Rhodoglobus vestalii gen. nov., sp. nov., a novel psychrophilic organism isolated from an Antarctic Dry Valley lake

Peter P. Sheridan,¹ Jennifer Loveland-Curtze,² Vanya I. Miteva² and Jean E. Brenchley²

Correspondence

Vanya I. Miteva

vim1@psu.edu

¹Department of Biological Sciences, PO Box 8007, Idaho State University, Pocatello, ID 83209, USA

²Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA 16802, USA

A novel, psychrophilic, Gram-positive bacterium (designated strain LV3^T) from a lake near the McMurdo Ice Shelf, Antarctica, has been isolated and characterized. This organism formed red-pigmented colonies, had an optimal growth temperature of 18 °C and grew on a variety of media between –2 and 21 °C. Scanning electron micrographs of strain LV3^T that showed small rods with unusual bulbous protuberances during all phases of growth were of particular interest. The G + C content of the genomic DNA was approximately 62 mol%. The cell walls contained ornithine as the diamino acid. The major fatty acids were anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0}. Cells grown at –2 °C contained significant amounts of anteiso-C_{15:1}. The major menaquinones found in strain LV3^T were MK-11 and MK-12. Phylogenetic analysis of the 16S rRNA gene sequence indicated that strain LV3^T was a member of the family *Microbacteriaceae* and related to, but distinct from, organisms belonging to the genera *Agreia*, *Leifsonia* and *Subtercola*. In addition, alignments of 16S rRNA sequences showed that the sequence of strain LV3^T contained a 13 bp insertion that was found in only a few related sequences. Based on the low growth temperature, unusual cell shape, distinct 16S rRNA gene sequence and structure and cell-wall amino acid and menaquinone compositions, *Rhodoglobus vestalii* gen. nov., sp. nov. is proposed, with the type strain LV3^T (= ATCC BAA-534^T = CIP 107482^T).

INTRODUCTION

Our research focuses on the isolation of psychrophiles, i.e. organisms that grow at 5 °C and have growth optima below 37 °C (Neidhardt *et al.*, 1990), and the characterization of their cold-active enzymes. We have isolated numerous psychrophilic micro-organisms and characterized several cold-active β -galactosidases (Coombs & Brenchley, 1999; de Prada *et al.*, 1996; Gutshall *et al.*, 1995, 1997; Loveland *et al.*, 1994; Loveland-Curtze *et al.*, 1999; Sheridan & Brenchley, 2000). Many of these strains were members of the high-G + C actinomycete–coryneform diverse group of bacteria (Stackebrandt & Woese, 1981). A recent psychrophilic isolate from a lake in the Dry Valley region of Antarctica, designated strain LV3^T, had unusual features. Scanning electron micrographs that showed cells with unusual, bulbous protrusions present during all stages of growth were of particular interest. Furthermore, unlike previous

isolates, these cells did not have traits typical of members of the genus *Arthrobacter*, such as a rod–coccus morphological cycle and lysine as the characteristic diamino acid in the peptidoglycan (Loveland-Curtze *et al.*, 1999).

Analyses of the 16S rRNA gene sequence from strain LV3^T showed that it was related to, but distinct from, genera such as *Agreia*, *Leifsonia*, *Subtercola*, *Agrococcus* and *Agromyces* in the family *Microbacteriaceae*. This group was recently revised with the addition of novel genera, because analysis of 16S rRNA gene sequences had demonstrated that many of the classical groupings were not monophyletic. The genus *Leifsonia* was proposed in an attempt to create order within this sub-branch of the actinomycete–coryneform bacterial phylogenetic tree (Evtushenko *et al.*, 2000). For example, the organisms formerly known as *Clavibacter xyli* subsp. *cynodontis* and subsp. *xyli* were reclassified as *Leifsonia xyli* subsp. *cynodontis* and *Leifsonia xyli* subsp. *xyli*, respectively. At the time of writing, there are five species with validly published names in the genus *Leifsonia*, which form a distinct phylogenetic branch close to the *Agromyces* cluster.

Abbreviation: DAB, diaminobutyric acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain LV3^T is AJ459101.

We describe here the unusual morphological, chemotaxonomic and phylogenetic characteristics of the psychrophilic strain LV3^T. Based on unique features and distinct phylogenetic placement, *Rhodoglobus vestalii* gen. nov., sp. nov. is proposed for strain LV3^T.

METHODS

Sampling and isolation. A cyanobacterial mat sample was collected in January 1993 from a meromictic pond (informally called 'Lake Vestal' in honour of the late J. Robie Vestal) located south of the Miers and Adams glaciers near Bratina Island on the McMurdo Ice Shelf, Antarctica. The sample was frozen at -80°C until approximately 2 g sample was inoculated into 5 ml instant ocean broth (Loveland-Curtze *et al.*, 1999) and incubated at 10°C until the culture became turbid. Organisms were isolated on instant ocean agar by using streak-planting techniques (Loveland-Curtze *et al.*, 1999) and colonies were passed at least three times to ensure purity. Isolates were streaked onto instant ocean agar with 100 mg X-Gal l^{-1} , to determine those that produced β -galactosidase. A red-pigmented colony designated strain LV3^T that hydrolysed X-Gal was chosen for further study.

