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Freshwater Bacteria Release Methane as a By-Product of Phosphorus Acquisition

Mengyin Yao,[a] Cynthia Henny,[b] Julia A. Maresca[a]

Department of Civil and Environmental Engineering, University of Delaware, Newark, Delaware, USA[a]; Research Center for Limnology, Indonesian Institute of Sciences, Cibinong, Indonesia[b]

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 *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.

Freshwater lakes may be responsible for as much as 20% of total annual methane emissions from natural sources (1–3), and supersaturation of methane in oxic water columns has been observed in many lakes (4–8). Some surface water methane is produced by methanogenesis in anoxic bottom waters, followed by upward diffusive transport or ebullition (9). Methanogenesis may also occur in oxic surface waters, either in anoxic microenvironments such as biofilms on phytoplankton or inside animal guts (5, 10) or potentially in oxygen-tolerant methanogens (11). Aerobic demethylation of organic compounds such as methylphosphonate (MPn) could also contribute to surface water methane release; however, this pathway has been observed only in marine systems (12–16). Because lakes may release as much methane to the atmosphere as the entire global ocean (8) and since the pathways for surface water methane production are poorly understood (17), a comprehensive characterization of the biological pathways responsible for methane production in these environments is critical to more-accurate predictions of global freshwater methane emissions (8, 9, 18–21).

Low-molecular-weight (LMW) phosphonates such as MPn, ethylphosphonate, and 2-aminoethylphosphonate (2-AEP) originate from the headgroups of phosphonolipids or are linked to exopolysaccharides and are produced in all three domains of life (22–25). Phosphonate biosynthetic pathways are encoded in ~5% of microbial genomes in freshwater environments (24), suggesting that phosphonate substrates could be available in these environments. Because breaking the C-P bond in phosphonates has a higher activation energy than breaking the equivalent phosphoester bond, phosphonate compounds tend to be chemically stable (26, 27). Organisms capable of cleaving the C-P bond could potentially utilize either the organic functional group or the phos-

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Address correspondence to Julia A. Maresca, jmaresca@udel.edu.

phorus, 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 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 with Titanium series reagents, as described earlier for water collected from the Lake Matano chemocline (40).

Analysis of Lake Matano metagenomic data. The raw reads were compared to the Clusters of Orthologous Groups (COG) database (41) using a BLASTx search with an E value cutoff of 10^{-10} and a minimum bit score of 50. The COG category of the best BLASTx hit was assigned to the raw reads. Odds ratios were calculated for each COG category identified 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 of the 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 were considered enriched (42), meaning that they are more highly represented in the metagenomic data than in the protein database. For comparison, odds ratios were calculated for the same COG categories in the metagenomic data from the chemocline in Lake Matano (40), where phosphate is not limiting (37).

Metagenomic reads were assembled into contigs using Newbler GS De Novo Assembler (software version 2.5.3) with the default settings for genomic projects. Contigs potentially including phosphonate degradation genes were identified by blasting the C-P lyase amino acid sequences from *Escherichia coli* (GenBank accession number NC_000913.3, locus tags b4092 to b4106 [https://www.ncbi.nlm.nih.gov/gene?LinkName=pubmed_gene&from_uid=1335942]) against the contigs. Contigs with at least three C-P lyase genes were analyzed using the open reading frame (ORF) finder (NCBI), BLAST (Basic Local Alignment Search Tool; NCBI), and CD-search (Conserved Domains; NCBI) to predict and annotate the genes on the contigs.

Analysis of other metagenomic data sets. The PhnJ amino acid sequences from *Pseudomonas stutzeri* (NCBI accession no. AAR91738) and *E. coli* (NCBI accession no. AAC77059.1) 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/mer/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 (NBRIP) without added P [per liter: $MgCl_2 \cdot 6H_2O$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KCl, 0.2 g; $(NH_4)_2SO_4$, 0.1 g; glucose, 10 g] (44). To test for MPn utilization, strains were grown in NBRIP 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, LM6-1, and LM-Y) capable of using MPn as their sole source of P. MPn, K_2HPO_4, or both were added to the P-free NBRIP medium as P sources. The pH of the medium was adjusted to 7.0 with 1 mM solutions of $NaHCO_3$ or acetic acid for the medium with added K_2HPO_4 or 1 mM solutions of $NaHCO_3$ or NaOH for medium with added MPn, as appropriate. Prior to measuring growth curves, cells were grown in NBRIP with K_2HPO_4 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 (OD_{660}) of ~0.05 and incubated horizontally at 30°C with shaking at ~150 rpm. Growth was monitored by measuring the OD_{660}.

