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ORIGINAL PAPER

Isolation and characterization of a tetramethylammonium-degrading *Methanococcoides* strain and a novel glycine betaine-utilizing *Methanolobus* strain

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Abstract Two novel strains of methanogens were isolated from an estuarine sediment with the capability to utilize quaternary amines. Based on the 16S rRNA analysis, strain B1d shared 99 % sequence identity with *Methanolobus vulcani* PL-12/M^T and strain Q3c shared 99 % identity with *Methanococcoides* sp. PM1 and PM2, but our current isolates display clearly different capabilities of growth on quaternary amines and were isolated based on these capabilities. Strain Q3c was capable of growth on tetramethylammonium and choline, while strain B1d was capable of growth on glycine betaine. *Ml. vulcani* PL-12/M^T was incapable of growth on glycine betaine, indicating an obvious distinction between strains B1d and PL-12/M^T. Strain Q3c now represents the only known tetramethylammonium-utilizing methanogen in isolation. Strain B1d is the first quaternary amine-utilizing methanogen from the genus *Methanolobus*. This study suggests that quaternary amines may serve as ready precursors of biological methane production in marine environments.

Keywords Methanogen · Glycine betaine · Tetramethylammonium · Choline · *Methanococcoides* · *Methanolobus*

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Introduction

Biological methane production, or methanogenesis, is a topic of considerable global and environmental interest due to the potential of methane to serve as a renewable energy source and its potency as a greenhouse gas (Rothman et al. 2014). Canonical methanogenesis is specific to the domain *Archaea*, and by studying methanogenesis, we can better understand global carbon and nitrogen cycling. Furthermore, studies related to quaternary amine degradation will give insights into the remediation of toxic compounds, i.e., tetramethylammonium (Tanaka 1994; Asakawa et al. 1998), or cardiovascular associated health factors such as choline and glycine betaine (Wang et al. 2011; Tang et al. 2013; Wang et al. 2014). Methanogenesis encompasses three defined pathways: hydrogenotrophic, aceticlastic, and methylotrophic (Thauer et al. 2008). Methylotrophic methanogenesis results in the mobilization and transfer of methyl groups from methylated compounds directly to the thiol group of coenzyme M (2-mercaptoethanesulfonic acid; CoM) (Harms et al. 1995; Burke and Krzycki 1997; Ferguson and Krzycki 1997; Sauer et al. 1997; Tallant and Krzycki 1997; Asakawa et al. 1998; Ferguson et al. 2000; Bose et al. 2008), which is the penultimate step of methanogenesis. Species of the order *Methanosarcinales* are some of the most adaptable methanogens to date with the potential to utilize 14 known substrates: H_2/CO_2,

H_2/CO, acetate, methanol, methylamine (MMA), dimethylamine (DMA), trimethylamine (TMA), tetramethylammonium (QMA), dimethyl sulfide, methanethiol, methylmercaptopropionate, glycine betaine (GB), choline, and dimethylethanolamine (DMEA).

Methylotrophic methanogenesis is a mechanism by which methanogens can compete with sulfate-reducing bacteria (SRB) in marine sediments (Purdy et al. 2003), as determined by the kinetics and thermodynamics in those environments (Winfrey et al. 1977; Banat et al. 1981; Lovley et al. 1982; Kristjansson and Schönheit 1983). Additionally, sulfate ions are inhibitory of hydrogenotrophic and aceticlastic methanogenesis in the presence of SRB, while negligible inhibition of methylotrophic pathways from sulfate ions has been observed (Oremland et al. 1982; King 1984; Garcia-Maldonado et al. 2012). Thus, under sulfate-reducing conditions, only methylotrophic methanogenic pathways are likely to account for significant production of methane. This raises the question of whether there are substrates for methylotrophic methanogens that are yet undiscovered and whether lesser-known substrates may play a larger role in methane production in marine sediments. Prior research showed that co-cultures of methanogens with SRB stimulated methane production with quaternary amines such as choline and glycine betaine; this activity was the direct result of trimethylamine production by fermenters in the co-culture (King 1984). In this way, the presence of fermenters, methanogens, and SRB could lead to a system of quaternary amine breakdown, which could explain their co-localization in the environment.

In 1994, *Methanococcoides* sp. NaT1, of the order *Methanosarcinales*, was isolated from Tokyo Bay in Japan (Tanaka 1994). Strain NaT1 was the first methanogen shown to be capable of utilizing QMA, the simplest quaternary amine, as a direct substrate for methanogenesis. This was also the first demonstration of QMA degradation in an anaerobic system. Furthermore, QMA utilization followed the canonical methylotrophic route, which directly methylates CoM (Asakawa et al. 1998). Unfortunately, viable cultures of strain NaT1 no longer exist, so there are no publicly available QMA-utilizing methanogen strains at this time. Consequently, the elucidation of QMA driven methanogenesis remains incomplete, as no sequenced genomic data are publicly available. This may be primarily due to the fact that strain NaT1 was isolated and described prior to the current age of rapid and inexpensive genome sequencing. A related observation was that novel *Methanococcoides* sp. strains isolated from marine sediments by growth on methylamine are capable of methanogenesis directly from choline, DMEA, and GB (Watkins et al. 2012, 2014); however, these strains are not known to degrade QMA. No quaternary amine-degrading methanogens have thus far been identified outside of the genus *Methanococcoides*.

The loss of the QMA-degrading methanogen strain NaT1, combined with our interest in anaerobic quaternary amine catabolism, led us to test for the presence of these methanogens in the environment. As we began this project, we used our GB methyltransferase enzyme from *Desulfitobacterium hafniense* Y51 (Ticak et al. 2014) coupled with prior studies of quaternary amine-dependent methanogenesis as a benchmark to formulate a hypothesis that GB and QMA methanogens are present environmentally and that we would be able to isolate different genera capable of this form of growth from brackish estuarine sediments. The significance of this work is threefold: (1) to show that quaternary amine-degrading methanogens inhabit estuarine and oceanic environments in numbers sufficient to allow facile isolation, (2) to obtain a QMA-degrading methanogen strain for further study of this pathway, and (3) to show that quaternary amine-degrading methanogens exist outside of the genus *Methanococcoides*. These substrates may well contribute more to the global carbon and nitrogen cycles than was previously understood. Furthermore, with the expanding knowledge of quaternary amines and abundance of these compounds in the environment, ecological studies will need to be focused on the amounts of methane formed from these compounds and the abundance of the organisms responsible for these processes.

Materials and methods

Growth and maintenance of enrichment and pure cultures

Anoxic sediment from the brackish Southwest Branch Back River in Hampton, Virginia, USA (GPS coordinates: 37.066444, −76.311639), was collected in May 2013, sealed in a polypropylene jar, and shipped overnight at ambient temperature to our laboratory. The jar was transferred to an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA), opened, and allowed to equilibrate overnight at 37 °C. Enrichment cultures were prepared in brackish medium (BM). This medium was identical to *Methanosarcina acetivorans* high-salt medium except that the concentration of added NaCl was lowered from 400 to 275 mM (Metcalf et al. 1997). BM is a chemically defined mineral salts medium that contains no carbon sources, such as yeast extract or acetate, other than the provided growth substrate. The cultures were grown in 27.2-mL sealed anaerobic Balch tubes with 10 mL of medium and 17.2 mL of headspace. The enrichment cultures were inoculated with sediment inside the anaerobic chamber, resealed with sterile butyl rubber stoppers and crimp seals, and the headspace of each tube was exchanged outside of the anaerobic chamber with filtered CO_2/N_2 (20/80 %) gas mix on a

