

Author version: *Int. J. Systemat. Evolut. Microbiol.*, vol.63(7); 2013; 2490-2496

Title ***Flavobacterium nitratireducens* sp. nov., an amyolytic bacterium of the family *Flavobacteriaceae* isolated from coastal surface sea water**

Authors **Nupur<sup>1</sup>., Bhumika, V., Srinivas, T. N. R<sup>2</sup>., Anil Kumar, P\*<sup>1</sup>**

Institution <sup>1</sup>MTCC-Microbial Type Culture Collection & Gene Bank, Institute of Microbial Technology, Chandigarh-160036, India

<sup>2</sup>National Institute of Oceanography, Regional Centre, Council of Scientific and Industrial Research (CSIR), Kochi-682018, India

Address for correspondence\* Dr. P. Anil Kumar

Microbial Type Culture Collection and Gene bank

Institute of Microbial Technology, Sector 39A,

Chandigarh - 160 036, INDIA

Email: [apinnaka@imtech.res.in](mailto:apinnaka@imtech.res.in)

Phone: +91-172-6665170

Running title *Flavobacterium nitratireducens* sp. nov.

Subject category *Bacteroidetes*

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain N1<sup>T</sup> is FR827898.

All authors are equally contributed to the present work.

A novel Gram-negative, rod shaped, non-motile bacterium, designated strain N1<sup>T</sup>, was isolated from marine water sample collected from sea shore, Bay Bengal, Visakhapatnam, India. The strain was positive for starch hydrolysis, nitrate reduction and ornithine decarboxylase activities and negative for citrate utilization, urease, oxidase, catalase and DNase activities. The predominant fatty acids were C<sub>16:0</sub> 3OH, iso-C<sub>15:0</sub>, iso-C<sub>15:0</sub> 3OH, iso-C<sub>17:0</sub> 3OH, anteiso-C<sub>15:0</sub>, C<sub>16:0</sub>, C<sub>15:0</sub> 3OH and C<sub>16:1</sub> ω7c and/or iso-C<sub>15:0</sub> 2-OH (summed feature 3). Strain N1<sup>T</sup> contained menaquinone 6 (MK-6) as the sole respiratory quinone. The only polyamine was homospermidine and the major polar lipids were phosphatidylethanolamine (PE), three unidentified aminolipids (AL1 to AL3) and two unidentified lipids (L1, L2). The DNA G + C content of the strain was 36.3 mol%. The 16S rRNA gene sequence analysis indicated that strain N1<sup>T</sup> was member of the genus *Flavobacterium* and closely related to *F. resistens* with pairwise sequence similarity of 96.5%. Phylogenetic analysis showed that strain N1<sup>T</sup> clustered with *F. glycines* and *F. daejeonense* with a distance of 4.8 and 6.0% (95.2 and 94.0% similarity), respectively. Based on the phenotypic characteristics and on phylogenetic inference, it appears that strain N1<sup>T</sup> represents a novel species of the genus *Flavobacterium*, for which the name *F. nitratreducens* sp. nov. is proposed. The type strain of *F. nitratreducens* sp. nov. is N1<sup>T</sup> (= MTCC 11155<sup>T</sup> = JCM 17678<sup>T</sup>).

The genus *Flavobacterium* (a member of the family *Flavobacteriaceae*, order *Flavobacteriales* and class *Flavobacteriia*, phylum “*Bacteroidetes*”) was established with the reclassification of two species *Bacillus aquatilis* and *Bacillus devorans* to *F. aquatile* and *F. devorans* by Bergey *et al.* (1923) and later emended by Bernardet *et al.* (1996). Members of the genus *Flavobacterium* are Gram-negative, rod shaped, yellow-pigmented bacteria, strictly aerobic, contains MK-6 as the sole respiratory quinone with DNA G + C contents in the range of 32-37 mol%. *Flavobacterium* species have been isolated from diverse group of habitats such as freshwater, river sediments, fish tissues and soil (Bernardet *et al.*, 1996, 2002; Bernardet & Bowman, 2006; Tamaki *et al.*, 2003; McCammon & Bowman, 2000), Antarctic lakes (McCammon *et al.*, 1998; McCammon & Bowman, 2000; Humphry *et al.*, 2001; Van Trappen *et al.*, 2003, 2004; Yi *et al.*, 2005), and a glacier (Zhu *et al.*, 2003). At the time of writing a total of 113 validly described species have been accommodated in the genus *Flavobacterium*. Out of which 23 species are reclassified based on the polyphasic taxonomic analysis to the other existing genera (*Chryseobacterium*, *Elizabethkingia*, *Empedobacter*, *Halomonas*, *Microbacterium*, *Myroides*, *Novosphingobium*, *Pedobacter*, *Planomicrobium*, *Psychroflexus*, *Salegentibacter*, *Sphingobacterium*, *Terrimonas* and *Zobellia*). In the present study we focused on the characterization and classification of a strain N1<sup>T</sup>, which was isolated from marine water sample, by polyphasic taxonomic approaches (Vandamme *et*

al. 1996) and proposing new species of the genus *Flavobacterium* for which the name *F. nitratireducens* sp. nov. is proposed.

Strain N1<sup>T</sup> was isolated from a marine surface water sample collected from sea shore, Bay of Bengal, Visakhapatnam, India. The sample (1 ml) was serially diluted (10 fold dilutions) and 100 µl of each dilution was plated on R2A medium and incubated at 30 °C. A shiny translucent yellow colony was observed upon five days incubation, which was purified and preserved as -70 °C glycerol stock for further characterization.

Cell morphology studies of the strain N1<sup>T</sup> were done by phase contrast microscopy (Olympus) and also using scanning electron microscope (SEM). Physiological properties such as growth at different temperatures 5, 10, 15, 20, 25, 30, 33, 35, 37, 40 and 45 °C and different NaCl concentrations (0, 1, 2, 3, 4 and 5% w/v) was tested by growing on R2A (Lányi, 1987). Growth of strain N1<sup>T</sup> at pH 5, 6, 7, 7.5, 8, 8.5, 9 and 10 was assessed on MA buffered with citric acid/NaOH (for pH 5 and 6), NaHPO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> (for pH 7 and 8), glycine/NaOH (for pH 9 and 10). Different biochemical tests listed in description of species and as well as in Table 1, Supplementary Table 1 were carried out using the culture that was grown at 30 °C on R2A medium as described by Lányi (1987) (catalase, oxidase activities, nitrate reduction, indole production and aesculin hydrolysis) and Smibert & Krieg (1994) (H<sub>2</sub>S production, gelatin and urea hydrolysis). The sensitivity of strain to antibiotics was tested on R2A medium using various antibiotic discs (HIMEDIA). Biochemical and enzymatic characterization of the strain was also performed using Vitek 2 GN (bioMérieux) with incubation at 30 °C, according to the manufacturer's protocol.