Physiological and biochemical characteristics. The ability of the strain to grow at different temperatures and on various media was tested by inoculating cells from a turbid culture grown in R2 broth (Difco) onto plates of R2 agar, Luria-Bertani agar, trypticase soy agar without added carbohydrate, nutrient agar, marine agar and instant ocean agar. The cultures were incubated aerobically at -2 , 10, 18, 25 and 37°C . Growth rates were determined by inoculating cells from a turbid culture grown in trypticase soy broth without added carbohydrate (TSB) into fresh TSB to a Klett reading of 10–15, and by incubating aerobically at -2 , 5, 10, 18, 21, 22 and 23°C . API CORYNE test strips (bioMérieux) were used for general characterization of enzymic and fermentation activities and gelatin hydrolysis. The strips were incubated at 18°C and the results were recorded after 24 and 48 h.

Electron microscopy. Cells were grown in TSB at 10 or 18°C for scanning electron microscopy (SEM). They were washed in 0.1 M sodium cacodylate buffer (pH 7.4), filtered onto a 0.2 μm pore polycarbonate filter and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 4 h at 4°C . After washing, the filters and cells were post-fixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.4) and dried in a BAL-TEC model CPD 030 critical point dryer. The preparations were mounted onto aluminium holders, sputter-coated with 10 nm Au/Pd and observed by SEM (JEOL model 5400) at 30 kV.

For transmission electron microscopy (TEM), cells were pelleted and resuspended in 1–2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) overnight at 4°C . After a secondary fixation with 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.4), the cells were dehydrated through an alcohol gradient series, infiltrated with an acetone-Spurrs mixture and polymerized in Spurr's resin. Ultrathin sections were taken, stained with 2% uranyl acetate in ethanol and lead citrate and observed by TEM (JEOL model 1200 EXII) at 80 kV. Both the SEM and TEM pictures were taken at the Penn State Electron Microscope Facility.

16S rRNA gene amplification and phylogenetic analyses. Genomic DNA was extracted from strain LV3^T using a modification of standard methods (Giovannoni *et al.*, 1990). The 16S rRNA gene

was amplified from chromosomal DNA by PCR using Ready-To-Go beads (Amersham Biosciences) and the universal primers 8F and 1492R (Pace *et al.*, 1986; Weisburg *et al.*, 1991). Both strands of the product were sequenced at the Penn State Nucleic Acid Facility on an ABI 370 sequencer. The 16S rRNA gene sequence was aligned with those from the Ribosomal Database Project (RDP) (Maidak *et al.*, 2000) and those obtained from a BLAST search of GenBank (<http://www.ncbi.nlm.nih.gov>), using the CLUSTAL W program found in the BioEdit platform (version 5.0.6). The alignment was based on 1642 nucleotide positions and was used in maximum-parsimony, maximum-likelihood and distance analyses utilizing the PAUP package (version 4.0b10; Swofford, 2002). The maximum-parsimony method utilized a heuristic search, and both the maximum-likelihood (with a transition–transversion ratio of 2) and distance methods were performed using the neighbour-joining algorithm. The initial distance analysis was performed using the neighbour-joining algorithm and an uncorrected 'p' distance in the PAUP program. To determine the effects of using various analysis methods on these results, we compared our results with distance trees generated using the Jukes–Cantor, F81, F84, Kimura two-parameter, Kimura three-parameter, Tamura–Nei, Tajima–Nei and HKY85 models with equal rates for variable sites. Unequal rates for variable sites were also examined for all eight models using Γ -parameters with shapes of 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0. Trees generated by all three methods were essentially congruent. A distance matrix was generated from the same alignment by the PAUP program, using the Jukes–Cantor, F81, F84, Kimura two-parameter, Kimura three-parameter, Tamura–Nei, Tajima–Nei and HKY85 models with equal rates for variable sites. The matrices were quite similar. Ten thousand bootstrap replicates were performed for the distance method, using the Jukes–Cantor model with equal rates for variable sites.

Cell-wall analysis. Cell-wall preparations were hydrolysed and the diamino acids were separated by using a TLC method (Bousfield *et al.*, 1985). Quantitative amino acid analyses were done on cell walls of strain LV3^T, prepared according to the short procedure of Schleifer & Kandler (1972), at the University of Michigan Carbohydrate and Structure Facility. For GC-MS analysis, the cell walls were hydrolysed in 6 M HCl at 110°C and the hydrolysates were dried for 12 h on a Speed Vac Concentrator (Savant Instruments). After derivatization with *N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA; Regis Technologies), the preparations were heated for 1 h at 60°C . GC-MS cell-wall analysis was carried out at the Penn State Intercollegiate Center for Mass Spectrometry, with a Hewlett Packard model 5972 mass spectrometer.

Fatty acid and lipoquinone analyses. Fatty acid determinations were done on cells of strain LV3^T grown in Luria–Bertani (LB) broth at -2°C and in TSB at 18°C . The cells were centrifuged and the pellets frozen at -20°C and sent to Microbial ID for fatty acid methyl ester analysis. Respiratory lipoquinone analyses were carried out by B. J. Tindall, Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, on freeze-dried cells cultivated in TSB at 18°C .

DNA base composition. The G+C content (mol%) was calculated for purified DNA extracted from cells of strain LV3^T by determining the melting temperature as described by Mandel & Marmur (1968). The melting temperature of purified chromosomal DNA extracted from *Escherichia coli* ATCC 23848 was also determined, to serve as an independent control.

