Isolation of a methane-oxidizing bacterium that bioremediates hexavalent chromium from a formerly industrialized Suburban River.

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Isolation of a methane oxidising bacterium that bioremediates hexavalent chromium from a formerly industrialised suburban river

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Running headline: Cr (VI) bioremediation by *Methylomonas*

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Significance and impact

Aerobic methanotrophic bacteria are known for bioremediation of an increasing range of organic and inorganic pollutants, using methane as carbon and energy source. Previously, one laboratory methanotroph strain, *Methylococcus capsulatus* Bath, was known to bioremediate toxic chromium (VI) by reducing it to chromium (III). Here, a newly isolated methanotroph strain, *Methylomonas koyamae* SHU1, has been shown able to remediate chromium (VI). This indicates that chromium (VI) bioremediation is not unique to *Methylococcus capsulatus* and moreover adds weight to the suggestion that methanotrophs may contribute directly to chromium (VI) detoxification in nature and in polymicrobial bioremediation fed with methane.

Abstract

Sediment samples were taken from sediment adjacent to an urban river in Sheffield in Northern England that had suffered heavy metal pollution due to previous activity of the steel industry (between the 17th and 19th centuries). The most abundant heavy metals found in the samples were lead, chromium, nickel, arsenic and cobalt, with maximum concentrations of 412.80 mg kg^{-1} 25.232 mg kg^{-1}, 25.196 mg kg^{-1}, 8.123 mg kg^{-1} and 7.66 mg kg^{-1}, respectively. Enrichment cultures were set up using methane as carbon and energy source, as a result of which a strain of methanotroph was isolated that was shown via 16S rRNA gene sequencing to be a strain *Methylomonas koyamae* and given the designation SHU1. *M. koyamae* SHU1 removed hexavalent chromium from an initial concentration of 10 ppm, which was inhibited by the metabolic inhibitor sodium azide or the methane monooxygenase inhibitor phenylacetylene. To the authors' knowledge this is the first description of a strain of the widely environmentally distributed genus *Methylomonas* that is capable of remediating hexavalent chromium.

Introduction

Methanotrophs are a subset of methylotrophic bacteria that utilise methane as sole carbon and energy source. More generally, methylotrophic bacteria use a range of one-carbon compounds, including methanol, methylated amines, halomethanes and methylated compounds containing sulphur, as their carbon and energy sources (Chistoserdova 2018; Smith and Murrell 2009). Methanotrophs are pervasive in the environment, including mesophilic and many severe conditions such as temperatures as low as 4^0C or as high as 72^0C (Jiang *et al.* 2010). Methanotrophs play an important role in global cycling of carbon (Semrau *et al.* 2010) and have been investigated for a wide range of biotechnological applications (Jiang *et al.* 2010). The key defining enzyme of methanotrophic microorganisms, methane monooxygenase (MMO), performs the oxidation of methane to methanol. The wide substrate range of the MMO enzymes for hydrocarbons and halogenated hydrocarbons has led them to be investigated for bioremediation of recalcitrant organic compounds (Semrau *et al.* 2013; Pandey *et al.* 2014). The interaction of metals with methanotrophs has been studied for some time, in particular the role of copper in regulating the expression of the two forms of MMO. In methanotrophs that are able to produce both the copper-dependent particulate MMO (pMMO) and broader substrate-range iron-containing soluble MMO (sMMO), the switch between expression of pMMO and sMMO is effected by available copper in the culture, with sMMO being expressed at low copper-to-biomass ratio and pMMO at high copper-to-biomass ratio (Stanley *et al.* 1983). A range of other heavy metals are known to interact with methanotrophs, including a range of metals via the copper acquisition peptide methanobactin (Semrau *et al.* 2013; Semrau *et al.* 2018) and reduction of Hg^{2+} ions to metallic mercury via a reductase activity (Boden and Murrell 2011). A previous study found that the common laboratory strain of methanotroph *Methylococcus capsulatus* Bath was able to reduce chromium (VI) to the less toxic and less bioavailable chromium (III), whist the model type II methanotroph *Methylosinus*

trichosporium OB3b did not under the conditions tested (Al Hasin *et al.* 2010). Other studies using mixed communities of microorganisms fed on methane have shown that methane can drive chromium (VI) reduction, probably in part indirectly, in microbial consortia (Lai *et al.* 2016; Long *et al.* 2017). Chromium bioremediation is important because chromium (VI) is a carcinogenic, toxic heavy metal affecting humans, aquatic life and posing threat to the environment (Jobby et al. 2018). In order to ascertain whether a chromium (VI) remediating methanotroph could be isolated from the environment, we have here investigated the environment of a river with a history of contamination by the steel industry. Substantial levels of heavy metal contamination were found. The microflora isolated included a new methanotroph strain, of the species *Methylomonas koyamae*, which is able to remediate chromium (VI) in a metabolically dependent fashion.

