Freshwater lakes emit large amounts of methane, some of which is produced in oxic surface waters. Two potential pathways for aerobic methane production exist: methanogenesis in oxygenated water, which has been observed in some lakes, and demethylation of small organic molecules. Although methane is produced via demethylation in oxic marine environments, this mechanism of methane release has not yet been demonstrated in freshwater systems. Genes related to the C-P lyase pathway, which cleaves C-P bonds in phosphonate compounds, were found in a metagenomic survey of the surface water of Lake Matano, which is chronically phosphorus starved and methane rich. We demonstrate that four bacterial isolates from Lake Matano obtain phosphorus from methylphosphonate and release methane and that this activity is repressed by phosphate. We further demonstrate that expression of \textit{phnJ}, which encodes the enzyme that releases methane, is higher in the presence of methylphosphonate and lower when both methylphosphonate and phosphate are added. This gene is also found in most of the metagenomic data sets from freshwater environments. These experiments link methylphosphonate degradation and methane production with gene expression and phosphate availability in freshwater organisms and suggest that some of the excess methane in the Lake Matano surface water, and in other methane-rich lakes, may be produced by P-starved bacteria.

**ABSTRACT**

Freshwater lakes emit large amounts of methane, some of which is produced in oxic surface waters. Two potential pathways for aerobic methane production exist: methanogenesis in oxygenated water, which has been observed in some lakes, and demethylation of small organic molecules. Although methane is produced via demethylation in oxic marine environments, this mechanism of methane release has not yet been demonstrated in freshwater systems. Genes related to the C-P lyase pathway, which cleaves C-P bonds in phosphonate compounds, were found in a metagenomic survey of the surface water of Lake Matano, which is chronically P starved and methane rich. We demonstrate that four bacterial isolates from Lake Matano obtain P from methylphosphonate and release methane and that this activity is repressed by phosphate. We further demonstrate that expression of \textit{phnJ}, which encodes the enzyme that releases methane, is higher in the presence of methylphosphonate and lower when both methylphosphonate and phosphate are added. This gene is also found in most of the metagenomic data sets from freshwater environments. These experiments link methylphosphonate degradation and methane production with gene expression and phosphate availability in freshwater organisms and suggest that some of the excess methane in the Lake Matano surface water, and in other methane-rich lakes, may be produced by P-starved bacteria.

**IMPORTANCE**

Methane is an important greenhouse gas and contributes substantially to global warming. Although freshwater environments are known to release methane into the atmosphere, estimates of the amount of methane emitted by freshwater lakes vary from 8 to 73 Tg per year. Methane emissions are difficult to predict in part because the source of the methane can vary: it is the end product of the energy-conserving pathway in methanogenic archaea, which live predominantly in anoxic sediments or waters but have also been identified in some oxic freshwater environments. More recently, methane release from small organic molecules has been observed in oxic marine environments. Here we show that demethylation of methylphosphonate may also contribute to methane release from lakes and that phosphate can repress this activity. Since lakes are typically phosphorus limited, some methane release in these environments may be a by-product of phosphorus metabolism rather than carbon or energy metabolism. Methane emissions from lakes are currently predicted using primary production, eutrophication status, extent of anoxia, and the shape and size of the lake; to improve prediction of methane emissions, phosphorus availability and sources may also need to be included in these models.
phosphorus, and indeed some pathogenic and enteric bacteria (28, 29), as well as bacteria isolated from soil (reviewed in references 26 and 27) and wastewater (30), are capable of using both the C and P moieties of phosphonate compounds.

Freshwater lakes are typically P limited (31), so in these environments, phosphonates would be more likely to provide P than C to organisms capable of phosphonate degradation. The organic functional group would then be released as a by-product. Thus, consumption of LMW phosphonates may result in the release of soluble organic compounds that can be used as C or energy sources (32) or of gaseous compounds that may escape consumption, such as ethane or methane (15, 33).

