Characterization of a novel facultative *Methylocystis* species capable of growth on methane, acetate and ethanol

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Summary

A non-motile strain of *Methylocystis*, strain SB2, isolated from a spring bog in southeast Michigan, had a curved rod morphology with a typical type II intracytoplasmic membrane system. This organism expressed the membrane-bound or particulate methane monooxygenase (pMMO) as well as a chalkophore with high affinity for copper and did not express the cytoplasmic or soluble methane monooxygenase (sMMO). Strain SB2 was found to grow within the pH range of 6–9, with optimal growth at 6.8. Growth was observed at temperatures ranging between 10°C and 30°C, with no growth at 37°C. The DNA G+C content was 62.9 mol%. Predominant fatty acids were 18:1ω7c (72.7%) and 18:1ω9c (24%) when grown on methane. Phylogenetic comparisons based on both *pmoA* and 16S rRNA sequences indicated that this organism belonged to the *Methylocystis* genus, and was closely related to *Methylocystis rosea* SV97T and *Methylocystis echinoides* IMET10491T (98% 16S rRNA gene sequence similarity to both strains). DNA : DNA hybridizations indicated that strain SB2 had 70% similarity with *M. rosea* SV97T. Unlike *M. rosea* SV97T, strain SB2 was able to utilize not only methane for growth, but also ethanol and acetate. Furthermore, the predominant fatty acids in strain SB2 were different from those found in *M. rosea* SV97T, i.e. 54.2% and 39.7% of fatty acids are 18:1ω8 and 18:1ω7 in *M. rosea* SV97T, while 18:1ω8 is completely absent in strain SB2.

Introduction

Methanotrophs, organisms that consume methane as their sole source of carbon and energy, are found in a wide variety of environments where methane : air interfaces develop, including forest and agricultural soils, wetlands, landfills, geothermal areas (e.g. hot springs), marine and freshwater sediments, among other locations. Most methanotrophs are physiologically and phylogenetically distinct organisms in either the *γ-Proteobacteria* (type I methanotrophs) and *α-Proteobacteria* (type II methanotrophs) (Semrau et al., 2010), although recently methanotrophs from *Verrucomicrobia* have been found (Dunfield et al., 2007; Pol et al., 2007; Islam et al., 2008).

Most of these bacteria are obligate methanotrophs, i.e. can only grow on C1 substrates, although *Methylocella* spp. are facultative, being able to grow on a variety of organic acids (Dedysh et al., 2005). These acidophilic organisms, growing between pH 4.5 and pH 7 (Dunfield et al., 2003) only express the cytoplasmic or soluble form of the methane monooxygenase (sMMO) (Dedysh et al., 2000; Dunfield et al., 2003). *Methylocapsa aurea* KYG1 has been isolated from a forest soil that is also facultative, being able to grow on acetate, but expresses only the membrane-bound or particulate methane monooxygenase (pMMO). This organism had an optimal growth pH of 6.0–6.2 (Dunfield et al., 2010). Recently, facultative methanotrophs able to grow on acetate have been reported in the *Methylocystis* genus, specifically the moderate acidophiles *Methylocystis* strain H2s and *Methylocystis heyeri* H2T, and the mesophile *Methylocystis echinoides* IMET10491T (Belova et al., 2010). Here we describe the isolation and characterization of another facultative mesophilic methanotroph, strain SB2, able to grow on methane, acetate or ethanol, that is a novel member of *Methylocystis*.

Results and discussion

Isolation of strain SB2

Strain SB2 was isolated from a spring bog located near Ann Arbor, Michigan (42°16′13.7″N, 83°39′36.5″W) in

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July 2006. Aqueous samples were spread onto nitrate mineral salts (NMS) Bacto-agar plates (Whittenbury et al., 1970). Plates were incubated with a CH₄/air mixture (1:2) at 30°C. Single colonies were selected from plates and continuously re-streaked onto fresh NMS plates and nutrient agar plates until no growth on nutrient agar was observed. Culture purity was confirmed using phase-contrast and electron microscopy. To further verify isolate purity, DNA was extracted from methane-grown organisms and 16S rRNA genes amplified via PCR and cloned using the TA cloning kit. Fifty recombinant clones of 16S rRNA gene PCR products were obtained and at least 1000 bp sequenced per clone, with all clones found to have identical sequences. Once pure cultures were obtained, the culture was maintained by transferring to fresh plates at least once a month.

