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Sphingobacterium antarcticus sp. nov., a Psychrotrophic Bacterium from the Soils of Schirmacher Oasis, Antarctica

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Two pure cultures of bacteria isolated from soil samples collected in Schirmacher Oasis, Antarctica, conformed to the definition of the genus *Sphingobacterium*. They differed from all of the known species of *Sphingobacterium* in being psychrotrophic. The G+C contents of the DNA of the two strains were found to be 39.3 and 40.3 mol%, and DNA-DNA hybridization studies indicated 7% homology with *S. multivorum* and *S. spiritivorum*. The name *Sphingobacterium antarcticus* sp. nov. is proposed for the two Antarctic strains. The type strain is 4BY (MTCC 675), and it has been deposited with the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India.

Until 1984, the genus *Flavobacterium* was extremely heterogeneous and included species which were gram negative, formed yellow or orange colonies, produced acid from carbohydrates, were either motile by peritrichous flagella or nonmotile, and which had either high (63 to 70 mol%) or low (26 to 34 mol%) G+C contents of their DNA (38). The genus was eventually restricted to only those gram-negative, nonmotile, yellow, rod-shaped bacteria that had a low G+C content (30 to 42%) (10, 25, 35). On the basis of these criteria, seven distinct species, namely *F. aquatile*, *F. breve*, *F. balustinum*, *F. meningosepticum*, *F. odoratum*, *F. multivorum*, and *F. spiritivorum* (9–11, 25, 39), were found to conform to the genus. Since then, at least three more new species of *Flavobacterium*, viz. *F. indologenes*, *F. yabuuchiae*, and *F. mizutaii*, have been identified (12, 41). A detailed study based on the phenotypic characteristics, cellular lipid composition, and G+C contents of DNA of 41 strains of *Flavobacterium* belonging to five different species (namely *F. spiritivorum*, *F. multivorum*, *F. meningosepticum*, *F. odoratum*, and *F. breve*) led to the formation of a new genus, *Sphingobacterium* (41). This genus was distinguished from the genus *Flavobacterium* by the presence of high concentrations of sphingophospholipids in its strains. On the basis of this criterion, *F. multivorum* and *F. spiritivorum* have been renamed *Sphingobacterium multivorum* and *S. spiritivorum* (41).

Our earlier studies on the terrestrial microorganisms of Schirmacher Oasis, Antarctica, indicated the presence of a heterogeneous population of bacteria and yeast (21, 26–29). The ten gram-negative, motile, rod-shaped bacteria were identified as *Pseudomonas fluorescens*, *Pseudomonas putida*, and *Pseudomonas syringae* (29), the thirteen gram-positive, nonmotile rods were identified as *Arthrobacter globiformis*, *Arthrobacter pascens*, and *Arthrobacter protophorimiae* (27), and the five gram-positive coccoid bacteria were identified as *Micrococcus roseus* and *Planococcus* spp. (28).

The present study describes two isolates of bacteria from the soils of Antarctica. Both isolates had all of the characteristics of *Sphingobacterium* but did not conform to any of the known species. We therefore propose that they be recognized as a new species, with the name *S. antarcticus*.

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To our knowledge, the present report is the first to describe *Sphingobacterium* spp. isolated from Antarctica.

MATERIALS AND METHODS

Source of the organisms, media, and growth conditions. Soil samples were collected around Lake Zub, Schirmacher Oasis (75°45′12″S, 11°46′E), Antarctica, during the third week of January, 1985. The soil temperature varied from +6°C to −6°C. These samples were appropriately diluted, plated on the surface of medium plates containing 0.5% peptone, 0.1% yeast extract, and 1.5% agar (pH 6.9), and incubated at 10°C. Details of the study site, sampling area, and mode of collection of the samples have been described earlier (21, 27–29). Fewer than 1% of the bacterial colonies that appeared after 1 to 2 weeks were yellow. These yellow colonies could be divided into two main types on the basis of colony size, shape, and intensity of color. Pure cultures of these two types of yellow bacteria—the pale yellow and the bright yellow colonies—isolated from two different soil sites close to one another were established and designated 4BY and 6BY, respectively. The optimum temperature, pH, and salt concentration for the growth of 4BY and 6BY were determined by using the medium described above.

