Methylocystis hirsuta sp. nov., a novel methanotroph isolated from a groundwater aquifer

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Strain CSC1^T, a Gram-negative, aerobic, methane-oxidizing bacterium, was isolated from an uncontaminated aquifer nearly 20 years ago. Based on 16S rRNA gene sequence similarity, this strain was identified as a member of the *Alphaproteobacteria*, most closely related to an uncultured member of the *Methylocystaceae* as well as two cultured organisms, *Methylocystis* sp. L32 and *Methylocystis* sp. SC2. This strain differed from extant species in cell shape, size, expression of soluble methane monooxygenase and its unique spiny surface layers, composed of polysaccharide. DNA–DNA hybridization results showed only 3.8 % relatedness with *Methylocystis echinoides* NCIMB 13100 and 41.1 % relatedness with *Methylocystis rosea* SV97^T. Based on these genotypic and physiological differences, this isolate is proposed as a member of a novel species of the genus *Methylocystis*, *Methylocystis hirsuta* sp. nov. (type strain CSC1^T =ATCC BAA-1344^T =DSM 18500^T).

Species of the genus *Methylocystis* are strictly aerobic, Gram-negative bacteria that are able to grow on one-carbon compounds (e.g. methane or methanol) (Bowman *et al.*, 1993). The genus *Methylocystis* belongs to the class *Alphaproteobacteria* and currently consists of three species with standing in nomenclature, *Methylocystis parvus*, *Methylocystis echinoides* and *Methylocystis rosea* (Whittenbury *et al.*, 1970; Gal'chenko *et al.*, 1977; Bowman *et al.*, 1993; Wartainen *et al.*, 2006). Numerous *Methylocystis* strains have been identified in a variety of environments, including lake, ocean, marsh and creek sediments and water, coal mine drainage water and the roots of plants (Whittenbury *et al.*, 1970; Gal'chenko *et al.*, 1977; Bowman *et al.*, 1993; Hanson & Hanson, 1996; Calhoun & King, 1998; Heyer *et al.*, 2002).

Species of the genus *Methylocystis* are type II methanotrophs, classified, in part, by their possession of paired membranes aligned with the cell periphery, the serine pathway and predominant fatty acids with 18 carbons (Hanson & Hanson, 1996; Graham *et al.*, 2002). Until recently, it was believed that all type II methanotrophs, including *Methylocystis* species, express the particulate form of methane monooxygenase (pMMO), and, with the exception of *Methylocystis parvus* OBBP^T and the recently reported *Methylocystis rosea* SV97^T, all express the soluble form of methane monooxygenase (sMMO) at low copper concentrations (Stanley *et al.*, 1983; Prior & Dalton, 1985; Choi *et al.*, 2003; Wartiainen *et al.*, 2006). *Methylocystis parvus* OBBP^T and *Methylocystis rosea* SV97^T do not possess genes encoding sMMO (Tsien & Hanson, 1992; McDonald *et al.*, 1997; Lloyd *et al.*, 1999; Wartiainen *et al.*, 2006). The genera *Methylocella* and *Methylocapsa* are classified as type II methanotrophs; however, they possess morphological and

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Abbreviations: PHB, poly-β-hydroxybutyrate; pMMO, particulate methane mononoxygenase; sMMO, soluble methane monooxygenase; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and partial *mxaF*, *mmoX* and *pmoA* gene sequences of strain CSC1^T are respectively DQ364433, DQ664499, DQ664498 and DQ364434.

Neighbour-joining trees based on partial *mmoX* and *mxaF* gene sequences and results of electron microscope cytochemistry of the S-layer of strain CSC1^T are available as supplementary material with the online version of this paper.

physiological characteristics that are distinctly different from those of other type II methanotrophs (Dedysh *et al.*, 2000, 2002). pMMO has not been observed in *Methylocella*, for example, and species in this genus have been shown to be facultatively methanotrophic, capable of growing on multicarbon substrates (Dedysh *et al.*, 2005; Theisen & Murrell, 2005). All *Methylocystis* species produce oxidase and catalase, are non-motile and are capable of fixing atmospheric nitrogen (Hanson & Hanson, 1996).

The focus of this paper is strain CSC1^T, a type II methanotroph isolated in the mid-1980s from an uncontaminated groundwater aquifer at Moffet Naval Air Station in Mountain View, CA, USA (Henry & Grbić-Galić, 1990). This methanotroph expresses sMMO under copper-limiting conditions and is capable of oxidizing aliphatic and aromatic compounds (Henry & Grbić-Galić, 1991; Adriaens, 1994; Adriaens & Grbić-Galić, 1994; Hršak, 1996; Hršak & Begonja, 1998). Despite its being the focus of these numerous studies aimed primarily at its contaminant degradation potential, strain CSC1^T has not been characterized and differentiated from other known type II methanotrophs. This study provides phenotypic and genotypic analysis of this groundwater isolate. The formal taxonomic description of this novel *Methylocystis* bacterium, strain CSC1^T, is reported. Differences in various characteristics of strain CSC1^T compared with other known methanotrophs are described, and its unique surface features broaden the observed physiological traits of methanotrophic bacteria.