Characterization of strain SB2

On NMS agar without copper, strain SB2 formed round, convex, red-orange-pigmented colonies. When SB2 was spread onto NMS agar with 10 or 40 mM copper (as CuCl₂), colonies were initially white-coloured, but darkened over time (Supplementary Fig. S1). Interestingly, strain SB2 grew in liquid cultures in the absence of copper with methane as the carbon source in NMS medium, but grew better in the presence of copper. Vitamins were also not required for growth (Supplementary Fig. S2). Strain SB2 was found to be Gram-negative and non-motile. Staining for *Azotobacter*-type cysts was negative using standard procedures (Vela and Wyss, 1964), and neither exospores nor rosettes were observed after 4-week incubation. Phase-contrast micrographs showed that they create large and tight aggregates of cells surrounded by a capsule (Fig. 1A). Electron microscopy showed that cells of SB2 were 0.7–1.0 μm wide and 0.9–2.0 μm in length when grown on methane with a well-developed system of type II intracytoplasmic membranes aligned parallel to the cytoplasmic membrane (Fig. 1B). Inclusions of low electron density were also occasionally observed, possibly comprising poly-β-hydroxyalkanoate granules. The DNA G+C content was measured to be 62.9 mol% as determined by DSMZ (Braunschweig, Germany) using standard protocols (Cashion et al., 1977; Tamaoka and Komagata, 1984; Mesbah et al., 1989). Predominant fatty acids were 18:1ω7c (72.7%), 18:1ω9c (24%) and 16:1ω7c (2.2%) when strain SB2 was grown on methane as determined by Microbial Insights (Rockford, Tennessee).

Strain SB2 grew at pH values ranging from 6 to 9 with methane as the carbon and energy source, with optimum growth at 6.8 when grown on methane in NMS medium. Growth on methane was also observed at temperatures ranging from 10°C to 30°C, but no growth was observed at 37°C. Many nitrogen sources were tested with methane as the carbon source: nitrate (as potassium nitrate), ammonia (as ammonium chloride), L-alanine, L-serine, L-isoleucine, L-proline, L-methionine, L-glutamine, L-asparagine, L-lysine, L-glycine, L-histidine and L-arginine. Of these, strain SB2 was able to utilize nitrate, ammonium, L-isoleucine, L-proline and L-glutamine as nitrogen sources, and was able to grow poorly in nitrogen-free mineral medium. The presence of nifH was confirmed using PCR using specific primers for nifH (Zehr and McReynolds, 1989; Auman et al., 2001). Sequencing of the nifH PCR product showed high similarity to nifH from *Methylocystis rosea* SV97T and *M. echinoides* IMET10491T (Supplementary Fig. S3). Furthermore, strain SB2 grew from an initial OD₆₀₀ of 0.037 to 0.12 after 3 days in nitrogen-free medium with methane as the carbon source (P < 0.01) (Supplementary Fig. S4). Collectively, these data indicate that strain SB2 can fix methane.
nucleotides. Finally, a recent plate assay developed for the screening of methanotrophs for chalkophore production. i.e. copper-binding compounds analogous to siderophores (Yoon et al., 2010) indicated that strain SB2 did produce a chalkophore (Supplementary Fig. S5).

PCR amplification of functional genes of the particulate methane monoxygenase (pmoA, encoding for the α-subunit of pMMO) using A189-mb661 (Costello and Lidstrom, 1999) revealed the presence of pMMO. Similar PCR assays using mmoXm-mmcXf (Auman et al., 2000), mmoX1882–mmox1403 (McDonald et al., 1995) and mmoX206f–mmox886r (Hutchens et al., 2004) failed to yield any PCR products for mmoX (encoding for the α-subunit of the sMMO hydroxylase). The lack of sMMO was also indicated by negative results of the naphthalene assay, specific for sMMO activity (Brusseau et al., 1990) in either the absence or the presence of copper.

Growth of strain SB2 on alternative carbon sources

Growth on methanol (0.05% v/v) and methylamine, glucose, fructose, sucrose, galactose, xylose, arabinose and maltose (all at a concentration of 0.05% w/v) was tested, as were the organic acids formate, pyruvate, succinate, malate, citrate and oxalate (all added as sodium salts at a concentration of 0.05% w/v). No growth was observed on any of these substrates. Some methanotrophs can utilize methanol as well as methane, but many methanotrophs have been shown to be unable to grow on methanol either due to its toxicity or the toxicity associated with the accumulation of formaldehyde from methanol oxidation (Whittenbury et al., 1970; Wilkinson et al., 1974; Linton and Vokes, 1978; Best and Higgins, 1981; Cornish et al., 1984). Here, the ability of strain SB2 to grow on methanol was tested at concentrations ranging from 0.01% to 1% (v/v), with no growth observed under any concentration (Supplementary Fig. S2).