vacuum-gassing manifold. Each 10 mL of enrichment culture was supplemented with one gram of anoxic sediment, filter sterilized (0.22-μm cellulose acetate filter) quaternary amine substrate (10 mM), vitamins, and streptomycin (200 μg/mL). The cultures were incubated at 37 °C statically in the dark and monitored daily for methane production by gas chromatography (GC). Once the cultures had reached an apparent maximum methane production, 1 % (v/v) of culture was transferred to 10 mL of fresh medium, supplemented as before. Subsequent transfers of the enrichment cultures were done into liquid medium lacking antibiotic. After three transfers in liquid BM, enrichment cultures were diluted 10^{-4}–10^{-5} and plated on BM containing 1.5 % (wt/v) agar and streptomycin (200 μg/mL) and incubated in anaerobic jars flushed with $CO_2/H_2S/N_2$ (20/0.1/79.9 %) gas mix inside of the Coy anaerobic chamber at 37 °C. After 1–2 weeks of incubation, the jars were opened, and the colonies were picked to liquid BM. Once grown, methanogenic cultures were subcultured on BM agar containing tetracycline (50 μg/mL) and incubated for 2 weeks as described above, then transferred to liquid BM. These cultures were examined for purity by differential interference contrast (DIC) microscopy, thioglycolate broth, as well as 16S rRNA PCR amplification and sequencing. Each culture was then transferred to BM containing 40 mM trimethylamine (TMA) or 62.5 mM methanol. Each strain was subcultured into BM with kanamycin (50 μg/mL) and ampicillin (100 μg/mL). Strain B1d was provided methanol (62.5 mM) and strain Q3c was provided TMA (40 mM) in the medium. Grown cultures were serially diluted in BM and plated onto BM supplemented with both kanamycin (50 μg/mL) and ampicillin (100 μg/mL) containing 1.5 % (wt/v) agar and incubated as described above. Isolated colonies of each culture were grown and maintained in liquid BM lacking antibiotics and used for identification and further characterization. Both strains were deposited in the Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Chiba, Japan, with the accession numbers NRBC 110459 (strain B1d) and NRBC 110458 (strain Q3c). The strains were also deposited in the RIKEN BioResource Center, Japan Collection of Microorganisms (JCM), with the accession numbers JCM 30225 (strain B1d) and JCM 30226 (strain Q3c). Actively growing *Methanolobus vulcani* PL-12/M^T (DSM 3029) was purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ) and grown and maintained in BM supplemented with 62.5 mM methanol and culturing was also attempted in BM supplemented with 40 mM GB.

Microscopic examination

DIC microscopy was performed on wet-mounts of strains B1d and Q3c at stationary phase using an Olympus AX70 microscope at 600× total magnification. The mounts were also observed under fluorescence microscopy with the same microscope with excitation wavelengths of 340–380 nm. Specimens for scanning electron microscopy (SEM) were prepared by using a 1:1 ratio of stationary-phase cultures with fixative (2 % paraformaldehyde, 2.5 % glutaraldehyde in BM lacking PO_4^{3-}) and allowing them to incubate anaerobically for 30 min at 25 °C. One hundred microliters of fixed cells was pipetted onto poly-L-lysine-coated coverslips and allowed to dry for 30 min. Excess medium was wicked away with a KimWipe™, and each sample was washed four times for 10 min with BM lacking PO_4^{3-}. Samples were then ethanol dehydrated, CO_2 critical point dried in a Tousimis Samdri-780A critical point dryer (Tousimis, Rockville, MD, USA) and gold sputter coated for 90 s at a current of 15 mA with a Denton gold sputter coater (Denton Vacuum, LLC, Moorestown, NJ, USA) (Fiester et al. 2014). Samples were examined at 25,000×, 50,000×, and 75,000× and an accelerating voltage of five kiloelectronvolts with a Zeiss 35VP scanning electron microscope (Carl Zeiss AG, Oberkochen, Germany).

Physiological characterization of the isolates

Both of the isolates were tested for their ability to grow in varying NaCl concentrations by amending the BM with the addition of zero, 0.05, 0.10, 0.275, 0.40, 0.60, 0.90, 1.20, or 1.40 M NaCl. BM with no additional NaCl contains 46 mM Na^+ from other sources. The temperature dependence of each strain was measured using BM at 4, 15, 18, 23, 30, 37, 40, and 45 °C. The cultures were grown twice using 1 % (v/v) inocula in triplicate at the respective NaCl and temperature conditions. These cultures contained 40 mM GB for strain B1d and 40 mM QMA for strain Q3c. To test for the ability to utilize different growth substrates, the cultures were transferred to BM containing the respective substrate; GB, dimethylglycine (DMG), choline, DMEA, carnitine, QMA, TMA, DMA, MMA, methanol, acetate, or H_2/CO_2. All substrates were supplemented at 40 mM except acetate (80 mM), methanol (62.5 mM), and H_2/CO_2 (80/20 %). Cultures with measureable growth were passaged three times in triplicate; with 1 % (v/v) inocula to ensure that growth was maintained and to limit substrate carryover. The cultures that did not grow were observed for 16 weeks.

Phylogenetic and DNA G+C (mol %) content analysis

Genomic DNA was isolated from strains B1d and Q3c as well as *Ml.* vulcani PL-12/M^T by phenol–chloroform extraction as described previously (Sambrook et al. 1989). To obtain G+C (mol %) content for each strain, extracted DNA from B1d and Q3c was prepared by using a Nextera

XT DNA Sample Preparation Kit and a MiSeq Reagent Kit version 3 (600 cycles), as per the manufacturer's instructions (Illumina, San Diego, CA, USA). The data were analyzed using the CLC Genomics Workbench version 7.0.4 (CLC Inc, Aarhus, Denmark).

PCR amplifications of archaeal and bacterial 16S rRNA were carried out using purified genomic DNA from each strain and three previously described sets of primers: 109f/A1492r, 1Af/A1492r, and 27f/1492r in addition to a known methanogenic marker, *mcrA*, with ME1/ME2 (Webster et al. 2006). The 25 μL of PCR reactions contained 1× Phusion DNA polymerase buffer, 1 U Phusion DNA polymerase (New England Biolabs), 200 μM dNTPs, 0.5 μM of each primer pair, and 100 ng purified genomic DNA quantitated via NanoDrop 2000 UV–Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The PCR reactions were performed by the following protocol: 5 min at 98 °C, followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 90 s, followed by a final extension step of 10 min at 72 °C. PCR products were cleaned using a Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and sequenced with PCR amplification primers by using a BigDye Terminator (version 3.1) Cycle Sequencing Kit (Life Technology Corporation, Carlsbad, CA, USA) on an Applied Biosystems 3730xl DNA analyzer (Life Technology Corporation). Sequencing was performed twice after initial isolation from the plates and twice again after final isolation to ensure the sequence validity for the isolates, while *Ml. vulcani* PL-12/M^T was sequenced as above three times using the above primers to replace *N*'s from the deposited NCBI sequence (GI:343206176).

The 16S rRNA sequence data for Q3c (1,330 nt) and B1d (1,330 nt) were used as queries for obtaining closely related sequences from both the GreenGenes database (DeSantis et al. 2006) and NCBI nucleotide BLAST database (Altschul et al. 1990), excluding uncultured sequences. The percent identities to the query sequences were recorded, and 28 sequences were obtained for phylogenetic analysis. The 28 sequences were aligned via MUSCLE (Edgar 2004) and then analyzed for the highest log likelihood value (−4,735.6805) for maximum likelihood analysis in MEGA6 (Tamura et al. 2013). The phylogenetic tree was reiterated 1,000 times using the general time-reversible model (Nei 2000) with a discrete gamma distribution among sites (+*G*, parameter = 0.1593) with any position less that 95 % site coverage being eliminated leaving 1,309-nt positions in the final analysis. *M. acetivorans* C2A^T (GI:470466243) and *Methanimicrococcus blatticola* PY-27 (GI:374719707) were used as outgroups that were truncated from the tree.

Amplified partial *mcrA* gene products were sequenced as stated above, and data for B1d and Q3c were used as queries for tBLASTx (Gish and States 1993) and used to search the non-redundant (nr) NCBI amino acid database. A total of 21 sequences were retrieved and used for the initial phylogenetic analysis. The sequences were aligned with MUSCLE and then truncated to 160 amino acids to eliminate bias of longer sequences. MEGA6 was used to calculate the highest log likelihood value (−1,124.629) for maximum likelihood analysis, which encompasses the Le–Gascuel model (LG model) (Le and Gascuel 2008). The LG model was used with a discrete gamma distribution among the different sites [5 categories (+*G*, parameter = 0.3718)] in addition to calculating the potential invariability of individual sites [(+*I*), 16.7476 %] in MEGA6 (Tamura et al. 2013). All positions that were less than 95 % site coverage were removed from the final analysis prior to tree construction yielding sequences with a final dataset of 160 amino acids. After tree construction, the outgroup sequences consisting of *M. acetivorans* C2A^T (GI:499333927), *Methanosarcina mazei* (GI:145370893), *Methanosarcina thermophila* TM-1^T (GI:154240554), and *Methanosarcina barkeri* (GI:499625182) were truncated from the final tree.