Strain CSC1^T was obtained from Dr Dubravka Hršak (Rudjer Boskovic Institute, Zagreb, Croatia) and *Methylosinus trichosporium* OB3b^T was obtained from Dr Jeremy Semrau (Department of Civil and Environmental Engineering, University of Michigan, Ann Arbor, MI, USA). *Methylocystis parvus* NCIMB 11129^T (=OBBP^T) and *Methylocystis echinoides* NCIMB 13100 were obtained from the NCIMB (Aberdeen, UK). *Methylocystis rosea* ATCC BAA-1196^T (=SV97^T) was obtained from the American Type Culture Collection. The basal medium used for growth when culturing for sMMO expression was nitrate mineral salts (NMS) medium with no added copper, as described previously (Whittenbury *et al.*, 1970; Lontoh & Semrau, 1998). Ultrapure water used in all culturing was obtained from a Barnstead NANOpure type I water purification unit (model D4751), which includes a spiral wound ultrafilter and a UV unit. Copper (II) nitrate [$Cu(NO_3)_2$; 10 μmol l^{-1}] was added to the NMS medium to provide conditions for pMMO expression. Liquid cultures were grown routinely at 250 r.p.m. and 30 °C in either 50 or 500 ml batches in 250 ml Erlenmeyer or 2800 ml Fernbach flasks, respectively. The flasks were fitted with rubber stoppers (Fisher Scientific) equipped with a resealable glass tube filled with glass wool to allow headspace removal and filling. A portion of the air headspace was removed and refilled with methane of 99.99 % purity (Strate Welding) using a vacuum pump assembly to achieve a headspace concentration of methane of 20 % (v/v).

For culturing on solid medium, 1.5 % (w/v) Bacto agar (Difco Laboratories) was added to the NMS medium. All plates were incubated in a sealed desiccator, containing anhydrous $CaSO_4$ (Drierite; W. A. Hammond Drierite Company) under an atmosphere of 20 % methane and 80 % air (by volume) at 30 °C, which was refreshed every 4–5 days. Purity of the cultures was verified by routine streaking on 2 % (w/v) nutrient agar in ultrapure water.

sMMO expression was verified qualitatively by a naphthalene oxidation assay modified from Brusseau *et al.* (1990). Four negative controls, autoclaved cells, cells cultured with 10 μmol $Cu(NO_3)_2$ l^{-1} (for expression of pMMO), cell-free medium and cells that had been subjected to addition of 1–2 ml acetylene gas (a known inhibitor of MMO; Prior & Dalton, 1985), were included with three live samples of active test culture diluted to an OD_{600} of 0.2 and transferred to autoclaved 10 ml capped test tubes. Seventy milligrams crushed naphthalene (Sigma) was added to each tube. After incubation at 30 °C and 250 r.p.m. for a minimum of 1 h, 0.1 ml of a freshly prepared solution of 4.21 mmol tetrazotized *o*-dianisidine l^{-1} (Sigma) was added. A subsequent pink-to-purple colour formation in the tubes indicated positive sMMO activity that was verified using UV/Vis spectrophotometry (Fisher Scientific) at 550 nm.

Genomic DNA was isolated from strain CSC1^T, grown to exponential phase, by a standard method (Ausubel *et al.*, 1989). The 16S rRNA gene was amplified by PCR using the universal bacterial primers 27f and 1492r (Lane, 1991). PCR primers used for sMMO were *mmoXA* (5′-ACCAAGGARCARTTCAAG-3′) and *mmoXB* (5′-TGGCACTCRTARCGCTC-3′) (Auman *et al.*, 2000); primers for methanol dehydrogenase (MDH) were *mxa* f1003 (5′-GCGGCACCAACTGGGGCTGGT-3′) and *mxa* r1561 (5′-GGGCAGCATGAAGGGCTCCC-3′) (McDonald & Murrell, 1997); and primers for pMMO were A189f (5′-GGNGACTGGGACTTCTGG-3′) and A682r (5′-GAASGCNGAGAAGAASGC-3′) (Holmes *et al.*, 1995).

All PCRs were carried out in a PTC-200 Thermo Cycler (MJ Research) using 25 μl reactions and Premix *Taq* polymerase (Takara). Conditions used for the different primer sets have been described previously (Auman *et al.*, 2000; Costello & Lidstrom, 1999; Holmes *et al.*, 1995). The PCR amplification products were ligated to vector pCR2.1 (Invitrogen) and transformed to competent *Escherichia coli* TOP10F′ cells according to the vendor's instructions. Plasmid DNA from transformants was isolated and the inserts were sequenced by the Biotechnology Resource Center at Cornell University (Ithaca, NY, USA).

Sequences were compared with previously identified sequences in the NCBI database using BLAST (Altschul *et al.*, 1990). Sequences identified in BLAST and sequences of extant methanotrophs were aligned and adjusted manually with CLUSTAL_X version 1.82 (Thompson *et al.*, 1997). Phylogenetic trees were generated by the neighbour-joining method in CLUSTAL_X and viewed using TreeView (Page, 1996).

DNA–DNA hybridizations between strain CSC1^T, *Methylocystis echinoides* NCIMB 13100 and *Methylocystis rosea* SV97^T were performed by the DSMZ (Braunschweig, Germany) using 2× SSC buffer (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) plus 10 % (v/v) formamide at an optimal renaturation temperature of 68 °C.

