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Francisella opportunistica sp. nov., isolated from human blood and cerebrospinal fluid

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Abstract

Two isolates of a Gram-negative, non-spore-forming coccobacillus cultured from the blood and cerebrospinal fluid of immunocompromised patients in the United States were described previously. Biochemical and phylogenetic analyses revealed that they belong to a novel species within the *Francisella* genus. Here we describe a third isolate of this species, recovered from blood of a febrile patient with renal failure, and formally name the *Francisella* species. Whole genome comparisons indicated the three isolates display greater than 99.9% average nucleotide identity (ANI) to each other and are most closely related to the tick endosymbiont *F. persica*, with only 88.6–88.8% ANI to the type strain of *F. persica*. Based on biochemical, metabolic and genomic comparisons, we propose that these three isolates should be recognized as *Francisella opportunistica* sp. nov, with the type strain of the species, PA05–1188^T, available through the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM 107100) and the American Type Culture Collection (ATCC BAA-2974).

The genus *Francisella* currently comprises seven named species, notably including *F. tularensis*, the causative agent of tularemia. It also includes the rare or opportunistic human pathogens *F. novicida* (also validly published as *F. tularensis* subsp. *novicida* [1–3]), *F.*

Ethical Approval

Patient follow-up was conducted as part of routine public health surveillance. Characterization of bacterial isolates derived from human specimens was approved by the CDC Institutional Review Board.

Conflict of Interest

The authors declare that there are no conflicts of interest. The findings and conclusions in this manuscript are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Sequence data available from GenBank. Accession numbers:

CP022375: 14–2155 whole genome

CP022376: MA06–7296 whole genome

CP022377: PA05–1188^T whole genome

EU031811: 16S rRNA sequence (identical for all three isolates)

philomiragia, and *F. hispaniensis*, as well as pathogens of aquatic organisms (*F. noatunensis* and *F. halioticida*) and tick endosymbionts (*F. persica* and other unclassified *Francisella*) [1, 2, 4–7].

We previously described two isolates of a novel *Francisella* species cultured from blood and cerebrospinal fluid of two US patients with immune compromising conditions [8]. One of these isolates, MA06–7296, was fully sequenced and provisionally named *F. opportunistica* [9]. The 16S rRNA and *sdhA* genes from the first isolate, PA05–1188, have also been sequenced and shown to be identical to those of MA06–7296 [8]. A third isolate, 14–2155, not previously described in the literature, was cultured in 2014 from blood of a febrile patient in Arizona with type II diabetes, renal failure, and cardiopulmonary failure.

As previously described, the *F. opportunistica* isolates are indole and catalase negative, oxidase positive, and urease negative (Table 1). All three are beta-lactamase negative, which is a unique characteristic among *Francisella* species described to date. Although MA06–7296 was previously reported as urease weak, this was not confirmed in subsequent testing [8]. *F. opportunistica* isolates do not grow in 6.5% NaCl and require cysteine supplementation for optimal growth [8, 9].

The PA05–1188 and MA06–7296 isolates were further tested for metabolic characteristics using the Biolog MicroLog system (Gram-negative GN2 MicroPlates), and compared to a selection of other *Francisella* isolates (Table 1). These included *F. novicida* (n = 11), *F. philomiragia* (n = 5), *F. tularensis* subsp. *tularensis* (n = 6, including five human and animal isolates and the attenuated strain ATCC 6223), and *F. tularensis* subsp. *holarctica* (n = 9, including eight human and animal isolates and the attenuated strain LVS). Results for the two subspecies of *F. tularensis* are combined in Table 1, as they cannot be consistently distinguished by any metabolic characteristic except the ability to metabolize glycerol. All tested *Francisella* strains were negative for tween-80, N-acetyl-D-galactosamine, D-arabitol, L-fucose, gentiobiose, m-inositol, α-D-lactose, D-mannitol, D-melibiose, D-raffinose, L-rhamnose, D-sorbitol, xylitol, citric acid, D-galactonic acid lactone, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, γ-hydroxybutyric acid, p-hydroxyphenylacetic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, glucuronamide, L-histidine, L-leucine, L-phenylalanine, γ-aminobutyric acid, phenylethylamine, putrescine, and 2-aminoethanol. All tested strains were positive for D-fructose, α-D-glucose, methyl pyruvate, α-ketobutyric acid, and L-serine. PA05–1188 was positive for α-ketovaleric acid and L-aspartic acid, while MA06–7296 was negative. Results that vary among *Francisella* species are shown in Table 1. No single metabolic reaction on the MicroLog GN2 plate is unique to *F. opportunistica*; however, the overall pattern of reactivity is characteristic.