Analytical methods

Methane measurements were performed using an Agilent Technologies model 6,890 N GC using a flame ionization detector (FID) with a ShinCarbon ST 80/100 column. Samples (200 μL) of headspace were aseptically removed from growing cultures using a one milliliter glass, gas-tight Hamilton syringe, fitted with a Mininert™ syringe valve and a sterile 25 gauge disposable needle, and injected into the GC. Methane was quantitated by comparison of the peak area values to standard curves of methane concentration generated at the time of each set of experimental measurements.

Quaternary amine (GB and QMA) concentrations were measured from 10 μL of culture samples, diluted 1:10, using the periodide method with standard curves specific to each substrate (Wall et al. 1960). The tertiary amines DMG and TMA were tested to ensure that there was no cross-reactivity in the periodide assay at the relevant concentrations. Methylamine and acetate concentrations in the media were measured by GC as described previously (Kremer et al. 1993; Burke and Krzycki 1995). A colorimetric assay was used to determine ammonium concentrations by first diluting culture media 1:300 prior to being assayed as described previously (Kandeler and Gerber 1988).

Results

Isolation of strains B1d and Q3c

Previous studies have shown the ability of methanogens to utilize quaternary amines such as QMA, choline, and GB

as carbon and energy sources (Tanaka 1994; Watkins et al. 2012, 2014). The lack of publicly available quaternary amine-degrading methanogenic strains along with our general interest in anaerobic quaternary amine metabolism led us to examine whether quaternary amine-degrading methanogens could be isolated from anaerobic estuarine sediment. We chose to examine sediment from a brackish tidal marsh on the coast of Virginia, USA, in the Southwest Branch Back River as a representation of the metabolic capabilities of organisms in such environments. In order to minimize the bacterial involvement in the degradation of the quaternary amines, we selected for methanogens using a modified medium (BM) optimized for marine methanogen growth and supplemented with antibiotics (200 μg/mL streptomycin). Methanogens have been shown to be resistant to many commonly used antibiotics and have been isolated in the presence of antibiotics in other studies (Whitman et al. 2006; Kumar et al. 2012). The QMA and GB enrichment cultures each showed rapid growth and methanogenesis after 2 days of incubation. Subsequent transfers were plated to obtain isolated colonies. We performed an additional selection step by plating dilutions of the cultures obtained from the initial platings onto BM agar containing tetracycline (50 μg/mL).

Analysis of the 16S rRNA sequence of these isolates indicated that the sequence of strain B1d from the GB enrichments appeared to be pure, but strain Q3c from the QMA enrichments still contained bacterial contamination, as evidenced by the amplification of a bacterial 16S rRNA sequence. In order to ensure purity of the methanogen cultures, we grew each isolate in either methanol or TMA in medium containing a cocktail of kanamycin and ampicillin on plates. Pure colonies of both strains were obtained at this step that were round, smooth, light brown, and slightly raised. The B1d colonies were translucent, and the Q3c colonies were opaque. Some colonies of each strain also formed bubbles after extended growth and produced cavities within the agar.

Identification and phylogenetic analysis of the isolates

We discovered the identity of the strains by 16S rRNA and *mcrA* gene analysis. We amplified a fragment of the 16S rRNA gene with *Archaea*-specific 109f/A1492r and A1f/A1492r primer pairs, described previously (Webster et al. 2006), and obtained single products from each primer pair (Fig. S1). We amplified a fragment of the *mcrA* gene product using the ME1/ME2 primer pair and obtained single products from each isolate. No PCR products were observed using the *Bacteria*-specific 27f/1492r primer pair for strains B1d and Q3c under the conditions tested. Sequencing of the 16S rRNA and *mcrA* products resulted in single homogeneous sequences for each of the products. A multiple sequence alignment was performed with available sequences from described species in the literature to compare these isolates to known cultivable strains (Fig. 1). The alignment of the 16S rRNA gene from strain B1d clustered strongly with *Ml. vulcani* PL-12/M^T, but a closer examination of the deposited sequence of *Ml. vulcani* PL-12/M^T revealed many unknown bases in the sequence, causing other *Methanolobus* strains to appear more similar, such as *Methanolobus profundi* MobM^T (Table 1). We therefore obtained *Ml. vulcani* PL-12/M^T from DSMZ and re-sequenced a 16S rRNA gene fragment generated using the A1f/A1492r primer pair. The newly sequenced fragment from *Ml. vulcani* PL-12/M^T was 99.1 % identical to that of strain B1d suggesting that B1d is a strain of *Ml. vulcani* PL-12/M^T. Strain Q3c showed approximately 99 % identity to *Methanococcoides* sp. PM1 and PM2 (Table 2), which belongs to an apparent cluster of *Methanococcoides methylutens* strains from several independent isolation studies. Within this cluster, *Mc. methylutens* strains PM1, PM2, AM1, NM1, DM1, and *Methanococcoides vulcani* SLH33 are capable of choline and DMEA degradation. *Mc. vulcani* SLH33 and *Mc. methylutens* PM2 and NM1 are capable of GB degradation. Only strain NaT1 was capable of QMA degradation (Watkins et al. 2012; L'Haridon et al. 2014; Watkins, et al. 2014). Therefore, strain Q3c was unique in its complement of substrates. Secondary phylogenetic analysis was done with the use of the conserved methanogen-specific marker methyl-coenzyme M reductase subunit A gene (*mcrA*) analyzed with tBLASTx (Gish and States 1993). The amino acid sequence of McrA for B1d clustered well within the *Methanolobus* products (Fig. 1b) but was more divergent from *Ml. vulcani* PL-12/M^T compared to the 16S rRNA tree (Fig. 1a). McrA data from Q3c (Fig. 1b) showed nearly identical sequence similarity to *Mc. methylutens* strains PM1 and PM2, much like the 16S rRNA analysis (Fig. 1a).

Characterization of the novel quaternary amine-utilizing strains

Microscopic analysis of strain B1d revealed small cells 0.6 ± 0.07 μm in diameter ($n = 15$) (Fig. 2a, c). The B1d cells were coccoid but showed some evidence of a lobed morphology as well. The B1d cells appeared primarily as single cells with some in aggregates, a common characteristic of *Methanolobus* members (Koenig and Stetter 1982; Mochimaru et al. 2009). Strain Q3c cells were 1.1 ± 0.14 μm in diameter ($n = 15$) and were also cocci arranged primarily as single cells, of which many had external protrusions resembling pili (Fig. 2b, d). All cells of each strain auto-fluoresced when viewed under a fluorescence microscope. No motility was observed in either strain when examined by DIC microscopy, and no flagella were observed in either strain using SEM. Both strains lysed easily with 0.01 % (wt/v) SDS or 0.01 % (v/v) Triton