Earlier studies reported strain CSC1^T as a Gram-negative, non-motile coccobacillus, possessing an internal membrane structure characteristic of type II methanotrophs (paired membranes inside the periphery of the cell), and forming lipid inclusions (Henry & Grbić-Galić, 1990, 1991; Hršak & Begonja, 1998). Fang *et al.* (2000) concluded that the intact phospholipids of strain CSC1^T clustered within the type II grouping, clearly distinct from groupings of type I methanotrophs. This study extended the previous phenotypic characterization studies by assessing exospore and rosette formation, growth at 37 °C, the presence of a surface (S-) layer, carbon and nitrogen source utilization and lysis by 2 % (w/v) SDS, all identified by Bowman *et al.* (1993) or Hanson & Hanson (1996) as differentiating characteristics among type II methanotrophic species.

Exospore formation was determined with 1- to 2-week-old broth cultures grown as described previously following the methods of Smibert & Krieg (1981). Five millilitres of culture was transferred in duplicate to fresh NMS medium for controls. A second set of duplicates was heated in a water bath at 80 °C for 20 min for pasteurization. Growth was monitored after streaking the controls and treated cultures onto solid NMS plates and incubation (as described previously) for 21 days. Exospores were monitored using light microscopy, also used to determine rosette formation and motility (Norris & Ribbons, 1971). Growth in liquid culture was monitored using 250 ml nephlos flasks with the same stopper assembly described above and a UV/Vis spectrophotometer (Fisher Scientific) at a wavelength of 600 nm.

Nitrogen and carbon sources were tested using NMS basal medium. To test for alternative nitrogen sources, KNO_3 was replaced with 0.1 % (w/v) anhydrous L-asparagine (MP Biomedicals), L-aspartate (Pfaltz & Bauer) or L-glutamine (MP Biomedicals) (all shown to support growth of *Methylocystis echinoides* IMET 10491^T and *Methylocystis parvus* OBBP^T; Bowman *et al.*, 1993) or L-lysine monohydrochloride (Sigma-Aldrich), L-ornithine hydrochloride (MP Biomedicals) or putrescine (MP Biomedicals) (all shown to support growth of *Methylosinus trichosporium* OB3b^T; Bowman *et al.*, 1993). NMS medium with added KNO_3 and without a nitrogen source respectively served as a positive and negative control, and the latter control also served as a test for growth in the absence of added nitrogen. To test for alternative carbon sources, 0.2 % (w/v) methylamine hydrochloride (Alfa Aesar), DMSO, methanol or glucose (Fisher Scientific) was added. Over the 30-day test period, flasks were prepared in duplicate and transfers were made to fresh medium supplied with the appropriate nitrogen or carbon source every 4 days. Growth measurements were performed as described previously.

Lysis by 2 % (w/v) SDS (Fisher Scientific) was determined by direct microscopic observation using cells harvested at three-quarters-exponential phase. Cells were centrifuged at 2460 ***g*** for 20 min, resuspended in the 2 % SDS stock solution for approximately 2 h and observed using an oil immersion phase-contrast microscope (Zeiss).

Transmission electron microscopy (TEM) was used to observe cells of strain CSC1^T expressing MMO, lipid inclusions and other fine structural features, including S-layers. Liquid cultures were incubated for 2–3 days and were fixed for 30 min at room temperature with cacodylate-buffered glutaraldehyde both with and without 0.1 % Alcian blue (Fassel *et al.*, 1992), stained for 30 min at room temperature with 1 % cacodylate-buffered osmium tetroxide and then stained for 50 min in 1 % aqueous uranyl acetate. After dehydrating in increasing strengths of ethanol, cells were embedded in both Spurr's and Epon resins (Dykstra, 1993). Thin sections were prepared and stained with lead citrate and examined on a Zeiss EM-10CA transmission electron microscope. *Methylocystis echinoides* NCIMB 13100 was observed by negative stain using 1 % aqueous uranyl acetate applied to cell suspensions on Formvar-coated grids.

In order to provide evidence that the observed S-layer is glycoprotein, two additional cytochemical approaches were utilized. Images of Alcian blue-stained specimens were compared with those with no Alcian blue in the glutaraldehyde fixative, since Alcian blue stains polysaccharide moieties (Lewis & Knight, 1977). Secondly, thin Epon sections on Formvar-coated nickel grids were first exposed to 3 % H_2O_2 for 15 min at room temperature to remove osmium and then exposed to 1 % aqueous Pronase solution (Sigma) for 60–90 min at 35 °C to remove protein components from the section (Lewis & Knight, 1977; Monneron & Bernhard, 1966). Controls included H_2O_2 alone and water substituted for the Pronase step.