Strain SB2 grew, however, on either acetate (as sodium acetate) or ethanol when the growth concentrations of these substrates were varied between 0.01% and 0.5% (w/v) and 0.05% and 1% (v/v) respectively (Supplementary Fig. S2). Optimal growth for both ethanol and acetate occurred at 0.1% (v/v and w/v respectively) with nitrate added as the nitrogen source (Fig. 2). Growth on methane and ethanol followed standard exponential kinetics, with growth rates of $0.052 \pm 0.004$ h$^{-1}$ and $0.022 \pm 0.002$ h$^{-1}$ for methane and ethanol respectively. Strain SB2 grew to a higher OD$_{600}$ on methane than on ethanol (0.83 and 0.45 respectively). Growth on acetate, however, was slower, and could be modelled as either exponential or linear growth, to a final OD$_{600}$ of 0.26. The possible linear growth on acetate is intriguing, and may be due to the pH of the growth medium, 6.8. At this pH, 99% of the added acetate exists as the dissociated form. It is believed that undissociated acetate is transferred across the cell membrane, thus the proton motive force is dissipated to transport acetate into the cell (Axe and Bailey, 1995), which inhibits microbial growth. It is interesting to note that other methanotrophs shown to be able to grow better on acetate, e.g. *Methylocapsa aureus*, *Methylocella silvestris* and *Methylocystis* sp. H2s, grow optimally at lower pH values (Dedysh et al., 2005; Belova et al., 2010; Dunfield et al., 2010) where more of the acetate exists in the undissociated form, while *M. echinoides* IMET10491T also recently found to grow on acetate on standard NMS medium, which has a pH of 6.8, does so relatively poorly (Belova et al., 2010). Furthermore, the growth yield and carbon conversion efficiency of *Methylocystis* strain SB2

Fig. 2. Growth of strain SB2 on various carbon sources: ○ = methane (10% v/v in the headspace); • = acetate (0.1% as sodium acetate, w/v); □ = ethanol (0.1%, v/v). Strain SB2 was incubated in 50 ml of NMS medium in 250 ml flask supplemented with 10 μM copper as CuCl$_2$. The flasks were incubated at 30°C with shaking at 250 rpm. Growth was monitored by measuring OD$_{600}$ using a Spectronic-20 spectrometer (Milton Roy Company, USA). Bars indicate the range of duplicate samples. Where bars are not apparent, the symbol size is greater than the measured range. The initial pH of the growth medium was 6.8 for all substrates, and after reaching the stationary phase, the pH was 6.7 for all substrates.

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and *M. silvestris* BL2 on methane were similar, but *M. silvestris* BL2 exhibited greater yield and carbon efficiency on acetate than *Methylocystis* strain SB2 (Table 1).

The purity of cultures grown on ethanol and acetate was verified by plating on nutrient agar, with no growth observed after incubation for 4 weeks. DNA was extracted from ethanol- and acetate-grown organisms with 16S rRNA genes amplified via PCR and cloned. Fifty recombinant clones of 16S rDNA PCR products were obtained for both ethanol- and acetate-grown organisms and at least 1000 bp sequenced per clone. All clones were found to have identical sequence as to that for methane-grown cultures, verifying culture purity. Transmission electron micrographs of strain SB2 grown on ethanol confirmed the presence of intracytoplasmic membranes aligned parallel to the cytoplasmic membrane, as well as inclusions of low electron density, possibly poly-β-hydroxyalkanoate granules (Supplementary Fig. S6).

As mentioned earlier, other *Methylocystis* strains have been recently found to grow on acetate, including the moderate acidophiles *M. heyeri* H2T, *Methylocystis* strain H2s, and the mesophile *M. echinoides* IMET10491T, but not *M. rosea* SV97T (Wartiaisen et al., 2006; Belova et al., 2010). The growth of *M. echinoides* IMET10491T and *M. heyeri* H2T on acetate, however, was less than that observed here for strain SB2, which was more similar to that found for *Methylocystis* strain H2s and *M. aurea* KYG7 (Belova et al., 2010), i.e. max OD_{410} of 0.09 for *M. echinoides* IMET10491T, max OD_{410} of 0.14 for *M. heyeri* H2T, max OD_{410} of ~0.23 for *Methylocystis* strain H2s, max OD_{600} of 0.3 for *M. aurea* KYG7 and a max OD_{600} of 0.26 for strain SB2.

