Pseudovibrio japonicus sp. nov., isolated from coastal seawater in Japan

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A Gram-negative, motile, rod-shaped bacterium (WSF2^T) was isolated from coastal seawater of the Boso Peninsula in Japan. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain WSF2^T represented a separate lineage within the genus *Pseudovibrio*. The DNA G + C content of strain WSF2^T was 51.7 mol%. DNA–DNA hybridization values between strain WSF2^T and the type strains of *Pseudovibrio* species were significantly lower than those accepted as the phylogenetic definition of a species. Furthermore, some biochemical characteristics indicated that strain WSF2^T differed from other *Pseudovibrio* species. Based on these characteristics, it is proposed that the isolate represents a novel species, *Pseudovibrio japonicus* sp. nov. The type strain is WSF2^T (=IAM 15442^T=NCIMB 14279^T=KCTC 12861^T).

The genus *Pseudovibrio* was proposed by Shieh *et al.* (2004) and originally contained one species, *Pseudovibrio denitrificans*, isolated from seawater in Taiwan. A second species, *Pseudovibrio ascidiaceicola*, isolated from a sea squirt, was described recently by Fukunaga *et al.* (2006). This genus contains halophilic, facultatively anaerobic bacteria that are motile by means of one to several lateral or subpolar flagella. Phylogenetic analyses based on 16S rRNA gene sequences have shown that the genus *Pseudovibrio* falls within the *Alphaproteobacteria* and is closely related to the genera *Pannonibacter*, *Roseibium* and *Stappia* (Shieh *et al.*, 2004; Fukunaga *et al.*, 2006).

In this study, the taxonomic position of strain WSF2^T, which was isolated from surface seawater off the coastline of Nojimazaki in the Boso Peninsula, Chiba Prefecture, Japan, was examined. A seawater sample (0.05 ml) was spread onto a plate containing modified SP5 agar [0.5 × artificial seawater (ASW; 1.5 % NaCl, 0.035 % KCl, 0.54 % MgCl₂ · 6H₂O, 0.27 % MgSO₄ · 7H₂O and 0.05 % CaCl₂ · 2H₂O), 0.9 % casitone, 0.1 % extract bonito (Wako Pure Chemical Industries) and 1.5 % agar] and incubated at 25 °C for a week. Strain WSF2^T was isolated using the dilution-plating technique at 25 °C. This isolate was maintained at 25 °C on marine agar 2216 (MA; Difco).

The 16S rRNA gene sequence was analysed as described by Hosoya *et al.* (2006). Sequences were edited and assembled using the program BIOEDIT (Hall, 1999). The most closely related sequences were found using the program BLAST of

the GenBank database (Altschul *et al.*, 1990). Multiple alignments were performed using the program CLUSTAL_X (version 1.83; Thompson *et al.*, 1997). Nucleotide substitution rates (K_{nuc} ; Kimura, 1980) were determined. Phylogenetic trees were constructed using the neighbour-joining method (Saitou & Nei, 1987) with CLUSTAL_X and the maximum-parsimony method (Fitch, 1971) with the software MEGA3 (Kumar *et al.*, 2004). Alignment gaps and unidentified base positions were not taken into consideration in the calculation. The topology of the phylogenetic trees was evaluated by performing a bootstrap analysis with 1000 bootstrapped trials. The dendrogram obtained by maximum-parsimony analysis showed essentially the same topography as data determined by neighbour-joining (data not shown). Phylogenetic analysis based on 16S rRNA gene sequences showed that strain WSF2^T falls into the genus *Pseudovibrio* (Fig. 1); 16S rRNA gene sequence similarity values with the type strains of *P. ascidiaceicola* and *P. denitrificans* were 98.9 and 98.3 %, respectively. For analysis of genetic relatedness, DNA–DNA hybridization was carried out at 45 °C for 4 h and measured fluorometrically using the method of Ezaki *et al.* (1989). Strain WSF2^T showed relatively low DNA–DNA relatedness values to *P. ascidiaceicola* IAM 15084^T (14.1–32.0 %) and *P. denitrificans* JCM 12308^T (34.9–35.4 %). These values are significantly lower than that accepted as the phylogenetic definition of a species (Wayne *et al.*, 1987).

