Aerobic bacterial methane synthesis

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Edited by David M. Karl, University of Hawaii at Manoa, Honolulu, HI, and approved May 19, 2021 (received for review September 14, 2020)

Reports of biogenic methane (CH4) synthesis associated with a range of organisms have steadily accumulated in the literature. This has not happened without controversy and in most cases the process is poorly understood at the gene and enzyme levels. In marine and freshwater environments, CH4 supersaturation of oxic surface waters has been termed the “methane paradox” because biological CH4 synthesis is viewed to be a strictly anaerobic process carried out by O2-sensitive methanogens. Interest in this phenomenon has surged within the past decade because of the importance of understanding sources and sinks of this potent greenhouse gas. In our work on Yellowstone Lake in Yellowstone National Park, we demonstrate microbial conversion of methylamine to CH4 and isolate and characterize an Acidovorax sp. capable of this activity. Furthermore, we identify and clone a gene critical to this process (encodes pyridoxal phosphate-dependent aspartate aminotransferase) and demonstrate that this property can be transferred to Escherichia coli with this gene and will occur as a purified enzyme. This previously unrecognized process sheds light on environmental cycling of CH4, contributing metabolite to biogenic CH4. Methylotrophic methanogens anaerobically convert MeA to CH4 (15, 16). Methylotrophic bacteria can metabolize MeA as a carbon source (17, 18), nonmethylyotrophs for nitrogen (17, 19) (20), a carbon and N source (21), or methyl- vores can use it as an energy source (22). MeA utilization is viewed to occur as an oxidation via either MeA dehydrogenase, MeA oxidase, or involving methyl group transfer to tetrahydrofolate involving the formation of formation of γ-glutamy-methylamidase (GMA) and N-methyl glutamate (NMG) (20, 23). In the current study, we report the discovery of an alternate, simpler route of MeA metabolism that yields CH4, involving a reaction catalyzed by a pyridoxal-phosphate–dependent aspartate aminotransferase.

Results

Water Column Characterization. Yellowstone Lake water column samples in 2016 shared similar EXO Sonde features (SI Appendix, Table S1 and Fig. S1). The PMEZs were located just below the approximate upper limit of the thermocline, although the July PMEZ was 0.5 m deeper (11.5 versus 11. m), and CH4 concentrations were approximately twofold greater (51 versus 26 nM) (Fig. L 4). Incubating July 29 PMEZ water samples (12 °C, initiated as aerobic) with 13C-labeled MPn and 13C-labeled substrates capable of supporting known methanogenesis pathways (acetate, formate, bicarbonate, H2 + bicarbonate) found no strong evidence of 13CH4 formation (SI Appendix, Fig. S24). Samples spiked with 13C-MeA produced 13CH4 and to a much lesser extent 13C-methionine as well (Fig. 1B and SI Appendix, Fig. S2). Lack of MPn metabolism was not anticipated and thus to relocate the MPn active region, follow-up sampling (August 5) of the PMEZ as well as 1 m above and below were spiked with 

Significance

Observations summarized herein contribute to an ongoing paradigm shift in microbial ecology, documenting an emergent property of ecosystem function that further challenges the perception that biogenic methane (CH4) production is strictly an anaerobic process. Relevant metabolites, a model bacterial isolate, gene, and enzyme are identified, and we show how this property can conceivably be broadly distributed in the biosphere and contribute to global CH4 emissions. Scientifically, this study will enable lines of investigation that will expand our understanding of CH4 synthesis and emission in nature and illustrates how CH4 synthesis may actually serve as a nexus for the C and N cycles in nature.


The authors declare no competing interest.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2019229118/-/DCSupplemental.

Published June 28, 2021.
However, MeA was present (detection limits at depths that correspond to the deeper PMEZ zone (0 to 9 m). PCRs using degenerate universal 16S rRNA gene sequence libraries was used to determine if specific taxa exhibited increased abundance at the 12-m depth PMEZ and hence might indicate which organism(s) could be involved in 13MeA → 13CH4 activity. Taxa classified as belonging to the order Burkholderiales (annotated as Acid A-1 and Acid A-2 in the Freshwater Database) exhibited a sharp peak (13% abundance, Fig. 2A) coinciding specifically with the MeA metabolic potential at 12 m (Fig. 2D). However, in agreement with the apparent absence of amplifiable mcrA (SI Appendix, Table S2), recognizable methanogen signatures were absent in all libraries throughout the water column.

To more closely examine the biology underpinning the MeA → CH4 activity, microorganisms from PMEZ samples were isolated on SAR-MeA agar containing MeA as the sole nitrogen source (Methods) and then screened in liquid SAR-MeA for CH4 generation. We isolated several bacteria capable of converting MeA to CH4. Genus-level classification based on 16S rRNA gene sequence includes the following: Acidovorax sp. (MK896843.1), Pseudomonas sp. (MK896839.1), Caulobacter sp. (MK896844.1), Mesorhizobium sp. (MK896845.1), and Dietzia sp. (MK896847.1). For further characterization, we selected one of the Acidovorax sp. isolates obtained from the 12-m depth because this genus is a member of the Burkholderiales order that was significantly abundant at the depth corresponding to the MeA metabolic potential (Fig. 2 A and D) and because it was found to be genetically tractable. Batch cultures in liquid SAR-MeA in sealed serum bottles (maintained aerobic with sterile air injections at each gas sampling) demonstrated robust growth (SI Appendix, Fig. S6A) and CH4 generation from MeA (Fig. 3A). Gas chromatography–mass spectrometry (GC–MS) analysis of headspace samples of the Acidovorax sp. cultured with 13C-MeA confirmed 13CH4 generation (SI Appendix, Fig. S7), demonstrating transfer of the MeA carbon to CH4. GB was also investigated in the context of the above hypothesized catabolic pathway and also found to support growth (SI Appendix, Fig. S6D) and CH4 generation (Fig. 3A).

