Description of *Francisella hispaniensis* sp. nov., isolated from human blood, reclassification of *Francisella novicida* (Larson *et al.* 1955) Olsufiev *et al.* 1959 as *Francisella tularensis* subsp. *novicida* comb. nov. and emended description of the genus *Francisella*

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Strain FhSp1^T, isolated from human blood in Spain in 2003, was studied for its taxonomic allocation. By 16S rRNA and *recA* gene sequencing, the strain was shown to belong to the genus *Francisella*. In the 16S rRNA gene sequence, *Francisella* sp. FhSp1^T shared similarity of more than 99 % with strains of *Francisella tularensis* subspecies and *Francisella novicida* U112^T, 98 % with *Francisella piscicida* GM2212^T and 98.4 % with *Francisella philomiragia* ATCC 25015^T. In the *recA* gene sequence, *Francisella* sp. FhSp1^T exhibited 91.6–91.7 % similarity to strains of *F. tularensis* subspecies, 91.2 % to *F. novicida* U112^T and 84 % to *F. philomiragia* ATCC 25017. The genus affiliation was supported by a quinone system typical of *Francisella* (Q-8 as the major component), a complex polar lipid profile similar to that of *F. tularensis* with the major components diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine and an unknown aminophospholipid (APL4) and a fatty acid profile consisting mainly of C_{10:0} (17.2 %), C_{14:0} (11.2 %), C_{16:0} (13.1 %), C_{18:0} 3-OH (14.2 %) and C_{18:1} ω9c (7.1 %). DNA–DNA hybridization, which showed unambiguously that FhSp1^T represents a novel species, and the results of biochemical tests allowed genotypic and phenotypic differentiation of the isolate from all hitherto-described *Francisella* species. A multiplex PCR developed in the course of this study discriminated FhSp1^T from representatives of all other *Francisella* species and subspecies, clades A.I and A.II of *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* biovar *japonica* and also between these representatives of the genus. Therefore, we propose the name *Francisella hispaniensis* sp. nov., with the type strain FhSp1^T (=FnSp1^T =FSC454^T =F62^T =DSM 22475^T =CCUG 58020^T). Furthermore, we formally propose the transfer of the species *Francisella novicida* to the species *Francisella tularensis* as *Francisella tularensis* subsp. *novicida* comb. nov. (type strain ATCC 15482^T =CCUG 33449^T =CIP 56.12^T). We also present an emended description of the genus *Francisella*.

Francisella tularensis is a facultatively intracellular bacterium, and all its subspecies cause the zoonotic disease tularemia in humans and animals. *Francisella philomiragia* and *Francisella*

novicida strains, although generally less virulent than strains of *F. tularensis*, may cause a tularemia-like disease in immunocompromised patients (Hollis *et al.*, 1989).

The GenBank/EMBL/DDJB accession numbers for the 16S rRNA gene and *recA* sequences of strain FhSp1^T are FN252413 and FN252412, respectively, and the accession number of the *recA* sequence of *F. tularensis* subsp. *tularensis* ATCC 6223^T is FN545355.

The genus *Francisella*, together with *Wolbachia persica*, *Fangia hongkongensis* and *Caedibacter taeniospiralis*, represents one of, if not the deepest branch in the class *Gammaproteobacteria* (Forsman *et al.*, 1994; Lau *et al.*, 2007; Gao *et al.*, 2009). At the time of writing, the genus *Francisella* comprises four recognized species, *F. tularensis*, with the three subspecies *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *mediasiatica*, *F. novicida*, *F. philomiragia* and *F. noatunensis* (formerly *F. philomiragia* subsp. *noatunensis*), with the two subspecies *F. noatunensis* subsp. *noatunensis* and *F. noatunensis* subsp. *orientalis* (McCoy & Chapin, 1912; Larson *et al.*, 1955; Olsufiev *et al.*, 1959; Olsufjev & Meshcheryakova, 1983; Hollis *et al.*, 1989; Ottem *et al.*, 2009; Mikalsen *et al.*, 2007). A fifth species, *Francisella piscicida* (Ottem *et al.*, 2007), was proposed to represent a later heterotypic synonym of *F. noatunensis* subsp. *noatunensis* (Ottem *et al.*, 2009) based on high degrees of sequence similarities in certain housekeeping genes and results from DNA–DNA hybridizations slightly below the threshold (70 % reassociation) for species delineation. However, the reliability of these taxonomic rearrangements cannot be considered to be certain and, hence, we use the name *F. piscicida* in this contribution.

In several publications, including the chapter on *Francisella* in the second edition of *Bergey's Manual of Systematic Bacteriology* (Sjöstedt, 2005), *F. novicida* is named *Francisella tularensis* subsp. *novicida* (Sjöstedt, 2005), but this name is not formally correct; although Sjöstedt (2005) proposed the transfer of this species to *F. tularensis* as the subspecies '*F. tularensis* subsp. *novicida*', this name has never been validly published.

Strain FhSp1^T was presumptively identified as a strain of *F. novicida* (Escudero *et al.*, 2003). However, recent analyses of five housekeeping genes and two genes encoding membrane proteins (Nübel *et al.*, 2006; Escudero *et al.*, 2010) as well as genomic fingerprinting using RAPD-PCR carried out in the course of our study (results not shown) revealed a clearly distant position of this strain from recognized *Francisella* species and subspecies. This uncertain taxonomic affiliation was further affirmed by the results of DNA typing methods employing 38 canonical insertion–deletion markers, in which strain FhSp1^T failed in amplification of seven tested loci (Larsson *et al.*, 2007). Escudero *et al.* (2008) named strain FhSp1^T an unusual *F. tularensis* strain. Here we report the classification of FhSp1^T as a member of a novel species of *Francisella*, applying biochemical, physiological, chemotaxonomic and genetic characterization. Furthermore, we formally propose to transfer the species *F. novicida* to the species *F. tularensis* as *Francisella tularensis* subsp. *novicida* comb. nov.

FhSp1^T showed distinct, convex, pale-white to grey colonies that reached maximum size (3–4 mm diameter) in 48 h on cysteine heart agar (HCA; BD) at 37 °C. Cysteine is not required for growth but enhances it. FhSp1^T could also be grown on Columbia blood agar, whereas no

growth occurred on MacConkey agar plates. Cell morphology was observed under an Axiostar microscope (Zeiss) at × 1000, with cells grown for 2 days at 37 °C on HCA. The pleomorphic and coccoid rods of FhSp1^T stained faintly Gram-negative.