For determination of the G + C content, DNA was extracted by the method of Saito & Miura (1963) and G + C content was determined by the method of Mesbah *et al.* (1989). The G + C content of strain WSF2^T was 51.7 mol%; those of the reference strains *P. ascidiaceicola* IAM 15084^T and *P. denitrificans* JCM 12308^T were 52.1 and 51.9 mol%, respectively.

Abbreviation: ASW, artificial seawater.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain WSF2^T (=IAM 15442^T=NCIMB 14279^T=KCTC 12861^T) is AB246748.

Fig. 1. Neighbour-joining phylogenetic tree of *Pseudovibrio japonicus* and other related genera of Alphaproteobacteria based on 16S rRNA gene sequence analysis. Bootstrap values greater than 500 are given at branch points. Bar, 0.01 K_{nuc} .

Phenotypic characteristics were determined as follows. Cells were grown for 48 h at 25 °C on MA and observed by TEM after negative staining with uranyl acetate. Gram staining was performed using the Hucker staining method described by Smibert & Krieg (1994). Spore staining was performed using the Schaeffer & Fulton staining method described by Barrow & Feltham (1993). Salt tolerance was tested on R2A agar (Difco) supplemented with 0–10 % NaCl. Growth at different temperatures (8–37 °C) and pH values (range pH 5.0–9.5), and cytochrome oxidase and catalase activities were determined by methods described previously (Hosoya *et al.*, 2006). Hydrolysis of DNA was determined on DNA agar (Nissui) supplemented with 0.5 × ASW by the discoloration of toluidine blue. Degradation of starch was tested on SP5 medium containing 0.2 % soluble starch by the method of Smibert & Krieg (1994). Acid production from carbon sources was tested on Leifson modified O/F medium incubated at 25 °C for a week by the method of Smibert & Krieg (1994). Hydrolysis of Tweens (20, 40, 60 and 80) was tested on modified Tween 80 medium [containing 0.1 % peptone, 0.5 × ASW, 1 % (v/v) Tweens and 2 % agar] by the method of Barrow & Feltham (1993). Degradation of L-tyrosine was tested on nutrient agar (Difco) containing 0.5 % L-tyrosine supplemented with 0.5 × ASW by the method of Barrow & Feltham (1993). Growth under anaerobic conditions was tested on MA at 25 °C for a week in an AnaeroPack (Mitsubishi Gas Chemical). Tests with the commercial systems API ZYM and API 20E (bioMérieux) were generally performed according to the manufacturer's instructions. Cells for inoculation to the API test strips were suspended in 0.5 × ASW. The API ZYM tests were read after 4 h incubation at 25 °C and API 20E tests were

read after 48 h at 25 °C. API 20E data pertaining to acid production from sugars were not utilized because reactions were weak. For analysis of cellular fatty acids, cells were grown for 48 h at 25 °C on trypticase soy agar (BBL) supplemented with 0.5 × ASW and then analysed by using the GC-based Microbial Identification system (MIDI).

Strain WSF2^T was a Gram-negative, facultatively anaerobic, non-sporulating bacterium. It formed beige colonies. Cells were approximately 2.0–4.0 µm long by 0.8–1.0 µm wide and motile by lateral or subpolar flagella (Fig. 2). Phenotypic characteristics are given in the species description. Phenotypically, strain WSF2^T, *P. ascidiaceicola* IAM 15084^T and *P. denitrificans* JCM 12308^T differed from each other in their chymotrypsin and α-glucosidase activities and their ability to produce acid from arabinose, galactose, inositol, maltose, raffinose and trehalose. The major cellular fatty acid of strain WSF2^T, *P. ascidiaceicola* IAM 15084^T and *P. denitrificans* JCM 12308^T was 18:1ω7c, although present in different amounts, but strain WSF2^T also contained 16:0 3-OH, which was not detected in *P. ascidiaceicola* or *P. denitrificans* (Table 1).

On the basis of DNA–DNA hybridization, phylogenetic analysis and phenotypic characteristics, strain WSF2^T is proposed as a novel species of the genus *Pseudovibrio*, *Pseudovibrio japonicus* sp. nov.

