Fulvivirga imtechensis sp. nov., a member of the phylum *Bacteroidetes*

Nupur, Shalley Sharma, Pradip Kumar Singh, Korpole Suresh and Pinnaka Anil Kumar

Correspondence
Pinnaka Anil Kumar
apinnaka@imtech.res.in

Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology (CSIR), Sector 39A, Chandigarh – 160 036, India

A novel, Gram-staining-negative, yellow-coloured, rod-shaped, obligately aerobic, non-motile bacterium, designated strain AK7^T, was isolated from seawater collected on the coast at Visakhapatnam, Andhra Pradesh, India. The predominant fatty acids of the novel strain were iso-C_{15:0}, iso-C_{15:0} 3-OH, C_{16:1}ω5c, iso-C_{17:0} 3-OH and summed features 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) and 4 (iso-C_{17:1} I and/or anteiso-C_{17:1} B). The major respiratory quinone was MK-7 and the polar lipid profile comprised phosphatidylethanolamine, two unidentified aminolipids and four other unidentified lipids. In phylogenetic analysis based on 16S rRNA gene sequences, strain AK7^T appeared most closely related to *Fulvivirga kasyanovii* KMM 6220^T (95.9% sequence similarity), a member of the family *Flammeovirgaceae* in the phylum *Bacteroidetes*. The genomic DNA G + C content of strain AK7^T was 55.1 mol%. Based on the morphological, biochemical, physiological, chemotaxonomic and phylogenetic evidence, strain AK7^T represents a novel species of the genus *Fulvivirga* for which the name *Fulvivirga imtechensis* sp. nov. is proposed. The type strain is AK7^T (=MTCC 11053^T =JCM 17390^T).

The genus *Fulvivirga*, which was described by Nedashkovskaya *et al.* (2007), lies within the family *Flammeovirgaceae* of the phylum *Bacteroidetes*. Members of the phylum *Bacteroidetes* are very diverse and, although most have been isolated from marine and freshwater habitats (O'Sullivan *et al.*, 2006), some have been isolated from soil, plants or the air (Buczolits *et al.*, 2002). At the time of writing, the genus *Fulvivirga* comprised only one species, *Fulvivirga kasyanovii*, which was initially isolated from seawater (Nedashkovskaya *et al.*, 2007). The aim of the present study was to use a polyphasic approach to determine the taxonomic position of a *Fulvivirga*-like bacterium that was recently isolated from seawater collected on the coast of south-eastern India.

Surface seawater (at pH 7.5 and 30 °C, with a salinity of 33 p.p.m.) was collected from the coast at Visakhapatnam, Andhra Pradesh, India. A sample of the water (1 ml) was serially diluted in saline [2% (w/v) NaCl] before 100 µl of each dilution was plated on ZoBell's marine agar (ZMA; Difco) and incubated at 30 °C. A yellowish colony was observed after 7 days of incubation; cells were purified, designated strain AK7^T, and preserved in 20% (v/v) glycerol at –70 °C.

The cell morphology of the novel strain was studied by phase-contrast microscopy (BX 51; Olympus). Gram staining was determined using a Gram staining kit (HiMedia)

according to the manufacturer's instructions. The strain was incubated on ZMA at 4, 10, 15, 20, 25, 30, 35, 37, 40 and 45 °C to determine the growth temperature. NaCl tolerance was determined at 30 °C and pH 7.5 on nutrient agar (NA) containing (l⁻¹) 5 g peptone, 3 g beef extract and 20 g agar, with 1, 2, 3, 4, 5, 6, 8, 10, 12, 15, 18 or 20% (w/v) NaCl. Growth at various pH values was assessed on tryptic soy agar (TSA; Difco) buffered with citric acid/NaOH (to give pH 5.0 and 6.0), NaHPO₄/Na₂HPO₄ (pH 7.0, 7.5, 8.0 and 8.5), glycine/NaOH (pH 9.0, 9.5 and 10.0) or Tris/HCl or NaOH (pH 11.0 and 12.0). The ability of strain AK7^T to utilize various compounds as sole carbon sources (at 25 mg) was checked on minimal medium (Cowan & Steel, 1965) supplemented with 2% (w/v) NaCl. Biochemical and enzymic characterization of the strain AK7^T was performed using Vitek 2 colorimetric GN cards (bioMérieux), at 30 °C and according to the manufacturer's instructions, except that sterile 2.0% (w/v) NaCl was used to prepare the inoculum.