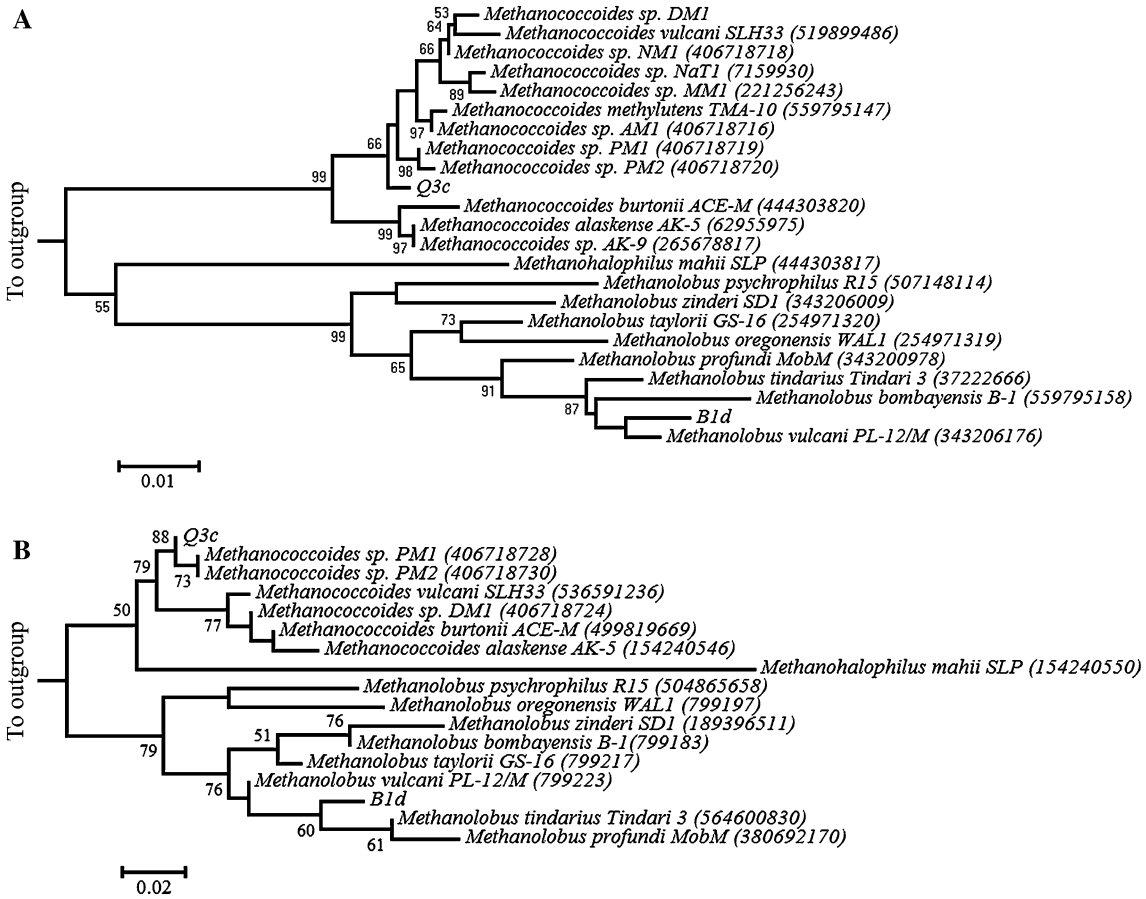

Fig. 1 Maximum likelihood trees showing the phylogenetic position of strains B1d and Q3c in relation to the most closely related organisms, based on the partial 16S rRNA gene sequence (**a**) or partial McrA amino acid sequence (**b**). These trees are based on 1,000 bootstrap repetitions with values equal to or greater than 50 % support being shown. Each tree is drawn to scale with distance being measured in substitutions per site. The *numbers* shown in *parentheses* are accession numbers for the indicated sequences

X-100 indicating proteinaceous cell walls. Both strains stained primarily gram-negative, although there was some variability in the reactions of each strain.

Growth experiments were conducted with each strain to determine its temperature and salt dependence as well as the substrate range. The results of the temperature and salt experiments are reported in Tables 1 and 2 for strains B1d and Q3c, respectively. The temperature optimum for each strain was approximately 37 °C, and the salt optimum was approximately 300 mM Na^+ for each strain. Strain B1d grew well in GB, TMA, and methanol, with slower growth in DMA and MMA. No growth of strain B1d was seen on any other substrate. *Ml. vulcani* PL-12/M^T does not grow in GB. Strain Q3c grew well in QMA and TMA with slower growth in DMA, MMA, and methanol. Interestingly, choline was found to support growth of strain Q3c as well, although choline-dependent growth was considerably slower than growth on other substrates, as seen with close relatives: strains PM1 and PM2 (Watkins et al. 2012). Subsequent transfers still showed a considerable lag time of 10–15 days prior to measurable growth. Neither strain PM1 nor PM2 is reported to use QMA as a methanogenic substrate. Neither B1d nor Q3c grew on acetate, H_2/CO_2, formate, carnitine, or DMEA. Strain Q3c did not grow on GB, unlike strain PM2. GC measurement of methylamines in the substrates and media confirmed that growth on QMA, GB, or choline was not due to the presence of contaminating TMA, DMA, or MMA. The lack of growth of strain Q3c on GB and the lack of growth of strain B1d on QMA or choline also supported the finding that no contaminating substrates were found in the substrates confounding the growth results. We tested the dependence of GB on growth of strain B1d and the dependence of QMA on the growth of strain Q3c and showed that no growth was observed in the absence of substrate and increased substrate concentration resulted in increased final OD_{600} up to the highest examined substrate concentration (80 mM) (Fig. S2). The rate of increase in OD_{600} did not appear to change considerably with increased substrate concentration for either strain. The

Table 1 Comparative analysis of several known *Methanolobus* species to strain B1d

	1	2	3	4	5	6	7	8	9
Characteristics									
Habitat	Estuarine sediments	Saline, deep subsurface coal seam	Saline, deep subsurface sand	Cold Wetlands	Sea sediments	Estuarine sediments	Sea sediments	Saline, alkaline aquifer	Marine black sediments
Cell diameter (μm)	0.5–0.7	0.5–1.0	0.9–1.2	0.9–1.2	1.0–1.25	0.5–1.0	1.0–1.5	1.0–1.5	0.8–1.25
Cell shape	Cocci	Irregular coccoides	Irregular coccoides	Diplococci	Cocci	Cocci	Irregular coccoides	Irregular coccoides	Irregular coccoides
Flagella	−	−	+	+	−	−	−	−	+
Growth conditions									
T (°C)	18–40	25–50	9–37	0–25	13–45	11–40	22–40	20–42	10–45
Na^+ concentration (M)	0.05–0.94	0.05–1.8	0.1–1	0.05–0.8	0.1–1.2	0.2–1.20	0.3–2.0	0.1–1.6	0.06–1.27
DNA G+C content (mol %)	40.2	42	42.4	44.9	39	40.8	39.2	40.9	45.9
16S rRNA gene sequence identity (%) compared to B1d	–	96.1	97.9	96.7	97.3 **(99.1)**	96.5	97.7	95.5	96.4

The bold value indicates 16S rRNA gene sequence identity (%) compared to B1d for the re-sequenced *Ml. vulcani* PL-12/M^T

Taxa: 1, B1d (this study); 2, *Ml. zinderi* $SD1^T$ (Doerfert et al. 2009); 3, *Ml. profundi* $MobM^T$ (Mochimaru et al. 2009); 4, *Ml. psychrophilus* $R15^T$ (Zhang et al. 2008); 5, *Ml. vulcani* PL-12/M^T (Kadam and Boone 1995); 6, *Ml. taylorii* GS-16^T (Oremland and Boon 1994); 7, *Ml. bombayensis* B-1^T (Kadam et al. 1994); 8, *Ml. oregonensis* $WAL1^T$ (Liu et al. 1990); 9, *Ml. tindarius* Tindari 3^T (Koenig and Stetter 1982)

Table 2 Comparative analysis of several known *Methanococcoides* species to strain Q3c

	1	2	3	4	5	6	7	8	9	10	11	12	13
Characteristics													
Habitat	Estuarine sediment	Volcanic sediment	Bay sediment	Volcanic sediment	Volcanic sediment	Bay sediment	Bay sediment	Mangrove sediment	Marine sediment	Marine sediment	Bay sediment	Antarctic lake	Marine sediment
Cell diameter (μm)	0.9–1.3	0.6–1.7	N.R.	N.R.	N.R.	N.R.	N.R.	0.8–2.0	1.5–2.0	1.0–1.5	0.5–1.2	0.8–1.8	1.0
Cell shape	Cocci	Irregular coccoides	N.R.	N.R.	N.R.	N.R.	N.R.	Irregular coccoides	Irregular coccoides	Irregular coccoides	Irregular coccoides	Cocci	Irregular coccoides
Flagella	–	+	N.R.	N.R.	N.R.	N.R.	N.R.	N.R.	–	+	–	+	–
Growth conditions													
T (°C)	15–40	4–35	N.R.	N.R.	N.R.	N.R.	N.R.	23–35	5–25	5–25	25–40	1.7–24	15–35
Na^+ concentration (M)	0.05–0.94	0.25–1.0	0.1–0.41	0.1–0.41	0.1–0.41	0.1–0.41	0.1–0.41	0.1–0.6	0.1–0.7	0.1–0.7	0.1–0.5	0.2–0.5	0.1–1.1
DNA G+C content (mol %)	43.3	43.4	N.R	N.R	N.R	N.R	N.R	N.R.	41.9	39.5	45	39.6	42
16S rRNA gene sequence identity compared to Q3c	–	98.5	99.2	98.9	98.9	99.3	99.2	98.7	98.3	98.3	99.0	97.9	99.2