Morphology, motility, and biochemical tests. Cultures in the logarithmic phase of growth were observed under the phase-contrast microscope to determine their shape and size. Motility was determined by direct observation of an overnight culture by using the hanging drop method and by the piercing of a soft-agar medium. The ability of the bacteria to glide was determined as described earlier by Holmes et al. (10). Staining of the flagella was done by the method of silver impregnation (1) by using *Pseudomonas aeruginosa* as a positive control.

The activities of catalase, oxidase, phosphatase, gelatinase, urease, arginine dihydrolyase, lysine decarboxylase, acylamidase, and β-galactosidase (8, 41); the production of indole; growth on MacConkey agar, cetrimide agar, citrate agar, or β-hydroxybutyrate; reduction of nitrate and nitrite; levan formation from sucrose; hydrolysis of starch, esculin, Tween 20, and Tween 80 (8, 32, 33); production of hydrogen sulfide; pigment production on King's A and B medium (14); production of acid and/or gas from different carbon compounds (13); and accumulation of poly-β-hydroxybutyrate (19) were monitored according to the references cited.

The sensitivity to antibiotics, the isolation of DNA, and

the G+C content of the DNA of the cultures was determined by standard methods (15, 24, 28, 29). DNA-DNA hybridization was performed by the membrane filter method (37). DNA was denatured by boiling in 0.2 M NaOH for 5 min and then quickly chilled on ice and neutralized with HCl. Denatured DNA was dotted onto nitrocellulose and immobilized by baking at 80°C for 2 h. The filters with the fixed DNA were prehybridized in a buffer containing 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt medium (4), calf thymus DNA (100 μg/ml), and 1% sodium dodecyl sulfate for 2 h at 65°C and then hybridized in the same buffer under similar conditions but in the presence of labelled DNA. DNA was labelled by nick translation (23).

Identification of fatty acids. Lyophilized 4BY and 6BY cells (100 mg) were hydrolyzed with methanolic KOH and converted to methyl esters in 0.2% sulfuric acid for 2 h at 70°C in a water bath. The resulting fatty acid methyl esters were extracted with chloroform and chromatographed on a Finnigan Mat 1020 automated gas chromatograph-mass spectrometer by using an SE30, 25-m capillary column in the temperature range of 120 to 200°C. The increase in temperature was 8°C/min. Fatty acids were identified on the basis of mass spectral data (36).

Identification of sphingolipids. Lipids were extracted from a lyophilized cell pellet (20) and used for the isolation of sphingolipids (5). Sphingolipids were resolved by thin-layer chromatography and detected by spraying with ninhydrin and/or Clorox-benzidine as described by Skispski and Barclay (31). Long-chain bases were prepared from the sphingolipids (42) and visualized with ninhydrin reagent. Infrared spectra of the long-chain bases were recorded in chloroform on a Perkin Elmer infrared spectrophotometer, and the mass spectra were recorded at 20 eV at a probe temperature of 210°C on a VG Micromass 7070-H mass spectrometer.

Reference strains. *Sphingobacterium multivorum* (earlier known as *F. multivorum* NCTC 11343^T and NCTC 11033^T) and *S. spiritivorum* (earlier known as *F. spiritivorum* NCTC 11386^T and NCTC 11387^T) were used as positive controls in studies related to morphology, motility, biochemical tests, and identification of fatty acids and sphingolipids.

Bacterial pigment. Lyophilized 4BY and 6BY cells (~20 mg) were suspended in methanol and extracted by vigorous vortexing for 30 min. The methanol extract was centrifuged, the yellow clear supernatant was recovered, and the absorption spectrum was recorded in an Hitachi 330 spectrometer.

RESULTS AND DISCUSSION

Colonies of 4BY and 6BY were isolated and purified from two different soil samples (collected around Lake Zub), which yielded about 1×10^3 colonies per g of soil. The colonies of 4BY and 6BY were yellow, translucent, round, smooth, and slightly convex. The two types of colonies differed in that 4BY was pale yellow in color and 1 to 2 mm in diameter, whereas 6BY was bright yellow and more than 2 mm in diameter. The yellow pigment could be extracted from both 4BY and 6BY with methanol. The absorption spectrum of the pigment indicated the presence of three absorption maxima in each case, but the positions of the absorption maxima varied. In 4BY the yellow pigment showed peaks at 396, 442, and 464 nm, whereas in 6BY the peaks were at 420, 447, and 472 nm. Cells from these colonies were gram-negative, nonsporeforming, straight rods, nonmotile in soft agar and in hanging drop preparations, and they did not possess any flagella. The cells also did not exhibit any gliding motility. Under optimum growth conditions (25°C and pH 6.9), the generation time of 4BY and 6BY was 2.5 h; at 30°C and pH 6.9 it was 6 h. At 5°C it was 20 h.