To further clarify the relationships among the three isolates and their position in the *Francisella* genus, we sequenced their full genomes using both PacBio and Illumina technology. For PacBio sequencing, 5 μg of input DNA was used for 10-kb fragment library preparation, and sequencing was performed on the PacBio RSII platform and assembled using the Hierarchical Genome Assembly Process (HGAP). For Illumina sequencing, libraries were prepared using the Nextera XT DNA Library Prep kit, and sequencing was performed on the MiSeq platform with a 300-cycle V2 reagent kit. Sequencing statistics and

GenBank accession numbers are listed in Table 2. *De novo* assembly of PacBio reads yielded a single complete circular contig encompassing the chromosome for each isolate. Illumina sequences were assembled both using PacBio sequences as a reference and *de novo* using SPAdes 3.10.1 [10]. No plasmids were identified by either technology. Six errors in the PacBio consensus, all in 14–2155, were corrected by assembly of Illumina sequences to the PacBio consensus. Final sequences used for analysis and submitted to GenBank represent the corrected sequence obtained by comparison of both methods. All three genomes are approximately 1.8 Mb in length, with 32.5% GC content.

Average nucleotide identity (ANI) was calculated using the ANI calculator tool (http://enve-omics.ce.gatech.edu/ani/) with default settings of 1000 bp window size, 200 bp step size, and 70% identity over 700 bp [11]. The three genomes displayed >99% ANI to each other and <90% ANI to any other *Francisella* species, consistent with their designation as three isolates of a distinct *Francisella* species (Figure 1) [11]. The most closely related species is *F. persica* (formerly *Wolbachia persica*), a tick endosymbiont, with approximately 89% ANI to each of the three isolates [7] (Figure 1). The three *F. opportunistica* genomes displayed approximately 87% ANI to *F. tularensis*, *F. novicida*, and *F. hispaniensis* genomes. These analyses are consistent with the previously described clustering of *F. opportunistica* MA06–7296 with *F. persica*, based on phylogenetic analysis of 36 conserved housekeeping proteins [9, 12].

Genomes were annotated using the NCBI Prokaryotic Genome Annotation Pipeline [13]. As previously described for PA05–1188^T and MA06–7296, the three isolates show 100% identity in their 16S rRNA and *sdhA* gene sequences [8]. The *F. opportunistica* 16S rRNA and *sdhA* genes display 97.6% and 84.0% pairwise nucleotide identity, respectively, to their homologs in *F. tularensis* Schu. Sporadic polymorphisms vary between the three genomes. Single nucleotide polymorphisms are largely concentrated in a few genomic regions, including the *Francisella* pathogenicity island (FPI; see below), a type I restriction-modification system, and a region that contains multiple genes annotated as being involved in sulfur metabolism. The MA06–7296 and 14–2155 nucleotide sequences are identical in all three of these regions, while PA05–1188^T contains multiple polymorphisms.

The three *F. opportunistica* genomes were aligned using the progressiveMauve algorithm within Geneious 11.0.4 software, and found to be collinear and extensively rearranged compared to other sequenced *Francisella* species (Figure 2) [14, 15]. IS elements were analyzed using ISFinder, and the sequences were deposited in the ISFinder database [16]. Six IS elements are polymorphic in *F. opportunistica*, with PA05–1188^T containing the largest number of unique IS element insertions (Table 3). The most frequent of these elements is 99.2% identical in its consensus sequence to ISFw2, an element previously described in the German environmental *Francisella* isolate W12–1067. Three additional elements are homologous to ISFtu elements named in *F. tularensis*. ISFop2 is a homolog of ISFtu2, with 69.5% amino acid similarity (52.2% identity). Interestingly, a single copy of a more closely related ISFtu2 element (93.5% similarity/87.1% identity) is present and conserved in all three genomes. A truncated element found only in PA05–1188 is homologous to ISFtu4, with 71.9% amino acid similarity (66.1% identity). ISFop1 shows some limited similarity to ISFtu1 (39.2% similarity/19.4% identity).