Amplification and sequencing of the 16S rRNA-encoding gene and *recA* were carried out as reported previously (Scholz *et al.*, 2006) using the primers listed in Table 1 (16S rRNA gene: 27f, 1492r, 341f, 518r, 926f; *recA*: *recA_tul_f*, *recA_tul_r*). Amplification of *recA* consisted of an initial denaturation step at 94 °C for 5 min and 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s and a final elongation step at 72 °C for 60 s. Primers *recA_tul_f* and *recA_tul_r* were constructed based on accessible genomic sequences of certain francisellae. 16S rRNA gene (1471 bp) and *recA* (965 bp) sequences of FhSp1^T were compared to entries in the EMBL database using the FASTA33 search tool (Pearson & Lipman, 1988). Strain FhSp1^T showed 99.7 % 16S rRNA gene sequence similarity to *F. novicida* U112^T and *F. tularensis* subsp. *tularensis* SCHU S4 and strain FSC198, 99.5–99.6 % to other *F. tularensis* subspecies, 98.2–98.4 % to strains of *F. philomiragia*, including the type strain ATCC 25015^T, and 98.0 % to *F. piscicida* GM2212^T. The *recA* gene sequence of FhSp1^T shared 91.2–91.7 % with the corresponding sequences of members of different *Francisella* subspecies, including *F. novicida* U112^T, and 84 % with that of *F. philomiragia* ATCC 25017 in 941 overlapping positions. *recA* sequences of recognized *Francisella* subspecies shared 99.9–100 % similarity with each other and 98.9–99.0 % with that of *F. novicida* U112^T. These data indicate a close relatedness of *F. novicida* to *F. tularensis* and a quite distant position of FhSp1^T from recognized *Francisella* species. Subsequent analysis of the *recA* gene was performed using the software package MEGA version 4.0 (Tamura *et al.*, 2007) after multiple alignment of sequences by CLUSTAL_X (Thompson *et al.*, 1997). Distance options were according to the maximum composite likelihood model and clustering with the neighbour-joining method was performed by using bootstrap values based on 2000 replications. A *recA*-based tree is shown in Fig. 1. In further analyses, the relatedness of FhSp1^T and *F. novicida* suggested by sequence similarities was confirmed.

For fatty acid, polar lipid and quinone analysis, cells were grown on HCA at 37 °C with 5 % CO₂ for 48 h, except *F. piscicida* DSM 18777^T, which was kept at 20 °C under ambient atmosphere, suspended in PBS, heat-killed and then lyophilized. Fatty acid methyl esters were prepared as described by Kämpfer & Kroppenstedt (1996).

The fatty acid profile of FhSp1^T consisted of C_{10:0} (17.2 %), C_{18:0} 3-OH (14.2 %), C_{16:0} (13.1 %), C_{14:0} (11.2 %), C_{18:1 ω 9c} (7.1 %), C_{24:1} (5.0 %), C_{18:0} (3.8 %), C_{16:0} 3-OH (2.8 %), C_{10:0} 2-OH (2.7 %), C_{22:0} (2.5 %), C_{24:0} (2.5 %), C_{20:0} (1.7 %), iso-C_{15:0} 2-OH and/or C_{16:1 ω 7c} (1.4 %), anteiso-C_{15:0} (1.6 %), iso-C_{15:0} (1.1 %), C_{12:0} (0.9 %), C_{20:2 ω 6,9c} (0.8 %), C_{17:0} 3-OH

Table 1. Oligonucleotide primers used in this study and expected amplicon sizes when used in multiplex PCR

Primers were designed in this study unless indicated otherwise.

Primer	Sequence (5'–3')	Reference*	Amplicon size (bp)
RAPD-PCR			
RAPD1a	GTGCCGACGG	–	–
RAPD1b	GTGCCGACGGC	–	–
RAPD2a	ATAGCCGACG	–	–
RAPD2b	ATAGCCGACGC	–	–
RAPD3a	TCTGACCACC	–	–
RAPD3b	TCTGACCACCG	–	–
RAPD4	CAGGCTTCCC	–	–
RAPD5	CGGCAACGGG	–	–
RAPD6	GACTGCGCAC	–	–
RAPD7	GCGTCAGGAG	–	–
RAPD9	AACAGGGCGG	–	–
RAPD10	GGACGCGGTGC	–	–
RAPD11	GCACGCATTGG	–	–
RAPD12-2	GTTCGTGCGGT	–	–
RAPD13	CCACCTCGCA	–	–
RAPD15	TGTGGAGCCC	–	–
RAPD16	GTTCGTGCGGT	–	–
RAPD17	CGTTTCAATG	–	–
RAPD18	GTTACATCAGTGC	–	–
RAPD19	TCAACAAGGTG	–	–
RAPD20	AAACATTATCTGC	–	–
RAPD21	CAACCAGCTATAG	–	–
RAPD22	CTTAAAAAGTT	–	–
RAPD23	AAGTTTGTTTGC	–	–
RAPD24	CAAAGTTTGTTTGC	–	–
RAPD27	AGTTTAGCTGG	–	–
RAPD28	TTGCTGGGG	–	–
RAPD29	AAGTTGTAGCTG	–	–
RAPD30	CAGCACCAGC	–	–
Multiplex PCR			
RD1_new_f	GCACATACCTATTTTATACTTCCAACCTG	–	629 (<i>F. tularensis</i> subsp. <i>holarctica</i>); 840 (<i>F. tularensis</i> subsp. <i>holarctica</i> bv. <i>japonica</i>); 1325 (<i>F. tularensis</i> subsp. <i>tularensis</i>)
RD1_r	GCCGAGTTTGATGCTGAAAA	1	
RD6_f	TGACCTGAGCCAGAGGTTAC	3	369
RD6_r	ACTTGCCAGCCTAATAATTAC	3	
F_nov_f	GCCATATAAAGGCAAAAGCTCCACTTC	–	1395
F_nov_r	GAGGGGATATGGCAATCATCTAGAGC	–	
F_med_f	GTCTTTGGTGGTCTTACGCCAATG	–	1132
F_med_r	GCTGGAATTACACTCTCTCTTAAATCCC	–	
F_philo_f	CGCACCCAGTCTTTGCGATAG	–	962
F_philo_r	CGCTAAATAATTCTCTTCGTAGCGATTG	–	
F_pisc_f	GCGGTCTATCCTGTTGTCGAGAAG	–	172
F_pisc_r	TTGCTTGAAATATTGGAGAAGAAATGG	–	
F_62_f	GTTGATCGGCGATAGTACTTCATCT	–	492
F_62_r	CCAAAGATCACAAGGCATTACCTAAG	–	
Amplification and sequencing			
<i>recA</i>			
recA_tul_f	CGCTAGAATCAGCCTTATCAC	–	965
recA_tul_r	TAAGCTCATCTTGAGTAACTGC	–	
16S rRNA gene			
27f	GCCCATTGAGGGGGATACC	2	1474
1492r	GGACTAAGAGTACCTTTTGTAGT	2	

Table 1. cont.

