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Francisella philomiragia subsp. *noatunensis* subsp. nov., isolated from farmed Atlantic cod (*Gadus morhua* L.)

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Seven bacterial isolates from farmed Atlantic cod displaying chronic granulomatous disease were characterized by phenotypic and molecular taxonomic methods. The isolates were Gram-negative, facultatively intracellular, non-motile, strictly aerobic coccobacilli which produced H_2S from cysteine-supplemented media and are therefore phenotypically consistent with members of the genus *Francisella*. Comparison of 16S rRNA gene sequences and six partial housekeeping gene sequences (*groEL*, *shdA*, *rpoB*, *rpoA*, *pgm* and *atpA*) confirmed the organism as a member of the genus *Francisella*, with *Francisella philomiragia* as its closest relative (99.3 % 16S rRNA gene sequence similarity, 92.2–99.0 % housekeeping gene sequence similarity). Despite the close relationship with *F. philomiragia*, isolates from Atlantic cod could be readily distinguished phenotypically and genetically from *F. philomiragia* ATCC 25015^T. DNA–DNA hybridization studies revealed a mean reassociation value of 68 %. Thus, on the basis of phenotypic and molecular genetic evidence, we propose that the strains isolated from Atlantic cod should be recognized as *Francisella philomiragia* subsp. *noatunensis* subsp. nov. with the type strain 2005/50/F292-6C^T (=NCIMB 14265^T=LMG 23800^T). *Francisella philomiragia* ATCC 25015^T (=DSM 735^T) is reclassified as *Francisella philomiragia* subsp. *philomiragia* subsp. nov.

Bacteria within the genus *Francisella* are non-motile, Gram-negative, strictly aerobic and facultatively intracellular coccobacilli that produce H_2S from cysteine-supplemented media. Currently, only three species are recognized within the genus *Francisella* (Larson *et al.*, 1955; Hollis *et al.*, 1989; Sjöstedt, 2005), *Francisella novicida*, *Francisella tularensis* and *Francisella philomiragia*, although evidence for other species exists (Barns *et al.*, 2005; Hsieh *et al.*, 2006; Kay *et al.*, 2006; Nylund *et al.*, 2006). It has been suggested that *F. novicida* represents a subspecies of *F. tularensis* (Sjöstedt, 2005), but this reclassification has not been formally proposed and the name '*Francisella tularensis* subsp. *novicida*' has not been validly published. In 2005, the National Veterinary Institute (NVI) in Norway isolated a Gram-negative, facultatively intracellular bacterium, genetically and phenotypically consistent with the genus *Francisella*, from farmed Atlantic cod (*Gadus morhua* L.) displaying granulomatous disease (Olsen *et al.*, 2006). The isolation procedures employed and the characteristics of the disease have been described previously (Olsen *et al.*, 2006) and strain 2005/50/F292-6C^T was deposited in the NCIMB and LMG culture collections (=NCIMB 14265^T=LMG 23800^T).

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *groEL*, *shdA*, *rpoB*, *rpoA*, *pgm* and *atpA* gene sequences reported in this paper are given in Supplementary Table S4 available with the online version of this paper.

Supplementary tables detailing the primers used in this study, the gene sequence similarities and Kimura two-parameter genetic distances between the isolates from Atlantic cod and other members of the genus *Francisella* and the GenBank accession numbers for the 16S rRNA, *groEL*, *shdA*, *rpoB*, *rpoA*, *pgm* and *atpA* gene sequences reported in this study are available with the online version of this paper.

In this study, seven isolates of an unidentified *Francisella* species isolated from different disease outbreaks in Atlantic cod along the Norwegian coast were characterized. In addition *F. philomiragia* strains CCUG 12603, CCUG 13404 and CCUG 19701 from the Culture Collection of the University of Göteborg, Sweden (CCUG) and *F. philomiragia* ATCC 25015^T (=DSM 735^T) were investigated.

Phenotypic characterizations were performed following standard protocols combined with enzymic reactions using the commercial kits API ZYM, Rapid ID 32A and Rapid ID 32E (bioMérieux) unless otherwise noted. These tests were performed at both 37 °C and 22 °C, reflecting the optimal growth temperatures of the isolates studied. Tests were read after 4 h and 24 h incubation, respectively.

