Cryobacterium mesophilum sp. nov., a novel mesophilic bacterium

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A novel mesophilic, Gram-positive, cream- to pale yellow-pigmented, aerobic bacterium, designated MSL-15^T, was isolated from a soil sample collected from Bigeum Island, South Korea. The cells were catalase-positive, motile and irregular rods. The diamino acid content of the cell-wall peptidoglycan was determined to be 2,4-diaminobutyric acid. Strain MSL-15^T grew well between 20 and 28 °C, with optimum growth occurring at 25–28 °C. The major fatty acids were anteiso-C_{15:0}, iso-C_{16:0}, anteiso-C_{17:0} and iso-C_{15:0}. The genomic DNA G+C content was 70.0 mol%. A phylogenetic analysis based on 16S rRNA gene sequences indicated that strain MSL-15^T was associated with the genus *Cryobacterium* and exhibited 96.6 % sequence similarity to *Cryobacterium psychrotolerans* JCM 13925^T and 94.8 % similarity to *Cryobacterium psychrophilum* JCM 1463^T. Phenotypic, chemotaxonomic and phylogenetic data revealed that strain MSL-15^T represents a novel species within the genus *Cryobacterium*, for which the name *Cryobacterium mesophilum* is proposed. The type strain is MSL-15^T (=DSM 19267^T=KCTC 19270^T).

The genus *Cryobacterium* was proposed by Suzuki *et al.* (1997) with only one species, the type species *Cryobacterium psychrophilum*, which was originally called '*Curtobacterium psychrophilum*'. This bacterium was isolated from a soil sample from Antarctica by Inoue (1976) and was assigned to the genus *Curtobacterium* on the basis of its cell-wall amino acid composition and its high DNA G+C content (Inoue & Komagata, 1976). However, the name '*Curtobacterium psychrophilum*' was not validly published. The chemotaxonomic features of this strain were examined by Suzuki *et al.* (1997) and diaminobutyric acid was found in the cell wall instead of ornithine. Phylogenetic analysis based on 16S rRNA gene sequences demonstrated that this bacterium represented a distinct lineage within the radiation of the family *Microbacteriaceae*, thus representing a novel genus (Suzuki *et al.*, 1997), and it was described as *Cryobacterium psychrophilum* on the basis of its phylogenetic and chemotaxonomic features. The genus *Cryobacterium* includes Gram-positive, aerobic, obligately psychrophilic bacteria that have a pleomorphic rod-shaped morphology. Very recently, an additional novel species of the genus *Cryobacterium*, *Cryobacterium psychrotolerans*, was proposed by Zhang *et al.* (2007) for an isolate from the China No.1 glacier. In this study, we report the isolation and identification of strain MSL-15^T, the first mesophilic strain placed in the genus *Cryobacterium*.

Strain MSL-15^T was isolated from soil collected from Bigeum Island after 2 weeks incubation at 28 °C on R2A agar (tenfold dilution) (Reasoner & Geldreich, 1985; Difco). Biomass for molecular systematic analysis and most of the chemotaxonomic studies was obtained from cultures grown in twofold-diluted R2A broth in shake flasks at 28 °C for 10 days. Morphological characteristics were examined by using light microscopy and scanning electron microscopy. Morphological features were observed on twofold-diluted R2A medium at 28 °C. Growth was tested over a range of temperatures (4–45 °C), pH values (5.0–12.0; using the addition of Na₂CO₃, prior to sterilization, to adjust the pH) and salt concentrations (0–15.0 %, w/v, NaCl). The optimal temperature and pH for growth were 28 °C and 7.0–8.0. The NaCl range for growth was 0–3 %. All tests were performed on twofold-diluted R2A agar.

Physiological and biochemical characteristics of strain MSL-15^T are given in the species description below and in Table 1. All the tests were performed at 28 °C and properties were recorded after 5, 7 and 10 days, except for the nitrate-reduction test, which was assessed after 1 and 2 days. Carbon-source utilization and acid production from sugars were determined according to the methods of Kämpfer *et al.* (1991). Citrate utilization, DNase, catalase and oxidase activities and hydrolysis of starch, casein, gelatin, hypoxanthine, Tween 80, tyrosine, urea and xanthine were determined as described by Cowan & Steel (1965). Hydrolysis of aesculin and nitrate reduction were

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MSL-15^T is EF466127.

