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Cometabolic Vinyl Chloride Degradation at Acidic pH Catalyzed by Acidophilic Methanotrophs Isolated from Alpine Peat Bogs

Munjeong Choi,[#] Taeho Yun,[#] Min Joon Song, Jisun Kim, Byoung-Hee Lee, Frank E. Löffler, and Sukhwan Yoon*

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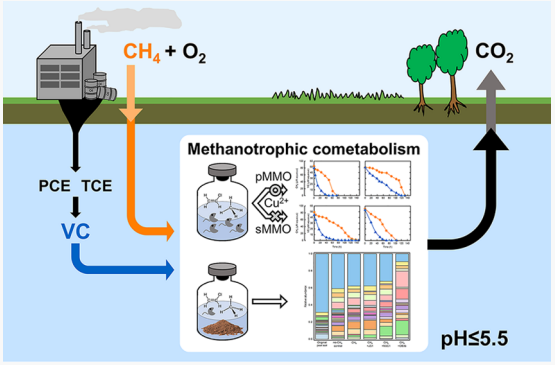
ABSTRACT: Remediation of toxic chlorinated ethenes via microbial reductive dechlorination can lead to ethene formation; however, the process stalls in acidic groundwater, leading to the accumulation of carcinogenic vinyl chloride (VC). This study explored the feasibility of cometabolic VC degradation by moderately acidophilic methanotrophs. Two novel isolates, *Methylomonas sp.* strain JS1 and *Methylocystis sp.* strain MJC1, were obtained from distinct alpine peat bogs located in South Korea. Both isolates cometabolized VC with CH_4 as the primary substrate under oxic conditions at pH at or below 5.5. VC cometabolism in axenic cultures occurred in the presence (10 μM) or absence (<0.01 μM) of copper, suggesting that VC removal had little dependence on copper availability, which regulates expression and activity of soluble and particulate methane monooxygenases in methanotrophs. The model neutrophilic methanotroph *Methylosinus trichosporium* strain OB3b also grew and cometabolized VC at pH 5.0 regardless of copper availability. Bioaugmentation of acidic peat soil slurries with methanotroph isolates demonstrated enhanced VC degradation and VC consumption below the maximum concentration level of 2 μg L^{-1}. Community profiling of the microcosms suggested species-specific differences, indicating that robust bioaugmentation with methanotroph cultures requires further research.



INTRODUCTION

Vinyl chloride (VC) is a human carcinogen often present in groundwater aquifers at concentrations above the maximum contaminant level (MCL) of 2 μg L^{-1}.[1] The major source of VC in soil and groundwater is reductive dechlorination of tetrachloroethene (PCE) and trichloroethene (TCE), which are common groundwater pollutants.[2] Members of the class *Dehalococcoidia* expressing VC reductive dehalogenases can reduce VC to innocuous ethene and are the basis for bioremediation at sites impacted with chlorinated ethenes.[3−6] Although monitored natural attenuation and enhanced anaerobic bioremediation have been successfully implemented at many contaminated sites, incomplete reductive dechlorination and VC stalls are not uncommon.[7−10] A particular challenge is sites with low pH groundwater because the known *Dehalococcoidia* do not grow under acidic pH conditions.[8,11] The pH of groundwater depends largely on the chemical composition of the local bedrock and soil, and moderately acidic pH (e.g., 5.0−6.0) is commonly observed.[12−15] Although microbial PCE and TCE reductive dechlorination to *cis*-1,2-dichloroethene at low pH has been demonstrated, sustained in situ reductive dechlorination to ethene in low pH groundwater has not been observed, presumably because organohalide-respiring *Dehalococcoidia* cannot grow at pH < 5.5.[8,9,11] Aggravating the pH problem is the release of protons during reductive dechlorination, which can contribute to further acidification.[16] Engineering approaches for buffering groundwater aquifers exist; however, the biogeochemical and hydrological complexity of in situ subsurface environments limits their implementation.[11,12,17]