The phylogenies of the 16S rRNA gene sequences and the *pmoA*, *mmoX* and *mxaF* partial gene sequences of strain CSC1^T and other methanotroph strains, respectively shown in Figs 1 and 2 and Supplementary Fig. S1(a, b) available in IJSEM Online, are consistent with placement of strain CSC1^T with other known type II methanotrophs. The 16S rRNA phylogeny of strain CSC1^T clearly places it within a branch of the *Alphaproteobacteria* dominated by *Methylocystis* species. Determination of sequence similarities revealed that the closest relatives with validly published names were *Methylocystis rosea* SV97^T (99 %), *Methylocystis parvus* OBBP^T (97 %) and *Methylocystis echinoides* IMET 10491^T (97 %) (Fig. 1). Lower 16S rRNA gene similarities of 96 and 95 %, respectively, were observed between strain CSC1^T and the *Methylosinus* type strains *Methylosinus sporium* NCIMB 11126^T and *Methylosinus trichosporium* OB3b^T. The nearest defined neighbours of strain CSC1^T are *Methylocystis* sp. IMET 10486 and two uncultured

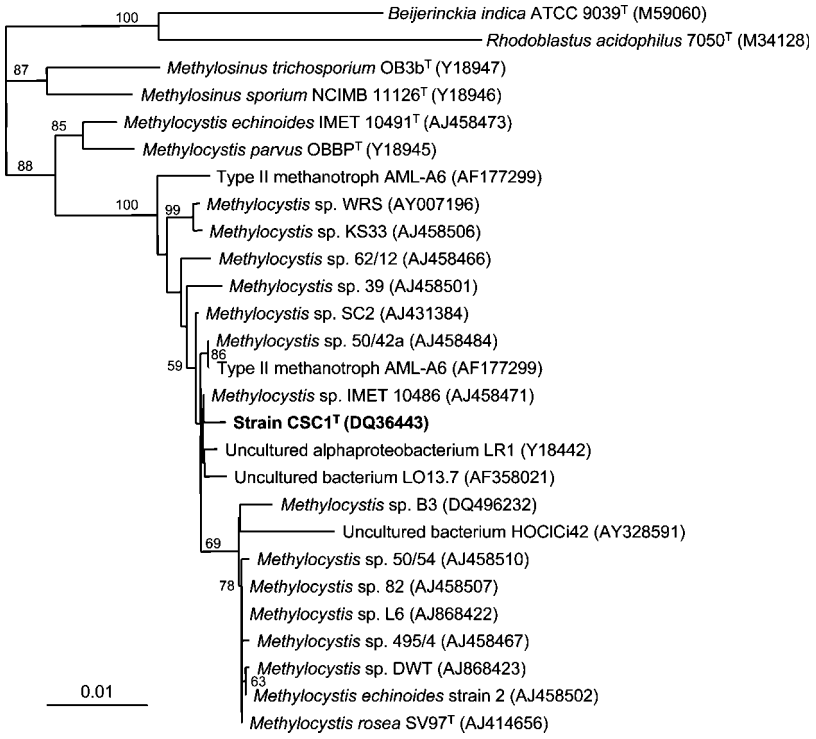


Fig. 1. Phylogenetic relationship of the 16S rRNA gene sequences of strain CSC1^T and other type II methanotrophs and related strains. The dendrogram shows the results of an analysis in which neighbour-joining was used. Numbers on branches represent bootstrap percentages greater than 50 %, derived from 1000 replicates. Bar, 0.01 substitutions per nucleotide site. 16S rRNA gene sequences from two non-methanotrophic alphaproteobacteria were used as an outgroup.

members of the *Methylocystaceae* (LR1 and LO13.7), each sharing 99 % 16S rRNA gene sequence similarity with strain CSC1^T. The greatest similarity of the partial *pmoA* gene sequence of strain CSC1^T was observed with various uncultured bacteria [uncultured bacterium DGGE band P5 (99 %), LOPB13.1 (99 %) and SL-1.52 (98 %)] (Fig. 2). Those strains from species with validly published names most closely matching the *pmoA* partial gene sequence of