Table 1. Characteristics that differentiate strain MSL-15^T from *Cryobacterium psychrotolerans* JCM 13925^T and *Cryobacterium psychrophilum* JCM 1463^T

Taxa: 1, strain MSL-15^T; 2, *C. psychrotolerans* JCM 13925^T; 3, *C. psychrophilum* JCM 1463^T. Comparative data were taken from Suzuki *et al.* (1997) and Zhang *et al.* (2007). All of the strains were negative for assimilation of hippurate. +, Positive; –, negative; w, weakly positive.

Characteristic	1	2	3
Colony colour	Cream to pale yellow	Yellow	Pink
Cell size (µm)	0.3–0.6 × 1.0–1.4	0.5–0.8 × 1.8–3.6	0.5–0.7 × 1.0–1.8
Motility	+	+	–
Growth temperature (°C)			
Range	20–28	4–27	4–17
Optimum	25–28	20–22	9–12
Hydrolysis of:			
Casein	+	+	–
DNA	–	–	+
Starch	+	+	w
Acid from:			
Arabinose	+	+	–
Fructose	–	–	+
Galactose	+	–	+
Maltose	–	+	–
Mannose	–	–	+
Sucrose	–	–	+
Assimilation of:			
Malate	+	–	+
Pyruvate	+	–	+
Nitrate reduction	–	+	–
Major menaquinones	MK-11 (48 %) and MK-12 (37 %)	MK-11 (39 %), MK-12 (38 %) and MK-10 (16 %)	MK-10 (50.4 %), MK-11 (17.3 %), MK-8 (14.2 %) and MK-9 (11.2 %)
DNA G + C content (mol%)	70.0	67.0	65.0

studied as described previously by Lányi (1987). Enzyme activity was determined using the API ZYM system (bioMérieux). The cells of strain MSL-15^T were Gram-positive, motile and irregular rods. Colonies on twofold-diluted R2A agar were light yellow, smooth and convex with entire margins.

The procedures used for the identification of cell-wall amino acids and sugars were those described by Komagata & Suzuki (1987). Polar lipids were extracted, examined by two-dimensional TLC and identified using the procedures of Minnikin *et al.* (1984). Menaquinones were isolated using the methods of Minnikin *et al.* (1984) and were separated by HPLC (Kroppenstedt, 1982), using *Agrococcus baldri* DSM 14216^T (Zlamala *et al.*, 2002) as a reference strain. The cellular fatty acid content was determined as described by Sasser (1990), using the Microbial Identification System (MIDI). The amino acids in the peptidoglycan layer of strain MSL-15^T comprised glutamic acid, glycine, alanine and 2,4-diaminobutyric acid in the molar ratio 1.0:0.8:1.0:1.6. The cell-wall sugars were glucose and ribose. The predominant menaquinones were MK-11 and MK-12. The phospholipids of strain MSL-15^T comprised diphosphatidylglycerol, phosphatidylglycerol and unidentified glycolipids. The cellular fatty acid content of strain MSL-15^T is given in the species description.

Chromosomal DNA from strain MSL-15^T was prepared by following the method of Marmur (1961). The DNA G + C content of strain MSL-15^T was determined by reversed-phase HPLC of nucleosides as described by Mesbah *et al.* (1989); a value of 70.0 mol% was obtained.

Amplification of the 16S rRNA gene sequence was performed as described by Cui *et al.* (2001). The almost-complete 16S rRNA gene sequence (1484 nt) of strain MSL-15^T was obtained and BLAST search comparisons were made against the GenBank/EMBL/DDBJ databases. The 16S rRNA gene sequence of strain MSL-15^T and previously published sequences were aligned using the CLUSTAL_X program (Thompson *et al.*, 1997). A phylogenetic tree was constructed from K_{nuc} values using the neighbour-joining method of Saitou & Nei (1987), (Kimura, 1980, 1983). The reliability of the phylogenetic tree was evaluated using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates. The phylogenetic tree shown in Fig. 1 includes sequences of representative members of the family *Microbacteriaceae*.

