Francisella guangzhouensis sp. nov., isolated from air-conditioning systems

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Four strains (08HL01032T, 09HG994, 10HP82-6 and 10HL1960) were isolated from water of air-conditioning systems of various cooling towers in Guangzhou city, China. Cells were Gram-stain-negative coccobacilli without flagella, catalase-positive and oxidase-negative, showing no reduction of nitrate, no hydrolysis of urea and no production of H2S. Growth was characteristically enhanced in the presence of L-cysteine, which was consistent with the properties of members of the genus Francisella. The quinone system was composed of ubiquinone Q-8 with minor amounts of Q-9. The polar lipid profile consisted of the predominant lipids phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylcholine, two unidentified phospholipids (PL2, PL3), an unidentified aminophospholipid and an unidentified glycolipid (GL2). The polyamine pattern consisted of the major compounds spermidine, cadaverine and spermine. The major cellular fatty acids were C10:0, C14:0, C16:0, C18:1 ω9c and C18:1 ω7c. A draft whole-genome sequence of the proposed type strain 08HL01032T was generated. Comparative sequence analysis of the complete 16S and 23S rRNA genes confirmed affiliation to the genus Francisella, with 95 % sequence identity to the closest relatives in the database, the type strains of Francisella philomiragia and Francisella noatunensis subsp. orientalis. Full-length deduced amino acid sequences of various housekeeping genes, recA, gyrB, groEL, dnaK, rpoA, rpoB, rpoD, rpoH, fopA and sdhA, exhibited similarities of 67–92 % to strains of other species of the genus Francisella. Strains 08HL01032T, 09HG994, 10HP82-6 and 10HL1960 exhibited highly similar pan-genome PCR profiles. Both the phenotypic and molecular data support the conclusion that the four strains belong to the genus Francisella but exhibit considerable divergence from all recognized Francisella species. Therefore, we propose the name Francisella guangzhouensis sp. nov., with the type strain 08HL01032T (=CCUG 60119T =NCTC 13503T).

Bacteria of the genus Francisella are usually considered as facultatively intracellular, Gram-negative, non-motile, pleomorphic coccobacilli, with a tendently fastidious nature and a common growth-dependency for L-cysteine. Historically, the genus consisted of three recognized species: Francisella tularensis, with the subspecies Francisella tularensis subsp. tularensis, Francisella tularensis subsp. tularensis and Francisella tularensis subsp. mediastica,
which cause the zoonotic disease tularaemia, and two species, *Francisella novicida* and *Francisella philomiragia*, with limited virulence that cause tularaemia-like diseases in immunocompromised patients (Clarridge et al., 1996; Hollis et al., 1989). More recently, *Francisella hispaniensis*, isolated from human blood, was added to the genus, and *F. novicida* was transferred to the species *F. tularensis* as a fourth subspecies, *F. tularensis* subsp. *novicida* (Huber et al., 2010), although this placement is controversial (Johansson et al., 2010; Busse et al., 2010). Genetic evidence showed that several additional species may belong to the genus *Francisella* (Barns et al., 2005), such as environmental *Francisella*-like endosymbionts and *Wolbachia persica* (Niebylski et al., 1997; Noda et al., 1997; Scoles, 2004), as well as some novel strains isolated from human cerebrospinal fluid, blood and urine (Kugeler et al., 2008; Escudero et al., 2010). The most recently proposed species is *Francisella halioticida*, isolated from giant abalone (*Haliotis gigantea*) in Japan (Brevik et al., 2011). Species of the genus *Francisella* can also infect fish in both marine and freshwater environments, and generally induce chronic granulomatous infections in the kidney, spleen, liver and heart. A wide range of fish hosts has been reported, such as three-line grunt (*Parapristipoma trilineatum*), tilapia (*Oreochromis niloticus*), hybrid striped bass (*Morone saxatilis*), Atlantic salmon (*Salmo salar*) and Atlantic cod (*Gadus morhua*) (Hsieh et al., 2006; Ottem et al., 2007a, b, 2009). Two types of fish pathogens phylogenetically close to *F. philomiragia* were characterized by sequencing of several housekeeping genes and determination of phenotypic traits (Mikalsen & Colquhoun, 2009; Ottem et al., 2009), and various species or subspecies have been proposed as *Francisella philomiragia* subsp. *noatunensis* (Mikalsen et al., 2007), later reclassified as *Francisella noatunensis* (Ottem et al., 2009), *Francisella piscicida* (Ottem et al., 2007b), *Francisella noatunensis* subsp. *orientalis* (Ottem et al., 2009) and ‘*Francisella asiatica*’ (Mikalsen & Colquhoun, 2009; although the latter name was effectively published in 2009, this paper has not yet appeared in print and the name ‘*Francisella asiatica*’ is not validly published).

