Sporosarcina macmurdoensis sp. nov., from a cyanobacterial mat sample from a pond in the McMurdo Dry Valleys, Antarctica

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Strain CMS 21w^T was isolated from a cyanobacterial mat sample taken from a pond located in the McMurdo Dry Valleys, Antarctica. Based on its phenotypic, chemotaxonomic and phylogenetic properties, strain CMS 21w^T was identified as a member of the genus *Sporosarcina*. At the 16S rRNA gene level, CMS 21w^T exhibited about 93–96 % similarity to all reported species of *Sporosarcina* and exhibited a maximum similarity of 96 % to both *Sporosarcina globispora* and *Sporosarcina psychrophila*. Based on more than 3 % difference at the 16S rRNA gene sequence level and the presence of distinct differences with respect to phenotypic, biochemical and chemotaxonomic features, strain CMS 21w^T (= MTCC 4670^T = DSM 15428^T = CIP 107784^T) is proposed as the type strain of a novel species of *Sporosarcina*, *Sporosarcina macmurdoensis* sp. nov.

The genus *Sporosarcina*, which belongs to the family *Bacillaceae*, was created by Kluyver & van Niel (1936) to accommodate bacteria that have spherical or oval-shaped cells, low DNA G + C content (40–42 mol%) and MK-7 as the major menaquinone. *Sporosarcina* species can be differentiated from other members of the *Bacillaceae* by their coccoid or rod-shaped cells, motility, sporulation and possession of MK-7 as the major menaquinone and A4 α as the peptidoglycan variant. The genus *Sporosarcina* originally comprised two species, *Sporosarcina ureae* and *Sporosarcina halophila* (Claus *et al.*, 1983; Claus & Fahmy, 1986). However, three species of the genus *Bacillus*, namely *Bacillus globisporus* (Larkin & Stokes, 1967), *Bacillus psychrophilus* (Nakamura, 1984) and *Bacillus pasteurii* (Miguel, 1889), which belonged to rRNA group 2 (Ash *et al.*, 1991) and contain L-lysine in their cell wall, have recently been transferred to the genus *Sporosarcina* (Yoon *et al.*, 2001) as *Sporosarcina globispora*, *Sporosarcina psychrophila* and *Sporosarcina pasteurii*. Yoon *et al.* (2001) also identified a novel species, *Sporosarcina aquimarina*. In continuation of our work on the heterotrophic bacteria associated with cyanobacterial mat samples from the lakes of Antarctica (Reddy *et al.*, 2000, 2002a, 2002b, 2003), seven psychrophilic bacteria from a pond in McMurdo Region, Antarctica, have been identified as strains of *Sporosarcina*.

Source of the organisms, media and growth conditions

Seven pure cultures (CMS 13, CMS 14, CMS 15, CMS 21w^T, CMS 89, CMS 91 and CMS 93) were isolated from cyanobacterial mat samples collected from ponds L4, L8 and E4 of Wright Valley, McMurdo Region, Antarctica (Matsumoto, 1993) according to a previously described procedure (Reddy *et al.*, 2000) using ABM agar (0.5 % peptone, 0.2 % yeast extract and 1.5 % agar, pH 7.2; Shivaji *et al.*, 1989). Optimum growth conditions were also determined using ABM agar plates.

All seven isolates were identical with respect to their colony and cell morphologies, growth requirements, salt tolerance and many biochemical characteristics, suggesting that they are probably strains of the same species. In fact, all seven had identical 16S rRNA gene sequences, implying that the seven isolates are clonal in origin. CMS 21w^T was chosen as a representative strain for detailed studies to establish its identity.

Morphology, motility and biochemical and chemotaxonomic characteristics

The morphology and motility of CMS 21w^T were determined by taking exponential-phase cells and observing them by phase-contrast microscopy (1000 $\times$). Biochemical tests were performed on cultures grown at 20 °C in nutrient agar (0.5 % peptone, 0.3 % beef extract, 0.8 % NaCl, pH 7.2). Catalase, oxidase, phosphatase, gelatinase, urease, lipase, arginine dihydrolase and β -galactosidase activities were

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determined according to standard methods (Holding & Collee, 1971). Production of indole, methyl red and Voges–Proskauer tests, reduction of nitrate to nitrite, hydrolysis of starch and aesculin and production of acid and gas were assessed according to standard procedures (Hugh & Leifson, 1953; Stanier *et al.*, 1966; Holding & Collee, 1971; Stolp & Gadkari, 1981). Antibiotic sensitivity and growth characteristics were analysed using nutrient agar plates and utilization of carbon compounds was determined on minimal medium. The DNA G+C content was determined as described previously (Shivaji *et al.*, 1991).