Standardization of the physiological age of strains N1<sup>T</sup>, *F. daejeonense* DSM 17708<sup>T</sup> and *F. glycines* ICMP 17618<sup>T</sup> was done based on the protocol ([http://www.microbialid.com/PDF/TechNote\\_101.pdf](http://www.microbialid.com/PDF/TechNote_101.pdf)) given by Sherlock Microbial Identification System (MIDI, USA). For cellular fatty acids analysis, strains N1<sup>T</sup>, *F. daejeonense* DSM 17708<sup>T</sup> and *F. glycines* ICMP 17618<sup>T</sup> were grown on R2A plates at 30 °C for 3 days (all three stains are at stationary phase of their growth). Cellular fatty acid methyl esters (FAMES) were obtained from cells by saponification, methylation and extraction following the protocol of MIDI. Cellular FAMES were separated by GC (6890) and identified and quantified using the Sherlock Microbial Identification System (MIDI-6890 with database TSBA6). Polyamines were extracted from strains N1<sup>T</sup>, *F. daejeonense* DSM 17708<sup>T</sup> and *F. glycines* ICMP 17618<sup>T</sup>, and analysis was carried out as described by Busse & Auling (1988) and Busse *et al.* (1997). The extracted samples were loaded on TLC plates (Silica gel 20x20 cm, Merck, Germany, 105553) and ethylacetate/cyclohexane (2:3) was used as the running solvent. Polar lipids were extracted from strains N1<sup>T</sup>, *F. daejeonense* DSM 17708<sup>T</sup>

and *F. glycines* ICMP 17618<sup>T</sup> following the method of Bligh & Dyer (1959) and analyzed by two-dimensional TLC after spraying with appropriate detection reagents (Komagata & Suzuki, 1987). Isoprenoid quinones were extracted from strains N1<sup>T</sup>, *F. daejeonense* DSM 17708<sup>T</sup> and *F. glycines* ICMP 17618<sup>T</sup> as described by Collins *et al.* (1977) and analyzed by HPLC (Groth *et al.*, 1997). Genomic DNA was isolated by using the procedure of Marmur (1961) and the mol% G + C content was determined from melting point ( $T_m$ ) curves (Sly *et al.*, 1986) obtained by using Lambda 35; Perkin Elmer spectrophotometer equipped with Templab 2.0 software package.

For 16S rRNA gene sequencing, DNA was prepared using a bacterial DNA isolation kit (Qiagen). The 16S rRNA gene was amplified by PCR using universal bacterial primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3'). The PCR product was purified using QIA quick PCR purification kit (Qiagen) and it was sequenced using an ABI PRISM model 3700 automatic DNA sequencer and Big Dye Terminator cycle sequencing kit (Applied Biosystems). The 16S rRNA gene sequence of strain N1<sup>T</sup> was subjected to BLAST sequence similarity search (Altschul *et al.*, 1990) and EzBioCloud (Kim *et al.*, 2012) to identify the nearest taxa. Based BLAST results all 16S rRNA gene sequences belong to the genus *Flavobacterium* were downloaded from the NCBI database (<http://www.ncbi.nlm.nih.gov>) and aligned using the CLUSTAL\_W program in MEGA5 (Tamura *et al.*, 2011). The evolutionary history was inferred by using the maximum-likelihood method (Tamura & Nei, 1993), neighbor-joining (NJ) method (Saitou & Nei, 1987) and maximum-parsimony method (Nei & Kumar, 2000) using the MEGA5 package (Tamura *et al.*, 2011). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 1185 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein, 1985).

Cells of the strain N1<sup>T</sup> were Gram-negative, rod shaped, non-motile, 0.2-0.3 wide x 1-1.5 long  $\mu\text{m}$  and multiply by binary fission. Colonies were circular, 2-3 mm in diameter, smooth, shiny, translucent, yellowish and raised with entire margin on R2A plates. Chemoorganoheterotrophic growth mode was observed. The strain grew between 15 to 45 °C temperature and optimum being 30 to 37 °C and from pH 6 to 10, with the optimum growth at pH 7.5-8.5. Optimum growth occurred at salinities from 0 to 1% (NaCl, w/v). Other phenotypic characteristics of the strain N1<sup>T</sup> are listed in the species description and in Tables 1, 2 and Supplementary Table 1.

The fatty acid profile was dominated by branched and hydroxy fatty acids, like C<sub>16:0</sub> 3OH (13.7%), iso-C<sub>15:0</sub> (13.4%), iso-C<sub>15:0</sub> 3OH (9.3%), iso-C<sub>17:0</sub> 3OH (8.1%), anteiso-C<sub>15:0</sub> (7.5%), C<sub>16:0</sub> (7.5%), C<sub>15:0</sub> 3OH (7.2%) and C<sub>16:1</sub> ω7c and/or iso-C<sub>15:0</sub> 2-OH (summed feature 3)(5.6%) (Table 2). Overall fatty acid composition of strain N1<sup>T</sup> was similar to those of the two reference strains and however, the isolate N1<sup>T</sup> could be clearly distinguished from the reference strains by the absence or low amount of C<sub>15:1</sub> ω6c, iso-C<sub>16:0</sub>, C<sub>17:0</sub> 3OH, C<sub>17:1</sub> ω6c, C<sub>17:1</sub> ω8c, C<sub>20:4</sub> ω6,9,12,15c and summed feature 3 (Table 2). The MK-6 is the sole menaquinone present in N1<sup>T</sup>. Both type strains are also contain MK-6 as sole menaquinone. Homospermidine was the sole polyamine of strain N1<sup>T</sup> in line with all other *Flavobacterium* species which polyamine composition was analyzed (Bernardet & Bowman, 2011; Bernardet *et al.*, 1996; Hosoya & Hamana, 2004; Kaur *et al.*, 2012; Lata *et al.*, 2012). The type strains *F. daejeonense* DSM 17708<sup>T</sup> and *F. glycines* ICMP 17618<sup>T</sup> were also contain homospermidine as sole polyamine. Strain N1<sup>T</sup> contained phosphatidylethanolamine (PE), three unidentified aminolipids (AL1 to AL3) and two unidentified lipids (L1, L2) as total polar lipids (Supplementary Fig. S1). In both type strains also nearly similar composition was observed (Supplementary Fig. S1). DNA G + C content of the strain N1<sup>T</sup> was 36.3 mol% (T<sub>m</sub>) (Table 1).