In August 2008, routine investigations to detect *Legionella* from water reservoirs of cooling towers in Guangzhou, China, led to the isolation of a Gram-negative cocco bacillus (strain 08HL01032T), initially suspected of belonging to the genus *Legionella*. However, subsequent phenotypic characterization and sequencing of the 16S rRNA gene placed this strain in the genus *Francisella* as potentially representing a novel species (Qu et al., 2009). Since then, three additional isolates, 09HG994, 10HPB2-6 and 10HL01960, with nearly identical phenotypic and genotypic properties have been isolated from other air-conditioning systems in Guangzhou city. In order to determine the phylogenetic position of these isolates, we performed a set of phenotypic and molecular analyses. Based on our results, we conclude that the four isolates belong to a single novel species within the genus *Francisella*, most closely related to *F. noatunensis* subsp. *orientalis* and *F. philomiragia*, for which the name *Francisella guangzhouensis* sp. nov. is proposed.

For initial cultivation, a total of 200 ml water was concentrated by filtration to a final volume of 10 ml. The concentrated samples were cultured on *Legionella BCYE* agar or BCYE-GVPC (BCYE supplemented with 3 g glycine l\(^{-1}\), 80 000 IU polymyxin B sulfate l\(^{-1}\), 1 mg vancomycin l\(^{-1}\) and 80 mg cycloheximide l\(^{-1}\)). Plates were incubated at 37 °C for 10 days under a 5 % CO\(_2\) atmosphere using a CO\(_2\) incubator (model D63450; Heraeus). Growth under anaerobic conditions was examined using an anaerobic incubation system (Forma 1025 anaerobic chamber; Thermo Scientific). Cell morphology was examined under an Olympus light microscope and a Hitachi H-7650 electron microscope with cells grown on cysteine heart agar supplemented with 9 % heated (chocolate) sheep red blood cells (CHAB) at 37 °C for 24 h under a 5 % CO\(_2\) atmosphere. Growth was examined at 4, 18, 25, 28, 30, 37 and 42 °C for 5 days of cultivation on CHAB medium. Oxidase and catalase activities were tested by using bioMérieux reagents. Other biochemical and enzymatic activities were tested with traditional methods according to the protocols of Kämpfer et al. (1991) and using Rapid ID 32A and API ZYM (bioMérieux).

All four strains grew strictly aerobically on CHAB and on BCYE and chocolate agar (both supplemented with cysteine) after 24–48 h incubation. Delayed or weak growth after 3–7 days was detected on sheep blood agar (SBA), BCYE without cysteine and Thayer–Martin agar. No growth was detected on KIA slant agar or MacConkey agar. Growth was observed at 18–37 °C, with an optimum at 25–28 °C. Cells showed characteristic growth for species of the genus *Francisella* on different agar media. On *Legionella BCYE* agar, colonies were convex, round and grey–blue–white, exhibiting regular and translucent edges, about 1.5–2.5 mm in diameter after 3–5 days of incubation (Fig. S1, available in IJSEM Online). Transparent, dew-drop-like colonies formed on *Haemophilus* chocolate agar, and typical greenish-grey mucoid-opalescent colonies were formed on CHAB under a 5 % CO\(_2\) atmosphere at 37 °C (Fig. S1). Cells were polymorphic, short, coccoid rods surrounded by a thin capsule-like structure, non-flagellated and devoid of endospores (Fig. 1).