Plasmid DNA isolation. The Wizard Plus SV Minipreps DNA purification kit (Promega) was used for plasmid DNA isolation.

RESULTS AND DISCUSSION

Morphological characteristics

Strain LV3^T formed smooth, round, convex, non-slimy, small (2–3 mm in diameter), reddish colonies on solid media. Strain LV3^T grew aerobically on most agar media (except nutrient agar) at temperatures of –2, 10 and 18 °C, but did not grow at 25 °C or higher. Colourless colonies were occasionally observed. Cells reached a higher density when grown in TSB or LB than when grown in R2 broth. The generation time in TSB was estimated to be 10, 20 and 29 h at 18, 10 and 5 °C, respectively. The cells grew more slowly at 21 °C than they did at 18 °C, indicating that 18 °C is the optimal temperature for growth (Fig. 1). The upper temperature limit for growth was 22 °C. The cells grew at –2 °C, even though there was an extended lag phase that was independent of the growth temperature of the inoculum. Members of only three other genera in the family *Microbacteriaceae* (*Subtercola*, *Cryobacterium* and *Frigoribacterium*) have optimal growth temperatures of 18 °C or below. *Cryobacterium* is the only genus with an upper growth temperature limit lower than that of strain LV3^T (Table 1). Formation of reddish intracellular pigment occurred soon after the start of the exponential phase in all cultures. Pigment formation is a common characteristic of representatives of the family *Microbacteriaceae* (Table 1). The cells also reached a high cell density and remained viable after prolonged incubation in TSB at –2 and 10 °C, with over 10¹⁰ c.f.u. ml^{–1} remaining after 4 months. The cell density dropped to 10⁹ c.f.u. ml^{–1} after 5 months incubation and there were no viable cells after 7 months.

Fig. 1. Influence of temperature on the growth of strain LV3^T in TSB. Specific growth rates were calculated from estimated generation time values at different temperatures and are the means of at least three experiments.

Microscopic observations showed that the cells were motile, Gram-positive, non-spore-forming, pleomorphic, short slender rods, about 0.15–0.2 µm in width and 1.0–1.2 µm in length. Cells from freshly inoculated cultures were longer than those from older cultures; however, no true rod–coccus cycle (as in species of the genera *Arthrobacter*, *Leifsonia* and *Frigoribacterium*) was observed. Clumps of cells were often formed in broth cultures. In addition, the cells frequently appeared swollen at the ends or in the middle. SEM showed extremely slender cells with enlarged, bulbous protuberances that were approximately 0.8 µm in diameter (Fig. 2a, b). Some of the cells were slightly curved, but lacked the typical fragmented shapes often seen with members of the genus *Arthrobacter*. The rods were slender and some appeared to enter the 0.2 µm filter pores (Fig. 2a). Most protuberances appeared to be in the central part of the cells. To determine whether the protuberances were formed during a certain growth stage or were due to growth at a specific temperature or in a particular medium, additional cultures that had been grown for different times, at various temperatures and in different media were examined. These structures were observed in pigmented and non-pigmented cells of all growth stages and culture conditions.

In order to determine whether these unique protuberances contained specialized material or inclusion bodies, we examined the cells using TEM (Fig. 2c and d). The micrographs showed uniform cytoplasmic material within the cells, without any disruption of cell-wall integrity or intracellular components in the protuberances. After 150 days incubation, the cells had the same morphology, which showed that the protuberances were not simply cell-wall ‘blebs’ being formed in preparation for cell lysis. Although the function of the protuberances is unknown, they appeared to be unrelated to any specific growth stage or condition. It may be that these structures provide an enhanced surface area for nutrient transport for the otherwise small cells, or are the result of an altered rod–coccus cycle, commonly observed among other Gram-positive, non-spore-forming aerobes.

Physiological characteristics

Initially, strain LV3^T was chosen for study because it hydrolysed X-Gal, indicating that it might have a cold-active β-galactosidase. Other enzymic characteristics were tested using API CORYNE test strips. The strain showed positive enzymic activities for pyrazinamidase, pyrrolidonyl arylamidase, alkaline phosphatase (weak), β-galactosidase (weak), α-glucosidase, β-glucosidase and catalase, and was negative for nitrate reductase, β-glucuronidase, *N*-acetyl-β-glucosidase and urease. It did not hydrolyse gelatin and did not form acid from glucose, ribose, xylose, mannitol, maltose, lactose, sucrose or glycogen. In other tests, the strain did not grow anaerobically or in the presence of 2.5, 5 or 10 % NaCl and did not form heat-resistant spores. A small (approx. 900 bp) plasmid was found in strain LV3^T. Further characterization of this plasmid is ongoing.