In permanently stratified lakes with deep chemoclines, where the anoxic bottom waters are essentially disconnected from the air-water interface, methane accumulation is more likely to be due to the production of methane in the surface water than to upward diffusion, since the rate of diffusion should be lower than the rate of methane consumption (8, 34). Lake Matano, Indonesia, is a deep, permanently stratified, chronically P-limited lake. Methane is supersaturated in Lake Matano surface water (7, 35), even though most of the methane produced in the anoxic sediments and bottom waters is oxidized in the chemocline (7) and diffusion of methane upward through the 120-m epilimnion should be slow enough to allow complete aerobic consumption of methane produced below the chemocline. Because of the degree of P limitation in Lake Matano (36–38), we hypothesize that additional methane may be produced in the surface water by P-starved organisms acquiring P from methylphosphonate. Here we use metagenomic analysis, physiological experiments, and gene expression analysis to test the hypothesis that P acquisition by aerobic heterotrophs in Lake Matano may account for some of the excess methane in Lake Matano surface water. We show that genes encoding phosphonate degradation are enriched in metagenomic data from surface water of Lake Matano (36–38), we hypothesize that additional methane may be produced in the surface water by P-starved organisms acquiring P from methylphosphonate. Here we use metagenomic analysis, physiological experiments, and gene expression analysis to test the hypothesis that P acquisition by aerobic heterotrophs in Lake Matano may account for some of the excess methane in Lake Matano surface water. We show that genes encoding phosphonate degradation are enriched in metagenomic data from surface water and widespread in other freshwater metagenomic data sets, that four isolates produce methane from MPn, and that both methane production and expression of phnJ, which encodes a subunit of the C-P lyase complex (27, 39), change in response to phosphate availability.

MATERIALS AND METHODS

Sample collection and sequencing. Surface water was collected from Lake Matano, Indonesia, for DNA extraction and sequencing from January to March 2009. Water (100 liters) was pumped through 0.22-μm Steripak filters, which were frozen on-site and kept frozen until processing. DNA was extracted and sequenced using a 454 Life Sciences GS-FLX instrument.

Analysis of other metagenomic data sets. The PhnJ amino acid sequences from Pseudomonas stutzeri (NCBI accession no. AAB91738) and E. coli (NCBI accession no. AAC770059.1) were used as BLAST queries against freshwater metagenomic data sets publicly available at the Joint Genome Institute’s Integrated Microbial Genomes website (https://img.jgi.doe.gov/cgi-bin/main.cgi) and at the European Nucleotide Archive (http://www.ebi.ac.uk/ena) (43). Data sets were scored as positive for phnJ if they had at least one hit with an E value of less than 1 × 10⁻10.

Strains and growth conditions. Seven heterotrophic bacteria isolated from the surface water of Lake Matano (Table 1) (38) were screened for their ability to acquire P from MPn. The base medium was the National Botanical Research Institute phosphate growth medium (NBRI) without added P [per liter: MgCl₂·6H₂O, 5 g; MgSO₄·7H₂O, 0.25 g; KCl, 0.2 g; (NH₄)₂SO₄, 0.1 g; glucose, 10 g (44). To test for MPn utilization, strains were grown in NBRI base medium with 0.2 mM MPn as the only P source. Production of methane from MPn was characterized in the four strains (LM-1, LM-5, LM-6, 1, and LM-Y) capable of using MPn as their sole source of P. MPn, K₂HPO₄, or both were added to the P-free NBRI medium as P sources. The pH of the medium was adjusted to 7.0 with 1 M solutions of NaHCO₃, or acetic acid for the medium with added K₂HPO₄, or 1 M solutions of NaHCO₃ or NaOH for medium with added MPn, as appropriate. Prior to measuring growth conditions, cells were grown in NBRI with K₂HPO₄ as the only P source. Serum bottles (60 ml) containing 30 ml medium and air in the headspace were inoculated with cells to an optical density at 660 nm (OD₆₆₀) of ~0.05 and incubated horizontally at 30°C with shaking at ~150 rpm. Growth was monitored by measuring the OD₆₆₀.

For the initial growth experiments, MPn, KH₂PO₄, or both were added to concentrations of 0.2 mM each. For experiments characterizing methane release with different concentrations of MPn, no phosphate was added to the medium, and MPn was added to concentrations of 5 μM, 10 μM, 50 μM, 100 μM, or 200 μM. To determine the effect of phosphate addition on MPn degradation, the initial MPn concentration was 50 μM and the starting K₂HPO₄ concentration was 0 μM, 4 μM, 10 μM, 50 μM, or 100 μM.

