***Microbacterium kitamiense* sp. nov., a new polysaccharide-producing bacterium isolated from the wastewater of a sugar-beet factory**

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Two strictly aerobic, heterotrophic and mesophilic new organisms, strains Kitami A1 and Kitami C2^T, were isolated from the wastewater of a sugar-beet factory in Kitami City, Hokkaido, Japan. In batch cultures, these organisms produced both insoluble and soluble exopolysaccharides (EPSs) utilizing sucrose as the sole carbon source. The G+C contents of the strains Kitami C2^T and Kitami A1 were 69.2 mol%. Both strains had anteiso-C15:0 acid, anteiso-C17:0 acid and iso-C16:0 as major components. The major isoprenoid quinones from these strains included menaquinone-11 and menaquinone-12. Physiological and biochemical characterization, phylogenetic analysis and DNA–DNA relatedness indicated that these two organisms are new species of the genus *Microbacterium*, for which the name *Microbacterium kitamiense* is proposed. The type strain of *M. kitamiense* is strain Kitami C2^T (= JCM 10270^T).

Keywords: *Microbacterium kitamiense*, polysaccharide, industrial wastewater

INTRODUCTION

There have been many reports on micro-organisms that produce exopolysaccharides (EPSs). Some of the bacterial polysaccharides are produced on an industrial scale and are used as raw materials in food processing and in medical and industrial preparations. It remains possible that new polysaccharide-producing bacteria could be found in various habitats. Recently, new polysaccharide-producing bacteria were isolated from a deep-sea hydrothermal vent (Raguenes *et al.*, 1996, 1997a, b).

We tried to find new polysaccharide-producing bacteria from wastewater obtained from several factories; we isolated numerous bacteria that produced polysaccharide in our laboratory. The strains Kitami C2^T and Kitami A1 were both isolated as polysaccharide producers from the wastewater of a sugar-beet factory in Kitami, Hokkaido, Japan.

In this study, we examined the physiological and

biochemical features, the chemotaxonomic characteristics and the phylogeny of these strains. DNA–DNA relatedness data showed that the strains should be classified as a new species of the genus *Microbacterium*.

METHODS

Bacterial strains and cultivation. EPS-producing bacteria, Kitami C2^T and Kitami A1, were isolated from the wastewater of a sugar-beet factory in Kitami City, Japan. The organisms were cultivated aerobically at 25 °C in tryptic soy agar (Difco), unless otherwise stated.

Production of EPS. A basal culture medium (pH 8.5) for EPS production contained 1.5% (w/v) sucrose, 0.075% CaCO₃, 0.05% MgSO₄, 0.1% K₂HPO₄, 0.0001% Na₂MoO₄, 0.01% KNO₃ and 1% trace-element solution (Patel, 1984). For the production of EPS, the bacteria were cultured at 25 °C for 7 d in a 500 ml Erlenmeyer flask containing 100 ml of the above medium by rotary shaking with 10 ml seed culture.

Isolation of EPS from culture fluid. For preparation of the EPS fraction, the culture broth from the Erlenmeyer flask was centrifuged at 11 000 g for 10 min to remove bacterial cells. The supernatant was again centrifuged at 14 000 g for 30 min. A clear supernatant and a gelatinous matrix were recovered. To the supernatant fraction, three volumes of ethanol were added and the precipitate was collected by centrifugation at 10 000 g for 10 min. The precipitate was

Abbreviation: EPS, exopolysaccharide.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA sequences reported in this paper are AB013907 (Kitami C2^T) and AB013920 (Kitami A1).

dissolved in distilled water and dialysed overnight against the distilled water; this was followed by lyophilization. The polymer obtained was designated 'soluble EPS'. The gelatinous matrix fraction was suspended and dialysed overnight against distilled water and then lyophilized. The polymer obtained was designated 'insoluble EPS'. The weights of the lyophilized EPSs were measured gravimetrically.

Chemical analysis of EPS. The total carbohydrate content was determined using the phenol/sulfuric acid method (Dubois *et al.*, 1956). The protein content was determined according to the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. EPS constituents were examined as their alditol acetate by GLC; this was followed by hydrolysis of the EPS. GLC analysis was performed on a Hitachi chromatograph model G-3000 equipped with a flame-ionization detector: a capillary column sp-2330 (0.25 mm $\times$ 40 m; Supelco) was used. For GLC analysis, the derivative procedure used was that of Albersheim *et al.* (1967). The uronic acid was analysed qualitatively by the method of Thomas & Albersheim (1972).