Table 1. Distinguishing characteristics of genera in the family *Microbacteriaceae*

Genera: 1, *Rhodoglobus*; 2, *Leifsonia*; 3, *Clavibacter*; 4, *Agromyces*; 5, *Agrococcus*; 6, *Subtercola*; 7, *Agreia*; 8, *Cryobacterium*; 9, *Leucobacter*; 10, *Rathayibacter*; 11, *Curtobacterium*; 12, *Frigoribacterium*; 13, *Microbacterium* (*Aureobacterium*); 14, *Mycetocola*; 15, *Okibacterium*. Data from Collins & Bradbury (1986), Collins & Keddie (1986), Evtushenko *et al.* (2000, 2001, 2002), Groth *et al.* (1996), Kämpfer *et al.* (2000), Komataga & Suzuki (1986), Männistö *et al.* (2000), Suzuki *et al.* (1997), Takeuchi & Hatano (1998), Takeuchi *et al.* (1996), Tsukamoto *et al.* (2001), Zgurskaya *et al.* (1992, 1993) and this study.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Colony colour*	R	Y,W	W, Y, O, P, BL	Y	W, Y, O	Y	Y, O	NR	B	NR	Y, O, W, P	Y	W, O	YW	Y
Growth temp. (°C):															
Range	−2–21	7–37	Up to 35	Up to 37	Up to 37	−2–28	Up to 37	<18	Up to 37	<37	Up to 35	0–28	10–40	>4–33	7–37
Optimum	18	24–28	21–26	26–30	28	15–17	24–26	9–12	28	24–28	24–27	4–10	30	25	24–27
DNA G+C content (mol%)	62	66–73	67–78	70–72	74	64–68	67	65	66	63–72	68–75	72	65–75	64–65	67
Diamino acid†	Orn	DAB	DAB	DAB	DAB	DAB	DAB/Orn	DAB	DAB	DAB	Orn	Lys	Lys/Orn	Lys	Lys
Major fatty acids	a-C _{15:0}	a-C _{15:0}	a-C _{15:0}	a,i	a-C _{15:0}	a-C _{15:0}	a-C _{15:0}	a-C _{15:0}	a-C _{15:0}	a-C _{15:0}	a-C _{15:0}	a-C _{15:0}	a-C _{15:0}	a-C _{15:0}	a-C _{15:0}
(≥5%)‡	i-C _{16:0}	i-C _{16:0}	i-C _{16:0}		i-C _{16:0}	i-C _{16:0}	i-C _{16:0}	i-C _{16:0}	i-C _{16:0}	i-C _{16:0}	i-C _{16:0}	i-C _{16:0}	i-C _{16:0}	i-C _{16:0}	i-C _{16:0}
	a-C _{17:0}	a-C _{17:0}	a-C _{17:0}		a-C _{17:0}	a-C _{17:0}	a-C _{17:0}	a-C _{17:0}	a-C _{17:0}	a-C _{17:0}	a-C _{17:0}	C _{16:0}	a-C _{17:0}	a-C _{17:0}	a-C _{17:0}
	a-C _{15:1}				a-C _{15:1}	a-C _{15:1}		i-C _{15:0}			a-C _{15:1}	a-C _{15:1}	i-C _{15:0}		i-C _{15:0}
					a-C _{14:0}			a-C _{15:1}			ch _{17:0}				
Major menaquinones	11, 12	10, 11	9	12	11, 12	9, 10	10	10	11	10	9	9	11, 12, 13	10	10, 11

*R, Red; W, white; Y, yellow; O, orange; P, pink; BL, blue; B, brown; YW, yellowish-white; NR, not reported.

†Orn, Ornithine; Lys, lysine.

‡a, Anteiso-branched acids; i, iso-branched acids; ch, ω -cyclohexyl acid. Except for *Agrococcus jenensis* ST54, monounsaturated 12-methyltetradecenoic acid (a-C_{15:1}) represents 5% or more of the total only when the strains were grown at lower temperatures ($\leq 10^\circ\text{C}$).

Fig. 2. Morphology of strain LV3^T cells: (a, b) scanning electron micrographs (bars, 1 µm); (c, d) transmission electron micrographs (bars, 200 nm).

16S rRNA gene sequence and phylogenetic analysis

The 16S rRNA gene sequence amplified from strain LV3^T DNA was analysed using the maximum-parsimony, maximum-likelihood and distance methods. Distance analysis results using the Jukes–Cantor model with equal rates for variable sites are presented (Fig. 3). These results showed that strain LV3^T is related to, but phylogenetically distinct from, organisms belonging to the genera *Agreia*, *Subtercola* and *Leifsonia*. The grouping of strain LV3^T with the uncharacterized strains '*Leifsonia rubeus*', *Corynebacterium* strain QSSC 3-5 and '*Leifsonia aureus*' was supported by high bootstrap values (Fig. 3).

A distance matrix table generated using the Jukes–Cantor model showed that the distances between sequences from strain LV3^T and the closest type strains (*Agreia bicolorata* VKM Ac-1804^T, *Subtercola pratensis* DSM 14246^T, *Subtercola boreus* K300^T, *Leifsonia naganoensis* DB 103^T, *Leifsonia poae* VKM Ac-1401^T, *Leifsonia aquatica* DSM 20146^T, *Leifsonia cynodontis* MDE1^T, *Leifsonia shinshuensis* DB 102^T and *Subtercola frigoramans* K265^T) were 4.92, 4.69, 5.12, 5.12, 5.13, 5.45, 5.98, 6.09 and 6.15 %, respectively. These values are either above or on the borderline of the