Results and Discussion

Characteristics of sampling sites

The site employed for study is a Suburban River in Sheffield in the North of England, with a history of steel manufacture. The samples were collected at nine different locations (Fig. S1). Samples for metal analysis were air dried and sieved and particle size fractions 5-8 as defined by Wentworth (1922) (Table S1; corresponding to particles of 600 µm diameter and finer) and then subjected to metal analysis by ICP-MS. The heavy metals found were lead (Pb), chromium (Cr), cobalt (Co), nickel (Ni) and arsenic (As). Maximum concentrations of these heavy metals at the various sample locations were chromium-25.232 mg kg^{-1}, cobalt-7.66 mg kg^{-1}, nickel-25.196 mg kg^{-1}, arsenic 8.123 mg kg^{-1} and lead 412.80 mg kg^{-1}.

Across the world, the average chromium concentration in pristine natural waters ranges from 0.2-1 µg l^{-1}. Natural chromium concentrations in sea water are found to be 0.04-0.5 µg l^{-1}. The

natural total chromium content in natural surface waters ranges from 0.5-2 μg l^{-1} approximately, while the dissolved total chromium content is 0.02-0.3 μg l^{-1} (World Health Organization, 2003). Groundwater in certain areas of California has been found to have chromium (VI) up to 1 μg l^{-1} (Hausladen *et al.* 2018). In light of these data, it is evident that if the chromium contained in the sediment samples from the River Sheaf were released into the surrounding river water, with a substantial proportion of the chromium in the hexavalent oxidation state, a substantial pollution problem could result.

Isolation and identification of environmental isolates

Isolates were obtained from sediment samples from the River Sheaf, on the basis of their ability to grow using methane as carbon and energy source, as described in the Materials and Methods section. Among the six isolates for which 16S rRNA gene sequence information was obtained, four were most similar to *Methylomonas koyamae*, one gave several BLAST hits within the genus *Methylophilus* and one was most similar to *Acidovorax facilis* (Table 1). The isolate SHU1, subsequently referred to as *M. koyamae* SHU1, was the isolate that could be assigned to a genus of known methanotrophs and for which the longest piece of 16S rRNA gene sequence was available. It was therefore selected for further characterization. The morphology of the isolated strain *M. koyamae* SHU1 was a Gram negative, motile rod (Figure 1a). The 16S rRNA gene sequence showed maximum similarity to *Methylomonas koyamae* Fw12E-Y (GenBank accession number NR_113033.1 with 99% identity across 973 base pairs and E value = 0.0). Its relationship to the 16S rRNA gene sequences from other methanotrophs can be seen in Figure S2.

The colonies of *M. koyamae* SHU1 appeared pink in colour on fresh nitrate minimal salts (NMS) agar plates and changed further to pinkish orange at longer incubation time (Figure 1b). The *M. koyamae* SHU1 could also grow using methanol as carbon and energy source (data not shown). *M. koyamae* belongs to type I methanotrophs of the *Methylomonas* family which are widespread in the environment. The sediments samples from the River Sheaf had near neutral pH and so isolation of a *Methylomonas* strain from these samples is consistent with the neutrophilic phenotype and optimal temperature 30˚C previously reported for members of the genus *Methylomonas* (Marco *et al.* 2004).

Methane oxidising bacteria of the genus *Methylomonas* have previously been found in fresh water lakes and rivers, wetland muds, activated sludge and waste water, and coal mine drainage water. The reason for the growth of type I methanotrophs in the present study can possibly be attributed to their faster growth compared with the type II methanotrophs (Chi *et al.* 2012) that are also likely to be present in the samples. The high oxygen concentration and low methane concentration in this top zone of soil provide a suitable environment for various type I strains of methanotrophs (Auman *et al.* 2000).

A. facilis is a constituent of soil microbiomes that is metabolically versatile (Willems *et al.* 1990), though not known to be methylotrophic. Members of the genus *Methylophilus* are known to be facultative methylotrophs that can be isolated from heavy-metal contaminated sites and can grow on a range of one-carbon compounds including methanol (Jenkins et al. 1987; Giri et al. 2013), though they are not known to be methanotrophic. The growth of both these organisms in the enrichments reported here may be result of an impure culture containing methanotrophs, or due to scavenging of carbon-containing nutrients from the agar in the media.