VC degradation has also been observed under oxic conditions. Some aerobic etheneotrophs utilize VC as the sole electron and carbon source at circumneutral pH, and their presence has been linked to in situ VC oxidation in groundwater.[18,19] VC-oxidizing etheneotrophs of the phyla *Actinobacteria* and *Proteobacteria* express alkene monooxygenase (AkMO) and epoxyalkane:coenzyme M transferase (EaCoMT) to metabolize VC.[20,21] A recent study reported recovery of *etnC* (encoding the alpha subunit of AkMO) and *etnE* (encoding the alpha subunit of EaCoMT) genes and transcripts from acidic (pH < 6), VC-contaminated groundwater; however, no direct evidence of etheneotrophic VC degradation activity in acidic aquifers has been reported to

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date.[22] Laboratory studies investigating etheneotroph activity at acidic pH are currently lacking.

Methanotrophs utilizing CH_4 as the sole source of energy and carbon and O_2 as the electron acceptor can also cometabolically oxidize VC.[23] Methanotrophic cometabolic oxidation of VC is mediated by both soluble (sMMO, with the catalytic subunit encoded by *mmoX*) and particulate methane monooxygenases (pMMO, with the presumed catalytic subunit encoded by *pmoA*), the enzyme systems catalyzing CH_4 oxidation to CH_3OH.[24,25] Methanotrophic oxidation of chlorinated ethenes has been previously examined at circumneutral pH with neutrophilic isolates such as *Methylosinus trichosporium* strain OB3b, *Methylococcus capsulatus* strain Bath, and *Methylomicrobium album* strain BG8.[24−26] Both sMMO and pMMO, reciprocally regulated by Cu availability, can catalyze cometabolic oxidation of chlorinated ethenes, albeit with different kinetics.[24,25] In general, sMMO, only expressed in strict absence of Cu, cooxidizes a broader range of nonmethane substrates at substantially higher rates than pMMO.[24,25]

Detailed studies of methanotrophic cometabolism of VC under acidic pH conditions are lacking. A recent laboratory study using a groundwater enrichment cultures demonstrated cometabolic degradation and suggested that VC cooxidation may be feasible under acidic pH conditions.[27] Diverse acidophilic methanotrophs inhabit various environments, ranging from extremely acidophilic geothermal sites to moderately acidophilic peat bogs.[28−31] The moderately acidophilic proteobacterial methanotrophs are promising candidates for bioremediation because their pH optima range between 5.0 and 6.2, coinciding with the groundwater pH observed in many aquifers.[22,32] Isolates affiliated with phylogenetically divergent methanotroph genera *Methylomonas*, *Methylocystis*, *Methylocapsa*, and *Methylocella* have been experimentally verified to grow with CH_4 under acidic pH conditions.[33−36] These acidophilic proteobacterial methanotrophs share highly similar MMO amino acid sequences with the neutrophilic methanotrophs, which have demonstrated to cometabolize chlorinated ethenes at circumneutral pH, suggesting that acidophilic methanotrophs expressing sMMO and/or pMMO may be capable of VC degradation.[37] Bioaugmentation and in situ stimulation of methanotrophic cometabolism are established technologies and could be readily implemented to remediate VC plumes in acidic groundwater.[38,39]

This study explores cometabolic VC degradation under low pH conditions by two novel acidophilic methanotrophs, *Methylomonas sp.* strain JS1 and *Methylocystis sp.* strain MJC1, isolated from acidic alpine peat bogs in South Korea, and a well-studied neutrophilic laboratory methanotroph *Methylosinus trichosporium* strain OB3b. Axenic culture and peat soil microcosm studies demonstrate effective VC cometabolism under low pH conditions and reveal that this activity can be mediated by acidophilic and neutrophilic methanotrophs. The new findings suggest that the stimulation of methanotrophic activity is a potential remedial approach to treat VC plumes in low pH aquifers.

