***Subtercola boreus* gen. nov., sp. nov. and *Subtercola frigoramans* sp. nov., two new psychrophilic actinobacteria isolated from boreal groundwater**

M. K. Männistö,¹ P. Schumann,² F. A. Rainey,³ P. Kämpfer,⁴ I. Tsitko,⁵
M. A. Tirola⁶ and M. S. Salkinoja-Salonen⁵

Author for correspondence: M. Männistö. Tel: +358 3 365 2947. Fax: +358 3 365 2869.
e-mail: mannisto@cc.tut.fi

¹ Institute of Water and Environmental Engineering, Tampere University of Technology, PO Box 541, 33101 Tampere, Finland

² DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Beutenbergstraße 11, 07745 Jena, Germany

³ Department of Biological Sciences, 508 Life Sciences Building, Louisiana State University, Baton Rouge, LA 70803, USA

⁴ Institut für Angewandte Mikrobiologie, Justus-Liebig Universität, Senckenbergstraße 3, D-35390 Giessen, Germany

⁵ Department of Applied Chemistry and Microbiology, PO Box 56 (Biocentre), 00014 University of Helsinki, Finland

⁶ Department of Bio and Environmental Sciences, University of Jyväskylä, PO Box 35, Jyväskylä, Finland

Psychrophilic actinobacterial isolates from permanently cold groundwater in Finland were characterized using a polyphasic approach. Growth on agar plates was observed at temperatures down to -2°C , with an optimum at $15\text{--}17^{\circ}\text{C}$, but no growth was observed at 30°C . The peptidoglycan type was B2 γ and the characteristic diamino acid was diaminobutyric acid. The cell wall sugars of strain K265^T were rhamnose, ribose, xylose and mannose and those of strain K300^T were glucose, rhamnose and xylose. The polar lipids included phosphatidylglycerol, diphosphatidylglycerol, one unknown phospholipid and two glycolipids. The main whole-cell fatty acids were 12-methyltetradecanoic acid, 14-methylpentadecanoic acid and 14-methylhexadecanoic acid. Large amounts of anteiso-1,1-dimethoxy-pentadecane and also iso-1,1-dimethoxy-hexadecane were present as diagnostic markers. The predominant menaquinones were MK-9 and MK-10. The G+C content of the DNA of strains K265^T and K300^T was 64.4 and 67.8 mol%, respectively. Comparison of 16S rRNA gene sequences revealed that strains K265^T and K300^T represent a new lineage among the type-B-peptidoglycan actinomycetes. The closest relatives were *Clavibacter michiganensis*, *Frigoribacterium faeni* and *Rathayibacter rathayi*. On the basis of 16S rDNA sequence, G+C content and chemotaxonomical and physiological characteristics, K265^T and K300^T clearly represent a new genus. The genus *Subtercola* gen. nov. is described, together with two species, namely *Subtercola boreus* sp. nov. (type strain K300^T = DSM 13056^T = CCUG 43135^T) and *Subtercola frigoramans* sp. nov. (type strain K265^T = DSM 13057^T = CCUG 43136^T).

Keywords: *Subtercola boreus* gen. nov., sp. nov., *Subtercola frigoramans* sp. nov., psychrophilic actinobacteria, 1,1-dimethyl acetal

INTRODUCTION

The few psychrophilic or psychrotolerant actinobacterial species so far described belong to the genera *Cryobacterium* (Suzuki *et al.*, 1997) and the recently described *Frigoribacterium* (Kämpfer *et al.*, 2000). Analysis of whole-cell fatty acid composition and partial 16S rRNA gene sequencing indicated that several taxa of actinomycetes were present among 190

isolates from Finnish groundwater in which the temperature is stable at 7°C (Männistö *et al.*, 1999; unpublished results). This paper is a polyphasic description of two novel actinomycetes, isolated from the groundwater, capable of growth on solid medium at temperatures down to -2°C . The phylogenetic, chemotaxonomic and physiological data show that the two isolates represent a new genus within B-type-peptidoglycan-containing members of the family *Microbacteriaceae*. We propose a new genus, *Subtercola* gen. nov., and the species *Subtercola boreus* sp. nov. and *Subtercola frigoramans* sp. nov. for these strains.

The EMBL accession numbers for the 16S rRNA gene sequences determined in this study are AF224723 (strain K265^T) and AF224722 (strain K300^T).

METHODS

Isolation. Strains K265^T and K300^T were isolated from the groundwater of a shallow aquifer located under a glacial gravel ridge in Southern Finland. The water was pumped from a subsurface depth of 18 m. It was highly humic (with 4–13 mg dissolved organic carbon l⁻¹), rich in iron (15 mg l⁻¹) and had a stable temperature of 7 ± 1 °C. The strains were isolated on PYGV agar plates (Staley, 1968) at 7 ± 1 °C. The cultures for characterization were grown on PYGV, TSA (BBL Microbiology Systems) or R2A (BBL Microbiology Systems) agar.

Morphology. The cultures were studied by phase-contrast microscopy (8, 24, 48 and 72 h at 20 °C) using an Olympus BH-2 light microscope. Gram-staining was performed using the Hucker method (Gerhardt *et al.*, 1994). For the preparation of thin sections, the cultures were grown for 7 d on TSA agar at 8 °C. The cells were prefixed with 4% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 2 h at room temperature and washed three times in the same buffer. Thin sections were prepared and examined as described elsewhere (Väisänen *et al.*, 1994).