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Closest related cultivated species</th>
<th>Ability to use</th>
</tr>
</thead>
<tbody>
<tr>
<td>LM-2</td>
<td>Microbacterium testaceum</td>
<td>−</td>
</tr>
<tr>
<td>LM-1</td>
<td>Agrobacterium tumefaciens</td>
<td>+</td>
</tr>
<tr>
<td>LM-5</td>
<td>Rhizobium sp.</td>
<td>+</td>
</tr>
<tr>
<td>LM-6</td>
<td>Agrobacterium tumefaciens</td>
<td>+</td>
</tr>
<tr>
<td>LM-P</td>
<td>Methylobacterium podarium</td>
<td>−</td>
</tr>
<tr>
<td>2-LM-22</td>
<td>Acinetobacter sp.</td>
<td>−</td>
</tr>
<tr>
<td>LM-Y</td>
<td>Pantoea ananatis</td>
<td>+</td>
</tr>
</tbody>
</table>

* All strains were isolated from water collected from Lake Matano, Indonesia, as described previously (38). The ability to use methylphosphonate (MPn) or 2-aminomethylphosphonate (2-AEP) was assessed by monitoring growth in NBRI medium with MPn or 2-AEP supplied as the sole phosphorus source at a concentration of 0.2 mM. The 2-AEP results were originally reported in reference 38.

Downloaded from http://aem.asm.org on October 15, 2017 by guest
To calculate the total methane production, we assumed it to be in equilibrium between the gas and aqueous phases. In serum bottles with a headspace-to-liquid ratio of 30 ml/30 ml, the percentage of methane in the gas phase ($f_g$) is 96.87% at 30°C based on equation 1 (45), using a value of 0.0013 mol liter$^{-1}$ atm$^{-1}$ for Henry’s constant, $K_H$ (35):

$$f_g = \frac{1}{K_H \times RT} \times \frac{V_g}{V_w} \times \frac{1}{1 + \frac{V_g}{V_w}}$$

(1)

where $V_g$ and $V_w$ are the volumes of the gas and aqueous phases, respectively, and $R$ is the gas constant. The amount of methane produced was calculated by dividing the quantity of methane in the headspace by $f_g$.

Percent repression of methane production by phosphate was calculated as follows:

$$\%\text{ repression} = \frac{(\text{total CH}_4)_{0P} - (\text{total CH}_4)_{aP}}{(\text{total CH}_4)_{0P}}$$

(2)

where $n = 4, 10, 50$, or 100 μM.

**Measurements of CH$_4$.** Methane in the culture headspace was measured by gas chromatography coupled with a flame ionization detector (GC-FID), Agilent 7890A (G3440A). Gas was sampled from the culture headspace (25 μl for the LM-Y initial growth experiment; 100 μl for all other experiments) using a gas-tight syringe and injected into the GC-FID at an inlet temperature of 250°C. The carrier gas was helium, at a rate of 25 ml min$^{-1}$. The oven temperature was 90°C for 4 min, and then the temperature was increased by 120°C min$^{-1}$ for 1.25 min, until a final temperature of 240°C was reached. The temperature was maintained at 240°C for 1.5 min to remove any residue in the column. The detector temperature was 200°C, with hydrogen and airflow of 35 and 350 ml min$^{-1}$, respectively. MSDChem was used to integrate the peaks. Methane standards with 99 ppm methane in nitrogen gas (item no. 01-04212; Scotty) and >99.9% methane (item no. GMT10015TC; Matheson) were used to prepare a standard curve of peak area and concentration (49.5 ppm, 999 ppm, and 1996.01 ppm). The methane concentration obtained from the standard curves was then converted from parts per million to ppm, 999 ppm, and 1,996.01 ppm. The methane concentration was calculated by dividing the amount of methane in the headspace by $f_g$.