**Gene Identification and Mutational Analysis.** Transposon Tn5B22 mutagenesis of the Acidovorax sp. isolate yielded two mutants (among ~8,000 transconjugates) capable of growth with ammonium.
chloride but not MeA nor GB as sole N source (SI Appendix, Fig. S6B) and were likewise defective for generating CH$_4$ (Fig. 3B). Cultures initiated at cell densities equivalent to mid log phase under these growth conditions (starting optical density = 0.15; SI Appendix, Fig. S6, compare D and E) also failed to generate CH$_4$, demonstrating lack of CH$_4$ synthesis in the mutants is due to defective...
MeA and/or GB metabolism, not lack of biomass. The two Tn5B22 insertion sites differed, but the same gene was affected in both mutants (SI Appendix, Fig. S8), providing strong evidence that the mutated gene is essential to MeA/GB metabolism and CH₄ synthesis in this organism. Basic local alignment search tool (BLAST)x analysis matched the gene to the family of pyridoxal 5′-phosphate-dependent aspartate aminotransferases (referred to herein as plp-aat). The specific plp-aat coding sequence without adjacent genomic DNA (GenBank accession no. MK170382) was PCR cloned from the Acidovorax isolate DNA into the broad host range plasmid pCPP30. Conjugation of the recombinant plasmid to the mutants reversed their negative growth and CH₄ synthesis phenotypes (Fig. 3C and SI Appendix, Fig. S6C), confirming the importance of this gene.

Introducing plp-aat to Escherichia coli strain BL21(DE3) allowed this enteric bacterium to grow in M9 broth containing MeA as the N source (Fig. 4A) and to synthesize CH₄ (Fig. 4B). However, growth in M9-MeA media required BL21 (pET28a::aat) to first be grown in lysogeny broth (LB) broth with antibiotic selection; that is, washed cells failed to grow in M9-MeA. Consequently, subsamples of stationary phase cells were transferred directly (~200-fold dilution) to the M9-MeA media (with isopropyl β-d-thiogalactopyranoside [IPTG] to induce aat transcription). The identity of the putative metabolite(s) apparently necessary for the recombinant E. coli to grow in M9-MeA is unknown at present, but it was not sufficient to allow the BL21 (pET28a+) negative control to grow in M9-MeA. Endpoint oxygen levels for the E. coli experiments were ~43% of saturation at 37 °C (SI Appendix, Fig. S9), illustrating the cultures had not gone anaerobic. For both Acidovorax (Fig. 3 and SI Appendix, Fig. S6A) and E. coli (Fig. 4), CH₄ synthesis was linked to growth on MeA, implying N acquisition and CH₄ release are somehow metabolically coupled.

PLP-Aat enzyme has been used as a model enzyme for understanding PLP-linked catalysis, and from these efforts, an essential catalytic lysine residue has been identified to be invariably conserved (33, 34). Prior work with the E. coli PLP-Aat enzyme has shown that mutation of this conserved lysine to alanine results in an enzyme with only 0.5% native aspartate aminotransferase activity (34). In an analogous approach, we changed the catalytic lysine (K237) to alanine in the cloned plp-aat and repeated the biocassays. Growth and CH₄ synthesis by BL21 carrying the K237A mutant clone was severely constrained in MeA media (Fig. 4).

**Discussion**

From experiments including analyses of microbial community composition and metabolic potential, targeted metabolomics, pure culture physiologic and genetic characterizations, and defined genetic transfer of this property, we put forth data that contributes to an ongoing paradigm shift for environmental CH₄ synthesis that is distinct from classical methanogenesis or other presently known mechanisms of CH₄ synthesis. We found MeA occurs at substantial concentrations in the pristine Yellowstone Lake environment in conjunction with GB and TMA (Fig. 2B and SI Appendix, Fig. S5), which are catabolic precursors of MeA. The extant microbial community exhibited the capacity to convert MeA to CH₄ (Fig. 2D), and depending on as of yet undefined environmental conditions, MeA is an important contributor to PMEZ formation (Fig. 1, Fig. 2D) or indeed the only contributor (Fig. 1A and B). The ability to convert MeA to CH₄ is heritable by a single gene (Figs. 3C and 4) and importantly, MeA→CH₄ conversion will occur under aerobic conditions (Figs. 3 and 4 and SI Appendix, Fig. S9). In sum, this study illustrates all the essential components of a relatively simple and ecologically relevant CH₄ synthesis pathway, that is, aerobic bacterial CH₄ synthesis.

