

Characterization of *Francisella* sp., GM2212, the first *Francisella* isolate from marine fish, Atlantic cod (*Gadus morhua*)

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Abstract A *Francisella* sp., isolate GM2212^T, previously isolated from diseased farmed Atlantic cod *Gadus morhua* in Norway is characterized. The complete 16S rDNA, 16S–23S intergenic spacer, 23S rDNA, 23S–5S intergenic spacer, 5S rDNA, FopA, lipoprotein TUL4 (LpnA), malate dehydrogenase and a hypothetical lipoprotein (LpnB) is sequenced and compared with *Francisella tularensis* and *Francisella philomiragia*. All these sequences support a close relationship between GM2212^T and *F. philomiragia*. The bacterium grows at 10–25°C with an optimum at about 20°C, a temperature range clearly different from *F. tularensis* and *F. philomiragia*. GM2212^T is catalase-positive, indole positive, oxidase-negative, do not produce H₂S in Triple Sugar Iron agar, and does not hydrolyze gelatin, is resistant to erythromycin and susceptible to ceftazidime, the latter five characteristics separating it from *F. philomiragia*. Cysteine enhances growth. Acid is produced from D-glucose, maltose, sucrose (weak) but not from lactose or glycerol. GM2212^T grows on both MacConkey agar and in nutrient broth (6% NaCl). The bacterium is resistant

to trimethoprim-sulfamethoxazole, penicillins, cefuroxime and erythromycin; but is susceptible to ceftazidime, tetracycline, gentamicin, ciprofloxacin. Based on the molecular and phenotypical characteristics, we suggest that this GM2212 isolate, may represent a new species of *Francisella*. Isolate GM2212^T (=CNCM I-3481^T = CNCM I-3511^T = DSM 18777^T).

Keywords *Francisella* sp. · GM2212 · rRNA genes · FopA · Lipoprotein TUL4 · LpnA · LpnB · Malate dehydrogenase · Antibiotics · Fatty acids

Introduction

The family Francisellaceae contains one genus, *Francisella*, and two recognized species, *F. tularensis* and *F. philomiragia* (Sjøstedt 2005). These have been shown to be widespread in some terrestrial and aquatic environments (Forsman et al. 1994; Anda et al. 2001; Barns et al. 2005; Petersen and Schriefer 2005; Sjøstedt 2005). However, based on similarities in the 16S rRNA gene sequence, several other potential new species of *Francisella* from ticks (Suitor and Weiss 1961; Niebylski et al. 1997; Noda et al. 1997; Scoles 2004), environmental samples (Barns et al. 2005) and fish (Kamaishi et al. 2005; Nylund et al. 2006; Olsen et al. 2006) have been reported. From diseased farmed Atlantic cod (*Gadus morhua*) in the county of Rogaland, western Norway, a *Francisella* sp. was isolated in 2004 (Nylund et al. 2006). Challenge experiments showed that this *Francisella* sp. was the cause for the new disease. The 16S rRNA gene sequence from the bacterium showed highest similarity to *Francisella* strains isolated from other fish species and to *F. philomiragia* strains, and based on

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SSU sequence data the *Francisella* sp. from cod was considered a likely new species (Nylund et al. 2006).

The objectives of this study were to compare the characteristics and a selection of genes from this *Francisella* sp. from cod (Nylund et al. 2006) with that of the recognized members of genus *Francisella*; *F. philomiragia* and *F. tularensis*. Based on these comparisons it is proposed that the *Francisella* isolate from cod could represent a new species within genus *Francisella*.

Materials and methods

In 2004 a *Francisella* sp. (GM2212^T) was isolated from the kidney of farmed Atlantic cod (*G. morhua*) in the county Rogaland, western Norway. GM2212^T was first isolated on blood agar-plates containing 0.1% cysteine and 1% D-glucose (BCG agar-plates) incubated at 15°C for 30 days (Nylund et al. 2006). The isolate has been deposited in the Collection Nationale de Cultures de Microorganismes, Institute Pasteur, Paris, France (Identification reference: *Francisella* n.sp. GM2212^T, Registration number: CNCM I-3481 and I-3511) and has been sent to the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (Identification reference: *Francisella* sp., GM2212^T, Registration number: DSM 18777).

