Cryobacterium psychrophilum gen. nov., sp. nov., nom. rev.,
comb. nov., an Obligately Psychrophilic Actinomycete
To Accommodate “*Curtobacterium psychrophilum*”
Inoue and Komagata 1976

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“*Curtobacterium psychrophilum*,” proposed by Inoue and Komagata in 1976, is a psychrophilic gram-positive irregular rod isolated from Antarctic soil. This organism grew optimally at 9 to 12°C and did not grow at higher than 18°C. Chemotaxonomic characteristics of this organism were the presence of 2,4-diaminobutyric acid in the cell wall and menaquinone-10 as the predominant respiratory quinone. The cellular fatty acid profile, which contained a significant amount of an anteiso-branched monounsaturated acid, 12-methyl tetradecenoic acid, was a distinctive characteristic of this organism and was reasonable for adaptation to low temperature. Phylogenetic analysis based on 16S ribosomal DNA sequences revealed that this organism was positioned at a separate branch in the family *Microbacteriaceae*, actinomycetes with group B peptidoglycan. We propose the name *Cryobacterium psychrophilum* gen. nov., sp. nov. for this organism. The type strain is JCM 1463 (=IAM 12024 =ATCC 43563 =IFO 15735 =NCIMB 2068).

In the course of an ecological study of microorganisms in Antarctica, Inoue isolated some obligately psychrophilic bacteria from soil in Antarctica (8), and the name “*Curtobacterium psychrophilum*” was proposed by Inoue and Komagata (9) in 1976 for an aerobic gram-positive pleomorphic rod. This bacterium grew optimally from 9 to 12°C and did not grow at temperatures higher than 18°C. The organism was classified in the genus *Curtobacterium* because of the presence of ornithine in the cell wall, the 66.5 mol% G+C content of the DNA, and the pleomorphic cell morphology. However, the name has not been validly published yet, and its further chemotaxonomic features have not been reported. Its position in the bacterial phylogenetic tree remained to be studied.

In the present study, we found a significant amount of anteiso-branched monounsaturated fatty acid, which has been generally reported to be at not more than a trace amount in bacterial cells (12, 16), except for in a plant pathogenic bacterium (23). Therefore, the cellular fatty acid composition of this organism is of interest in terms of the effect of the cultivation temperature, considering the psychrophilic property, as well as a chemotaxonomic marker.

We also found 2,4-diaminobutyric acid (DAB) in the cell wall peptidoglycan instead of ornithine, which was reported in the original paper (9). This paper deals with the taxonomy of “*C. psychrophilum*” and the proposal of the name *Cryobacterium psychrophilum* gen. nov., sp. nov., nom. rev., comb. nov.

MATERIALS AND METHODS

Bacterial strains studied and cultivation. “*C. psychrophilum*” JCM 1463^T originated from Inoue strain 27-O-b isolated from soil in Antarctica (9). This is the proposed monotype strain of this invalid species. This bacterium was cultivated for 7 days at 10°C on R agar (32) unless otherwise stated. *Curtobacterium*

albidum JCM 1344^T, *Curtobacterium citreum* JCM 1346^T, and *Curtobacterium pusillum* JCM 1350^T were cultivated at 10 and 17°C on R agar for comparison of the cellular fatty acid compositions.

Chemotaxonomy. The amino acid composition of the cell wall peptidoglycan, the cell wall acyl type, and the isoprenoid quinones were determined by methods described previously (15). The cell wall sugar profile was determined by high-performance liquid chromatography (HPLC) with derivatization as described by Takeuchi and Yokota (28). The polar lipid profile was determined by the integrated method for lipid analysis described by Minnikin et al. (17).