Primer	Sequence (5'–3')	Reference*	Amplicon size (bp)
341f	CCTACGGGAGGCAGCAG	4	– (Sequencing primer)
518r	ATTACCGCGGCTGCTGG	4	– (Sequencing primer)
926f	AAACTYAAKGAATTGACGG	2	– (Sequencing primer)

*1, Broekhuijsen *et al.* (2003); 2, Lane (1991); 3, Molins-Schneekloth *et al.* (2007); 4, Muyzer *et al.* (1993).

(0.7 %), anteiso- $C_{17:1\omega9c}$ (0.6 %), $C_{13:1}$ at 12–13 (0.5 %), anteiso- $C_{17:0}$ (0.4 %), $C_{9:0}$ (0.1 %) and an unidentified acid with an equivalent chain-length of 14.969. This fatty acid profile shares the majority of characteristics reported for different *F. tularensis* and *F. novicida* strains (Jantzen *et al.*, 1979; Hollis *et al.*, 1989), including relatively large amounts of $C_{10:0}$, $C_{14:0}$, $C_{16:0}$, $C_{24:1}$ and the long-chain hydroxy acid $C_{18:0}$ 3-OH and presence of significant amounts of $C_{10:0}$ 2-OH, $C_{16:0}$ 3-OH, $C_{20:0}$, $C_{22:0}$ and $C_{24:0}$. A fatty acid profile containing these components is unique among bacteria and, hence, it supports the assignment of FhSp1^T to the genus *Francisella*.

Quinones were extracted and analysed by HPLC as described by Tindall (1990a) and Altenburger *et al.* (1996). The HPLC was equipped as described by Stolz *et al.* (2007). Strain FhSp1^T exhibited a quinone system with the major compound ubiquinone Q-8 (98 %) and traces of Q-9 (2 %). The quinone system supports the affiliation of strain FhSp1^T to the genus *Francisella*; several strains of the genus analysed for this trait also showed a quinone system with the major ubiquinone Q-8 (Hollis *et al.*, 1989). Our analysis of the quinone systems of strain FhSp1^T, *F. tularensis* subsp. *holarctica* strains F76 and ATCC 29684, *F. tularensis* subsp. *mediasiatica* F65, *F. tularensis* subsp. *tularensis* strains ATCC 6223^T and SCHU S4, *F. tularensis* subsp. *novicida* ATCC 15482^T, *F. philomiragia* ATCC 25015^T and *F. piscicida* DSM 18777^T showed similar results. The main quinone was ubiquinone Q-8 (95–100 %), and minor amounts of ubiquinone Q-9 were also detected.

Polar lipids were extracted and analysed by two-dimensional TLC after Tindall (1990b). The polar lipid profile of strain FhSp1^T was almost identical to that of *F. tularensis*

subsp. *tularensis* ATCC 6223^T (Fig. 2a), differing only by the absence of trace amounts of an unidentified aminophospholipid (APL2). The polar lipid profile of *F. tularensis* subsp. *tularensis* ATCC 6223^T was composed of the major lipids phosphatidylethanolamine and diphosphatidylglycerol, moderate amounts of phosphatidylcholine and an unidentified aminophospholipid (APL4) and phospholipid (PL3) and minor to trace amounts of phosphatidylglycerol, unidentified phospholipids (PL1, PL2), an unidentified lipid with highly hydrophobic chromatographic behaviour (L) and unidentified aminophosphoglycolipids (APGL1–2), aminophospholipids (APL1–3) and an aminolipid (AL2). The polar lipid profile of *F. novicida* ATCC 15482^T was indistinguishable from that of *F. tularensis* subsp. *tularensis* ATCC 6223^T (not shown). The most striking difference in the polar lipid profile of *F. philomiragia* ATCC 25015^T was the absence of aminophospholipid APL4 (Fig. 2b). Furthermore, aminophospholipid APL2 was not detectable, but minor amounts of an unidentified glycolipid (GL) and an unidentified aminolipid (AL1) were found. *F. piscicida* DSM 18777^T exhibited a polar lipid profile most similar to that of *F. philomiragia* subsp. *philomiragia* ATCC 25015^T but with trace amounts of APL2 and no detectable APGL2, AL1, AL2 and PL1 (not shown). These polar lipid profiles reflect quite well the close phylogenetic relationship between FhSp1^T and *F. tularensis* subsp. *tularensis* on the one hand and between *F. philomiragia* ATCC 25015^T and *F. piscicida* DSM 18777^T on the other.

Metabolic fingerprinting was done using the Biolog MicroLog system (Gram-negative identification test panel GN2 MicroPlates) according to the instructions of the manufacturer. All strains were first grown on HCA at 37 °C

Fig. 1. Phylogenetic tree reconstruction with *recA* sequences (941–944 bp) using MEGA neighbour-joining analysis. Bar, 0.05 substitutions per nucleotide position. The significance of branches is indicated by bootstrap percentages calculated for 2000 subsets. The *recA* sequence of *Rickettsia bellii* RML 369-C^T was used as an outgroup.

Fig. 2. Total polar lipid profiles of *F. tularensis* subsp. *tularensis* ATCC 6223^T (a) and *F. philomiragia* ATCC 25015^T (b) after two-dimensional TLC and detection with molybdatophosphoric acid. DPG, Diphosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; AL1–2, unknown aminolipids; PL1–3, unknown phospholipids; APL1–4, unknown aminophospholipids; APGL1–2, unknown aminophosphoglycolipids; GL, unknown glycolipid; L, unknown polar lipid.

with 5% CO₂, except for *F. piscicida* DSM 18777^T, which was kept at 20 °C under ambient atmosphere. All strains were tested at least three times in independent test series. MICs of antimicrobial agents were determined by Etest (AB Biodisk; Tec-Laim) on HCA supplemented with 9% chocolate red blood cells (CHAB). From 48 h cultures on CHAB plates, cells of strain FhSp1^T were resuspended in physiological saline to achieve a turbidity equivalent to that of a McFarland standard 0.5 and applied to the agar with the help of a sterile swab before application of Etest strips.

