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Sphingobacterium canadense sp. nov., an isolate from corn roots ☆

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Abstract

A free-living Gram-negative bacterial strain CR11^T was isolated from corn roots. Polyphasic taxonomy was performed, including API20 NE and API50 CH bacterial identification kits, Biolog analysis, lipids and fatty acid analysis, DNA–DNA hybridization, 16S rRNA and *cpn60* gene sequence analyses. 16S rRNA gene sequence analysis indicated that strain CR11^T belonged to the genus *Sphingobacterium* and was closely related to *Sphingobacterium multivorum* IFO 14947^T (98% similarity) and *Sphingobacterium. thalpophilum* ATCC 43320^T (97% similarity). DNA–DNA hybridization showed 11% and 13% DNA re-association with *S. multivorum* LMG 8342^T and *S. thalpophilum* LMG 11520^T, respectively. Major fatty acids (16:0, 15:0 iso and 17:0 iso 3-OH) and the G+C content of the DNA (40.5 mol%), were also similar to those of the genus *Sphingobacterium*. The predominant respiratory quinone was MK-7. In all analyses, including phenotypic characterization, this isolate was found to be different from the closely related species, *S. multivorum* and *S. thalpophilum*. On the basis of these results, this strain represents a new species within the genus *Sphingobacterium*. The name *Sphingobacterium canadense* sp. nov. is suggested and the type strain is CR11^T (= NCCB 100125^T = LMG 23727^T).

Keywords: *Sphingobacterium*; Biolog analysis; 16s rDNA sequence analysis; DNA–DNA hydridization; *cpn60* gene; Corn

Introduction

A detailed study based on phenotypic characteristics, cellular lipid composition and the G+C DNA contents of 41 strains of *Flavobacterium* belonging to five different species (namely *Flavobacterium spiritivorum*, *Flavobacterium multivorum*, *Flavobacterium meningosepticum*, *Flavobacterium odoratum*, *Flavobacterium breve*) led to the formation of a new genus, *Sphingobacterium*. This genus was distinguished from the genus *Flavobacterium* by the presence of high concentrations of sphingolipids in its strains. On the basis of this criterion, *F. multivorum* and *F. spiritivorum* have been re-named *Sphingobacterium multivorum* and *Sphingobacterium spiritivorum*. [14]. The genus *Sphingobacterium* was first described by Yabuuchi et al. [17], with three new species *S. spiritivorum*, *S. multivorum* and *Sphingobacterium mizutae*. At present, the genus is comprised of nine species, including *Sphingobacterium antarcticum* [14], *Sphingobacterium faecium* [15], *Sphingobacterium heparinum* [15], *Sphingobacterium piscium* [15], *Sphingobacterium deajeonense* [7] and *Sphingobacterium thalpophilum* [15].

Several plant growth promoting rhizobacteria were isolated from washed roots and rhizosphere of corn growing in Western Ontario, Canada. Strain CR11^T was

☆ *Note*: The Genbank/EMBL/DDBJ accession numbers for the 16S rRNA and *cpn60* gene sequences of strain CR11^T are AY787820 and DQ914834, respectively.

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also isolated from washed corn roots and was initially identified as *S. multivorum* based on cell and colony morphology, as well as the results of the API20 NE bacterial identification kit. However, the 16S rRNA sequence of this strain showed 98% similarity with this species and 97% similarity with *S. thalpophilum*. Therefore, the purpose of this study was to clarify the taxonomic position of this strain by using a polyphasic approach based on detailed phenotypic analysis, lipids and fatty acid analysis, phylogenetic analyses of 16S rRNA and *cpn60* genes and DNA–DNA hybridization.

Material and methods

Isolation, morphology and phenotypic characterization

Isolate CR11^T was isolated on M medium [16], except that biotin was not added and pH 7.2–7.4 was used. Sub cultivation was carried out on the same medium at 30 °C for 48–72 h. Cell morphology was observed using a scanning electron microscope (SEM). Temperature (20–41 °C), pH (4–10) and NaCl concentration ranges (0.5–3%) were determined on M medium. The Biolog analysis system, as well as the API 20NE and API CH50 bacterial identification kits were used for physiological characterization. The flexirubin pigment test was carried out as described by Fautz and Reichenbach [2]. The analysis of quinones was carried out by using the identification services of DSMZ, Braunschweig, Germany. For the phosphate solubilization assay, aliquots (10 µl) of bacterial culture grown in M medium over night were spot inoculated onto NBRIP medium [11] and plates were incubated for 14 days at 28 °C. Formation of a clear zone in the agar around the growing bacterial colonies was considered as a positive result. Indole acetic acid (IAA) production in the presence of 100 mg/l L-tryptophan in CCM [13] was determined as mentioned by Mehnaz and Lazarovits [8].