For the initial growth experiments, MPn, KH_2PO_4, 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 K_2HPO_4 concentration was 0 μM, 4 μM, 10 μM, 50 μM, or 100 μM.

TABLE 1 Bacterial strains used in this study[a]

Strain designation	Closest related cultivated species	Ability to use: MPn	Ability to use: 2-AEP
LM-2	*Microbacterium testaceum*	−	−
LM-1	*Agrobacterium tumefaciens*	+	+
LM-5	*Rhizobium* sp.	+	+
LM6-1	*Agrobacterium tumefaciens*	+	+
LM-P	*Methylobacterium podarium*	−	−
2-LM-22	*Acinetobacter* sp.	−	−
LM-Y	*Pantoea ananatis*	+	+

[a] All strains listed were isolated from water collected from Lake Matano, Indonesia, as described previously (38). The ability to use methylphosphonate (MPn) or 2-aminoethylphosphonate (2-AEP) was assessed by monitoring growth in NBRIP 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.

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

Target (strain[s])	Application	Forward primer (sequence)	Reverse primer (sequence)	Source or references
phnJ	PCR	PhnJoc1 (AARGTRATMGAYCARGG)	PhnJoc2 (CATYTTYGGATTRTCRAA)	46, 47
phnJ1 (LM-1, LM-5, and LM6-1)	RT-PCR	phnJ-F1 (ACCATCATCCAGACGCGGCA)	phnJ-R1 (AGCTTGACGTGCATCAGGCC)	This study
phnJ2 (LM-Y)	RT-PCR	phnJ-F2 (TCAGACGCGTCACCGTATT)	phnJ-R2 (CTTCGTACAGTTTGACCTGC)	This study

[a] 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.

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 law constant, K_H (35):

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

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:

$$\%\,\text{repression} = \frac{(\text{total CH}_4)_{0\,\text{P}_i} - (\text{total CH}_4)_{n\,\text{P}_i}}{(\text{total CH}_4)_{0\,\text{P}_i}} \quad (2)$$

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 sample residue in the column. The detector temperature was 200°C, with hydrogen and airflows 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. 01-04-212; Scotty) and >99.9% methane (item no. GMT10015TC; Matheson) were used to prepare a standard curve of peak area and concentration (49.5 ppm, 99 ppm, 999 ppm, and 1,996.01 ppm). The methane concentration obtained based on the standard curves was then converted from parts per million to micromolar using the molar gas volume 24.87 liters mol^{-1} at 30°C for ideal gas at 1 atm of pressure.

Amplification and sequencing of *phnJ* in isolates. Initially, the primers PhnJoc1 and PhnJoc2 (Table 2) were used to amplify the *phnJ* genes from isolates (46, 47). Each PCR volume (25 μl) included 1× Mg-free buffer (item number D4545; Sigma-Aldrich), 1.5 mM $MgCl_2$, 0.25 μM each primer, 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.75 U SigmaTaq (Sigma-Aldrich item number D1806), and 0.5 μl (7 to 30 ng) template DNA. PCR conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 52.5°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min.

PCR products (~400 bp) were extracted from agarose gels and sequenced by Sanger sequencing at the University of Delaware Sequencing and Genotyping Center. Primers for RT-PCR were designed based on these sequences (phnJ-F1/phnJ-R1 for strains LM-1, LM-5, and LM6-1 and phnJ-F2/phnJ-R2 for strain LM-Y; see Table 2 for sequences).