Physiological characteristics. Growth on TSA plates at -2, 0, 2, 4, 8, 10, 15, 20, 25, 28 and 30 °C was observed and recorded after 14 d and the growth rates on PYGV and trypticase soy broth were determined at 13, 15, 17, 20 and 23 °C by automated kinetic turbidometry (Bioscreen; Lab-Systems). The inoculum (50 µl) was grown on PYGV plates for 3–4 d and suspended in PYGV broth to an optical density (600 nm) of 0.3; 300 µl PYGV or TSA was pipetted into each well. Growth, with medium shaking, was measured as turbidity using a wide-band filter (450–580 nm) and BIOLINK (Labsystems) software. Physiological tests were performed on microtitre plates as described elsewhere (Kämpfer *et al.*, 1991) and read after 3 d at 20 °C.

Chemotaxonomic analyses. For whole-cell fatty acid analysis, the strains were grown on TSA at 4, 10, 15, 20 and 25 °C for 3–12 d. Fatty acid methyl esters were prepared and analysed as described previously (Väisänen *et al.*, 1994). Fatty acid methyl esters were identified using the MIDI aerobic library (TSBA, version 3.9; MIDI). 1,1-Dimethyl acetals were identified as described by Kämpfer *et al.* (2000). Peaks in the whole-cell methanolysate not identified with the MIDI aerobic library version 3.9, were rerun using the BHIBLA anaerobic library and also analysed by GC-MS using the Wiley 138K and NIST mass-spectral libraries. Methanolysates of *Propionibacterium freudenreichii* DSMZ 20271^T and *Propionibacterium jensenii* DSMZ 20535^T were used as references for 1,1-dimethyl acetals.

Purified cell wall preparations were obtained as described by Schleifer & Kandler (1972). Amino acids and peptides in cell wall hydrolysates were analysed by two-dimensional TLC on cellulose plates, using the solvent systems described by Schleifer & Kandler (1972). The molar ratios of cell wall amino acids were determined by GC and GC-MS of *N*-heptafluorobutyryl amino acid isobutyl esters (MacKenzie, 1987). Cell wall sugars were determined by GC of alditol acetals as described by Groth *et al.* (1996).

Menaquinones were extracted as described by Collins *et al.* (1977) and analysed by HPLC (Groth *et al.*, 1996). Polar lipids extracted by the method of Minnikin *et al.* (1979) were identified by two-dimensional TLC followed by spraying with specific reagents (Collins & Jones, 1980).

Base composition of DNA. DNA was extracted after lysis with proteinase K and purified with phenol/chloroform and

chloroform-isoamyl alcohol extractions, 2-propanol precipitation and caesium chloride gradient purification as described by Wilson (1994). Hydrolysis, dephosphorylation and HPLC measurement were performed as described by Johnson (1994). The HPLC column was a Purospher RP-18 endcapped reversed-phase column (250 mm × 4.0 mm i.d., 5.0 µm particle size; Merck). The mobile phase was 20 mM triethylamine phosphate (pH 5.1) in 12% methanol. The flow rate was 1 ml min⁻¹. Hydrolysed lambda phage DNA was used as a standard.

16S rRNA gene sequence determination and phylogenetic analyses. The extraction of genomic DNA, PCR amplification of the 16S rRNA gene and sequencing of the purified PCR products were carried out as described previously (Rainey *et al.*, 1996). Sequence reaction products were purified by ethanol precipitation and electrophoresed with a model 310 Genetic Analyzer (Applied Biosystems). The 16S rRNA gene sequences obtained in this study were aligned against the previously determined actinobacterial sequences available from the public databases, using the ae2 editor (Maidak *et al.*, 1999). The programs of the PHYLIP package, including DNADIST and NEIGHBOR, were used for the phylogenetic analyses (Felsenstein, 1993). The method of Jukes & Cantor (1969) was used to calculate evolutionary distances. The tree topology was re-analysed using 1000 bootstrapped data sets and the programs SEQBOOT, DNADIST and CONSENSE of the PHYLIP package (Felsenstein, 1993).

Nucleotide sequence accession numbers. The accession numbers and strain designations of the reference 16S rRNA gene sequences used in the phylogenetic analyses are as follows: *Agrococcus jenensis* DSM 9580^T (X92492), *Agromyces ramosus* DSM 43045^T (X77447), *Agromyces mediolanus* DSM 20152^T (X77449), *Arthrobacter globiformis* DSM 20124^T (M23411), '*Brevibacterium helvolum*' DSM 20419 (X77440), *Clavibacter michiganense* subsp. *michiganense* DSM 46364^T (X77435), '*Corynebacterium aquaticum*' DSM 20146^T (X77450), *Cryobacterium psychrophilum* IAM12024^T (D45058), *Curtobacterium citreum* DSM 20528^T (X77436), *Curtobacterium luteum* DSM 20542^T (X77437), *Frigoribacterium faeni* DSM 46346^T (Y18807), *Leucobacter komagatae* IFO 15245^T (D17751), *Microbacterium aurum* IFO 15204^T (D21340), *Microbacterium barkeri* DSM 20145^T (X77446), *Microbacterium imperiale* DSM 20530^T (X77442), *Microbacterium lacticum* DSM 20427^T (X77441), *Microbacterium liquefaciens* DSM 20638^T (X77437), *Rathayibacter rathayi* DSM 7458^T (X77439), *Rathayibacter toxicus* JCM 9669^T (D84127).

