Larkinella insperata gen. nov., sp. nov., a bacterium of the phylum 'Bacteroidetes' isolated from water of a steam generator

Marc Vancanneyt,¹ Olga I. Nedashkovskaya,² Cindy Snauwaert,¹ Stefanie Mortier,¹ Katrien Vandemeulebroecke,¹ Bart Hoste,¹ Peter Dawyndt,³ Galina M. Frolova,² Danielle Janssens¹ and Jean Swings^{1,3}

Correspondence

Marc Vancanneyt

Marc.Vancanneyt@UGent.be

^{1,3}BCCM/LMG Bacteria Collection¹ and Laboratory of Microbiology³, Ghent University, Ledeganckstraat 35, B-9000 Ghent, Belgium

²Pacific Institute of Bioorganic Chemistry of the Far-Eastern Branch of the Russian Academy of Sciences, Pr. 100 Let Vladivostoku 159, 690022, Vladivostok, Russia

A Gram-negative bacterium, designated strain LMG 22510^T, was isolated from water of a pharmaceutical company steam generator. The cells had a ring-like and horseshoe-shaped morphology and possessed gliding motility. Phylogenetic analysis of the 16S rRNA gene sequence showed that the strain was a member of the *Flexibacter* group within the phylum 'Bacteroidetes'; its nearest neighbour was *Spirosoma linguale* (88.8% sequence similarity). DNA base content, fatty acid composition and biochemical characteristics were determined. Genotypic and phenotypic data indicated that strain LMG 22510^T could not be assigned to any recognized genus; therefore, a novel genus and species is proposed, *Larkinella insperata* gen. nov., sp. nov., with LMG 22510^T (= NCIMB 14103^T) as the type strain.

The *Flexibacter* group within the phylum 'Bacteroidetes' (previously *Cytophaga-Flavobacterium-Bacteroides* or CFB) is a phylogenetically diverse group of micro-organisms. One of the lineages in this group comprises the genera *Spirosoma*, *Runella* and *Flectobacillus*, currently classified in the family *Spirosomaceae* (Larkin & Borral, 1984). The genera *Spirosoma* and *Flectobacillus* each comprise one species, *Spirosoma linguale* and *Flectobacillus major*, respectively. The genus *Runella* includes two species, *Runella slithyformis* (type species) and *Runella zeae* (Chelius *et al.*, 2002). Other more recently discovered taxa related to species of the latter family are the distantly related genera *Dyadobacter* and *Arcicella* (Chelius & Triplett, 2000; Nikitin *et al.*, 2004; Reddy & Garcia-Pichel, 2005). A characteristic morphological feature of the majority of members of the above-mentioned genera is the formation of ring-like or horseshoe-shaped cells, except for cells of *R. zeae* and *Dyadobacter* species, which are straight to curved rods. In the present report, the genotypic and phenotypic properties of strain LMG 22510^T, which was isolated from water of a steam generator, are described; data show that the organism

should be classified as a novel species in a new genus in the *Flexibacter* group of the phylum 'Bacteroidetes'.

Strain LMG 22510^T was isolated and purified from cooled water produced by a steam generator in a pharmaceutical company in Belgium in 2004. The Gram-negative cells have ring-like and horseshoe-shaped morphology and gliding motility. Colonies produce non-diffusible pale-pink pigments on tryptic soy agar (BBL) at 28 °C under aerobic conditions. The latter cultivation conditions were used for maintenance of the cultures and further experiments, unless indicated otherwise.

The phylogenetic position of strain LMG 22510^T was determined by complete 16S rRNA gene sequence analysis. Genomic DNA was prepared according to the protocol of Niemann *et al.* (1997). 16S rRNA gene amplification, purification and sequencing were performed as described by Vancanneyt *et al.* (2004) except that 16S rRNA gene amplicons were purified using a NucleoFast 96 PCR Clean-up kit (Macherey-Nagel). Sequencing reactions were performed using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and purified using a Montage SEQ₉₆ Sequencing Reaction Clean-up kit (Millipore). Electrophoresis of sequence reaction products was performed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Sequence assembly was performed using the program AutoAssembler (Applied Biosystems). The 16S rRNA gene

Published online ahead of print on 16 September 2005 as DOI 10.1099/ijs.0.63948-0.