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_2HPO_4, or both) and stored at −20°C in RNALater until processing. For RNA extraction, cells were stabilized in RNA Protect Reagent (catalog number 76506; Qiagen) and then digested with lysozyme (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 RNEasy 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 nuclease-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 RNA 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 subunits of the phosphonate transporter (*phnDEC*), activation of the phosphonate by ATP (*phnIGHL*), 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

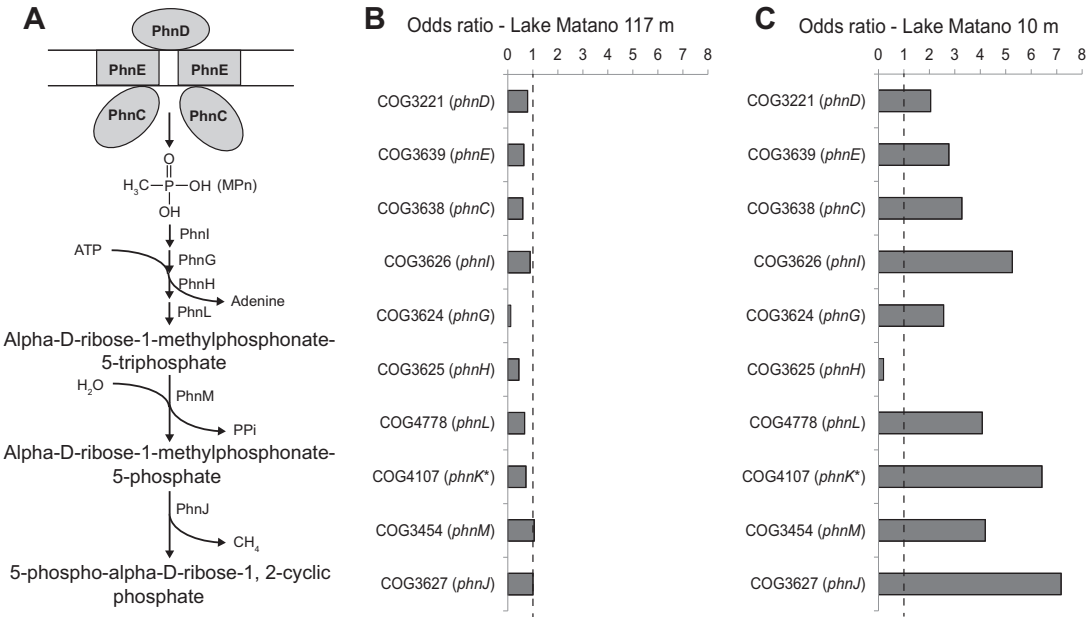

FIG 1 C-P lyase pathway in Lake Matano. (A) C-P lyase proteins that transport phosphonates (PhnCDE) and cleave the C-P bond (PhnGHIJKLM), based on data in references 48 and 66). PhnJ is responsible for the release of methane (or another organic group) from the intermediate. (B) Odds ratios of genes encoding 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).

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 from *E. coli* (50) were used to query the contigs, and Contig00268 and contig00117 were chosen for further annotation. Contig00268 has 5,357 nucleotides and carries a partial *phnI* sequence, *phnJKLM*, a predicted inositol monophosphatase (IMPase), and a partial gene encoding the ATP-binding motif of an ABC transporter. Contig00117 has 7,056 nucleotides and carries the phosphonate transporter-encoding *phnDCE*, along with an alkaline phosphatase gene and a partial penicillin-binding protein-encoding gene (Fig. 2).