MICs were read after 48 h of incubation at 37 °C in an atmosphere with 5% CO₂. For quality assurance, each batch of plates was tested with the control strains *F. tularensis* subsp. *tularensis* ATCC 6223^T and *F. tularensis* subsp. *holarctica* NCTC 10857^T.

Results of physiological and biochemical tests and susceptibility tests for antimicrobial agents are given in Table 2 or listed in the species description. Strain FhSp1^T differed from *F. novicida* ATCC 15482^T in 13 traits and from the other type strains of recognized *Francisella* species and subspecies in at least 12 traits.

For environmental studies aiming at identifying potential zoonotic reservoirs of *F. tularensis* or related species as well as in epidemiology, rapid differentiation of bacterial strains belonging to the genus *Francisella* is mandatory. Known differences in virulence between the distinct subspecies of *F. tularensis* may be critical for the clinical management of tularemia patients. Moreover, the unusual natural resistance of strains affiliated to the genus *Francisella* against a wide range of antibiotics necessitates the rapid identification of these strains in critically ill people. There are several lines of evidence to show that immunocompromised patients in particular are prone to infections with uncommon *Francisella* strains that are not detected or identified by classical microbiological methods (Kugeler *et al.*, 2008) and which might even occur in regions that were not considered to be endemic for *F. tularensis* or related species (Leelaporn *et al.*, 2008).

PCR-based identification of *Francisella* strains at the genus, species and subspecies levels has been developed by Forsman *et al.* (1994) on the basis of differences in the 16S rRNA gene. Johansson *et al.* (2000) designed primers to differentiate between *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* isolates in a multiplex PCR that, however, needs further analyses in order to distinguish between these subspecies and *F. tularensis* subsp. *mediasiatica* and *F. novicida*. Broekhuijsen *et al.* (2003) developed a PCR based on the region of difference RD1, which was identified by a genome-wide DNA microarray analysis. This PCR allows discrimination between all three subspecies of *F. tularensis*, *F. novicida* and *F. tularensis* subsp. *holarctica* biovar japonica with one primer pair. However, it cannot discriminate between the two genotypes of *F. tularensis* subsp. *tularensis*, *F. philomiragia*, *F. piscicida* and FhSp1^T and, since the characteristic amplicon that identifies *F. novicida* is larger than 3000 bp, the PCR assay is suboptimal. In the course of this study, examinations were carried out in order to develop a comprehensive multiplex-PCR assay that allows convenient and fast discrimination between *Francisella* subspecies and species, including FhSp1^T.

PCR template DNA was prepared by three cycles of freeze-thawing (Wieser & Busse, 2000) from heat-inactivated biomass. In order to find genomic differences suitable for the design of specific primers, comparative genomic fingerprinting was carried out with representatives of each

Table 2. Differential metabolic characteristics of strain FhSp1^T and the type strains of other francisellae

Strains: 1, FhSp1^T; 2, *F. novicida* ATCC 15482^T; 3, *F. philomiragia* ATCC 25015^T; 4, *F. piscicida* DSM 18777^T; 5, *F. tularensis* subsp. *tularensis* ATCC 6223^T; 6, *F. tularensis* subsp. *mediasiatica* GIEM543; 7, *F. tularensis* subsp. *holarctica* GIEM503^T. The tests were carried out with the Biolog GN2 MicroPlate system; tests with *F. piscicida* DSM 18777^T were carried out with 24 h of incubation at 20 °C; other tests were carried out for 24 h at 37 °C. +, Positive; −, negative; (+), weakly positive. All strains were positive for oxidation of D-fructose, L-serine, L-proline, L-glutamic acid, D-mannose, pyruvic acid methyl ester and α-D-glucose. All strains were negative for oxidation of water (control), α-cyclodextrin, Tweens 40 and 80, N-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, i-erythritol, L-fucose, gentiobiose, myo-inositol, lactose, lactulose, D-mannitol, melibiose, methyl β-D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, turanose, xylitol, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, γ-hydroxybutyric acid, p-hydroxyphenylacetic acid, itaconic acid, α-ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, glucuronamide, L-histidine, L-leucine, L-phenylalanine, D-serine, DL-carnitine, urocanic acid, phenylethylamine, putrescine, 2-aminoethanol and 2,3-butanediol. Identification results according to Biolog were *F. philomiragia* for strains 1–4 and *F. tularensis* for strains 5–7.

Test	1	2	3	4	5	6	7
Activity of:							
Catalase	(+)	(+)	+	+	+	+	(+)
Oxidase	+	−	+	−	−	−	−
Oxidation of:							
Dextrin	+	−	−	−	−	−	−
Glycogen	+	−	−	−	−	−	−
N-Acetyl-D-glucosamine	+	+	+	+	+	−	+
Cellobiose	+	+	−	−	−	−	−
D-Galactose	+	+	−	−	−	+	−
Maltose	−	−	+	−	−	−	−
Sucrose	+	+	+	−	−	−	−
Trehalose	+	−	+	−	−	−	−
Succinic acid monomethyl ester	+	+	+	+	−	+	+
Acetic acid	+	−	−	−	−	−	−
α-Hydroxybutyric acid	+	−	+	−	−	−	−
β-Hydroxybutyric acid	+	+	+	−	−	−	−
α-Ketobutyric acid	+	+	+	−	+	+	+
α-Ketoglutaric acid	+	−	−	−	−	−	−
DL-Lactic acid	+	+	+	−	−	−	+
Succinic acid	+	+	−	−	−	−	−
Bromosuccinic acid	+	−	−	−	−	−	−
Succinamic acid	−	−	+	−	−	−	−
L-Alaninamide	+	+	+	+	−	−	−
D-Alanine	+	+	+	−	−	−	−
L-Alanine	+	+	+	+	−	+	+
L-Alanyl glycine	+	+	+	+	−	−	−
L-Asparagine	+	+	+	+	−	−	+
L-Aspartic acid	+	+	+	−	−	−	+
Glycyl L-aspartic acid	+	−	−	−	−	−	−
Glycyl L-glutamic acid	+	+	+	−	−	−	−

Table 2. cont.

