. Phylogenetic Differentiation Associated with Gene Gain and Loss.

| Presence of gene (Intact, disrupted, or absent) and predicted protein family name subcategory | Subspecies strains | LocusID (SchuS4 Reference) | Description of predicted protein product (gene name) |
|---|---|---|---|
| **Intact** | Type A strains only | FTT0496 | hypothetical protein |
|  |  | FTT0677c | hypothetical protein |
|  |  | FTT0813c | adenosine deaminase (add) |
|  |  | FTT068c | hypothetical protein (AI subspecies specific) |
|  |  | FTT1080c | hypothetical membrane protein |
|  |  | FTT1122c | hypothetical lipoprotein |
|  |  | FTT1766 | O-methyltransferase |
|  |  | FTT1791 | hypothetical protein |
| **Intact** | Type A and B strains only | FTT0524 | hypothetical protein |
|  |  | FTT1172c | cold shock protein (csp) |
| **Intact** | Type A and B strains and disrupted or absent in at least one non- or rarely-human pathogenic strain | FTT0755 | hypothetical membrane protein |
|  |  | FTT1011 | hypothetical protein |
|  |  | FTT1580c | hypothetical protein |
|  |  | FTT1175c | hypothetical membrane protein |
| **Disrupted or absent** | Type B strains only | FTT0214 | pseudogene, transporter protein |
|  |  | FTT0514 | L-lactate dehydrogenase (ldhD1) |
|  |  | FTT0529c | DNA polymerase IV, devoid of proofreading, damage inducible protein P (dipP) |
|  |  | FTT0652c | ferritin-like protein (ftnA) |
|  |  | FTT1378 | pseudogene, hypothetical protein |
|  |  | FTT1429c | pseudogene, hypothetical protein |
|  |  | FTT1516c | mercuric reductase (merA) |
|  |  | FTT1619 | pseudogene, acetyltransferase |
|  |  | FTT1661 | thiopurine S-methyltransferase (tmpT) |
|  |  | FTT1768c | chitinase |
|  |  | FTT1786 | pseudogene, hypothetical protein |
|  |  | FTT1793c | aminopeptidase N (pepN) |
|  |  | FTT1799c | pseudogene, hypothetical protein |
| **Transporters: The ATP binding Cassette (ABC) Superfamily** |  | FTT0276c | cyclohexadienyl dehydratase precursor |
|  |  | FTT0445 | ABC transporter, ATP-binding component |
| **Transporters: The Major Facilitator Superfamily (MFS)** |  | FTT0657 | major facilitator superfamily (MFS) transporter |
|  |  | FTT0775c | major facilitator superfamily (MFS) transporter (bcz2) |
|  |  | FTT1380 | major facilitator superfamily (MFS) transporter |
|  |  | FTT1618 | major facilitator superfamily (MFS) transporter |
| **Disrupted** | Type B strains only | FTT0178c | 30S ribosomal protein S6 modification protein-related protein (rnmK) |
|  |  | FTT0221 | acid phosphatase precursor (acpA) |
|  |  | FTT0544 | phosphonoacetate hydratase (phnA) |
|  |  | FTT0553 | hypothetical protein |
|  |  | FTT0568 | hypothetical protein |
|  |  | FTT0747c | hypothetical protein |
|  |  | FTT0783 | Arylsulfatase (ars) |
|  |  | FTT0786 | hypothetical protein |
|  |  | FTT0846 | deoxyribonucleotide photolyase |
|  |  | FTT0898c | hypothetical protein |
|  |  | FTT0902 | hypothetical protein |
| Presence of gene (Intact, disrupted, or absent) and predicted protein family name subcategory | Subspecies strains | LocusID (SchuS4 Reference) | Description of predicted protein product (gene name) |
|---|---|---|---|
| FTTO949c | hypothetical membrane protein |
| FTTO107c | hypothetical protein |
| FTTO1109 | choioylglycine hydrolase family protein |
| FTTO1171c | DNA-methyltransferase, Type I restriction-modification Enzyme subunit M (hsdM) |
| FTTO1202 | transcriptional regulator lysR family |
| FTTO1267 | transcriptional regulator lysR family |
| FTTO1293c | hypothetical protein , sua5_yciO_yrdC family protein |
| FTTO1383 | sun protein |
| FTTO1413 | aminotransferase |
| FTTO1428c | acetyltransferase |
| FTTO1591 | lipoprotein |
| FTTO1623c | hypothetical protein |
| FTTO1625c | hypothetical protein |
| FTTO1796c | hypothetical protein |
| **Transporters: The ATP binding Cassette (ABC) Superfamily** | FTTO017 | ABC transporter ATP-binding protein for toxin secretion |
| FTTO125 | oppD, oligopeptide transporter, subunit D |
| FTTO475 | the small conductance mechanosensitive ion channel (MscS) family transporter |
| FTTO775c | the chloride channel family transporter |
| **Transporters: The Major Facilitator Superfamily (MFS)** | FTTO129 | major facilitator superfamily (MSF) transporter |
| FTTO487 | major facilitator superfamily (MSF) transporter |
| FTTO488c | major facilitator superfamily (MSF) transporter |
| FTTO671 | major facilitator superfamily (MSF) transporter |
| **Transporters: Proton-dependent oligopeptide transport (POT) family** | FTTO651 | proton-dependent oligopeptide transport (POT) family protein |
| FTTO1005c | proton-dependent oligopeptide transport (POT) family protein (yhiP) |
| **Disrupted or absent** | **Type A and Type B strains only** | FTTO262 | hypothetical lipoprotein |
| FTTO495 | hypothetical protein |
| FTTO706 | (gki), glucose kinase |
| FTTO865 | pseudogene, hypothetical protein |
| FTTO883 | pseudogene, alcohol dehydrogenase |
| FTTO1577 | hypothetical protein |
| **Disrupted or absent** | **Type B strains and closely related rarely-human pathogenic F. tularensis subsp. mediasiatica FSC147** | FTTO095 | hypothetical protein |
| FTTO122 | (oppA), oligopeptide transporter, subunit A * intact in strain FSC022 |
| FTTO177c | acetyltransferase |
| FTTO223c | hypothetical protein (ybgL) |
| FTTO464 | (ansB), periplasmic L-asparaginase II precursor |
| FTTO673c | hypothetical protein |
| FTTO829c | aspartatealanine antipporter |
| FTTO850 | hypothetical protein |
| FTTO864c | transcriptional regulator lysR family |
| FTTO911 | hypothetical protein |
| FTTO961 | (mdaB), modulator of drug activity B |
| FTTO995 | major facilitator superfamily (MSF) transporter |
pathogenicity, are not intact or known to be functional in Francisella subspecies (Tables 4 and 5). As a result, comparison of highly, moderately, and rarely/non-virulent strains has provided a more comprehensive list of potential virulence factors mediating human infection by Francisella (Tables 3–6 and Table S1a–h).