Using API ZYM and Rapid ID 32A, all strains showed very similar profiles, resembling the profiles of *F. philomiragia*, *F. philomiragia* subsp. *noatunensis* 2005/50/F292-6C\(^\dagger\), *F. noatunensis* subsp. *orientalis* Ehime-1\(^\dagger\) and *F. halioticida* Shimane-1\(^\dagger\) (Mikalsen et al., 2007; Ottem et al., 2009; Brevik et al., 2011). They were all positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), acid phosphatase, alanine arylamidase and naphthol-AS-BI-glucosidase, a, b-glucosidase, a-glucosidase-6-phosphatase, a-glucosidase, b-glucosidase, a-arabinosidase and...
β-glucuronidase, raffinose fermentation and nitrate reduction. In contrast to \textit{F. philomiragia}, they were negative for leucine arylamidase, β-galactosidase, \textit{N}-acetyl-β-glucosaminidase, proline arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, pyroglutamic acid arylamidase, glycine arylamidase, glutamyl glutamic acid arylamidase and serine arylamidase. Detailed results are provided in the species description. The main differentiating physiological characteristics of the four strains in comparison with other species of the genus \textit{Francisella} are summarized in Table 1.

To obtain the profile of fatty acid methyl esters, cells were harvested after 48 h of incubation on CHAB agar at 37 °C under a 5 % CO₂ atmosphere. Extracts were analysed according to the standard protocol of the Sherlock Microbial Identification System (MIDI version 5.0). \textit{F. philomiragia} ATCC 25015\textsuperscript{T} was used as control under identical conditions. In these analyses, \textit{F. philomiragia} ATCC 25015\textsuperscript{T} was confirmed as \textit{F. philomiragia} with a similarity of 0.842; however, 08HL01032\textsuperscript{T}, 09HG994, 10HP82-6 and 10HL1960 were identified as \textit{F. tularensis} with a similarity index ranging from 0.559 to 0.670. As shown in Table 2, the novel isolates were rich in three even-chain saturated fatty acids, C\textsubscript{10:0}, C\textsubscript{14:0} and C\textsubscript{16:0}, and two long-chain fatty acids, C\textsubscript{18:1ω9c} and C\textsubscript{18:0} 3-OH; this profile was similar but different from those of other member of the genus \textit{Francisella}.