Taxa: 1, Q3c (this study); 2, *Mc. vulcani* SLH33 (L'Haridon et al. 2014); 3, *Mc. sp.* AM1; 4, *Mc. sp.* DM1; 5, *Mc. sp.* NM1; 6, *Mc. sp.* PM1; 7, *Mc.* sp. PM2 (Watkins et al. 2012); 8, *Mc. sp.* MM1 (Lyimo et al. 2009); 9, *Mc. alaskense* AK-5, 10, *Mc. alaskense* AK-9 (Singh et al. 2005); 11, *Mc. sp.* NaT1 (Tanaka 1994); 12, *Mc. burtonii* (Franzmann et al. 1992); 13, *Mc. methylutens* TMA-10^T (Sowers and Ferry 1983)

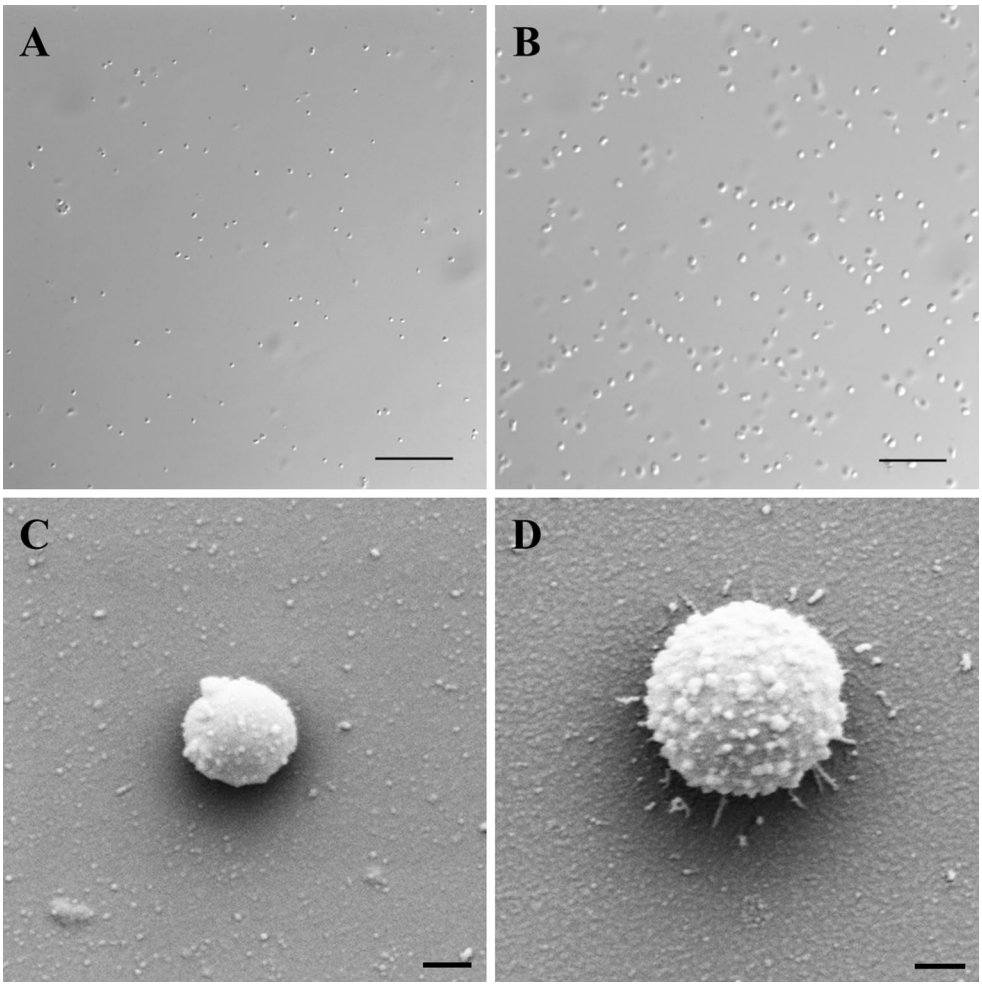

Fig. 2 Microscopic examination of strains B1d and Q3c. **a** DIC micrograph of strain B1d at ×600 magnification. *Scale bar* indicates 10 μm. **b** DIC micrograph of strain Q3c at ×600 magnification. *Scale bar* indicates 10 μm. **c** Scanning electron micrograph of strain B1d at ×75,000 magnification. *Scale bar* indicates 200 nm. **d** Scanning electron micrograph of strain Q3c at ×75,000 magnification. *Scale bar* indicates 200 nm. On average, strain B1d measured 0.60 ± 0.07 μm ($n = 15$) in diameter and strain Q3c measured 1.10 ± 0.14 μm ($n = 15$) in diameter

methanogenic inhibitor bromoethanesulfonic acid (BES) completely inhibited the growth of both strains. Strain Q3c cells entered death phase shortly after reaching stationary phase when grown in 80 mM QMA. This could be due to increased ammonia in the medium or change in pH, as ammonium did accumulate during growth and the pH of the culture dropped from an initial pH of 6.8 to approximately 6. Strain Q3c utilized all four methyl groups of QMA without excretion of simpler methylamines during the growth cycle, as evidenced by a complete lack of TMA, DMA, and MMA in the medium during growth (data not shown) and the stoichiometry of mol methane produced per mol of QMA (Fig. 3). We also measured ammonium concentration in the medium at points during the growth of the cultures and found that Q3c cultures showed a net increase of approximately 5 mM ammonium when comparing T_0- and stationary-phase cultures. Strain B1d showed a net decrease in ammonium concentration from T_0- to stationary-phase of approximately 5 mM. Given that all of the QMA (10 mM) in the strain Q3c culture had been mineralized by the time it reached stationary phase, it would appear that each strain consumed 5 mM ammonium for biosynthesis and growth. Consistent with previously described methylotrophic methanogenic pathways, each strain disproportionated its quaternary amine growth substrate. Strain B1d showed a ratio of 0.71:1 (CH_4:GB) (Fig. 3a) and strain Q3c showed a ratio of 2.8:1 (CH_4:QMA) which indicates a ratio 0.70:1 (CH_4:methyl group), since there are four available methyl groups on QMA (Fig. 3b). These ratios are close to the theoretical 0.75:1 (CH_4:methyl group) ratio for methylotrophic methanogenesis.