Culture media and growth conditions

Two different liquid media have been successfully used for culturing of GM2212^T: (a) Bacto™ Eugon Broth (Becton, Dickinson and Company) was made following the manufacturers recommendation, and added FeCl₃·6H₂O (Merck™) to a final concentration of 2 mM as described by Kamaishi et al. (2005). (b) Growth medium B1817 consists of 900 ml of Marine Broth 2216 (Difco), added 100 ml of Fetal Calf Serum (Gibco/BRL™), 60 ml of Yeastolate Ultrafiltrate (Gibco/BRL), 40 ml L-cysteine·HCl (Merck) sol 6.3 g/l dH₂O (distilled water, filter sterilized) and 40 ml D-glucose (Merck) sol 200 g/l dH₂O. Medium B1817 were also made as agar plates where 900 ml Marine Broth 2216 was added 15 g Agar-agar (Mikrobiologie) and boiled until the agar was dissolved. The mixture was cooled to 60°C and added the same amount of Fetal Calf Serum, Yeastolate Ultrafiltrate, L-cysteine and D-glucose as mentioned above. Portions of 25 ml were dispensed into sterile petridishes.

Tubes with 10 ml aliquots of the liquid medium Bacto Eugon Broth or B1817 were inoculated with GM2212^T, from BCG agar-plates. The tubes were incubated in Infors Unitron incubators at 10, 15, 20, 25, 29

and 37°C and with 250 rpm shaking for testing of optimal growth temperatures. Growth was monitored using optical density measurements at 550 nm (OD₅₅₀). After the optimal growth temperature was determined GM2212^T were grown to the mid-logarithmic phase (OD₅₅₀ = 0.6), pelleted at 2,500×g for 30 min, before the growth media was removed and the bacteria eluted in sterile filtered sea-water. The eluted bacteria were progressively frozen in liquid nitrogen in 500 µl aliquots and stored at –80°C.

If not otherwise stated, all inoculations with GM2212^T were performed with the isolate grown in B1817 to the mid-logarithmic phase, or with the isolate grown on B1817 agar-plates. All liquid media were incubated at 20°C with shaking at 250 rpm in an Infors Unitron incubator and the agar-plates and agar-tubes were incubated at 20°C in a Memmert GTR0214 incubator.

GM2212^T was also grown on Trypton Soy Agar (TSA)- and Brain Heart Infusion Agar (BHIA)-plates without any supplement of L-cysteine. These were made following the manufacturers recommendation (Difco). Plates were inoculated with GM2212^T grown in liquid medium or on agar-plates, incubation as mentioned above.

Growth on MacConkey (MAC) agar was also tested. MAC agar-plates were prepared following the manufacturers recommendation (Difco), inoculated and incubated as mentioned above.

To test if CO₂ enhanced the growth of GM2212^T, B1817, chocolate and MAC agar-plates were incubated in an ASSAB Medicine CO₂-incubator in an atmosphere supplemented with 5% CO₂.

Growth was also tested in nutrient broth both with and without 6% NaCl. Nutrient broth was prepared following the manufacturers recommendation (Difco). Nutrient broth with 6% NaCl was added 60 g/l of NaCl. Tubes containing 10 ml of nutrient broth were inoculated and incubated as mentioned above.

Oxygen requirements of GM2212^T, was tested in thioglycollate medium. This medium was prepared following the manufacturers recommendation (Difco) and 5 ml aliquots were made in glass tubes. Tubes were stab inoculated with GM2212^T and incubated as mentioned above.

Phenotyping

The size and morphology was described by Nylund et al. (2006). Gram staining and studies on motility was performed on the bacterium grown on B1817 agar-plates. The Gram-staining procedure was performed using the crystal-violet, iodine, ethanol, safranin

method. Motility tests were performed using wet mounts. The same procedures were also performed with *F. philomiragia* (DSM 7535, ATCC 25015^T).