Fatty acid methyl esters (Sato & Murata, 1988) were analysed as described previously (Reddy *et al.*, 2002a). Isoprenoid quinones were extracted according to the method described by Collins *et al.* (1977) and separated by HPLC using an SB-C₁₈ Zorbax reverse-phase column (4.6 × 250 mm) fixed to a Hewlett-Packard series 1100 HPLC connected to a Hewlett-Packard programmable fluorescence detector (Tamaoka *et al.*, 1982). An isocratic gradient of methanol/isopropyl ether (3:1, v/v) was used for elution; the flow rate and filter used were respectively 0.5 ml min⁻¹ and 270 nm. Peptidoglycan was prepared and analysed according to the method described by Komagata & Suzuki (1987).

Phenotypic and chemotaxonomic characteristics of CMS 21w^T are described in the species description. From these characteristics, it is apparent that CMS 21w^T, which is rod-shaped, non-motile and Gram-positive with subterminal spores, is phenotypically similar to species of the genus *Sporosarcina*. This was confirmed by chemotaxonomic characteristics such as A4α peptidoglycan variant (with L-Lys–D-Glu peptidoglycan type), MK-7 as the major menaquinone, anteiso-C_{15:0} as the major fatty acid and a DNA G+C content of 44 mol%. The phenotypic and chemotaxonomic characteristics that differentiate CMS 21w^T from the five reported type strains of *Sporosarcina* and *Bacillus insolitus* are listed in Table 1.

Phylogenetic analysis

16S rDNA was amplified, sequenced and analysed as described previously (Shivaji *et al.*, 2000). The partial sequence of the 16S rRNA gene containing 1488 bp was aligned with closely related sequences using CLUSTAL W (Thompson *et al.*, 1994). Pairwise evolutionary distances were computed using the DNADIST program with Kimura's two-parameter model (Kimura, 1980) and phylogenetic trees were constructed using four tree-making algorithms (UPGMA, KITCH, FITCH and DNAPARS) (Felsenstein, 1993). Phylogenetic trees were also made using DNADIST, UPGMA and CONSENSE after resampling the data 1000 times using SEQBOOT and bootstrap analysis (Felsenstein, 1993).

BLAST sequence similarity analysis using the 16S rRNA gene sequence indicated that CMS 21w^T is closely related to *Sporosarcina* species. This was further confirmed by phylogenetic analysis of the 1488 bp 16S rRNA gene sequence of CMS 21w^T and sequences from species of the genera

Sporosarcina, *Bacillus*, *Caryophanon*, *Kurthia* and *Planococcus* and by constructing a UPGMA-based phylogenetic tree. The evolutionary distance calculated using Kimura's two-parameter model indicated that CMS 21w^T is related to species of the genus *Sporosarcina* (similarity of 93–96 %) and is most closely related to *S. globispora* (96 %), *S. psychrophila* (96 %) and *B. insolitus* (96 %). The topology of the phylogenetic tree showed that CMS 21w^T is closely related to *S. globispora* (Rüger, 1983), *S. psychrophila* (Nakamura, 1984), *S. aquimarina* (Yoon *et al.*, 2001), *S. ureae* (Claus & Fahmy, 1986), *S. pasteurii* (Yoon *et al.*, 2001) and *B. insolitus* (Rüger, 1983). However, within the cluster, only *S. psychrophila* and *S. globispora* had a high bootstrap value (100 %); this value varied from 40 to 60 % with the other species (Fig. 1). All the different algorithms used for tree construction gave comparable branching patterns. However, CMS 21w^T cannot be grouped with *B. insolitus* since the latter strain has ornithine in its peptidoglycan, unlike all reported species of *Sporosarcina*, which contain lysine (Yoon *et al.*, 2001). Furthermore, inclusion of *B. insolitus* in the cluster is not very robust, having a low bootstrap value of 55 % with strain CMS 21w^T.