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequences of *Larkinella insperata* LMG 22510^T and *Spirosoma linguale* LMG 10896^T are AM000022 and AM000023, respectively.

sequences (continuous stretch of 1466 bp) and sequences retrieved from GenBank/EMBL of related reference strains were aligned and a phylogenetic tree was constructed by the neighbour-joining method using the BioNumerics software package (version 3.5; Applied Maths). Unknown bases were discarded for the analyses. Bootstrapping analysis was undertaken to test the statistical reliability of the topology of the neighbour-joining tree using 500 bootstrap resamplings of the data (Fig. 1). Comparison of the sequences revealed highest sequence similarity (88.1%) between strain LMG 22510^T and *S. linguale* ATCC 23276.

Because no 16S rRNA gene sequence was publicly available for the type strain of *S. linguale*, the nearly complete gene sequence (continuous stretch of 1460 bp) was determined for *S. linguale* LMG 10896^T using the same methodology and approach as described above. A similarity of 97.8% was calculated between the sequences of *S. linguale* strains LMG 10896^T and ATCC 23276. *S. linguale* LMG 10896^T and strain LMG 22510^T shared a similarity of 88.8%. Similarity values with the type strains of the type species of the genera *Arcicella*, *Dyadobacter*, *Flectobacillus* and *Runella*, more distantly related taxa, were lower (83.6–86.3%).

For determination of DNA base content, DNA was extracted following the protocol of Pitcher *et al.* (1989) as modified by Leisner *et al.* (2002). DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was separated by HPLC using a Waters Symmetry Shield C8 column maintained at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5%

acetonitrile. Non-methylated phage lambda DNA (Sigma) was used as the calibration reference. The DNA G+C content of strain LMG 22510^T was 53 mol%.

Determination of the fatty acid methyl ester composition of strain LMG 22510^T and its nearest neighbour *S. linguale* LMG 10896^T was performed using the standard protocol of the Sherlock Microbial Identification System (version 5.0; MIDI). Dominant fatty acids for both strains studied are summarized in Table 1 and included 15:0 iso, 16:1 ω 5c, 17:0 iso 3-OH and summed feature 3 (comprising 15:0 iso 2-OH, 16:1 ω 7c and/or 16:1 ω 7t; cf. Table 1). Major quantitative differences between LMG 22510^T and *S. linguale* LMG 10896^T were found in the amounts of the above-mentioned fatty acids. Apart from the latter differences, strain LMG 22510^T can be distinguished from *S. linguale* by the presence of small amounts of 14:0 and 16:0 iso 3-OH and by the absence of 13:0 iso.

Isoprenoid quinones were extracted from lyophilized cells and analysed as described by Akagawa-Matsushita *et al.* (1992). Menaquinones were detected by monitoring at 270 nm and were identified by comparison with known quinones from the reference strain, *Cyclobacterium marinum* LMG 13164^T. The main isoprenoid quinone was MK-7.

Analysis of polar lipids was carried out as described previously (Ivanova *et al.*, 2000). Phospholipids of LMG 22510^T were phosphatidylethanolamine and phosphatidylserine. Also, one glycolipid and four unidentified lipids were detected.

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences of *Larkinella insperata* LMG 22510^T and *Spirosoma linguale* LMG 10896^T and representative members of related genera within the *Flexibacter* group. *Rhodothermus marinus* was used as outgroup. Numbers at nodes indicate bootstrap values (%).

Table 1. Fatty acid content (mean percentages of total) of *Larkinella insperata* LMG 22510^T and *S. linguale* LMG 10896^T

Strains: 1, *L. insperata* LMG 22510^T; 2, *S. linguale* LMG 10896^T. Those fatty acids for which the amount for both taxa is less than 1 % are not given; —, not detected; tr, less than 1 %.