Based on blastn analysis, Contig00268 has more than 83% coverage and 65% identity to gene clusters from three actinobacterial strains: *Rhodococcus opacus* strain PD630, *R. opacus* strain R7, and *Mycobacterium simiae* strain MO323. The three actinobacterial strains lack the accessory *phnNP* and the regulatory gene *phnO* that are part of the C-P lyase gene cluster in *E. coli* (50). The three actinobacterial strains all carry an inositol monophosphatase (IMPase)-encoding gene downstream of the C-P lyase gene clusters, and *M. simiae* encodes a penicillin-binding protein and an inositol 1-phosphate synthase upstream of the C-P lysase, while the *R. opacus* strains encode it downstream of the C-P lyase. In the two *R. opacus* strains, a predicted ABC transporter lies immediately downstream of the C-P lyase cluster (not shown). The best hit of blastn analysis for Contig00117 was also most similar to an *Actinobacterium*, *Isoptericola dokdonensis* DS-3, with 32% coverage and 71% identity. The predicted PhnE amino acid sequences in Contig00117, *R. opacus*, and *M. simiae* appear to have two PhnE domains (TIGR01097), in contrast to the *E. coli* PhnE, which has only one (Fig. 2). Several glyphosate- and phosphite-utilizing microbes have *phn* gene clusters with two copies of *phnE* (51, 52). In the gene cluster on Contig00117, these appear to be fused: no stop codon between the two *phnE* 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 phosphonate 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 phosphonate bond cleavage may occur in a variety of freshwater environments, including lakes, lake sediments, bogs, rivers, and streams.

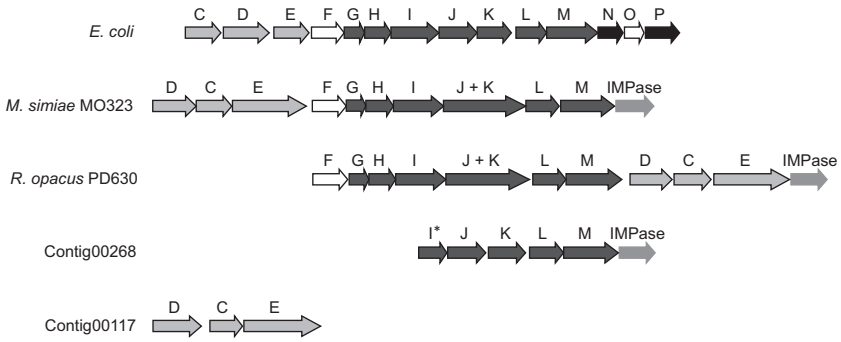

FIG 2 Phosphonate-related gene clusters in Lake Matano metagenomic data. Contig00268 has high identity (66%) to the gene cluster encoding the multienzyme C-P lyase complex, *phnIJKLM*, from *Rhodococcus opacus* strains PD630 and R7 (only *R. opacus* strain PD630 is shown) and organization similar to 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 (*phnCDE*) are light gray, regulatory genes (*phnFO*) are white, phosphonate degradation genes (*phnGHIJKLM*) are dark gray, and the *phnNP* accessory genes are black.

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 (5S, 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 methanogen genera *Methanococcus* and *Methanomicrobium*; however, when these reads were used as blastn 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 *Methanosarcina barkeri* DSM804 (RefSeq: WP_011305916.1) 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, K_2HPO_4, or both and reach stationary phase at approximately the same time (Fig. 3, dashed lines). These isolates do not

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

Sample source	Environment type	*phnJ*	Study (reference[s])
Lake Damariscotta, Maine, USA	Lake	+	ENA-PRJEB4844 (43)
Lake Ekoln, Sweden	Lake	+	ENA-PRJEB4844 (43)
Lake Erken, Sweden	Lake	−	ENA-PRJEB4844 (43)
Lake Mendota, Wisconsin, USA	Lake	+	ENA-PRJEB4844 (43)
Lake Vättern, Sweden	Lake	+	ENA-PRJEB4844 (43)
Sparkling Lake, Wisconsin, USA	Lake	+	ENA-PRJEB4844 (43)
Trout Bog Lake, Wisconsin, USA	Lake	+	ENA-PRJEB4844 (43)
Lake Erie, Canada/USA	Lake	+	GOLD project ID Gp0111910
Lake Sakinaw, British Columbia, Canada	Lake	+	GOLD project ID Gp0052015 (68, 69)
Lake Grosse Fuchskuhle, Germany	Mixed culture from lake	+	GOLD project ID: Gp0057572 (70)
Lake Superior, Canada/USA	Lake	+	GOLD study ID Gs0053068
Sandusky Bay, Ohio, USA	Lake	−	GOLD study ID Gs0053068 (71)
Lake Ontario, Canada/USA	Lake sediment	−	GOLD study ID Gs0053068
Lake Washington, Washington, USA	Lake sediment	+	GOLD study ID Gs0060820 (72)
Laguna de Carrizo, Spain	Lake sediment	−	GOLD study ID Gs0063259 (73)
Delaware River, Delaware/New Jersey, USA	River	+	GOLD study ID Gs0063440
Lake Michigan, Canada/USA	Lake	+	GOLD study ID Gs0110155 (74)
Crystal Bog, Wisconsin, USA	Bog	+	GOLD study ID Gs0110170
Loktak Lake, India	Lake	+	GOLD study ID Gs0111445 (75)
Notre Dame University, Indiana, USA	Anoxic pond sediments	+	GOLD study ID Gs011357
University of Edinburgh, Edinburgh, UK	Pond	+	GOLD study ID Gs0114295
Streams affected by fracking, Pennsylvania, USA	Stream	+	GOLD study ID Gs0114818 (76)