For production of biomass for extraction of quinones, polar lipids and polyamines, cells were grown aerobically at 37 °C in Bacto heart infusion broth (BD) supplemented with 10 g glucose l\textsuperscript{-1} and 1 g l-cysteine l\textsuperscript{-1}. Quinones and polar lipids were extracted and analysed as described previously (Tindall, 1990a, b; Altenburger et al., 1996). Biomass subjected to polar lipid analysis was harvested at the late exponential growth phase as recommended by Busse & Auling (1988). HPLC analyses of the quinone system and polyamine pattern were carried out with the equipment described by Stolz et al. (2007). The quinone system of strain 08HL01032\textsuperscript{T} was composed of ubiquinones Q-8 (98 %) and Q-7 (2 %). Similar quinone systems have also been reported for the subspecies of \textit{F. tularensis}, \textit{F. philomiragia}, \textit{F. piscicida} and \textit{F. hispaniensis} (Huber et al. 2010). The polar lipid profile (Fig. 2) was composed of the predominant lipids phosphatidylethanolamine, diphostatidyglycerol, phosphatidylglycerol, phosphatidycholine, two unidentified phospholipids (PL2, PL3), an unidentified aminophospholipid (AP4) and an unidentified glycolipid (GL2). In addition, minor amounts of two further unidentified glycolipids (GL1, GL3) and two unidentified aminophospholipids (AP1, AP2) were detectable. This polar lipid profile was rather similar to those of \textit{F. tularensis} subsp. tularensis and \textit{F. philomiragia} (Huber et al. 2010). However, the presence of the three glycolipids and the absence of aminoglycolipids and other minor lipids clearly distinguished strain 08HL01032\textsuperscript{T} from the latter two taxa. Additionally, the presence of AP4 has been reported for \textit{F. tularensis} subsp. \textit{tularensis} but not for \textit{F. philomiragia}. The polyamine pattern of strain 08HL01032\textsuperscript{T} consisted of the major compounds spermidine [27.6 μmol (g dry weight)\textsuperscript{-1}], cadaverine [14.5 μmol (g dry weight)\textsuperscript{-1}] and spermine [14.4 μmol (g dry weight)\textsuperscript{-1}]. This polyamine pattern, with large amounts of cadaverine and spermidine, is rare among bacteria, but was also detected in \textit{F. piscicida} (H.-J. Busse, unpublished results). The quinone system, polar lipid profile and polyamine pattern showed a high degree of similarity to those of other representatives of the genus \textit{Francisella}, but the presence of three glycolipids reflected the phylogenetic distance of the novel strains from other representatives of the genus.

Isolates 08HL01032\textsuperscript{T}, 09HG994, 10HP82-6 and 10HL1960 were initially confirmed to belong to the genus \textit{Francisella} by amplifying a 1200 bp fragment of the 16S rRNA gene using the genus-specific primer pair F11/F5 (Forssman et al., 1994). The specific 1200 bp fragment was amplified successfully from all four isolates (not shown). In subsequent analyses, nearly entire 16S rRNA gene sequences (1504 bp) of strains 08HL01032\textsuperscript{T}, 09HG994, 10HP82-6 and 10HL1960 were obtained using the universal bacterial primers 27f (5’-AGAGTTTGATCMGCTGAG-3’) and 1492r (5’-GGYTACCTTGTTACGACTT-3’), corresponding to positions 8–27 and 1512–1492, respectively, of the \textit{Escherichia coli} 16S rRNA gene sequence (Lane, 1991). PCR products were sequenced using an Applied Biosystems ABI 3730XL automatic sequencer. The 16S rRNA gene sequences of all four strains were identical. Comparative sequence analysis (trimmed sequences of 1447 nt) was performed using the
The BLAST searches showed that the four isolates shared the highest similarity of 95.6% with *Francisella* sp. MA067296 (GenBank accession no. EU031810) and *Francisella* sp. PA051188 (EU031811), and 94.7–95.3% similarity with all other species of the genus *Francisella* with validly published names. A 16S rRNA gene sequence-based (1402 nt) phylogenetic reconstruction (maximum-parsimony tree with bootstrap analysis of 1000 replicates) was generated using PAUP* (Swofford, 2002). As shown in Fig. 3(a), the four novel isolates form a well-separated cluster within the genus *Francisella*. This topology was entirely consistent with neighbour-joining and maximum-likelihood-based reconstructions also done in PAUP* (not shown). The long branch of the novel isolates, together with 100% bootstrap support, confirm the conclusion that the four isolates belong to a single separate species.