accepted values for a genus (Rosselló-Mora & Amann, 2001). The 16S rDNA sequence of strain LV3^T also groups with some less well-characterized strains and is 0.7 % distant from '*L. rubeus*', 2.12 % from *Corynebacterium* strain QSSC 3-5 and 2.79 % from '*L. aureus*'. Both '*L. rubeus*' and '*L. aureus*' were isolated from a pond in the Wright Valley, Antarctica, which is within 100 miles of the lake from which strain LV3^T was isolated. Strains QSSC 3-5 and QSSC 9-20 were isolated from an Antarctic quartz stone subglacial community (Smith *et al.*, 2000) and *Cryobacterium psychrophilum* JCM 1463^T was isolated from Antarctic soil (Suzuki *et al.*, 1997). Although *S. boreus* K300^T, *S. frigoramans* K265^T (Männistö *et al.*, 2000) and *Frigoribacterium faeni* 801^T (Kämpfer *et al.*, 2000) were not isolated from Antarctic samples, they are psychrophilic. Based on the distances between '*L. rubeus*' and '*L. aureus*' and the other members of the genus *Leifsonia*, as well as the distance between strain QSSC 3-5 and the other members of the genus *Corynebacterium*, these organisms may need to be included in the newly proposed genus *Rhodoglobus*.

In addition, during analysis of the 16S rRNA gene sequences, we observed that the sequence for strain LV3^T contained a 13 bp insertion that was present in only a few related 16S

Fig. 3. Phylogenetic tree based on distance analysis (neighbour-joining algorithm using the Jukes–Cantor model with equal rates for variable sites) of the 16S rRNA gene sequence of isolate LV3^T compared with 54 closely related sequences. Bar, 0.005 substitutions per site.

rRNA genes. Insertions with an almost-identical sequence were found in '*L. rubeus*' and '*L. aureus*', and insertions with similar sequences were found in *S. frigoramans* K265^T,

C. psychrophilum JCM 1463^T and strains QSSC 9-20 and WCHB 1-18 (Dojka *et al.*, 1998). However, insertions were not found in *S. boreus* K300^T, *S. pratensis* DSM 14246^T,

Fig. 4. Diagrams illustrating the inserted nucleotides in the rRNA gene sequence of strain LV3^T in comparison with that of *A. globiformis*. (a) Part of the secondary structure of the 16S rRNA gene of *A. globiformis* corresponding to nucleotides 436–476; (b, c) proposed secondary structures of the inserted 16S rRNA gene sequence for strain LV3^T.

Table 2. Fatty acid composition (%) of strain LV3^T

Strain LV3^T was grown in LB at -2°C and in TSB at 18°C .

Fatty acid	Growth temperature ($^{\circ}\text{C}$)	
	-2	18
i-C _{14:0}	1.03	1.11
i-C _{15:0}	1.94	0.79
a-C _{15:0}	34.54	53.16
i-C _{15:1}	0.20	0.0
a-C _{15:1}	23.19	2.25
C _{16:0}	0.33	0.55
i-C _{16:0}	23.18	18.76
i-C _{17:0}	0.87	0.58
a-C _{17:0}	14.72	22.62
C _{17:1} ω 5c	0.0	0.18

Agreia bicolorata VKM Ac-1804^T (Evtushenko *et al.*, 2001), strain QSSC 3-5 or any of the type strains in the genus *Leifsonia*. The presence of insertions did not correspond to the isolates' geographical origin or to their mode of growth (psychrophilic). The insertion sequence of strain LV3^T was mapped (Fig. 4a) onto the secondary structure of the most closely related organism (*Arthrobacter globiformis* DSM 20124^T) for which a structure has been generated (Gutell, 1994; <http://www.rna.icmb.utexas.edu>). The partial secondary structure presented in Fig. 4a corresponded to nucleotides 436–476 of the 16S rRNA gene sequence of *Arthrobacter globiformis* DSM 20124^T. As the inserted nucleotides can form a number of possible hairpin structures that would stabilize the insertion (Fig. 4b), it is unlikely that they were artefacts. The combination of the distance of the 16S rRNA gene sequence for strain LV3^T from that of other type strains, plus its insertion sequence, supports the designation of strain LV3^T as a representative of a novel genus.

Chemotaxonomic characteristics

The predominant fatty acids in cells of strain LV3^T cultured at 18°C were branched-chain saturated fatty acids: anteiso-C_{15:0}, iso-C_{16:0} and anteiso-C_{17:0} (Table 2). Most species closely related to strain LV3^T also contain significant amounts of branched-chain fatty acids (Table 2). When strain LV3^T was cultured at -2°C , the amount of anteiso-C_{15:0} decreased by 1.5-fold and the amount of the unsaturated fatty acid anteiso-C_{15:1} increased 10-fold. These results are consistent with changes in the ratio of unsaturated and saturated fatty acids found in other Gram-positive non-spore-forming rods grown at different temperatures. For example, *Cryobacterium psychrophilum* (previously known as '*Curtobacterium psychrophilum*'), which does not grow at above 18°C , exhibits a decrease in anteiso-C_{15:0} and anteiso-C_{17:0} and a sixfold increase in anteiso-C_{15:1} when cultured at 4°C (Suzuki *et al.*, 1997). Anteiso-C_{15:1} was found in cells of *S. frigoramans* when grown at temperatures $\leq 15^{\circ}\text{C}$ (Männistö *et al.*, 2000).

Adjustment of the fatty acid composition may increase membrane fluidity at lower temperatures. The majority of bacterial species contain straight-chain saturated and unsaturated fatty acids, and maintaining membrane fluidity may be accomplished by increasing the amount of unsaturated and short-chain fatty acids at lower temperatures (Kaneda, 1991).