**Phylogenetic comparison of strain SB2 with other methanotrophs**

The 16S rRNA sequence (1132 bp, GenBank Accession No. GU734136) of strain SB2 was found to be most similar to *M. rosea* SV97T (98%) and *M. echinoides* IMET10491T (98%). Lower similarity values were found with *Methylocystis parvus* OB3bT (94%), as well as with *Methylosinus trichosporium* OB3bT (94%) and *Methylosinus sporium* NCIMB 11126T (95%). Using a Neighbour-Joining analysis, strain SB2 clustered very closely with *M. rosea* SV97T based on nucleotide sequences of partial 16S rDNA genes (Fig. 3). Similar patterns were observed with pmoA sequences (437 bp; GenBank Accession No. GU734137), with strain SB2 having 99% similarity to pmoA from *M. rosea* SV97T, 98% similarity to pmoA from *M. echinoides* IMET10491T, 94% similarity to *M. parvus* OB3bT, and 87% similarity to pmoA from both *M. trichosporium* OB3bT and *M. sporium* NCIMB 11126T. Phylogenetic analysis based on partial pmoA sequence data supported the placement of strain SB2 within the *Methylocystis* genus of the α-Proteobacteria (Fig. 4). Given the highest similarity between strain SB2 and *M. rosea* SV97T for both 16S rRNA and pmoA gene sequences, DNA–DNA hybridizations were performed between these strains by DSMZ (Braunschweig, Germany) using 2× SSC buffer (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) with 5% (v/v) formamide at a renaturation temperature of 70°C. It was found that strain SB2 had an average 70% DNA–DNA similarity with *M. rosea* SV97T from duplicate samples (range of 68.8–71.3%).

Although strain SB2 showed relatively high DNA–DNA similarity to *M. rosea* SV97T as well as high 16S rRNA sequence similarity, it cannot be definitively declared as belonging to the same species as *M. rosea* SV97T given the recommendation of a threshold value of 70% DNA–DNA similarity for the definition of bacterial species by the *ad hoc* committee (Wayne et al., 1987). In such a situation, other physiological data must be considered, e.g. range of growth substrates, growth conditions, etc. As outlined in Table 2, there are significant differences between strain SB2 and *M. rosea* SV97T as well as with *M. echinoides* IMET10491T, which has been recently
Methylosinus trichosporum OB3b$^T$ (Y18947)
Methylosinus sporum NCTC 11126$^T$ (Y18946)
Methylcystis sp. LW5 (AF150790)
Methylcystis parvis OBPP$^T$ (Y18945)
Methylcystis echinoides IMET 1049$^T$ (AJ458473)
Methylcystis sp. H2s (FN422003)
Methylcystis sp. KS8a (AJ458493)
Methylcystis sp. KS7 (AJ458498)
Methylcystis heyera H2$^T$ (AM283543)
Methylcystis sp. M42/2 (AJ458499)
Methylcystis sp. 62/12 (AJ458466)
Methylcystis IMET 10489 (AJ458472)
Methylcystis sp. AML-A3 (AF177298)
Methylcystis sp. 42/22 (AJ458479)
Methylcystis sp. KS33 (AJ458506)
Methylcystis sp. 39 (AJ458501)
Methylcystis sp. SC2 (AJ431384)
Methylcystis sp. 50/42a (AJ458484)
Methylcystis sp. AML-A6 (AF177299)
Methylcystis sp. IMET 10486 (AJ458471)
Methylcystis echinoides 2 (AJ459502)
Methylcystis rosea SV97$^T$ (AJ414656)
Methylcystis sp. SB2 (GU734136)

Fig. 3. Phylogenetic relationship of the 16S rRNA gene sequence of strain SB2 with other methanotrophs. The evolutionary history was inferred using the Neighbor-Joining method based on nucleotide sequences of partial 16S rDNA genes (Saitou and Nei, 1987). Bootstrap values derived from 100 replicates are shown. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). Scale bar indicates 0.005 changes per nucleotide position.