The two isolates had the following characteristics in common. They were nonfermentative; were catalase, oxidase, urease, gelatinase, β-galactosidase, and phosphatase positive; did not accumulate poly-β-hydroxybutyrate; hydrolyzed Tween 20, Tween 80, esculin, and gelatin but not starch; did not reduce nitrate or nitrite; did not produce indole; produced hydrogen sulfide; and were capable of growth on β-hydroxybutyrate and MacConkey agar but not on cetrimide agar. Citrate, acetate, and malonate were not utilized; casein was not digested; arginine dihydrolase and lysine decarboxylase were not produced. Both isolates produced acid from glucose (1% and 10%), lactose (1% and 10%), cellulose, raffinose, and mannose; acid was not produced from fructose, sucrose, galactose, maltose, arabinose, melezitose, rhamnose, sorbitol, glycerol, mesoinositol, xylose, salicin, ethanol, and mannitol. The isolates did not accumulate poly-β-hydroxybutyrate, produced yellow nondiffusible pigments, and did not produce fluorescent pigment on King's A and B medium.

4BY and 6BY had very similar nutritional requirements. They could grow on Minimal A medium supplemented with a carbon source such as L-arabinose, D-xylose, L-rhamnose, D-ribose, glucose, D-fructose, D-mannose, D-galactose, sucrose, maltose, lactose, raffinose, mannitol, glycerol, *m*-inositol, sorbitol, succinate, pyruvate, lactate, glutamate, formate, β-hydroxybutyrate, and malate. Starch, cellulose, melibiose, glycogen, dextrin, and inulin were not utilized as a carbon source.

The main cellular fatty acid components of 4BY and 6BY were identified as $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{17:1}$, and $iC_{15:0}$. Of these, $iC_{15:0}$ and $C_{16:1}$ were the most predominant, constituting 29 and 56% of the total cellular fatty acid content, respectively. By using the solvent system chloroform-methanol-acetic acid-water (100:20:12:5), it was possible to resolve the sphingolipids of 4BY and 6BY into two major spots (with R_fs of 0.26 and 0.32, respectively) and two minor spots (with R_fs of 0.41 and 0.51, respectively). In *S. multivorum*, sphingolipids with R_fs of 0.32, 0.41, and 0.51 were present but the sphingolipid with an R_f of 0.26 was not detected. In 4BY and 6BY, sphingophospholipids constituted 23% of the total phospholipid. This is in close agreement with the 21% reported for *S. multivorum* (41). The long-chain bases obtained from the sphingolipids of 4BY, 6BY, and *S. multivorum* (NCTC 11343^T) resolved into three spots on silica gel thin-layer chromatography plates (0.5 mm thick) by using the solvent system chloroform-methanol-water (65:25:4, vol/vol/vol). Long-chain bases with R_fs of 0.32 and 0.57 were detected in 4BY, 6BY, and *S. multivorum*, but the third long-chain base had an R_f of 0.09 in 6BY and 4BY and 0.18 in *S. multivorum*. The presence of an amino group in the long-chain bases was confirmed by a positive ninhydrin reaction and a broad band at 3,280 cm^{-1} in the infrared spectrum. The OH group was not clearly detected, probably because of an overlap of the amino and OH group bands. Preliminary mass spectral studies of the three long-chain bases obtained by scraping preparative thin-layer chromatography plates were conducted, and the results based on the molecular ion peaks

$$(M^+),\ M^+\text{-}H_2O,\ \text{and}\ M^+\text{-}(HO\text{-}CH_2\text{-}\underset{\displaystyle NH_2}{\underset{|}{CH}})$$

indicate the presence of long-chain bases corresponding to $C_{16:0}$, $C_{17:0}$, and $C_{18:1}$ with a molecular mass of 273, 287, and

TABLE 1. Characteristics in which the two strains of *S. antarcticus* differed from each other or from *S. multivorum*