RESULTS

Morphological and physiological characteristics

Strains K265^T and K300^T formed yellow colonies within 3–4 d at 20 °C. K265^T formed large, mucoid, pale yellow colonies on PYGV or R2A agar; K300^T was less slimy. On TSA agar, the strains formed smooth, round, convex, non-slimy, yellow colonies. The microscopic investigations showed that the cells of strain K300^T were irregular, short, sometimes slightly curved rods 0.2–0.3 µm in width and 0.6–1.0 µm in length. The cells of strain K265^T were irregular, pleomorphic rods 0.3–0.4 µm in width and 0.9–1.5 µm in length. Young cells of K265^T were frequently swollen at the pole or in the middle. The non-motile cells stained Gram-positive at the early stages of

Fig. 1. Electron micrographs of thin sections of strains K300^T (top) and K265^T (bottom) grown on tryptic soy agar plates for 7 d at 8 °C. Bar, 200 nm.

growth (24 h at 20 °C). Both strains turned into short, coccoid rods in the late stages of growth (> 48 h at 20 °C). When grown in liquid medium, v- and y-forms were frequently seen. Micrographs of thin sections of strains K300^T and K265^T are shown in Fig. 1. Cells of both strains had the typical morphology of Gram-positive bacteria, i.e. a cell membrane surrounded by an amorphous layer. The cells exhibited a perpendicular type of division. Cells of strain K300^T had an irregular, electron-dense outer layer.

Growth was optimal at 15–17 °C. The growth rates of strain K265^T on PYGV medium were 0.017 and 0.018 1/h at 15 and 17 °C, respectively; the growth rate of strain K300^T was 0.023 1/h at both temperatures. Visible colonies formed within 14 d at +2 °C and, in the case of strain K265^T, even at –2 °C. The colony morphology was similar at –2 °C and 20 °C.

The metabolic properties of strains K265^T and K300^T are shown in Table 1. A wide variety of sugars were assimilated but none of the 11 amino acids tested was used. The strains differed from each other in their ability to assimilate substrates such as rhamnose, ribose, acetate, propionate, *cis*-aconitate and citrate.

Table 1. Physiological characteristics of the strains K265^T and K300^T

Both strains assimilated L-arabinose, D-cellobiose, D-fructose, D-galactose, gluconate, D-glucose, D-mannose, D-maltose, sucrose, D-trehalose, D-xylose, adonitol, D-mannitol, fumarate, DL-3-hydroxybutyrate and 4-hydroxybenzoate. Neither strain utilized *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, α -D-melibiose, maltitol, D-sorbitol, putrescine, *trans*-aconitate, adipate, 4-aminobutyrate, azelate, glutarate, itaconate, DL-lactate, L-malate, mesaconate, oxoglutarate, pyruvate, suberate, L-alanine, β -alanine, L-aspartate, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-tryptophan or phenylacetate. Both strains hydrolysed aesculin, pNP- α -D-glucopyranoside and pNP- β -D-glucopyranoside. Neither strain hydrolysed pNP-phenylphosphonate, pNP- β -D-galactopyranoside, pNP-phosphorylcholine, 2-deoxythymidine-5'-pNP-phosphate, L-alanine-*para*-nitroanilide, L-glutamate- γ -3-carboxy-pNA or L-proline-*para*-nitroanilide. Abbreviation: pNP, *para*-nitrophenyl.

Characteristic	Reaction of:	
	K265 ^T	K300 ^T
Assimilation of:		
<i>p</i> -Arbutin	+	–
L-Rhamnose	–	+
D-Ribose	–	+
Salicin	+	–
i-Inositol	–	+
Citrate	–	+
3-Hydroxybenzoate	–	+
Acetate	–	+
Propionate	–	+
<i>cis</i> -Aconitate	–	+
Hydrolysis of:		
pNP- β -D-Glucuronide	–	+
pNP- β -D-Xylopyranoside	+	–
Bis-pNP-phosphate	–	+

Chemotaxonomic characteristics

The polar lipids in K265^T and K300^T consisted of phosphatidylglycerol, diphosphatidylglycerol, one unidentified phospholipid and two unidentified glycolipids.

The diamino acid in the peptidoglycan of strains K265^T and K300^T was diaminobutyric acid (DAB). The molar ratio of alanine:glycine:glutamic acid:DAB was, for strain K265^T, 0.6:1.2:0.1:2.0; for strain K300^T, the ratio was 0.6:1.1:0.1:2.0. The amino acid ratio (except that of glutamic acid) is consistent with peptidoglycan type B2 γ (Schleifer & Kandler, 1972). This peptidoglycan type was confirmed by characteristic two-dimensional thin-layer chromatographic peptide patterns of partial hydrolysates of cell walls (data not shown). The low content of glutamic acid in the hydrolysates was due to nearly complete substitution of D-glutamic acid at position 2 of the

Table 2. Composition of whole-cell methanolysates of strains K265^T and K300^T grown at different temperatures

Strains were grown on trypticase soy agar for 3–14 d.