The FPI in each of the *F. opportunistica* genomes is present in a single copy and lacks the *pdpC* and *pdpE* genes, as described previously [9]. Sequences corresponding to these genes were found elsewhere in the genomes, outside the FPI, by BLAST search. The *pdpE* gene is intact in all three genomes, but *pdpC* is pseudogenized. Notably, in *F. persica* and other *Francisella*-like endosymbionts, *pdpC* and *pdpE* are present in the FPI region, but flanked by IS elements not present in either copy of the *F. tularensis* FPI, suggesting that these two genes have been subject to transposition [17].

The absence of beta-lactamase activity detected by biochemical testing is unusual among *Francisella* species. *F. tularensis* genomes include two annotated class A beta-lactamase genes, one of which is disrupted by insertion sequences in *F. opportunistica*. Interestingly, the same gene is also disrupted in *F. persica*, suggesting that this species may also lack beta-lactamase activity.

Taken together, these phenotypic and whole genome analyses reveal a novel *Francisella* species, most closely related to the endosymbiont *F. persica*. The genomes of the three isolates display >99% ANI to each other and are collinear, but contain differences in IS element content and nucleotide sequence in certain genomic regions, including the FPI. The isolation of *F. opportunistica* from three ill patients with immune compromising conditions in different regions of the United States (Pennsylvania, Massachusetts, and Arizona) suggests that the species is widespread. Correct identification of rare *Francisella* species is critical in order to avoid biosafety and biosecurity concerns associated with *F. tularensis*. Clinical laboratories should be aware of the possibility of infection with non-*tularensis Francisella* spp., especially in patients with immune compromising conditions [8, 18]. *F. opportunistica* can be distinguished from *F. tularensis* by beta-lactamase, antigen detection (DFA and slide agglutination) and by molecular methods including PCR and sequencing [8].

Description of *Francisella opportunistica* sp. nov.

F. opportunistica (op.por.tu.nis'ti.ca N.L. fem. adj. *opportunistica*, opportunistic, referring to the organism's ability to opportunistically infect immunocompromised humans). The type strain, PA05–1188, was isolated in 2005 from a patient with hemophagocytic syndrome and juvenile rheumatoid arthritis; the MA06–7296 and 14–2155 strains were isolated in 2006 and 2014, respectively, from patients with end-stage renal disease. Cells are small Gram-negative coccobacilli. They are catalase negative, oxidase positive, and beta-lactamase negative. Other biochemical and metabolic characteristics are as shown in Table 1. Slide agglutination and direct fluorescent antibody assay for *F. tularensis* are both negative. [8, 9]. Whole genome sequence information for all three strains is available from GenBank with accession numbers CP022375 (14–2155), CP022376 (MA06–7296), and CP022377 (PA05–1188^T). The genome length is approximately 1.8 Mb, with a GC content of 32.5%. The three isolates display >99% ANI to each other and <89% ANI to any other species, with the most closely related species being *F. persica*. They are 100% identical across the 16S rRNA and *sdhA* genes, with 97.6% identity to *F. tularensis* 16S rRNA and 84.0% identity to *F. tularensis sdhA*. The type strain, PA05–1188^T, is available through the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM 107100) and the American Type Culture Collection (ATCC BAA-2974).

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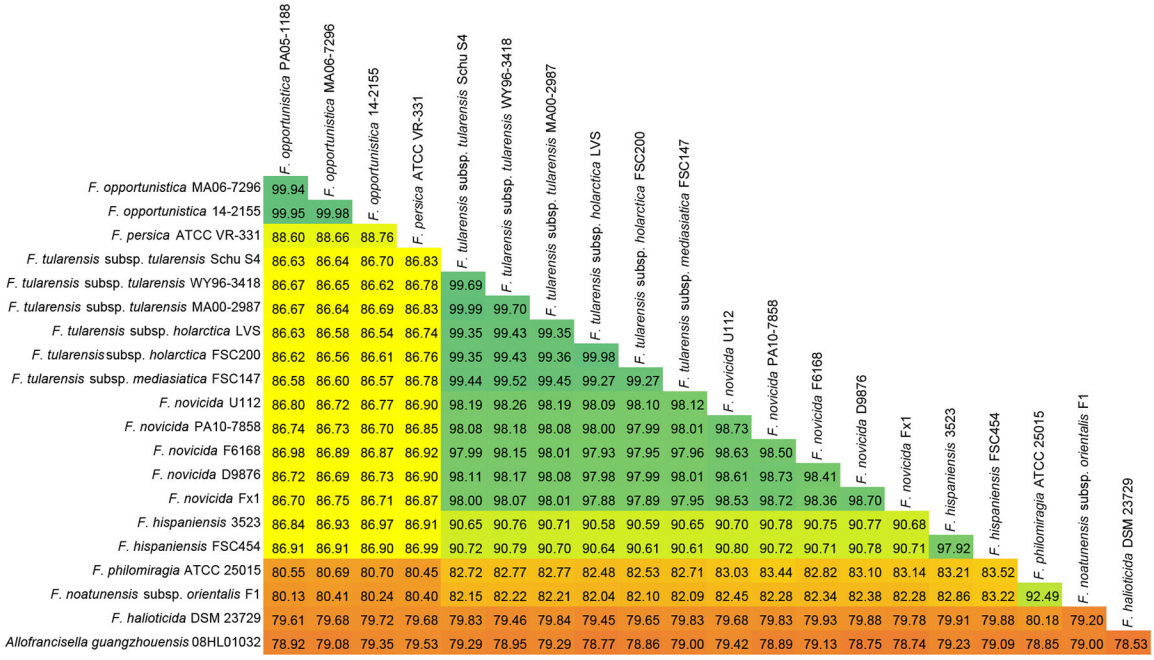