**Gene functions specific to human-pathogenic Francisella strains.** In total, we have identified fourteen genes that are intact only in the Francisella human pathogenic strains (Type A and Type B strains), eight of these are specific to the more virulent Type A strains and are either absent or disrupted in other subspecies (Table 3). We have found that previously identified genes encoding hypothetical proteins and the O-antigen cluster in Type A and B strains are also intact in the F. tularensis subsp. mediasiatica FSC147 strain, and this O-antigen cluster is distinct from the cluster type characterized in isolates of F. tularensis subsp. novicida and F. tularensis subsp. philomiragia [17].

With the exception of add (FTT0939), an adenosine deaminase and a gene (FTT1766) encoding O-methyltransferase, all of the eight genes specific to Type A strains are predicted to encode either proteins of unknown function or potential membrane proteins (Table 3). These ORFs are intact in all of the Type A strains, but disrupted in strains of the Type B subtype as well as in strains of the non-human pathogenic subspecies. In addition, we note the presence of a gene (FTT1068c) encoding a hypothetical protein that is intact specifically in the Type A I clade and was previously identified by Beckstrom-Sternberg SM et al. [10]. All of the Type A specific loci have been previously reported [17], but not previously identified to be disrupted in F. tularensis subsp. mediasiatica. There is a collection of genes that are intact only in Type A and/or B strains and although their functions are not known, a few are predicted to encode membrane proteins (Table 3). In addition, we find that a gene (FTT0604) encoding a CPA1 family antporter has an in-frame stop upstream of a transmembrane domain in Type A strains that is not present in Type B strains (data not shown) [17]. This large protein family is highly conserved, and functionally characterized to catalyze Na+:H+ exchange. Although much less is known regarding their primary physiological function in bacteria, there is evidence that CPA transport systems act to regulate cytoplasmic pH and mediate electrophile resistance [38].

The metabolic pathways and growth requirements for the Type A Schu S4 strain have been characterized previously and include

### Table 3. Cont.

| Presence of gene (Intact, disrupted, or absent) and predicted protein family name subcategory | Subspecies strains | LocusID (SchuS4 Reference) | Description of predicted protein product (gene name) |
| --- | --- | --- | --- |
| Membrane proteins | FTT1426c | hypothetical membrane protein |
| Membrane proteins | FTT1626c | hypothetical membrane protein |

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### Table 4. Phylogenetic Differentiation Associated with Gain and Loss of Genes with Sequence Similarity to T3SS Effectors.

| Disrupted or absent | Type A and/or B strains only | FTT003c | lipase/acyltransferase |
| --- | --- | --- | --- |
| Disrupted or absent | F. tularensis subsp. mediasiatica, Type A and B strains only | FTT0612 | hypothetical protein (present in three copies in novicida strains) |
| Present | All subspecies strains | FTT0211c | outer membrane lipoprotein |
| | | FTT0393 | methionine aminopeptidase (map) |
| | | FTT0541c | haloacid dehalogenase (yqB) |
| | | FTT0659 | DNA recombination protein (rmUC) |
| | | FTT0910 | hypothetical protein |
| | | FTT1132c | glycerophosphoryl diester phosphodiesterase (gpdQ) |
| | | FTT1156c | Type IV pilin multimeric outer membrane protein (pilQ) |
| | | FTT1268c | chaperone protein (dnaJ) |
| | | FTT1376 | acyl carrier protein (acpP) |
| | | FTT1512c | chaperone protein (dnaJ) |
| | | FTT1671 | riboflavin biosynthesis protein (riBD) |