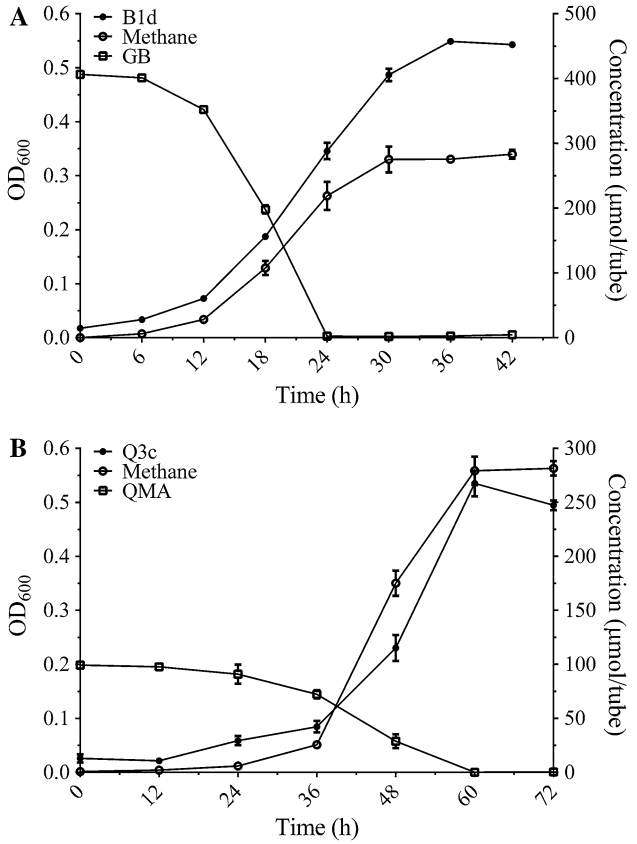

Fig. 3 Growth curves are presented showing changes in OD_{600} as well as quaternary amine and methane concentrations over time for strains B1d (**a**) and Q3c (**b**). Strains B1d and Q3c disproportionate their quaternary amine substrates to produce approximately 0.75 mol methane per mol methyl group utilized. The *error bars* indicate one standard deviation from the mean

Discussion

The isolation of *Ml. vulcani* B1d by direct enrichment on GB has expanded the number of methanogenic genera capable of quaternary amine utilization, and the isolation of strain *Mc. methylutens* Q3c from estuarine sediment implies a more widespread ecology of QMA degradation. Given that methylotrophic substrates, such as methylamines, are commonly referred to as non-competitive substrates in these anaerobic communities, it is interesting to see how quaternary amines may fit into this model. Our laboratory has demonstrated that *D. hafniense*, an organism known to occupy similar environments to methylotrophic methanogens, is also capable of methylotrophic growth when coupled with fumarate, nitrate, or thiosulfate reduction (Ticak et al. 2014). This suggests that methylotrophic substrates are not always non-competitive. Furthermore, in a recent study involving microcosm experiments, investigation of non-competitive substrate usage was performed for methanogens and SRB from microbial mats in hypersaline environments. Interestingly, members of the genus *Methanococcoides*, *Methanohalophilus*, and *Methanolobus* were observed in these communities (Garcia-Maldonado et al. 2012). It is plausible that quaternary amines may serve as either competitive or non-competitive substrates in soil sediments; hence, further studies on the ecology of quaternary amine distribution and their anaerobic degradation are warranted. There are relatively few known natural sources of QMA in the environment, with some sources known in marine environments being from the phyla Mollusca, Cnidaria, and Bryozoa (Barceloux 2008). It is unknown whether any other sources of QMA exist in marine and estuarine environments, but it is a possibility worth examining. It is also interesting to note that the ability of strain Q3c to grow in media lacking added NaCl suggests that this organism may be able to grow in freshwater sediments as well. All previous species of quaternary amine-utilizing methanogens have been isolated from marine or brackish environments, but quaternary amines, especially choline, would exist in freshwater systems as well. Our isolation of strain Q3c allows for a more complete physiological and genetic characterization of QMA-dependent methanogenesis to build upon the work done by the laboratories of Tanaka, Thauer and their coworkers (Tanaka 1994; Asakawa et al. 1998).

GB is an abundant quaternary amine in marine and estuarine environments that often serves as a compatible solute in a variety of organisms to combat the osmotic stress of saline environments (Heijthuijsen and Hansen 1989; Oren 1990; Ziegler et al. 2010). GB is known to contribute to methanogenesis by serving as a substrate for fermentative bacteria that cleave it via a Stickland reaction to produce the methanogenic precursors TMA and acetate (Naumann et al. 1983). In this study, we isolated strain B1d based on its ability to grow on GB directly, and the speed at which the enrichment cultures grew suggests that GB may readily serve as a substrate for strain B1d, and possibly other methanogens, in situ.

We can infer from previous work by Thauer and coworkers (Asakawa et al. 1998) that demethylation of QMA in strain Q3c proceeds via a methylotrophic pathway analogous to other methylamine breakdown pathways, as shown in Fig. 4. MtqA, MtqB, and MtqC, the enzymes for QMA demethylation were isolated from strain NaT1 and characterized previously and the N termini were reported (Asakawa et al. 1998). However, we have been unable to identify genes in the NCBI database that could encode MtqABC. Further research in our laboratory currently focusing on the genome sequence of strain Q3c may pinpoint the genes responsible for this pathway. Alternative pathways for QMA degradation in Q3c still remain a possibility since the genes responsible for QMA degradation in any methanogen have yet to be discovered.

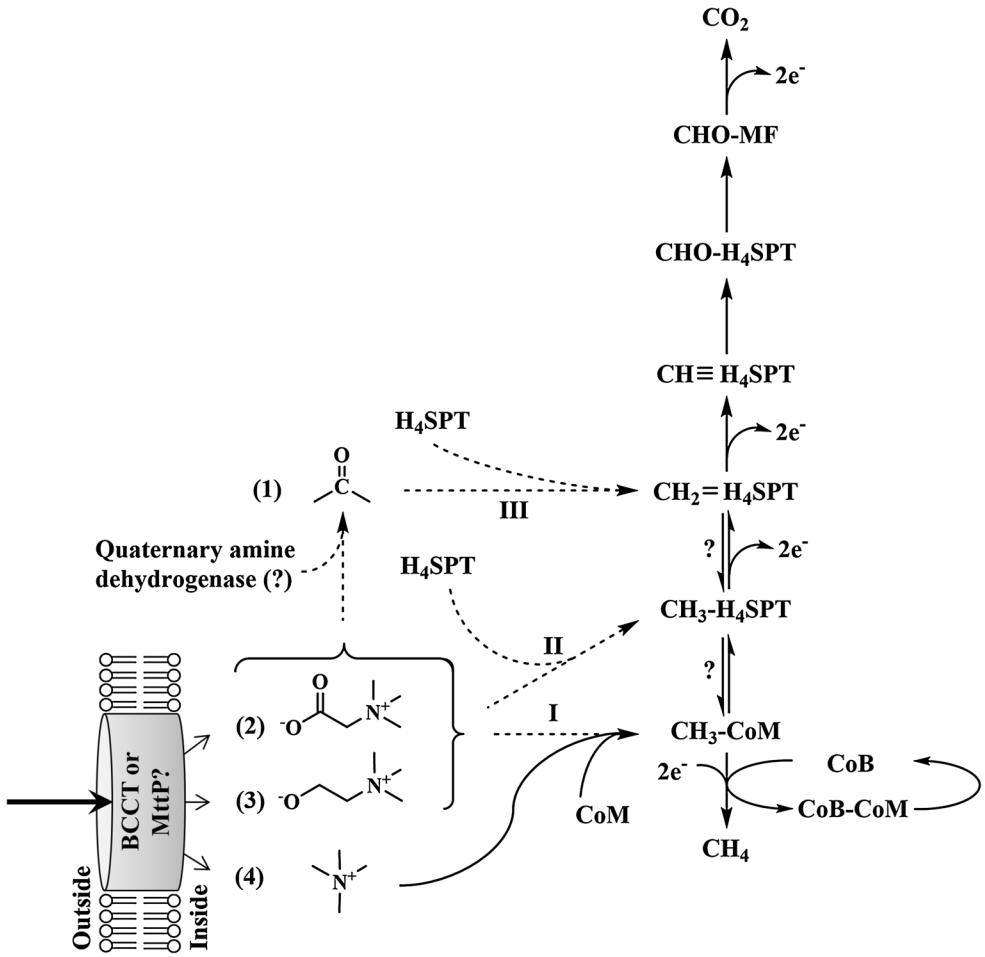

Fig. 4 Proposed quaternary amine metabolic schema for methanogens. (*I*) In this route, quaternary amines are demethylated by a three-component enzyme system including a substrate-specific methyltransferase, a corrinoid-binding protein, and a 2-mercaptoethanesulfonate (CoM) methyltransferase, which is supported by Asakawa et al. (1998). (*II*) Quaternary amines could potentially serve as substrates for a route of direct or indirect methylation of tetrahydrosarcinapterin (H_4SPT). (*III*) The proposed Mtr/Mer bypass proposed by Welander and Metcalf (2008) may also present a route of quaternary amine degradation through the formation of formaldehyde from a quaternary amine dehydrogenase followed subsequently by an enzymatic mediated condensation with H_4SPT to yield methylene tetrahydrosarcinapterin (CH_2=H_4SPT). *Arrows* indicate experimentally verified pathways of quaternary amine-dependent methanogenesis. *Dashed lines* indicate hypothetical pathways of quaternary amine demethylation and electron flow through oxidation and reduction of one-carbon groups are also indicated. Transport of quaternary amines is also proposed via betaine/choline/carnitine transporter (BCCT) or a homologue of the predicted trimethylamine permease (MttP) seen in other sequenced methylamine-utilizing methanogens. CHO–MF, formyl–methanofuran; CHO–H_4SPT, formyl–H_4SPT; CH≡H_4SPT, methenyl–H_4SPT; CH_3–H_4SPT, methyl–H_4SPT; CoB, 7-mercaptoheptanoylthreoninephosphate; CH_3–CoM, 2-methylthioethanesulfonate; CoB–CoM, mixed disulfide of CoB and CoM; CO_2, carbon dioxide; CH_4, methane. *Arabic numerals* 1, formaldehyde; 2, glycine betaine; 3, choline; 4, tetramethylammonium