Cellular fatty acid analysis. Fatty acid methyl esters were liberated from 50 mg of lyophilized cells by methanolysis at 100°C for 3 h with 3 ml of 5% methanolic HCl and were extracted three times each with 3 ml of petroleum ether (15, 25). Hydrogenation of unsaturated fatty acids was carried out by bubbling of hydrogen gas in the presence of platinum black for 60 min. A Shimadzu GC-14A gas chromatograph with a fused silica capillary column, OV-1 (0.25 mm by 25 m; Nihon Chromato Co., Tokyo, Japan), was used for the separation of fatty acid methyl esters. The temperature of the injection port and detector block was 250°C; the column oven was kept at 180°C. Helium was used as the carrier gas. Gas-liquid chromatography-mass spectrometry (GC-MS) was carried out with a Shimadzu gas-liquid chromatograph-mass spectrometer, model QP-1000. The conditions for GC were the same as those mentioned above. For the MS, the ionizing current was 60 mA, the electron-accelerating voltage was 70 eV, and the ion-source temperature was 250°C. Standard methyl esters of 12-methyl tetradecanoic (a-15:0), 14-methyl hexadecanoic (a-17:0), 12-methyl tridecanoic (i-14:0), 13-methyl tetradecanoic (i-15:0), 14-methyl pentadecanoic (i-16:0), and 15-methyl hexadecanoic (i-17:0) acids were purchased from GL Sciences, Inc. (Tokyo, Japan), as were those of straight-chain acids.

Biochemical and physiological characteristics. Acid production from 28 carbohydrates, assimilation of 16 organic acids, nitrate reduction, and hydrolysis of DNA, starch, gelatin, and casein were tested by the methods described by Yamada and Komagata (32).

DNA base composition. DNA was extracted from the biomass by the methods of Saito and Miura (20) with some modifications. DNA base composition was determined by the method of Tamaoka and Komagata (30) by HPLC after enzymatic digestion of DNA to deoxyribonucleosides. An equimolar mixture of four deoxyribonucleotides in the Yamasa GC kit (Yamasa Shoyu Co., Ltd., Choshi, Japan) was used as the quantitative standard.

Phylogenetic analysis. The 16S ribosomal DNA (rDNA) sequence of “*C. psychrophilum*” JCM 1463^T was determined by a method previously described (26) and deposited in DDBJ under accession number D45058. Nucleotide substitution rates (K_{nuc} values) were calculated (13), and the phylogenetic tree was constructed by the neighbor-joining method (21). The topology of trees was evaluated by bootstrap analysis of the sequence data with CLUSTAL W software (31). This sequence was aligned with the following published sequences from DDBJ, GenBank, and EMBL: *Agrococcus jenensis* DSM 9580^T, X92492; *Agromyces cerinus* subsp. *cerinus* JCM 9083^T, D45060; *Agromyces fucosus* subsp.

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FIG. 1. Gas-liquid chromatogram of cellular fatty acid methyl esters extracted from *C. psychrophilum* JCM 1463^T. The fatty acids used are abbreviated as follows: i-14:0, 12-methyl tridecanoic acid; i-15:1, 13-methyl tetradecenoic acid; a-15:1, 12-methyl tetradecenoic acid; i-15:0, 13-methyl tetradecanoic acid; a-15:0, 12-methyl tetradecanoic acid; i-16:0, 14-methyl pentadecanoic acid; i-17:0, 15-methyl hexadecanoic acid; a-17:0, 14-methyl hexadecanoic acid.

hippuratus JCM 9085^T, D45061; *Agromyces mediolanus* DSM 20152^T, X77449; *Agromyces ramosus* DSM 43045^T, X77447; *Aureobacterium barkeri* DSM 20145^T, X77446; *Aureobacterium testaceum* DSM 20166^T, X77445; "*Brevibacterium helvolum*" DSM 20419, X77440; *Clavibacter michiganensis* subsp. *insidiosus* JCM 1369, D45051; *Clavibacter michiganensis* subsp. *michiganensis* DSM 46364^T, X77435; *Clavibacter michiganensis* subsp. *nebraskensis* DSM 7483^T, X77434; *Clavibacter xyli* subsp. *cynodontis*, M60935; "*Corynebacterium aquaticum*" JCM 1368, D45057; *Curtobacterium citreum* DSM 20528^T, X77436; *Curtobacterium luteum* DSM 20542^T, X77437; *Leucobacter komagatae* JCM 9414^T, D45063; *Microbacterium imperiale* DSM 20530^T, X77442; *Microbacterium lacticum* DSM 20427^T, X77441; *Rathayibacter rathayi* DSM 7485^T, X77439; and *Rathayibacter tritici* DSM 7486^T, X77438. *Brevibacterium linens* DSM 20425^T (X77451) was used as an out-group organism.