[a] 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.

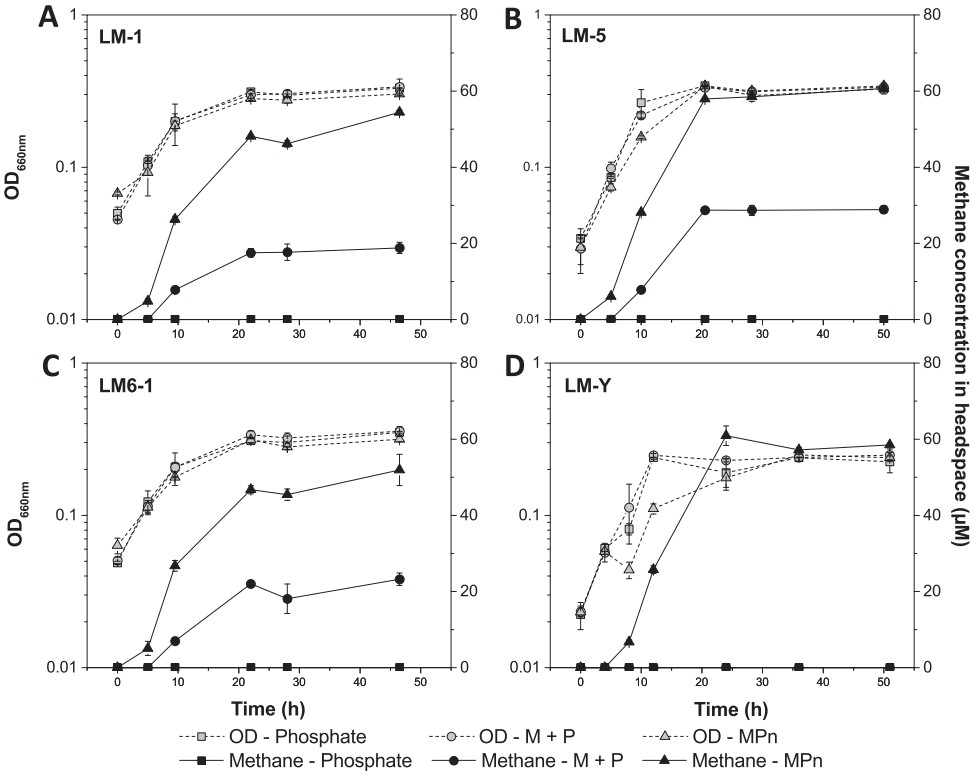

FIG 3 Growth and methane production of isolates grown with phosphate, MPn, or both. Cells were grown at 30°C in serum bottles in NBRIP medium (44) with MPn (0.2 mM), K_2HPO_4 (0.2 mM), or both (0.2 mM each) supplied as P sources. Prior to measuring growth curves, cells were grown in NBRIP with K_2HPO_4 as the only P source. Growth was measured by monitoring the optical density at 660 nm, and methane in the headspace was measured by gas chromatography coupled with a flame ionization detector (GC-FID), Agilent 7890A (G3440A). All isolates grew at similar rates on all P sources (dashed lines). Methane was produced by LM-1, LM-5, and LM6-1 when MPn was present (A, B, C, solid lines). LM-Y produced methane only when MPn was present and phosphate was absent (D, solid lines).