One could speculate that GB and choline are catabolized through methylotrophic pathways as well. This hypothesis is supported by collaborative work in our laboratory, in which we have shown that a pyrrolysine-lacking homologue of MttB, the TMA methyltransferase from methylotrophic methanogens, functions as a GB methyltransferase in the bacterium *D. hafniense* Y51 (Ticak et al. 2014). This enzyme, MtgB, is a non-pyrrolysine member of a superfamily of enzymes (COG5598) that also includes the pyrrolysine-containing enzymes from both the *Bacteria* and *Archaea*. We propose that constituents of this family catalyze a variety of corrinoid-dependent methyl transfer reactions with quaternary and tertiary *N*-methylated amines as substrates. Another possible route is that demethylation of GB and choline could occur through the action of substrate-specific dehydrogenases that demethylate the substrate and generate formaldehyde and reducing power; the formaldehyde could enzymatically react with H_4SPT to generate methylene–H_4SPT (Fig. 4) (Welander and Metcalf 2005, 2008). However, the methanol bypass pathway was unable to support growth on methanol alone in the absence of the *mtr*/*mer* operon in *M. barkeri*, so this possibility is

not likely to account for the growth on quaternary amines. It is possible, however, that an analogous quaternary amine bypass pathway exists in these organisms. A third route is also envisioned in which the direct methylation of H_4SPT from the substrate is carried out by an unknown enzyme or pathway. Therefore, future work in our laboratory, guided by the genome data from strains B1d and Q3c that we have recently obtained, will be critical in gaining insight into the genes and enzymes responsible for quaternary amine-dependent methanogenesis.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Altschul SF, Gish W et al (1990) Basic local alignment search tool. J Mol Biol 215:403–410. doi:10.1016/S0022-2836(05)80360-2

Asakawa S, Sauer K, Liesack W, Thauer RK (1998) Tetramethylammonium:coenzyme M methyltransferase system from *Methanococcoides* sp. Arch Microbiol 170:220–226. doi:10.1007/s002030050636

Banat IM, Lindstrom EB, Nedwell DB, Balab MT (1981) Evidence for coexistence of two distinct functional groups of sulfate-reducing bacteria in salt marsh sediment. Appl Environ Microbiol 42:985–992

Barceloux DG (2008) Medical toxicology of natural substances: foods, fungi, medicinal herbs, plants, and venomous animals. Wiley. doi:10.1002/9780470330319

Bose A, Pritchett MA et al (2008) Genetic analysis of the methanol- and methylamine-specific methyltransferase 2 genes of *Methanosarcina acetivorans* C2A. J Bacteriol 190:4017–4026. doi:10.1128/JB.00117-08

Burke SA, Krzycki JA (1995) Involvement of the "A" isozyme of methyltransferase II and the 29-kilodalton corrinoid protein in methanogenesis from monomethylamine. J Bacteriol 177:4410–4416

Burke SA, Krzycki JA (1997) Reconstitution of monomethylamine:coenzyme M methyl transfer with a corrinoid protein and two methyltransferases purified from *Methanosarcina barkeri*. J Biol Chem 272(26):16570–16577. doi:10.1074/jbc.272.26.16570

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 72:5069–5072. doi:10.1128/AEM.03006-05

Doerfert SN, Reichlen M, Iyer P, Wang M, Ferry JG (2009) *Methanolobus zinderi* sp. nov., a methylotrophic methanogen isolated from a deep subsurface coal seam. Int J Syst Evol Microbiol 59:1064–1069. doi:10.1099/ijs.0.003772-0

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797. doi:10.1093/nar/gkh340

Ferguson DJ Jr, Krzycki JA (1997) Reconstitution of trimethylamine-dependent coenzyme M methylation with the trimethylamine corrinoid protein and the isozymes of methyltransferase II from *Methanosarcina barkeri*. J Bacteriol 179:846–852

Ferguson DJ Jr, Gorlatova N et al (2000) Reconstitution of dimethylamine:coenzyme M methyl transfer with a discrete corrinoid protein and two methyltransferases purified from *Methanosarcina barkeri*. J Biol Chem 275:29053–29060. doi:10.1074/jbc.M910219199

Fiester SE, Evangelista SF, Arivett BA, Adams DJ, Redfern RE, Woolverton CJ (2014) The role of the liquid crystalline state in the bundling of *Salmonella enterica* serovar Typhimurium flagella. Liq Cryst 41:1277–1285. doi:10.1080/02678292.2014.919667

Franzmann PD, Springer N, Ludwig W, Conway de Macario E, Rohde M (1992) A methanogenic archaeon from Ace Lake, Antarctica: *Methanococcoides burtonii* sp.nov. Syst Appl Microbiol 15:573–581

Garcia-Maldonado JQ, Bebout BM, Celis LB, Lopez-Cortes A (2012) Phylogenetic diversity of methyl-coenzyme M reductase (*mcrA*) gene and methanogenesis from trimethylamine in hypersaline environments. Int Microbiol 15:33–41. doi:10.2436/20.1501.01.155

Gish W, States DJ (1993) Identification of protein coding regions by database similarity search. Nat Genet 3:266–272. doi:10.1038/ng0392-266

Harms U, Weiss DS et al (1995) The energy conserving *N*5-methyl tetrahydromethanopterin:coenzyme M methyltransferase complex from *Methanobacterium thermoautotrophicum* is composed of eight different subunits. Eur J Biochem 228:640–648. doi:10.1111/j.1432-1033.1995.0640m.x

Heijthuijsen JHFG, Hansen TA (1989) Betaine fermentation and oxidation by marine *Desulfuromonas* strains. Appl Environ Microbiol 55:965–969

Kadam PC, Boone DR (1995) Physiological characterization and emended description of *Methanolobus vulcani*. Int J Syst Bacteriol 45:400–402

Kadam PC, Ranade DR, Mandelco L, Boone DR (1994) Isolation and characterization of *Methanolobus bombayensis* sp. nov., a methylotrophic methanogen that requires high concentrations of divalent cations. Int J Syst Bacteriol 44:603–607

Kandeler E, Gerber H (1988) Short term assay of soil urease activity using colorimetric determination of ammonium. Biol Fertil Soils 6:68–72. doi:10.1007/BF00257924

King GM (1984) Utilization of hydrogen, acetate, and "non-competitive" substrates by methanogenic bacteria in marine sediments. Geomicrobiol J 3:276–301

Koenig H, Stetter KO (1982) Isolation and characterization of *Methanolobus tindarius*, sp. nov., a coccoid methanogen growing only on methanol and methylamines. Zentralbl Bakteriol Mikrobiol Hyg A 3:478–490

Kremer JD, Cao X et al (1993) Isolation of two novel corrinoid proteins from acetate-grown *Methanosarcina barkeri*. J Bacteriol 175:4824–4833

Kristjansson JK, Schönheit P (1983) Why do sulfate-reducing bacteria outcompete methanogenic bacteria for substrates? Oecologia 60:264–266. doi:10.1007/BF00379530

Kumar S, Dagar SS, Puniya AK (2012) Isolation and characterization of methanogens from rumen of Murrah buffalo. Ann Microbiol 62:345–350. doi:10.1007/s13213-011-0268-8