Conclusions

CMS 21w^T is a Gram-positive, non-motile rod with sub-terminal spores, L-Lys–D-Glu peptidoglycan, MK-7 as the menaquinone, anteiso-C_{15:0} as the major fatty acid and a DNA G+C content of 44 mol%, thus exhibiting phenotypic and chemotaxonomic characteristics of the genus *Sporosarcina*. However, at the 16S rRNA gene level, CMS 21w^T exhibits about 96 % similarity to both *S. globispora* and *S. psychrophila* and the similarity is even lower to *S. aquimarina* (94.6 %), *S. pasteurii* (93 %) and *S. ureae* (95 %). Therefore, considering that CMS 21w^T differs from all reported species by more than 3 % at the 16S rRNA gene level, it is unlikely to have DNA–DNA similarities of more than 70 % (Stackebrandt & Goebel, 1994). Therefore, it would appear that CMS 21w^T represents a novel species of *Sporosarcina*. Further evidence that CMS 21w^T represents a novel species comes from differences in phenotypic characteristics (Table 1). CMS 21w^T is unique in that it has both anteiso-C_{15:0} and iso-C_{16:1} as major fatty acids, whereas other species of the genus *Sporosarcina* have only anteiso-C_{15:0} as the major fatty acid (Yoon *et al.*, 2001). CMS 21w^T also contains C_{15:1}, which is absent in other species of the genus *Sporosarcina*. Furthermore, in *B. insolitus*, the major fatty acids are iso-C_{15:0}, anteiso-C_{15:0} and C_{16:1} (Kaneda *et al.*, 1983). Therefore, CMS 21w^T is proposed as the type strain of a novel species of the genus *Sporosarcina*, *Sporosarcina macmurdoensis* sp. nov.

Description of *Sporosarcina macmurdoensis* sp. nov.

Sporosarcina macmurdoensis (mac.mur.do.en'sis. N.L. fem. adj. *macmurdoensis* pertaining to the McMurdo Region, Antarctica, where the isolates were collected).

Table 1. Phenotypic characteristics that differentiate *S. macmurdoensis* sp. nov. CMS 21w^T from type strains of related species

Strains/species: 1, CMS 21w^T; 2, *S. psychrophila* (Nakamura, 1984); 3, *S. globispora* (Nakamura, 1984; Rüger, 1983); 4, *S. aquimarina* (Yoon *et al.*, 2001); 5, *S. pasteurii* (Yoon *et al.*, 2001); 6, *S. ureae* (Claus & Fahmy, 1986); 7, *B. insolitus* (Claus & Berkeley, 1986). +, Positive; –, negative; NA, data not available. For all strains, the major menaquinone is MK-7.

Characteristic	1	2	3	4	5	6	7
Colony colour	White	White	White	Light orange	White	White	NA
Cell shape	Rods	Rods	Rods	Rods	Spherical	Spherical	Rods
NaCl (%) tolerance	3	4	5	13	10	3	<2
Optimum pH for growth	7	7	7	6.5–7.0	9	7	6–7
Growth at (°C):							
4	+	+	+	+	NA	NA	+
30	–	+	–	+	+	+	–
37	–	–	–	+	+	+	–
Optimum growth temperature (°C)	20	25	20	25	30	25	25
Presence of:							
β-Galactosidase	–	+	+	NA	NA	NA	NA
Phosphatase	+	NA	NA	NA	NA	–	NA
Urease	–	+	+	+	+	+	–
Gelatinase	+	+	+	+	+	–	–
Starch hydrolysis	+	–	+	–	–	–	–
Nitrate reduction	+	+	–	+	+	–	–
DNA G + C content (mol%)	44	44.1	40	40	39	40–42	35.9
Peptidoglycan type	L-Lys–D–Glu	L-Lys–D–Glu	L-Lys–D–Glu	L-Lys–Ala–Asp	L-Lys–D–Asp	L-Lys–Gly–Glu	D–Orn–D–Glu
Peptidoglycan variant	A4α	A4α	A4α	A4α	A4α	A4α	A4β
Major fatty acids*	aiC _{15:0} , iC _{16:1}	aiC _{15:0}	aiC _{15:0}	aiC _{15:0}	aiC _{15:0}	aiC _{15:0}	iC _{15:0} , aiC _{15:0} , C _{16:1}
Acid production from:							
D-Fructose	–	+	–	+	NA	NA	NA
D-Galactose	–	+	–	–	NA	NA	NA
D-Glucose	–	+	+	–	NA	NA	–
Lactose	–	–	+	–	NA	NA	–
D-Mannitol	–	+	–	–	NA	NA	–
Sucrose	–	+	+	–	NA	NA	NA
D-Xylose	–	+	–	–	NA	NA	–
Utilization of:							
Acetate	–	+	NA	NA	NA	NA	NA
Adonitol	–	+	–	NA	NA	NA	NA
Fumaric acid	–	+	+	NA	NA	NA	NA
D-Glucose	+	+	–	NA	NA	NA	NA
D-Maltose	+	–	–	NA	NA	NA	NA
D-Xylose	+	+	–	NA	NA	NA	NA