Phylogenetic relationships of strain N1<sup>T</sup> based on the 16S rRNA gene sequence indicated that strain N1<sup>T</sup> was close to the *F. resistens* with pair-wise sequence similarity of 96.5%. Phylogenetic analysis based on maximum-likelihood and neighbor-joining trees indicated that strain N1<sup>T</sup> clustered with species of the genus *Flavobacterium*, viz. clustered with *F. glycines* and *F. daejeonense* with a distance of 4.8 and 6.0% (95.2 and 94.0% similarity), respectively (Fig. 1).

The main features of the strain N1<sup>T</sup> are in line with the original and emended descriptions of the genus *Flavobacterium* but could be distinguished from published closely related species *F. glycines* and *F. daejeonense* (Tables 1 and 2). Thus, the cumulative differences of strain N1<sup>T</sup> from the closely related type strains unambiguously classify the strain as a new species of the genus *Flavobacterium* for which the name *F. nitratireducens* sp. nov. is proposed.

#### **Description of *Flavobacterium nitratireducens* sp. nov.**

*Flavobacterium nitratireducens* (ni.tra.ti.re.du'cens. N.L. n. nitras -atis, nitrate; L. part. adj. reducens, leading back, bringing back and in chemistry converting to a different oxidation state; N.L. part. adj. *nitratireducens*, reducing nitrate).

Cells are Gram-negative, rod shaped, 0.2-0.3 μm wide and 1.0-1.5 μm long. Cells form chains, multiply by binary fission and are non-motile. Colonies grown for 3 days at 30 °C on R2A plates are circular, 2-3 mm in diameter, smooth, shiny, translucent, yellowish and raised with entire

margin. Flexirubin-type pigment is absent. Grows at 15-37 °C (optimum, 30-37 °C), and at pH 7-9 (optimum, 7.5-8.5). Optimum growth occurred at salinities up to 0-1% (w/v). Positive for ornithine decarboxylase activity and negative for catalase, oxidase, lysine decarboxylase and arginine dihydrolase activities. Indole and H<sub>2</sub>S production is absent. Methyl red and Voges Proskauer's reactions are negative. Nitrate reduction is positive. Hydrolyzed starch but not agar, aesculin, gelatin and Tween 20/40/60/80. Positive reactions (from VITEK 2 GN) in tests for Ala-Phe-Pro-arylamidase, L-pyrrolydonyl-arylamidase,  $\beta$ -N-acetylglucosaminidase, glutamyl arylamidase, L-proline arylamidase, tyrosine arylamidase,  $\alpha$ -glucosidase,  $\beta$ -N-acetylgalactosaminidase, urease,  $\alpha$ -galactosidase, phosphatase, D-glucose, D-maltose, D-mannose, sucrose and D-trehalose; negative reactions were observed for  $\beta$ -galactosidase,  $\gamma$ -glutamyl-transferase,  $\beta$ -glucosidase,  $\beta$ -xylosidase,  $\beta$ -alanine arylamidase, lipase, glycine arylamidase,  $\beta$ -glucoronidase and glu-gly-arg-arylamidase, adonitol, L-arabitol, D-cellobiose, D-mannitol, fermentation of glucose, palatinose, D-sorbitol, D-tagatose, citrate, malonate, 5-keto-D-gluconate, L-histidine, L-lactate, L-malate, L-lactate and succinate alkalisation, courmarate, ELLMAN and 0/129 resistance. Susceptible to antibiotics ( $\mu$ g/disc), kanamycin (30), chloramphenicol (30), lincomycin (2), bacitracin (8 units/disc), polymixin-B (300), gentamycin (10), novobiocin (30), ciprofloxacin (10), cephadoxil (30), chlorotetracycline (30), neomycin (30), penicillin-G (2 units/disc), tetracycline (30), spectinomycin (100) and amoxicillin (30); resistant to ampicillin (25), ceftazidime (3) and vancomycin (30). The predominant fatty acids were C<sub>16:0</sub> 3OH (13.7%), iso-C<sub>15:0</sub> (13.4%), iso-C<sub>15:0</sub> 3OH (9.3%), iso-C<sub>17:0</sub> 3OH (8.1%), anteiso-C<sub>15:0</sub> (7.5%), C<sub>16:0</sub> (7.5%), C<sub>15:0</sub> 3OH (7.2%) and C<sub>16:1</sub>  $\omega$ 7c and/or iso-C<sub>15:0</sub> 2-OH (summed feature 3)(5.6%). The isoprenoid quinone present is MK-6. The polyamine present is homospermidine. The polar lipids consisted of phosphatidylethanolamine, three unidentified aminolipids (AL1-AL3) and two unidentified lipids (L1, L2). The G + C content of the genomic DNA is 36.3 mol%.

The type strain, N1<sup>T</sup> (= MTCC 11155<sup>T</sup> = JCM 17678<sup>T</sup>) was isolated from a marine water sample collected from sea shore, Bay Bengal, Visakhapatnam, India.

### Acknowledgements

We thank Council of Scientific and Industrial Research (CSIR) and Department of Biotechnology, Government of India for financial assistance. We would like to thank Mr. Deepak for his excellent help in sequencing DNA. The laboratory facility was extended by MMRF of NIO, RC, Kochi funded by the Ministry of Earth Sciences, New Delhi. TNRS is thankful to CSIR SIP project (SIP1302) for providing samples, facilities and funding. NIO contribution number is XXXXX.