Fig. 1.

Distance matrix showing ANI among Francisellaceae family members. Matrix is color-coded according to ANI value, with the highest values shown in green and the lowest in orange.

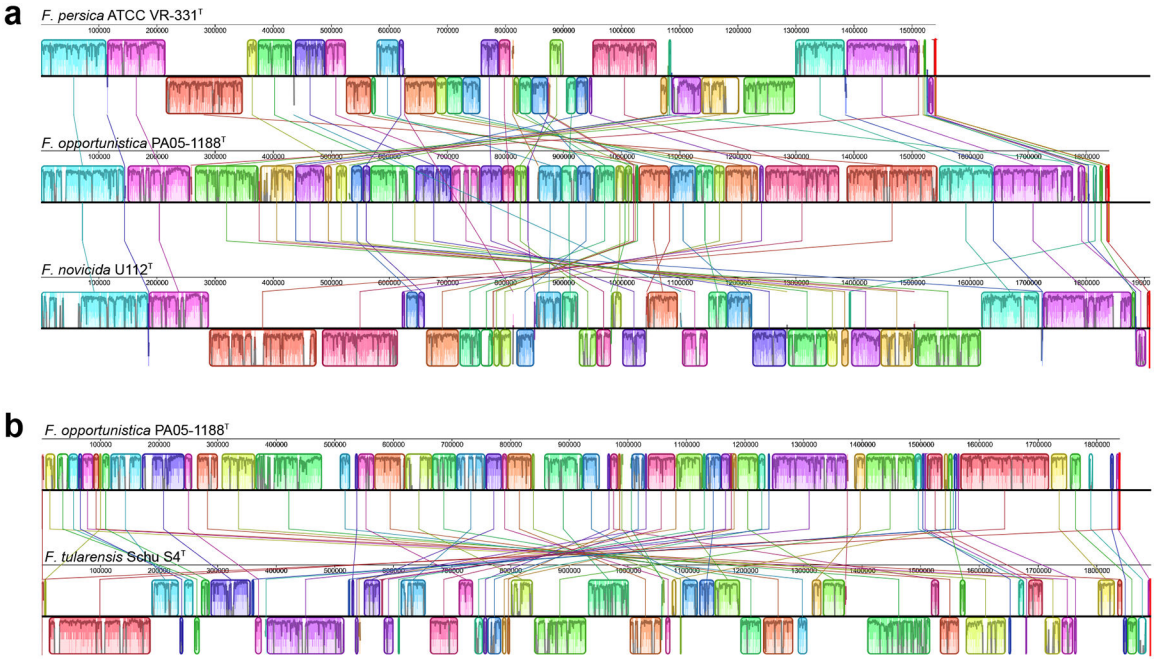

Fig. 2.

Genome structure comparisons showing extensive rearrangement. (A) *F. persica*, *F. opportunistica*, and *F. novicida*. (B) *F. opportunistica* and *F. tularensis* (shown separately to highlight more extensive rearrangement). Locally collinear blocks are color coded, and local average nucleotide conservation is shown in graphs within the blocks. Homologous blocks are connected by colored lines. Blocks above the black line are in forward orientation compared to *F. opportunistica*, and those below the line are in reverse orientation. Figure was generated using progressiveMauve and Geneious 11.0.4 [14, 15, 19].