Strain	Growth temp. (°C)	Saturated/monounsaturated fatty acids (%)*						1,1-Dimethyl acetals (%)*					Unknown compounds (%)† ECL		
		i-14:0	i-15:0	i-16:0	a-15:0	a-17:0	a-15:1	a-15:0	a-17:0	i-15:0	i-16:0	16:0	13:56	14:26	14:57
K265 ^T	4	3.4	—	10.6	45.0	2.1	10.4	14.4	—	—	9.1	—	3.0	—	1.5
	10	6.1	—	8.4	46.0	3.5	2.2	16.7	1.2	—	10.9	—	2.5	—	1.5
	15	10.9	—	9.7	44.1	3.9	tr	13.9	1.4	—	12.3	—	—	—	1.8
	20	7.5	—	9.8	44.8	3.9	—	13.5	1.8	—	13.2	—	1.6	—	2.3
	25	6.7	tr	10.2	46.1	6.8	—	10.3	2.9	—	13.3	—	—	—	2.1
K300 ^T	4	—	6.9	3.2	38.2	1.0	6.4	19.6	0.6	3.4	6.3	—	4.7	—	9.1
	10	3.3	1.1	5.1	49.5	1.2	tr	19.4	0.8	tr	10.5	1.3	3.0	tr	1.5
	15	1.5	0.9	4.4	53.8	2.1	—	20.6	0.9	tr	7.9	tr	3.1	1.4	1.3
	20	—	1.7	4.2	54.5	3.0	—	18.2	1.5	0.9	7.8	tr	3.5	1.4	1.1
	25	tr	4.3	4.2	51.6	3.5	tr	11.0	4.0	1.7	11.9	tr	2.0	—	2.6

* Per cent of total peak area of the ion chromatogram; tr, traces (< 1%); a, anteiso-branched acid; i, iso-branched acid.

† Unknown compounds not identified by the MIDI fatty acid methyl ester database (version 3.9) or by the mass-spectral libraries Wiley 138K or NIST (% of total peak area of the total-ion chromatogram); ECL, equivalent chain length.

peptide subunit by *threo*-3-hydroxyglutamic acid, as revealed by two-dimensional TLC and GC-MS.

The cell wall sugars of strain K265^T included rhamnose, ribose and small amounts of xylose and mannose. Glucose, rhamnose and small amounts of xylose were present in the cell wall of strain K300^T.

The major isoprenoid quinones of K265^T were MK-9 (36%), MK-10 (32%), MK-12 (18%), MK-13 (4%), MK-11 (4%) and MK-8 (4%) and those of K300^T were MK-10 (58%), MK-9 (30%), MK-11 (8%) and MK-8 (2%). The G + C content of the genomic DNA of strain K265^T and strain K300^T was 64.4 and 67.8 mol %, respectively.

Cellular fatty acid compositions

The results of the gas chromatographic data for whole-cell methanolysates of strains K265^T and K300^T are shown in Table 2. It shows that in both strains all identified significant fatty acids were of the branched-chain type, mainly 12-methyl tetradecanoic acid (a-15:0) and 14-methyl pentadecanoic acid (i-16:0). In addition the strain K265^T contained 12-methyl tridecanoic acid (i-14:0) and both strains contained unsaturated 12-methyl tetradecanoic acid (a-15:1) when grown at temperatures of ≤ 15 °C. In addition to fatty acids, several major peaks in the whole-cell methanolysates (Fig. 2a) were identified as branched-chain C15, C16 and C17 1,1-dimethyl acetals. The BHBLA anaerobic library and mass-spectral analysis, using *P. freudenreichii* DSMZ 20271^T and *P. jensenii* DSMZ 20535^T as standards for 1,1-dimethyl acetals, proved that the major peak of K265^T and K300^T (with an

Fig. 2. (a) Total-ion gas chromatogram of a whole-cell methanolysate of strain K265^T grown on tryptic soy agar plates at 6 °C; i, iso-branched; a, anteiso-branched; DMA, 1,1-dimethyl acetal. (b) Mass fragmentogram of 1,1-dimethoxy-hexadecane (i-16:0 DMA).

Fig. 3. Phylogenetic tree based on 16S rRNA gene sequence comparison, demonstrating the positions of strains K265^T and K300^T. Bar, 10 nucleotide substitutions per 100 nucleotides.

equivalent chain length of 15:197) was 1,1-dimethoxy-anteiso-pentadecane (Fig. 2a). Methanolysates of the strains K265^T and K300^T contained several peaks with the mass fragment at m/z 75 diagnostic for branched-chain 1,1-dimethyl acetals (Fig. 2b). Such peaks in the K265^T methanolysates were identified, on the basis of their mass fragments, as 1,1-dimethoxy-anteiso-pentadecane (a-15:0 DMA), 1,1-dimethoxy-iso-hexadecane (i-16:0 DMA) and 1,1-dimethoxy-anteiso-heptadecane (a-17:0 DMA) (Table 2). In addition, small amounts of 1,1-dimethoxy-iso-pentadecane (i-15:0 DMA) and a straight-chain 1,1-dimethoxy-hexadecane (16:0 DMA) were identified in strain K300^T. Three peaks in the methanolysates of strains K265^T and K300^T remained unidentified.