Primers targeting the *groEL*, *atpA*, *rpoA* and *pgm* genes were constructed from the draft whole genome sequence of *F. philomiragia* ATCC 25017 (https://maple.lsd.ornl.gov/microbial) and supplemented by primers previously

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described for the detection of the *shdA* gene (Barns *et al.*, 2005) and the variable region of the *rpoB* gene from *Legionella pneumophila* (Ko *et al.*, 2002). The 16S rRNA sequences were generated as reported by Olsen *et al.* (2006), following standard protocols. Primers and PCR programs developed during the present study are presented in Supplementary Table S1 (available with the online version of this paper). Gene fragments were amplified and sequenced in both directions on separate days. DNA–DNA hybridization of Atlantic cod strain 2005/50/F292-6C^T with *F. philomiragia* ATCC 25015^T was performed in 2 × SSC at 62 °C at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany) in duplicate experiments as described by Lopez-Cortes *et al.* (2006).

Sequences were aligned and compiled using BioEdit software (Hall, 1999). Two matrices were constructed for phylogenetic analyses. The first was concatenated, encompassing the *groEL*, *atpA*, *pgm*, *rpoB*, *shdA* and *rpoA* gene sequences from seven Atlantic cod isolates, five *F. philomiragia* isolates and five isolates of *F. tularensis*, including two recognized subspecies and '*F. tularensis* subsp. *novicida*'. The other matrix covered the majority of the small-subunit rRNA gene (16S rRNA) from a larger number of strains. Both matrices were subjected to maximum-likelihood analysis in PAUP version 4.0b10 (Swofford, 2002) using GTR + I + G and HKY + G models for concatenated and 16S rRNA gene sequence matrices, respectively, as selected by MODELTEST 3.7 software (Posada & Crandall, 1998). Trees were constructed using 10 × random addition of the sequences in heuristic searches with tree bisection-reconnection branch swap. To assess the stability of tree branching patterns, bootstrap analyses (100 pseudoreplicates) were performed using evolutionary models and tree building strategy as described above.

Results and Discussion

Phenotypical characterization of established *Francisella* species is somewhat limited due to their fastidious nature. No reaction was registered on inoculation of the strains isolated from Atlantic cod into phenol red broth base (Difco) supplemented with 1 % (w/v) glucose, ribose, maltose, mannitol, sorbitol, trehalose, sucrose or arabinose, respectively, after incubation for 14 days at 22 °C. Production of *β*-lactamase was detected by the Gots test (Gots, 1945), a feature shared by nearly all isolates of the genus *Francisella* (Sjöstedt, 2005). Using Rapid ID 32A and Rapid ID 32E test kits, all strains isolated from Atlantic cod were positive for the degradation of glucose, mannose, alanine and proline, features shared with the *F. philomiragia* strains tested (Table 1). However, in contrast to *F. philomiragia*, maltose, sucrose, leucine, glycine and trehalose were not degraded. The incubation temperature did not affect the outcome of these tests. In common with *F. tularensis*, and in contrast to *F. philomiragia*, the novel isolates tested cytochrome oxidase-negative. The novel isolates, in common with *F. philomiragia*, did not show cross-reactivity toward *F. tularensis* antiserum (Clarridge *et al.*, 1996; Olsen *et al.*, 2006).

Absolute requirement for cysteine was not tested. In contrast to *F. philomiragia* and '*F. tularensis* subsp. *novicida*', for which cysteine, although enhancing growth,