Agglutination tests with two polyclonal rabbit antisera were performed: (a) antiserum against *F. tularensis* (Becton, Dickinson and Company) and, (b) rabbit antiserum against GM2212^T. The suspension used in immunization of the rabbits consisted of formaline-inactivated bacteria. These bacteria had been grown on BCG agar-plates, suspended in PBS to a final concentration of 5.0×10^9 bacteria/ml, and inactivated by addition of formalin to a final concentration of 0.5%. Undiluted anti *F. tularensis* rabbit antiserum 50 μ l was placed on a micro-slide and added 50 μ l GM2212^T (grown in B1817). The solution was mixed well and the slide was gently agitated for 5–10 min before investigated under a microscope. The same procedure was also performed with antiserum against GM2212^T. Antisera against both *F. tularensis* and GM2212^T were also tested in an agglutination-test on the type strain of *F. philomiragia* (DSM 7535, ATCC 25015^T) and on ethanol-inactivated *F. philomiragia* (strain 1951) and GM2212^T.

Biochemical characterization

Bergey's Manual give the characteristics for members of the genus *Francisella*, its species and subspecies (Sjøstedt 2005). Tests to determine these characteristics were performed with GM2212^T, along side with the type strain of *F. philomiragia* (DSM 7535, ATCC 25015^T). All inoculations with *F. philomiragia* (DSM 7535, ATCC 25015^T) were performed with the bacterium grown in B1817 or on B1817 agar-plates as described for GM2212^T.

The presence of the enzymes catalase and oxidase were tested on GM2212^T and *F. philomiragia* (DSM 7535, ATCC 25015^T) grown on B1817 agar-plates using a 3% H₂O₂ solution and an oxidase-reagent (bio-Mérieux[®]sa), respectively. Indole tests were performed on the bacteria grown in TSB or on TSA agar-plates using Kovacs test reagent (Merck).

Tests to determine the nitrate reduction, urease activity and gelatin hydrolysis were performed as described by Smibert and Krieg (1994) with some modifications. In the gelatin hydrolysis test, B1817 was used as the growth medium. H₂S production was tested on Triple Sugar Iron (TSI) agar (Difco) and in B1817 using lead acetate paper strips (Fluka). Acidification of carbohydrates was tested in B1817 without D-glucose. The different substrates were added to a final concentration of 1%. Each tube was added 50 μ l of a pH indicator, Bromocresol-purple [0.2% Bromocresol-purple

(Sigma) in 50% ethanol]. Tubes were inoculated and incubated as mentioned above together with control tubes without any substrate.

The antibiotic susceptibility of GM2212^T and *F. philomiragia* (DSM 7535, ATCC 25015^T) was tested on B1817 agar-plates. Plates were inoculated with 200 μ l of bacteria and incubated at 20°C. Tablets of trimethoprim-sulfamethoxazole (23.75–1.25 μ g), penicillin (10 μ g), ampicillin (10 μ g), cefuroxime (30 μ g), ceftazidime (30 μ g), erythromycin (15 μ g), gentamicin (10 μ g), ciprofloxacin (5 μ g) and tetracycline (30 μ g) was added to the inoculated B1817-plates. The plates were incubated as mentioned above. Growth in liquid B1817 added ampicillin and oxolinic acid was also tested. Tubes with 10 ml B1817 containing 100 μ g/ml ampicillin or 15 μ g/ml oxolinic acid were inoculated with the bacteria and incubated as mentioned above.

For fatty acid analysis, 500 μ l of GM2212^T were inoculated in 80 ml B1817 and incubated at 20°C with 250 rpm for 7 days. After the incubation period the bacterial cultures were harvested at $2,500 \times g$ for 50 min followed by removal of the growth media. The bacterial pellets were weighted and stored at -80°C prior to fatty acid analysis at NIFES (National Institute of Nutrition and Seafood Research). The fatty acids were analyzed using a gas liquid chromatography flame ionization detector as described by Lie and Lambertsen (1991). No hydroxy fatty acids were determined using this procedure.

DNA extraction and gene sequencing

DNA from GM2212^T and *F. philomiragia* (DSM 7535, ATCC 25015^T and strain 1951) was extracted as described by Nylund et al. (2006). The following genes and spacers in GM2212^T, and both of the *F. philomiragia* strain genomes were sequenced: 16S, 16S–23S intergenic spacer, 23S rRNA, 23S–5S intergenic spacer, 5S, FopA, lipoprotein TUL4 (LpnA), malate dehydrogenase (mdh) and a hypothetical lipoprotein (LpnB). The PCR reaction mixture was as described by Nylund et al. (2006). Primers used to amplify the rRNA and FopA genes from the *F. philomiragia* strains are the same as previously described by Nylund et al. (2006). New primers used to amplify additional protein genes are presented in Table 1. Amplification was performed in a GeneAmp PCR system 9700 [Applied Biosystems (ABI)] at 95°C for 5 min; 40 cycles of 94°C for 30 s, X°C (50°C for the protein primers, and 55°C for the rRNA primers) for 45 s, 72°C for 1 min and 30 s followed by extension at 72°C for 10 min and a short storage at 4°C.