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350 enzymes involved in 137 predicted pathways of small molecule metabolism [9]. These studies also established that the growth requirements for this strain include a supplemental supply of 14 essential amino acids as well as cysteine, for sulfate assimilation. Our comparative analysis confirms that most, if not all, pathways for amino acid synthesis seem to be inactivated in assimilation. Our comparative analysis confirms that most, if not all, of 14 essential amino acids as well as cysteine, for sulfate growth requirements for this strain include a supplemental supply molecule metabolism [9]. These studies also established that the enzymes involved in 137 predicted pathways of small metabolism [9]. Consistent with mediiasiatica parasites, which like important role of adenosine editing in regulating survival of is unknown [17], however, studies have characterized the significance of a Type A specific intact adenosine deaminase ORF inactivation was found in pyrimidine metabolism pathway. The pathway is interrupted, however, no subspecies-specific gene previous studies, we find that most of the purine metabolism inactivation of these genes in the closely related non-human pathogenic subspecies strains has provided new insight into how gene decay in different gene functional classes may have contributed to the attenuation of pathogenicity in Francisella.

**DNA metabolism and oxidative stress.** In agreement with previous studies, we find that genes encoding members of the LysR transcriptional regulator family as well as other proteins involved in transcription, such as acetyltransferases, are specifically disrupted or absent in Type B strains [17]. Not previously known, was the inactivation of these genes in the closely related non-human pathogenic F. tularensis subsp. mediiasiatica FSC147 strain (Table 5). The range of known LysR transcriptional regulator targets includes DNA metabolism and oxidative stress. In agreement with previous studies, we find that genes encoding members of the LysR transcriptional regulator family as well as other proteins involved in transcription, such as acetyltransferases, are specifically disrupted or absent in Type B strains [17]. Not previously known, was the inactivation of these genes in the closely related non-human pathogenic F. tularensis subsp. mediiasiatica FSC147 strain (Table 5). The range of known LysR transcriptional regulator targets includes

| Disrupted or absent | F. tularensis subsp. mediiasiatica, Type A, and B strains only | FTT0046 | magnesium chelatase family protein (comM) |
|--------------------|--------------------------------|--------|---------------------------------|
| Disrupted or absent | F. tularensis subsp. mediiasiatica only | FTT1797c | peroxiredoxin (alkyl hydroperoxide reductase subunit C) (prdx1ahpc) |
| Disrupted or absent | Type A strains only | FTT0542 | peptidyl-prolyl cis-trans isomerase D |
| Present | All subspecies strains | FTT0458 | stringent starvation protein A regulator of transcription (sspA) |
| | | FTT0557 | hypothetical protein ahpC/TSA family |
| | | FTT0623 | trigger factor (Tf) protein (peptidyl-prolyl cis/trans isomerase) |
| | | FTT0628 | peptidyl-prolyl cis-trans isomerase D |
| | | FTT0633 | membrane protease subunit (fapX) |
| | | FTT0634 | membrane protease subunit (fapC) |
| | | FTT0832 | FKBP-type 16 kDa peptidyl-prolyl cis-trans isomerase (FkpB) |
| | | FTT0878c | peptide methionine sulfoxide reductase (msrB) |
| | | FTT1186 | SrrA (tmRNA)-binding protein (smpB) |
| | | FTT1422 | SM-20-related protein |
| | | FTTT1725c | protein-L-isoaspartate O-methyltransferase (pcm) |

**Table 5.** Phylogenetic Differentiation Associated with Gain and Loss of Genes with Sequence Similarity to T4SS Components.

| Disrupted or absent | At least one strain of a human pathogenic subspecies | FTT0046 | magnesium chelatase family protein (comM) |
|--------------------|---------------------------------|--------|---------------------------------|
| | | FTT0179 | DNA internalization-related competence protein (rec2), *Not present in Type B, OSU18 strain |
| | | FTT0830c | DNA uptake protein (dpA) |
| | | FTT1301c | amidophosphoribosyltransferase (similar to comF) |
| Present | All subspecies strains | FTTT1057c | fimbrial biogenesis and twitching motility protein (fimB), *In novicida subspecies, present only in U112 |
| | | FTTT1156c | Type IV pilin multimeric outer membrane protein |

**Table 6.** Phylogenetic Differentiation Associated with Gain and Loss of Genes Regulating Competence (E values > 1e-10).
genes whose roles include niche adaptation and virulent responses [17,41]. The functional importance of the LysR transcriptional family in mediating pathogenicity, and their notable decay in the F.t. holarctica and F.t. mediasiatica subspecies lends support to the notion that they may be responsible for attenuation of virulence.