Characteristic	Result for:		
	4BY	6BY	*S. multivorum* (NCTC 11343^T)
Colony size (diam)	1–2 mm	>2 mm	1 mm
Colony color	Pale yellow	Bright yellow	Yellow
Growth at 37°C	−	−	+
Growth at 2°C	+	+	−
Acid production from:			
Melezitose	−	−	+
Fructose	−	−	+
Sucrose	−	−	+
Arabinose	−	−	+
Glycerol	−	−	+
Xylose	−	−	+
Galactose	−	−	+
Salicin	−	−	+
Maltose	−	−	+
Gelatinase	+	+	−
Major fatty acid	$C_{16:1}$	$C_{16:1}$	i-2-OH-$C_{15:0}$
Mol% G+C	39.3 ± 1	40.3 ± 1	40.5

TABLE 2. Sensitivity of *S. antarcticus* from Schirmacher Oasis, Antarctica, to antibiotics[a]

Antibiotic	Concn	Resistance (R) or sensitivity (S) of:			
		S. multivorum	*S. spiritivorum*	4BY	6BY
Streptomycin	10 μg	R	R	R	R
Ampicillin	10 μg	R	R	R	R
Gentamycin	10 μg	R	R	R	R
Kanamycin	40 μg	R	R	R	R
Polymyxin B	300 units	R	ND[b]	R	R
Tobramycin	15 μg	R	ND	R	R
Erythromycin	15 μg	R	R	R	R
Penicillin	10 units	ND	ND	R	R
Carbenicillin	50 μg	R	R	R	R
Nystatin	100 μg	ND	ND	R	R
Rifamycin	10 μg	S	S	S	S
Tetracycline	30 μg	R	S	S	S
Nalidixic acid	30 μg	S	ND	S	S
Novobiocin	10 μg	ND	ND	S	S
Bacitracin	10 units	ND	ND	S	R
Vancomycin	30 μg	ND	ND	S	R
Chloramphenicol	30 μg	S	ND	R	S
Colistin	10 μg	ND	ND	R	S
Trimethoprim	10 μg	S	ND	S	R

[a] The concentration refers to the quantity of antibiotic per disc.
[b] ND, not done.

299, respectively. The G+C contents of the DNA of 4BY and 6BY were 39.3 and 40.3 mol%, respectively. DNA-DNA hybridization studies indicated 100% homology between 4BY and 6BY. On the basis of the characteristics described above, 4BY and 6BY were identified as belonging to the genus *Sphingobacterium* (12, 41). Earlier studies had indicated the presence of the closely related genus *Flavobacterium* in the soils and the marine environment of Antarctica (2, 3).

Members of the genus *Sphingobacterium* differ from *Flavobacterium* spp. in that they contain sphingolipids, do not produce indole, do not grow on skim milk acetate agar, and are gelatinase negative (41); they can be distinguished from *Cytophaga* and *Flexibacter* spp. in that they have a carotenoidlike pigment and do not exhibit gliding motility.

So far, three species of mesophilic *Sphingobacterium*, namely *S. spiritivorum*, *S. multivorum*, and *S. mizutaii*, have been recognized (12, 41). These three species could be distinguished from one another on the basis of their ability to produce acid aerobically from ethanol, mannitol, and rhamnose and on their ability to hydrolyze starch (41). Since 4BY and 6BY give a negative reaction for all of the tests described above, they closely resemble *S. multivorum*, not *S. spiritivorum* (which is positive for all of the tests) or *S. mizutaii* (which could produce acid from rhamnose but is negative with respect to the other three tests). Furthermore, 4BY and 6BY had G+C contents of 39.3 and 40.3 mol%, respectively, and produced acid aerobically only from glucose, raffinose, cellobiose, lactose, and mannose, thus closely resembling *S. multivorum*.