For analysis of fatty acids and polar lipids, cell biomass of the novel strain and, for comparison, *Fulvivirga kasyanovii* JCM 16186^T was produced by aerobic culture for 24 h on ZMA at 30 °C. Fatty acid methyl esters were prepared and analysed by following the protocol of version 4.0 of the Sherlock Microbial Identification System (MIDI) and using the TSBA50 peak-naming database. Polar lipids were extracted and separated, by two-dimensional TLC, as described by Collins & Jones (1980). Molybdatophosphoric acid, molybdenum blue, ninhydrin and α-naphthol spray

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AK7^T is FR687203.

Fig. 1. Phase-contrast micrograph of cells of strain AK7^T. Bar, 5 μ m.

reagents were then used for the detection of total lipids, phospholipids, aminolipids and glycolipids on the TLC plates, respectively. Isoprenoid quinones were extracted as described by Collins *et al.* (1977) and analysed by HPLC (Groth *et al.*, 1996). Genomic DNA was extracted and purified according to the method of Marmur (1961) so that its G + C content could be determined, by the melting-point method (Sly *et al.*, 1986), in a Lambda 35 spectrophotometer (Perkin Elmer).

For the phylogenetic analysis, DNA was extracted from the novel strain and purified by using a genomic DNA extraction kit (Qiagen). The 16S rRNA gene was then amplified by PCR, using the universal bacterial primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3'). After the amplicon was purified, using a QIA quick PCR purification kit (Qiagen), it was

sequenced in an ABI Prism 3700 automatic DNA sequencer (Applied Biosystems) by the use of a BigDye Terminator Cycle Sequencing kit (Applied Biosystems). Phylogenetic neighbours were identified and pairwise 16S rRNA gene sequence similarities were calculated using a BLAST sequence similarity search (Altschul *et al.*, 1990) and the EzTaxon server (Chun *et al.*, 2007). 16S rRNA gene sequences that were similar to that of AK7^T were downloaded from the database of the United States *National Center for Biotechnology Information* (<http://www.ncbi.nlm.nih.gov>) so that they could be aligned, along with the sequence of the novel strain, using CLUSTAL W (Thompson *et al.*, 1994) within version 5 of the MEGA package (Tamura *et al.*, 2011). The alignment was then corrected manually with the BioEdit sequence alignment editor (Hall, 1999). Phylogenetic trees were constructed, using both the maximum-likelihood and neighbour-joining algorithms, again using the MEGA package, with evolutionary distances calculated in the Kimura two-parameter model and the tree topologies evaluated by bootstrap analyses with 1000 replications.

The cells of strain AK7^T were non-motile rods (Fig. 1). The predominant fatty acid was iso-C_{15:0} and the major respiratory quinone was MK-7. The polar lipid profile (Fig. 2) comprised phosphatidylethanolamine, two unidentified aminolipids and four other unidentified lipids. The genomic DNA G + C content was 55.1 ± 0.06 mol%. Further details and other characteristics of the novel strain are given in the species description.

In the phylogenetic analysis based on 16S rRNA gene sequences, strain AK7^T appeared to be most closely related to *F. kasyanovii* KMM 6220^T (95.9% sequence similarity) and clustered with this type strain in both the maximum-likelihood and neighbour-joining trees (Fig. 3). However, strain AK7^T could be distinguished from *F. kasyanovii* JCM 16186^T by its cell size, NaCl requirement and tolerance, temperature range for growth, fatty acid and polar lipid

Fig. 2. Two-dimensional TLCs of the polar lipids of strain AK7^T (a) and *Fulvivirga kasyanovii* JCM 16186^T (b), after staining with molybdatophosphoric acid. PE, phosphatidylethanolamine; AL1–AL5, unidentified aminolipids; L1–L4, unidentified lipids. Directions of first and second dimensions are indicated.

Fig. 3. Maximum-likelihood (a) and neighbour-joining (b) phylogenetic trees based on 16S rRNA gene sequences, showing the relationship between strain AK7^T and members of the family *Flammeovirgaceae*. Bootstrap values $\geq 50\%$ (based on 1000 replications) are given at branch points. *Flavobacterium aquatile* ATCC 11947^T was used as an outgroup. Bars, 0.02 substitutions per nucleotide position.

profiles and genomic DNA G + C content (Tables 1 and 2). Based on the phenotypic, chemotaxonomic and phylogenetic evidence, strain AK7^T represents a novel species of the genus *Fulvivirga* for which the name *Fulvivirga imtechensis* sp. nov. is proposed.