Table 1. Physiological characteristics of strains of *Francisella philomiragia*

Strains: 1, *F. philomiragia* CCUG 12603; 2, *F. philomiragia* CCUG 13404; 3, *F. philomiragia* CCUG 19701; 4, *F. philomiragia* ATCC 25015^T; 5, *F. philomiragia* subsp. *noatunensis* subsp. nov. strain 2005/50/F292-6C^T. +, Positive; −, negative. All strains were negative in tests for: urease, *α*-galactosidase, *β*-galactosidase-6-phosphate, *α*-glucosidase, *β*-glucosidase, *α*-arabinosidase, *β*-glucuronidase, raffinose fermentation, glutamic acid decarboxylase, *α*-fucosidase, reduction of nitrates, aesculin, arabinose, adonitol, rhamnose, mannitol, cellobiose, melibiose, glucuronate, malonate, para-phenylalanine deaminase, 5-ketogluconate, palatinose, galacturonate, tetrathionate reductase, galactosidase, raffinose, glutamyl glutamic acid arylamidase, lipase C14, valine arylamidase, cystine arylamidase, trypsin, *α*-chymotrypsin, *β*-glucuronidase, *α*-glucosidase, *β*-glucosidase, *α*-mannosidase, *α*-fucosidase and motility. All isolates were positive for: *N*-acetyl-*β*-glucosaminidase, alkaline phosphatase, esterase C4, esterase lipase C8, acid phosphatase, naphthol-AS-BI-phosphohydrolase, D-glucose, mannose, H_2S production, *β*-lactamase production, proline arylamidase and alanine arylamidase. All isolates were weakly positive for indole and catalase.

Characteristic	1	2	3	4	5
Optimal growth temperature (°C)	37	37	37	37	22
Cysteine requirement for growth	−	−	−	−	+*
Oxidase	+	+	+	+	−
Enzymic degradation of maltose, sucrose, trehalose, sorbitol, colistin and coumarate	+	+	+	+	−
Detection of arylamidases:					
Arginine, leucyl glycine, phenylalanine, leucine, pyroglutamic acid, tyrosine, glycine, histidine and serine	+	+	+	+	−
Enzymic activity:					
o-Nitrophenyl *N*-acetyl-*β*-D-glucosaminide (ONAG), p-nitrophenyl-*β*-D-galactopyranoside (PNPG) and indoxyl phosphate (IDP)	+	+	+	+	−
β-Galactosidase	+	−	−	+	−

*No growth on blood agar.

is not a strict requirement (Sjöstedt, 2005), no visible growth of the novel strains from Atlantic cod was detected on media without cysteine supplement after 14 days incubation. The isolates from Atlantic cod grew relatively poorly on cysteine heart agar (CHA, Difco); after 6 days incubation at 22 °C, no single colonies could be detected. On addition of 5 % sheep blood (CHAB), single colonies of approximately 1 mm in diameter were observed after 4 days of incubation at 22 °C. To determine the optimal growth temperature for the novel isolates, six different temperatures (4, 10, 15, 22, 30 and 37 °C) were tested. Optimal growth was observed at 22 °C, while only slight growth was detected at 30 °C on CHAB plates. All isolates of the genus *Francisella* previously recovered from various fish species appear to have upper cardinal temperatures in this range (Ottem *et al.*, 2007; Hsieh *et al.*, 2006). The *F. philomiragia* isolates grew well at 37 °C on both blood agar and CHAB in clear contrast to the isolates from Atlantic cod. Although Hollis reported three *F. philomiragia*-like bacteria with optimum growth temperatures of 25 °C (Hollis *et al.*, 1989), these strains were able to grow at 35 °C in contrast to the isolates from Atlantic cod.

BLAST searches (Altschul *et al.*, 1997) of the 16S rRNA gene sequence (1416 bp) of strain 2005/50/F292-6C^{T} from Atlantic cod (GenBank accession number DQ295795) revealed similarities of 99.3 % and 99.2 % with *F. philomiragia* ATCC 25015^{T} (AY928394) and *F. philomiragia* ATCC 25017 (AY928395), respectively. The remaining *F. philomiragia* strains studied had 16S rRNA gene sequences identical to *F. philomiragia* ATCC 25017. The isolates from Atlantic cod showed highest similarity to '*F. tularensis* subsp. *novicida*' (AY968237) with 97.9 % gene sequence similarity. Further, 16S rRNA gene sequence similarities of 100 % (DQ309246, 1416/1416), 99.9 % (AM403242, 1414/1416) and 99.2 % (AF385857, 1404/1416) were identified with *Francisella* spp. reported from Atlantic cod from Norway, Atlantic salmon (*Salmo salar*) from Chile and tilapia (*Oreochromis* sp.) from Asia, respectively (Hsieh *et al.*, 2006; Nylund *et al.*, 2006). A 2 bp insertion was observed in the strains isolated from Atlantic cod compared with those of the *F. philomiragia* strains and this indel was shared with all *Francisella tularensis* subspecies and the three aforementioned unidentified *Francisella* species from fish.