Removal of chromium by *M. koyamae* SHU1

Isolated organisms from metal contaminated sites can often be employed to study the metal detoxification process. *M. koyamae* SHU1 was therefore tested for chromium (VI) bioremediation and could remove hexavalent chromium from an initial concentration of 10 mg l^{-1} when cultivated on methane as carbon and energy source (Figure 2a). There was no removal of hexavalent chromium by *M. koyamae* SHU1 in the cultures with added sodium azide, which is a metabolic inhibitor. Removal of hexavalent chromium was not observed with the blank and in heat killed cells indicating that the live, metabolically active cells of *M. koyamae* SHU1 are solely responsible for the removal of the chromium.

The hexavalent chromium inhibited the growth of *M. koyamae* SHU1 (data not shown), in spite of which these cells remain to some extent metabolically intact and metabolically remove the hexavalent chromium. As was found previously with *Methylococcus capsulatus* Bath (Al Hasin *et al.* 2010), the heat killed cells of *M. koyamae* SHU1 did not show any removal of hexavalent chromium indicating that chromium (VI) removal is likely to be an enzymatic process since heat killing inactivates enzymes. Heat killing of the cells is also expected to interfere with membrane structure, protein synthesis machinery (Narayani and Shetty 2013) and labile small molecules, any of which may be necessary for chromium (VI) reduction.

Certain methanotrophs, including *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b, are able to produce two forms of MMO, the copper-dependent particulate MMO (pMMO) and the iron-containing soluble MMO (sMMO), according to the concentration of available copper in the medium (Stanley *et al.* 1983). Whilst strains of *M. koyamae* are only known to produce pMMO, copper availability is known to affect the production of a large number of proteins in *Methylococcus capsulatus* (Kao *et al.* 2004) and

responses to metals are increasingly recognised as important in controlling gene expression in a wide range of methanotrophs (Semrau *et al.* 2018). The effect of copper concentration and MMO on chromium removal by cells of *M. koyamae* SHU1 was investigated by growing the organism in high copper concentration medium and low copper concentration medium and then adding the MMO suicide substrate phenyl acetylene (Lontoh et al. 2008) at the same time as chromium (VI). The cells grown in low copper concentration medium performed chromium (VI) removal in this experiment to a lesser extent than those grown in high medium (Figure 2b).

The chromium reduction carried out by *Methylococcus capsulatus* (Bath) showed that it can reduce hexavalent chromium between concentrations of 1.4 to 1000 mg l^{-1} and inhibition of organism by sodium azide caused loss of 57% of chromium removal. Metabolic inhibition by azide is generally considered to be at the level of cytochrome *c* oxidase, where the azide ion is a mimic for dioxygen. Hence, the effect of azide on chromium (VI) removal by both *Mc. capsulatus* and *M. koyamae* is presumably an indirect one due to general metabolic disorder when the principal electron transport chain is inhibited (Al Hasin *et al.* 2010).

Conclusions

Here, it has been shown possible to isolate a chromium (VI)-bioremediating methanotroph from an environment with significant current chromium content and a history of heavy metal pollution. The organism isolated has a 16S rRNA genome sequence that is most similar to that of *M. koyamae*, a methanotroph previously isolated from a rice field (Ogiso *et al.* 2012). Since methanotrophic chromium (VI) bioremediation had only previously been shown in one strain grown for many decades in the laboratory, the results reported here confirm that methanotrophs can be added to the wide diversity of microorganisms and plants known to be able to

bioremediate chromium (VI) (Cervantes *et al.* 2001). They are also an encouragement to endeavours to isolated environmental microorganisms for methane-fuelled chromium (VI) bioremediation under a range of conditions.

Materials and Methods

Site and Sampling:- The site employed for the study is the River Sheaf which is a river in Sheffield 53° 23′ N 1° 28′ W53.383° N 1.467° W, South Yorkshire, England. Situated in the Pennine foothills in the extreme south west corner of Yorkshire, Sheffield city is built on seven hills and watered by five rivers. The Rivers Sheaf, Porter, Loxley and Rivelin finally join into the River Don. Millhouses is a public urban park located in Millhouses neighbourhood in the south of Sheffield. It is a 12.87 hectare park stretching along 1.2 km along the floor of the River Sheaf valley sandwiched between Abbeydale Road South (A 621) and the railway tracks of the Midland Mainline Railway. Prior to the construction of the park it was used for farmland and industrial purposes due to various power mills located on the river (Sheffield Libraries Archives and Information 2006).