## References

- Ali, Z., Cousin, S., Frühling, A., Brambilla, E., Schumann, P., Yang, Y. & Stackebrandt, E. (2009).** *Flavobacterium rivuli* sp. nov., *Flavobacterium susaxonicum* sp. nov., *Flavobacterium swingsii* sp. nov. and *Flavobacterium reichenbachii* sp. nov. isolated from a hard water rivulet. *Int J Syst Evol Microbiol* **59**, 2610-2617.
- 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.
- Aslam, Z., Im, W.-T., Kim, M. K. & Lee, S.-T. (2005).** *Flavobacterium granuli* sp. nov., isolated from granules used in a wastewater treatment plant. *Int J Syst Evol Microbiol* **55**, 747-751.
- Bergey, D. H., Harrison, F. C., Breed, R. S., Hammer, B. W. & Huntoon, F. M. (editors) (1923).** Genus II. *Flavobacterium* gen. nov. In *Bergey's Manual of Determinative Bacteriology*, 1st edn, pp. 97-117. Baltimore: Williams & Wilkins.
- Bernardet, J. F. & Bowman, J. P. (2006).** The genus *Flavobacterium*. In *The Prokaryotes*. pp. 481-453. Edited by M. Dworkin, S. Flakow, E. Rosenberg, K. H. Schleifer & E. Stackebrandt. New York: Springer-Verlag.
- Bernardet J.-F. & Bowman J. P. (2011).** Genus I. *Flavobacterium* Bergey et al. 1923. In *Bergey's Manual of Systematic Bacteriology*, 2<sup>nd</sup> ed., Vol. 4, pp. 112-154. Edited by W. Whitman. The Williams & Wilkins Co., Baltimore.
- Bernardet, J. F., Nakagawa, Y. & Holmes, B. (2002).** Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. *Int J Syst Evol Microbiol* **52**, 1049-1070.
- Bernardet, J. F., Segers, P., Vancanneyt, M., Berthe, F., Kersters, K. & Vandamme, P. (1996).** Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov. (basonym, *Cytophaga aquatilis* Strohl and Tait 1978). *Int J Syst Bacteriol* **46**, 128-148.
- Bligh, E. G. & Dyer, W. J. (1959).** A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**, 911-917.
- Busse, H. J. & Auling, G. (1988).** Polyamine pattern as chemotaxonomic marker within *Proteobacteria*. *Syst Appl Microbiol* **11**, 1-8.
- Busse, H.-J., Bunka, S., Hensel, A. & Lubitz, W. (1997).** Discrimination of members of the family *Pasteurellaceae* based on polyamine patterns. *Int J Syst Bacteriol* **47**, 698-708.

- 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., Pirouz, T., Goodfellow, M. & Minnikin, D. E. (1977).** Distribution of menaquinones in actinomycetes and corynebacteria. *J Gen Microbiol* **100**, 221-230.
- Cousin, S., Pauker, O. & Stackebrandt, E. (2007).** *Flavobacterium aquidurense* sp. nov. and *Flavobacterium hercynium* sp. nov., from a hard-water creek. *Int J Syst Evol Microbiol* **57**, 243-249.
- Dong, K., Liu, H., Zhang, J., Zhou, Y. & Xin, Y. (2012).** *Flavobacterium xueshanense* sp. nov. and *Flavobacterium urumqiense* sp. nov., two psychrophilic bacteria isolated from glacier ice. *Int J Syst Evol Microbiol* **62**, 1151-1157.
- Felsenstein, J. (1985).** Confidence limits on phylogenies: An approach using the bootstrap. *Evol* **39**, 783-791.
- Groth, I., Schumann, P., Rainey, F. A., Martin, K., Schuetze, B. & Augsten, K. (1997).** *Demetria terragensis* gen. nov., sp. nov., a new genus of actinomycetes isolated from compost soil. *Int J Syst Bacteriol* **47**, 1129-1133.
- Hosoya, R. & Hamana, K. (2004).** Distribution of two triamines, spermidine and homospermidine, and an aromatic amine, 2-phenylethylamine, within the phylum *Bacteroidetes*. *J Gen Appl Microbiol* **50**, 255-260.
- Humphry, D. R., George, A., Black, G. W. & Cummings, S. P. (2001).** *Flavobacterium frigidarium* sp. nov., an aerobic psychrophilic, xylanolytic and laminarinolytic bacterium from Antarctica. *Int J Syst Evol Microbiol* **51**, 1235-1243.
- Kämpfer, P., Lodders, N., Martin, K. & Avendano-Herrera, R. (2012).** *Flavobacterium chilense* sp. nov. and *Flavobacterium araucanum* sp. nov., isolated from farmed salmonid fish. *Int J Syst Evol Microbiol* **62**, 1402-1408.
- Kaur, I., Kaur, C., Khan, F. & Mayilraj, S. (2012).** *Flavobacterium rakeshii* sp. nov., isolated from marine sediment and emended description of *Flavobacterium beibuense* Fu et al. 2011. *Int J Syst Evol Microbiol* doi:10.1099/ijs.0.035691-0.
- Kim, O.S., Cho, Y.J., Lee, K., Yoon, S.H., Kim, M., Na, H., Park, S.C., Jeon, Y.S., Lee, J.H., Yi, H., Won, S. & Chun, J. (2012).** Introducing EzTaxon-e: a prokaryotic 16S rRNA Gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* **62**, 716-721.
- Kim J. H., Kim K. Y. & Cha C. J. (2009).** *Flavobacterium chungangense* sp. nov., isolated from a freshwater lake. *Int J Syst Evol Microbiol* **59**, 1754-1758.



- Kim, B. Y., Weon, H. Y., Cousin, S., Yoo, S. H., Kwon, S. W., Go, S. J. & Stackebrandt, E. (2006).** *Flavobacterium daejeonense* sp. nov. and *Flavobacterium suncheonense* sp. nov., isolated from greenhouse soils in Korea. *Int J Syst Evol Microbiol* **56**, 1645-1649.
- Komagata, K. & Suzuki, K. (1987).** Lipid and cell wall analysis in bacterial systematics. *Methods Microbiol* **19**, 161-206.
- Lányi, B. (1987).** Classical and rapid identification methods for medically important bacteria. *Methods Microbiol* **19**, 1-67.
- Lata, P., Lal, D. & Lal, R. (2012).** *Flavobacterium ummariense* sp. nov., isolated from hexachlorocyclohexane (HCH) contaminated soil and emended description of *Flavobacterium ceti* Vela et al. 2007. *Int J Syst Evol Microbiol* **62**, 2674-2679.
- Madhaiyan, M., Poonguzhali, S., Lee, J. S., Lee, K. C. & Sundaram, S. (2010).** *Flavobacterium glycines* sp. nov., a facultative methylotroph isolated from the rhizosphere of soybean. *Int J Syst Evol Microbiol* **60**, 2187-2192.
- Marmur, J. (1961).** A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208-218.
- McCammon, S. A. & Bowman, J. P. (2000).** Taxonomy of Antarctic *Flavobacterium* species: description of *Flavobacterium gillisiae* sp. nov., *Flavobacterium tegetincola* sp. nov. and *Flavobacterium xanthum* sp. nov., nom. rev. and reclassification of [*Flavobacterium*] *salegens* as *Salgentibacter salegens* gen. nov., comb. nov. *Int J Syst Evol Microbiol* **50**, 1055-1063
- McCammon, S. A., Innes, B. H., Bowman, J. P., Franzmann, P. D., Dobson, S. J., Holloway, P. E., Skerratt, J. H., Nichols, P. D. & Rankin, L. M. (1998).** *Flavobacterium hibernum* sp. nov., a lactose-utilizing bacterium from a freshwater Antarctic lake. *Int J Syst Bacteriol* **48**, 1405-1412.
- Nei, M. & Kumar, S. (2000).** *Molecular Evolution and Phylogenetics*. NY: Oxford University Press.
- Nogi, Y., Soda, K. & Oikawa, T. (2005).** *Flavobacterium frigidimaris* sp. nov., isolated from Antarctic seawater. *Syst Appl Microbiol* **28**, 310-315.
- Ryu, S. H., Park, J. H., Moon, J. C., Sung, Y., Lee, S. S. & Jeon, C. O. (2008).** *Flavobacterium resistens* sp. nov., isolated from stream sediment. *Int J Syst Evol Microbiol* **58**, 2266-2270.
- Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406-425
- Sly, L. I., Blackall, L. L., Kraat, P. C., Tao, T.-S. & 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.