In order to confirm the conclusion that strains 08HL01032T, 09HG994, 10HP82-6 and 10HL1960 belong to a single novel species, genomic fingerprinting was performed with six strains (08HL01032T, 09HG994, 10HP82-6 and 10HL1960, another proposed strain of *F. guangzhouensis*, 10HP82-2, and a distantly related strain, 10HL1970, as a control) using two random amplification polymorphic DNA (RAPD) PCRs and a repetitive element primed (rep) PCR, the (GTG)5-PCR. All PCRs were performed in a total volume of 15 μl including 75 ng genomic DNA, 1× DreamTaq buffer, 0.2 mM each dNTP, 1.0 μM each primer and 0.8 U DreamTaq DNA polymerase (Fermentas GmbH). RAPD-PCRs were performed with primers A (5′-ATCTGGGCAGC-3′) and B (5′-ATCTGGGCAGC-3′) (Ziemke et al., 1997) and the rep-PCR with primer (GTG)5 (5′-GTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGT
Francisella chaperone protein dnaK, sdhA subunit (gyrB heat-shock protein 60 family chaperone merase sigma factors rpoB 972 nt) and beta subunit (DNA-directed RNA polymerase alpha subunit (rpoA) 4254 nt), the 23S rRNA gene sequence similarity of strains of the genus Francisella was detected with fopA (67 %); the highest value of 92 % was detected with dnaK. Other deduced amino acid sequence similarities were in the range 80–90 %. It should be noted that the calculated similarities for deduced amino acid sequences of strains of F. guangzhouensis sp. nov. are low when compared with the corresponding similarities within the F. tularensis subspecies group and even among the various species of the genus Francisella. For example, the similarity of dnaK within the various F. tularensis subspecies (including the recently transferred subspecies F. tularensis subsp. novicida) is 99 %. The lowest similarity of dnaK (F. tularensis subsp. tularensis SCHU4) is 96 %, to F. noatunensis subsp. orientalis, which is still high compared with F. guangzhouensis sp. nov. (92 %). This relationship was detected with all deduced amino acid sequences investigated and thus confirms the separate position of F. guangzhouensis sp. nov. as a novel species.

From the annotated genome sequence, the following genes (entire coding sequences) were extracted and compared with existing entries in the EMBL nucleotide and protein database: 16S and 23S rRNA genes (1523 and 2889 nt, respectively), recombinase A (recA, 1146 nt), the DNA-directed RNA polymerase alpha subunit (rpoA, 972 nt) and beta subunit (rpoB, 4254 nt), RNA polymerase sigma factors rpoD (1734 nt) and rpoH (882 nt), heat-shock protein 60 family chaperone groEL (1635 nt), chaperone protein dnaK (1929 nt), DNA gyrase subunit B (gyrB, 2406 nt), succinate dehydrogenase flavoprotein subunit sdhA (1794 nt) and outer-membrane protein A (fopA, 1186 nt). Sequences were translated to primary amino acid sequences and subjected to BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis (Thompson et al., 1994).

The 23S rRNA gene sequence similarity of strains of F. guangzhouensis sp. nov. in comparison to all other known strains of species of the genus Francisella was 95 %, confirming the separate position of the novel species. The lowest similarity at the deduced amino acid sequence level in comparison to other species of the genus Francisella was detected with fopA (67 %); the highest value of 92 % was detected with dnaK. Other deduced amino acid sequence similarities were in the range 80–90 %. It should be noted that the calculated similarities for deduced amino acid sequences of strains of F. guangzhouensis sp. nov. are low when compared with the corresponding similarities within the F. tularensis subspecies group and even among the various species of the genus Francisella. For example, the similarity of dnaK within the various F. tularensis subspecies (including the recently transferred subspecies F. tularensis subsp. novicida) is 99 %. The lowest similarity of dnaK (F. tularensis subsp. tularensis SCHU4) is 96 %, to F. noatunensis subsp. orientalis, which is still high compared with F. guangzhouensis sp. nov. (92 %). This relationship was detected with all deduced amino acid sequences investigated and thus confirms the separate position of F. guangzhouensis sp. nov. as a novel species.