PCR products were purified using QIAquick PCR purification kit (Qiagen) as described by the manufac-

Table 1 Overview of primers used in amplification and sequencing of the 17-kDa lipoprotein TUL4 (LpnA), hypothetical lipoprotein (LpnB), malate dehydrogenase (mdh) and outer membrane protein (FopA) from GM2212^T and *F. philomiragia* (DSM 7535, ATCC25015^T, strain 1951)

Primer	Sequence	Location
17 kDa lipoprotein gene TUL4 (LpnA)		
Lipo-F3	5'-AGT CTT TTA TCT TTG TCA ATC GCA G	22–47
Lipo-F4	5'-TCT TGC TAG TTG TTC TAC AGT	48–69
Lipo-R3	5'-GGA GCT TGC CAT TGT AAT CT	267–247
Lipo-R4	5'-CAC TTC CTT GAG GAT TAT TA	245–225
Lipo-R5	5'-AKT KAT TGA ATC AGA AGC RAT TAC	436–412
Hypothetical lipoprotein gene (LpnB)		
Lipo-F2	5'-GTT AAT CCT GAT GAT AAC YTT G	121–143
Lipo-R1	5'-GCT GTC CAT GTC CCA TTA CA	426–406
Malate dehydrogenase gene (mdh)		
mdh-F1	5'-GCT TRT TGG TGC TGG TAA TA	21–41
mdh-R1	5'-RCT TTC WGC CAT TTG RAT WC	736–716
Outer membrane protein gene (FopA)		
FMP-F1	5'-GCT TCA ATG TAC TAA GGT TTG CCC	Outside
FMP-F2	5'-CAA GAT AGA ACT GGY CAG TGG	202–223
FMP-R4	5'-ATA KAT MTC AAA YTC GCT WCC AG	985–962

The location of the primers is given in relation to the open reading frame of the protein encoding genes from *F. philomiragia* (DSM 7535, ATCC 25015^T)

turer. Sequencing was then performed with ABI PRISM BigDye terminator chemistry (version 2) according to ABI. All sequences were assembled using the Vector NTI Suite 7.0 program (InforMax Inc.). The different genes were identified by BLASTn search. Sequences obtained in the present study were submitted to the GenBank.

Phylogenetic analysis

The Vector NTI Suite software package was used for the multiple alignments of nucleotide sequences. To perform pair wise comparisons between the different gene-sequences the multiple sequence alignment editor GeneDoc was used (Nicholas et al. 1997). Sequences of *F. tularensis* subsp. *tularensis* (accession no.: NC_006570) already available on the EMBL nucleotide database were included in the comparisons.

Results

Culture media and growth conditions

Growth of GM2212^T, in the two liquid media Eugon Broth and B1817 was tested at 10, 15, 20, 25, 29 and 37°C. Growth was observed in the temperature range 10–25°C in both liquid media. The growth was very slow at 10°C and even less evident at 25°C, while no growth was observed at 29 and 37°C. The best growth was obtained at 20°C.

The bacterium grew best in B1817 medium and the mid-logarithmic phase was reached within 2–3 days. Growth in Eugon Broth was slower and the mid-logarithmic phase was first reached 4–5 days after inoculation, consequently, B1817 was used in the further growth-studies of GM2212^T, at 20°C.

When inoculated onto B1817, and chocolate agar-plates (20°C) colonies were produced that were distinct, punctiform, convex, slightly transparent to grayish-white after 2–3 days. Colonies grew in size to 1–2 mm within 4–5 days. Growth of the isolate on TSA- and BHIA-plates was also observed, but was very slow compared to growth on media supplemented with L-cysteine. The colonies described above were not observed, instead, a very thin transparent layer was evident at the rim of the inoculated area after 7–10 days.