Effect of temperature on cellular fatty acid compositions

The effect of cultivation temperature on the whole-cell fatty acid composition of the new isolates was unusual. Strain K300^T reduced its anteiso-15:0 content and both strains reduced their anteiso-17:0 content as the growth temperature was reduced (Table 2). When grown at 4 °C, K265^T and K300^T contained 6–10% anteiso-15:1. Moreover, cultivation temperature influenced the 1,1-dimethyl acetal composition of strains K265^T and K300^T. As the growth temperature was lowered from 25 to 4 °C, the contribution of the anteiso-15:0 DMA, the main DMA in the whole-cell

methanolysates, increased in both strains from 10–11 to 14–20%, whereas the contributions of anteiso-17:0 DMA and iso-16:0 DMA fell accordingly. Thus, it appears that a low growth temperature favoured the synthesis of DMAs with shorter carbon chain lengths, a shift from iso-branched DMAs to anteiso-branched congeners, or both.

16S rRNA gene sequence comparison

Almost complete 16S rRNA gene sequences 1467 (strain K300^T) and 1472 (strain K265^T) nucleotides in length were determined. Phylogenetic analyses based on a data set comprising 1330 unambiguous nucleotides between positions 38 and 1478 but excluding positions 97–193 [due to the absence of this region in the reference sequence of *L. komagatae* (D17751)] (*Escherichia coli* positions; Brosius *et al.*, 1978) showed that the new isolates cluster together as distinct lineages within the radiation of the actinomycete genera with group-B-peptidoglycan that comprises the family *Microbacteriaceae* (Stackebrandt *et al.*, 1997) (Fig. 3). Strains K300^T and K265^T share 97.0% 16S rRNA gene sequence similarity (data not shown). The new isolates show the highest levels of 16S rRNA gene sequence similarity with species of the genera *Frigoribacterium* (95.5–96.7%), *Clavibacter* (95.1–97.1%) and *Rathayibacter* (94.3–96.7%) (data not shown). Species of the genera *Clavibacter*, *Frigoribacterium* and *Rathayibacter* share 96.1–97.0% 16S rRNA gene

sequence similarity (data not shown). The bootstrap analyses indicate no significance in the branching pattern of the lineage comprising the new isolates and any previously described generic lineage of this family (Fig. 3).

DISCUSSION

The characteristics that differentiate the strains K265^T and K300^T from the genera *Clavibacter*, *Rathayibacter*, *Cryobacterium* and *Frigoribacterium* are shown in Table 3. *Clavibacter* and *Frigoribacterium* species contain MK-9 as the predominant menaquinone, whereas *Rathayibacter* and *Cryobacterium* species contain MK-10 (Sasaki *et al.*, 1998; Kämpfer *et al.*, 2000; Suzuki *et al.*, 1997). Strains K265^T and K300^T contained both MK-9 and MK-10 in almost equal proportions. In addition, K265^T contained MK-12 and MK-13, which are absent in strain K300^T and in species of *Clavibacter*, *Frigoribacterium* and *Rathayibacter*. The principal phospholipids of K265^T and K300^T were similar to those of *Clavibacter* (Collins & Jones, 1980), *Rathayibacter* (Zgurskaya *et al.*, 1993), *Frigoribacterium* (Kämpfer *et al.*, 2000) and *Cryobacterium* (Suzuki *et al.*, 1997).

The whole-cell methanolysates of strains K265^T and K300^T contained predominantly iso- and anteiso-branched fatty acids and 1,1-dimethyl acetals. The whole-cell methanolysates of strains K265^T and K300^T were quite similar to that of *F. faeni*, only differing in their content of several different iso- and anteiso-branched 1,1-dimethyl acetals (Table 3). However, the

peptidoglycan type of *Frigoribacterium* was B2β, whereas that of strains K265^T and K300^T was B2γ.

The amino acid ratio in the peptidoglycan of strains K265^T and K300^T differed from that of other DAB-containing genera (Table 3) in the low levels of glutamic acid. In strains K265^T and K300^T, nearly all of the glutamic acid residues at position 2 of the peptide subunit were replaced by hydroxy-glutamic acid. More or less complete hydroxylation of D-glutamic acid has been observed also in other representatives of the peptidoglycan-B-type cross-linkage, e.g. in *Microbacterium* species (Schleifer *et al.*, 1967).

The optimal growth temperatures of strains K265^T and K300^T were around 10 degrees lower, down to −2 °C for K265^T, than those in the genera *Clavibacter* (Davis *et al.*, 1984) and *Rathayibacter* (Zgurskaya *et al.*, 1993). The new genus *Frigoribacterium* (Kämpfer *et al.*, 2000), recently described and isolated from a Finnish farming environment, showed a similar growth-temperature range, whereas *Cryobacterium* (Suzuki *et al.*, 1997) did not grow above 18 °C. Psychrophilic actinobacteria proposed as species of *Arthrobacter* have been reported recently (Vasil'eva *et al.*, 1998; Loveland-Curtze *et al.*, 1999). 'Arthrobacter crygenae' strain Z-0064 had an optimal growth temperature of 6 °C and did not grow above 15 °C (Vasil'eva *et al.*, 1998), whereas 'Arthrobacter psychrolactophilus' strains had growth ranges of 0–30 °C (Loveland-Curtze *et al.*, 1999).

Decreases in temperature influenced both the fatty acid and dimethyl acetal compositions of the strains

Table 3. Diagnostic and differentiating characteristics of the genera *Clavibacter*, *Rathayibacter*, *Frigoribacterium*, *Cryobacterium* and strains K265^T and K300^T

+, Positive reaction; −, negative reaction; (+), varies among different strains; A, anteiso-branched; I, iso-branched; S, straight-chain saturated.