- Smibert, R. M. & Krieg, N. R. (1994).** Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607-654. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Tamaki, H., Hanada, S., Kamagata, Y., Nakamura, K., Nomura, N., Nakano, K. & Matsumura, M. (2003).** *Flavobacterium limicola* sp. nov., a psychrophilic, organic-polymer-degrading bacterium isolated from freshwater sediments. *Int J Syst Evol Microbiol* **53**, 519-526.
- Tamura, K. & Nei, M. (1993).** Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* **10**, 512-526.
- 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.
- Van Trappen, S., Mergaert, J. & Swings, J. (2003).** *Flavobacterium gelidilacus* sp. nov., isolated from microbial mats in Antarctic lakes. *Int J Syst Evol Microbiol* **53**, 1241-1245.
- Van Trappen, S., Vandecandelaere, I., Mergaert, J. & Swings, J. (2004).** *Flavobacterium degerlachei* sp. nov., *Flavobacterium frigoris* sp. nov. and *Flavobacterium micromati* sp. nov., novel psychrophilic bacteria isolated from microbial mats in Antarctic lakes. *Int J Syst Evol Microbiol* **54**, 85-92.
- Van Trappen, S., Vandecandelaere, I., Mergaert, J. & Swings, J. (2005).** *Flavobacterium fryxellicola* sp. nov. and *Flavobacterium psychrolimnae* sp. nov., novel psychrophilic bacteria isolated from microbial mats in Antarctic lakes. *Int J Syst Evol Microbiol* **55**, 769-772.
- Vandamme, P., Pot, B., Gillis, M., de Vos, P., Kersters, K. & Swings, J. (1996).** Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol Rev* **60**, 407-438.
- Xin, Y. H., Liang, Z. H., Zhang, D. C., Liu, H. C., Zhang, J. L., Yu, Y., Xu, M. S., Zhou, P. J. & Zhou, Y. G. (2009).** *Flavobacterium tiangeerense* sp. nov., a cold-living bacterium isolated from a glacier. *Int J Syst Evol Microbiol* **59**, 2773-2777.
- Yi, H., Oh, H.-M., Lee, J.-H., Kim, S.-J. & Chun, J. (2005).** *Flavobacterium antarcticum* sp. nov., a novel psychrotolerant bacterium isolated from the Antarctic. *Int J Syst Evol Microbiol* **55**, 637-641.
- Zhu, F., Wang, S. & Zhou, P. J. (2003).** *Flavobacterium xinjiangense* sp. nov. and *Flavobacterium omnivorum* sp. nov., novel psychrophiles from the China No. 1 glacier. *Int J Syst Evol Microbiol* **53**, 853-857.

**Legends to Figures**

**Fig. 1.** Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain N1<sup>T</sup> and closely related members of the family *Flavobacteriaceae*. Numbers at the nodes are bootstrap values >50%. Bootstrap values (>50%) based on 1000 resamplings are shown for the neighbour-joining, maximum-likelihood and maximum-parsimony methods. *Myroides odoratus* ATCC 4651<sup>T</sup> (M58777) was used as an outgroup. Bar, 0.01 substitution per nucleotide position.

**Supplementary Fig. S1.** Two-dimensional thin-layer chromatogram of the total polar lipids of the strain N1<sup>T</sup> (a), *F. daejeonense* DSM 17708<sup>T</sup> (b) and *F. glycines* ICMP 17618<sup>T</sup> (c) after spraying the plates with molybdotophosphoric acid. Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; L, unidentified lipid; AL, unidentified aminolipid. The spots identified as phospho-, amino- or glyco-lipids by spraying molybdenum blue, ninhydrin and  $\alpha$ -naphthol reagents, respectively.

**Table 1.** Features that distinguish strain N1<sup>T</sup> from the closely related species *F. daejeonense* and *F. glycines*

Data were from present study. All strains were rod-shaped, yellow-pigmented, positive for  $\alpha$ -glucosidase and D-glucose and sucrose utilization. All strains were negative for indole production, methyl red and Voges Proskauer's reactions, agar and Tween 20/40/60/80 hydrolysis, lysine decarboxylase, arginine dihydrolase,  $\gamma$ -glutamyl-transferase,  $\beta$ -xylosidase,  $\beta$ -alanine arylamidase, glycine arylamidase,  $\beta$ -glucuronidase and glu-gly-arg-arylamidase activities, L-lactate and succinate alkalisation, palatinose, adonitol, L-arabitol, D-tagatose, malonate, 5-keto-D-gluconate, L-histidine, L-malate and L-lactate, coumarate, ELLMAN and 0/129 resistance. +, positive; -, negative. All strains were susceptible to antibiotics ( $\mu$ g/disc) gentamicin (10), ciprofloxacin (10), cephadroxil (30), chlortetracycline (30), tetracycline (30), neomycin (30) and amoxicillin (30); resistant to ceftazidime (3). +, positive; -, negative; S, sensitive; R, resistance; PE, phosphatidylethanolamine; AL, unidentified aminolipid; L, unidentified lipid.