All housekeeping loci were analysed individually and as concatenated sequences. Identical sequences were identified for all seven isolates from Atlantic cod. Sequencing and alignment of genes generated in-frame fragments of 1461 bp, 775 bp, 575 bp, 261 bp, 289 bp and 502 bp from *groEL*, *atpA*, *pgm*, *shdA*, *rpoB* and *rpoA* genes, respectively. Sequences were concatenated in the aforementioned order (total length: 3863 bp). The housekeeping gene sequence alignment was expanded to include homologous gene sequences from '*F. tularensis* subsp. *novicida*' U112 (CP000439), *F. tularensis* subsp. *holarctica* OSU18 (CP000437), *F. tularensis* subsp. *holarctica* LVS (AM233362), *F. tularensis* subsp. *tularensis* Schu 4 (AJ49949), *F. tularensis* subsp. *tularensis* FCS 198 (AM286280) and *F. philomiragia* ATCC 25017. The ratio of mean nonsynonymous (dN) to synonymous substitutions (dS) per site (dN : dS ratio) within the selected genes was calculated using START software (Jolley *et al.*, 2001). Housekeeping genes are believed to be either selectively neutral or subject to purifying selection. Thus, the rate of synonymous substitutions (dS) should be equal to or slightly greater than the rate of nonsynonymous substitutions (dN), giving a dN : dS ratio of <1 (Viscidi & Demma, 2003). The dN : dS ratio varied from 0.0071 (*rpoB*) to 0.0706 (*groEL*), indicating purifying selection in all loci investigated and demonstrating their suitability for use in phylogenetic analysis. No gaps were observed in the alignments of the housekeeping genes. Sequence similarity values were calculated for individual gene fragments and for a concatenated sequence (see Supplementary Table 2 in IJSEM Online). The *groEL* gene sequence obtained for the isolates from Atlantic cod shared between 96.6 and 97.3 % similarity with the *F. philomiragia* strains examined, whereas the *atpA* gene sequences shared between 96.8 and 97.2 % similarity, *pgm* gene sequences between 95.0 and 96.3 % similarity, *shdA* gene sequences between 97.7 and 98.5 % similarity, *rpoB* gene sequences shared between 97.9 and 99.0 % similarity and the *rpoA* gene sequences shared between 92.2 and 92.8 %. These levels of average nucleotide identity (ANI), with the exception of *rpoA*, lie just above the 95 % level suggested by Konstantinidis *et al.* (2006) to represent separate species and can be compared with similarity values of 98.7–98.9 % for *groEL*, 98.5–99.2 % for *atpA*, 95.1–98.4 % for *pgm*, 97.7–100 % for *shdA*, 97.7–99.3 % for *rpoB* and 99–99.8 % for *rpoA* observed among established *F. philomiragia* strains. The concatenated sequence obtained for the strains from Atlantic cod shared between 96.1 and 96.7 % similarity with those of *F. philomiragia*, whereas the concatenated sequences obtained from different *F. philomiragia* strains shared between 98.3 and 99.2 % similarity with one another.

Phylogenetic analysis confirmed the close relationship between the isolates from Atlantic cod and *F. philomiragia* (Fig. 1). In the 16S rRNA gene sequence analysis, there was 92 % bootstrap support for the separation of the *Francisella* species isolated from fish, including the isolates from Atlantic cod, from established *F. philomiragia* isolates (Fig. 1a). Maximum-likelihood analysis of concatenated housekeeping gene fragments (not including other fish isolates as this information is not available) verified the separation of the isolates from Atlantic cod from *F. philomiragia* (Fig. 1b), with 100 % bootstrap support. Analyses of individual genes did not identify congruous relationships in every case (data not shown). Nevertheless, concatenation of housekeeping genes has been shown to produce more reliable phylogenetic trees than single gene approaches (Gadagkar *et al.*, 2005). Further, a Kimura two-parameter distance matrix (see Supplementary Table S3 in IJSEM Online) based upon the concatenated sequence was generated in PAUP version 4.0b10 (Swofford, 2002). The