Analyses showed that the diamino acid in the peptidoglycan is ornithine (Table 1). Significant amounts of alanine, glycine and glutamic acid were also found, and only small amounts of lysine were detected. GC-MS results confirmed that lysine was present only in small quantities and homoserine was present only in trace amounts. Based on the results of the quantitative amino acid analyses, the molar ratio of glutamic acid to glycine to alanine to ornithine was 1:1.5:1.1:1.4. Strain LV3^T peptidoglycan seems to be closest to type B2 α (Schleifer & Kandler, 1972), although the relative amounts of the amino acids are not totally consistent with this type.

Species closely related to strain LV3^T have cell walls comprised of type-B peptidoglycan, although the diamino acid differs between the genera of the family *Microbacteriaceae*. The closest characterized phylogenetic relatives of strain LV3^T, including *S. boreus* and *S. frigoramans* (Männistö *et al.*, 2000), contain diaminobutyric acid (DAB) as the diamino acid. *Agreia bicolorata* (Evtushenko *et al.*, 2001) contains both DAB and ornithine; another organism that contains this unusual combination is *S. pratensis* (Behrendt *et al.*, 2002), which is currently being reclassified. Many *Microbacterium* species, including reclassified *Aureobacterium* species (Takeuchi & Hatano, 1998) and *Curtobacterium* species (Komataga & Suzuki, 1986), contain ornithine. *Microbacterium* species, *Mycetocola saprophilus*, *Mycetocola tolaasinivorans*, *Mycetocola lacteus* (Tsukamoto *et al.*, 2001) and *Okibacterium fritillariae* (Evtushenko *et al.*, 2002) contain lysine.

Cells of strain LV3^T contained 65 % MK-12, 35 % MK-11 and small amounts of MK-10 and MK-13. The presence of saturated menaquinones is characteristic of members of the family *Microbacteriaceae*, but close relatives of strain LV3^T do not have the same menaquinone composition. For example, MK-10 is the major menaquinone in *Agreia bicolorata* (Evtushenko *et al.*, 2001) and MK-10 and MK-11 are the major menaquinones in *S. pratensis* (Behrendt *et al.*, 2002). *S. boreus* and *S. frigoramans* (Männistö *et al.*, 2000) have predominantly MK-9 and MK-10. The menaquinone composition of strain LV3^T also distinguishes it from other genera in the family *Microbacteriaceae*.

DNA G + C content

The G + C content for the genomic DNA of strain LV3^T was calculated to be 62 mol%, based on its melting temperature. This value is consistent with the G + C content for the 900 bp plasmid found in strain LV3^T, which was found to be 59 mol% (V. I. Miteva & J. E. Brenchley, unpublished data).

The DNA G + C content for strain LV3^T may be another distinguishing feature, as it was slightly lower than those reported for related organisms such as *L. poae* at 66–73 mol% (Evtushenko *et al.*, 2000) and *Agreia bicolorata* at 67 mol% (Evtushenko *et al.*, 2001).

Description of *Rhodoglobus* gen. nov.

Rhodoglobus (Rho.do.glo'bus. Gr. n. *rhodon* the rose; L. masc. n. *globus* ball; N.L. masc. n. *rhodoglobus* red ball).

Cells are aerobic, Gram-positive, easily decolourized, motile and non-spore-forming. Colonies are smooth, round, convex, non-slimy, translucent, reddish and small (2–3 mm in diameter). Individual cells are slender rods (mean cell size of 0.15–0.2 $\times$ 1–1.2 μ m), with bulbous protuberances (mean diameter 0.8 μ m) seen at all stages of growth. Cells grow aerobically at –2–21 °C. Cell walls contain ornithine as the diagnostic amino acid. Predominant fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}. Major menaquinones are MK-11 and MK-12. The type species is *Rhodoglobus vestalii*.

Description of *Rhodoglobus vestalii* sp. nov.

Rhodoglobus vestalii [ves.ta'li.i. N.L. gen. n. *vestalii* in honour of the late J. Robie Vestal, who studied Antarctic micro-organisms and was a mentor to one of the authors (P. P. S.)].

The following characteristics are shown in addition to those that define the genus. Cells do not exhibit a marked rod–coccus cycle and grow optimally at 18 °C. G + C content of the DNA is approximately 62 mol%. Major cellular fatty acids are anteiso- and iso-branched fatty acids. When cells are grown at –2 °C, the amount of the unsaturated fatty acid anteiso-C_{15:1} increases substantially and the amount of anteiso-C_{15:0} decreases. Cells show positive enzymic activities for pyrazinamidase, pyrrolidonyl arylamidase, alkaline phosphatase (weak), β -galactosidase (weak), α -glucosidase, β -glucosidase and catalase, but are negative for nitrate reductase, β -glucuronidase, *N*-acetyl- β -glucosidase and urease. Cells do not hydrolyse gelatin and do not form acid from glucose, ribose, xylose, mannitol, maltose, lactose, sucrose or glycogen. The 16S rRNA gene sequence contains an insertion of 13 nucleotides that is shared with some of its most closely related neighbours.

The type strain, LV3^T (= ATCC BAA-534^T = CIP 107482^T), was isolated from an Antarctic Dry Valley lake.