RESULTS

Morphological characteristics. Cells of "*C. psychrophilum*" JCM 1463^T were gram-positive, non-endospore-forming irregular rods. Cell size was 0.5 to 0.7 μ m by 1.0 to 1.8 μ m in the culture on R agar for 5 days at 10°C. Motility was not observed.

Chemotaxonomy. The peptidoglycan hydrolysate contained DAB, glycine, glutamic acid, and alanine. The molar ratio of

glutamic acid to alanine to glycine to DAB in the cell wall peptidoglycan was 1.0:0.6:1.0:1.3. The cell wall acyl type was acetyl. Fucose and rhamnose were found in the cell wall hydrolysate. The major isoprenoid quinone was menaquinone (MK), and the composition was MK-10 (50.4%), MK-11 (17.3%), MK-8 (14.2%), and MK-9 (11.2%). Diphosphatidylglycerol, phosphatidylglycerol, and one glycolipid were detected by thin-layer chromatography and specific visualization.

Cellular fatty acid composition. The gas chromatogram of the fatty acid methyl esters from the cells of "*C. psychrophilum*" JCM 1463^T cultivated at 10°C for 7 days is shown in Fig. 1. The predominant peaks corresponding to the standard compounds were a-15:0, i-15:0, a-17:0, and i-16:0 (Table 1). The identities of these peaks were also confirmed by MS. In addition to those, a significant amount of 12-methyl tetradecenoic acid (a-15:1) was found. The mass spectrum of this peak was considered to be that of methyl pentadecenoate because of the relatively large peak of 55 and the presence of $M - 32$, $M - 74$, and 254 (M^+), in spite of the base peak at 74. It is true that the methyl esters of anteiso-branched, iso-branched, and straight-chain saturated fatty acids with the same numbers of carbon were not distinguished from one another only by their mass spectra. However, the peak disappeared by hydrogenation, and the ratio of the acid in the cellular fatty acids was accurately added to that of the corresponding saturated fatty acid, a-15:0. Therefore the peak was identified as a-15:1. The peak of 13-methyl tetradecenoic acid (i-15:1) was also identified by the same procedure.

Effect of cultivation temperature on cellular fatty acid composition. The cellular fatty acid compositions of "*C. psychrophilum*" JCM 1463^T cultivated at different temperatures were examined (Table 1). The proportion of a-15:1 was increased in the cells grown at the lower temperature. The cellular fatty acid compositions of some mesophilic *Curtobacterium* strains grown at 10 and 17°C were examined for comparison as shown in Table 1. They are reported to grow optimally at 25 to 30°C (7, 14) and did not produce enough biomass for fatty acid analysis below 10°C. Although a small amount of a-15:1 was detected, they did not show the same composition as "*C. psychrophilum*" JCM 1463^T.

Biochemical and physiological characteristics. The results of tests to determine biochemical and physiological characteristics are shown in Table 2.

TABLE 1. Effect of growth temperature on the cellular fatty acid composition of *C. psychrophilum* and some mesophilic *Curtobacterium* strains