Test	1	2	3	4	5	6	7
Hydroxy-L-proline	+	−	−	−	−	−	−
L-Ornithine	+	+	−	−	−	−	−
L-Pyroglutamic acid	+	+	+	−	−	−	−
L-Threonine	+	+	+	−	−	−	+
γ-Aminobutyric acid	+	−	−	−	−	−	−
Inosine	+	+	+	−	−	−	−
Uridine	+	+	+	−	−	−	+
Thymidine	+	+	+	−	−	−	+
Glycerol	+	+	+	−	+	+	−
DL-α-Glycerol phosphate	+	+	+	−	+	+	+
α-D-Glucose 1-phosphate	+	−	+	−	−	−	−
D-Glucose 6-phosphate	+	−	+	−	−	−	−

Francisella taxon (results not shown). Primers used for RAPD-PCR fingerprinting and their sequences are listed in Table 1. Reactions were carried out in a final volume of 25 µl containing 1× Green GoTaq Flexi buffer, 2 mM MgCl₂, 200 µM each dNTP, 0.6 U GoTaq DNA polymerase (Promega), 8 µM RAPD primer and approximately 5 ng DNA. Reactions were performed using an initial 5 min denaturation step at 95 °C followed by 45 cycles of 1 min at 95 °C, 1 min at 32–38 °C and 2 min at 72 °C and a final extension step of 5 min at 72 °C. The resolution of bands was improved by using a ramp rate of 0.3 °C s^{−1}. Amplification products (5 µl each) were separated by 1.5 % (w/v) agarose gel electrophoresis in 1× TAE running buffer at a constant voltage of 110 V for at least 90 min. Fragments were visualized with UV light after staining with ethidium bromide. Bands of interest were excised from the gel and DNA was eluted with the QIAquick gel extraction kit according to the instructions of the manufacturer (Qiagen). PCR products were cloned using the Promega pGEM-T Easy vector system and *Escherichia coli* strain DH10B according to the manufacturer's manual. Plasmid insert sequences (sequencing was done at VBC-genomics, Vienna) were subjected to database searches and compared to genome sequences of *Francisella* strains (accession nos AM233362, CP000803, CP000437, AJ749949, CP000439, AM286280 and CP000608).

Primers used in the multiplex PCR were constructed based on comparative genomic fingerprinting and information from the literature. By means of RAPD-PCR, sequence fragments potentially suitable for the design of specific primers were identified and sequenced for strains of *F. piscicida* (RAPD12 band of 201 bp) and *F. philomiragia* (RAPD6 band of 1259 bp) and FhSp1^T (RAPD13 band of 770 bp) (not shown). For *F. tularensis* subsp. *mediasiatica*, a specific primer pair was designed using a variable region that was detected after comparison of larger sequence regions of the *F. tularensis* subsp. *mediasiatica* genome sequence with other accessible *Francisella* genome sequences from the databases. The region of difference RD1 described by Broekhuijsen *et al.* (2003) provided a

primer pair that resulted in specific products for *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica*, including biovar *japonica* [the reverse primer was taken from Broekhuijsen *et al.* (2003) and the forward primer was redesigned], and was also used to design a primer pair specific for *F. novicida* (for amplicon sizes, see Table 3). The primer pair RD6_f/r (Molins-Schneekloth *et al.*, 2007) allowed differentiation between *F. tularensis* subsp. *tularensis* A.I and A.II by lack of the *F. tularensis* species-specific band of 369 bp in ATCC 6223^T (A.II).

The primer pairs were tested in individual PCRs (results not shown) and then integrated into one multiplex-PCR assay. The 25 µl reaction mixture consisted of 5 µl 5 ×

buffer, 2.5 µl MgCl₂, 0.5 µl dNTPs, 0.2 µl each of primers RD1_new_f, RD1_r, F_nov_f and F_nov_r, 0.1 µl each of primers RD6_f, RD6_r, F_pisc_f, F_pisc_r, F_philo_f, F_philo_r, F_62_f, F_62_r, F_med_f and F_med_r, 0.12 µl Promega GoTaq polymerase and 0.5 µl template DNA. Reactions were performed in an Eppendorf Mastercycler using an initial 5 min denaturation step at 95 °C followed by 35 cycles of 1 min at 95 °C, 1 min at 60 °C and 1 min 30 s at 72 °C and a final extension step of 5 min at 72 °C. The products were separated in a 1.5 % agarose gel as described above, stained with ethidium bromide and visualized with UV light. Applying the developed multiplex PCR, all test strains could be correctly identified (Table 3 and Fig. 3). The assay uses 14 primers

Table 3. *Francisella* strains examined in this study and their multiplex PCR profiles

Strain	Taxon as received	Multiplex PCR profile (bp)
<i>F. tularensis</i> subsp. <i>holarctica</i>		
F124 ^T = GIEM503 ^T	<i>F. tularensis</i> subsp. <i>holarctica</i>	369, 629
F1	<i>F. tularensis</i> subsp. <i>holarctica</i>	369, 629
F4	<i>F. tularensis</i> subsp. <i>holarctica</i> BVII	369, 629
F7	<i>F. tularensis</i> subsp. <i>holarctica</i> BVII	369, 629
F9	<i>F. tularensis</i> subsp. <i>holarctica</i> BVII	369, 629
F11	<i>F. tularensis</i> subsp. <i>holarctica</i> BVII	369, 629
F12	<i>F. tularensis</i> subsp. <i>holarctica</i> BVII	369, 629
F13	<i>F. tularensis</i> subsp. <i>holarctica</i> BVII	369, 629
F17	<i>F. tularensis</i> subsp. <i>holarctica</i> BVII	369, 629
F19	<i>F. tularensis</i> subsp. <i>holarctica</i> BVII	369, 629
F33	<i>F. tularensis</i> subsp. <i>holarctica</i> BVII	369, 629
F57	<i>F. tularensis</i> subsp. <i>holarctica</i>	369, 629
F76	<i>F. tularensis</i> subsp. <i>holarctica</i>	369, 629
F89	<i>F. tularensis</i> subsp. <i>holarctica</i> BVI	369, 629
F88	<i>F. tularensis</i> subsp. <i>holarctica</i> BVI	369, 629
F100	<i>F. tularensis</i> subsp. <i>holarctica</i>	369, 629
F101	<i>F. tularensis</i> subsp. <i>holarctica</i>	369, 629
F49 = ATCC 29684	<i>F. tularensis</i> subsp. <i>holarctica</i> LVS	369, 629
F119 = FSC021	<i>F. tularensis</i> subsp. <i>holarctica</i> bv. <i>japonica</i>	369, 840
F120 = FSC022	<i>F. tularensis</i> subsp. <i>holarctica</i> bv. <i>japonica</i>	369, 840
<i>F. tularensis</i> subsp. <i>tularensis</i>		
F66 ^T = ATCC 6223 ^T = GIEM Schu ^T	<i>F. tularensis</i> subsp. <i>tularensis</i> , avirulent; A.II	1325
FSC237 = SCHU S4	<i>F. tularensis</i> subsp. <i>tularensis</i> ; A.I	369, 1325
<i>F. tularensis</i> subsp. <i>mediasiatica</i>		
F63 ^T = GIEM543 ^T	<i>F. tularensis</i> subsp. <i>mediasiatica</i>	369, 1132
F65	<i>F. tularensis</i> subsp. <i>mediasiatica</i>	369, 1132
<i>F. tularensis</i> subsp. <i>novicida</i>		
F61 ^T = ATCC 15482 ^T	<i>F. tularensis</i> subsp. <i>novicida</i>	369, 1395
F59	<i>F. tularensis</i> subsp. <i>novicida</i>	369, 1395
F60	<i>F. tularensis</i> subsp. <i>novicida</i>	369, 1395
<i>F. hispaniense</i> sp. nov.		
FhSp1 ^T = FnSp1 ^T = F62 ^T = FSC454 ^T = DSM 22475 ^T = CCUG 58020 ^T	<i>F. hispaniense</i>	492
<i>F. philomiragia</i>		
F51 ^T = ATCC 25015 ^T	<i>F. philomiragia</i>	962
F50 = ATCC 25017	<i>F. philomiragia</i>	962
<i>F. piscicida</i>		
F165 ^T = DSM 18777 ^T	<i>F. piscicida</i>	172