ACKNOWLEDGEMENTS

We thank the members of our research group for helpful discussions. This work was supported by Department of Energy grant DE-FG02-93ER20117. P.P.S. was also supported by an Alfred P. Sloan Foundation Fellowship in Molecular Evolution from the National Science Foundation and by partial funding from grant NSF/IGERT DGE-9972759 for the Biogeochemical Research Initiative for

Education (BRIE). P. P. S. and V. I. M. received partial support from the Penn State Astrobiology Center NASA-Ames co-operative agreement NCC2-1057. We thank Rosemary Walsh for preparation of the EM samples and helpful assistance.

REFERENCES

- Behrendt, U., Ulrich, A., Schumann, P., Naumann, D. & Suzuki, K. (2002). Diversity of grass-associated *Microbacteriaceae* isolated from the phyllosphere and litter layer after mulching the sward; polyphasic characterization of *Subtercola pratensis* sp. nov., *Curtobacterium herbarum* sp. nov. and *Plantibacter flavus* gen. nov., sp. nov. *Int J Syst Evol Microbiol* **52**, 1441–1454.
- Bousfield, I. J., Keddle, R. M., Dando, T. R. & Shaw, S. (1985). Simple rapid methods of cell wall analysis as an aid in the identification of aerobic coryneform bacteria. In *Chemical Methods in Bacterial Systematics*, pp. 221–236. Edited by M. Goodfellow & D. E. Minnikin. London: Academic Press.
- Collins, M. D. & Bradbury, J. F. (1986). Plant pathogenic species of *Corynebacterium*. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1276–1283. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Collins, M. D. & Keddle, R. M. (1986). Genus *Microbacterium* Orla-Jensen 1919, 179^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1320–1322. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Coombs, J. M. & Brenchley, J. E. (1999). Biochemical and phylogenetic analyses of a cold-active β -galactosidase from the lactic acid bacterium *Carnobacterium piscicola* BA. *Appl Environ Microbiol* **65**, 5443–5450.
- de Prada, P., Loveland-Curtze, J. & Brenchley, J. E. (1996). Production of two extracellular alkaline phosphatases by a psychrophilic *Arthrobacter* strain. *Appl Environ Microbiol* **62**, 3732–3738.
- Dojka, M. A., Hugenholtz, P., Haack, S. K. & Pace, N. R. (1998). Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. *Appl Environ Microbiol* **64**, 3869–3877.
- Evtushenko, L. I., Dorofeeva, L. V., Subbotin, S. A., Cole, J. R. & Tiedje, J. M. (2000). *Leifsonia poae* gen. nov., sp. nov., isolated from nematode galls on *Poa annua* and reclassification of '*Corynebacterium aquaticum*' Leifson 1962 as *Leifsonia aquatica* (ex Leifson 1962) gen. nov., nom. rev., comb. nov. and *Clavibacter xyli* Davis *et al.* 1984 with two subspecies as *Leifsonia xyli* (Davis *et al.* 1984) gen. nov., comb. nov. *Int J Syst Evol Microbiol* **50**, 371–380.
- Evtushenko, L. I., Dorofeeva, L. V., Dobrovolskaya, T. G., Streshinskaya, G. M., Subbotin, S. A. & Tiedje, J. M. (2001). *Agreia bicolorata* gen. nov., sp. nov., to accommodate actinobacteria isolated from narrow reed grass infected by the nematode *Heteroanguina graminophila*. *Int J Syst Evol Microbiol* **51**, 2073–2079.
- Evtushenko, L. I., Dorofeeva, L. V., Krausova, V. I., Gavrish, E. Yu., Yashina, S. G. & Takeuchi, M. (2002). *Okibacterium fritillariae* gen. nov., sp. nov., a novel genus of the family *Microbacteriaceae*. *Int J Syst Evol Microbiol* **52**, 987–993.
- Giovannoni, S. J., DeLong, E. F., Schmidt, T. M. & Pace, N. R. (1990). Tangential flow filtration and preliminary phylogenetic analysis of marine picoplankton. *Appl Environ Microbiol* **56**, 2572–2575.
- 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.
- Gutell, R. R. (1994). Collection of small subunit (16S- and 16S-like) ribosomal RNA structures: 1994. *Nucleic Acids Res* **22**, 3502–3507.
- Gutshall, K. R., Trimbur, D. E., Kasmir, J. J. & Brenchley, J. E. (1995). Analysis of a novel gene and β -galactosidase isozyme from a psychrotrophic *Arthrobacter* isolate. *J Bacteriol* **177**, 1981–1988.
- Gutshall, K., Wang, K. & Brenchley, J. E. (1997). A novel *Arthrobacter* β -galactosidase with homology to eucaryotic β -galactosidases. *J Bacteriol* **179**, 3064–3067.
- Kämpfer, P., Rainey, F. A., Andersson, M. A., Nurmiäho-Lassila, E.-L., Ulrych, U., Busse, H.-J., Weiss, N., Mikkola, R. & Salkinoja-Salonen, M. (2000). *Frigoribacterium faeni* gen. nov., sp. nov., a novel psychrophilic genus of the family *Microbacteriaceae*. *Int J Syst Evol Microbiol* **50**, 355–363.
- Kaneda, T. (1991). Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. *Microbiol Rev* **55**, 288–302.
- Komata, K. & Suzuki, K.-I. (1986). Genus *Curtobacterium* Yamada and Komagata 1972 425^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1313–1317. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Loveland, J., Gutshall, K., Kasmir, J., Prema, P. & Brenchley, J. E. (1994). Characterization of psychrotrophic microorganisms producing β -galactosidase activities. *Appl Environ Microbiol* **60**, 12–18.
- Loveland-Curtze, J., Sheridan, P. P., Gutshall, K. R. & Brenchley, J. E. (1999). Biochemical and phylogenetic analyses of psychrophilic isolates belonging to the *Arthrobacter* subgroup and description of *Arthrobacter psychrolactophilus*, sp. nov. *Arch Microbiol* **171**, 355–363.
- Maidak, B. L., Cole, J. R., Lilburn, T. G. & 9 other authors (2000). The RDP (Ribosomal Database Project) continues. *Nucleic Acids Res* **28**, 173–174.
- Mandel, M. & Marmur, J. (1968). Use of ultraviolet absorbance-temperature profile for determining the guanine plus cytosine content of DNA. *Methods Enzymol* **12B**, 195–206.
- Männistö, M. K., Schumann, P., Rainey, F. A., Kämpfer, P., Tsitko, I., Tirola, M. A. & Salkinoja-Salonen, M. S. (2000). *Subtercola boreus* gen. nov., sp. nov. and *Subtercola frigoramans* sp. nov., two new psychrophilic actinobacteria isolated from boreal groundwater. *Int J Syst Evol Microbiol* **50**, 1731–1739.
- Neidhardt, F. C., Ingraham, J. L. & Schaechter, M. (1990). *Physiology of the Bacterial Cell: a Molecular Approach*. Sunderland, MA: Sinauer Associates.
- Pace, N. R., Stahl, D. A., Lane, D. J. & Olsen, G. J. (1986). The analysis of natural microbial populations by ribosomal RNA sequences. *Adv Microb Ecol* **9**, 1–55.
- Rosselló-Mora, R. & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev* **25**, 39–67.
- Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.
- Sheridan, P. P. & Brenchley, J. E. (2000). Characterization of a salt-tolerant family 42 β -galactosidase from a psychrophilic Antarctic *Planococcus* isolate. *Appl Environ Microbiol* **66**, 2438–2444.
- Smith, M. C., Bowman, J. P., Scott, F. J. & Line, M. A. (2000). Sublithic bacteria associated with Antarctic quartz stones. *Antarct Sci* **12**, 177–184.
- Stackebrandt, E. & Woese, C. R. (1981). Towards a phylogeny of the actinomycetes and related organisms. *Curr Microbiol* **5**, 197–202.
- Suzuki, K., Sasaki, J., Uramoto, M., Nakase, T. & Komagata, K. (1997). *Cryobacterium psychrophilum* gen. nov., sp. nov., nom. rev., comb. nov., an obligately psychrophilic actinomycete to accommodate '*Curtobacterium psychrophilum*' Inoue and Komagata 1976. *Int J Syst Bacteriol* **47**, 474–478.