No difference in growth on B1817 or chocolate agar-plates could be observed between those incubated in an atmosphere supplemented with 5% CO₂ and those incubated in normal atmosphere. Growth on MAC agar, however, was evident after 14 days (colorless colonies <1 mm) only in an atmosphere supplemented with 5% CO₂, while incubation without CO₂ did not support growth of GM2212^T on MAC agar.

Growth was evident on top of and beneath the uppermost layer in the thioglycollate medium. Weak growth was evident after 7–14 days of incubation in nutrient broth added 6% NaCl, while no growth was evident in nutrient broth without NaCl.

Phenotypic and biochemical characteristics

Cells were Gram-negative and non-motile. Antiserum against *F. tularensis* subsp. *tularensis* did not agglutinate GM2212^T, in contrast to antiserum against GM2212^T. *F. philomiragia* (DSM 7535, ATCC 25015^T and strain 1951) was slightly agglutinated by the GM2212^T antiserum, but not by the *F. tularensis* subsp. *tularensis* antiserum (Table 2).

H₂S production was detected by the lead acetate paper strips in GM2212^T cultures supplemented with L-cysteine grown for 7–14 days (B1817), but not on TSI-agar. H₂S production was detected by both methods after 5–7 days in cultures with the type strain of *F. philomiragia* (DSM 7535, ATCC 25015^T).

GM2212^T was resistant to trimethoprim-sulfamethoxazole, penicillin, ampicillin, cefuroxime, erythromycin and susceptible to ceftazidime, tetracycline, gentamicin and ciprofloxacin. Reduced growth in

B1817 tubes containing oxolinic acid was observed, while growth in B1817 tubes with ampicillin was similar to the growth in tubes without any supplement of antibiotics. *F. philomiragia* (DSM 7535, ATCC 25015^T) was resistant to trimethoprim-sulfamethoxazole, penicillin, ampicillin, cefuroxime, ceftazidime and susceptible to erythromycin, tetracycline, gentamicin and ciprofloxacin. Growth of *F. philomiragia* in B1817 tubes containing ampicillin was similar to the growth in tubes without any supplement of antibiotics, while growth in B1817 with oxolinic acid was reduced.

An overview of the relative fatty acid composition (except the hydroxy fatty acids) of GM2212^T is given in Table 3. Of the total fatty acids examined in GM2212^T 52.6% was completely identified and 47.4% partly determined. The identified fatty acids were predominantly made up of long-chain saturated and mono-saturated acids ranging from C₁₈ to C₂₄, and the saturated even-chain acids C_{14:0} and C_{16:0}. The partly determined

Table 2 Differential physiological and biochemical characteristics at 20°C of strains 1; *Francisella* sp., GM2212^T and 2; *F. philomiragia* (DSM 7535, ATCC 25015^T)

Characteristic	1	2	3	4	5	6
Size	<1.5 µm	<1.5 µm	<0.5 µm	<0.5 µm	<0.5 µm	<1.5 µm
Gram stain	+	+	w+	w+	w+	w+
Cysteine required for growth	– ^a	–	+	+	+	–
H ₂ S production in cysteine-supplemented medium	w+ (7–14 days)	+	+	+	+	+
β-Lactamase	+	+	+	+	–	+
Acid production from:						
Maltose	+	+	+	+	–	w
Lactose	–	–	–	–	–	–
Sucrose	w+	+	–	–	–	+
D-Glucose	+	+	+	+	–	+
Glycerol	–	–	+	–	+	w
Agglutination of <i>F. tularensis</i> antiserum	–	–	+	+	+	w
Agglutination of strain GM2212 ^T antiserum	+	– ^b	– ^c	– ^c	– ^c	– ^c
Presence of <i>F. tularensis</i> 17-kDa lipoprotein	+	+	+	+	+	+
Aerobic, microaerophilic	+	+	+	+	+	+
Growth in nutrient broth 0% NaCl	–	–	–	–	–	–
Growth in nutrient broth 6% NaCl	w+	+	–	–	–	w
Catalase	+	+	w	w	w	w
Oxidase	–	+	–	–	–	–
Indole	+	+	–	–	–	–
Urease	–	–	–	–	–	–
Nitrate reduction	–	–	–	–	–	–
H ₂ S slant, TSI	–	+	–	–	–	–
Gelatin hydrolysis	–	+	–	–	–	–
Motility	–	–	–	–	–	–