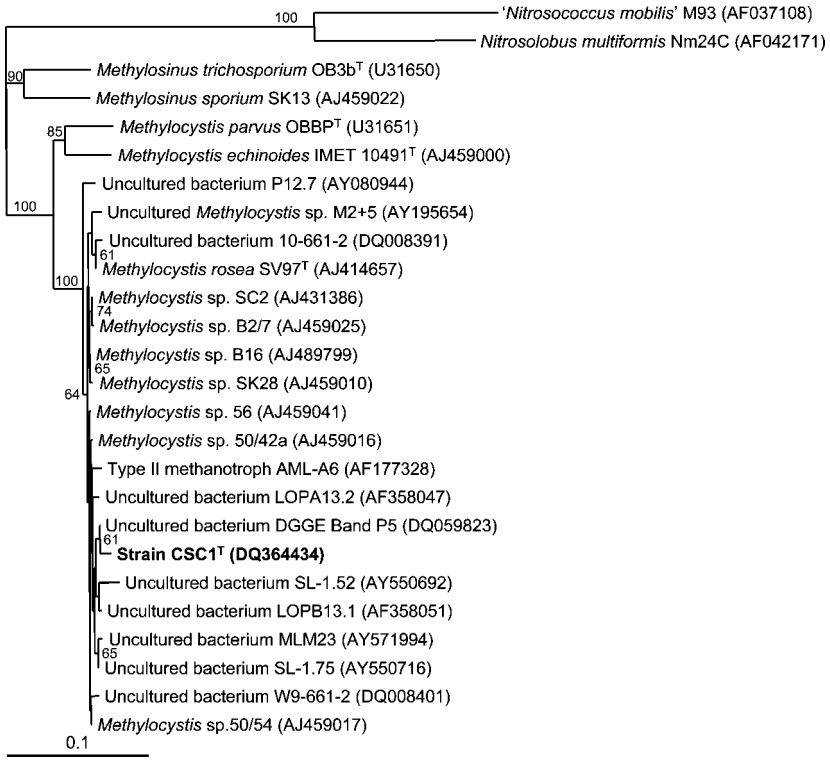


Fig. 2. Phylogenetic relationship of the partial *pmoA* gene sequences of strain CSC1^T and members of related genera. Numbers on branches represent bootstrap percentages greater than 50 %, derived from 1000 replicates. Bar, 0.1 substitutions per nucleotide site. *amoA* sequences of two ammonia-oxidizing bacteria were used as an outgroup.

strain CSC1^T were again *Methylocystis rosea* SV97^T (98 %), *Methylocystis echinoides* IMET 10491^T (92 %) and *Methylocystis parvus* OBBP^T (92 %).

Given the suspected placement in the genus *Methylocystis*, DNA–DNA hybridization was performed with *Methylocystis echinoides* NCIMB 13100 and *Methylocystis rosea* SV97^T, yielding relatedness values of 3.8 and 41.1 %, respectively. Strain CSC1^T therefore does not belong to the species *Methylocystis echinoides* or *Methylocystis rosea*, according to the 70 % threshold value recommendation of Wayne *et al.* (1987).

As shown in Table 1, rosette formation by cells of strain CSC1^T was not observed. No growth was evident after pasteurization, indicating that this methanotroph is not resistant to heat, and growth was also not observed at 37 °C. Optimum growth was observed at approximately 30 °C. As reported previously for *Methylosinus trichosporium* OB3b^T, *Methylocystis echinoides* IMET 10491^T and *Methylocystis parvus* OBBP^T (Bowman *et al.*, 1993), strain CSC1^T was not lysed by a 2 % solution (w/v) of SDS, but 10 % (w/v) SDS did lyse the cells. It was shown to be capable of growing on alternative nitrogen sources L-asparagine, L-aspartate, L-glutamine, L-ornithine and putrescine; however, no growth was visible in the presence of L-lysine. Growth was also observed in nitrogen-free NMS medium. As reported for *Methylosinus trichosporium* OB3b^T, *Methylocystis echinoides* IMET 10491^T and *Methylocystis parvus* OBBP^T by Bowman *et al.* (1993), of the four alternative carbon sources methylamine, dimethylsulfide, methanol and glucose tested, only methanol supported growth of strain CSC1^T.

Expression of sMMO upon culturing strain CSC1^T in NMS medium with no copper was confirmed by formation of a purple colour after incubation with naphthalene and addition of *o*-dianisidine, whereas controls with acetylene and with cells cultured in the presence of copper yielded no colour. These results strongly suggest that sMMO was expressed in strain CSC1^T when grown without copper and was responsible for the naphthalene oxidation.

TEMs of strain CSC1^T grown in the presence of copper verify the type II membrane structure of paired membrane lamellae in the peripheral cytoplasm (Fig. 3a, b). In thin section, a variety of cell shapes were visible at low magnification (Fig. 3a), but elongated or dumbbell shapes

Table 1. Phenotypic characteristics that differentiate strain CSC1^T from related type strains

Strains: 1, strain CSC1^T (unless indicated, data obtained from Henry & Grbić-Galić, 1990, 1991; Hršak & Begonja, 1998); 2, *Methylosinus trichosporium* OB3b^T (Whittenbury *et al.*, 1970; Fassel *et al.*, 1990, 1992; Bowman *et al.*, 1993; Hanson & Hanson, 1996); 3, *Methylocystis echinoides* IMET 10491^T (Gal'chenko *et al.*, 1977; Bowman *et al.*, 1993; Hanson & Hanson, 1996); 4, *Methylocystis parvus* OBBP^T (Whittenbury *et al.*, 1970; Bowman *et al.*, 1993; Hanson & Hanson, 1996).

Characteristic	1	2	3	4
Colony colour	Cream	White/buff/yellow	White/buff/pale pink	White/tan/pale pink
Cell morphology				
Width (μm)	0.3–0.6	0.5–1.5	0.6	0.3–0.5
Length (μm)	0.7–1	2–3	0.8–1.2	0.5–1.5
Shape	Dumbbell	Pear	Coccobacillus, rod	Coccobacillus
S-layers	Sharp, solid spines*	Bead-like/filamentous	Tubular spines	−
Accumulation of:				
Polyphosphate	+*	−	−	+
PHB	+	+	−†	+
Motility	−	Polar flagella	−	−
Rosettes	−*	+	−	−
Heat resistance	−*	+	−	−
Growth at 37 °C	−*	+	−†	+
Usage of nitrogen sources				
L-Asparagine	+*	−	+	+‡
L-Aspartate	+*	−	+	+‡
L-Glutamine	+*	−	+	+‡
L-Lysine	−*	+	−	+‡
L-Ornithine	+*	+	−	+‡
Putrescine	+*	+	−	+‡

*Data from this study.