Le SQ, Gascuel O (2008) An improved general amino acid replacement matrix. Mol Biol Evol 25:1307–1320. doi:10.1093/molbev/msn067

Liu Y, Boone DR, Choy C (1990) *Methanohalophilus oregonense* sp. nov., a methylotrophic methanogen from an alkaline, saline aquifer. Int J Syst Bacteriol 40:111–116

L'Haridon SM, Chalopin MM et al (2014) *Methanococcoides vulcani* sp. nov., a novel marine methylotrophic methanogen; using betaine, choline, and *N*,*N*-dimethylethanolamine for methanogenesis, isolated from the Napoli Mud Volcano in the Eastern Mediterranean Sea; and emendation of the genus *Methanococcoides*. Int J Syst Evol Microbiol 64:1978–1983. doi:10.1099/ijs.0.058289-0

Lovley DR, Dwyer DF, Klug MJ (1982) Kinetic analysis of competition between sulfate reducers and methanogens for hydrogen in sediments. Appl Environ Microbiol 43:1373–1379

Lyimo TJ, Pol A, Jetten MS, Op den Camp HJM (2009) Diversity of methanogenic archaea in a mangrove sediment and isolation of a new *Methanococcoides* strain. FEMS Microbiol Lett 291:247–253. doi:10.1111/j.1574-6968.2008.01464.x

Metcalf WW, Zhang JK et al (1997) A genetic system for Archaea of the genus *Methanosarcina*: liposome mediated transformation and construction of shuttle vectors. Proc Natl Acad Sci USA 94:2626–2631. doi:10.1073/pnas.94.6.2626

Mochimaru H, Tanaki H, Hanada S, Imachi H, Nakamura K, Sakata S, Kamagata Y (2009) *Methanolobus profundi* sp. nov., a methylotrophic methanogen isolated from deep subsurface sediments in a natural gas field. Int J Syst Evol Microbiol 59:714–728. doi:10.1099/ijs.0.001677-0

Naumann E, Hippe H et al (1983) Betaine: new oxidant in the Stickland reaction and methanogenesis from betaine and L-alanine by a *Clostridium sporogenes-Methanosarcina barkeri* coculture. Appl Environ Microbiol 45:474–483

Nei MKS (2000) Molecular evolution and phylogenetics. Oxford University Press, Oxford

Oremland RS, Boone DR (1994) *Methanolobus taylorii* sp. nov., a new methylotrophic, estuarine methanogen. Int J Syst Bacteriol 44:573–575

Oremland RS, Marsh LM, Polcin S (1982) Methane production and simultaneous sulfate-reduction in anoxic, salt marsh sediments. Nature 296:143–145. doi:10.1038/296143a0

Oren A (1990) Formation and breakdown of glycine betaine and trimethylamine in hypersaline environments. Antonie Van Leeuwenhoek 58:291–298. doi:10.1007/BF00399342

Purdy KJ, Nedwell DB, Embley TM (2003) Analysis of the sulfate-reducing bacterial and methanogenic archaeal populations in contrasting Antarctic sediments. Appl Environ Microbiol 69:3181–3191. doi:10.1128/AEM.69.6.3181-3191.2003

Rothman DH, Fourier GP, French KL, Alm EJ, Boyle EA, Changqun C, Summons RE (2014) Methanogenic burst in the end-Permian carbon cycle. Proc Natl Acad Sci USA 111:5462–5487. doi:10.101073/pnas.1318106111

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor

Sauer K, Harms U et al (1997) Methanol:coenzyme M methyltransferase from *Methanosarcina barkeri*. Purification, properties and encoding genes of the corrinoid protein MT1. Eur J Biochem 243:670–677. doi:10.1111/j.142-1033.1997.t01-1-00670.x

Singh N, Kendall MM, Liu YT, Boone DR (2005) Isolation and characterization of methylotrophic methanogens from anoxic marine sediments in Skan Bay, Alaska: description of *Methanococcoides alaskense* sp. nov., and emended description of *Methanosarcina baltica*. Int J Syst Evol Microbiol 55:2531–2538. doi:10.1099/ijs.0.63886-0

Sowers KR, Ferry JG (1983) Isolation and characterization of a methylotrophic marine methanogen, *Methanococcoides methylutens* gen. nov., sp. nov. Appl Environ Microbiol 45:684–690

Tallant TC, Krzycki JA (1997) Methylthiol:coenzyme M methyltransferase from *Methanosarcina barkeri*, an enzyme of methanogenesis from dimethylsulfide and methylmercaptopropionate. J Bacteriol 179:6902–6911

Tamura K, Stecher G et al (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729. doi:10.1093/molbev/mst197

Tanaka K (1994) Anaerobic degradation of tetramethylammonium by a newly isolated marine methanogen. J Ferment Bioeng 78:386–388. doi:10.1016/0922-338X(94)90287-9

Tang WH, Wang Z et al (2013) Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med 368:1575–1584. doi:10.1056/NEJMoa1109400

Thauer RK, Kaster AK et al (2008) Methanogenic archaea: ecologically relevant differences in energy conservation. Nat Rev Microbiol 6:579–591. doi:10.1038/nrmicro1931

Ticak T, Kountz DJ, Girosky KE, Krzycki JA, Ferguson DJ Jr. (2014) A nonpyrrolysine member of the widely distributed trimethylamine methyltransferase family is a glycine betaine methyltransferase. Proc Nat Acad Sci USA. doi:10.1073/pnas.1409642111

Wall JS, Christianson DD, Dimler RJ, Senti FR (1960) Spectrophotometric determination of betaines and other quaternary nitrogen compounds as their periodides. Anal Chem 32:870–874

Wang Z, Klipfell E et al (2011) Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 472:57–63. doi:10.1038/nature09922

Wang Z, Tang WH et al (2014) Prognostic value of choline and betaine depends on intestinal microbiota-generated metabolite trimethylamine-*N*-oxide. Eur Heart J 35:904–910. doi:10.1093/eurheartj/ehu002

Watkins AJ, Roussel EG et al (2012) Choline and *N*,*N*-dimethylethanolamine as direct substrates for methanogens. Appl Environ Microbiol 78:8298–8303. doi:10.1128/AEM.01941-12

Watkins AJ, Roussel EG et al (2014) Glycine betaine as a direct substrate for methanogens (*Methanococcoides* spp.). Appl Environ Microbiol 80:289–293. doi:10.1128/AEM.03076-13

Webster GPR, Cragg BA, Newberry CJ, Weightman AJ, Fry JC (2006) Prokaryotic community composition and biogeochemical processes in deep subseafloor sediments from the Peru Margin. FEMS Microbiol Ecol 58:65–85. doi:10.1111/j.1574-6941.2006.00147.x

Welander PV, Metcalf WW (2005) Loss of the *mtr* operon in *Methanosarcina* blocks growth on methanol, but not methanogenesis, and reveals an unknown methanogenic pathway. Proc Natl Acad Sci USA 102:10664–10669. doi:10.1073/pnas.0502623102

Welander PV, Metcalf WW (2008) Mutagenesis of the C1 oxidation pathway in *Methanosarcina barkeri*: new insights into the Mtr/Mer bypass pathway. J Bacteriol 190:1928–1936. doi:10.1128/JB.01424-07

Whitman W, Bowen TL, Boone DR (2006) The methanogenic bacteria. Prokaryotes 3:165–207. doi:10.1007/0-387-30743-5_9

Winfrey MR, Nelson DR et al (1977) Association of hydrogen metabolism with methanogenesis in Lake Mendota sediments. Appl Environ Microbiol 33:312–318

Ziegler C, Bremer E et al (2010) The BCCT family of carriers: from physiology to crystal structure. Mol Microbiol 78:13–34. doi:10.1111/j.1365-2958.2010.07332.x

Zhang G, Jiang N, Liu X, Dong X (2008) Methanogenesis from methanol at low temperatures by a novel psychrophilic methanogen, "*Methanolobus psychrophilus*" sp. nov., prevalent in Zoige wetland of the Tibetan plateau. Appl Environ Microbiol 74:6114–6120. doi:10.1128/AEM.01146-08