A stratified random sampling method was employed to collect the samples at the River Sheaf site passing along Millhouses Park. The total site was broken into two areas and samples were collected randomly from each area depending on the accessibility to sediment samples at the location (Figure S1). Nine samples were collected in October, 2011 for chemical and microbiological analysis, at approximately equal spacing along the river from where the River enters Millhouses Park with grid reference SK 332835 to the point at which the River leaves the Park, according to a risk assessment approved by the University.

212 **Isolation, enrichment and cultivation of methanotrophs**

213 Enrichment and growth of methanotrophic microorganisms was performed in NMS medium
214 (Smith and Murrell, 2011) containing Cu as $CuSO_4.5H_2O$ at 1 mg L^{-1} or (where expression of
215 sMMO was required) 0.1 mg L^{-1} of $CuSO_4.5H_2O$. Single colonies were obtained by streaking
216 on NMS agar plates. Aliquots (0.5 g) of sediment samples collected from the River Sheaf were
217 inoculated into 50 ml portions of NMS medium in 200 ml Erlenmeyer flasks. Methane gas
218 was introduced at 1:4 v/v with air into the culture flasks at regular intervals using hypodermic
219 syringes and the flasks were sealed with Subaseals (Fisher) to prevent methane loss. The flasks
220 were incubated at 30^0C on a rotary incubator for 1 week to 10 days for the growth of
221 methanotrophs in the flask.

222 The growth of methanotrophs was observed by monitoring the turbidity of the enrichments in
223 flasks. The cultures in the flasks were sub cultured into fresh NMS medium and incubated at
224 30^0C on a rotary incubator for 1 week to 10 days of growth. A loopful of the resulting
225 enrichment was streaked on fresh NMS plates and incubated at 30^0C in a methane air
226 atmosphere until single colonies of the isolate were obtained. The isolated organisms were
227 streaked several times on fresh NMS plates, prior to sequencing of the 16S rRNA genes as
228 described by Murrell *et al.* (1998).

229 **Analytical Methods**

230 Heavy metal concentrations in soil samples were determined by means of extraction with nitric
231 acid followed by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) using a model
232 Hewlett Packard (HP) 4500 system (Yokogawa Corporation, Japan) that was calibrated with
233 ICP-MS Calibration standard (XXI) (Sigma). Cells and other particulate material were
234 removed from liquid culture samples by centrifugation (5000 × g; 5 min; room temperature).
235 The hexavalent chromium in the supernatant was then quantified spectrophotometrically via

the diphenyl carbazide assay as described in Al Hasin *et al.* (2010) and the concentrations of chromium VI in the bacterial cultures were determined by reference to a standard curve (1-10 mg l^{-1} of chromium VI). The removal of chromium by *M. koyamae* (SHU1) in the presence of the metabolic inhibitor sodium azide (0.5%) and heat killed (autoclaved) cells were performed similarly as described above.

Bioinformatics

Similarity searches using nucleotide query sequences were performed using BLAST; phylogenetic trees were constructed from multiple sequence alignments using Clustal Omega, both via the EBI website (Madeira *et al.* 2019).

Acknowledgements

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Conflict of interest

No conflict of interest declared.

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340 **Table 1** Molecular identification of isolated microorganisms using amplified partial 16S rRNA gene
341 sequences

Isolate	Colony appearance	Sequence length (bp)	Closest match	Genbank accession no.	Identity	E Value
SHU1	Pinkish white	973	*Methylomonas koyamae*	NR113033.1	99%	0.0
SHU2	Creamish orange	315	*Methylophilus leisingeri*	AB 193725.1	99%	2e-157
			Methylophilus methylotrophus	NF 911346.1	99%	1e-155
			Methylophilus rhizospharerae	AB 698737.1	99%	1e-155
SHU3	Creamish Orange	366	*Methylomonas koyamae*	NR 113033.1	99%	0.0
SHU4	Pink	467	*Methylomonas koyamae*	NR 113033.1	99%	0.0
SHU5	Creamish orange	819	*Methylomonas koyamae*	NR 113033.1	99%	0.0
SHU6	Orange	980	*Acidovorax facilis*	JQ236816.1	99%	0.0

342

343

344

346 **Figure legends**

347

348 **Figure 1** *M. koyamae* SHU1: (a) phase contrast microscopy of culture (1,000 × magnification),
349 the organisms are short rods, which were seen to be motile; (b) morphology of the colonies on
350 an NMS agar plate.