Some of the characteristics in which the present isolates differed from *S. multivorum* are listed in Table 1. Apart from these characteristics, distinct differences are observed in the fatty acid compositions. In the type strain of *F. multivorum*, the predominant cellular fatty acids were the branched and hydroxy fatty acids, with i-2-OH-$C_{15:0}$, i-3-OH-$C_{17:0}$, and i-$C_{15:0}$ constituting 35, 10, and 27%, respectively (41), and $C_{16:1}$, which constituted 13%. But in 4BY and 6BY the predominant fatty acids were i-$C_{15:0}$ (29%) and $C_{16:1}$ (56%) and contained only trace amounts of hydroxy fatty acids. Psychrotrophic bacteria are known to possess a greater proportion of unsaturated fatty acids so as to increase the membrane fluidity at low temperatures (7, 18). This may be the reason for the high proportion of $C_{16:1}$ (56%) in 4BY and 6BY as compared to *S. multivorum* (13%). However, it is difficult to give reasons as to why hydroxy fatty acids are present in only minimal amounts in 4BY and 6BY. A detailed study of the cellular fatty acid composition of nine isolates of *Sphingobacterium* belonging to three different species also indicated some prominent differences. For instance, in two isolates of *S. multivorum*, NCTC 11343^T and GIFU 926, fatty acids $C_{16:1}$, i-$C_{15:0}$, i-2-OH-$C_{15:0}$, and i-3-OH-$C_{17:0}$ constituted 13 and 29%, 27 and 18%, 35 and 19%, and 10 and 1%, respectively (41). Furthermore, NCTC 11343^T did not possess the fatty acids $C_{14:0}$, 2-OH-$C_{14:0}$, and 3-OH-$C_{16:0}$, and GIFU 926 did not possess i-$C_{17:1}$. DNA-DNA homology studies indicated 100% homology between 4BY and 6BY but both showed only about 10% hybridization with *S. multivorum* and about 5% with *S. spiritivorum*, respectively. The sensitivity of the isolates to antibiotics was also slightly different from that of *S. multivorum* (Table 2). The present isolates of *S. antarcticus* also differ from *F. yabuuchiae* (which shows about 64% homology with *S. spiritivorum*) and *F. thalpophilum* (which is capable of growth at 42°C and produces acid from arabinose, rhamnose, glycerol, and mannitol and reduces nitrate) (12). Since the two isolates of *Flavobacterium* described above possess characteristics of the genus *Sphingobacterium*, it was suggested that the isolates be transferred to the genus *Sphingobacterium* (12) provided that taxonomists are convinced. We are of the opinion that isolates which differ in their fatty acid compositions due to their psychrotrophic or thermophilic nature compared with the type strains of *Sphingobacterium* should be included in *Sphingobacterium*.

Earlier studies had also indicated that a number of bacterial isolates from Antarctica possess unusual characteristics and thus cannot be readily identified as existing species (17, 22, 27–30, 34, 40). When the differences between the Antarctic isolates and the known species have been many and distinct, the former have been assigned to new species such

as *Halomonas subglaciescola* (6) and *Flectobacillus glomeratus* (16). Taking all the characteristics described for 4BY and 6BY into account, it seems appropriate to assign them to a new species of *Sphingobacterium* for which the name *S. antarcticus* is proposed. The present study is the first report on the taxonomy of *Sphingobacterium* isolates from the soils of continental Antarctica.

Description of *Sphingobacterium antarcticus* sp. nov. *Sphingobacterium antarcticus* (ănt.ärk'ti.kŭs. N.L. *antarcticus*, pertaining to the antarctic) cells are aerobic gram-negative rods, length 2 to 3 μm, width 0.5 to 1 μm. Nonmotile. Colony morphology on peptone-yeast extract agar: round, yellow, smooth, slightly convex, 1 to 2 mm in diameter. No growth factors required. Optimal growth temperature, 25°C; can grow between 2 and 30°C, but not at temperatures above 30°C. Grow between pH 6 and 8, with optimum growth at pH 6.9. NaCl not required for growth; can tolerate up to 0.5 M NaCl.

Utilizes a wide variety of carbon sources and can acidify glucose, lactose, cellobiose, raffinose, and mannose. Does not produce gas from carbohydrates.

Catalase, oxidase, phosphatase, gelatinase, urease, and β-galactosidase positive. Tween 20, Tween 80, and esculin are hydrolyzed. Starch is not hydrolyzed. The major fatty acid is $C_{16:1}$; the long-chain bases of the sphingophospholipid are $C_{16:1}$, $C_{17:0}$, and $C_{16:0}$. DNA base composition is 39.3 to 40.3 mol% G+C. Habitat is soil from Schirmacher Oasis, Antarctica. The type strain is 4BY (= MTCC 675).

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