Also of interest is the subspecies specific loss of the hsdM gene (FTT1171c) in the more moderately human pathogenic Type B strains, which encodes a DNA methyltransferase component of the Type I restriction-modification defense system (Table 3). Previous studies have established that the Type I restriction-modification defense system
A

Figure 4. Multiple alignment of Francisella genomes of the Type B lineage identifies conserved sequence regions with rearrangements; pseudogenes are found proximal to predicted breakpoints. A comparison of genome rearrangement patterns between the more ancestral F. tularensis subsp. holarctica FSC022 japonica strain and representative strains of the main holarctica group (F. tularensis subsp. holarctica FSC022 and F. tularensis subsp. holarctica OSU18) was done using MAUVE (A). MAUVE uses an anchored alignment algorithm that permits reordering of the alignment anchors for identification of rearrangements. Colored Local Collinear Blocks (LCBs) are regions of sequence alignment that are free of rearrangements. Each LCB is defined by the anchor regions or predicted sites of rearrangement. A default LCB cutoff of 175, and a filtering for larger blocks containing 10 kb or larger was done in Mauve. Sequence inversions are denoted by differential positioning of the LCBs relative to a reference axis. A zoomed-in section of the whole-genome alignment is shown so that the annotated ORFs are visible (black outlined boxes). The small red ORFs are tRNA genes. ORFs proximal to predicted rearrangement breakpoints (red circles) have been color coded and labeled (FWD, INV). A summary of the predicted protein products for these genes is provided in (B). These include genes that have been identified as being either disrupted or absent in the F. tularensis subsp. holarctica subspecies.

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regulates resistance of horizontal gene transfer in *S. aureus* [42]. And a recent study reports the presence of a strong restriction barrier in the *F. tularensis* subsp. *novicida* subspecies that is mediated by numerous restriction/modification systems, which have been lost during the evolution of the human pathogenic subspecies [35].

**Secretion systems.** Generally, the cell envelope of gram-negative bacteria is composed of an outer membrane, peptidoglycan cell wall, periplasm and an inner membrane. Secretion systems acting across the cell envelope mediate transfer of virulence factors, and are therefore fundamental regulators of bacterial pathogenesis [43]. Interestingly, early studies that identified virulence factors characterized a particular set of genes homologous to components of the Type III secretion system in *Yersinia*. These genes are frequently associated with pathogenicity islands present in animal pathogens as well as in plants [44,45]. In addition, two Type III effector genes of the gram-negative pathogenic bacteria that causes blight in rice have been shown to mediate host transcription factor induction [46].

Previous studies have reported the presence of many components of different secretion systems in *F. tularensis* [25,47]. Overall, our analysis of the recently sequenced *F. tularensis* genomes is consistent with previous characterization of the genes (and pseudogenes) that encode components of the Twin Arginine Translocation (TAT), and secretion systems of Type I (T1SS), Type II (T2SS), Type V (T5SS), and VI (T6SS) (Tables 4–6 and Table S1c,d,f) [44,48,49] in the *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *novicida* strains. There is presently no existing evidence to support the presence of intact Type III (T3SS) or Type IV (T4SS) secretion systems, or their functional role in *Francisella*, with the exception of the Type IV pilus biogenesis system. Although this system shares components of the T3SS, it is primarily associated with the T2SS [9,25,47,49] (Table 4).
The Type III secretion system (T3SS) mediates transfer of virulence factors into host cells, and the T4SS may mediate transfer of DNA or virulence factors into eukaryotic target cells. Both systems are major pathogenicity factors in other bacteria [46,30,31]. And the secreted T3SS effectors have been shown to be important contributors to virulence in bacterial pathogenesis [48]. However, we did identify a small number of known T3SS effectors (Table 4). This same approach did not identify any significant hits (e<1e-5) to Agrobacterium tumefaciens C58 T4SS proteins that were queried against the Francisella genomes [52,53]. However, we did identify a small number of Francisella genes, using the KEGG system, which were assigned to T4SS [54,55]. It is also worth noting that comM, a gene disrupted in F. tularensis subsp. tularensis, F. tularensis subsp. holarctica, and F. tularensis subsp. mediasiatica, has been previously characterized in Haemophilus influenzae and is also a highly conserved component of systems regulating competence in gamma-proteobacteria and E. coli [62]. Although the biological significance of this merging of the T2SS and T3SS system in Francisella is not fully understood, recent studies have shown a relationship between the two systems [63]. The T3SS component proteins and a T3SS effector were previously characterized in Francisella genomes [52,53]. One of these genes, hpDA (FTT1524c), encodes for an ATP-dependent helicase homologous to the E. coli HrpA DEAH-box RNA helicase previously shown to be involved in mRNA processing of an operon involved in fimbrial biogenesis [54], and the other gene, (FTT0023), encodes for a lipase/acyltransferase. In novicida strains, three genes (FTN_1069, FTN_1070, FTN_1071) are similar to the T3SS effector, OspD3_Sfx. These genes are truncated in Type A (FTT0612), Type B, and mediasiatica subspecies strains.