Description of *Fulvivirga imtechensis* sp. nov.

Fulvivirga imtechensis (im.tech.en'sis. N.L. fem. adj. *imtechensis* an arbitrary adjective formed from IMTECH, the acronym of the Institute of Microbial Technology in Chandigarh, where the isolate was characterised).

Cells are Gram-staining-negative, non-spore-forming, non-motile rods (0.3–0.5 × 5–6 µm) that multiply by binary fission. Colonies on ZMA are circular, 1–2 mm in diameter, smooth, yellowish in colour, translucent and raised. Grows

well at 30–37 °C and at pH 7.0–8.0. Growth occurs with 2–12 % (w/v) NaCl (optimum between 2 % and 6 %). Positive for aesculin hydrolysis, oxidase and catalase activities, and nitrate reduction but negative for DNase, urease, amylase and caseinase activities, indole production and hydrolysis of Tweens 20, 40, 60 and 80. Utilizes maltose, D-mannose, sucrose, trehalose, galactose, xylose, cellobiose and fructose but not D-sorbitol, melibiose, D-lactose, dulcitol, salicin, raffinose, L-rhamnose, inulin or inositol. Positive (on Vitek 2 GN cards) for Ala-Phe-Pro arylamidase, adonitol, glutamyl arylamidase-*p*-nitroanilide, L-proline arylamidase and tyrosine arylamidase activities but negative for citrate utilization and *N*-acetyl-β-glucosaminidase, *N*-acetyl-β-galactosaminidase, phosphatase, lysine decarboxylase, ornithine decarboxylase, β-glucosidase, β-xylosidase, glycine arylamidase, Glu-Gly-Arg arylamidase, galactosidase, α-glucosidase,

Table 1. Characteristics that distinguish strain AK7^T from *Fulvivirga kasyanovii* JCM 16186^T

Strains: 1, AK7^T; 2, *F. kasyanovii* JCM 16186^T. Both strains are positive for Ala-Phe-Pro arylamidase, glutamyl arylamidase-*p*-nitroanilide, L-proline arylamidase, tyrosine arylamidase, oxidase, catalase and urease activities, growth on MacConkey agar and citrate utilization. Both are negative for Gram staining, indole production, alkalization of L-lactate and succinate, acidification of L-arabitol, cellobiose, coumarate, D-glucose, maltose, D-mannitol, D-mannose, palatinose, D-sorbitol, sucrose, D-tagatose, trehalose, malonate and 5-keto-D-gluconate, assimilation of trisodium citrate, L-histidine, L-malate and L-lactate, glucose fermentation, and β-galactosidase, *N*-acetyl-β-glucosaminidase, γ-glutamyl transferase, β-glucosidase, β-xylosidase, β-alanine arylamidase-*p*-NA, lipase, urease, α-glucosidase, *N*-acetyl-β-galactosaminidase, galactosidase, glycine arylamidase, ornithine decarboxylase, lysine decarboxylase and β-glucuronidase activities. +, Positive; –, negative; PE, phosphatidylethanolamine; AL1–AL5, unidentified aminolipids; L1–L4, unidentified lipids.

Characteristic	1	2
Cell shape	Rod	Rod
Cell width (µm)	0.3–0.5	0.2–0.3
Cell length (µm)	5–6	2.3–2.5
Motility	–	+
NaCl concentration for growth (% w/v)		
Range	0–15	0–10
Optimum	2–6	2–3
Growth temperature (°C)		
Range	20–40	15–45
Optimum	30–35	35–37
Optimum pH for growth	7–8	7.5–8.0
Growth on TSA	–	+
Hydrolysis of:		
Tween 40	–	+
Tween 80	–	+
Aesculin	+	–
Nitrate reduction	+	–
Activity of:		
DNase	–	+
Gelatinase	–	+
Caseinase	–	+
L-Pyrrolydonyl arylamidase	–	+
Phosphatase	–	+
Glu-Gly-Arg arylamidase	–	+
Adonitol acidification	+	–
Quinone	MK-7	MK-7
Polar lipids	PE, AL1, AL2, L1–L4	PE, AL1–AL5, L1–L3
Genomic DNA G + C content (mol%)	55.1	60.7

Table 2. Cellular fatty acid contents (%) of strain AK7^T and *Fulvivirga kasyanovii* JCM 16186^T

Strains: 1, AK7^T; 2, *F. kasyanovii* JCM 16186^T. All data are from the present study. —, Not detected.