Some have argued that lateral transport of CH₄ derived from near shore–based methanogenesis to be the basis for metalimnetic CH₄ (35–37). However, evidence supporting in situ CH₄ synthesis in oxic lake waters is now clear and overwhelming (e.g., refs. 1, 12

![Fig. 4.](https://doi.org/10.1073/pnas.2019229118) PLP-Aat enables E. coli to grow with MeA as a nitrogen source and synthesize CH₄. (A) Growth profiles of E. coli BL21 with MeA or NH₄⁺ and carrying the empty plasmid (control), cloned aat (pET28a::aat), or mutant aat wherein the catalytic lysine (K237) was replaced by alanine. (B) CH₄ generation by BL21 with the same constructs. All data and error bars (where visible) are the mean ± SD of three replicate cultures.
MPn to CH₄ was present in both PMEZs and consistently at the water (10). Indeed, the microbiome metabolic capacity to convert alkyphosphonates covalently bound to organic matter in marine assay methodology would not detect methylphosphonate (or any substrates (ref. 12 and Fig. 2) reduction versus lateral transport concluded that oxic biogeneic CH₄ photosynthetic primary producers in aqueous environments (42, 43). MeA metabolism has likewise been examined in some detail (23, 25), although this prior characterization work did not include synthesis of CH₄ as an end product.

Ecological-based evidence in support of an aerobic GB → CH₄ pathway includes research which infers CH₄ production associated with methylamines derived from zooplankton grazing of phytoplankton (6) and recent studies showing TMA enhances CH₄ synthesis in N-depleted incubations of Lake Stechlin oxic PMEZ water samples (65). Furthermore, the current study provides direct genetic evidence as illustrated by the inability of Acidovorax aurt.:TnB22 mutants to synthesize CH₄ from GB nor use GB as an N source (Fig. 3B and SI Appendix, Fig. S6 B and E). By contrast, the transcript counts were reverted back to wild-type levels when cultured with the plp-aat gene (Fig. 3C). This indicates that, at least for this organism, acquiring N from GB must include the biochemical step catalyzed by the enzyme encoded by plp-aat described herein.

Several studies speak to the general importance of GB and MeA beyond simply their ubiquity and abundance in marine environments. Potential fates include assimilation as a carbon source, oxidized for energy, or used as a nitrogen source (19, 22, 25, 66–68). These examples represent metabolisms that would compete with a MeA → CH₄ pathway, but it is still reasonable to consider its potential for CH₄ synthesis. At the moment, information on marine MeA dynamics is limited, but MeA assimilation (0.005 to 54 nmol L⁻¹ day⁻¹), oxidation (0.09 to 3.43 nmol L⁻¹ day⁻¹) and turnover rates (40 to 57 nmol L⁻¹ day⁻¹) have been reported (60, 67), facilitating at least a preliminary estimate. Conservatively assuming a PLP-Aat catalyzed MeA turnover rate of 1 nmol L⁻¹ day⁻¹, this would account for 11.6% of annual marine CH₄ emission (2.1 Mt CH₄ yr⁻¹ (69), Methods).

This mode of CH₄ synthesis could be highly relevant to any situation where GB and or MeA occur, including environments where CH₄ synthesis has traditionally been interpreted to derive from methanogens. There is no obvious reason why this PLP-Aat reaction would be constrained by reedox conditions per se because aminotransferase reactions are important for maintaining cellular amino acid equilibria in all self-replicating organisms. Indeed, numerous aerobic or anaerobic organisms may be involved with varying efficiency, and at present, we are unable to offer any specific criteria for excluding hundreds of more-remote aat homologs in metagenome libraries that may encode the same function, perhaps with greater efficiency. However, we draw attention to the fact that the PLP-Aat encoded in the genome of E. coli strain BL21(DE3) (GenBank genome accession no. AM946981, used herein for bioassays) shares only 21.6% identity with the Acidovorax enzyme and did not allow E. coli to grow with MeA nor make CH₄ from MeA. Thus, apparently not all proteins annotated as PLP-dependent Aat enzymes are equal in regard to catalyzing the MeA → CH₄ reaction. Characterizing homologs capable of this reaction will enhance our understanding of the universality of this process within and across environments. A cursory search identified thousands of homologs with a high degree of homology with the PLP-Aat, suggesting the possibility that MeA → CH₄ activity occurs in other aqueous and terrestrial environments as well. Examples of highly homologous Aat proteins occurring in a

We predict compounds such as GB as well as choline (55–57) play an important role in generating methylated amines and thus are likely to contribute to this aerobic CH₄ synthesis pathway, particularly in marine environments. GB is a ubiquitous osmylate in bacteria, archaea, and eukaryotes (23, 58) and a precursor to the equally ubiquitous MeA, which is also recognized as an important N source (19, 23, 59) occurring at concentration ranges from nM to μM (60–64). Methylated amines are indicator metabolites of GB degradation (28), are common in algae (6, 32) or phototrophic mats (28, 29), and consistent with the documented biochemistry and genetics underlying GB conversion to TMA and TMA conversion to MeA in various organisms (17) (30, 31) (32). MeA metabolism has likewise been examined in some detail (23, 25), although this prior characterization work did not include synthesis of CH₄ as an end product.