Characteristic	<i>Clavibacter</i> * [†]	<i>Rathayibacter</i> †	<i>Frigoribacterium</i> ‡	<i>Cryobacterium</i> §	K265 ^T	K300 ^T
Peptidoglycan type	B2γ	B2γ	B2β	B2γ	B2γ	B2γ
Cell wall diamino acid	DAB	DAB	D-Lysine	DAB	DAB	DAB
Major menaquinone(s)	MK-9	MK-10	MK-9	MK-10	MK-9, MK-10	MK-9, MK-10
G + C content (mol %)	67–78	67–78	71	66.5	64	68
Main cell wall sugars:						
Glucose	+	+	—	—	—	+
Galactose	+	(+)	—	—	—	—
Mannose	+	+	—	+	+	—
Ribose	—	—	—	—	+	—
Rhamnose	+	+	—	+	+	+
Fucose	—	—	—	+	—	—
Xylose	—	(+)	—	—	+	+
Major fatty acid types	A, I, S	A, I, S	A, I, S	A, I, S	A, I	A, I
Major 1,1-dimethyl acetals	—	—	a-15:0	—	a-15:0, i-16:0, a-17:0	a-15:0, i-16:0, i-15:0, a-17:0, 16:0
Growth temp. range (°C)	20–29	24–28	0–28	< 18	−2–28	2–28
Optimal growth temp. (°C)	20–29	24–28	0–28	9–12	15–17	15–17
Isolation source	Plant material	Plant material	Farm air	Antarctic soil	Boreal groundwater	Boreal groundwater

* Data from Groth *et al.* (1996).

† Data from Zgurskaya *et al.* (1993).

‡ Data from Kämpfer *et al.* (2000).

§ Data from Suzuki *et al.* (1997).

K265^T and K300^T. Strains K265^T and K300^T increased their content of anteiso 15:1 fatty acid at cold temperatures, as reported for *C. psychrophilum* (Suzuki *et al.*, 1997) and *F. faeni* (Kämpfer *et al.*, 2000).

1,1-Dimethyl acetals are formed by the methanolysis of alk-1'-enyl glyceryl ethers (plasmalogens) (Jantzen & Hofstad, 1981). Plasmalogens are known to occur as significant components of animal cell membranes and in many obligately anaerobic bacteria such as *Fusobacterium* spp. (Jantzen & Hofstad, 1981), clostridia (Johnston & Goldfine, 1994), *Megasphaera elsdenii* (Kaufman *et al.*, 1990) and *Eubacterium lentum* (Verhulst *et al.*, 1987). 1,1-Dimethyl acetals were detected in the anaerobic actinobacterial species *Propionibacterium freudenreichii* and *P. jensenii* (Kämpfer *et al.*, 2000). 1,1-Dimethyl acetals were recently detected also in the aerobic psychrophilic *F. faeni* belonging to the same actinobacterial family (*Microbacteriaceae*) as strains K265^T and K300^T (Kämpfer *et al.*, 2000).

The biological function of plasmalogens is not known but Kaufman *et al.* (1990) showed that membranes of *M. elsdenii* (with high plasmalogen content) were more ordered than membranes devoid of plasmalogens. This may indicate that the changes in the plasmalogen-derived 1,1-dimethyl acetal composition may reflect a role in membrane fluidity.

On the basis of the presence of several unusual 1,1-dimethyl acetals, other distinctive chemotaxonomic features and the 16S rRNA gene sequence, we propose that strains K265^T and K300^T should be placed in a new genus, namely *Subtercola* gen. nov. The 16S rRNA gene sequence comparisons clearly indicate that the strains K265^T and K300^T represent new taxa within the family *Microbacteriaceae*. It should be noted that the degree of 16S rRNA gene sequence divergence between these two strains (3%) is more than that found between strain K265^T and *Clavibacter michiganense* subsp. *michiganense* (which share 97.1% similarity) and is the same as that found between *F. faeni* and *C. michiganense* subsp. *michiganense* (3%). The influence of this sequence divergence is also seen in the results of phylogenetic analyses in which the bootstrap values indicate that there is no significance in the branching point joining the K265^T and K300^T lineages. Based on the results of 16S rRNA gene sequence analyses it could be proposed that the two strains represent different genera. However, there is little chemotaxonomic and phenotypic data to support this and we therefore propose, at this time, that these two strains should be included in a single genus as two novel species, for which we propose the names *Subtercola boreus* gen. nov., sp. nov. and *Subtercola frigoramans* gen. nov., sp. nov.

Description of *Subtercola* gen. nov.

Subtercola (sub.ter'co.la. L. prep. *subter* below, underneath; L. masc. suffix n. *-cola* inhabitant; M.L. masc. n. *subtercola* the one who lives underneath).

Aerobic, Gram-positive, irregular rods. Cells occur singly or in v-forms. Endospores are not produced. The colonies are pale to bright yellow depending on the culture medium. The colonies are circular, convex and smooth. Grows optimally at 15–17 °C. Growth is observed in the temperature range 2–28 °C. The cell wall diamino acid is DAB. The main cell wall amino acids are alanine, glycine, *threo*-3-hydroxy-glutamic acid and DAB. The peptidoglycan type is B2γ. The polar lipids are phosphatidylglycerol, diphosphatidylglycerol, one unknown phospholipid and two glycolipids. The main cellular fatty acids are 12-methyltetradecanoic acid (a-15:0), 14-methylpentadecanoic acid (i-16:0) and 14-methylhexadecanoic acid (a-17:0). The whole-cell methanolysates of *Subtercola* sp. contain 1,1-dimethoxy-anteiso-pentadecane (a-15:0 DMA) and 1,1-dimethoxy-iso-hexadecane (i-16:0 DMA) as major components in amounts comparable to those of fatty acids. The major isoprenoic quinones are MK-9 and MK-10. The G + C content of the DNA is 64–68 mol%. No mycolic acids are present. The type species is *Subtercola boreus*.