Sphingolipid assay and fatty acid analysis

Lipids were extracted from a bacterial cell pellet by using Folch's method [3] and they were used for the isolation of sphingolipids. Sphingolipid analysis was carried out by Avantis polar lipids, Inc., Alabama, USA. Sphingolipids were resolved by 2D-TLC [6]. Sphingomyelin, D-erythro-sphingosine and D-erythro-sphinganine, and ceramide were used as standards. Standards and samples were plated on TLC silica gel plates. All plates were chromatographed using chloroform–methanol–6.7 M ammonium hydroxide (33:15:1.25;v/v/v) in the first dimension and chloroform–methanol–acetic acid–water (42:12:3.15:1; v/v/v/v) for the second dimension. The sample and standards were detected using a combination of iodine, ninhydrin, acid molybdate, and charring.

For fatty acid analysis, bacterial cultures were grown in tryptic soy broth at 28 °C for 24–48 h. Cellular fatty acid profiles of isolate CR11^T, *S. multivorum* LMG 8342^T and *S. thalpophillum* LMG 11520^T were determined by using a Microbial identification system equipped with a gas chromatograph (Agilent Technology, CA, USA; Model #6890N) and version 5.0 of the aerobe library (Microbial identification system (1993) Operating manual, MIDI, Inc., Newark, Del.), according to a standard protocol [12].

DNA base composition, DNA–DNA hybridization and phylogenetic analysis

Genomic DNA was extracted and the DNA base composition was determined using the HPLC technique [10]. DNA–DNA hybridization was performed at 38.5 °C, according to the method described by Cleenwerck et al. [1].

The 16S rRNA gene was amplified by using the primers and PCR conditions as previously described by Mehnaz et al. [9]. The sequence was deposited in the GenBank (Accession No. AY787820). Phylogenetic analysis was performed using the software package Bionumerics (Applied Maths, Belgium) after including the consensus sequence in an alignment of small ribosomal subunit sequences collected from the international nucleotide sequence library EMBL. The alignment was pair wise, which was calculated by using an open gap penalty of 100% and a unit gap penalty of 0%. A similarity matrix was created by identity calculation with a gap penalty of 0% after discarding unknown bases. A resulting tree based on comparison of 1528 bases was constructed using the neighbour-joining method. Bootstrap analysis was performed using the same software package to test the statistical reliability of the topology of the neighbour-joining tree with 1000 bootstrap re-samples of the data.

Amplification of chaperonin 60 (*cpn60*) genes

The chaperonin gene (*cpn60*) was amplified from the bacterial genomic DNA of isolate CR11^T, *S. multivorum* LMG 8342^T and *S. thalpophilum* LMG 11520^T, by using universal *cpn60* degenerate primers. The primers were H729, H730 [4], H1610 (5′-CGCCAGGGTTTTCCCAGTCACGACGAIIIIGCIGGYGACGGYACSACSAC-3′) and H1611 (5′-AGCGGATAACAATTTCACACAGGACGRCGRTCRCCGAAGCCSGGIGCCTT-3′). For PCR, a primer mixture (forward mix: 1 part H729 and 3 parts H1610; reverse mix: 1 part H730 and 3 parts H1611) was used. The PCR conditions were: 5 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 50 °C, 45 s at 72 °C and 5 min at 72 °C. The sequences were deposited

in the GenBank (CR11^T, Accession No. DQ914834; *S. multivorum* LMG 8342^T, Accession No. DQ914835; *S. thalpophilum* 11520^T, Accession No. DQ985196).