Physiological and biochemical characterization. The physiological and biochemical characterization of the two strains isolated was determined as described by Komagata (1985).

Cell wall analysis. Cell wall analysis was performed as described by Schleifer & Kandler (1972). The glycolate test was performed according to the method of Uchida & Aida (1977).

Quinone composition. Cells cultivated overnight in tryptic soy broth at 25 °C were used to determine the quinone composition, as described by Komagata & Suzuki (1987).

Cellular fatty acid composition. Cells cultivated overnight in tryptic soy broth at 25 °C were used for cellular fatty acid analysis according to the method described by Suzuki & Komagata (1983).

DNA base composition and DNA-DNA hybridization. Chromosomal DNA was prepared from bacterial cells according to the method of Marmur (1961). The G+C content of DNA was determined according to the method of Tamaoka & Komagata (1984).

Levels of DNA relatedness were determined by the method of Ezaki *et al.* (1989). Probes for DNA-DNA hybridization were prepared from Kitami C2^T, Kitami A1 and type strains of *Microbacterium aurantiacum* IFO 15234^T and *Microbacterium chocolateum* IFO 3758^T.

Determining and comparing 16S rRNA gene sequences. Cloning and sequencing of 16S rRNA genes were carried out by the method described previously (Shida *et al.*, 1997). The phylogenetic tree was created by the neighbour-joining method (Saito & Nei, 1987), using K_{nuc} values (Kimura, 1980). The topology of the tree was evaluated by performing a bootstrap analysis using the CLUSTAL W version 1.5 program (Thompson *et al.*, 1994).

RESULTS AND DISCUSSION

Production and characterization of the EPS

In batch cultures in the presence of sucrose (Erlenmeyer flasks at 25 °C for 7 d), these organisms produced both insoluble and soluble EPSs. The yields

Table 1. Characteristics of strains Kitami C2^T and Kitami A1

NC, No change; +, positive; −, negative. Both strains were negative for the oxidase test, and for hydrolysis of casein, urea, Tweens 20, 40, 60 and 80, and for utilization of citrate, succinate, fumarate, propionate, and for formation of indole and production of hydrogen sulfide, and acid production from inositol, sorbose, lactose, ribose, salicin, sorbitol, melibiose and adonitol, and for gas production from glucose, arabinose, xylose and mannitol, and for growth in 0.02 % Na₂S₂O₃ or 0.01 % lysozyme. Both strains were positive for catalase, hydrolysis of gelatin, starch, DNA, and for decomposition of tyrosine, and for utilization of ammonium and nitrate, and for acid production from glucose, fructose, galactose, mannose, maltose, arabinose, sucrose, xylose, trehalose, glycerol, mannitol and raffinose, and for growth in 2, 5 or 7 % NaCl.

Characteristic	Kitami C2 ^T	Kitami A1
Litmus milk	NC	NC
Nitrate reduction	−	+
Voges-Proskauer	−	−
pH in Voges-Proskauer	5.2	5.3
pH range	6.0–9.0	6.0–9.0
Temperature range (°C)	15–37	15–37
Acid production from:		
Cellobiose	−	+
Rhamnose	−	+

of soluble and insoluble EPSs of Kitami C2^T were about 0.5 g l^{−1} and 1.8 g l^{−1}, respectively; yields of soluble and insoluble EPSs of Kitami A1 were 0.8 g l^{−1} and 5.0 g l^{−1}, respectively. These EPS contained trace amounts of protein and no uronic acid. The sugar constituents of soluble EPSs of Kitami C2^T were 14 % (w/w) rhamnose, 19 % mannose, 25 % galactose and 42 % glucose, while those of insoluble EPSs were 27 % rhamnose, 13 % mannose, 9 % galactose and 51 % glucose. The sugar constituents of the soluble EPS of Kitami A1 were 31 % rhamnose, 19 % mannose and 50 % glucose, while those of insoluble EPS were 25 % rhamnose, 11 % mannose, 21 % galactose and 43 % glucose. These data suggested that the sugar constituents of Kitami C2^T bear a close resemblance to those of Kitami A1.