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 μM K_2HPO_4, strain LM-Y did not produce any methane (Fig. 3D). However, the other isolates produced some methane in the presence of K_2HPO_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 μM MPn is added, suggesting that at concentrations of >100 μM MPn, sufficient P is available to maximize growth yield in NBRIP and the excess P_i in solution may even inhibit further degradation of MPn (54).

Effect of phosphate on methane release from MPn. Addition of 200 μM phosphate to the cultures with initial MPn concentrations of 200 μM 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 μM MPn and 0 to 100 μM

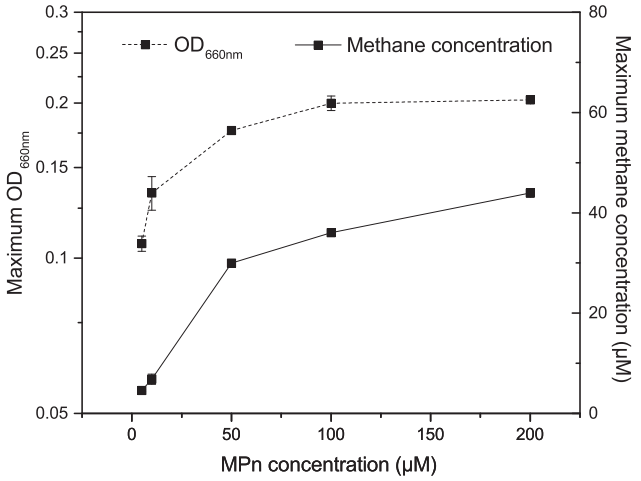

FIG 4 Growth and methane production of LM-Y grown on different MPn concentrations. MPn was added to final concentrations of 5 to 200 μM. Cells were grown ~36 h, and OD_{660} and methane concentrations in the headspace were measured at different times during incubation. The maximum methane concentration and OD_{660} were reached after about 24 h of incubation.

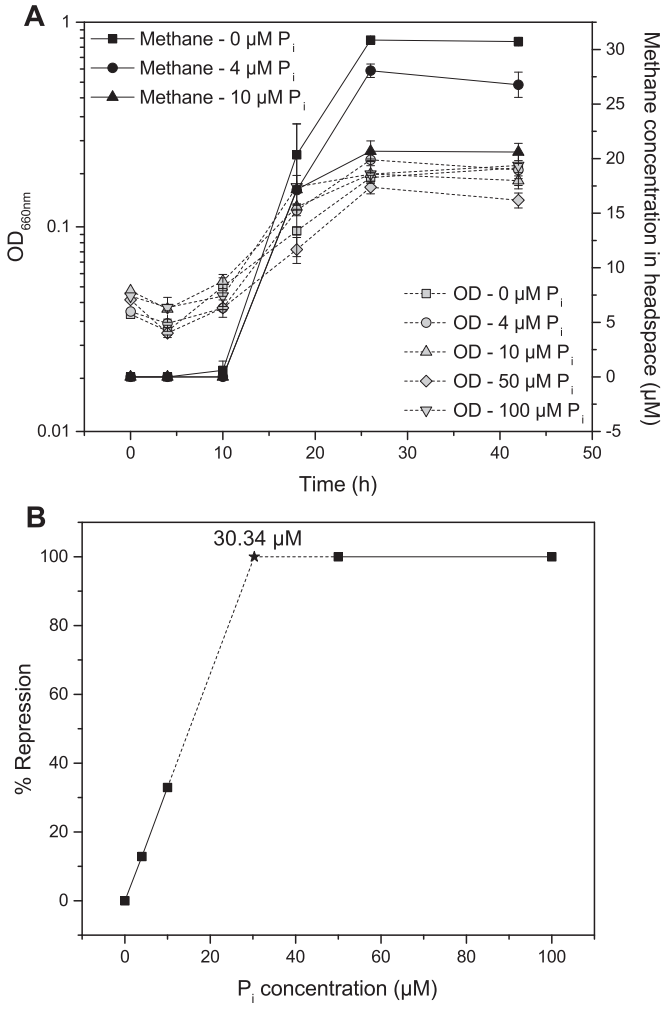

FIG 5 Phosphate represses methane production from MPn in LM-Y. All cultures were grown with 50 μM MPn and the indicated concentration of KH_2PO_4. Since no CH_4 production was observed when KH_2PO_4 was used, we assume that there is no inhibition in the absence of KH_2PO_4, and 100% inhibition in the presence of ≥50 μM KH_2PO_4. Percent repression was calculated as described in equation 2.