Type IV pilus secretion systems form a trans-envelope channel and an extracellular pilus structure. Type IV pili are known to mediate bacterial adhesion and twitching motility, and possibly other cellular functions necessary for bacterial growth and pathogenesis [55]. Genes required for Type IV pili biogenesis have been previously identified in Francisella tularensis genomes and several of these encode components shared by the T3SS (i.e. flagella), or are homologous to genes also required for T2SS [9,25]. Consistent with previously characterized Type B strains, we identified an early in-frame stop in the pilT gene (FTT0088, T2SS) present in FSC022 and FSC257. This gene is also disrupted in the F. tularensis subsp. mediasiatica FSC147 strain, indicating that this genetic disruption occurred after the phylogenetic split between the Type A and F. tularensis subsp. mediasiatica/Type B Francisella strains [56], Table S1f. FTT0861c, encoding a Type IV pil fiber block protein, has a large internal deletion and frame-shift in all of the Type A strains and in the FSC257 and FSC200 Type B strains. All of the Type B strains isolated from Russia and Sweden have a 3-bp deletion, which restores the reading frame without introducing an internal stop codon (data not shown). The prepilin peptidase encoded by the pilD (FTT0683c) gene has an in-frame stop near the C-terminus in all Type A and B strains, making them 9aa shorter. The impact of this change with regards to pathogenic capacity of different Francisella strains is not known, however, Legionella pneumophila pilD mutants are known to be greatly reduced in virulence [57]. In addition, we report that the pilA gene in all of the novicida strains is a single gene corresponding to a merge of FTT0888, FTT0889 and FTT0890, each about the size of pilA genes in other bacterial species. Although the biological significance of this merging of pilA genes is unknown, previous studies have established a functional importance of gene fusions in mediating virulence. For example, the fusion of the partial ORFs, FTT0918 and FTT0919, has been shown to be a significant contributing factor to virulence attenuation in the FSC043 strain [50]. Recent studies have also characterized the presence of fibres resembling type IV pili on the surface of the LVS and demonstrated the importance of pilD and the other genes of the Type IV pilus biogenesis system in mediating F. tularensis virulence in mice [59,60].

Most of the genes identified by their similarity to known T4SS genes are present in all of the Francisella subspecies. Of interest are two genes, FTT1797c and FTT0542; the former is disrupted only in the F. tularensis subsp. mediasiatica FSC147 strain and the latter is specifically disrupted in the F. tularensis subsp. tularensis subspecies. The disruption of a gene (FTT1797c), encoding a peptide methionine-S-sulfoxide reductase, only in the F. tularensis subsp. mediasiatica FSC147 strain is notable given emerging evidence that the absence of the MsrA2 enzyme results in decreased bacterial pathogen survival in host organisms [61]. Also of interest is the subspecies specific loss of the gene (FTT0542) encoding the peroxiredoxin oxidative response protein in Type A strains (Table 5).

The Twin Arginine Translocation System. The Twin Arginine Translocation (TAT) secretion system mediates cytoplasmic membrane transport of folded proteins regulating bacterial pathogenesis. Our analysis using the TatP program identified potential substrates for the (TAT) secretion pathway that were disrupted or absent in the F. tularensis subsp. tularensis subspecies (Table S1f). The ber2 transporter gene (FTT0775c), is specifically disrupted in the Type A1 strains and genes encoding a short-chain dehydrogenase/reductase family protein (FTT0723) and an amino acid transporter (FTT0361) are specifically disrupted in all of the Type B strains. The (FTT0723) gene encoding a short-chain dehydrogenase/reductase family protein and (FTT1510c), which encodes a HAAP family transporter are also disrupted in the F. tularensis subsp. mediasiatica strain (Table S1f).
We have reported the sequencing of five globally diverse strains of Francisella and their comparative analysis with all other publicly available Francisella genome sequences, including the geographically restricted and rare F. tularensis subsp. mediasiatica FSC147 isolate from the Central Asian region. Our analysis of these whole genome sequences has provided novel insights into the genomic attributes that underlie the attenuated virulence of the F. tularensis subsp. holarctica and F. tularensis subsp. mediasiatica lineages, in comparison to F. tularensis subsp. tularensis strains. These subspecies are more closely related to each other phylogenetically than to their more distant F. philomiragia and novicida relatives, however, they inhabit geographically distinct regions. Although the origin of the Type B lineage is debatable, recent evidence suggests that F. tularensis subsp. holarctica originated in Asia, as evident from the phylogenetic basal positioning of the F. tularensis subsp. holarctica FSC022 japonica strain, and this clade diverged from the Type-B radiation lineage proposed to have originated in North America. Molecular approaches prior to the availability of whole genome sequence analysis has characterized the Type B-radiation lineage as a genetically homogeneous clade. These findings together with the wide distribution of Type B isolates across the Northern hemisphere has lead to speculations that the Type B-radiation group recently emerged through a genetic bottleneck, resulting in niche adaptation and attenuation of virulence. Consequently, increased survival in a range of vertebrate and invertebrate hosts likely facilitated the spread of Type B Francisella across the Northern hemisphere. The findings that Type B strains can survive in watercourses also reflects this broadening of the host range.