*Fig. 5.* CH₄ synthesis by purified Aat. CH₄ synthesis by purified Aat in E. coli cytoplasmic extract. Complete reactions contained purified enzyme (typically ∼3 μg), MeA (4 mM), α-ketoglutarate (4 mM), and 5′-pyridoxal phosphate (1 mM). Other reactions shown were conducted wherein a single substrate was omitted in order to illustrate the requirement of each for the generation of methane. All data are the mean ± SD of three replicate reactions.

<table>
<thead>
<tr>
<th>Produced methane (nmol / mg-protein)</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete reaction mix</td>
<td>0</td>
</tr>
<tr>
<td>No PLP</td>
<td>0</td>
</tr>
<tr>
<td>No α-ketoglutarate</td>
<td>0</td>
</tr>
<tr>
<td>No 5′-pyridoxal phosphate</td>
<td>0</td>
</tr>
<tr>
<td>Complete, heat treated Aat</td>
<td>0</td>
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The presence of GB in aquatic environments makes it an attractive candidate for generating CH₄. Indeed, the metabolic capacity to convert alkyphosphonates covalently bound to organic matter in marine environments (42, 43), the Yellowstone Lake PMEZ is consistently (across four sampling seasons) not associated with a Chl a peak (e.g., Fig. 24 and SI Appendix, Fig. S1). Subsurface chlorophyll maxima result from phytoplankton sinking/migration behaviors associated with nutrient depletion or from photodapptation at deeper locations (44). Metabolite exchange between sinking phytoplankton and bacteria (45–47) may be an active (potentially mutualistic) process or derived from algal cell lysis caused by bacterial algaeicides or virus lytic events (48–50). Increased water density at colder temperature transitions in the water column could result in transient accumulation of substrates (i.e., live functioning or lysed algae, particulate organic matter) (51) at specific depths. Enhanced residence time would facilitate CH₄ synthesis and accumulation, resulting in PHEME formation. CH₄ concentration at these depths does not necessarily correlate with synthesis rate and may be more easily explained as synthesis exceeding consumption. The absence of dissolved MPn does not necessarily mean that alkylphosphonates are absent (detection limit 1 μM, SI Appendix, Fig. S4). Our assay methodology would not detect methylphosphonate or (any alkylphosphonates) covalently bound to organic matter in marine water (10). Indeed, the microbiome metabolic capacity to convert MPn to CH₄ was present in both PMEZs and consistently at the more shallow PMEZ. Presumably, to some extent the occurrence and separation of MPn and MeA metabolic potentials in the water column is tied to bioavailability of relevant substrates. Methionine conversion to CH₄ was limited relative to MPn and MeA but nevertheless detectable. Prior studies have documented methionine conversion to CH₄ although under anaerobic conditions (52–54).

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https://doi.org/10.1073/pnas.2019229118
variety of aquatic environments are provided in SI Appendix, Fig. S11.

The PLP-Aat catalytic mechanism resulting in CH₄ release is unknown at present. In particular, the source of redundant represents a mechanistic enigma that further contributes to the methane paradox (70). Recreating the reaction milieu in E. coli cytoplasmic extract amended with MeA, α-ketoglutarate, PLP, and live purified PLP-Aat enzyme resulted in methane generation (Fig. S5). Methane was not generated when enzyme was heat inactivated before addition. Prefiltering the extract using a 3 kDa cutoff eliminates a direct contribution of other enzyme(s) but would not remove other metabolite(s), which may contribute or may be associated with the requirement for E. coli to be first grown in a complex medium. The functional importance of the catalytic lyse that is conserved across all PLP-Aat enzymes appears to be involved for the MeA → CH₄ reaction and thus implies some basic similarities; that is, the Schiff base formation between the Aat catalytic lyse and the aldehyde carbon of the coenzyme pyridoxal 5'-phosphate (33, 34). However, beyond this initial step it is also not possible at this juncture to predict reaction mechanism, or efficiency, or any other metabolite/gene product apparently involved in the functioning cell, though it is relevant to point out that CH₄ synthesis in both Acidovorax (Fig. 3) and E. coli (Fig. 4) appears connected to growth, implying a link between CH₄ release and N acquisition to support growth. And furthermore, for at least organisms such as the Acidovorax sp. isolate detailed herein, we view this organism as a robust, though almost certainly not unique, participant in PMEZ sp. isolate. Based on the characterization of the metabolic potentials reveals a route of CH₄ synthesis occurring under fully oxygenated conditions. This also elucidates a nexus for C fixation, which is also consistent with the absence of genes annotated as aat for at least organisms such as the Acidovorax sp. isolate. Thus, the isotopic signature (δ¹³C) of the CH₄ of the headspace was measured using a modification of the protocols described by Wang et al. (12) on a Picarro G2201-i cavity ring-down spectrometer (CRDS) equipped with a Small Sample Introduction Module 2 (SSIM2). Each vial was first pressurized with 20 mL of <0.2 µm filtered ambient air (δ¹³C = ~−48‰ versus Vienna Pee Dee Belemnite) and then 20 mL was removed via gastight syringe and injected into the SSIM2. Factory calibration was used for the CRDS, and a tank of compressed ambient air was used as a reference material between injections to verify lack of instrument drift. For samples taken in 2017, the isotopic signature (δ¹³C) of the CH₄ of the headspace was determined using GC-MS.