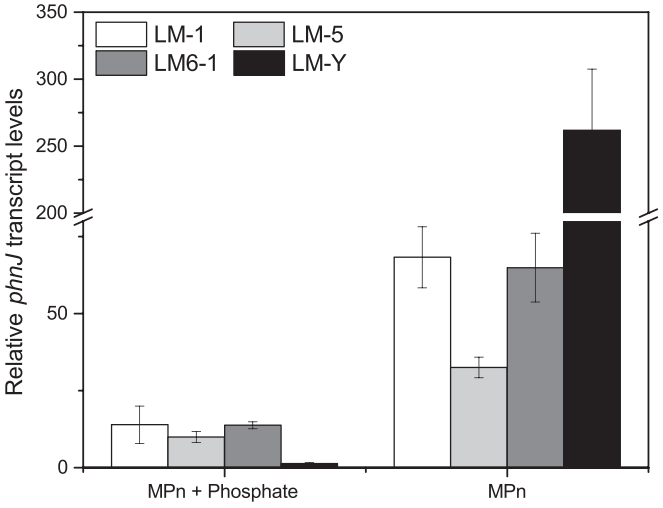

FIG 6 Transcript abundance of *phnJ* in isolates grown with phosphate, MPn, or both. Liquid subsamples (1.5 or 3.0 ml) were removed from all cultures during mid-exponential and stationary phases of growth on each P source (MPn, K_2HPO_4, or both). Transcript levels were calculated relative to *phnJ* 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 LM6-1, 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.

KH_2PO_4 (Fig. 5). Methane production was completely inhibited in cultures grown with 50 μM MPn and 50 or 100 μM KH_2PO_4 (Fig. 5A). In cultures grown with 50 μM MPn and 0 μM, 4 μM, or 10 μM P_i, the amount of methane produced decreased as KH_2PO_4 increased (Fig. 5A). The relationship between added KH_2PO_4 concentration and percent repression of methane production is linear (Fig. 5B), and we extrapolate that methane production would be completely repressed at P_i 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 phosphate, 0.2 mM MPn, 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 P_i. Similarly, the *phnJ* gene is expressed in these strains only in the presence of MPn, and its expression level is modulated by addition of P_i. 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

TABLE 4 Relative expression levels of *phnJ* expression in isolates grown under different conditions

Phase	P source[a]	Avg ± SD of relative levels of *phnJ* transcripts			
		LM-1	LM-5	LM6-1	LM-Y
Exponential growth	Phosphate	1.00	1.00	1.00	1.00
	MPn + phosphate	13.92 ± 6.05	9.97 ± 1.77	13.75 ± 1.09	1.35 ± 0.32
	MPn	68.26 ± 9.94	32.50 ± 3.33	64.90 ± 11.16	261.81 ± 45.67
Stationary	Phosphate	0.35 ± 0.06	0.06 ± 0.01	0.19 ± 0.06	0.17 ± 0.01
	MPn + phosphate	0.6 ± 0.01	0.54 ± 0.21	0.52 ± 0.04	0.17 ± 0.03
	MPn	0.94 ± 0.32	1.60 ± 0.28	0.70 ± 0.04	1.36 ± 0.06

[a] Relative levels of *phnJ* transcripts in cells grown with phosphate (0.2 mM), phosphate + MPn (0.2 mM each), or MPn alone (0.2 mM) were determined, normalized based on total RNA, and compared to levels in cells grown on phosphate during exponential growth phase. For each sample, two biological replicates and two technical replicates were analyzed.

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_i 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).

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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.

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