**Expression of phnJ during growth on different P sources.** Liquid subsamples (1.5 or 3.0 ml) were removed from all cultures during the mid-exponential and stationary phases of growth on each P source (MPn, K$_2$HPO$_4$, or both) and stored at $-20^\circ$C in RNA later until processing. For RNA extraction, cells were stabilized in RNA Protect Reagent (catalog number 76506; Qiagen) and then digested with lysosomes (15 mg ml$^{-1}$) and 20 μl proteinase K (800 U ml$^{-1}$, catalog no. P8107S; New England BioLabs) at room temperature for 15 min. RNA was then purified from the bacterial lysate using the Qiagen RNaseasy minikit (catalog no. 74104; Qiagen) according to the manufacturer’s instructions. Genomic DNA contamination was removed by Turbo DNase treatment (catalog no. AM1907; Ambion). RNA was used as the template for reverse transcription using the RETROscript kit (catalog no. AM1710; Ambion). RNA (~1 μg) and random decamers (2 μl, 50 μM) were denatured at 75°C for 3 min and then immediately placed on ice. RT buffer (2 μl, 10×), dNTP mix (4 μl, 2.5 mM each), RNase inhibitor (1 μl, 10 U μl$^{-1}$), and Moloney murine leukemia virus reverse transcriptase (MMLV-RT; 1 μl, 100 U μl$^{-1}$) were added to the mixture to a volume of 20 μl. This solution was incubated at 44°C for 1 h and then at 92°C for 10 min to inactivate the reverse transcriptase (MMLV-RT). The single-stranded cDNA product was used as the template for quantitative PCR (qPCR).

The abundance of the phnJ transcript was quantified in the cDNA products using qPCR. The qPCRs were performed on an Eppendorf real-time Mastercyclerep and processed in duplicates with 0.5 μl cDNA, 10 μl SYBR green FastMix for iQ (catalog no. 95071; Quanta), 0.25 μl each primer (25 mM), and 9 μl nuclelease-free water. The reaction protocol was as follows: 95°C for 2 min 30 s, followed by 40 cycles of 95°C for 15 s, 62°C for 15 s, and 68°C for 30 s.

**Accession number(s).** The metagenomic data obtained in this study have been deposited in the NCBI database with accession number SAMN03292416.

**RESULTS AND DISCUSSION**

**Potential for phosphonate degradation in Lake Matano and other freshwater environments.** Heterotrophic bacteria isolated from Lake Matano were recently shown to utilize a diverse array of P sources, including 2-AEP (Table 1) and to make large changes in the DNA content and lipid composition of the cells when P starved (38). To assess the probability of methane production from MPn degradation in Lake Matano surface water, reads mapping to genes encoding the C-P lyase pathway (Fig. 1A) were quantified. All of the genes necessary for cleavage of MPn to methane and phosphate are present in the metagenomic data set from the surface water of Lake Matano (Fig. 1C). This pathway includes sub-units of the phosphonate transporter (phnDEC), activation of the phosphonate by ATP (phnlGH), release of diphosphate (phnM), and cleavage of the C-P bond, which results in release of methane (phnJ) (48).

The odds ratios compare the frequency of each protein in the metagenomic data set to its frequency in the COG database (42). An odds ratio greater than 1 indicates that a predicted protein is

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**TABLE 2 Primers used for phnJ sequencing and RT-PCR analysis**

<table>
<thead>
<tr>
<th>Target (strain[s])</th>
<th>Application</th>
<th>Forward primer (sequence)</th>
<th>Reverse primer (sequence)</th>
<th>Source or references</th>
</tr>
</thead>
<tbody>
<tr>
<td>phnJ</td>
<td>PCR</td>
<td>PhnJoc1 (AARGTATMGAYGACGG)</td>
<td>PhnJoc2 (CATTTTGAAGGATGTRCAAA)</td>
<td>46, 47</td>
</tr>
<tr>
<td>phnJ1 (LM-1, LM-5, and LM6-1)</td>
<td>RT-PCR</td>
<td>phnJ-F1 (ACATCATCAGACGGGCGCAG)</td>
<td>phnJ-R1 (AGCTTGAGTGCATCAGGCC)</td>
<td>This study</td>
</tr>
<tr>
<td>phnJ2 (LM-Y)</td>
<td>RT-PCR</td>
<td>phnJ-F2 (TCAGACGCGGTACCGGTATT)</td>
<td>phnJ-R2 (CTCTGTAGGTTTGCAGCCGC)</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Degenerate primers were used to amplify and sequence phnJ from all isolates; specific primers were designed based on the sequences to use for qPCR. Strains LM-1, LM-5, and LM6-1 are members of Alphaproteobacteria that are closely related to each other, and the same primer pair was used for all three. Primers for LM-Y were slightly different but were complementary to the same region of the gene and generated a product of the same size.*
present in the metagenomic data set at a higher frequency than expected based on its frequency in protein databases (42). Below the chemocline in Lake Matano, the phosphate concentration increases to ~10 μM, making the microbes at this depth less P starved than those in the surface water (37, 49). Because the odds ratios for nearly all of the genes required for MPn transport and degradation are less than 1 in the metagenomic data from the P-replete chemocline (Fig. 1B) but greater than 1 in the P-limited surface water (Fig. 1C), we hypothesized that MPn could serve as a P source for some organisms in Lake Matano, which would release methane as a by-product.