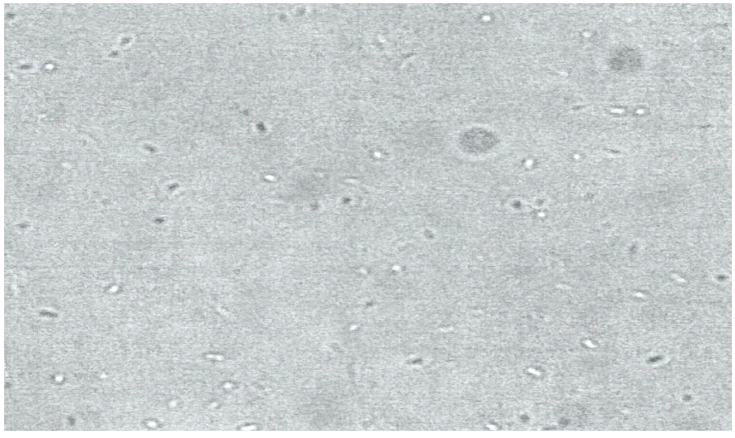
351

352 **Figure 2** Removal of hexavalent chromium by *M. koyamae* SHU1 under various conditions
353 measured via the DPC assay. (a) Effect of sodium azide and heat treatment: (♦) concentration
354 of Cr (VI) in the culture of *M. koyamae* SHU1 without inhibitor; (■) concentration of Cr (VI)
355 by the culture in the presence of sodium azide (0.5%) (▲) concentration of Cr (VI) in the
356 presence of heat killed cells. (b) Effect of different copper concentrations: (♦) concentration
357 of Cr (VI) in high copper concentration (1 mg/L of $CuSO_4.5H_2O$) (■) concentration of Cr (VI)
358 in low copper concentration (0.1 mg/L) in the presence of phenylacetylene. The organisms
359 were grown into mid-log phase before the addition of acetylene (0.05% v/v) and 10 ppm of
360 hexavalent chromium.

361

Figure 1

(a)



(b)

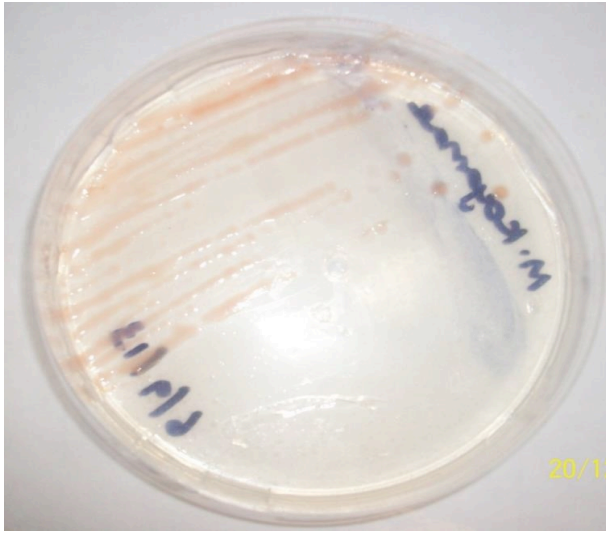
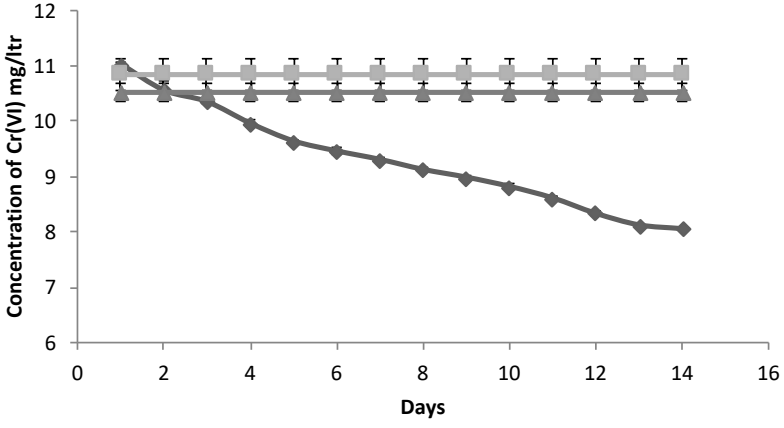
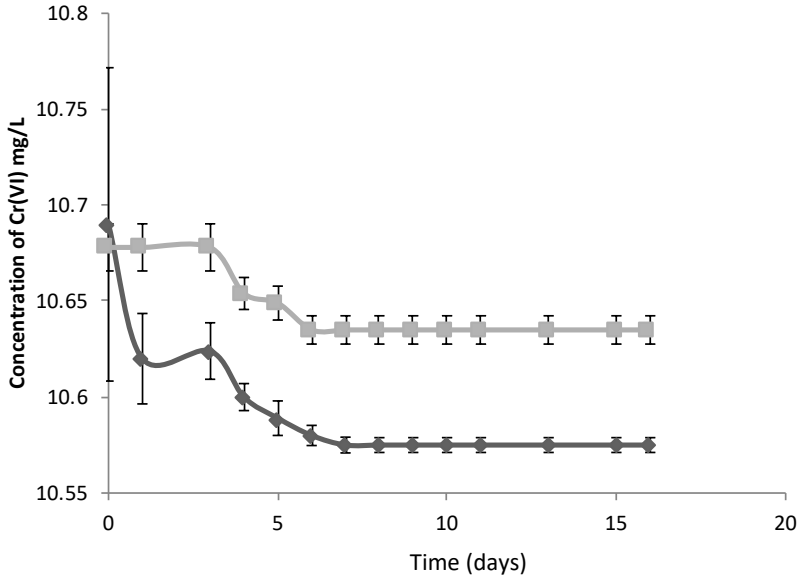