found to also grow on acetate. Strain SB2 can grow on multi-carbon compounds while *M. rosea* SV97$^T$ cannot. Additional differences between *M. rosea* SV97$^T$ and strain SB2 include the findings that strain SB2 cannot grow either at 37°C, or below pH 6, that the pigmentation of strain SB2 varies with copper concentration, as well as large differences in the predominant fatty acids. Strain SB2 also is different from *M. echinoides* IMET10491$^T$ in that strain SB2 lacks spinae, has variable red coloration and has different predominant fatty acids. It is interesting to note that the predominant fatty acids in *M. rosea* SV97$^T$ and *M. echinoides* IMET10491$^T$ are 18:1o8 and 18:1o7, while strain SB2 they are 18:1o7c and 18:1o9c, and that the presence of 18:1o9c is rare in *Methylcystis* spp. (Bowman et al., 1993).

In summary, a novel facultative methanotroph, *Methylcystis* strain SB2, similar to both *M. rosea* SV97$^T$ and *M. echinoides* IMET10491$^T$, was found that can utilize multi-carbon substrates for growth. At this time, it is unclear whether strain SB2 is a novel species or subspecies within *Methylcystis*, and this strain should be characterized further to resolve this issue, e.g. DNA : DNA hybridizations with other methanotrophs. The finding that strain SB2 can utilize multi-carbon compounds for growth supports the suggestion of Belova and colleagues (2010) that some methanotrophs, particularly *Methylcystis* spp., utilize such compounds to generate reducing equivalents that enhance methanotrophic growth *in situ*, particularly in bogs where the concentration of acetate can be appreciable and acidic conditions can cause the undissociated
Fig. 4. Phylogenetic relationship of the pmoA gene sequences of strain SB2 with other methanotrophs. Neighbour-Joining method [Jukes–Cantor correction (Jukes and Cantor, 1969)] was used, and bootstrap values derived from 100 replicates are shown. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). Scale bar indicates 0.01 changes per nucleotide position.

Table 2. Characteristics distinguishing Methylocystis sp. SB2 from Methylocystis rosea SV97T and Methylocystis echinoides IMET10491T.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Methylocystis sp. SB2</th>
<th>Methylocystis rosea SV97T</th>
<th>Methylocystis echinoides IMET10491T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell morphology</td>
<td>Curved rods</td>
<td>Straight and curved rods</td>
<td>Coccibacilli/rods/vibroid</td>
</tr>
<tr>
<td>Pigmentation</td>
<td>Varies with copper concentration (white, pink, red and brown)</td>
<td>Pink-red</td>
<td>White/buff</td>
</tr>
<tr>
<td>Spinae</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Growth</td>
<td>–</td>
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<td>–/a</td>
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<tr>
<td>37°C</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>pH 5.0</td>
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<td>+</td>
<td>–</td>
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<tr>
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<td>+</td>
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</tr>
<tr>
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<td>+</td>
</tr>
<tr>
<td>Predominant fatty acids</td>
<td>18:1ω7c; 18:1ω6c; 16:1ω7c</td>
<td>18:1ω8; 18:1ω7; 16:1ω7</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>62.9</td>
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<td>62</td>
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<tr>
<td>DNA–DNA hybridization with SB2 (range of duplicate samples)</td>
<td>ND</td>
<td>70% (68.8–71.3)</td>
<td>ND</td>
</tr>
</tbody>
</table>

a. Conflicting findings reported in the literature (Bowman et al., 1993; Lindner et al., 2007).

Data for M. rosea SV97T are from Wartiainen and colleagues (2006) and data for M. echinoides IMET10491T are from Bowman and colleagues (1993), Lindner and colleagues (2007) and Belova and colleagues (2010).

ND, not determined.

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form of acetate to predominate. Further work is warranted to determine how broadly distributed facultative methanotrophy is and what compounds other than ethanol and acetate can be utilized by these organisms.

Acknowledgements

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References


Facultative methanotrophy in a Methylocystis sp. 180


Vela, G.R., and Wyss, O. (1964) Improved stain for visual


Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Coloration of Methylcystis sp. SB2 after 4 weeks of incubation on NMS medium with either no added copper; or 10 μM or 40 μM Cu added as CuCl2.