Here, we provide evidence suggesting that genome rearrangements and gene decay may have played a prominent role in the pathoadaptation of different Francisella subspecies, including the Type B-radiation lineage. Our findings therefore suggest that a greater genetic diversity exists between isolates of the Type B group than previously known. Since these strains are more recently diverged in comparison to other Francisella strains, our findings also suggest that perhaps this lineage is undergoing an evolutionary process of further diversification. Evidence of genomic rearrangements and diversity is predominant from our comparisons between the more ancestral FSC022 strain and all of the other Type B strains of the radiation lineage. Surprisingly, we also report similar evidence of significant rearrangements from comparisons specifically between the OSU18 and FSC257 strains of the Type B-radiation lineage. In addition, we report cases of pseudogenes that are specific to Type A.II and Type B strains isolated from the Russian and Sweden regions, which include the FSC257 strain.

Like the Type B lineage, F. tularensis subsp. mediasiatica has also previously been characterized as a subspecies with very little genetic diversity. In contrast, however, F. tularensis subsp. mediasiatica is geographically very restricted and isolates are rare which may limit our knowledge of the true genetic diversity of this lineage. Interestingly, despite the distinction of their environmental niches, the F. tularensis subsp. holarctica and F. tularensis subsp. mediasiatica lineages share a similar pathogenicity profile and we find that the genomes of these subspecies share intriguing genomic profiles with respect to key genes known to play a role in bacterial pathogenesis. Specifically, the F. tularensis subsp. holarctica and F. tularensis subsp. mediasiatica strains contain polymorphisms in a shared set of genes that contain known factors important for virulence and niche adaptation in other bacteria. These genes encode protein products that include important transcriptional regulators (ie. LysR family), structural components (ie. PIIT),

(Tables 3 and Table S1b,f). We identified a total of 14 MFS and ABC encoding genes that were specifically disrupted or absent in F. tularensis subsp. holarctica and/or F. tularensis subsp. mediasiatica (Table 3 and Table S1b). Our comparative analysis also confirmed previous findings regarding specific loss in Type A.II and Type B strains of the FTT0727 and FTT0729 genes that encode components of the DRI/YHH family ABC exporter, as well as loss of genes encoding members of the OCTN family of ABC transporters, the REG family of ABC transporters and the oppABCDF system [36,66,67], Table S1b). The opp operon is an important regulator of oligopeptide transport and bacterial growth [68]. We find that two genes, oppD (FTT0125) and oppF (FTT0126), are separate loci in Type A strains, but a 960 bp internal deletion in Type B strains results in a fused ORF with disrupted oppD and oppF coding sequences (Table 3 and Table S1g).

Two Component Systems. Bacterial Two Component Systems (TCSs) have been found to be important regulators of growth and virulence [69,70]. We identified three known paired TCSs that exhibit differences between the Francisella subspecies. The ntrXY regulator (unique to F. philomiragia), the vicRK system, and the kdpABCDEF system [9] (Table S1f). Studies in bacterial species other than Francisella have provided evidence that the vicRK system plays a key role in mediating virulence by actively detecting changes in temperature, oxidative stress and osmotic pressure [71,72]. We find that two genes of the vicRK system are specifically disrupted in strains of the human pathogenic Type A and B lineage (Table S1f) [17,73].

The kdp genes function in a turgor pressure response system that is sensitive to low potassium levels. Interestingly, we find that all of the genes in the kdp operon, with the exception of kdpC and a small ORF we have identified as kdpF (FTT1740c), are differentially inactivated in Type A and B strains, consistent with previous studies [17] as well as in the F. tularensis subsp. mediasiatica strain, FSC147. Previous work has shown that a transposon mediated disruption of the kdpD gene in the F. tularensis subsp. novicida U112 strain leads to attenuation of virulence in mice [34].

Overall, the biological significance of the subspecies specific genomic differences that we report are largely unknown, except in those cases also identified and verified experimentally by other studies. It is worth mentioning that there are cases, like the kdpD gene, where gene loss in other species of bacteria (M. tuberculosis) results in increased virulence rather than attenuation [69]. It is known that regulation of highly conserved genetic pathways important in determining virulence and host tropism are dependent upon the overall biology of the organism, and are also influenced by the host environment. In regards to mechanisms of host tropism and Francisella infection, much has been gleaned from a recent study suggesting that F. tularensis subsp. holarctica bacterial dissemination post-infection is accomplished by regulation of dendritic cells migration in mice [74]. Along these lines, it should be mentioned that all Francisella subspecies assayed to date are virulent in cultured cells and in animal models, even though the pathogenicity of different subspecies in the human host varies considerably. The F. tularensis subsp. novicida U112 strain, for example, rarely affects humans even though this strain is highly virulent in animal models. This, together with its genetic tractability, makes F. tularensis subsp. novicida U112 a commonly used model system in studies of Francisella pathogenesis. Although there is much to be learned from these studies, we are still challenged by the inherent limitations that exist in assaying how subspecies genetic differences determine virulence in various host environments, especially in humans.
metabolic regulators (i.e., MdaB), membrane proteins, and transporters of the Major facilitator superfamily. Also disrupted specifically in the *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *mediasiatica* strains is a gene of unknown function that is a component of the Francisella pathogenicity island (FTI1340) and encodes a protein fusion product of two hypothetical proteins in *F. tularensis* subsp. *novicida*. We also find that a gene encoding a subunit of the OppA oligopeptide transporter is disrupted in all of the radial lineage Type B strains and the *F. tularensis* subsp. *mediasiatica* strain, but is intact in the more ancestral FSC022 strain. Also of note is the specific disruption of the msrA2 gene in *F. tularensis* subsp. *mediasiatica* that encodes a peptide methionine sulfoxide reductase.