The Newbler GS De Novo Assembler was used to assemble the metagenomic reads from the Lake Matano surface water, and 530,076 reads (66.39% of the total number of reads) were assigned to 18,727 contigs. Amino acid sequences of C-P lyase proteins in the metagenomic data set were assigned to 1,474 contigs, or 9,530,076 reads (66.39% of the total number of reads) were assigned to 1,474 contigs, and 60 contigs contained a partial gene encoding the ATP-binding motif of an ABC transporter. Contig00117 has 7,056 nucleotides and carries the phnJKLM gene cluster on Contig00117, these appear to be fused: no stop codon between the two genes could be found, so we predict that they encode a single polypeptide.

The product of phnJ is responsible for cleavage of the C-P bond in phosphate compounds and thus for release of methane from MPn (53), so the distribution of this gene in freshwater metagenomic data sets was assessed. Metagenomic data derived from freshwater samples and archived at the Integrated Microbial Genomes resource (https://img.jgi.doe.gov/) or the European Nucleotide Archive (http://www.ebi.ac.uk/ena) were queried for the presence of phnJ. Eighteen of the 23 data sets (78%) had homologs of phnJ (Table 3), suggesting that phosphate bond cleavage may occur in a variety of freshwater environments, including lakes, lake sediments, bogs, rivers, and streams.

FIG 1 C-P lyase pathway in Lake Matano. (A) C-P lyase proteins that transport phosphonates (PhnCDE) and cleave the C-P bond (PhnGHIJKLM), based on data in references 48 and 66. PhnC is responsible for the release of methane (or another organic group) from the intermediate. (B) Odds ratios of genes encoding proteins in the C-P lyase pathway in metagenomic data from the Lake Matano chemocline (40), where P is not limiting (37). Odds ratios were calculated for each COG category in the metagenomic data set by calculating the ratio (A/B)/(C/D), where A is the number of reads in the metagenome that are in a given COG category, B is the number of reads in the metagenome that are in all other COG categories, C is the number of proteins in the COG database that are in a given COG category, and D is the number of proteins in the COG database that are in all of the other COG categories. COG categories with odds ratios of <1 are not considered enriched; none of the genes involved in transport or degradation of phosphonates are enriched in the chemocline. (C) Odds ratios of genes encoding proteins in the C-P lyase pathway in metagenomic data from Lake Matano surface water, where P is limiting. COG categories with odds ratios of >1 are considered enriched in the Lake Matano data set compared to the COG database (42).
Methanogenesis has been observed in oxic surface waters of lakes (4–6, 8, 10). However, it has not been directly observed in Lake Matano surface waters, so we assessed the metagenomic data set from 10 m to determine whether surface water methanogenesis seemed likely. No reads mapping to archaeal rRNA sequences (55, 16S, or 23S) are present in this data set. Ninety-five reads (of a total of 798,463 reads) mapped to archaeal genomes. Most of these (75 of 95 reads) mapped to the class Halobacteria, the members of which are not methanogenic. The remaining 20 reads were initially mapped to the methanogenic genera Methanococcus and Methanomicrobium; however, when these reads were used as blast queries against the NBCI nonredundant database, only 4 were identified as similar to sequences from methanogen genomes (Methanoculleus and Methanococcus). After the metagenomic reads were used as queries in a blast search against the COG database, the results were screened for reads mapping to methanogenesis pathways, but none were identified. Additionally, the amino acid sequence of McrA from Methanococcoides burkii DSM804 (RefSeq: WP_001304916.1) was used as a query against the metagenomic data set, and no reads with homology to McrA were identified. We thus conclude that if water column methanogenesis occurs in the epilimnion of Lake Matano, it is either localized deeper than 10 m or carried out by a very small number of organisms not detected in our metagenomic survey.