Fig. 3. Multiplex PCR results. Lanes: 1 and 12, 100 bp and 1.5 kb markers; 2, *F. piscicida* DSM 18777^T; 3, *F. philomiragia* ATCC 25017; 4, *F. philomiragia* ATCC 25015^T; 5, *F. hispaniensis* sp. nov. FhSp1^T; 6, *F. novicida* ATCC 15482^T; 7, *F. tularensis* subsp. *mediasiatica* GIEM543^T; 8, *F. tularensis* subsp. *tularensis* SCHU S4 (A.I); 9, *F. tularensis* subsp. *tularensis* ATCC 6223^T (A.II); 10, *F. tularensis* subsp. *holarctica* GIEM503^T; 11, *F. tularensis* subsp. *holarctica* bv. *japonica* F119. Aliquots of 5 µl PCR product were loaded onto the 1.5% agarose gel and visualized after staining with ethidium bromide.

and allows the differentiation of all *Francisella* species and subspecies including biovar *japonica* of *F. tularensis* subsp. *holarctica* and biovars A.I and A.II of *F. tularensis* subsp. *tularensis*.

Strain FhSp1^T was shown to be distant from the other established *Francisella* species by sequence analyses of different housekeeping genes (Nübel *et al.*, 2006) as well as in RAPD-PCR and the developed multiplex PCR. Hence, these results indicate that this strain is not a representative of *F. novicida* or of any other recognized *Francisella* species, but a representative of a novel species of the genus *Francisella*.

In order to support the rank of a separate species, DNA reassociation experiments were carried out comparing FhSp1^T with the type strains of two *F. tularensis* subspecies, *F. novicida* ATCC 15482^T, *F. philomiragia* ATCC 25015^T and *F. piscicida* DSM 18777^T. DNA–DNA hybridization experiments were carried out according to Ziemke *et al.* (1998). Nick translation was done with 2 µg DNA for 90 min at 15 °C. High-molecular-mass DNA was extracted and purified according to Pitcher *et al.* (1989). DNA concentrations were estimated chemically according to Richards (1974). The results are means from at least six hybridizations. In hybridization experiments, strain FhSp1^T exhibited the following levels of reassociation: 52% (9.7% SD), reciprocal 54% (4.7% SD), with *F. novicida* ATCC 15482^T; 61% (8.4% SD), reciprocal 35% (3% SD), with *F. tularensis* subsp. *mediasiatica* GIEM543^T; 57% (7.3% SD), reciprocal 38% (7.5% SD) with *F.*

tularensis subsp. *holarctica* GIEM503^T; 39% (7.7% SD), reciprocal 43% (5.9% SD), with *F. philomiragia* ATCC 25015^T; and 47% (3.4% SD), reciprocal 28% (6.2% SD), with *F. piscicida* DSM 18777^T. DNA–DNA hybridization was not carried out successfully with the type strain of *F. tularensis* subsp. *tularensis* because DNA from repeated purifications of differently grown biomass was heavily fragmented. However, hybridizations between all other subspecies of *F. tularensis* and *F. novicida* have shown that the subspecies of *F. tularensis* and *F. novicida* exhibit reassociation values higher than 70%, demonstrating that they belong to a single species (Hollis *et al.*, 1989). Hence, the strains of *F. tularensis* employed in our hybridization experiments are useful for reference and demonstrate unambiguously that FhSp1^T can be considered as a representative of an as-yet undescribed species of *Francisella*.

The data presented here (polar lipids, quinone system, fatty acids) as well as molecular data from Nübel *et al.* (2006), Larsson *et al.* (2007) and Escudero *et al.* (2010) demonstrate that strain FhSp1^T is a member of the genus *Francisella*. Results from DNA–DNA hybridization and unique physiological traits show that FhSp1^T is a representative of a novel *Francisella* species. Therefore, we propose a novel *Francisella* species, *Francisella hispaniensis* sp. nov., to accommodate strain FhSp1^T.

The taxonomic status of *F. novicida* as one of four *Francisella* species has been questioned repeatedly. A range of different molecular data, including results of DNA–DNA hybridizations, has demonstrated a relatedness to *F. tularensis* at the species level. Sjöstedt (2005) discussed the matter in detail and proposed to reclassify *F. novicida* (Larson *et al.* 1955) Olsufiev *et al.* 1959 as '*F. tularensis* subsp. *novicida*'. This new combination is used in several papers; however, this combination has not been validly published by publication in the *International Journal of Systematic and Evolutionary Microbiology* or by inclusion in a Validation List to satisfy Rule 27 of the Bacteriological Code and, hence, has no standing in nomenclature.

Here, we propose to reclassify *F. novicida* as a subspecies of *F. tularensis*, *Francisella tularensis* subsp. *novicida* comb. nov., and therefore give the already widely adopted classification a formal basis. Furthermore, we provide an emended description of the genus *Francisella*.