The collection of genes that we have identified as likely regulators of niche adaptation and virulence in *Francisella* also includes genes encoding factors exhibiting weak sequence similarity to known genes of the Type III and Type IV secretion systems. The biological significance of the sequence similarity of some *Francisella* loci to genes encoding effector and component proteins characteristic of either the T3SS or T4SS, respectively, is unclear. The absence of intact Type III or Type IV secretion systems might be indicative of evolutionary decay, or a mechanism of cross-talk between components of partial secretion systems that mediate Francisella virulence; An aspect which emphasizes the uniqueness of the mechanism of virulence in Francisella in comparison to other bacteria.

Our findings shed light on the evolutionary process of Francisella pathogenicity, and also provide broader insight into the general evolutionary process of bacterial pathoadaptation. A model of evolution of *Francisella* subspecies can be proposed from comparative analysis of genomic features of *F. tularensis* subspecies and what is known regarding mechanisms of pathoadaptation in recently obligate intracellular pathogens: As strains underwent geographical dispersion and adaptation to new niches, their genomes acquired more transposable elements and experienced higher frequencies of rearrangements. These events led to an increase of genomic polymorphisms that promoted functional acquisition required for environmental adaptation and virulence, as evident in the highly pathogenic *tularensis* subspecies. The high abundance of pseudogenes in more recently emerged subspecies is likely a reflection of the *Francisella* genomes in decay, especially in the strains that are human pathogens, where the more nutritionally rich environment in the host (e.g., rabbits and humans) made it unnecessary to maintain many of the genes required as a free-living organism. Interestingly, mechanisms of genomic pathoadaptation seems to have promoted a more benign pathogenic biology in the more recently emerged Type B *Francisella* strains, and attenuation in rarely human pathogenic *F. tularensis* subsp. *mediasiatica*.

**Materials and Methods**

**Sequencing and Assembly**

*F. tularensis* subsp. *holarctica* type B strain FSC257 (FSC257) was isolated from the tick Dermacentor pictus in 1949 from the area of Moscow, Russia. A different lineage of *holarctica* subspecies, strain FSC022, was isolated in Japan in 1950. The CDC standard for Type A strains, FSC033 (399), was isolated from a squirrel in Georgia, USA and the genomic DNA of this strain, as well as strains FSC257 and FSC022 were kindly provided by Mats Forsman of Swedish Defense Research Agency, Sweden.

Two antibiotic sensitive *F. tularensis* subsp. *novicida* strains, GA99-3548 and GA99-3549(F6168) [75], were isolated from human patient samples in Louisiana and California, respectively and the DNA sequenced in this project was provided by Scott Bearden at the CDC Fort Collins, Colorado, USA.

8× draft assemblies of 5 strains using 454 Technology and ABI Hybrid Assembly was done as described at http://www.broad.mit.edu/seq/msc/. The sequence coverage generated is shown in Table 1 and Arachne [76] was used for sequence assembly.

**Accession Numbers**

The *Francisella* genomes were annotated as described on the Broad Institute *Francisella tularensis* group database: [http://www.broadmit.edu/annotation/genome/Francisella_tularensis_group/MultiHome.html](http://www.broadmit.edu/annotation/genome/Francisella_tularensis_group/MultiHome.html)

**Genomic Comparative Maps**

Genomic Comparative Maps were constructed using CGview software [77] and scripts for mapping blast analysis provided courtesy of Paul Stothard and customized by M.Champion. Five sequenced draft genomes (*F. tularensis* subsp. *holarctica* FSC257 and FSC022; *F. tularensis* subsp. *mediasiatica* FSC033; *F. tularensis* subsp. *novicida* GA99-3548, and GA99-3549 strains) were aligned to the *F. tularensis* subsp. *holarctica* OSU18 reference sequence using the blastn program (minimum percent identity = 95 and expected threshold = 1e⁻⁵).

**Genome Alignments and Analysis of Predicted Genome Rearrangements**

A comparison of genome rearrangement patterns between the more ancestral FSC022 Type B strain (reference) and the clonal strains of the *holarctica* subspecies was done using the alignment program, PatternHunter and for certain cases, visual comparisons were also done using Mauve [78]. PatternHunter was utilized at default settings; Except the maximum distance between spans for the spans to be merged on both the reference and the query sequence was set to 200 bp and the alignments were filtered for overlap percentages > = 90 [79]. Predicted rearrangements were identified from sequence alignments and breakpoint sites were further analyzed for proximal gene decay (see methods below for further description of how pseudogenes were identified). Specifically, total predicted ORF counts were binned according to distance (kb) from the predicted breakpoint. The number of pseudogenes in each bin was used to determine the percentage present. The statistical significance of pseudogene enrichment was determined using Fisher’s Exact Test to derive *p*-values. PatternHunter whole genome alignments were also used to generate dotplots. For confirmation of select breakpoints in Mauve, a default LCB cutoff of 175, and filtering for blocks > = to 10 kb.