In the 16S rRNA gene tree (Fig. 3a), the four novel isolates formed a well-separated cluster with 100 % bootstrap support, forming a basal lineage in the family Francisellaceae. A phylogenetic tree of sequences from whole genomes from members of the genus Francisella was generated using concatenated housekeeping gene sequences [dnaK, gyrB, rpoA, rpoB, groEL, mdh (first 472 nt only), recA, rpoD, rpoH and sdhA] as well as genes encoding the
Gene sequences were downloaded from whole genomes at PATRIC (http://patricbrc.vbi.vt.edu/; Gillespie et al. 2011) and aligned using Sequencher 5.01 (Gene Codes). The total length of the concatenated sequences was 21 633 nt, including gaps, for the alignment of the 20 genomes containing all of these genes. The tree was generated in PAUP* 4.0b using maximum-parsimony and contained 3093 parsimony-informative characters, with the basal position of *F. guangzhouensis* sp. nov. based on the 16S rRNA gene sequence phylogeny. The resulting phylogeny shows the relationship of *F. guangzhouensis* sp. nov. compared with other members of the *Francisellaceae* based on partial sequencing (1402 nt) of the 16S rRNA gene. The tree was reconstructed using maximum-parsimony and rooted with *Legionella pneumophila* Philadelphia 1T. Numbers at branching nodes are percentage bootstrap support from 1000 replicates. Bar, 30 characters. (b) Maximum-parsimony tree reconstructed from concatenated gene sequences (*dnaK, gyrB, rpoA, rpoB, groEL, mdh, recA, rpoD, rpoH, sdhA, 16S rRNA gene* and *23S rRNA gene*) totalling 21 633 nt. Bootstrap values are percentages based on 1000 replicates; values <90% are not shown. Bar, 300 characters.

**Fig. 3.** (a) Phylogenetic tree of strains 08HL01032T, 09HG994, 10HP82-6 and 10HL1960 and other members of the *Francisellaceae* based on partial sequencing (1402 nt) of the 16S rRNA gene. The tree was reconstructed using maximum-parsimony and rooted with *Legionella pneumophila* Philadelphia 1T. Numbers at branching nodes are percentage bootstrap support from 1000 replicates. Bar, 30 characters. (b) Maximum-parsimony tree reconstructed from concatenated gene sequences (*dnaK, gyrB, rpoA, rpoB, groEL, mdh, recA, rpoD, rpoH, sdhA, 16S rRNA gene* and *23S rRNA gene*) totalling 21 633 nt. Bootstrap values are percentages based on 1000 replicates; values <90% are not shown. Bar, 300 characters.

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other members of the genus, with 100 % bootstrap support based on 1000 replicates, unless indicated otherwise (Fig. 3b). Phylogenetic trees generated with neighbour-joining and maximum-likelihood algorithms in PAUP* gave nearly identical topologies and high branch support for all relevant nodes (not shown).

Together, the phenotypic data as well as the molecular analyses support the affiliation of the novel strain 08HL01032T with the genus Francisella within a novel species, which we name Francisella guangzhouensis sp. nov. Strains 09HG994, 10HP82-6 and 10HL1960 are members of this novel species.

**Description of Francisella guangzhouensis sp. nov.**

_Francisella guangzhouensis_ (guang.zhou.en’sis. N.L. fem. adj. _guangzhouensis_ of or pertaining to Guangzhou, the city in Guangdong province, China, where the first strains were isolated).

Gram-negative, non-motile coccobacillus. Does not grow on KIA slant agar or MacConkey agar. No growth on triple-sugar iron agar. Slow or delayed growth is observed on sheep blood agar without cysteine. Good growth occurs within 24–48 h at 37 °C in the presence of 5 % CO₂ on CHAB, BCYEa and chocolate agar when supplemented with L-cysteine, with maximum-likelihood algorithms in PAUP* gave nearly identical topologies and high branch support for all relevant nodes (not shown).

Together, the phenotypic data as well as the molecular analyses support the affiliation of the novel strain 08HL01032T with the genus Francisella within a novel species, which we name Francisella guangzhouensis sp. nov. Strains 09HG994, 10HP82-6 and 10HL1960 are members of this novel species.

_Francisella guangzhouensis_ (guang.zhou.en’sis. N.L. fem. adj. _guangzhouensis_ of or pertaining to Guangzhou, the city in Guangdong province, China, where the first strains were isolated).