GC-MS Analysis. For CH₄ isotope analysis, 800 µL headspace gas was injected directly into an Agilent 7890 GC–MS equipped with Carboxen-1010 porous-layer open-tubular (PLOT) capillary GC column in splitless mode. The injection temperature was 200 °C, the ion source was set to 230 °C, helium was the carrier gas, and the flow rate was 1 mL/min. The oven temperature was held at 35 °C for 7.5 min and then heated at 25 °C/min to 250 °C and held at that temperature for 6.5 min. The mass spectrometer was operated in scanselected ion monitoring (SIM) mode; scan range (10 to 100 m/z) for SIM m/z 14, 15, 16, 17 were used. The ¹³CH₄ standard was purchased from Sigma-Aldrich, and for ¹²CH₄, a standard high purity methane tank was purchased from American Welding and Gas.

Detection of CH₄ culture headspace oxygen, 250 µL headspace gas was injected directly into an Agilent 7890 GC–MS equipped with a Carboxen-1010 PLOT capillary GC column in splitless mode. The injector temperature was 200 °C, with the ion source at 230 °C and a helium flow rate of 1 mL/min. An isotopic GC program at 65 °C with the mass spectrometer in SIM mode for m/z 32 was used.

Detection of GB, TMA, and MeA. Methylamine hydrochloride, trimethylamine hydrochloride, and glycine betaine hydrochloride standards were purchased from Sigma-Aldrich. For detection of MeA in lake waters, dansyl chloride (Dns-Cl, N,N-dimethylaminonaphthalene-5-sulfonfyl chloride) was used to label the compounds of interest prior to analysis by LC–MS. The dansylation procedure was performed as described by Guo et al. (72). Briefly, a Hamilton gastight syringe was used to draw 50 µL water from the sample serum bottle, which was then added to a 250 µL screw-top vial. The sample analysis via and pH adjusted to ~9.5 with 2 µL 160 mM sodium hydroxide. Dns-Cl prepared in acetonitrile (20 mg/mL) was added to the sample in a volume of 46 µL. Samples were then incubated for 30 min at room temperature. After the incubation period, pH was adjusted to ~4 with 2 µL 10% formic acid. At this point, the sample was ready for analysis. Chromatography experiments were done on an Agilent 6538 quadrupole time-of-flight (Q-TOF) mass spectrometer, positive mode, equipped with a reversed-phase Agilent Zorbax Eclipse Plus C18 column (2.1 x 150 mm). Solvent A was 0.1% formic acid in high-performance liquid chromatography (HPLC) water, and solvent B was 0.1% formic acid in acetonitrile. The 15 min binary gradient elution profile was as follows: t₀ 1 min, 0% B; t₀ 11 min, 55% B; t₀ 14 min, 100% B; and t₀ 15 min, 0% B. The wavelength was 320 nm, the flow rate was 600 µL/min, and the sample injection volume was 10 µL. Limit of detection was determined to be 20 nM.

For detection of trimethylamine, ethyl bromoacetate was used to label the compound of interest prior to analysis by LC–MS. The labeling procedure was performed as described by Johnson (73). A Hamilton gastight syringe was used to draw 90 µL water from the sample serum bottle and transferred to a 250 µL polypropylene analysis vial and then 10 µL (20 mg/mL acetonitrile) ethyl bromoacetate was added. Samples were then incubated for 30 min at room temperature. At this point, the sample was ready for analysis. Chromatography was done on an Agilent 6538 Q-TOF mass spectrometer, positive mode, equipped with a normal-phase Waters ACQUITY BEH HILIC 1.7 µm column (2.1 x 100 mm). Solvent A was 0.1% formic acid in HPLC water, and solvent B was 0.1% formic acid in acetonitrile. The 4.7 min binary gradient elution

Upon identifying the PMEZs from the GC analysis (~24 h post-lake acquisition), untreated cold room stored samples were then used to initiate enrichment cultures (Fig. 3). Labeled ¹³C-labeled formate, acetate, NaHCO₃, NaHCO₃ + H₂, Mph, Mea, or methionine. PMEZ samples taken in 2016 were incubated in the dark at 12 °C to mimic in situ conditions. ¹³C-enrichment of samples taken in 2017 focused only on ¹³C-Mph and ¹³C-Mea. Also, by that time we had found all functionally relevant lake isolates to have mesophilic temperature optima and thus incubations were conducted at room temperature, which was more optimal for assessing the potential of lake microbial community samples to convert these substrates to ¹³CH₄. The isotopic signature (δ¹³C) of the CH₄ of the headspace was measured using a modification of the protocols described by Wang et al. (12) on a Picarro G2201-i cavity ring-down spectrometer (CRDS) equipped with a Small Sample Introduction Module 2 (SSIM2). Each vial was first pressurized with 20 mL of <0.2 µm filtered ambient air (δ¹³C = ~−48‰ versus Vienna Pee Dee Belemnite) and then 20 mL was removed via gastight syringe and injected into the SSIM2. Factory calibration was used for the CRDS, and a tank of compressed ambient air was used as a reference material between injections to verify lack of instrument drift. For samples taken in 2017, the isotopic signature (δ¹³C) of the CH₄ of the headspace was determined using GC-MS.