Literature data from Sjøstedt (2005), strains: 3, *F. tularensis* subsp. *tularensis*; 4, *F. tularensis* subsp. *holarctica*; 5, *F. tularensis* subsp. *mediasiatica*; 6, *F. tularensis* subsp. *Novicida*

+ stands for positive

– stands for negative

w stands for weak

^a Grows very slowly on TSA- and BHIA-agars without added cysteine

^b Negative or slight agglutination of *F. philomiragia* (DSM 7535, ATCC 25015^T and strain 1951)

^c Not tested, since these are literature data

Table 3 Relative fatty acid composition of *Francisella* sp., GM2212^T

Fatty acid	Abbreviation	<i>Francisella</i> sp., GM2212 ^T
Unidentified	<C ₁₄	17.8
Unidentified	<C ₁₄	1.3
Tetradecanoic acid	14:0	10.3
Pentadecanoic acid	15:0	0.1
Hexadecanoic acid	16:0	4.0
9-Hexadecenoic acid	16:1 _n - 7	1.0
Heptadecanoic acid	17:0	0.1
Octadecanoic acid	18:0	4.5
9-Octadecenoic acid	18:1 _n - 9	9.9
11-Octadecenoic acid	18:1 _n - 7	0.1
9,12-Octadecadienoic acid	18:2 _n - 6	0.1
Eicosanoic acid	20:0	3.4
9-Eicosenoic acid	20:1 _n - 11	2.4
Docosanoic acid	22:0	4.9
5,8,11, 14-Eicosatetraenoic acid	20:4 _n - 6	0.1
11-Docosanoic acid	22:1 _n - 11	4.7
Tetracosanoic acid	24:0	6.0
15-Tetracosanoic acid	24:1 _n - 9	0.5
7,10,13,16, 19-Docosapentaenoic acid	22:5 _n - 3	0.1
4,7,10,13,16, 19-Docosahexaenoic acid	22:6 _n - 3	0.2
Unidentified	>C ₂₀	22.7
Unidentified	>C ₂₂	1.1
Unidentified	>C ₂₂	4.5
SUM		100

The amount of each fatty acid is shown as a percentage of the total content of fatty acids

fatty acids were seen in the gas chromatographic profile as five distinct peaks, of which peaks 1 and 2 represented saturated fatty acids shorter than C₁₄ in length (probably C_{10:0} and C_{12:0}). Peaks 3–5 were observed late in the chromatogram and represented fatty acids longer than C₂₀ in length.

Table 4 Nucleotide similarities in the rRNA- and protein-genes between *Francisella* sp., GM2212^T, *F. philomiragia* (DSM 7535, ATCC 25015^T), *F. philomiragia* (strain 1951) and *F. tularensis* (SCHU4)

	16S rRNA	16S–23S rRNA spacer	23S rRNA	FopA	Malate dehydrogenase	TUL4 (LpnA)	Lipoprotein (LpnB)
<i>F. philomiragia</i> (DSM 7535, ATCC 25015 ^T)	99.1/1,498nt	98/328nt	99.3/2,862nt	98.5/1,098nt	96/653nt	98/420nt	89/272nt
<i>F. philomiragia</i> (strain 1951)	99.1/1,498nt	98/328nt	99.3/2,862nt	98.5/955nt	97/653nt	97/373nt	89/272nt
<i>F. tularensis</i> (SCHU4)	97.5/1,498nt	91/328nt	97.8/2,862nt	81.9/955nt	83/653nt	70/382nt	78/272nt
<i>Francisella</i> sp., GM2212 ^T	1,498nt	328nt	2,862nt	955nt	653nt	383nt	272nt