Strain	Growth temp (°C)	Fatty acid composition (%) ^a									
		Monounsaturated				Saturated					
		a-15:1	a-17:1	i-15:1	i-16:1	a-15:0	a-17:0	i-14:0	i-15:0	i-16:0	ch-17:0
<i>Cryobacterium psychrophilum</i> JCM 1463 ^T	17	4.4		0.8		50.9	15.0	0.7	16.9	9.4	1.7
	10	19.8		2.3		33.6	13.4		18.4	10.2	1.7
	8	22.3		1.8		34.3	14.6		16.5	8.3	1.3
	4	26.3		1.8		31.2	11.2		18.5	8.4	1.1
<i>Curtobacterium albidum</i> JCM 1344 ^T	17	1.9				67.3	20.7	1.7	1.9	8.3	
	10	6.3	tr			67.0	14.1	1.9	2.1		
<i>Curtobacterium citreum</i> JCM 1346 ^T	17	tr				60.2	23.8	1.4	1.7	11.4	
	10	8.0	3.3		3.6	51.8	21.1	1.3	0.8	10.0	
<i>Curtobacterium pusillum</i> JCM 1350 ^T	17					46.0	25.6	0.4	3.3	6.3	18.5
	10	1.7				54.9	23.8	0.3	2.2	2.4	14.7

^a a, anteiso-branched acid; i, iso-branched acid; ch, ω -cyclohexyl acid; tr, trace amount.

TABLE 2. Physiological and biochemical characteristics of *C. psychrophilum* JCM 1463^T

Characteristic	Reaction ^a
Acid from:	
Amygdalin	—
L-Arabinose	—
Galactitol	—
Erythritol	—
Esculin	—
Fructose	+
Galactose	+
D-Glucitol	—
D-Gluconate	—
Glucose	+
Glycerol	—
Inositol	—
Inulin	—
D-Lactose	—
Maltose	—
Mannitol	—
Mannose	+
D-Melezitose	—
Melibiose	—
D-Raffinose	—
L-Rhamnose	—
Ribitol	—
Ribose	—
Salicin	—
Sucrose	+
Tagatose	—
D-Trehalose	—
D-Xylose	—
Assimilation of:	
n-Butyrate	—
Citrate	—
Formate	—
Fumarate	+
Glutamate	—
Glyoxylate	+
Hippurate	—
Lactate	+
Malate	+
Maleate	—
Nicotinate	—
Propionate	+
Pyruvate	+
Succinate	+
Tartrate	—
Uric acid	—
Hydrolysis of:	
Casein	—
Gelatin	—
DNA	+
Starch	w
Nitrate reduction	—

^a —, negative reaction; +, positive reaction; w, weakly positive reaction.

DNA base composition. The DNA base composition of “*C. psychrophilum*” JCM 1463^T was 65 mol% G+C by the HPLC-nucleoside method.

16S rDNA sequence analysis. The 16S rDNA of “*C. psychrophilum*” JCM 1463^T was determined for 1,501 bases, as shown in the DDBJ database under accession number D45058. The sequence showed high similarities to those of actinomycetes with group B peptidoglycan, and the phylogenetic tree created with the representative strains is shown in Fig. 2. This organism was positioned in the cluster of actinomycetes with

group B peptidoglycan but isolated from any of the subclusters corresponding to the established genera.

DISCUSSION

An obligately psychrophilic gram-positive bacterium, “*C. psychrophilum*” JCM 1463^T, showed interesting and unique characteristics, particularly in the cellular fatty acid profile. The presence of a significant amount of 12-methyl pentadecenoic acid (i.e., a-15:1) is characteristic for the cellular fatty acids of “*C. psychrophilum*” JCM 1463^T. The cellular fatty acid profile of this organism is principally the anteiso- and iso-branched type, which is frequently found in gram-positive bacteria (11, 12, 24). In this type of cellular fatty acid, a-15:0, the melting point is low enough to maintain the fluidity of the membrane as monounsaturated acids do in the straight-chain type (12). Therefore, generally trace or smaller amounts of unsaturated acids are found in this type of cellular fatty acid of mesophilic bacteria (11, 12, 25). Even though unsaturated fatty acids have been found in some psychrophilic bacilli, they were straight-chain monounsaturated fatty acids (2, 10). Although Stead et al. reported that the ratio of a-15:1 accounted for approximately 10% in the cells of the strains of *Clavibacter michiganensis* subsp. *sepedonicus* (23), our analysis of the type strain of this subspecies showed a cellular fatty acid composition containing a-15:1 in 1% of the acids (unpublished data).