**Methods**

**Study Site, Sampling, and Initial Sample Analyses.** The Yellowstone Lake sampling location and YSI EXO1 multiparameter Sonde water column characterizations were as previously described (12). For 2016 samplings, PMEZ localizations were determined using a dissolved gas equilibration system also as described (12). In 2017, the more extensive water column sampling did not allow for the use of the more time-consuming flow-through gas equilibration device for PMEZ identification. Instead, the thermocline was identified and characterized using the EXO Sonde, which then allowed us to approximate the depth of the anticipated PMEZ (i.e., just below the upper limit of the thermocline) (12). Extensive sampling was then conducted above and below this depth. On the day of sampling in 2017, lake conditions were exceptionally placid, allowing for sampling in 0.5-m increments.

For in situ CH₄ analysis, duplicate lake water samples for each depth were collected in 250 mL serum bottles, filling from the bottom and overflowing with at least one volume to expel bubbles. Vials were immediately sealed with gray chlorobutyl rubber stoppers and secured with aluminum crimps. Upon reaching shore, samples were killed by injecting 200 µL saturated HgCl₂ solution and stored on ice for transport to the laboratory where they were stored at 4 °C. CH₄ analysis was by GC of an introduced headspace (using ultra-high purity N₂) on a Hewlett-Packard HP5890A (for 2016 work) or a Varian CP-3800 gas chromatograph, both with flame ionization detection. The original CH₄ concentration in solution was calculated using Henry’s law and solubility equations (71). All other water samples were stored on ice in the dark for transport to the laboratory and then stored in a cold room (5 °C).

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profile was as follows: t) 0 min, 90%; t) 2 min, 70%; t) 3.6 min, 60%; t) and t) 4.7 min, 90%. The flow rate was 400 μL/min, and the sample injection volume was 10 μL. Limit of detection was determined to be 1 nm.

**Isolate Enrichment, Cultivation, and Characterization.** All bacteria used in this study are described in **Table S2**. To isolate Acidovorax from the lake water, we used the same SAR media described previously as SAR-Mn (12), except modified such that 10 mM glucose replaced pyruvate, 1 mM MgO or GB was provided as the sole N source instead of NH4SO4, and inorganic phosphate was used instead of MPn as a P source (agar media referred to herein as SAR-MeA). Sample aliquots from the positive 16S rDNA sample were plated directly onto SAR-MeA solidified with Agar Noble. Following a 2-wk incubation at 25 °C, visible unique colonies were identified based on morphological differences and selected for several rounds of subculture on SAR-MeA agar to obtain pure cultures.

DNA from each culture was extracted using Wizard Genomic DNA Purification Kits (Promega) as per manufacturer’s instructions. The 16S rRNA genes were PCR cloned using 27F and 1492R PCR primers (SI Appendix, Table S2). Amplicons were individually cloned into pCR2.1 (Invitrogen); clone-bearing plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen) and then sequenced by the Brigham Young University Central DNA sequencing facility. Resulting sequences were compared with GenBank sequences to identify closely related cultured organisms via BLASTn.

For culture characterizations, Acidovorax was grown in liquid SAR-MeA media containing glucose as the sole carbon source (4 g/L). Growth was tracked based on culture optical absorbance (A595) using a Molecular Dynamics microtiter plate reader. MEA → CH4 synthesis experiments were conducted in 70 mL sealed serum bottles in order to quantify CH4 synthesis, which was measured via GC as described in Study Site, Sampling, and Initial Sample Analyses.

**DNA Isolation, PCR, and Sequencing.** DNA was extracted from all water column depths for use as PCR templates to determine whether the methanogen indicator gene, mcrA, was detectable. In each case, biomass from triplicate 1 L samples was separately collected on Sterivex-GV 0.22 μm filter cartridges (Millipore Sigma) and then DNA extracted using the PowerWater DNA isolation kit (MO BIO Laboratories, Inc.) following the manufacturer’s instructions.

PCR primers for near full-length amplification of the Acidovorax pure culture 16S rRNA gene used the primers described by Lane (74), and for the mcrA gene, we used the universal primers described by Luton et al. (75) (SI Appendix, Table S2). For community 16S rRNA gene sequencing, DNA extractions were quantified using a SpectraMax Plus Microplate Reader (Molecular Devices), then sequenced by the Brigham Young University Central DNA sequencing facility. Resulting sequences were compared with GenBank sequences to identify closely related cultured organisms via BLASTn.

**Results.** PCRs were conducted using 27F and 1492R PCR primers (SI Appendix, Table S2). Amplicons were individually cloned into pCR2.1 (Invitrogen); clone-bearing plasmids were purified using QIAprep Spin Miniprep Kit (Qiagen) and then sequenced by the Brigham Young University Central DNA sequencing facility. Resulting sequences were compared with GenBank sequences to identify closely related cultured organisms via BLASTn.