MATERIALS AND METHODS

Peat Sampling and Isolation of the Acidophilic Methanotrophs. Peat samples were collected from two alpine peat bogs in South Korea: the Sohwangbyeonsan peat bog located at the shoulder of Mt. Odae (N 37° 46′ 09″ E 128° 41′ 07″, altitude of 1170 m) and the Moojechi peat bog located at the shoulder of Mt. Jeongjok (N 35° 27′ 43″ E 129° 8′ 34″, altitude of 748 m). Both peat bogs are protected areas with no history of anthropogenic chlorinated solvent contamination. The cover soil was carefully removed, and peat was sampled from 10−20 cm depth. Discernable plant materials were carefully removed, and the peat was transferred into Mason jars, which were immediately filled with peat bog water and closed. The samples were transported to the laboratory in coolers filled with ice and stored at 4 °C. The Sohwangbyeonsan and Moojechi peat bog samples were moderately acidic at the time of sampling, with measured pH values of 5.70 and 5.79, respectively.

Cultivation of Methanotrophs. Diluted nitrate mineral salts (DNMS) medium was used for all experiments performed in this study.[40] The DNMS medium contained, per liter, 0.2 g of $MgSO_4{\cdot}7H_2O$, 0.2 g of KNO_3, 0.04 g of $CaCl_2{\cdot}2H_2O$, 0.02 mL of a 3.8% (w/v) Fe(III)-EDTA solution, 0.1 mL of 0.1% (w/v) $Na_2MoO_4{\cdot}2H_2O$ solution, and 0.2 mL of a concentrated trace element solution.[41] The medium was buffered to the target pH with Na_3PO_4 and K_2HPO_4, with their respective concentrations calculated for the total phosphate concentration of 5 mM. After autoclaving, 1 L of DNMS medium received 5 mL of a filter-sterilized 200-fold concentrated vitamin stock solution.[41] Unless otherwise mentioned, the DNMS medium was distributed to 250 mL glass serum bottles (Wheaton, Millville, NJ) in 40 mL aliquots. The serum bottles used for the VC degradation experiments were washed in a 2.2 M HNO_3 bath overnight before use to remove any adsorbed Cu. The Cu concentration in the DNMS medium without added Cu did not exceed 0.01 μM.[42] After the bottles were sealed with butyl rubber stoppers (Geo-Microbial Technologies, Inc., Ochelata, OK), 20% of the headspace was replaced with >99.999% CH_4 gas (Deokyang, Ulsan, South Korea), which was provided as the sole electron donor and carbon source. After inoculation, the bottles were incubated on a shaker set to 200 rpm in dark at room temperature (25 °C).

Enrichment, Isolation, and Characterization of Acidophilic Peat Bog Methanotrophs. Batch enrichment cultures were prepared by adding 10 g (wet weight) peat soil into 40 mL DNMS medium dispensed in 250 mL glass serum bottles. Headspace of the culture bottles initially consisted of 20% CH_4 and 80% air (v/v). The serum bottles were incubated at room temperature with shaking at 200 rpm until the CH_4 concentration dropped to below 10% v/v. The enrichments were then diluted 10-fold into serum bottles with fresh DNMS medium. After CH_4 consumption was verified in the initial transfer cultures, 0.3 mL aliquots served as the inoculum for dilution-to-extinction cultivation. A 10-fold serial dilution series of each methanotrophic enrichment was prepared in 22-mL glass test tubes sealed with butyl rubber stoppers, each containing 3 mL of fresh DNMS medium and 20% v/v CH_4 in the headspace. After incubation for 10 days, aliquots of the highest dilution with visible turbidity and evidence for CH_4 consumption were spread onto solid DNMS medium (1.5% w/v agar) with the pH adjusted to 5.0 with 5 mM phosphate buffer. The agar plates were incubated inside a GasPak 150 jar filled with 1 atm mixed gas with a 1:1 (v/v) CH_4-to-air ratio (BD, Franklin Lakes, NJ). After three consecutive transfers of isolated colonies on solid medium, a single colony was inoculated into fresh liquid DNMS medium with CH_4 as the sole carbon source. The primer pair 27f-1492r was used to amplify 16S rRNA gene fragments, which were

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sequenced to obtain a line of evidence for culture purity (indicated by the absence of the contaminating peak in the electropherogram) and reveal the isolates' phylogenetic affiliations.