**Phylogenetic Analysis**

The evolutionary history of 20 *Francisella* strains was inferred from whole genome SNP data using the Maximum Parsimony method and MEGA4 software [80]. PatternHunter was utilized (as described above) to perform pairwise local alignments of 200 bp segments on a sliding window. This analysis compared sequence segments and did not require synteny, therefore rearrangements were not a factor in SNP discovery. Whole genome SNPs at least 20 bp apart and present in more than one *Francisella* genome were identified using custom scripts and clustalw alignments of these sequences were selected for further analysis using MEGA4.
Table 7. GenBank Accession Numbers for Francisella Strains Used in This Study.

| GenBank accession numbers | Strain name (strains sequenced by the Broad in bold) |
|---------------------------|-----------------------------------------------------|
| AJ749949                  | F. tularensis subspecies tularensis strain SCHU S4 |
| AAYE00000000              | F. tularensis subspecies tularensis FSC033         |
| AM286280                  | F. tularensis subsp. tularensis FSC198             |
| CP000608                  | F. tularensis subsp. tularensis WY96-3418          |
| CP000437                  | F. tularensis subsp. holarctica OSU18              |
| CP000803                  | F. tularensis subsp. holarctica FTA                |
| AAU0D00000000             | F. tularensis subsp. holarctica FSC257             |
| AAYD00000000              | F. tularensis subsp. holarctica FSC022             |
| NC_007880                 | F. tularensis subsp. holarctica LV5                |
| AASP00000000              | F. tularensis subsp. holarctica FSC200             |
| CP000439                  | F. tularensis subsp. novicida U112                 |
| ABAAH00000000             | F. tularensis subsp. novicida GA99-3548            |
| AAYF00000000              | F. tularensis subsp. novicida GA99-3549            |
| CP000937                  | F. philomiragia subsp. philomiragia ATCC 25017     |

Strain sequences available from the Microbial Genomes Projects at BCM-HGSC: [86]

Francisella tularensis subsp. tularensis ATCC6223
Francisella tularensis subsp. holarctica KD971026
Francisella tularensis subsp. holarctica MI001730
Francisella tularensis subsp. holarctica OR96246
Francisella tularensis subsp. holarctica RC503

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Comparative Analysis of Pathogenic Francisella

We performed an extensive comparative analysis of ORFs and whole genome sequences to identify genes unique to one or more subspecies, but absent in others. Briefly, we did an all versus all blast comparison (e = 10^{-10}) of all CDS (including pseudogenes) from four completely sequenced genomes: SCHU S4 (Type A1); WY96-3418 (Type A2), OSU18 (Type B) and U112 (spp. novicida), to identify an initial list of ORFs not found in ORFs from one or more of the other genomes. We then used this list of ORFs to search against all 20 genomes in order to evaluate conservation profiles using both nucleotide as well as protein sequence alignments (clustalw).

We used a similar approach to search for likely gene fusion/split events. Specifically, we first identified candidate gene pairs via an all versus all blast comparison (e = 10^{-10}) of coding sequences. We used custom scripts to identify candidates and then reviewed these candidates in the context of genome alignments and targeted gene sequence alignment from all the available Francisella genome sequences. This allowed us to distinguish real gene fusion/split event from artifacts due to annotation or sequencing errors.

We assigned all protein coding genes (including pseudogenes) to KEGG pathways using KAAS [81]. Additional information about transporters were added from [36], and from membrane transportDB and TransAAP analysis [82,83]. Members of the bacterial two component systems were identified using PFAM domains for the response regulators (PF00072) and histidine kinase (PF00512, PF07536, PF07568 and PF00773), similar to the approach used by Kiiil et al. [94].

Search for similarity to proteins in the T3SS and T4SS pathways. We used P. aeruginosa T3SS component protein sequences and a repertoire of effector protein sequences as queries to search a translated nucleotide Francisella database with the TBLASTN algorithm [85] (BLOSUM62 matrix, default parameters with the exception of setting the expected threshold to e<1e^{-3}) similar to the approach of Tobe, et. al. [48]. The same approach, using Agrobacterium tumefaciens C58 T4SS protein sequences as queries was also done [52,55]. In addition, we also used the Francisella nucleotide sequences as queries to search the KEGG peptide database using blastx (BLOSUM62 matrix, default parameters with the exception of setting the expected threshold to e<1e^{-5}). Hits were filtered based on the expectation threshold, however in general, those identified also exhibit a <40% identity to the query and therefore are defined as ‘weakly similar’.

Supporting Information

Figure S1 Whole genome sequence alignments and dotplot comparisons between the F. tularensis subsp. mediasiatica FSC147 strain and other subspecies strains. FSC147 is the reference genome.
Comparison of Pathogenic Francisella

Table S1: Subspecies specific disruption of genes encoding proteins of major secretory pathways, membrane proteins and components of known metabolic pathways.

Table S2: Summary of genes identified as candidates for mediating pathogenicity in F. tularensis subsp. holarctica (Type A), F. tularensis subsp. holarctica (Type B), and F. tularensis subsp. novicida by previous studies.

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