Description of *Pseudovibrio japonicus* sp. nov.

Pseudovibrio japonicus (ja.po'ni.cus. N.L. masc. adj. *japonicus* pertaining to Japan, where the isolate originated).

Cells are Gram-negative, facultatively anaerobic, rod-shaped, approximately 2.0–4.0 × 0.8–1.0 µm. Motile by

Fig. 2. TEM of a negatively stained cell of strain WSF2^T. Bar, 1 μ m.

lateral or subpolar flagella. Cytochrome oxidase- and catalase-positive. Optimal growth temperature is 25–30 °C; grows at 15 °C, but not at 8 or 35 °C. The pH range for growth is 6.0–9.0. Growth occurs at NaCl concentrations of 1–6 %; no growth occurs at 7 % NaCl. No growth occurs in the absence of NaCl. Positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and β -galactosidase. Positive for degradation of DNA, gelatin

Table 1. Fatty acid profiles of *Pseudovibrio* species

Strains: 1, *P. japonicus* strain WSF2^T; 2, *P. ascidiaceicola* IAM 15084^T; 3, *P. denitrificans* JCM 12308^T. Values are percentages (w/w) of total fatty acids. —, Not detected or trace amount (<1.0 %).

Fatty acid	1	2	3
16:0	8.1	1.3	7.1
16:0 3-OH	2.0	—	—
17:0	—	—	1.0
18:1 ω 7c	80.2	74.6	83.0
18:0	2.0	8.8	1.7
19:0 cyclo ω 8c	—	3.9	—
18:0 3-OH	—	2.3	1.4
Summed feature 2*	2.3	2.2	1.9
Summed feature 3†	1.9	2.2	1.3

*Summed feature 2 contains 14:0 3-OH and/or 16:1 iso I.

†Summed feature 3 contains 15:0 iso 2-OH and/or 16:1 ω 7c.

and Tweens (20, 40, 60 and 80). Indole is produced from tryptophan. Tyrosine is degraded. Nitrate is reduced to nitrogen (API 20E). Acid is produced by fermentation from cellobiose, fructose, glucose, mannose, sucrose and xylose. Acid is produced by oxidation from glycerol. Does not decompose citrate, starch or urea. Does not produce acetoin or H₂S. Negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, lipase (C4), cystine arylamidase, chymotrypsin, α -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. Does not produce acid from arabinose, dulcitol, galactose, inositol, lactose, maltose, mannitol, raffinose, rhamnose, sorbitol or trehalose. The major fatty acid is 18:1 ω 7c. The DNA G+C content of the type strain is 51.7 mol%.

The type strain is WSF2^T (=IAM 15442^T=NCIMB 14279^T=KCTC 12861^T), isolated from coastal seawater of the Boso Peninsula in Japan.

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.
- Barrow, G. I. & Feltham, R. K. A. (1993). *Cowan and Steel's Manual for the Identification of Medical Bacteria*, 3rd edn. Cambridge: Cambridge University Press.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.
- Fukunaga, Y., Kurahashi, M., Tanaka, K., Yanagi, K., Yokota, A. & Harayama, S. (2006). *Pseudovibrio ascidiaceicola* sp. nov., isolated from ascidians (sea squirts). *Int J Syst Evol Microbiol* **56**, 343–347.
- 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.
- Hosoya, S., Arunpairojana, V., Suwannachart, C., Kanjana-Opas, A. & Yokota, A. (2006). *Aureispira marina* gen. nov., sp. nov., a gliding, arachidonic acid-containing bacterium isolated from the southern coastline of Thailand. *Int J Syst Evol Microbiol* **56**, 2931–2935.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Saito, H. & Miura, K. (1963). Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim Biophys Acta* **72**, 619–629.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Shieh, W. Y., Lin, Y.-T. & Jean, W. D. (2004). *Pseudovibrio denitrificans* gen. nov., sp. nov., a marine, facultatively anaerobic, fermentative

bacterium capable of denitrification. *Int J Syst Evol Microbiol* **54**, 2307–2312.

Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Manual of Method 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.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible

strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.