*ai, Anteiso; i, iso.

Colonies are circular, flat, irregular, opaque and white with a diameter of 2–3 mm. Cells are rod-shaped, single and non-motile and form subterminal spores. Cells can tolerate a maximum of 3 % (w/v) NaCl and grow at pH 6–9 (pH 7 is optimum for growth). Psychrophilic; grows at 4–25 °C (optimum growth temperature 18–20 °C). Positive for catalase, phosphatase, gelatinase and starch hydrolysis and negative for lipase, urease, oxidase, β-galactosidase, arginine dihydrolase, arginine decarboxylase, lysine decarboxylase,

indole production, methyl red test, Voges–Proskauer test, aesculin hydrolysis and nitrate reduction to nitrite. Does not produce acid or gas from L-arabinose, D-fructose, D-galactose, lactose, D-mannose, D-mannitol, L-rhamnose, sucrose or D-xylose. Utilizes dulcitol, D-fructose, D-galactose, D-glucose, *meso*-inositol, lactose, D-maltose, D-mannose, pyruvate, D-raffinose, D-xylose and L-glutamic acid as sole carbon sources, but not acetate, adonitol, L-arabinose, D-cellobiose, cellulose, citrate, dextran, glucose,

Fig. 1. Phylogenetic relationship between *Sporosarcina macmurdoensis* sp. nov. CMS 21w^T and other related reference micro-organisms in the genera *Bacillus*, *Caryophanon*, *Kurthia* and *Planococcus* based on 16S rRNA gene sequence analysis using UPGMA. Bootstrap values (%) are given at nodes. Branch lengths are not to scale.

meso-erythritol, fumaric acid, glycerol, inulin, lactic acid, D-mannitol, D-melibiose, melezitose, L-rhamnose, D-ribose, sorbitol, D-sorbose, sucrose, succinic acid, trehalose, thioglycollate, L-alanine, L-arginine, L-aspartic acid, L-asparagine, L-glutamine, L-lysine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, L-tryptophan or L-valine. Sensitive to amikacin, ampicillin, amphotericin, bacitracin, carbenicillin, cefazoline, cefaperazone, cephotaxime, chloramphenicol, chlorotetracycline, co-trimoxazole, ciprofloxacin, erythromycin, furazolidone, furoxone, gentamicin, kanamycin, lomefloxacin, nalidixic acid, neomycin, nitrofurazone, nitrofurantoin, norfloxacin, novobiocin, nystatin, oxytetracycline, penicillin, polymyxin-B, rifampicin, roxithromycin, streptomycin, tetracycline, tobramycin, trimethoprim and vancomycin, but resistant to cefuroxime, colistin and lincomycin. Peptidoglycan type is L-Lys-D-Glu of the A4 α variant and MK-7 is the major menaquinone. When grown in nutrient broth at 22 °C, the fatty acid composition is as follows (proportions of total fatty acids in parentheses): C_{13:0} (0.5%), C_{14:0} (0.5%), iso-C_{14:0} (11.1%), C_{15:0} (1.1%), iso-C_{15:0} (4.0%), anteiso-C_{15:0} (37.4%), C_{15:1} (1.5%), C_{16:0} (0.7%), iso-C_{16:0} (3.2%), C_{16:1} ω 7c (1.1%), iso-C_{16:1} (22.6%), iso-C_{17:0} (3.0%) and anteiso-C_{17:0} (13.0%).

The DNA G+C content of the type strain, CMS 21w^T (= MTCC 4670^T = DSM 15428^T = CIP 107784^T), is 44 mol%.

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