†Contrary to the reported characteristics of *Methylocystis echinoides* IMET 10491^T (Bowman *et al.*, 1993), our experiments showed *Methylocystis echinoides* NCIMB 13100 to accumulate PHB and to grow at 37 °C.

‡Bowman *et al.* (1993) reported 75–87 % of strains positive.

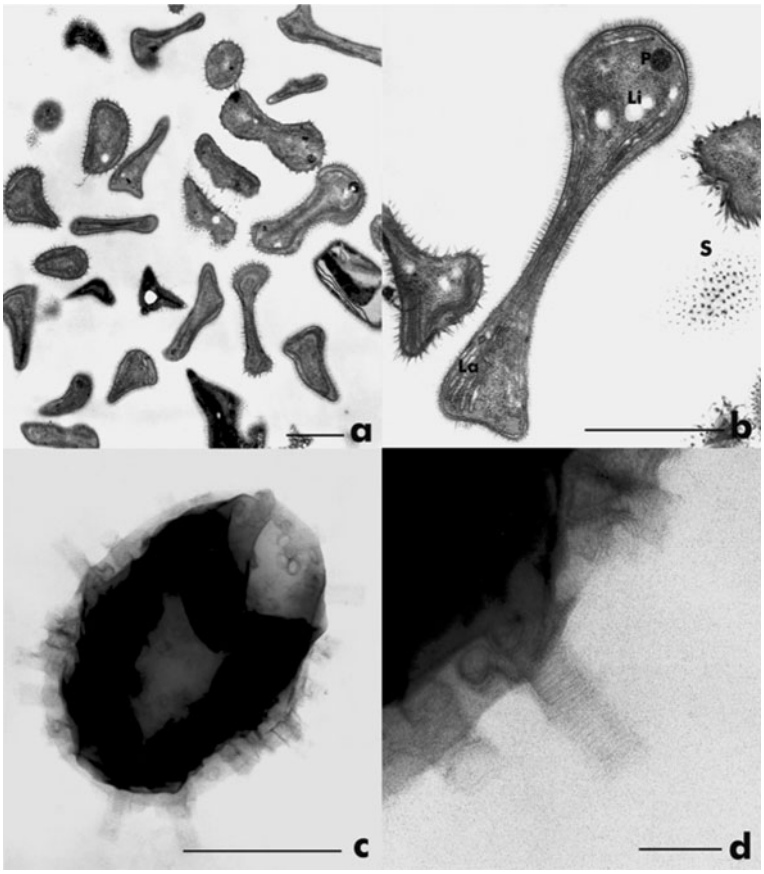


Fig. 3. Transmission electron micrographs of strain CSC1^T (a, b) and *Methylocystis echinoides* NCIMB 13100 (c, d). (a, b) Morphology of cells of strain CSC1^T grown with 10 μM copper. In (b), numerous lamellae (La) are present. Lipid inclusions (Li) and polyphosphate (P) storage inclusions are also present. S indicates a surface view of the spiny surface of a cell. (c, d) Cells of *Methylocystis echinoides* NCIMB 13100 viewed with negative stain are elliptical in profile and have numerous tubular projections from the surface. In (c), some may be seen in circular end-on profile at the upper right. The high-magnification view (d) shows that the tubes are striated. Bars, 1 μm (a and b), 0.5 μm (c) and 0.1 μm (d).

of cells predominated. Many of the other profiles could represent dumbbell shapes sectioned in different planes. Cells grown without copper contained only a few internal membranous lamellae (data not shown). Polyphosphate bodies and lipid inclusions were common.

As shown in Fig. 3(a, b), distinctive S-layers, probably composed of glycoprotein, were revealed with TEM of ultrathin sections of strain CSC1^T fixed with Alcian blue. These spiked S-layer structures, 50–75 nm in height, covered the entire surface of the cell wall. We have seen that the cytoplasm of cells of strain CSC1^T embedded in Spurr resin will sometimes shrink away from the wall, lending support to the idea that the S-layer is more rigid than the rest of the wall (data not shown). This shrinkage does not occur in cells embedded in Epon resin (e.g. cells in Fig. 3a, b).

S-layers have been observed in both type I and II methanotrophs isolated from a wide range of environments, including members of the genera *Methylomicrobium*, *Methylomonas*, *Methylosinus* and *Methylocystis* (Fassel *et al.*, 1992; Sorokin *et al.*, 2000; Trotsenko & Khmelenina, 2002). Type II *Methylosinus trichosporium* OB3b^T was found to have bead-like S-layer structures and occasional filamentous material in the outer envelope (Fassel *et al.* 1990, 1992). Similar bead-like S-layer structures were observed by Fassel *et al.* (1992) in the cell envelope of *Methylocystis* sp. strain Lake Washington, but not in a strain the authors referred to as '*Methylocystis paris*', and the authors concluded that the absence of these structures in the latter species could be a species variation. Both *Methylocystis* species possessed considerable filamentous material, however (Fassel *et al.*, 1992). *Methylocystis echinoides* was reported to have rigid tubular structures arranged radially at the cell surface (Gal'chenko *et al.*, 1977), features that are absent from *Methylocystis parvus* OBBP^T (Heyer *et al.*, 2002). In this study, negative-stain preparations of *Methylocystis echinoides* NCIMB 13100 show ellipsoid cells with square-ended tubular projections (Fig. 3c) that appear striated at high magnification (Fig. 3d), as reported by Haubold (1977) for this strain but not in the original description of Gal'chenko *et al.* (1977). Haubold (1977) concluded that the tubes of *Methylocystis echinoides* IMET 10491^T were smaller in diameter than those described by Gal'chenko *et al.* (1977). The tubular appearance of the S-layers of *Methylocystis echinoides* NCIMB 13100 (Fig. 3c, d) and the isolate described by Gal'chenko *et al.* (1977) is considerably different from the solid-sharp spines of strain CSC1^T.