Similarities are shown in percent and in number of nucleotide (nt). rRNA genes [*Francisella* sp., GM2212^T, accession no.: DQ309246, *F. philomiragia* (DSM 7535, ATCC 25015^T) accession no.: EF153479, *F. philomiragia* (strain 1951) accession no.: DQ813266], FopA [*Francisella* sp., GM2212^T, accession no.: DQ333226, *F. philomiragia* (DSM 7535, ATCC 25015^T) accession no.: EF153476, *F. philomiragia* (strain 1951) accession no.: DQ825766], mdh [*Francisella* sp., GM2212^T, accession no.: DQ825768, *F. philomiragia* (DSM 7535, ATCC 25015^T) accession no.: EF153478, *F. philomiragia* (strain 1951) accession no.: DQ813265], TUL4 [*Francisella* sp., GM2212^T, accession no.: DQ825765, *F. philomiragia* (DSM 7535, ATCC 25015^T) accession no.: EF153475, *F. philomiragia* (strain 1951) accession no.: DQ813267], LpnB [*Francisella* sp., GM2212^T, accession no.: DQ825767, *F. philomiragia* (DSM 7535, ATCC 25015^T) accession no.: EF153477, *F. philomiragia* (strain 1951) accession no.: DQ813268]

Gene sequences

The rRNA genes from GM2212^T have been sequenced by Nylund et al. (2006). This study provides the same genes (4,841 nt) from *F. philomiragia* (strain 1951, accession no.: DQ813266) and (4,512 nt) from the type strain of *F. philomiragia* (DSM 7535, ATCC 25015^T, accession no.: EF153479). The 16S and 23S sequence from GM2212^T is more similar to the *F. philomiragia* strains than to *F. tularensis*. In the more variable 16S–23S spacer region, the similarity is lower (<98%) between GM2212^T and the two *F. philomiragia* strains, but differ markedly from *F. tularensis* (Table 4).

Results from the comparisons of the putative outer membrane protein (FopA), mdh, lipoprotein LpnA (TUL4) and the conserved hypothetical lipoprotein (LpnB) gene-sequences from GM2212^T, *F. philomiragia* (DSM 7535, ATCC 25015^T and strain 1951) and *F. tularensis* subsp. *tularensis* (SCHU4) is also listed in Table 4. In all proteins the nucleotide sequence similarity between the two *F. philomiragia* strains and GM2212^T (89–99%) are clearly higher than to *F. tularensis* (70–98%). The maximum divergence of GM2212^T from the *F. philomiragia* strains is seen in the LpnB gene (11%).

Discussion

Genus *Francisella* contains two recognized species, *F. tularensis* and *F. philomiragia* (Sjøstedt 2005). The most closely related bacterium to these is *Wolbachia persica*, an obligate intracellular bacterium from a tick that should be transferred to *Francisella* (Niebylski et al. 1997; Noda et al. 1997). Several other bacteria, clearly related to or members of *Francisella*, have also

been detected as endosymbionts in a wide range of tick species (Niebylski et al. 1997; Noda et al. 1997; Scoles 2004) and in environmental samples (Barns et al. 2005). Several strains of *Francisella* have also been isolated from diseased fish in culture, tilapia (*Oreochromis niloticus*), three-line grunt (*Parapristipoma trilineatum*) and from cod in Norway (Kamaishi et al. 2005; Kay et al. 2006; Nylund et al. 2006; Olsen et al. 2006), but no validly published descriptions or names are yet available for these. As shown by Nylund et al. (2006), it also appears that *Francisella* sp. from cod in Norway is distinct from the *Francisella* sp. causing disease in Asian fish culture.

The present study confirm a high 16S rRNA and FopA gene sequence similarity between GM2212^T and *F. philomiragia* (DSM 7535, ATCC 25015^T and strain 1951), found by Nylund et al. (2006). Our previous study (Nylund et al. 2006) was hampered by the lack of spacer and 23S sequence information for *F. philomiragia*, obtained in the present study from strain 1951 and DSM 7535, ATCC 25015^T. These comparisons support a closer relation of GM2212^T to *F. philomiragia* than to *F. tularensis*. However, the differences suggest that GM2212^T could be a new species. This is supported by comparison of the additional protein genes sequenced (TUL4 = LpnA, LpnB and mdh).

In the biochemical characters there are similarities between GM2212^T and *F. philomiragia* (DSM 7535, ATCC25015^T). However, the most striking differences are that GM2212^T is oxidase-negative, do not produce H₂S in TSI agar, and lack the ability to hydrolyze gelatin (present study). GM2212^T also grows slower than *F. philomiragia* and clearly differ by its low optimal growth temperature and growth temperature range. The susceptibility of GM2212^T to some antibiotics is also different from that of *F. philomiragia*.