Characteristic	<i>Flavobacterium nitratireducens</i> N1 <sup>T</sup>	<i>Flavobacterium daejeonense</i> DSM 17708 <sup>T</sup>	<i>Flavobacterium glycines</i> ICMP 17618 <sup>T</sup>
<b>Vitek 2 GN tests:</b>			
Ala-Phe-Pro-arylamidase	+	+	-
L-pyrrolydonyl-arylamidase	+	-	-
$\beta$ -N-acetylglucosaminidase	+	-	-
glutamyl arylamidase	+	-	-
L-proline arylamidase	+	-	-
tyrosine arylamidase	+	-	-
Urease	+	-	-
$\beta$ -N-acetylgalactosaminidase	+	-	-
<b>Susceptibility to Antibiotics (<math>\mu</math>g/disc):</b>			
Chloramphenicol (30)	S	S	R
Lincomycin (2)	S	S	R
Bacitracin (8units/disc)	S	R	R
Polymyxin-B (300)	S	R	R
Novobiocin (30)	S	R	R
Spectinomycin (100)	S	S	R
Penicillin-G (2units/disc)	S	R	R
Major fatty acids	C <sub>15:0</sub> 3OH, iso-C <sub>15:0</sub> , anteiso-C <sub>15:0</sub> , iso-C <sub>15:0</sub> 3OH, C <sub>16:0</sub> , C <sub>16:0</sub> 3OH, iso-C <sub>17:0</sub> 3OH and SF 3	iso-C <sub>15:0</sub> , anteiso-C <sub>15:0</sub> , iso-C <sub>15:0</sub> 3OH, C <sub>15:1</sub> $\omega$ 6c, iso-C <sub>15:1</sub> G, C <sub>16:0</sub> , C <sub>16:0</sub> 3OH, iso-C <sub>17:0</sub> 3OH and SF 3	C <sub>15:0</sub> 3OH, iso-C <sub>15:0</sub> , anteiso-C <sub>15:0</sub> , iso-C <sub>15:0</sub> 3OH, C <sub>15:1</sub> $\omega$ 6c, C <sub>16:0</sub> , C <sub>16:0</sub> 3OH, iso-C <sub>17:0</sub> 3OH and SF 3
Polar lipids	PE, AL1-AL3, L1, L2	PE, AL1-AL3, L1, L2	PE, AL2, AL3, L1, L2
Habitat	Marine water	<sup>a</sup> Greenhouse soil	<sup>a</sup> Rhizosphere of field-grown soybean

SF 3, Summed feature 3. \*Summed features are groups of two or three fatty acids that could not be separated by GC with the MIDI system. Summed feature 3 comprised C<sub>16:1</sub>  $\omega$ 7c and/or iso-C<sub>15:0</sub> 2-OH

**Table 2.** Fatty acid composition of the strain N1<sup>T</sup>, *F. daejeonense* DSM 17708<sup>T</sup> and *F. glycines* ICMP 17618<sup>T</sup>

Results are presented as a percentage of the total fatty acids. Fatty acids amounting to 5% or more of the total fatty acids are in bold. Data were from present study. ND, not detected. The strains were grown on R2A plates at 30°C for three days. ND, not detected. Values of less than 1% for all strains are not shown. tr, traces (<1%). Data in the parenthesis is taken from Kim *et al.*, 2006 and Madhaiyan *et al.*, 2010.

Fatty acid	<i>Flavobacterium nitratireducens</i> N1 <sup>T</sup>	<i>Flavobacterium daejeonense</i> DSM 17708 <sup>T</sup>	<i>Flavobacterium glycines</i> ICMP 17618 <sup>T</sup>
C <sub>13:1</sub> at 12-13	1.3	tr (ND)	tr (ND)
C <sub>14:0</sub>	2.2	1.1 (1.0)	2.6 (3.0)
iso-C <sub>14:0</sub>	1.4	tr (tr)	1.0 (1.9)
C <sub>15:0</sub> 2OH	1.0	tr (tr)	tr (1.3)
C <sub>15:0</sub> 3OH	<b>7.2</b>	3.6 (2.6)	<b>6.2</b> (2.8)
iso-C <sub>15:0</sub>	<b>13.4</b>	<b>13.4</b> (20.1)	<b>12.9</b> (15.5)
anteiso-C <sub>15:0</sub>	<b>7.5</b>	4.8 (5.2)	<b>6.4</b> (9.2)
iso-C <sub>15:0</sub> 3OH	<b>9.3</b>	<b>6.1</b> (8.6)	<b>5.4</b> (6.4)
C <sub>15:1</sub> ω6c	ND	4.6 (6.0)	<b>5.2</b> (5.9)
iso-C <sub>15:1</sub> G	3.2	<b>6.3</b> (5.1)	2.5 (3.3)
iso-C <sub>15:1</sub> ω6c	1.0	ND (ND)	ND (ND)
C <sub>16:0</sub>	<b>7.5</b>	<b>10.2</b> (6.5)	<b>6.2</b> (6.2)
C <sub>16:0</sub> 3OH	<b>13.7</b>	<b>8.1</b> (7.5)	<b>12.3</b> (11.2)
iso-C <sub>16:0</sub>	ND	tr (tr)	1.0 (tr)
iso-C <sub>16:0</sub> 3OH	4.4	1.7 (1.2)	3.5 (3.8)
C <sub>17:0</sub> 2OH	1.5	1.0 (ND)	1.7 (ND)
C <sub>17:0</sub> 3OH	tr	2.1 (1.2)	ND (1.4)
iso-C <sub>17:0</sub> 3OH	<b>8.1</b>	<b>10.1</b> (9.4)	<b>6.2</b> (4.5)
anteiso-C <sub>17:0</sub>	tr	tr (ND)	1.0 (ND)
C <sub>17:1</sub> ω6c	tr	1.3 (2.6)	1.7 (2.5)
C <sub>17:1</sub> ω8c	ND	1.3 (tr)	tr (tr)
C <sub>18:0</sub>	1.5	1.0 (ND)	2.3 (ND)
C <sub>18:0</sub> 10-methyl, TBSA	1.9	ND (ND)	ND (ND)
C <sub>18:1</sub> 2OH	ND	1.3 (ND)	ND (ND)
C <sub>18:1</sub> ω9c	ND	1.6 (ND)	ND (ND)
C <sub>18:3</sub> ω6,9,12c	tr	tr	3.1 (ND)
C <sub>20:4</sub> ω6,9,12,15c	ND	1.3 (ND)	2.3 (ND)
Summed feature 2	1.4	1.1 (1.0)	1.4 (1.1)
Summed feature 3	<b>5.6</b>	<b>9.4</b> (17.2)	<b>11.8</b> (15.8)
Summed feature 8	tr	1.7	ND (ND)
Summed feature 9	ND	1.5	ND (ND)

Summed features are groups of two or three fatty acids that could not be separated by GC with the MIDI system. Summed feature 2 comprised C<sub>12:0</sub> ALDE and/or unknown ECL 10.928; summed feature 3 comprised C<sub>16:1</sub> ω7c and/or iso-C<sub>15:0</sub> 2-OH; Summed feature 8 comprised C<sub>18:1</sub> ω7c and/or C<sub>18:1</sub> ω6c; Summed feature 9 comprised iso-C<sub>17:1</sub> ω9c and/or C<sub>16:0</sub> 10-methyl.