Gram-negative, non-motile coccobacillus. Does not grow on KIA slant agar or MacConkey agar. No growth on triple-sugar iron agar. Slow or delayed growth is observed on sheep blood agar without cysteine. Good growth occurs within 24–48 h at 37 °C in the presence of 5 % CO₂ on CHAB, BCYEa and chocolate agar when supplemented with L-cysteine, with single colonies approximately 2 mm in diameter. Catalase-positive and oxidase-negative. No reduction of nitrate, hydrolysis of urea or production of H₂S from cysteine-supplemented media. Gelatin and aesculin are not hydrolysed. Variable Voges–Prokauer reaction (strains 08HL01032T, 10HP82-6 and 10HL1960 positive, strain 09HG994 negative). Positive for alkaline phosphatase, arginine arylamidase, tyrosine arylamidase, esterase (C4), esterase lipase (C8), acid phosphatase and naphthol-AS-BI-phosphohydrolase. Negative for indole production, leucine arylamidase, β-galactosidase, glutamic acid decarboxylase, histidine arylamidase, N-acetyl-β-glucosaminidase, proline arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, pyrog glutamic acid arylamidase, glycine arylamidase, glutamyl glutamic acid arylamidase and serine ary lamidase. Ferments D-glucose, sucrose, malto se, D-mannose and lactose; no fermentation of D-mannitol, dulcitol, salicin, adonitol, myo-inositol, D-sorbitol, L-arginine, raffinose, L-rhamnose, D-xylose, trehalose, cellobiose, erythritol, melibiose or D-arabitol. Only a few compounds (D-glucose, sucrose, malto se and D-mannose) can be used as sole sources of carbon. The following compounds are not utilized as sole sources of carbon: N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, L-arginine, L-lysine, cellobiose, D-fructose, D-galactose, gluconate, glycerol, D-mannitol, maltool, melibiose, L-rhamnose, D-ribose, sucrose, salicin, trehalose, D-xylose, adonitol, myo-inositol, D-sorbitol, putrescine, acetate, propionate, cis- and trans-aconitate, 4-aminobutyrate, adipate, azelate, fumarate, glutarate, DL-3-hydroxybutyrate, itaconate, DL-lactate, 2-oxoglutarate, pyruvate, suberate, citrate, mesaconate, L-alanine, β-alanine, L-ornithine, L-phenylalanine, L-serine, L-aspartate, L-histidine, L-leucine, L-proline, L-tryptophan, 3-hydroxybenzoate, 4-hydroxybenzoate and phenylacetate. The chromogenic substrates p-nitrophenyl (pNP) β-D-glucopyranoside, pNP β-D-galactopyranoside, bis-pNP phosphate and L-alanine p-nitroanilide (pNA) are hydrolysed, but pNP β-D-gluco pyranoside, pNP β-D-xylpyranoside, pNP β-D-fructuronide, bis-pNP phenylphosphonate, bis-pNP phosphorycholine, 2-deoxythymidine-2′-pNP phosphate, γ-L-glutamate pNA and L-proline pNA are not. The quinone system contains the major compound Q-8, with minor amounts of Q-9. The polar lipid profile consists of the predominant lipids phosphatidylethanolamine, diphosphatidylglycerol, phosphatidyglycerol, phosphatidylcholine, two unidentified phospholipids (PL2, PL3), an unidentified aminophospholipid (APL4) and an unidentified glycolipid (GL2). Minor amounts of two additional unidentified glycolipids (GL1, GL3) and two unidentified aminophospholipids (APL2, APL3) are also present. The polyamine pattern is composed of spermidine, cadaverine and spermine (2 : 1 : 1).

The type strain is 08HL01032T (=CCUG 60119T=NCTC 13503T), isolated from water of a cooling tower in Guangzhou city, China. The DNA G+C content of the type strain is 32.5 mol%.
References