The differentiating biochemical characters of GM2212^T generally differ from literature data of *F. tularensis*, the highest similarity evident is to *F. tularensis* subsp. *novicida*. However, the most striking differences between GM2212^T and literature data on *F. tularensis* subsp. *novicida* (Sjøstedt 2005), is that the former show tryptophanase activity (indole positive), do not degrade glycerol, is not agglutinated by antisera against *F. tularensis* subsp. *tularensis* and grows best at lower temperatures. On the basis of 16S rRNA, 16S–23S spacer, 23S rRNA, TUL4 = LpnA, LpnB and mdh gene sequence analysis and phenotypic characteristics, GM2212^T could indeed represent a novel species within the genus *Francisella*.

Due to the high similarity in the rRNA genes, a DNA–DNA hybridization between GM2212^T and the type strain of *F. philomiragia* (DSM 7535,

ATCC25015^T) is currently being performed. This should clarify if *Francisella* sp., GM2212^T, isolate from cod represent a species distinct from *F. philomiragia*. DNA–DNA hybridization has previously been used to delineate *F. philomiragia* from *F. tularensis* in studies by Hollis et al. (1989). In these studies *F. philomiragia* showed 39% average relatedness to the type strain of *F. tularensis* and four other *F. tularensis* strains.

A characteristic shared by *Francisella* sp., GM2212^T, *F. philomiragia* and *F. tularensis* is an association with or presence in water. Strains of *F. tularensis* have been associated with lakes, streams and rivers and there are cases where people have become infected after direct contact with or by ingestion of contaminated water (Karpoff and Antonoff 1936; Greco et al. 1987; Whipp et al. 2003). In most of the cases where such water-borne infections with *F. tularensis* has occurred, carcasses of rodents or lagomorphs have been found in or near the water source (Anda et al. 2001; Reintjes et al. 2000; Hoel et al. 1991). Strains of *F. philomiragia* have so far only been found in saline-, brackish- and seawater (Jensen et al. 1969; Hollis et al. 1989; Friis-Møller et al. 2004), where infection in humans may occur in immunocompromised individuals and in near drowning victims (Wenger et al. 1989; Friis-Møller et al. 2004). In contrast to *F. tularensis* there is no evidence that animal carcasses were involved in cases where *F. philomiragia* have caused disease. So far, *Francisella* sp., GM2212^T, has been detected in tissues of cod, and with a specific real-time PCR also in saithe (*Pollachius virens*), blue mussels (*Mytilus edulis*) and crabs (*Cancer pagurus*) from the environment of affected cod farms (pers. obs.). In contrast to the related *F. tularensis* and *F. philomiragia*, *Francisella* sp., GM2212^T, have not been reported to cause disease in humans despite that people working in the cod-farming industry have been in contact with infected cod, and despite that infected cod frequently must have been used for human consumption. It is unlikely that humans can acquire *Francisella* sp., GM2212^T, infections due to fact that the bacterium does not grow at 37°C. Nevertheless, if disease is suspected in immunocompromised patients who have been in contact with infected cod, diagnostic laboratories should use appropriate growth media, incubation temperature and time which could support growth of the bacterium to rule out infection with *Francisella* sp., GM2212^T.

Description of *Francisella* sp., GM2212^T

Aerobic, microaerophilic; Gram-negative, coccoid to short rods, single cells, typically 0.5 µm, maximum 1.5 µm. Growth at 10–25°C, optimal at c. 20°C.

Oxidase-negative, catalase-positive. Do not produce H₂S in TSI agar, does not hydrolyze gelatin. Cysteine enhances growth. Acid production from D-glucose, maltose, sucrose (weak) but not from lactose or glycerol. Indole positive. Growth on MAC agar and in nutrient broth (6% NaCl). Resistant to trimethoprim-sulfamethoxazole, penicillines, cefuroxime, erythromycin; susceptible to ceftazidime, tetracycline, gentamicin and ciprofloxacin. The predominant non-hydroxy fatty acids are C_{<14} unidentified (17.8%), C₁₄ (10.3%), C_{18:1n-9} (9.9%), C₂₄ (6.0%), C₂₀₊ unidentified (22.7%).

Isolate GM2212^T (=CNCM I-3481^T = CNCM I-3511^T = DSMZ 18777^T) was isolated from the kidney of farmed Atlantic cod (*G. morhua*) from Rogaland County, western Norway.

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