**Discussion.** CH4 synthesis experiments were conducted in 70 mL sealed serum bottles in order to quantify CH4 synthesis, which was measured via GC as described in Study Site, Sampling, and Initial Sample Analyses.
56. E. Jameson et al., Deltaproteobacteria (Pelobacter) and Methanothepicococcoides are responsible for choline-dependent methanogenesis in a coastal saltmarsh sediment. ISME J. 13, 277–289 (2019).
**Table S1.** EXO Sonde measurements for 2016 Yellowstone Lake PMEZ samplings

<table>
<thead>
<tr>
<th>Date</th>
<th>Depth</th>
<th>Conductivity</th>
<th>Chlorophyll a</th>
<th>Phycocyanin</th>
<th>Temperature</th>
<th>pH</th>
<th>Dissolved Oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td>29-Jul-17</td>
<td>11.5 m</td>
<td>66.0 μS/cm</td>
<td>1.08 μg/L</td>
<td>0.03 μg/L</td>
<td>12.6 °C</td>
<td>8.01</td>
<td>8.28 (100)</td>
</tr>
<tr>
<td>5-Aug-17</td>
<td>11.0 m</td>
<td>67.4 μS/cm</td>
<td>0.81 μg/L</td>
<td>0.03 μg/L</td>
<td>13.6 °C</td>
<td>7.98</td>
<td>8.17 (100)</td>
</tr>
</tbody>
</table>