Fatty acid	1	2
13:0 iso	—	3.8
14:0	1.0	—
15:0 iso	13.9	7.6
15:0 anteiso	1.4	tr
15:0 iso 3-OH	2.1	4.9
16:0	3.9	7.2
16:1 ω 5c	48.7	17.5
16:0 3-OH	3.6	4.7
16:0 iso 3-OH	1.8	—
17:0 iso 3-OH	14.3	6.5
SF3*	8.3	47.9

*SF3, Summed feature 3 (summed features consist of one or more fatty acids that cannot be separated by the Microbial Identification System): 15:0 iso 2-OH, 16:1 ω 7c and/or 16:1 ω 7t.

Phenotypic analysis was performed using previously described methods (Nedashkovskaya *et al.*, 2003, 2004). The physiological, biochemical and morphological characteristics of strain LMG 22510^T are given in the species description and in Tables 2 and 3. Strain LMG 22510^T and its nearest neighbour, *S. linguale*, have many similar properties, but also a significant number of distinct features, e.g. differences in hydrolysis of gelatin, starch, Tweens 20 and 80, utilization and acid production from particular carbohydrates and susceptibility to antibiotics (Table 2). Phenotypic features, summarized in Table 3, demonstrate that the novel taxon also differs from other more distantly related genera.

Phylogenetic, genomic and phenotypic data clearly indicate that LMG 22510^T represents a novel genus and species within the *Flexibacter* group, for which the name *Larkinella insperata* gen. nov., sp. nov. is proposed.

Description of *Larkinella* gen. nov.

Larkinella (Lar.ki.nel'la. N.L. fem. n. *Larkinella* named in honour of the American microbiologist John M. Larkin, who described the family *Spirosomaceae* in co-authorship with Renée Borrall).

Ring-like and horseshoe-shaped cells, motile by gliding. Gram-negative. Do not form endospores. Strictly aerobic. Produce non-diffusible pale-pink pigments. Chemo-organotroph. Cytochrome oxidase-, catalase- and alkaline phosphatase-positive. Dominant cellular fatty acids are 15:0 iso, 16:1 ω 5c, 17:0 iso 3-OH and summed feature 3 (comprising 15:0 iso 2-OH, 16:1 ω 7c and/or 16:1 ω 7t).

Table 2. Phenotypic properties of *L. insperata* LMG 22510^T and its nearest phylogenetic neighbour *S. linguale* LMG 10896^T

Strains: 1, *L. insperata* LMG 22510^T; 2, *S. linguale* LMG 10896^T. Both strains produce ring-like and horseshoe-shaped cells. Both strains are positive for respiratory type of metabolism, motility by gliding, oxidase, catalase, β -galactosidase and alkaline phosphatase activities, hydrolysis of Tween 40, utilization of mannose and sucrose, susceptibility to ampicillin, carbenicillin and doxycycline and resistance to benzylpenicillin, chloramphenicol, gentamicin, neomycin and polymyxin B. Both strains are negative for nitrate reduction, flexirubin and H₂S production, NaCl requirement for growth, indole and acetoin (Voges-Proskauer reaction) production, hydrolysis of agar, casein, DNA, urea, cellulose (CM-cellulose and filter paper) and chitin, acid production from adonitol, L-arabinose, dulcitol, L-fucose, N-acetylglucosamine, D-glucose, glycerol, inositol, D-lactose, D-maltose, mannitol, D-melibiose, L-raffinose, L-rhamnose, sorbitol, L-sorbose, D-xylose and L-xylose and utilization of citrate, inositol, malonate, mannitol and sorbitol. w, Weakly positive.

Characteristic	1	2
Cell size (μ m)		
Outer diameter	1.5–3.0	1.5–6.0
Width	0.5–0.9	0.5–1.0
NaCl range for growth (%)	0–2	0–1.25
Temperature range for growth (°C)	6–40	5–39
Hydrolysis of:		
Gelatin	w	—
Starch	—	+
Tween 20	—	+
Tween 80	—	+
Acid from:		
D-Cellobiose	—	+
D-Fructose	—	+
D-Galactose	—	+
D-Sucrose	—	+
Utilization of:		
L-Arabinose	+	—
D-Glucose	+	—
D-Lactose	+	—
Susceptibility to:		
Erythromycin	—	+
Kanamycin	—	+
Lincomycin	—	+
Oleandomycin	—	+
Streptomycin	—	+
Tetracycline	—	+

The main isoprenoid quinone is MK-7. The genus is a member of the phylum '*Bacteroidetes*'. The type species is *Larkinella insperata*.