Emended description of the genus *Francisella* Dorofe'ev 1947

Francisella (Fran.ci.sel'la. N.L. dim. ending -ella; N.L. fem. n. *Francisella* named after Edward Francis, American bacteriologist, who extensively studied the aetiological agent and pathogenesis of tularaemia and is credited with naming the disease).

In addition to the description of the genus provided by Sjöstedt (2005), species of the genus are characterized by a complex polar lipid profile consisting of the major

components diphosphatidylglycerol and phosphatidylethanolamine, moderate to major quantities of phosphatidylcholine and minor amounts of phosphatidylglycerol, three unidentified phospholipids, an aminolipid, two aminophosphoglycolipids and two aminophospholipids. Additional lipids may be present. The quinone system is ubiquinone Q-8. The type species is *Francisella tularensis*.

Description of *Francisella hispaniensis* sp. nov.

Francisella hispaniensis (his.pa.ni.en'sis. L. fem. adj. *hispaniensis* from Hispania, the Latin name for Spain, the country where the type strain was isolated).

Cells are pleomorphic or coccoid rods (approx. $0.5 \times 1.5 \mu\text{m}$). Gram-negative, oxidase-positive and showing weak catalase activity. Good growth occurs on HCA and Columbia blood agar at 20–42 °C with an optimum at 37 °C. Forms distinct, mucoid, pale-white to grey colonies within 24 h with a diameter of approx. 1–2 mm on HCA. Resistant to meropenem, amoxicillin plus clavulanic acid, ampicillin and aztreonam; susceptible to chloramphenicol, tetracycline, doxycycline, gentamicin, tobramycin, streptomycin, nalidixic acid, ciprofloxacin, levofloxacin, erythromycin and rifampicin. Does not produce indole or H_2S . Negative in the Voges–Proskauer test, nitrate reduction and growth on triple-sugar iron agar. Gelatin and aesculin are not hydrolysed. Negative for arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase, urease and β -galactosidase. The quinone system consists of Q-8 and traces of Q-9. Predominant polar lipids are phosphatidylethanolamine and phosphatidylcholine, as well as an unknown *F. tularensis*-specific aminophospholipid (APL4). Other polar lipids are diphosphatidylglycerol, phosphatidylglycerol, unknown phospholipids (PL1–3), an unknown lipid (L) and some unknown aminophosphoglycolipids (APGL1–2), aminophospholipids (APL1–2) and aminolipids (AL2). The fatty acid profile is mainly composed of the major compounds $\text{C}_{10:0}$, $\text{C}_{14:0}$, $\text{C}_{16:0}$ and the hydroxy acid $\text{C}_{18:0}$ 3-OH, as well as minor amounts of $\text{C}_{18:1}\omega 9\text{c}$, $\text{C}_{18:0}$, $\text{C}_{16:0}$ 3-OH and $\text{C}_{10:0}$ 2-OH. Results of biochemical tests are indicated in Table 2.

The type strain, FhSp1^T (=FnSp1^T =FSC454^T =F62^T =DSM 22475^T =CCUG 58020^T), was isolated in Spain in 2003 from blood of a patient suffering severe septicaemia secondary to acute obstructive pyelonephritis (Escudero *et al.*, 2010).

Description of *Francisella tularensis* subsp. *novicida* comb. nov.

Francisella tularensis subsp. *novicida* (no.vi.ci'da. L. adj. *novus* new; L. suff. *-cida* from L. v. *caedo* to cut, kill; N.L. n. *novicida* new killer).

Basonym: *Francisella novicida* (Larson *et al.* 1955) Olsufiev *et al.* 1959.

Other synonym: '*Pasteurella novicida*' Larson *et al.* 1955.

The species shares the characteristics listed in the emended genus description. Further characteristics were summarized by Larson *et al.* (1955) and Sjöstedt (2005). Biochemical characteristics determined with the Biolog GN2 MicroPlate are shown in Table 2.

The type strain, ATCC 15482^T =CCUG 33449^T =CIP 56.12^T, was isolated from a water sample from Ogden Bay, Utah, USA, in 1951 (Larson *et al.*, 1955).

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References

- Altenburger, P., Kämpfer, P., Makristathis, A., Lubitz, W. & Busse, H.-J. (1996). Classification of bacteria isolated from a medieval wall painting. *J Biotechnol* **47**, 39–52.
- Broekhuijsen, M., Larsson, P., Johansson, A., Byström, M., Eriksson, U., Larsson, E., Prior, R. G., Sjöstedt, A., Titball, R. W. & Forsman, M. (2003). Genome-wide DNA microarray analysis of *Francisella tularensis* strains demonstrates extensive genetic conservation within the species but identifies regions that are unique to the highly virulent *F. tularensis* subsp. *tularensis*. *J Clin Microbiol* **41**, 2924–2931.
- Escudero, R., Elia, M., Sáez-Nieto, J. A., Herrera, L., Galán, J. A., Ruiz, M., Menéndez, V., Royo, G. & Anda, P. (2003). Preliminary characterization of a human isolate of *Francisella novicida*. In *Abstracts of the 4th International Conference on Tularemia*, 15–18 September 2003, Bath, UK, p. 30.
- Escudero, R., Toledo, A., Gil, H., Kováčsová, K., Rodríguez-Vargas, M., Jado, I., García-Amil, C., Lobo, B. & Bhide, M. (2008). Molecular method for discrimination between *Francisella tularensis* and *Francisella*-like endosymbionts. *J Clin Microbiol* **46**, 3139–3143.
- Escudero, R., Elia, M., Sáez-Nieto, J. A., Menéndez, V., Toledo, A., Royo, G., Rodríguez-Vargas, M., Whipp, M. J., Gil, H. & other authors (2010). A possible novel *Francisella* genomic species isolated from blood and urine of a patient with severe illness. *Clin Microbiol Infect* **16**, 1026–1030.
- Forsman, M., Sandstrom, G. & Sjöstedt, A. (1994). Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. *Int J Syst Bacteriol* **44**, 38–46.
- Gao, B., Mohan, R. & Gupta, R. S. (2009). Phylogenomics and protein signatures elucidating the evolutionary relationships among the *Gamma*proteobacteria. *Int J Syst Evol Microbiol* **59**, 234–247.
- Hollis, D. G., Weaver, R. E., Steigerwalt, A. G., Wenger, J. D., Moss, C. W. & Brenner, D. J. (1989). *Francisella philomiragia* comb. nov. (formerly *Yersinia philomiragia*) and *Francisella tularensis* biogroup *novicida* (formerly *Francisella novicida*) associated with human disease. *J Clin Microbiol* **27**, 1601–1608.
- Jantzen, E., Berdal, B. P. & Omland, T. (1979). Cellular fatty acid composition of *Francisella tularensis*. *J Clin Microbiol* **10**, 928–930.
- Johansson, A., Ibrahim, A., Göransson, I., Eriksson, U., Gurycova, D., Clarridge, J. E. & Sjöstedt, A. (2000). Evaluation of PCR-based methods for discrimination of *Francisella* species and subspecies and development of a specific PCR that distinguishes the two major subspecies of *Francisella tularensis*. *J Clin Microbiol* **38**, 4180–4185.