- Swofford, D. L. (2002).** PAUP (Phylogenetic Analysis Using Parsimony), version 4.0b10. Sunderland, MA: Sinauer Associates.
- Takeuchi, M. & Hatano, K. (1998).** Union of the genera *Microbacterium* Orla-Jensen and *Aureobacterium* Collins *et al.* in a redefined genus *Microbacterium*. *Int J Syst Bacteriol* **48**, 739–747.
- Takeuchi, M., Weiss, N., Schumann, P. & Yokota, A. (1996).** *Leucobacter komagatae* gen. nov., sp. nov., a new aerobic gram-positive, nonsporulating rod with 2,4-diaminobutyric acid in the cell wall. *Int J Syst Bacteriol* **46**, 967–971.
- Tsukamoto, T., Takeuchi, M., Shida, O., Murata, H. & Shirata, A. (2001).** Proposal of *Mycetocola* gen. nov. in the family *Microbacteriaceae* and three new species, *Mycetocola saprophilus* sp. nov., *Mycetocola tolaasinivorans* sp. nov. and *Mycetocola lacteus* sp. nov., isolated from cultivated mushroom, *Pleurotus ostreatus*. *Int J Syst Evol Microbiol* **51**, 937–944.
- Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991).** 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.
- Zgurskaya, H. I., Evtushenko, L. I., Akimov, V. N., Voyevoda, H. V., Dobrovolskaya, T. G., Lysak, L. V. & Kalakoutskii, L. V. (1992).** Emended description of the genus *Agromyces* and description of *Agromyces cerinus* subsp. *cerinus* sp. nov., subsp. nov., *Agromyces cerinus* subsp. *nitratus* sp. nov., subsp. nov., *Agromyces fucosus* subsp. *fucosus* sp. nov., subsp. nov., and *Agromyces fucosus* subsp. *hippuratus* sp. nov., subsp. nov. *Int J Syst Bacteriol* **42**, 635–641.
- Zgurskaya, H. I., Evtushenko, L. I., Akimov, V. N. & Kalakoutskii, L. V. (1993).** *Rathayibacter* gen. nov., including the species *Rathayibacter rathayi* comb. nov., *Rathayibacter tritici* comb. nov., *Rathayibacter iranicus* comb. nov., and six strains from annual grasses. *Int J Syst Bacteriol* **43**, 143–149.