Morphological, physiological and biochemical characterization

Strains Kitami C2^T and Kitami A1 were strictly aerobic, non-motile, non-sporing, Gram-positive irregular rods (0.5–0.8 $\times$ 1.0–2.0 μ m). After 5 d growth at 25 °C, colonies on tryptic soy agar were circular, round, capitate, smooth, orange in colour, glistening and about 1.5–3.0 mm in diameter. The physiological and biochemical characteristics of these strains are shown in Table 1.

Fig. 1. Phylogenetic tree derived from 16S rRNA sequence data for strain Kitami C2^T, strain Kitami A1 and other species. Bar, 0.007 K_{nuc} units.

Chemotaxonomic characteristics

The cell wall peptidoglycan of strains Kitami C2^T and Kitami A1 contained D-ornithine, as diamino acid, and the glycan moiety of the cell wall contained glycolate residues.

The major isoprenoid quinones from these strains contained menaquinone 11 (MK-11, 67–68 % of total quinones) and menaquinone 12 (MK-12, 18–24 % of total quinones).

In strains Kitami C2^T and Kitami A1, anteiso-C15:0 acid (40–47 % of total cellular fatty acids), anteiso-C17:0 acid (32–39 %) and iso-C16:0 acid (10–13 %) were major components.

Phylogenetic analysis

The 16S rRNA gene-similarity value for Kitami C2^T and Kitami A1 was 100 %. The sequence-similarity values (> 94 %) placed these strains within the realm of the genus *Microbacterium*. A phylogenetic tree of strains Kitami C2^T and Kitami A1, based on 16S rRNA gene sequences, is shown in Fig. 1. These two strains were members of a robust, monophyletic cluster containing the *Microbacterium* species (Fig. 1). Re-

cently, the genus *Microbacterium* was emended; additional species have been described by Takeuchi & Hatano (1998a, b).

DNA base composition and DNA relatedness

The G + C content of strains Kitami C2^T and Kitami A1 was 69.2 mol % (Table 2). The two isolates were located on the same branch as *M. aurantiacum* and *M. chokolatum* under the high bootstrap value in the phylogenetic tree based on 16S rRNA sequences (Fig. 1). Therefore, we estimated the DNA–DNA relatedness value by using strains *M. aurantiacum* and *M. chokolatum* as the references. DNA relatedness values for Kitami C2^T and Kitami A1 were 100 % (Table 2). The values for relatedness to *M. aurantiacum* and *M. chokolatum* (60–69 %) were not high enough to permit identification as the same species (Table 2).

Differential characteristics of Kitami C2^T and the 26 previously described species of the genus *Microbacterium* are summarized in Table 3. According to the phylogenetic analysis based on the 16S rRNA sequence and DNA–DNA relatedness data, the isolates were not very distant from *M. aurantiacum* and *M. chokolatum*. However, on the basis of the physio-

Table 2. DNA base compositions and levels of DNA relatedness

Strain*	G + C content (mol %)	Reassociation (%) with DNA from:†		
		<i>M. chocolateum</i> IFO 3758 ^T	<i>M. aurantiacum</i> IFO 15234 ^T	Kitami C2 ^T
<i>M. chocolateum</i> IFO 3758 ^T	69.2	100	68	69
<i>M. aurantiacum</i> IFO 15234 ^T	69.3	65	100	67
Kitami C2 ^T	69.2	60	68	100
Kitami A1	69.2	63	68	100

* IFO, Institute for Fermentation, Osaka, Japan.

† Reassociation values are means of two determinations; the maximum difference noted between two determinations was 9%.

Table 3. Differential characteristics of *Microbacterium* species

Species: 1, *M. kitamiense*; 2, *M. lacticum*; 3, *M. dextranolyticum*; 4, *M. imperiale*; 5, *M. arborescens*; 6, *M. aurum*; 7, *M. laevaniformans*; 8, *M. liquefaciens*; 9, *M. flavescens*; 10, *M. terregens*; 11, *M. saperdae*; 12, *M. barkeri*; 13, *M. testaceum*; 14, *M. esteraromaticum*; 15, *M. arabinogalactanolyticum*; 16, *M. keratanolyticum*; 17, *M. terrae*; 18, *M. trichohecanolyticum*; 19, *M. luteolum*; 20, *M. schleiferi*; 21, *M. aurantiacum*; 22, *M. chocolateum*; 23, *M. halophilum*; 24, *M. hominis*; 25, *M. ketosireducens*; 26, *M. maritimum*; 27, *M. thalassium*. Y, yellow; W/Y, white or yellow; W, white; O, orange; w, weakly positive; d, different reactions; +, positive; -, negative.