To elucidate further the nature of the spiked S-layer in strain CSC1^T, cells fixed with glutaraldehyde alone (Supplementary Fig. S2a) were compared with those fixed with an Alcian blue/glutaraldehyde mixture

(Supplementary Fig. S2b). Alcian blue is a differential stain for polysaccharide (Lewis & Knight, 1977), and the spines visible in Supplementary Fig. S2(b) were considerably darker, longer and more distinct than the same structures visible in Supplementary Fig. S2(a), even though Supplementary Fig. S2(b) is at a lower magnification. This strongly indicates polysaccharide content. After treatment with H_2O_2 to remove osmium from the Epon sections, sections treated with Pronase, a broad-spectrum protease, lost the entire S-layer (Supplementary Fig. S2c, d), indicating that the layer contains considerable protein.

Sequence analysis of the 16S rRNA gene (Fig. 1) and partial gene sequences of *pmoA* (Fig. 2), *mmoX* (Supplementary Fig. S1a) and *mxaF* (Supplementary Fig. S1b) supports placement of strain CSC1^T within the closely related genera *Methylocystis* and *Methylosinus*. Phenotypic results (Table 1) show that strain CSC1^T can be differentiated from *Methylosinus trichosporium* OB3b^T, *Methylocystis echinoides* IMET 10491^T and *Methylocystis parvus* OBBP^T. All of the strains included in Table 1 have been reported to be oxidase- and catalase-positive, possess colonies that are of opaque transparency, smooth edge and convex elevation and grow on methanol and in nitrogen-free medium. Unlike the known strains, strain CSC1^T was not capable of growth at 37 °C; however, all of the methanotrophs grow optimally near 30 °C. Unlike *Methylocystis echinoides* IMET 10491^T, strain CSC1^T was observed to accumulate poly-β-hydroxybutyrate (PHB). Gal'chenko *et al.* (1977) reported the ability of their isolate to accumulate PHB and grow at 37 °C, whereas Bowman *et al.* (1993) reported that *Methylocystis echinoides* IMET 10491^T did not accumulate PHB or grow at 37 °C. Our TEM and growth studies using *Methylocystis echinoides* NCIMB 13100 agree with the results of Gal'chenko *et al.* (1977) (data not shown), suggesting that *Methylocystis echinoides* strains NCIMB 13100 and IMET 10491^T in fact belong to different species.

The elongated dumbbell shape of strain CSC1^T, its lack of motility and its ability to form polyphosphate separate it from *Methylosinus trichosporium* OB3b^T. Other distinguishing characteristics between strain CSC1^T and *Methylosinus trichosporium* OB3b^T include smaller cell size, S-layer morphology and lack of heat resistance. Also, unlike reported observations of *Methylosinus trichosporium* OB3b^T, strain CSC1^T can use L-asparagine, L-aspartate and L-glutamine and cannot use L-lysine as a nitrogen source. The two strains share the ability to use L-ornithine and putrescine.

Greater similarity, however, is shared with the two *Methylocystis* strains, including the absence of motility, heat resistance and rosette formation. The cell shape of strain CSC1^T, its ability to form polyphosphate and its cream-coloured colonies differ from *Methylocystis echinoides* IMET 10491^T and *Methylocystis parvus* OBBP^T (Table 1). Unlike *Methylocystis echinoides* IMET 10491^T, strain CSC1^T is capable of using L-ornithine and putrescine as nitrogen sources, whereas, unlike *Methylocystis parvus* OBBP^T, strain CSC1^T is not capable of using L-lysine. In addition, as reported for *Methylocystis trichosporium* OB3b^T, *Methylocystis echinoides* IMET 10491^T and *Methylocystis parvus* OBBP^T, cells of strain CSC1^T are not lysed by 2 % (w/v) SDS.

While strain CSC1^T has previously been shown by TEM to contain characteristic type II membranes (Henry & Grbić-Galić, 1990; Hršak & Begonja, 1998), it was revealed here to accumulate both polyphosphate bodies (Fig. 2) and PHB storage granules, consistent with the properties of *Methylocystis parvus* OBBP^T (Bowman *et al.*, 1993). No study has reported the structure of the cell envelope of strain CSC1^T in comparison with that of other well-characterized methanotrophs. Of special interest are the S-layers, regular crystalline surface layers in archaea and bacteria, composed of protein or glycoprotein subunits (Sleytr *et al.*, 1993; Sidhu & Olsen, 1997).