Description of *Subtercola boreus* sp. nov.

Subtercola boreus (bo.re'us. L. adj. *boreus* Northern, referring to the boreal groundwater aquifer in Finland, from which the organism was isolated).

Cells are short, irregular rods 0.2–0.3 µm in width and 0.6–1.0 µm in length. Colonies are yellow, circular, convex and smooth. Growth occurs at 2–28 °C. The major cellular fatty acids are 12-methyltetradecanoic acid (a-15:0), 14-methylpentadecanoic acid (i-16:0), 14-methylhexadecanoic (a-17:0) acid and 13-methyltetradecanoic acid (i-15:0). 1,1-Dimethoxy-iso-pentadecane (i-15:0 DMA), 1,1-dimethoxy-anteiso-heptadecane (a-17:0 DMA) and 1,1-dimethoxy-hexadecane (16:0 DMA) are found in whole-cell methanolysates. The cell wall contains glucose, rhamnose and xylose. Other chemotaxonomic characteristics are as described for the genus. Good carbon sources include L-arabinose, D-cellobiose, D-fructose, D-galactose, gluconate, D-glucose, D-mannose, D-maltose, L-rhamnose, D-ribose, sucrose, D-trehalose, D-xylose, adonitol, i-inositol, D-mannitol, acetate, propionate, *cis*-aconitate, citrate, fumarate, DL-3-hydroxybutyrate, 3-hydroxybenzoate and 4-hydroxybenzoate. Amino acids are mostly not utilized. The G + C content of the DNA is 68 mol%. Isolated from groundwater. The type strain is K300^T (= DSM 13056^T = CCUG 43135^T).

Description of *Subtercola frigoramans* sp. nov.

Subtercola frigoramans (fri.gor.a'mans. L. neut. n. *frigus* the cold; L. gen. neut. n. *frigoris* of the cold; L. part. pres. *amans* loving; M.L. part. pres. *frigoramans* loving the cold).

Cells are pleomorphic, irregular rods 0.3–0.4 µm in width and 0.9–1.5 µm in length. In liquid culture the cells may be arranged in v- or y-forms. Colonies are

pale yellow to bright yellow depending on the culture medium. Large, mucoid colonies are formed on glucose-containing medium. Growth occurs at -2°C to 28°C . The major cellular fatty acids are 12-methyltetradecanoic acid (a-15:0), 14-methylpentadecanoic acid (i-16:0), 14-methylhexadecanoic acid (i-17:0) and 12-methyltridecanoic acid (i-14:0). 1,1-Dimethoxy-anteiso-heptadecane (a-17:0 DMA) is found in whole-cell methanolysates. The cell wall contains rhamnose, ribose, xylose and mannose. The main isoprenoid quinones are MK-10 and MK-9. Other chemotaxonomical characteristics are the same as described for the genus. Good carbon sources include L-arabinose, *p*-arbutin, D-cellobiose, D-fructose, D-galactose, gluconate, D-glucose, D-mannose, D-maltose, sucrose, salicin, D-trehalose, D-xylose, adonitol and D-mannitol. Acetate, propionate, aconitate and most amino acids are not assimilated. Fumarate, but not citrate, is assimilated. The G+C content of the DNA is 64 mol%. Isolated from groundwater. The type strain is K265^T (= DSM 13057^T = CCUG 43136^T).

ACKNOWLEDGEMENTS

This work was supported by the Academy of Finland (grant nos 34519 and 46569). We thank Maria Anderson for advice on whole-cell fatty acid analysis, Raimo Mikkola for expertise with the GC-MS analysis and Arja Strandell for thin-section preparation. We thank the Division of Electron Microscopy at the Institute of Biotechnology at Helsinki University for the lease of equipment. We are grateful to H. G. Trüper for the Latin construction of the genus and species names. We thank Jani Salminen for the water-quality monitoring data for the aquifer.