Phylogenetic analysis of the 16S rRNA gene sequence revealed that the novel isolate fell within the cluster of the family *Microbacteriaceae* and represented a line of descent distinct from recognized actinomycetes of this family (Fig. 1), belonging to a cluster of species that included

Fig. 1. Neighbour-joining phylogenetic dendrogram, based on 16S rRNA gene sequences, for strain MSL-15^T and species of the family *Microbacteriaceae*. *Frigoribacterium faeni* DSM 10309^T (Y18807) served as the outgroup. Numbers at nodes are bootstrap percentages (based on 1000 resampled datasets) $\geq 50\%$. Bar, 0.005 substitutions per nucleotide position.

Cryobacterium psychrophilum JCM 1463^T and *Cryobacterium psychrotolerans* JCM 13925^T (Fig. 1) (with 16S rRNA gene sequence similarities of 94.80 and 96.61 %, respectively). The relationship was confirmed in all three tree-making analyses [least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981) and neighbour-joining (Fig. 1)]. Additionally, there were significant differences in the morphology, physiology and chemotaxonomic characteristics of strain MSL-15^T and those of its closest phylogenetic neighbours (Table 1). On the basis of phenotypic and genotypic data, strain MSL-15^T is distinguishable from its closest neighbours in the family *Microbacteriaceae*. On the basis of the data from this polyphasic study, strain MSL-15^T represents a novel species within the genus *Cryobacterium*, for which the name *Cryobacterium mesophilum* sp. nov. is proposed.

Emended description of the genus *Cryobacterium* Suzuki et al. 1997

Cryobacterium [Cry.o.bac.te'ri.um. Gr. n. *kryos* cold; Gr. n. *bakterion* a small rod; N.L. neut. n. *Cryobacterium* a cold (preferring) rod].

Cells are motile or non-motile, pleomorphic rods (0.3–0.8 × 1.0–3.6 µm). Gram-positive (may be Gram-variable in old cultures). Strictly aerobic. Grows optimally between 9 and 28 °C. DNase and nitrate reduction are variable; catalase-positive. Negative for gelatin hydrolysis and the assimilation of hippurate, but variable for casein hydrolysis. The DNA G + C content ranges from 65 to 70 mol%.

Rhamnose and fucose or glucose and ribose are the characteristic cell-wall sugars. The major fatty acid is anteiso-C_{15:0}. The major menaquinones are MK-10, MK-11 and MK-12. The polar lipids are diphosphatidylglycerol, phosphatidylglycerol and unidentified glycolipids. The type species is *Cryobacterium psychrophilum*.

Description of *Cryobacterium mesophilum* sp. nov.

Cryobacterium mesophilum [me.so.phi'lum. Gr. adj. *mesos* middle; Gr. adj. *philos* loving; N.L. neut. adj. *mesophilum* middle (temperature)-loving, mesophilic].

Cells are Gram-positive, motile, irregular rods of 0.3–0.6 × 1.0–1.4 µm. Colonies are light yellow, smooth, circular and convex with entire margins. Strictly aerobic and mesophilic. Tests for DNase and citrate utilization are negative. Catalase-positive. Growth occurs at 20–28 °C and pH 6.0–11.0, with optima at 28 °C and pH 7.0–8.0. Growth occurs in the presence of 0–3 % (w/v) NaCl. Negative for nitrate reduction. Hydrolyses starch and casein, but not aesculin, gelatin or DNA. Galactose, glucose, inositol, lactose, mannose, mannitol, melibiose, raffinose and rhamnose are utilized as sole sources of carbon and energy, but arabinose, fructose, maltose and ribose are not. The major menaquinones are MK-11 and MK-12. The main polar lipids are diphosphatidylglycerol and phosphatidylglycerol. The amino acids of the cell-wall peptidoglycan are 2,4-diaminobutyric acid, alanine, glycine

and glutamic acid. Glucose and ribose are the main sugar components of the whole-cell sugars. The major fatty acids are anteiso-C_{15:0} (35.4 %), iso-C_{16:0} (34.6 %), anteiso-C_{17:0} (17.0 %) and iso-C_{15:0} (5.7 %). Fatty acids constituting less than 5 % are iso-C_{17:0} (2.3 %), C_{16:0} (2.0 %) and iso-C_{14:0} (1.7 %). The genomic DNA G + C content is 70.0 mol%.

The type strain, MSL-15^T (=DSM 19267^T=KCTC 19270^T), was isolated from soil from Bigeum Island, South Korea.

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