Description of *Larkinella insperata* sp. nov.

Larkinella insperata (in.spe.ra'ta. L. fem. adj. *insperata* unexpected, referring to the unexpected source from which the bacterium was isolated).

Table 3. Phenotypic properties that distinguish the genus *Larkinella* from related taxa

Data from Larkin & Borrall (1984), Chelius & Triplett (2000), Chelius *et al.* (2002), Reddy & Garcia-Pichel (2005) and this study. w, Weakly positive.

Characteristic	<i>Larkinella</i>	<i>Spirosoma</i>	<i>Dyadobacter</i>	<i>Runella</i>
Habitat	Water	Freshwater, soil	Plants, soil	Freshwater, plants
Colony colour	Pale pink	Yellow	Yellow	Salmon
Cell morphology	Ring-like and horseshoe-shaped cells	Ring-like and horseshoe-shaped cells, filaments, coils and helices may be present	Straight to curved cells, filaments	Slightly curved, often horseshoe-shaped cells, filaments, rarely coils of two to three turns, or straight to slightly bent rods
Gliding motility	+	+	—	+
Flexirubin reaction	—	—	+	—
Highest NaCl concentration tolerated for growth (%)	2	1.25	1.0–1.5	0.75–1
Growth on peptone water	—	—	+	+
Maximum growth temperature (°C)	40	39	30–37	37–42
Hydrolysis of:				
Gelatin	+	—	—	—
Starch	—	+	—	w
DNA G + C content (mol%)	53	51–53	48	49

Main characteristics are the same as those given for the genus. In addition, cells have an outer diameter of 1.5–3.0 µm and width of 0.5–0.9 µm. Colonies are 1–2 mm in diameter, circular and shiny with entire edges. Growth is observed at 10–40 °C and with 0–2 % NaCl. β-Galactosidase-positive. Decomposes gelatin and Tween 40. Does not degrade agar, casein, starch, DNA, Tweens 20 or 80, urea, cellulose (CM-cellulose or filter paper) or chitin. Does not produce acid from adonitol, L-arabinose, D-cellobiose, dulcitol, D-fructose, L-fucose, D-galactose, N-acetylglucosamine, D-glucose, glycerol, inositol, D-lactose, D-maltose, mannitol, D-melibiose, L-raffinose, L-rhamnose, sorbitol, L-sorbose, D-sucrose, D-xylose or L-xylose. Utilizes L-arabinose, D-glucose, D-lactose, D-mannose and D-sucrose as sole carbon sources for growth, but not citrate, inositol, malonate, mannitol or sorbitol. Flexirubin-type pigments are absent. Does not reduce nitrate. No production of H₂S, indole or acetoin (Voges–Proskauer reaction). Susceptible to ampicillin, carbenicillin and doxycycline; resistant to benzylpenicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, lincomycin, neomycin, oleandomycin, polymyxin B, streptomycin and tetracycline. Major fatty acid components (> 1.0 %) include 14:0, 15:0 iso, 15:0 anteiso, 15:0 iso 3-OH, 16:0, 16:1ω5c, 16:0 3-OH, 16:0 iso 3-OH, 17:0 iso 3-OH and summed feature 3 (comprising 15:0 iso 2-OH, 16:1ω7c and/or 16:1ω7t). The G + C content of the DNA is 53.0 mol%.

The type strain is LMG 22510^T (= NCIMB 14103^T), isolated from water produced by a steam generator in a pharmaceutical company in Belgium.

Acknowledgements

This research was supported by the Belgian Federal Public Planning Service – Science Policy; O. I. N. was supported by grants of the Federal Agency for Sciences and Innovations of the Ministry for Education and Sciences of the Russian Federation no. 2-2.16, the Russian Foundation for Basic Research no. 05-04-48211 and the Program of Fundamental Investigations of the Presidium of the Russian Academy of Sciences ‘Molecular and Cell Biology’.