Table 1.

Phenotypic characterization.

W, weak. V, variable among tested strains. See text for reactions identical among all *Francisella* tested.

	F. opportunistica	F. tularensis	F. novicida	F. philomiragia
Oxidase *	+	−	−	+
Catalase *	−	W	W	W
Indole *	−	−	−	+
Urease *	−	−	−	−
β-lactamase *	−	+	+	+
α-cyclodextrin	−	−	V	−
Dextrin	+	V	V	V
Glycogen	+	V	V	V
Tween-40	+	−	V	−
N-acetyl-D-glucosamine	+	V	+	+
Adonitol	−	−	V	−
L-arabinose	−	V	V	V
D-cellobiose	−	V	V	V
i-erythritol	−	−	V	V
D-galactose	+	V	+	V
lactulose	−	−	V	−
maltose	−	−	V	V
D-mannose	+	V	+	+
β-methyl-D-glucoside	−	−	−	V
D-psicose	−	V	V	V
sucrose	−	−	+	+
D-trehalose	−	−	+	V
turanose	−	−	V	−
mono-methyl-succinate	+	V	+	+
acetic acid	+	V	+	V
cis-aconitic acid	−	−	V	V
formic acid	−	−	V	−
D-galacturonic acid	−	−	−	V
α-hydroxybutyric acid	−	V	+	V
β-hydroxybutyric acid	+	−	+	+
itaconic acid	−	−	V	V
α-keto glutaric acid	+	V	V	V
α-keto valeric acid	V	−	−	−
D,L-lactic acid	+	V	+	+
succinic acid	+	V	+	V

	F. opportunistica	F. tularensis	F. novicida	F. philomiragia
bromosuccinic acid	+	V	V	V
succinamic acid	−	V	−	V
L-alaninamide	+	V	+	+
D-alanine	+	V	+	+
L-alanine	+	V	+	+
L-alanyl-glycine	+	V	+	+
L-asparagine	+	V	+	+
L-aspartic acid	+	V	+	+
L-glutamic acid	+	V	+	+
glycyl-L-aspartic acid	−	V	V	V
glycyl-L-glutamic acid	−	V	+	V
hydroxy-L-proline	+	−	V	−
L-ornithine	+	−	+	V
L-proline	+	V	+	+
L-pyroglutamic acid	+	−	+	+
D-serine	−	V	−	−
L-threonine	+	V	+	+
D,L-carnitine	−	−	−	V
urocanic acid	−	V	−	−
inosine	+	−	+	V
uridine	−	V	+	+
thymidine	−	V	V	V
2,3-butanediol	−	−	V	−
glycerol	+	V	+	V
D,L-α-glycerol phosphate	+	+	+	V
glucose-1-phosphate	−	−	V	V
glucose-6-phosphate	+	V	V	V

*Reactions marked with an asterisk are not on the Biolog MicroLog GN2 plates, and were run separately for *F. opportunistica* isolates only. Results of these reactions for other *Francisella* species were compiled from the literature.

Table 2.

Genome sequencing

Strain	GenBank Accession #	Genome length	Average PacBio coverage	Average PacBio read length	Average Illumina coverage
PA05–1188^T	CP022377	1,839,223	308	16516	701
MA06–7296	CP022376	1,831,298	352	18239	711
14–2155	CP022375	1,824,971	225	18982	1051

Table 3.

IS elements polymorphic in *F. opportunistica*

Name	Family	Group	# conserved	# unique to PA05–1188	# unique to MA06–7296	# unique to 14–2155	Closest Francisellaceae homolog[a]
ISFw2	IS5	IS5	9	5	0	0	*Francisella* sp. W12–1067 element (99% nt/100% aa identity)
ISFop1	IS630	N/A	1	1	0	0	Unnamed *Allofrancisella* element
ISFop2	IS5	IS427	11	1	0	1	ISFtu2 (69.5% aa similarity)
ISFop3	IS110	N/A	5	2	0	0	Unnamed element in *F. novicida* (some strains), and *F. uliginis*
ISFop4	IS3	IS150	2	2	3	0	None
Truncated element[b]	IS982	N/A	0	1	0	0	ISFtu4 (71.9% aa similarity)

[a]Homologs were found by BLAST searches limited to Francisellaceae. Amino acid similarity is given for defined, named elements listed in ISFinder.

[b]This element is truncated and was not able to be annotated within ISFinder.