It is not known why S-layers develop in some bacteria and not in other closely related strains. However, hypotheses include suggestions that formation of these structures reflects adaptation to an ecological niche (Easterbrook & Alexander, 1983; Easterbrook, 1989) or a response to exposure to harsh environments (Minsky *et al.*, 2002). Others suggest that S-layers may provide micro-organisms with a selective advantage by serving as a protective coating or as molecular porins or sieves and traps for substrates, in maintaining the rigidity of the cell envelope or in providing a means of cell adhesion and surface recognition (Sára & Sleytr, 1987; Sleytr & Messner, 1988; Sára *et al.*, 1992; Sidhu & Olsen, 1997). Easterbrook & Sperker (1982) hypothesized that spinae may simultaneously fulfil many fortuitous roles, analogous to 'arms' with multipotential activities, including attachment, distance-keeping and protection. However, why some species are prone to spine formation and others are not, why S-layers exist in a variety of shapes and symmetries and why these structures develop among species of methanotrophs is not clearly understood.

The original report of Gal'chenko *et al.* (1977) referred to an isolate from lake mud in Russia, whereas the type strain *Methylocystis echinoides* IMET 10491^T was isolated from sewage sludge (Meyer *et al.*, 1986), both more nutrient-rich environments than the sediments of the uncontaminated groundwater aquifer in California where strain CSC1^T was isolated. The epithet *echinoides* is a Neo-Latin adjective derived from the Greek word *echinos*, meaning hedgehog, named for the hedgehog-like appearance of this bacterium. However, as reported by Gal'chenko *et al.* (1977) and Haubold (1977) and verified in this study (Fig. 3c, d), the spines on these methanotrophs appear to be tubular and less dense in comparison with the spikes observed on strain CSC1^T, which would be more aptly named for a hedgehog. Despite the different originating environments of *Methylocystis echinoides* IMET 10491^T and strain CSC1^T, proximity in the grouping of the genus *Methylocystis*, as strongly suggested by the results of this study, adds credence to the hypothesis that phylogeny and ecology may both play a

role in S-layer formation. Similar clustering of S-layer-producing strains of *Bacillus cereus* has been observed and, similar to these results, some strains in this cluster do not possess S-layers, while others do (Mignot *et al.*, 2001). These authors concluded that ecological pressure is associated with the acquisition and maintenance of S-layers in hosts that fall into a phylogenetic cluster.

Phylogenetically, strain CSC1^T is most closely related to *Methylocystis* species. Its cell size, rosette formation and presence of surface layers are most similar to *Methylocystis echinoides* IMET 10491^T. However, DNA–DNA hybridization revealed only 3.8 % relatedness between strain CSC1^T and *Methylocystis echinoides* NCIMB 13100 and 41.1 % relatedness with *Methylocystis rosea* SV97^T. Strain CSC1^T and *Methylocystis echinoides* IMET 10491^T showed differences in surface-layer morphology, cell shape, colony colour, formation of polyphosphate and ability to use L-ornithine or putrescine as a nitrogen source. Characteristics of cell shape and the presence of surface layers, genes encoding sMMO and the ability to use L-lysine as a nitrogen source are divergent from *Methylocystis parvus* OBBP^T. The lack of polar flagella, smaller cell size, different cell shape, lack of heat resistance, presence of polyphosphate, ability to use L-asparagine, L-aspartate or L-glutamine and inability to use L-lysine as a nitrogen source differentiate strain CSC1^T from *Methylosinus trichosporium* OB3b^T. Accepting these differences, strain CSC1^T could be described as a member of a novel species in the genus *Methylocystis*.

Description of *Methylocystis hirsuta* sp. nov.

Methylocystis hirsuta (hir.su′ta. L. fem. adj. *hirsuta* hairy, rough, shaggy, bristly).

Cells are aerobic, Gram-negative, 0.3–0.6 × 0.7–1 μm in size and occur singly or in clusters. Reproduces by normal cell division; budding division does not occur. Cells are not motile but possess a spiny surface layer composed of polysaccharide. Produces oxidase and catalase. Forms lipid cysts. PHB accumulates. Contains type II intracytoplasmic membranes which are aligned parallel to the cell wall. Type II methanotroph. Methane and methanol are the sole sources of carbon and energy. Capable of using KNO_3, L-asparagine, L-aspartate, L-glutamine, L-ornithine and putrescine. Capable of growth in nitrogen-free medium. Expresses sMMO under low copper concentrations. Capable of cometabolically oxidizing a variety of aliphatic and aromatic compounds. Not resistant to pasteurization. Is not lysed by 2 % (w/v) SDS, but is lysed by 10 % (w/v) SDS. Colonies are cream-coloured, slow-growing and 0.8–1.5 mm in diameter after 17–18 days at 30 °C on NMS agar plates, incubated in the presence of 20 % (v/v) methane in the headspace of a sealed desiccator. No growth on complex organic media. Optimal pH for growth is 7.0; does not grow at pH 4.0 or 9.0. Is not capable of growth at 37 °C. Optimal temperature for growth is approximately 30 °C.

The type strain, strain CSC1^T (=ATCC BAA-1344^T =DSM 18500^T), was isolated from an uncontaminated groundwater aquifer in the mid-1980s from Moffett Naval Air Station in Mountain View, CA, USA.

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