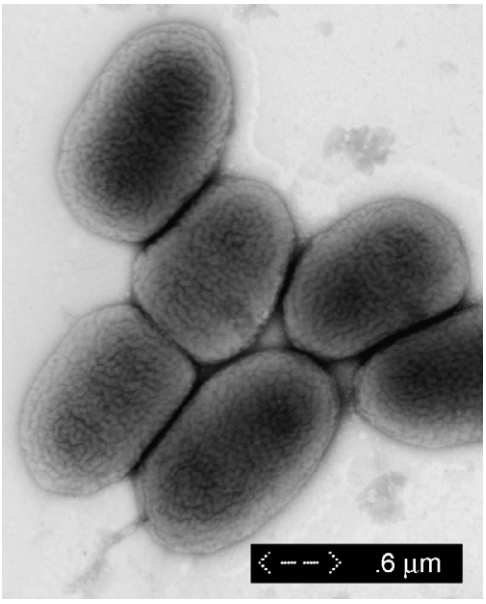

Fig. 1. Cell morphology of corn isolate CR11^T under the electron microscope.

Results and discussion

Morphology and phenotypic characterization

Isolate CR11^T formed round, wet, off-white colonies which turn yellow after a few days. Growth was observed at 20–37 °C, pH 5–10 and concentrations of 0.5–3% NaCl. Cells of the bacterium were short rods, 0.9–1.0 µm wide and 1.0–1.9 µm long (Fig. 1.). Results of the physiological characterization are given in the species description. A summary of the results for the differentiation of isolate CR11^T from *Sphingobacterium* species is presented in Table 1. Flexirubin pigment was not detected. MK-7 was detected as the predominant menaquinone (98%) and traces of menaquinones 6 and 8 were also detected. Phosphate solubilization on NBRIP medium was not observed. IAA production in the presence of 100 mg/l tryptophan was ~277 ng/ml.

Sphingolipid assay and fatty acid composition

For sphingolipid analysis, a spot was detected with ninhydrin and acid molybdate, which had similar retention and characteristics as the ceramide standard. A trail of eight lipids was observed to be ninhydrin

Table 1. Differential phenotypic characteristics of strain CR11^T and other type strains of *Sphingobacterium* species

Characteristics	1	2	3	4	5	6	7	8	9	10
Growth at:										
5 °C	–	–	–	–	–	–	+	+	+	+
41 °C	–	+	–	–	–	+	–	–	–	–
Hydrolysis of:										
Esculin	+	–	+	+	+	+	+	+	+	+
Gelatin	+	–	–	–	–	–	–	+	–	–
Urease	+	–	+	+	+	+	+	+	–	–
Assimilation of:										
L-rhamnose	–	–	+	+	–	+	+	+	ND	ND
L-arabinose	+	–	–	+	V	+	+	+	+	–
D-mannitol	–	–	+	–	–	–	–	+	ND	ND
D-melibiose	+	+	+	+	+	+	+	–	ND	ND
Glycerol	+	–	V	–	–	+	+	+	ND	ND
L-glutamate	–	–	–	+	+	–	+	+	ND	ND
Acid production from:										
L-rhamnose	–	–	–	V	–	+	–	–	ND	ND
L-arabinose	+	–	–	+	+	+	+	–	+	V
Sucrose	+	–	+	+	+	+	+	–	+	+
DNA G+C content (mol%)	40.5	38.7	39.0	39.9-40.5	39.3-40.0	44.0-44.2	37.3	39.3	42.3	41.1–42.1

Strains: 1, *Sphingobacterium canadense* sp. nov. CR11^T; 2, *S. daejeonense* TR6-04^T; 3, *S. spiritivorum* NBRC 14948^T; 4, *S. multivorum* NBRC 14947^T; 5, *S. mizutaii* NBRC 14946^T; 6, *S. thalpophilum* NBRC 14963^T; 7, *S. faecium* NBRC 15299^T; 8, *S. antarcticum* MTCC 675^T; 9, *S. heparinum* IFO 12017^T; 10, *S. piscium* IFO 14985^T.

All strains are positive for aerobic growth at 30 °C, catalase, oxidase, negative for Gram staining, sporulation, indole production, motility. Data for *S. daejeonense* TR6-04^T, *S. spiritivorum* NBRC 14948^T, *S. multivorum* NBRC 14947^T, *S. mizutaii* NBRC 14946^T, *S. thalpophilum* NBRC 14963^T, *S. faecium* NBRC 15299^T, and *S. antarcticum* MTCC 675^T is taken from Kim et al. [7], and for *S. piscium* and *S. heparinum* from Takeuchi and Yokota [15].

+, positive; –, negative; v, variable, ND, no data available.

positive, and five of them were shown to be acid molybdate positive. These five are believed to be phospholipids. There appeared to be no lipids coinciding with D-erythro-sphingosine and D-erythro-sphinganine.