The presence of a-15:1 is unusual but reasonable for psychrophilic gram-positive bacteria such as “*C. psychrophilum*” JCM 1463^T to maintain membrane fluidity even at low temperatures. The amount of a-15:1 was increased by cultivation at lower temperatures, as shown in Table 1. The cells of some mesophilic *Curtobacterium* strains grown at 10°C also contained a-15:1 acid as shown in Table 1. Although unusually low temperature was employed for cultivation of these strains, which grew optimally at 25 to 30°C (7, 14), the amount of a-15:1 was so small (8% at the utmost) that they could not show a composition similar to that of “*C. psychrophilum*” JCM 1463^T. This fact indicates the distinctiveness of “*C. psychrophilum*” JCM 1463^T. The shift of cellular fatty acid composition in mesophilic anteiso-dominant bacteria is observed as the increase in the ratio of a-15:0 to a-17:0 in response to the change of cultivation temperature from 37°C to 20°C and not as the appearance of anteiso monounsaturated acids (25). The increase in the proportion of unsaturated straight-chain fatty acids was observed in psychrophilic gram-negative bacteria (5).

The diamino acid in the peptidoglycan of “*C. psychrophilum*” JCM 1463^T was DAB instead of ornithine as reported previously (9). The molar ratio of amino acids was almost similar to those of the strains of the known DAB-containing genera, *Agromyces*, *Clavibacter*, and *Rathayibacter* (4, 22, 26), and was different from those of *Agrococcus jenensis* (6) and *Leucobacter komagatae* (27). Therefore, the peptidoglycan structure of this organism can be estimated to be that of B2γ of Schleifer and Kandler (22). Comparison of the ratio with that of *Agromyces ramosus* JCM 3108^T (glutamic acid to alanine to glycine to DAB = 1.0:0.8:1.1:1.8) (4, 26), “*C. psychrophilum*” JCM 1463^T contains a rather low level of alanine and DAB. The substitute ratio of positions 3 (DAB) and 4 (alanine) of the peptide subunit might be relatively low. The cell wall acyl type of this strain was an acetyl type like that of the other DAB-containing taxa.

The major MK of “*C. psychrophilum*” JCM 1463^T is MK-10 with significant amounts of MK-8 and MK-11. Although the strains of the genus *Rathayibacter* also possess MK-10 as the predominant MK, the composition is mostly MK-10, unlike in “*C. psychrophilum*” JCM 1463^T. The polar lipid profile of “*C.*

FIG. 2. Unrooted phylogenetic tree derived from 16S rDNA sequences. The tree was created by using the neighbor-joining method and K_{nuc} values. The numbers on the tree indicate bootstrap values (percent) for the branch points. The strains used and the nucleotide sequence accession numbers are indicated in the text.

psychrophilum" JCM 1463^T, consisting of diphosphatidylglycerol, phosphatidylglycerol, and glycolipid, is identical to those of the strains of the genera *Agromyces*, *Clavibacter*, and *Rathayibacter* (1, 3, 33, 34), except for the variation in the number of spots corresponding to glycolipids.

The cell wall sugar profile of "*C. psychrophilum*" JCM 1463^T is distinct in the presence of fucose and rhamnose. Takeuchi and Yokota reported the presence of fucose in only one strain of *Clavibacter michiganensis* subsp. *michiganensis* among the coryneform bacteria which they studied (29). However, it is different from "*C. psychrophilum*" JCM 1463^T in containing a significant amount of galactose. The cell wall of "*C. psychrophilum*" JCM 1463^T is clearly differentiated from *Rathayibacter* strains in containing a large amount of fucose, which is lacking in the cell wall of *Rathayibacter* strains (34).

The phylogenetic analysis of "*C. psychrophilum*" JCM 1463^T based on 16S rDNA sequences revealed that this organism was accommodated in the cluster corresponding to actinomycetes with group B peptidoglycan, i.e., the family *Microbacteriaceae* (18, 19, 29). In the cluster of *Microbacteriaceae*, DAB-containing taxa such as *Agrococcus*, *Agromyces*, *Clavibacter*, *Leucobacter*, and *Rathayibacter* formed independent subclusters corresponding to the genera (6, 19, 26). "*C. psychrophilum*" JCM 1463^T showed a lineage independent from that of any of the genera in the cluster *Microbacteriaceae*.