Fig. S2. A. Growth of Methylcystis sp. SB2 on methane in NMS medium with either 10 μM (■) or no added copper (□). For growth in copper-free NMS medium, strain SB2 was first grown on NMS medium in the presence of 10 μM copper to the mid-exponential phase, harvested by centrifugation, washed twice with copper-free fresh NMS medium to remove residual copper, and then resuspended into copper-free fresh media.

B. Growth of Methylcystis sp. SB2 on methane in NMS medium with 10 μM copper and with (■) or without (□) vitamins.

C. Growth of Methylcystis sp. SB2 on varying amounts of methanol [■ = 0.05%; □ = 0.1%; ▲ = 0.5%; ◊ = 1.0% (v/v)] in NMS medium with 10 μM copper.

D. Growth of Methylcystis sp. SB2 on varying amounts of acetate [■ = 0.01%; □ = 0.05%; ▲ = 0.1%; △ = 0.2%; ◊ = 0.5% (v/v)] in NMS medium with 10 μM copper.

E. Growth of Methylcystis sp. SB2 on varying amounts of ethanol [■ = 0.05%; □ = 0.1%; ▲ = 0.5%; ◊ = 1.0% (v/v)] in NMS medium with 10 μM copper.

Bars indicate the range of duplicate samples.

Fig. S3. Phylogenetic relationship of the nifH PCR product (320 bp) of Methylcystis sp. SB2 with other methanotrophs. Neighbour-Joining method [Jukes–Cantor correction (Jukes and Cantor, 1969)] was used, and bootstrap values derived from 100 replicates are shown. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). Scale bar indicates 0.1 changes per nucleotide position.

Fig. S4. Growth of Methylcystis sp. SB2 on nitrogen (■) or nitrogen-free (□) mineral salts medium. For growth on nitrogen-free medium, strain SB2 was first grown to the mid-exponential phase (OD600 = 0.5) in 50 ml of NMS media in 250 ml flasks. Strain SB2 was then harvested by centrifuging 10 ml of the culture at 4000 rpm for 5 min. The cell pellet was then washed twice by resuspending it in 10 ml of fresh nitrogen-free mineral salts medium and centrifuging again at 4000 rpm for 5 min. The final cell pellet was then resuspended in 50 ml of fresh nitrogen-free mineral salts medium in 250 ml flask and incubated with methane (10% v/v in the headspace) at 30°C. Bars indicate the range of duplicate samples.

Fig. S5. Split NMS/50 μM Cu–CAS plates for detection of chalkophore production over time by Methylcystis sp. SB2. A. Strain SB2 incubated for 6 days at 30°C.

B. Strain SB2 incubated for 21 days at 30°C.

NMS agar was supplemented with 1 μM copper as CuCl2. 50 μM Cu–CAS agar was prepared by adding 50 ml of 1.05 mM CAS solution to 10 ml of a 5 mM CuCl2 solution. This solution was then added to 40 ml of 2.625 mM HDTMA under stirring to give final concentrations of 0.5 mM, 0.525 mM and 1.05 mM of Cu, CAS and HDTMA respectively. 450 ml of NMS was prepared separately. Concentrations of salts were adjusted for 500 ml of NMS medium, considering later addition of the Cu–CAS solution. The purple-coloured Cu–CAS stock solution and NMS agar preparation were then autoclaved separately. After cooling to ~50°C, 50 ml of the purple-coloured Cu–CAS solution was carefully pipetted into NMS agar medium. Vitamin and phosphate buffer solutions were then added to the medium. After the agar plates cooled and solidified, half of the agar gel was carefully excised with a heat-sterilized razor. The empty space was then filled with sterilized NMS agar.

Fig. S6. Transmission electron micrograph of Methylcystis sp. SB2 grown on ethanol. Strain SB2 was incubated in liquid NMS (Whittenbury et al., 1970) with 0.1% ethanol (v/v) supplemented with 10 μM copper as CuCl2 for 2 days, and then harvested by centrifugation at 12 000 rpm for 10 min at 4°C. The sample was fixed in 2.5% glutaraldehyde in 0.1 M Sorensen’s buffer, post-fixed in 1% osmium tetroxide in the same buffer, and then stained with uranyl acetate and lead citrate. The sections were examined using a Philips CM100 electron microscope (Philips/FEI, Hillsboro, OR) at 60 kV. Images were recorded digitally using a Hamamatsu ORCA-HR digital camera system operated using AMT software (Advanced Microscopy Techniques Corp., Danvers, MA).

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