REFERENCES

- Brosius, J., Palmer, M. L., Kennedy, J. P. & Noller, H. F. (1978). Complete nucleotide sequence of the ribosomal RNA gene from *Escherichia coli*, *Proc Natl Acad Sci USA* **75**, 4801–4805.
- Collins, M. D. & Jones, D. (1980). Lipids in the classification and identification of coryneform bacteria containing peptidoglycans based on 2,4-diaminobutyric acid, *J Appl Bacteriol* **48**, 459–470.
- Collins, M. D., Pirouz, T., Goodfellow, M. & Minnikin, D. E. (1977). Distribution of menaquinones in actinomycetes and corynebacteria, *J Gen Microbiol* **100**, 221–230.
- Davis, M. J., Gillespie, A. G., Jr, Vidaver, A. K. & Harris, R. W. (1984). *Clavibacter*: a new genus containing some phytopathogenic coryneform bacteria, including *Clavibacter xyli* subsp. *xyli* sp. nov., subsp. nov. and *Clavibacter xyli* subsp. *Cynodontis* subsp. nov., pathogens that cause ratoon stunting disease of sugarcane and bermudagrass stunting disease, *Int J Syst Bacteriol* **34**, 107–117.
- Felsenstein, J. (1993). PHYLIP (Phylogenetic Inference Package) version 3.5.1 Department of Genetics, University of Washington, Seattle, WA, USA.
- Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (1994). *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology.
- 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.
- Jantzen, E. & Hofstad, T. (1981). Fatty acids of *Fusobacterium* species: taxonomic implications, *J Gen Microbiol* **123**, 163–172.
- Johnson, J. L. (1994). Similarity analysis of DNAs. In *Methods for General and Molecular Bacteriology*, pp. 655–682. Edited by P. R. Gerhardt, G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Johnston, N. C. & Goldfine, H. (1994). Isolation and characterization of new phosphatidylglycerol acetals of plasmalogens. A family of ether lipids in clostridia, *Eur J Biochem* **223**, 957–963.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Kämpfer, P., Steiof, M. & Dott, W. (1991). Microbiological characterization of a fuel-oil contaminated site including numerical identification of heterotrophic water and soil bacteria, *Microb Ecol* **21**, 227–251.
- Kämpfer, P., Rainey, F. A., Andersson, M. A., Nurmiaho-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 Bacteriol* **50**, 355–363.
- Kaufman, A. E., Goldfine, H., Narayan, O. & Gruner, S. M. (1990). Physical studies on the membranes and lipids of plasmalogen-deficient *Megasphaera elsdenii*, *Chem Phys Lipids* **55**, 41–48.
- 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.
- MacKenzie, S. L. (1987). Gas chromatographic analysis of amino acids as the *N*-heptafluorobutyl isobutyl esters, *J Assoc Off Anal Chem* **70**, 151–160.
- Maidak, B. L., Cole, J. R., Parker, C. T., Jr & 11 other authors (1999). A new version of the RDP (Ribosomal Database Project). *Nucleic Acids Res* **27**, 171–173.
- Männistö, M. K., Tirola, M. A., Salkinoja-Salonen, M. S., Kulomaa, M. & Puhakka, J. A. (1999). Diversity of chlorophenol-degrading bacteria isolated from contaminated boreal groundwater, *Arch Microbiol* **171**, 189–197.
- Minnikin, D. E., Collins, M. D. & Goodfellow, M. (1979). Fatty acid and polar lipid composition in the classification of *Cellulomonas*, *Oerskovia* and related taxa, *J Appl Bacteriol* **47**, 87–95.
- Rainey, F. A., Ward-Rainey, N., Kroppenstedt, R. M. & Stackebrandt, E. (1996). The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of *Nocardiopsiaceae* fam. nov., *Int J Syst Bacteriol* **46**, 28–96.
- Sasaki, J., Chijimatsu, M. & Suzuki, K. (1998). Taxonomic significance of 2,4-diaminobutyric acid isomers in the cell wall peptidoglycan of actinomycetes and reclassification of *Clavibacter toxicus* as *Rathayibacter toxicus* comb. nov., *Int J Syst Bacteriol* **48**, 403–410.
- Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications, *Bacteriol Rev* **36**, 407–477.
- Schleifer, K. H., Plapp, R. & Kandler, O. (1967). Identification of threo-3-hydroxyglutamic acid in the cell wall of *Microbacterium lacticum*, *Biochem Biophys Res Commun* **28**, 566–570.
- Stackebrandt, E., Rainey, F. A. & Ward-Rainey, N. L. (1997).

Proposal for a new hierarchic classification system, *Actinobacteria* classis nov, *Int J Syst Bacteriol* **47**, 479–491.

Staley, J. T. (1968). *Prosthecomicrobium* and *Ancalomicrobium*: new prosthecate freshwater bacteria, *J Bacteriol* **95**, 1921–1942.

Suzuki, K.-I., Sasaki, J., Uramoto, M., Nakase, T. & Komagata, K. (1997). *Cryobacterium psychrophilum* gen. nov., sp. nov., nov. rev., comb. nov., an obligately psychrophilic actinomycete to accommodate '*Curtobacterium psychrophilum*' Inoue and Komagata 1976, *Int J Syst Bacteriol* **47**, 474–478.

Väisänen, O. M., Nurmiäho-Lassila, E. L., Marmo, S. A. & Salkinoja-Salonen, M. S. (1994). Structure and composition of biological slimes of paper and board machines, *Appl Environ Microbiol* **60**, 641–653.

Vasil'eva, L. V., Savel'eva, N. D., Omel'chenko, M. V., Lysenko, A. M., Pusheva, M. A., Trotsenko, Y. A. & Zavarzin, G. A. (1998).

Arthrobacter crygenae sp. nov. and *Acidovorax psychrophilic* sp. nov., psychrophilic hydrogen bacteria from tundra soil, *Microbiology* **64**, 195–200.

Verhulst, A., Van Hespén, H., Symons, F. & Eyssen, H. (1987). Systematic analysis of the long-chain components of *Eubacterium lentum*, *J Gen Microbiol* **133**, 275–282.

Wilson, K. (1994). Preparation of genomic DNA from bacteria. In *Current Protocols in Molecular Biology*, pp. 2.4.1–2.4.5. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. New York: Wiley.

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 iranica* comb. nov., and six strains from annual grasses, *Int J Syst Bacteriol* **43**, 143–149.