**Supplementary Table 1.** Features that distinguish strain N1<sup>T</sup> from the phylogenetically related species of the genus *Flavobacterium*

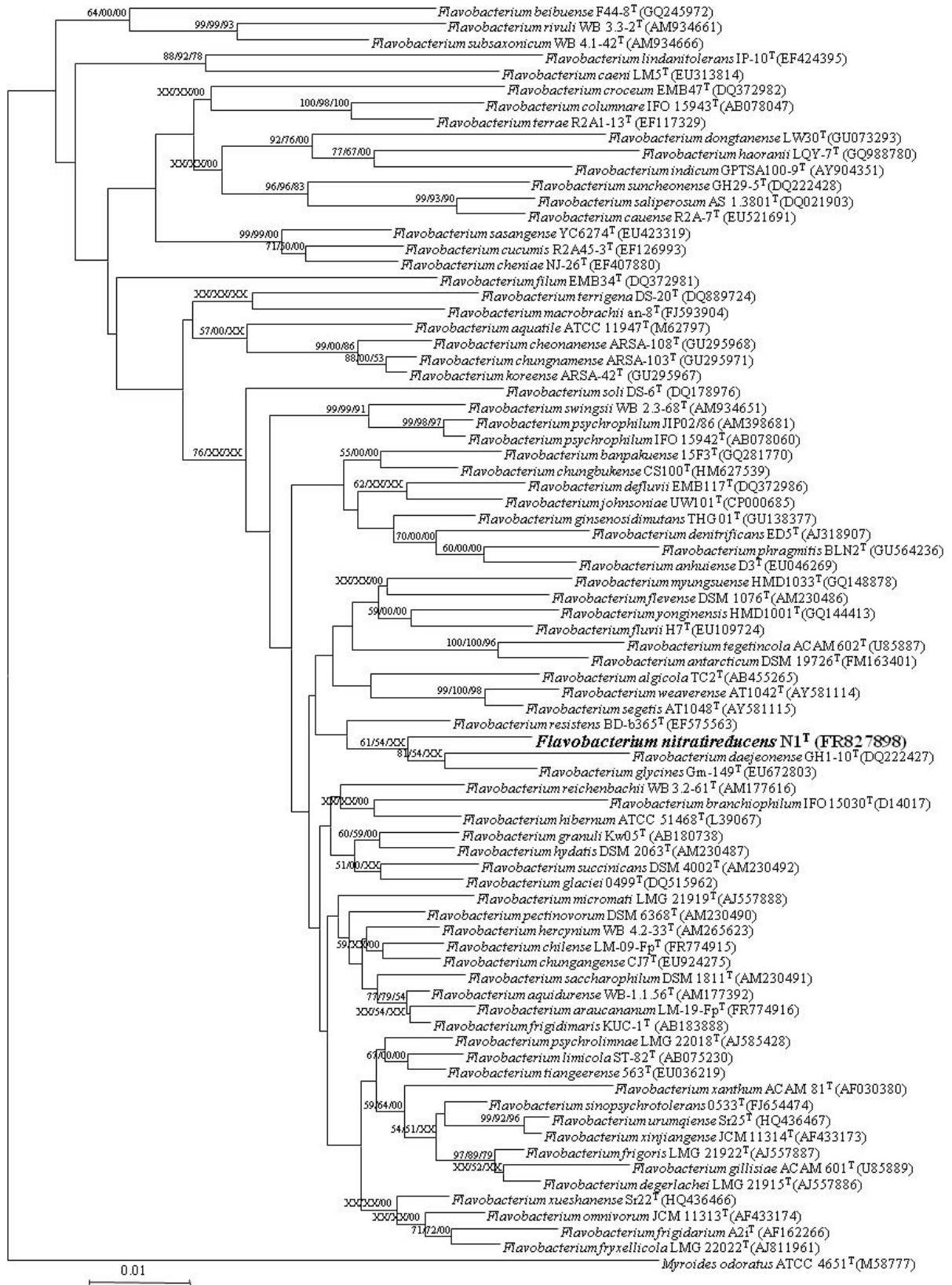
Taxa, 1. Strain N1<sup>T</sup> (*F. nitratireducens* sp. nov.); 2, *F. daejeonense* DSM 17708<sup>T</sup>; 3, *F. glycines* ICMP 17618<sup>T</sup> (Data for taxa 1-3 were from present study); 4, *F. resistens* BD-b365<sup>T</sup> (Data taken from Ryu *et al.*, 2008); 5, *F. tiangeerense* 0563<sup>T</sup> (Data taken from Xin *et al.*, 2009); 6, *F. xueshanense* Sr22<sup>T</sup> (Data taken from Dong *et al.*, 2012); 7, *F. pectinovorum* ATCC 19366<sup>T</sup> (Data taken from Bernardet *et al.*, 1996); 8, *F. hibernum* ATCC 51468<sup>T</sup> (Data taken from McCammon *et al.*, 1998); 9, *F. omnivorum* JCM 11313<sup>T</sup> (Data taken from Zhu *et al.*, 2003); 10, *F. micromati* LMG 21919<sup>T</sup> (Data taken from Van Trappen *et al.*, 2004); 11, *F. frigidimaris* KUC-1<sup>T</sup> (Data taken from Nogi *et al.*, 2005); 12, *F. granuli* Kw05<sup>T</sup> (Data taken from Aslam *et al.*, 2005); 13, *F. chilense* LM-09-Fp<sup>T</sup> (Data taken from Kämpfer *et al.*, 2012); 14, *F. reichenbachii* WB 3.2-61<sup>T</sup> (Data taken from Ali *et al.*, 2009); 15, *F. aquidurensis* WB-1.1.56<sup>T</sup> (Data taken from Cousin *et al.*, 2007); 16, *F. chungangense* CJ7<sup>T</sup> (Data taken from Kim *et al.*, 2009); 17, *F. fryxellicola* LMG 22022<sup>T</sup> (Data taken from Van Trappen *et al.*, 2005); 18, *F. limicola* ST-82<sup>T</sup> (Data taken from Tamaki *et al.*, 2003).