The results of analyses of the biochemical and physiological characteristics of "*C. psychrophilum*" JCM 1463^T were similar to those of the previous report by Inoue and Komagata (9), except for the acid from sucrose and glycerol and assimilation of formate.

On the basis of the data described above, we conclude that "*C. psychrophilum*" JCM 1463^T should belong to a new genus in the family *Microbacteriaceae*. No strain other than the strain first isolated by Inoue and Komagata has been known. However, considering the unusual properties of the organism as shown above and the difficulty of reproducible isolation, we propose here a new genus, *Cryobacterium*, for "*C. psychrophilum*" JCM 1463^T and propose that it be renamed as *Cryobacterium psychrophilum* gen. nov., sp. nov., nom. rev., comb. nov.

Description of *Cryobacterium* gen. nov. *Cryobacterium* (Cry.o.bac.te'ri.um Gr. n. kryos, cold; Gr. n. bakterion, a small rod; M. L. neut. n. *Cryobacterium*, a cold [preferring] rod). Pleomorphic nonmotile rod. Non endospore forming. Branching occurs in the early growth phase. Rod forms occur in old culture. Occasionally gram variable in the old culture. Grows optimally at 9 to 12°C and not at 18°C. Aerobic. DNase and catalase are positive. Gelatin and casein are not hydrolyzed. Acid is produced from several sugars. The G+C content of DNA is approximately 65 mol%. The amino acids of cell wall peptidoglycan are 2,4-diaminobutyric acid, alanine, glycine, and glutamic acid. Rhamnose and fucose are the characteristic cell wall sugars. The main cellular fatty acids are iso- and anteiso-branched acids. A significant amount of 12-methyl tetradecenoic acid (anteiso-C15:1) is contained. The major MK is MK-10. Mycolic acids are absent. The polar lipids are diphosphatidylglycerol, phosphatidylglycerol, and glycolipid.

The type species is *C. psychrophilum*.

Description of *Cryobacterium psychrophilum* sp. nov., nom. rev., comb. nov. (basonym "*Curtobacterium psychrophilum*" Inoue and Komagata 1976). *Cryobacterium psychrophilum* (psy.chro'phi.lum. Gr. adj. psychros, cold; Gr. adj. philus, loving; M. L. fem. adj. psychrophilum, cold loving). Nonmotile, non-endospore-forming, pleomorphic rod 0.5 to 0.7 µm by 1.0 to 1.8 µm in culture on R agar for 5 days at 10°C. Circular, opaque pink colonies are formed in 5 days of culture on R agar at 10°C. Branching occurs in the early growth phase. Rod forms occur in old culture. Occasionally gram variable in the old culture. Aerobic. DNase and catalase are positive. Acid is produced from fructose, mannose, glucose, galactose, and sucrose but not from arabinose, mannitol, and glycerol. Lactate, pyruvate, fumarate, and hippurate are utilized. Gelatin and casein are not hydrolyzed. The G+C content of the DNA is 65 mol%. 2,4-Diaminobutyric acid, alanine, glycine, and glutamic acid are the component amino acids of cell wall peptidoglycan, in the ratio 1.3:0.6:1.0:1.0. Rhamnose and fucose are the characteristic cell wall sugars. The main cellular fatty acids are iso- and anteiso-branched acids. 12-Methyl tetradecenoic acid (a-C15:1) accounts for approximately 20% of the cellular fatty

acids in cells grown at 10°C. The MK composition is predominantly MK-10, followed by MK-11 and MK-8. The polar lipids are diphosphatidylglycerol, phosphatidylglycerol, and glycolipid. The type strain of *C. psychrophilum* is JCM 1463 (= K. Inoue and K. Komagata 27-O-b = IAM 12024 = ATCC 43563 = IFO 15735 = NCIMB 2068).

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