Characteristic	1	2*	3*	4*	5*	6*	7*	8†	9†	10†	11†	12†	13†	14†	15†	16†	17†	18†	19†	20†	21†	22†	23†	24†	25†	26†	27†
Colour of colonies	O	Y	Y	O	O	Y	Y	Y	Y	Y	W/Y	Y	O	Y	W	Y	Y	Y	W/Y	Y	O	O	Y	W/Y	Y	Y	Y
Motility	-	-	-	+	-	-	-	-	-	-	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	+	-
Growth at 37 °C	+	-	-	+	-	-	+	-	-	-	-	+	-	d	-	-	-	-	-	w	+	+	+	+	-	+	-
H ₂ S formation	-	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Voges-Proskauer reaction	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-
Hydrolysis of:																											
Gelatin	+	-	-	-	+	+	+	+	+	-	-	+	+	-	+	+	+	-	-	-	-	-	w	-	+	+	w
Starch	+	+	+	+	-	+	+	-	+	-	+	+	-	+	+	-	+	+	-	-	+	+	+	-	+	-	+
Tween 20	-	-	+	-	-	-	-	-	-	+	+	+	+	d	-	-	d	+	+	d	+	+	w	+	+	+	w
Tween 40	-	-	+	-	-	-	-	+	-	+	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	+	+
Tween 60	-	-	+	-	-	w	-	+	-	+	+	+	+	+	+	-	+	+	-	d	+	+	+	+	+	+	+
Tween 80	-	-	+	-	-	-	-	+	-	+	-	+	+	+	+	-	+	+	-	d	+	+	+	+	+	+	+
Assimilation of:																											
Malate	-	+	-	+	-	-	+	+	-	+	+	+	+	+	+	+	w	+	+	-	d	d	-	+	d	+	-
Fumarate	-	+	+	+	+	-	+	+	w	+	+	+	+	+	+	+	+	+	+	-	+	d	-	+	d	-	-
Propionate	-	+	-	-	-	-	+	+	w	+	+	w	w	+	+	+	d	w	+	-	+	d	-	+	d	-	-

* Data from the description by Yokota *et al.* (1993a).† Data from the description by Yokota *et al.* (1993b).

‡ Data from the description by Takeuchi & Hatano (1998b).

logical and biochemical characteristics, the isolates were obviously different from these two strains. These characteristics in Table 3 also indicated that strain Kitami C2^T can be distinguished easily from all previously described species of the genus *Microbacterium*. In our opinion, this strain warrants the status of a new taxon; therefore, we propose that strains Kitami C2^T and Kitami A1 should be classified as new species in the genus *Microbacterium*, namely *Microbacterium kitamiense* sp. nov.

Description of *Microbacterium kitamiense* sp. nov.

Microbacterium kitamiense (kita'mi.ense. M.L. adj. *kitamiense* referring to Kitami, a city in Hokkaido, Japan, where the organism was isolated).

The cells are irregular rods (0.5–0.8 µm in width, 1.0–2.0 µm in length). Gram-positive, non-motile and non-sporing. Strictly aerobic, catalase-positive and oxidase-negative. The pH in Voges–Proskauer broth is 5.7. Hydrogen sulfide and indole are not produced. Gelatin and starch are hydrolysed. Casein, DNA, Tweens 20, 40, 60 and 80 and urea are not hydrolysed. Tyrosine is decomposed. Citrate, propionate, fumarate, L-malate and succinate are not utilized. Nitrate and ammonium are utilized. Growth occurs at temperatures between 15 and 37 °C and at pH 6.0–9.0. Growth occurs in the presence of 7% NaCl. Growth is inhibited by 0.02% NaN₃ and by 0.01% lysozyme. Acid, but no gas, is produced from glucose, arabinose, xylose and mannitol. The cell wall peptidoglycan contains D-ornithine as diamino acid and there are

high levels of glycolate in the glycan moiety of the cell wall. The major fatty acids are anteiso-C15:0, anteiso-C17:0 and iso-C16:0. The major quinones are menaquinone 11 and menaquinone 12. The G + C content of type strain is 69.2 mol %. The type strain, Kitami C2^T, has been deposited at the Institute of Physical and Chemical Research, Wako, Japan, as strain JCM 10270^T.

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