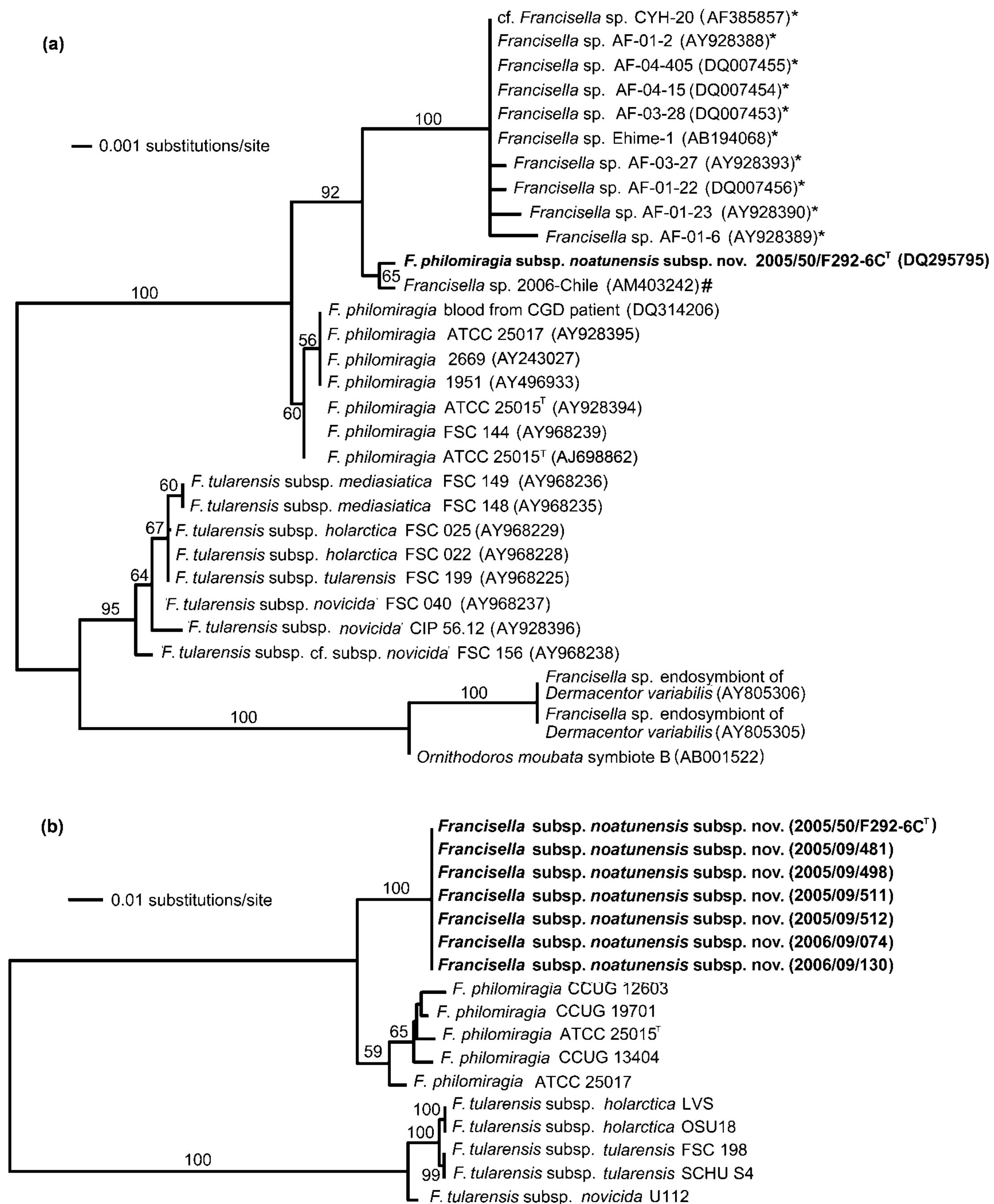

Fig. 1. Maximum-likelihood analysis. (a) 16S rRNA gene sequence data; total length of the alignment was 1348 characters. GenBank accession numbers are in parentheses. Bar, 0.001 substitutions per site. (b) Concatenated *groEL*, *atpA*, *pgm*, *shdA*, *rpoB* and *rpoA* gene sequences; total length of the alignment was 3863 characters. Bootstrap values >50 % are indicated (maximum-likelihood). Strain reference numbers are in parentheses. Bar, 0.01 substitutions per site. *Isolated from fish in Asia. # Isolated from fish in Chile.