Figure 2

(a)


Concentration of Cr(VI) mg/ltr
12
11
10
9
8
7
6
0
2
4
6
8
10
12
14
16
Days

(b)


Concentration of Cr(VI) mg/L
10.8
10.75
10.7
10.65
10.6
10.55
0
5
10
15
20
Time (days)

Isolation of a methane oxidising bacterium that bioremediates hexavalent chromium from a formerly industrialised suburban river

Supporting information

Swapnika Challa and Thomas J. Smith

Scale 1:5000

Red dots denote sampling locations

Figure S1 Map showing the sampling locations along the River Sheaf

Table S1. Size classification of sample fractions

Fraction	Size range	Wentworth grade	Phi(Φ) scale
1	≥5 mm	Pebble	-2
2	≤5.0 mm ≥2.36 mm	Granule	-1
3	≤2.36 mm ≥1.18 mm	Very coarse sand	0
4	≤1.18 mm ≥600 µm	Coarse sand	1
5	≤600 µm ≥300 µm	Medium sand	2
6	≤300 µm ≥150 µm	Fine sand	3
7	≤150 µm ≥75 µm	Very fine sand	4
8	≤75 µm	Silt	5-8

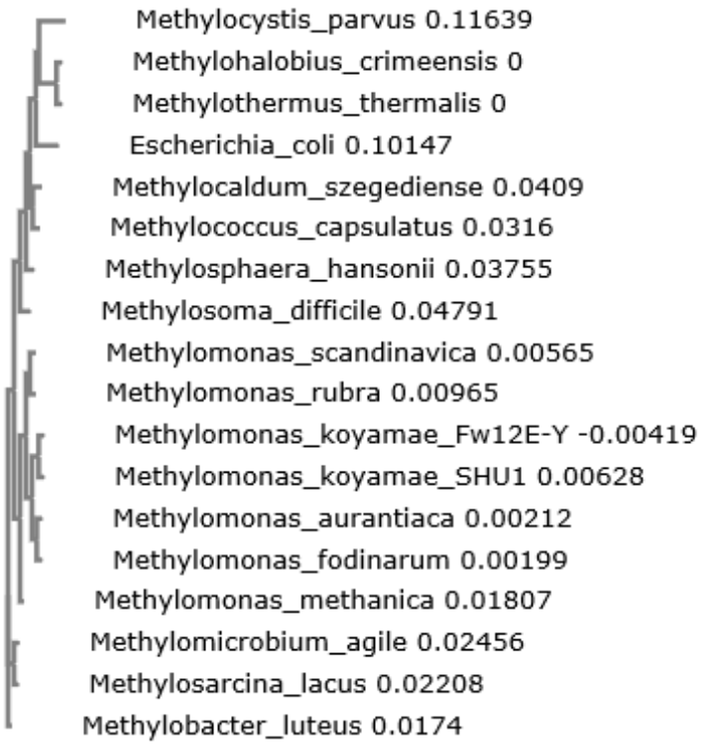


Figure S2 Phylogenetic tree of partial 16S rRNA gene sequences generated by CLUSTAL omega, showing the new isolate *Mtm. koyamae* SHU1.