All strains were rod-shaped, yellow-pigmented. +, positive; -, negative; ND, data not available; w, weak growth.

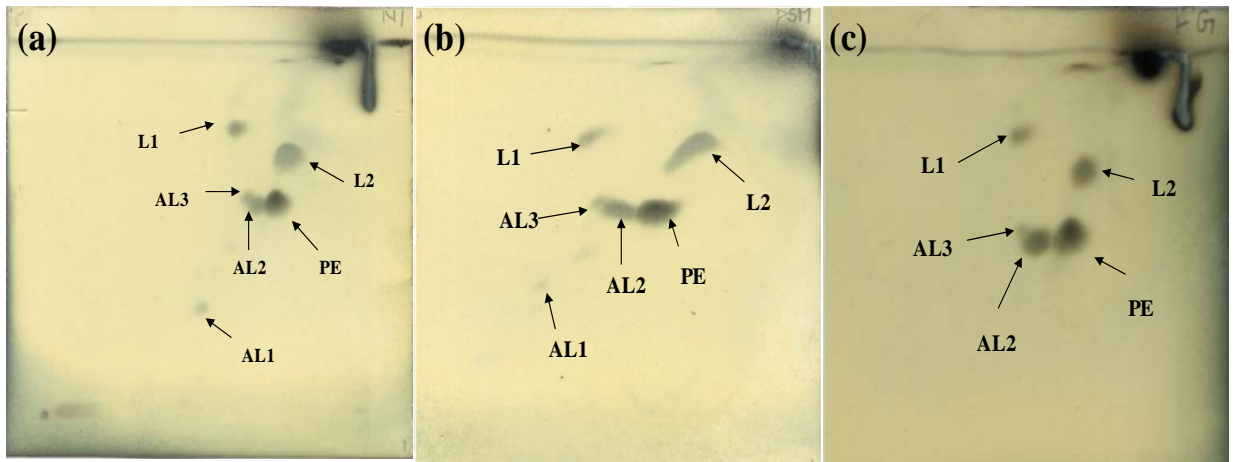
Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Gliding motility	-	-	+	+	-	-	+	+	-	-	-	-	+	+	-	-	-	-
Flexirubin-type pigment	-	-	+	+	-	-	-	+	-	-	+	-	+	+	+	-	-	-
NaCl growth range (% w/v) (optimum)	0-1 (0-1)	0-3 (0-1)	0-1 (0)	0-2 (0-0.5)	0-0.5 (0)	0-0.5 (0)	0-1 (0)	ND	0-3.5 (0)	0-2 (0)	0-3 (0)	0-2 (0-1)	0-3	0-2 (0)	0-1 (0)	0-4 (0)	0-2 (0)	0-1.5 (0)
Growth temperature range (°C) (optimum)	15-37 (30-37)	5-37 (30)	10-37 (28-30)	15-40 (20-30)	4-26 (22-23)	2-18 (14)	20-40 (20-25)	4-31 (26)	ND (11)	5-25 (ND)	2-26 (18)	15-37 (25-30)	4-30 (25-28)	6-30 (20-26)	13-30 (19-28)	5-35 (25)	5-25 (20)	0-25 (15-20)
pH growth range (optimum)	7-9 (7.5-8.5)	6-8 (7)	6-8 (7-7.5)	6-9.5 (7-8)	6.5-9 (7-8)	6-9 (7)	5-10 (ND)	ND	ND	ND	ND	ND	6.5-8.5 (7-8)	5.8-8 (6.2)	6-6.6 (6.4)	5-8 (6)	ND	ND
Oxidase	-	+	w	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+
Catalase	-	+	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+
Nitrate reduction	+	+	+	-	-	-	+	+	+	-	-	-	ND	-	-	-	-	-
H <sub>2</sub> S production	-	-	-	ND	-	-	-	-	-	-	-	-	-	w	ND	-	-	-
Ornithine decarboxylase	+	+	-	-	-	ND	ND	-	-	-	ND	-	-	ND	ND	ND	-	-
β-galactosidase	-	-	+	+	-	-	ND	ND	ND	-	ND	+	ND	+	+	-	-	-
Phosphatase	+	-	-	+	+	+	+	ND	ND	+	ND	ND	ND	+	+	+	+	+
α-glucosidase	+	+	+	+	ND	-	ND	ND	ND	+	ND	ND	ND	-	+	-	w	-
α-galactosidase	+	+	+	w	-	-	ND	ND	ND	-	ND	ND	ND	+	-	-	w	+
β-glucosidase	-	-	-	-	-	-	ND	ND	ND	-	ND	+	ND	-	-	-	-	+
Lipase	-	-	-	w	+	-	ND	ND	ND	-	ND	ND	ND	-	-	-	-	+
Fermentation glucose	-	-	-	-	-	+	+	+	-	-	+	-	+	-	ND	-	-	-

<b>Hydrolysis of:</b>																		
Aesculin	-	-	-	+	+	+	ND	+	+	+	+	+	+	+	+	+	+	+
Gelatin	-	-	-	-	-	-	+	+	-	-	+	-	+	+	-	+	-	+
Starch	+	+	+	+	-	-	+	+	+	-	ND	-	+	+	+	+	-	+
<b>Utilization of:</b>																		
D-maltose	+	-	+	+	-	+	+	ND	-	-	+	+	+	+	+	-	+	+
D-mannose	+	-	+	-	(+)	+	ND	+	+	-	+	+	+	w	+	-	-	+
D-trehalose	+	-	+	ND	-	-	ND	+	+	ND	+	ND	+	+	-	ND	ND	ND
D-cellobiose	-	-	-	ND	+	ND	+	+	+	ND	+	ND	+	+	-	ND	ND	ND
D-mannitol	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	ND
D-sorbitol	-	-	-	ND	-	ND	ND	+	-	ND	-	-	-	-	-	ND	ND	ND
Citrate	-	-	-	-	-	-	ND	-	-	ND	ND	ND	-	-	-	-	-	-
DNA G + C content (mol%)	36.3	<sup>a</sup> 38.7	<sup>a</sup> 35.6	35.4	34.8	37.2	34.0	34.0	35.0	33.0	34.5	36.2	ND	34.3	33.5	34.5	35.0	34.0

<sup>a</sup>Data taken from Kim *et al.*, 2006 and Madhaiyan *et al.*, 2010.





**Fig. 1****Supplementary Fig. S1**