**Table S2.** Bacterial strains, plasmids and primers used in this study.

<table>
<thead>
<tr>
<th>Plasmid, strain, primers</th>
<th>Relevant markers and characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCPP30</td>
<td>TetR, Tra−, Mob+, IncP replicon, broad host range</td>
<td>Micheal Kahn, Washington St. Univ.</td>
</tr>
<tr>
<td>pCPP30::oat</td>
<td>pCPP30 carrying the wild type oat for complementation of the oat::Tn5-B22 mutant</td>
<td>This study</td>
</tr>
<tr>
<td>pET-28a(+)</td>
<td>Control expression plasmid for bioassay experiments</td>
<td>Novagen</td>
</tr>
<tr>
<td>pET-28a(+)::oat</td>
<td>Expression expression plasmid carrying the oat gene for bioassays</td>
<td>This study</td>
</tr>
<tr>
<td>pET-28a(+)::oat (K241A)</td>
<td>Expression expression plasmid carrying the K241A mutant oat gene for bioassays</td>
<td>This study</td>
</tr>
<tr>
<td>pSUP102</td>
<td>Suicide plasmid, Tn5·B22 delivery shuttle</td>
<td>80</td>
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<tr>
<td>pCR2.1</td>
<td>PCR TA cloning vector</td>
<td>Invitrogen</td>
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<td><strong>Strain</strong></td>
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<tr>
<td>Acidovorax sp.</td>
<td>Wild type Acidovorax strain YL-MeA-13 used for characterizations</td>
<td>This study</td>
</tr>
<tr>
<td>Mutant 3-29</td>
<td>YL-MeA-13, oat::Tn5·B22; GentR</td>
<td>This study</td>
</tr>
<tr>
<td>Complemented Mutant 3-29</td>
<td>YL-MeA-13, oat::Tn5·B22 mutant carrying pCPP30::oat; GenR, TetR</td>
<td>This study</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>Lake isolate (MK896839.1)</td>
<td>This study</td>
</tr>
<tr>
<td>Caulobacter sp.</td>
<td>Lake isolate (MK896844.1)</td>
<td>This study</td>
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<tr>
<td>Mesorhizobium sp.</td>
<td>Lake isolate (MK896845.1)</td>
<td>This study</td>
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<tr>
<td>Dietzia sp.</td>
<td>Lake isolate (MK896847.1)</td>
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<tr>
<td><strong>Escherichia coli</strong></td>
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<tr>
<td>S17-1</td>
<td>ProMob+, conjugation donor</td>
<td>Lab stock</td>
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<tr>
<td>BL21 (DE3)</td>
<td>F′omp T hsdSΔr6Km rB gal dcm rne 131 (DE3) pLysS (CamR)</td>
<td>Invitrogen</td>
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<td><strong>Primers</strong></td>
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<td>Universal mcrA</td>
<td>mcrA F, 5′-GGTGGGTGATMGGATTACACARTAYGCWACAGC-3′</td>
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<tr>
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<td>mcrA R, 5′-TTCTATGCRETTTGATTGGTATGTTAGT-3′</td>
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<tr>
<td>16S rRNA gene, near full length</td>
<td>27 F, 5′-AGAGTTTTACGATGCCTTACG-3′</td>
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<td>1492 R, 5′-TACCGTACCTTCGTTACGAC-3′</td>
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<td>16S rRNA gene Illumina sequencing</td>
<td>515F, 5′-GTGBCAGCMGCCGCTGAA-3′</td>
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<tr>
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<td>806R, 5′-GGACTACHVGGGTWTCTAAT-3′</td>
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Fig. S1. EXO Sonde characterization of water column properties in 2016 samplings. Note $O_2$ saturation (or nearly so) at both PMEZ depths (July, 11 m; August, 11.5 m).
Fig. S2. $^{13}$CH$_4$ derived from lake water incubated with $^{13}$C-labeled methylated substrates that are potentially used by methanogens for methane synthesis. Substrates used were acetate, formate, methylamine (MeA), methionine (Methio), methylphosphonate (MPn), bicarbonate (Bicarb) or bicarbonate plus hydrogen (Bicarb + H$_2$). Data are mean ± range of n=2 distinct water samples, INT, refers to interference from an unknown (sulfur-containing) gas.
Fig. S3. PCR-based probing of Yellowstone Lake DNA for the mcrA gene as a proxy for presence or absence of methanogens. Universal mcrA primers were as described by Luton et al. (75). M, molecular weight markers, T1W1, positive control DNA from riparian environment (26) and as previously used (12). “*” denotes PMEZ depths.
Fig. S4. LCMS analysis of methylphosphonate in Yellowstone Lake water. Lake samples failed to show any detectable signal. The black trace shows lake water without addition of exogenous MPn. Lake water was spiked with MPn at concentrations ranging from 1-103 µM. Level of detection was 1 µM. Extracted ion chromatograms (97.004 m/z) show high MPn (Panel A) and low MPn (panel B) spike concentrations from the standard addition experiment.
Fig. S5. Best fit regression analysis examining metabolite concentration relationships within the water column. (A) Correlations between glycine betaine or trimethylamine (TMA) and methylamine (MeA). (B) Semi Log_{10} relationship between TMA and MeA. Open symbols indicate methylamine or trimethylamine concentrations which were below detection.
Fig. S6. Growth characteristics of the wild type, mutant, and complemented mutant strains grown with NH$_4^+$, methylamine (MeA), or glycine betaine (GB) as a sole N source. Cultures in panels A, B, and C were initiated at O.D.s 0.011-0.014 to illustrate how methane production from MeA is linked to growth (compare to Fig. 3). Cultures depicted in panels D and E were initiated at ~10-fold higher O.D. (Ab$_{595} = 0.15$) that was roughly equivalent to mid log phase of cultures in A,B, C, to illustrate that lack of methane production in the mutant is not due to lack of biomass. All data points and error bars (where visible) are the mean ± range from duplicate cultures.
Figure S7. Transfer of $^{13}$C from MeA to CH$_4$. (A) Overlaid extracted ion chromatograms corresponding to $^{13}$CH$_4$ (m/z=17) in headspace gas sample ($^{13}$C-MEA) from Acidovorax sp. cultured with $^{13}$C-MeA as a sole N source, a $^{12}$CH$_4$ standard (m/z=16), and $^{13}$CH$_4$. (B) Mass spectra of CH$_4$ peaks from Acidovorax sp. cultures supplied with $^{12}$CH$_4$ or $^{13}$CH$_4$. 
**Fig. S8.** Physical description of the *plp-aat* gene, adjacent DNA, and Tn5B22 insertion sites. Positions of the Tn5B22 insertion sites are indicated by inverted red arrowheads. Gene annotation as described in the *Acidovorax* sp. YL-MeA13-2016 genome (DOE IMG Gold Project ID Gp0440505): *tolA*, colicin import membrane protein; *tolR*, biopolymer transport TolR; *tolQ*, biopolymer transport TolQ; *ybgC*, Tol-Pal system-associated acyl-CoA thiotolerase; *plp-aat*, pyrodixal phosphate-dependent aspartate aminotransferase; *nusB*, Nutilization substance B; *ribH*, dimethyl-8-ribitllumazine synthase; and *rib*, dihydroxy-2-butanone-4-phosphate synthase; *fer2*, ferredoxin-like. Note that the gene we describe as *plp-aat* is annotated by the JGI as “aspartate/methionine/tyrosine aminotransferase. Genbank annotation and manual BLAST searches conclude that aspartate aminotransferase is a better annotation.
Fig. S9. Oxygen profile of CH4 synthesizing E.coli BL21 carrying the recombinant aat cloned from Acidovorax. Negative controls were not inoculated. Data and error bars (where visible) represent the mean ± SD of three replicate cultures.
Fig. S10. SDS-PAGE profile of purified Aat protein. The soluble protein fractions from *E. coli* BL21 were purified as a His-tagged proteins using a nickel column. Lane 1, non-induced protein. Lane 2, induced total soluble protein. Lane 3, proteins eluted with 40 mM imidazole. Lane 4, protein eluted with 100 mM imidazole. Lane 5, Ni purified fraction containing Aat.
Figure S11. PLP-Aat homologs occur in other aquatic environments. Examples of PLP-Aat amino acid sequences sharing significant homology with the *Acidovorax* PLP-Aat. Aquifer, metagenome accession classified as a *Burkholderiales* protein (OGA61428); Lake, *Limnohabitans* isolate (WP_108287182); River, a *Polaromonas* sp. river isolate (TAG32972); and Marine metagenome, hypothetical protein GOS_617153 from The Sorcerer II Global Ocean Sampling Expedition (EDH37539). Inverted blue arrowhead denotes the catalytic lysine (K) that is invariant among PPL-Aat enzymes of plants, bacteria, archaea, and animals. Yellow highlighted residues designate PLP binding sites or the active site (K).