The data for fatty acid analysis of CR11^T, *S. multivorum* LMG8342^{T}and *S. thalpophilum* LMG11520^T is given in Table 2.

DNA base composition, DNA–DNA hybridization and phylogenetic analysis

The G+C content of the DNA of strain CR11^T was 40.5 mol% which is in accordance with the values for the genus *Sphingobacterium* (39–42 mol%; [15]). DNA–DNA hybridization results showed that isolate CR11^T had 11% DNA re-association with *S. multivorum* LMG 8342^T and 13% re-association with *S. thalpophilum* LMG 11520^T.

On the basis of the distance matrix, the percentage 16S rDNA sequence similarity indicated that the closest relatives to strain CR11^T were *S. multivorum* IFO14947^T (98.0%), *S. thalpophilum* ATCC 43320^T (97.1%) and *S. faecium* DSM 11690^T (94.6%). Similarity with other *Sphingobacterium* spp. was 90–93%. The phylogenetic tree based on the 16S rRNA gene sequence, constructed by using the neighbour-joining method, is shown in Fig. 2.

Table 2. Fatty acid analysis of CR11^T, *Sphingobacterium multivorum* LMG 8342^T and *Sphingobacterium thalpophilum* LMG 11520^T

Fatty acid	CR11^T (%)	*S. multivorum* (%)	*S. thalpophilum* (%)
13:1 at 12-13	ND	0.80	0.25
14:0	1.42	3.02	3.06
16:0	8.70	9.37	9.26
18:0	0.58	ND	ND
13:0 iso	0.27	ND	0.14
15:0 iso	21.96	18.70	21.42
15:1 iso F	0.28	ND	ND
15:0 anteiso	0.27	ND	0.09
16: 0 anteiso	0.18	ND	ND
17:0 iso	0.17	ND	0.15
14:0 2-OH	0.41	0.99	0.57
16:0 2-OH	0.42	0.71	1.69
16:0 3-OH	3.16	5.63	4.12
16:1 2-OH	0.26	0.74	0.36
15:0 iso 3-OH	2.78	2.51	3.66
16:0 iso 3-OH	0.31	0.48	0.37
17:0 iso 3-OH	9.76	6.12	9.47
18:1 w7c	1.03	0.42	0.32
18:1 w9c	0.42	ND	0.33
Feature 1	0.32	0.37	ND
Feature 2	0.27	0.44	0.57
Feature 3	45.90	47.63	43.31
Feature 4	1.13	0.72	0.55

Summed Feature 1 = 13:0 3-OH/15:1 isoH; Summed Feature 2 = 12:0 aldehyde?16:1 iso I/14:0 3-OH; Summed Feature 3 = 16:1 ω7c/16:1 ω6c; Summed Feature 4 = 10 Methyl 16:0; 17:1 iso ω9c; ND = not detected.

Amplification of chaperonin 60 (*cpn60*) genes

The DNA sequence was compared with the *cpn60* sequences of *S. multivorum* LMG 8342^T (Accession No. DQ914835) and *S. thalpophilum* LMG 11520^T (Accession No. DQ985196). CR11^T showed 89.8% sequence similarity with *S. multivorum* LMG 8342^T and 85.2% with *S. thalpophilum* LMG 11520^T.

Conclusion

Based on phenotypic and genotypic results, strain CR11^T has to be regarded as a new species of *Sphingobacterium*. The 16S rRNA sequence of *S. multivorum* and *S. thalpophilum* showed very close similarity with this strain but they are clearly discriminated by DNA–DNA re-association values below 70%. This study also showed the importance of *cpn60* gene analysis for discrimination between CR11^T, *S. multivorum* and *S. thalpophilum*. The strain can also be phenotypically differentiated from the rest of the *Sphingobacterium* species (Table 1). Therefore, we are proposing this strain as a new species of *Sphingobacterium*.

Description of *Sphingobacterium canadense* sp. nov.