**Utilization of MPn as a P source by Lake Matano isolates.**

Seven isolates were screened for growth on MPn. Four were capable of growth on MPn as the sole P source and were selected for further analysis (Table 1). All four strains grow at the same rates on MPn, K2HPO4, or both and reach stationary phase at approximately the same time (Fig. 3, dashed lines). These isolates do not

![FIG2 Phosphonate-related gene clusters in Lake Matano metagenomic data. Contig00268 has high identity (66%) to the gene cluster encoding the multienzyme C-P lyase complex, phnJKL, from Rhodococcus opacus strain PD630 and R7 (only R. opacus strain PD630 is shown) and organization similar to that of the phn gene cluster from E. coli, whose C-P lyase pathway has been well studied (50, 67). Genes are colored based on their functions: transporter genes (phnCDIJKL) are light gray, regulatory genes (phnFO) are white, phosphonate degradation genes (phnGHIJKLM) are dark gray, and the phnNP accessory genes are black.](http://aem.asm.org/)

**TABLE 3 Distribution of phnJ in freshwater metagenomic data sets deposited at IMG or ENA**

<table>
<thead>
<tr>
<th>Sample source</th>
<th>Environment type</th>
<th>phnJ</th>
<th>Study (reference[s])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake Damariscotta, Maine, USA</td>
<td>Lake</td>
<td></td>
<td>ENA-PRJEB4844 (43)</td>
</tr>
<tr>
<td>Lake Ekoln, Sweden</td>
<td>Lake</td>
<td></td>
<td>ENA-PRJEB4844 (43)</td>
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<tr>
<td>Lake Erken, Sweden</td>
<td>Lake</td>
<td></td>
<td>ENA-PRJEB4844 (43)</td>
</tr>
<tr>
<td>Lake Mendota, Wisconsin, USA</td>
<td>Lake</td>
<td></td>
<td>ENA-PRJEB4844 (43)</td>
</tr>
<tr>
<td>Lake Vattern, Sweden</td>
<td>Lake</td>
<td></td>
<td>ENA-PRJEB4844 (43)</td>
</tr>
<tr>
<td>Sparkling Lake, Wisconsin, USA</td>
<td>Lake</td>
<td></td>
<td>ENA-PRJEB4844 (43)</td>
</tr>
<tr>
<td>Trout Bog Lake, Wisconsin, USA</td>
<td>Lake</td>
<td></td>
<td>ENA-PRJEB4844 (43)</td>
</tr>
<tr>
<td>Lake Erie, Canada/USA</td>
<td>Lake</td>
<td></td>
<td>GOLD project ID: Gp0011910</td>
</tr>
<tr>
<td>Lake Sakinaw, British Columbia, Canada</td>
<td>Lake</td>
<td></td>
<td>GOLD project ID: Gp0052015 (68, 69)</td>
</tr>
<tr>
<td>Lake Grosse Fuchskuhle, Germany</td>
<td>Mixed culture from lake</td>
<td></td>
<td>GOLD project ID: Gp0057572 (70)</td>
</tr>
<tr>
<td>Lake Superior, Canada/USA</td>
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<tr>
<td>Lake Washington, Washington, USA</td>
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<tr>
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<td>GOLD study ID: Gs0063259 (73)</td>
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<td>Delaware River, Delaware/New Jersey, USA</td>
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<td>GOLD study ID: Gs0063440</td>
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<tr>
<td>Lake Michigan, Canada/USA</td>
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<td>Crystal Bog, Wisconsin, USA</td>
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<td>GOLD study ID: Gs0111445 (75)</td>
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<tr>
<td>Notre Dame University, Indiana, USA</td>
<td>Anoxic pond sediments</td>
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<td>GOLD study ID: Gs011357</td>
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<td>University of Edinburgh, Edinburgh, UK</td>
<td>Pond</td>
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<td>GOLD study ID: Gs0114295</td>
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<tr>
<td>Streams affected by fracking, Pennsylvania, USA</td>
<td>Stream</td>
<td></td>
<td>GOLD study ID: Gs0114818 (76)</td>
</tr>
</tbody>
</table>