Fatty acid	1	2
iso-C _{13:0}	—	0.36
C _{14:0}	0.61	0.48
iso-C _{15:1} G	4.10	5.50
iso-C _{15:0}	54.61	56.39
anteiso-C _{15:0}	1.19	1.71
C _{16:1} ω5c	14.35	7.57
iso-C _{15:0} 3-OH	5.11	8.43
C _{16:0}	1.03	—
C _{16:0} 3-OH	—	0.69
iso-C _{17:0} 3-OH	9.87	11.56
Summed features*		
3	3.93	3.53
4	3.94	1.16

*Summed features represent groups of two fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained C_{16:1}ω7c and/or iso-C_{15:0} 2-OH. Summed feature 4 contained iso-C_{17:1} I and/or anteiso-C_{17:1} B.

lipase, palatinose, β-alanine arylamidase-*p*-nitroanilide and γ-glutamyl transferase activities. Fatty acid profile is dominated by iso-C_{15:0}, iso-C_{15:0} 3-OH, C_{16:1}ω5c, iso-C_{17:0} 3-OH, summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) and summed feature 4 (iso-C_{17:1} I and/or anteiso-C_{17:1} B). The major isoprenoid quinone is MK-7. The polar lipid profile comprises phosphatidylethanolamine, two unidentified aminolipids and four other unidentified lipids.

The type strain, AK7^T (=MTCC 11053^T =JCM 17390^T), was isolated from seawater collected on the coast at Visakhapatnam, Andhra Pradesh, India. The genomic DNA G+C content of the type strain is 55.1 ± 0.06 mol%.

Acknowledgements

The authors thank Dr J. Euzéby, for his expert suggestion for the correct species epithet and Latin etymology, and Mr Deepak Bhatt, for his excellent help in the DNA sequencing. They are also grateful to the Indian Council of Scientific and Industrial Research (CSIR) and Department of Biotechnology, Government of India, for financial assistance.

References

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Buczolits, S., Denner, E. B. M., Vybiral, D., Wieser, M., Kämpfer, P. & Busse, H.-J. (2002). Classification of three airborne bacteria and proposal of *Hymenobacter aerophilus* sp. nov. *Int J Syst Evol Microbiol* **52**, 445–456.
- Chun, J., Lee, J.-H., Jung, Y., Kim, M., Kim, S., Kim, B. K. & Lim, Y. W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol* **57**, 2259–2261.
- Collins, M. D. & Jones, D. (1980). Lipids in the classification and identification of coryneform bacteria containing peptidoglycans based on 2,4-diaminobutyric acid. *J Appl Bacteriol* **48**, 459–470.
- Collins, M. D., Pirouz, T., Goodfellow, M. & Minnikin, D. E. (1977). Distribution of menaquinones in actinomycetes and corynebacteria. *J Gen Microbiol* **100**, 221–230.
- Cowan, S. T. & Steel, K. J. (1965). *Manual for the Identification of Medical Bacteria*. London: Cambridge University Press.
- Groth, I., Schumann, P., Weiss, N., Martin, K. & Rainey, F. A. (1996). *Agrococcus jenensis* gen. nov., sp. nov., a new genus of actinomycetes with diaminobutyric acid in the cell wall. *Int J Syst Bacteriol* **46**, 234–239.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.
- Nedashkovskaya, O. I., Kim, S. B., Shin, D. S., Beleneva, I. A. & Mikhailov, V. V. (2007). *Fulvivirga kasyanovii* gen. nov., sp. nov., a novel member of the phylum *Bacteroidetes* isolated from seawater in a mussel farm. *Int J Syst Evol Microbiol* **57**, 1046–1049.
- O'Sullivan, L. A., Rinna, J., Humphreys, G., Weightman, A. J. & Fry, J. C. (2006). Culturable phylogenetic diversity of the phylum 'Bacteroidetes' from river epilithon and coastal water and description of novel members of the family *Flavobacteriaceae*: *Epilithonimonas tenax* gen. nov., sp. nov. and *Persicivirga xylanidelens* gen. nov., sp. nov. *Int J Syst Evol Microbiol* **56**, 169–180.
- Sly, L. I., Blackall, L. L., Kraat, P. C., Tian-Shen, T. & Sangkhobol, V. (1986). The use of second derivative plots for the determination of mol% guanine plus cytosine of DNA by the thermal denaturation method. *J Microbiol Methods* **5**, 139–156.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731–2739.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.