Sphingobacterium canadense (ca.na.den'se. N.L. neut. adj. canadense, pertaining to Canada; region of isolation, referring to its isolation from Canada)

Cells are Gram negative, short rods, 0.8–1.0 × 1.0–1.9 μm in size, non-motile. Growth occurs on LB at 20–37 °C but not at 41 °C, at pH 5–10 and concentrations of 0.5–3% NaCl are tolerated. After 24–48 h growth on LB, colonies are circular, entire, low convex, smooth, off-white and turn yellow (non-fluorescent pigment) after a few days. Bacterial cultures are positive for IAA production and negative for phosphate solubilization. Dextrin, α-cyclodextrin, *N*-acetyl-D-glactosamine, *N*-acetyl-D-glucosamine, D-cellobiose, D-fructose, D-galactose, gentiobiose, D-glucose, D-lactose, lactulose, maltose, D-mannose, D-melbiose, D-raffinose, sucrose, D-trehalose, succinic acid mono-methyl ester, acetic acid, formic acid, α-keto valeric acid, propionic acid, L-asparagine, L-proline and glycerol are oxidized but D-mannitol, L-arabinose, L-fucose, myo-inositol, maltose, L-rhamnose, D-sorbitol, xylitol, citric acid, D, L-lactic acid, malonic acid, succinic acid, D-alanine, L-alanine, potassium gluconate, trisodium citrate, capric acid, adipic acid, phenylacetic acid, L-aspartic acid, L-glutamic acid, L-histidine, L-leucine, L-ornithine, L-phenylalanine, D-serine,

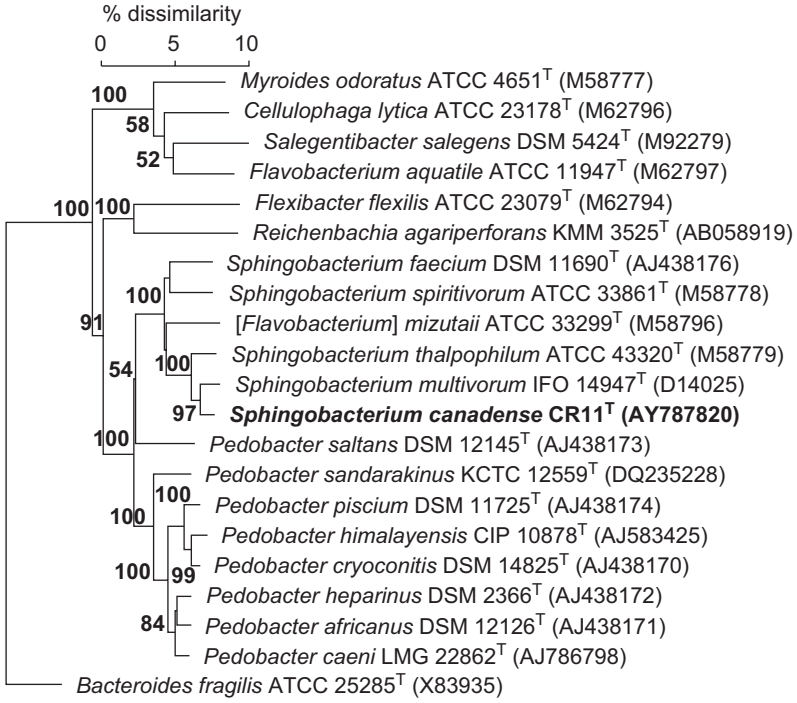

Fig. 2. The phylogenetic tree based on the 16S rRNA gene sequences, constructed by using the neighbour-joining method, showing the close relationship between strain CR11^T and the nearest relatives of the genus *Sphingobacterium*. Numbers at the nodes indicate percentages of occurrence in 1000 bootstrapped trees and only the values greater than 60% are shown.

L-serine and L-threonine are not oxidized. Positive for acid production from D- and L-arabinose, D-xylose, D-glucose, D-fructose, D-mannose, methyl alpha D-glucopyranoside, esculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-sucrose, inulin, D-melezitose, D-raffinose, starch, D-turanose and L-fucose. Negative for glycerol, erythritol, D-ribose, L-xylose, D-adonitol, methyl beta D-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl alpha D-mannopyranoside, arbutin, D-trehalose, *N*-acetyl glucosamine, amygdalin, glycogen, xylitol, gentibiose, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. Positive for catalase, oxidase, urease, nitrate reduction, *ß*-glucosidase, *ß*-galactosidase, gelatine hydrolysis and negative for indole production, glucose fermentation, arginine dihydrolase. Major cellular fatty acids are 16:0, 15:0 iso, 17:0 iso 3-OH and summed feature 3 (16:1 ω7c/16:1 ω6c). The G + C content of the DNA is 40.5 mol%.

The type strain, CR11^T (= NCCB100125 ^{T} = LMG 23727 ^{T}), was isolated from roots of corn (*Zea mays*) in London, Ontario, Canada.

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