References

- Akagawa-Matsushita, M., Itoh, T., Katayama, Y., Kuraishi, H. & Yamasato, K. (1992). Isoprenoid quinone composition of some marine *Alteromonas*, *Marinomonas*, *Deleya*, *Pseudomonas* and *Shewanella* species. *J Gen Microbiol* **138**, 2275–2281.
- Chelius, M. K. & Triplett, E. W. (2000). *Dyadobacter fermentans* gen. nov., sp. nov., a novel Gram-negative bacterium isolated from surface-sterilized *Zea mays* stems. *Int J Syst Evol Microbiol* **50**, 751–758.
- Chelius, M. K., Henn, J. A. & Triplett, E. W. (2002). *Runella zeae* sp. nov., a novel Gram-negative bacterium from the stems of surface-sterilized *Zea mays*. *Int J Syst Evol Microbiol* **52**, 2061–2063.
- Ivanova, E. P., Zhukova, N. V., Svetashev, V. I., Gorshkova, N. M., Kurilenko, V. V., Frolova, G. M. & Mikhailov, V. V. (2000). Evaluation of phospholipid and fatty acid compositions as chemotaxonomic markers of *Alteromonas*-like proteobacteria. *Curr Microbiol* **41**, 341–345.
- Larkin, J. M. & Borrall, R. (1984). Family I. *Spirosomaceae* Larkin & Borrall 1978, 595^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 125–126. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

- Leisner, J. J., Vancanneyt, M., Lefebvre, K., Vandemeulebroecke, K., Hoste, B., Euras Vilalta, N., Rusul, G. & Swings, J. (2002).** *Lactobacillus durianis* sp. nov., isolated from an acid-fermented condiment (tempoyak) in Malaysia. *Int J Syst Evol Microbiol* **52**, 927–931.
- 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.
- Nedashkovskaya, O. I., Suzuki, M., Vysotskii, M. V. & Mikhailov, V. V. (2003).** *Reichenbachia agariperforans* gen. nov., sp. nov., a novel marine bacterium in the phylum *Cytophaga-Flavobacterium-Bacteroides*. *Int J Syst Evol Microbiol* **53**, 81–85.
- Nedashkovskaya, O. I., Kim, S. B., Han, S. K., Rhee, M. S., Lysenko, A. M., Falsen, E., Frolova, G. M., Mikhailov, V. V. & Bae, K. S. (2004).** *Ulvibacter litoralis* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae* isolated from the green alga *Ulva fenestrata*. *Int J Syst Evol Microbiol* **54**, 119–123.
- Niemann, S., Puhler, A., Tichy, H.-V., Simon, R. & Selbitschka, W. (1997).** Evaluation of the resolving power of three different DNA fingerprinting methods to discriminate among isolates of a natural *Rhizobium meliloti* population. *J Appl Microbiol* **82**, 477–484.
- Nikitin, D. I., Strömpl, C., Oranskaya, M. S. & Abraham, W.-R. (2004).** Phylogeny of the ring-forming bacterium *Arcicella aquatica* gen. nov., sp. nov. (ex Nikitin *et al.* 1994), from a freshwater neuston biofilm. *Int J Syst Evol Microbiol* **54**, 681–684.
- Pitcher, D. G., Saunders, N. A. & Owen, R. J. (1989).** Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* **8**, 151–156.
- Reddy, G. S. N. & Garcia-Pichel, F. (2005).** *Dyadobacter crusticola* sp. nov., from biological soil crusts in the Colorado Plateau, USA, and an emended description of the genus *Dyadobacter* Chelius and Triplett 2000. *Int J Syst Evol Microbiol* **55**, 1295–1299.
- Vancanneyt, M., Mengaud, J., Cleenwerck, I., Hoste, B., Dawyndt, P., Degivry, M. C., Ringuet, D., Janssens, D. & Swings, J. (2004).** Reclassification of *Lactobacillus kefirgranum* Takizawa *et al.* 1994 as *Lactobacillus kefirnofaciens* subsp. *kefirgranum* subsp. nov. and emended description of *L. kefirnofaciens* Fujisawa *et al.* 1988. *Int J Syst Evol Microbiol* **54**, 551–556.