mean genetic distance between the isolates from Atlantic cod and *F. philomiragia* isolates was 0.03766 and was 0.01275 between the *F. philomiragia* isolates. In comparison, the greatest genetic distance between subspecies of *F. tularensis* was 0.0136, detected between '*F. tularensis* subsp. *novicida*' and *F. tularensis* subsp. *holarctica* strain LVS.

DNA–DNA reassociation values between strain 2005/50/F292-6C^T and *F. philomiragia* ATCC 25015^T gave a mean value of 68 % (duplicate experiments, 65.2 + 70.8 %), values close to those found by Hollis *et al.* (1989) when comparing different *F. philomiragia* isolates (72–83 % at 65 °C). Thus, this novel strain lies in a 'grey area' regarding

the recommendations of Stackebrandt *et al.* (2002) in relation to recognition as an independent species.

F. philomiragia strains have been linked to water-borne transmission and are considered as opportunistic pathogens in humans, causing disease in immunocompromised individuals (Sjöstedt, 2005). Strains of *F. philomiragia* have less fastidious metabolic requirements than the isolates from Atlantic cod, suggesting a different ecological niche for the pathogenic fish isolates. All *F. philomiragia* isolates are able to grow at 35 °C, whereas the isolates from Atlantic cod did not thrive at 30 °C or above. Thus, the novel strains are not expected to cause disease in warm-blooded animals and are therefore not considered to be a zoonotic threat. All isolates belonging to the same 16S rRNA gene clade as the isolates from Atlantic cod are directly linked to diseases in fish (Ottem *et al.*, 2007; Hsieh *et al.*, 2006) and it is likely that this phylogenetic clade represents an ecological separation from previously recognized *F. philomiragia* strains.

Considering differences in growth requirements, DNA–DNA reassociation values, phylogenetic analysis and the genetic distance of evolutionarily conserved genes, we consider that the isolates from Atlantic cod merit subspecies status within *F. philomiragia*. The name *Francisella philomiragia* subsp. *noatunensis* subsp. nov. is proposed for the isolates from Atlantic cod. According to Rule 40b of the Bacteriological Code, the creation of this subspecies automatically creates the subspecies *Francisella philomiragia* subsp. *philomiragia* subsp. nov. Tests for the differentiation of related species are listed in Table 1.

Description of *Francisella philomiragia* subsp. *noatunensis* subsp. nov.

Francisella philomiragia subsp. *noatunensis* [no.at.un.en.sis. N.L. n. *noatun* (enclosure of ships) was the coastal abode of the Norse god of fisheries and seamanship; L. fem. suffix -*ensis* suffix meaning 'belonging to'; N.L. fem. adj. *noatunensis* belonging to the coast/sea].

Cells are weakly Gram-negative, facultatively intracellular, weakly catalase positive and strictly aerobic coccobacilli. Cells do not grow on blood agar and colonies on CHAB are low convex, whitish, slightly translucent and mucoid in appearance. Single colonies of approximately 1 mm in diameter are observed after 4 days incubation at 22 °C. Growth is observed in the temperature range 10–30 °C, with limited growth in the upper limits of the range and no growth is observed at 37 °C. Optimal growth takes place at 22 °C on CHAB agar. Using the Rapid ID 32A and Rapid ID 32E tests, degradation of glucose and mannose and hydrolysis of proline and alanine is detected. API ZYM tests indicate the presence of alkaline phosphatase, esterase C4, esterase lipase C8, acid phosphatase and naphthol-AS-BI-phosphohydrolase.

The type strain, strain 2005/50/F292-6C^T (=NCIMB 14265^T=LMG 23800^T), was isolated from Atlantic cod.

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