*The nucleotide sequence of phnJ from Pseudomonas stutzeri (accession no. AAR91738.1) or E. coli (accession no. AAC77059.1) was used to query all of these data sets; a data set was scored as positive if it had at least one hit with an E value of <10−10. Approximately 25% of samples from Yellowstone National Park also had phnJ homologs. IMG, Integrated Microbial Genomes & Microbiomes.*
appear to be capable of consuming the methane released as a carbon or energy source, since they do not grow when MPn is provided as the sole P, C, and energy source (data not shown). Methane was produced only in the presence of MPn (Fig. 3, solid lines). In the presence of both MPn and 200 $\mu$M K$_2$HPO$_4$, strain LM-Y did not produce any methane (Fig. 3D). However, the other isolates produced some methane in the presence of K$_2$HPO$_4$ when MPn was also present (Fig. 3A to C). No methane was produced by any strain when MPn was not provided, indicating that the methane is a product of MPn metabolism.

To further investigate the relationship between MPn concentration and methane production, strain LM-Y was grown in NBRIP with different concentrations of MPn (Fig. 4). This strain has been shown to decrease RNA content and replace its phospholipids with amino- and glycolipids when P is limited, so the cells may increase their P content without necessarily altering the growth yield (38). As the MPn concentration increases, the cell yield and total methane production also increase, suggesting that availability of additional P promotes additional growth, as expected (Fig. 4). Both yield and methane production are similar when 100 or 200 $\mu$M MPn is added, suggesting that at concentrations of >100 $\mu$M MPn, sufficient P is available to maximize growth yield in NBRIP and the excess P in solution may even inhibit further degradation of MPn (54).

**Effect of phosphate on methane release from MPn.** Addition of 200 $\mu$M phosphate to the cultures with initial MPn concentrations of 200 $\mu$M reduced the amount of methane produced by strains LM-1, LM-5, and LM6-1 by 40 to 60% but completely repressed methane production in the culture of LM-Y. To better understand the effect of phosphate on phosphonate consumption by this strain, LM-Y was grown with 50 $\mu$M MPn and 0 to 100 $\mu$M K$_2$HPO$_4$ for 10 days.
KH₂PO₄ (Fig. 5). Methane production was completely inhibited in cultures grown with 50 μM MPn and 50 or 100 μM KH₂PO₄ (Fig. 5A). In cultures grown with 50 μM MPn and 0 μM, 4 μM, or 10 μM P, the amount of methane produced decreased as KH₂PO₄ increased (Fig. 5A). The relationship between added KH₂PO₄ concentration and percent repression of methane production is linear (Fig. 5B), and we extrapolate that methane production would be completely repressed at P₃ concentrations above 30 μM.

Expression of phnJ during growth on different P sources. The phnJ gene encodes the enzyme that catalyzes release of methane from the intermediate α-D-ribose-1-methylphosphonate-5-phosphate (53). We examined the expression of this gene in all four bacterial strains grown with 0.2 mM MPn and 0.2 mM, 0.4 mM, or 10 μM P, or both in the medium. LM-Y, the only strain in which methane production from MPn is abolished in the presence of phosphate (Fig. 3), appears to express phnJ only when MPn is present and phosphate is absent (Fig. 6). The other three strains, which produce methane when MPn is provided, whether or not it is the only P source (Fig. 6), express phnJ under both conditions. However, the phnJ transcript is less abundant in the presence of phosphate (Fig. 6 and Table 4).

Environmental significance. Here, we show that freshwater heterotrophic bacteria are capable of producing methane from MPn and that the pathway for phosphonate degradation is widespread in freshwater lakes. We further demonstrate that in Lake Matano, Alphaproteobacteria, Gammaproteobacteria, and likely Actinobacteria can take up phosphonate compounds and cleave the C-P bond to acquire P and that this activity is repressed in the presence of P₃. Similarly, the phnJ gene is expressed in these strains only in the presence of MPn, and its expression level is modulated by addition of P₃. In sum, this work shows that methane production in freshwater systems may occur as a result of phosphate limitation, as microbes acquire P from phosphonates.