- Kämpfer, P. & Kroppenstedt, R. M. (1996). Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* **42**, 989–1005.
- Kugeler, K. J., Mead, P. S., McGowan, K. L., Burnham, J. M., Hogarty, M. D., Ruchelli, E., Pollard, K., Husband, B., Conley, C. & other authors (2008). Isolation and characterization of a novel *Francisella* sp. from human cerebrospinal fluid and blood. *J Clin Microbiol* **46**, 2428–2431.
- Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.
- Larson, C. L., Wicht, W. & Jellison, W. L. (1955). A new organism resembling *P. tularensis* isolated from water. *Public Health Rep* **70**, 253–258.
- Larsson, P., Svensson, K., Karlsson, L., Guala, D., Granberg, M., Forsman, M. & Johansson, A. (2007). Canonical insertion-deletion markers for rapid DNA typing of *Francisella tularensis*. *Emerg Infect Dis* **13**, 1725–1732.
- Lau, K. W. K., Ren, J., Fung, M.-C., Woo, P. C. Y., Yuen, K.-Y., Chan, K. K. M., Qian, P.-Y., Wong, P.-K. & Wu, M. (2007). *Fangia hongkongensis* gen. nov., sp. nov., a novel gammaproteobacterium of the order *Thiotrichales* isolated from coastal seawater of Hong Kong. *Int J Syst Evol Microbiol* **57**, 2665–2669.
- Leelaporn, A., Yongyod, S., Limsrivanichakorn, S., Yungyuen, T. & Kiratisin, P. (2008). *Francisella novicida* bacteremia, Thailand. *Emerg Infect Dis* **14**, 1935–1937.
- McCoy, G. W. & Chapin, C. W. (1912). Further observations on a plague-like disease of rodents with a preliminary note on the causative agent, *Bacterium tularensis*. *J Infect Dis* **10**, 61–72.
- Mikalsen, J., Olsen, A. B., Tengs, T. & Colquhoun, D. J. (2007). *Francisella philomiragia* subsp. *noatunensis* subsp. nov., isolated from farmed Atlantic cod (*Gadus morhua* L.). *Int J Syst Evol Microbiol* **57**, 1960–1965.
- Molins-Schneekloth, C. R., Belisle, J. T. & Petersen, J. M. (2007). Genomic markers for differentiation of *Francisella tularensis* subsp. *tularensis* A.I and A.II. *Appl Environ Microbiol* **74**, 336–341.
- Muyzer, G., de Waal, E. C. & Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**, 695–700.
- Nübel, U., Reissbrodt, R., Weller, A., Grunow, R., Porsch-Özcürümez, M., Tomaso, H., Hofer, E., Splettstoesser, W., Finke, E.-J. & other authors (2006). Population structure of *Francisella tularensis*. *J Bacteriol* **188**, 5319–5324.
- Olsufjev, N. G., Emelyanova, O. S. & Dunaeva, T. N. (1959). Comparative studies of strains of *B. tularensis* in the Old and New World and their taxonomy. *J Hyg Epidemiol Microbiol Immunol* **3**, 138–149.
- Olsufjev, N. G. & Meshcheryakova, I. S. (1983). Subspecific taxonomy of *Francisella tularensis* McCoy and Chapin 1912. *Int J Syst Bacteriol* **33**, 872–874.
- Ottem, K. F., Nylund, A., Karlsbakk, E., Friis-Møller, A., Krossoy, B. & Knappskog, D. (2007). New species in the genus *Francisella* (Gammaproteobacteria; Francisellaceae); *Francisella piscicida* sp. nov. isolated from cod (*Gadus morhua*). *Arch Microbiol* **188**, 547–550.
- Ottem, K. F., Nylund, A., Karlsbakk, E., Friis-Møller, A. & Kamaishi, T. (2009). Elevation of *Francisella philomiragia* subsp. *noatunensis* Mikalsen et al. (2007) to *Francisella noatunensis* comb. nov. [syn. *Francisella piscicida* Ottem et al. (2008) syn. nov.] and characterization of *Francisella noatunensis* subsp. *orientalis* subsp. nov., two important fish pathogens. *J Appl Microbiol* **106**, 1231–1243.
- Pearson, W. R. & Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proc Natl Acad Sci U S A* **85**, 2444–2448.
- Pitcher, D. G., Saunders, N. A. & Owen, R. J. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* **8**, 151–156.
- Richards, G. M. (1974). Modifications of the diphenylamine reaction giving increased sensitivity and simplicity in the estimation of DNA. *Anal Biochem* **57**, 369–376.
- Scholz, H. C., Tomaso, H., Al Dahouk, S., Witte, A., Schlöter, M., Kämpfer, P., Falsen, E. & Neubauer, H. (2006). Genotyping of *Ochrobactrum anthropi* by *recA*-based comparative sequence, PCR-RFLP, and 16S rRNA gene analysis. *FEMS Microbiol Lett* **257**, 7–16.
- Sjöstedt, A. B. (2005). Genus I. *Francisella* Dorof'ev 1947, 176^{AL}. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 2, part B, pp. 200–210. Edited by D. J. Brenner, N. R. Krieg, J. T. Staley & G. M. Garrity. New York: Springer.
- Stolz, A., Busse, H.-J. & Kämpfer, P. (2007). *Pseudomonas knackmussii* sp. nov. *Int J Syst Evol Microbiol* **57**, 572–576.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Tindall, B. J. (1990a). A comparative study of the lipid composition of *Halobacterium saccharovororum* from various sources. *Syst Appl Microbiol* **13**, 128–130.
- Tindall, B. J. (1990b). Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* **66**, 199–202.
- Wieser, M. & Busse, H.-J. (2000). Rapid identification of *Staphylococcus epidermidis*. *Int J Syst Evol Microbiol* **50**, 1087–1093.
- Ziemke, F., Höfle, M. G., Lalucat, J. & Rosselló-Mora, R. (1998). Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179–186.