Phosphonates, including MPn, may comprise up to 10% of dissolved organic phosphorus (DOP) in some lakes (55–57), though many freshwaters have no detectable phosphonates (58, 59) or only very small quantities thereof (60, 61). However, homologs of phnJ are present in 18 of 23 metagenomic data sets from freshwater environments (water and sediment), including bogs, lakes, ponds, rivers, and streams (Table 3). The number of data sets in which phnJ is found and the diversity of environments indicate that the ability to degrade phosphonates is widespread in freshwater environments. Additionally, based on the abundance of phnD, which encodes one subunit of the phosphonate transporter, picocyanobacteria in the Great Lakes are predicted to be capable of phosphonate uptake (62). Given the broad distribution of phosphonate uptake and degradation pathways, phosphonates may be undetectable in freshwater systems not because they are not present but because they are rapidly broken down. In fact, phosphonates in marine systems have been shown to be highly

FIG 5 Phosphate represses methane production from MPn in LM-Y. All cultures were grown with 50 μM MPn and the indicated concentration of KH₂PO₄. Since no CH₄ production was observed when KH₂PO₄ was used, we assume that there is no inhibition in the absence of KH₂PO₄, and 100% inhibition in the presence of ≥50 μM KH₂PO₄. Percent repression was calculated as described in equation 2.

FIG 6 Transcript abundance of phnJ in isolates grown with phosphate, MPn, or both. Liquid subsamples (1.5 or 3.0 ml) were removed from all cultures during mid-exponential and stationary phases of growth on each P source (MPn, K₂HPO₄, or both). Transcript levels were calculated relative to phnJ in cells grown with phosphate and harvested during the exponential growth phase. Expression of phnJ was highest in cells grown with MPn as the sole P source; in strains LM-1, LM-5, and LM-6-1, expression was lower but still detectable when both MPn and phosphate were supplied as P sources. Expression of phnJ in LM-Y in the presence of both MPn and phosphate is indistinguishable from its expression in the presence of phosphate only.
reactive (63), and the same may be true in freshwater environments, which are typically P limited (64). This P limitation may result in microbial acquisition of P from a wide variety of sources (38), ultimately leading to release of organic by-products such as methane.

The steady-state concentration of methane at 40 m in Lake Matano is ~3 μM, 3 orders of magnitude greater than the saturation concentration (7). Many freshwater lakes are supersaturated with regard to methane, often more than can be explained by methanogenesis in anoxic sediments or bottom waters (5, 6, 10). In some of these systems, surface water methane is produced by methanogenesis in oxic surface waters (5, 10). However, in severely P-limited systems, demethylation of P-containing compounds may also contribute to surface water methane production. Because Lake Matano has less than 50 nM total P in the surface water (37), planktonic organisms there must be capable of immediate uptake and utilization of P in any available form (38). Both physiological experiments with isolates and bioinformatics analysis of metagenomic data indicate that heterotrophic bacteria in Lake Matano surface water are capable of producing methane as a by-product of acquisition of P from phosphonate compounds. Given the prevalence of genes encoding phosphonate biosynthesis and degradation in freshwater metagenomic data sets, P metabolism in freshwater systems may have unexpectedly large effects not only on P cycling but also on release of greenhouse gases. The current models for methane cycling in fresh waters use lake size, shape, nutrient status, temperature, and primary production to predict rates of methane production (19, 21, 65) but do not include methane production by any pathway in oxic water columns, even though this phenomenon has been observed in many lakes (4–6, 8, 10) and is known to be important in the ocean (12, 13). Because lakes may release as much as ~100 Tg methane globally each year, or ~20% of total annual natural methane emissions, which is more than the emissions from the world’s oceans (1, 2), understanding the environmental and biological factors contributing to methane emissions from freshwater systems is critical to making more-accurate predictions of both freshwater and global methane emissions (9, 18–21).

ACKNOWLEDGMENTS

We gratefully acknowledge Thomas Hanson at the Delaware Biotechnology Institute for the use of the GC-FID, Brewster Kingham at the University of Delaware Sequencing and Genotyping Center for sequencing the phnJ products, and Nicole Donofrio in the University of Delaware Plant & Soil Sciences Department for technical assistance with the qRT-PCR.

We declare that we have no conflicts of interest.

Author contributions: M.Y. analyzed metagenomic data, monitored growth and methane production, quantified gene expression rates using qPCR, made the figures, and contributed to the manuscript writing. C.H. provided essential logistical support for the field expedition and critical commentary on the manuscript. J.A.M. extracted DNA from field samples, analyzed metagenomic and biochemical data, supervised experiments, and coordinated the manuscript writing and editing.

FUNDING INFORMATION

This research was supported by the Department of Civil and Environmental Engineering at the University of Delaware but received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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