Growth and physiological characteristics of the two methanotroph isolates were examined in batch culture incubations with and without 10 μM Cu^{2+} provided as $CuCl_2$. To determine the pH optima, growths of the methanotroph isolates were monitored in medium with the pH adjusted between 4.5 and 7.0 in 0.5 pH unit increments. In incubations showing exponential growth, specific growth rates were calculated. Maximum CH_4 consumption rates ($V_{max,app}$) were measured at the optimum medium pH for each isolate. Batch cultures of strain JS1 and strain MJC1 were grown to OD_{600nm} = 0.2, and cells were collected by centrifugation (10 min, 10,000 × g) and the pellets were suspended in 40 mL of fresh medium in 250 mL serum bottles. To prevent synthesis of new proteins, chloramphenicol was added at a final concentration of 30 μg mL^{-1}. After sealing the bottles, 1% of the headspace was replaced with CH_4, and the serum bottles were agitated at 200 rpm at room temperature. CH_4 concentrations in the headspace were monitored to calculate the linear CH_4 consumption rates ($V_{max,app}$).

Quantification of *pmoA* and *mmoX* Transcripts in Methanotroph Isolates. Copper regulation of *pmoA* and *mmoX* transcription was verified in the two axenic methanotroph cultures using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). *Methylomonas sp.* strain JS1 and *Methylocystis sp.* strain MJC1 cultures were grown in DNMS medium with an initial pH of 5.0 and 5.5, respectively, and cells were harvested during late exponential growth phase (OD_{600nm} between 0.15 and 0.2). RNA extraction and RT-qPCR followed the established procedures (see Supplementary Information).[43]

Genome Sequencing and Bioinformatic Analysis. The genomes of the two new acidophilic methanotroph isolates were sequenced and analyzed. The genome of *Methylomonas sp.* strain JS1 was sequenced with a PacBio RS II System (Menlo Park, CA) at Macrogen Inc. (Seoul, South Korea). The quality-trimmed raw data were assembled *de novo* into a closed genome using the RS HGAP Assembly v3.0 software.[44] The genome of *Methylocystis sp.* strain MJC1 was sequenced with a HiSeq 2500 Illumina sequencer at Cosmogentech Inc. (Seoul, South Korea). The draft genome was assembled *de novo* from the quality-trimmed raw data using the SPAdes v3.13.0 software, and the completeness of the draft genome was assessed with CheckM software.[44,45] The genomes were annotated with Prokka v.1.12b and the RAST server.[46,47] The annotated genome sequences of strain JS1 and strain MJC1 were deposited in NCBI's GenBank database with BioSample accession numbers SAMN13025342 and SAMN13025341, respectively.

For phylogenetic analyses, the sequences of 16S rRNA gene and the catalytic subunits of the two key enzymes of aerobic methanotrophy, PmoA and MmoX, of the new isolates were compared to the respective sequences of known proteobacterial methanotrophs deposited in NCBI database. The full 16S rRNA gene and the PmoA and MmoX sequences of 19 gammaproteobacterial methanotrophs and 3 alphaproteobacterial methanotrophs, each affiliated with a distinct genus, were downloaded from the NCBI database (Table S1). These 16S rRNA gene sequences and PmoA and MmoX amino acid sequences were aligned using MUSCLE, and maximum-likelihood phylogenetic trees were constructed using MEGA v10.1.7 using the default settings.[48,49] The digital DNA−DNA hybridization (dDDH) values between the genomes of the new isolates and 27 proteobacterial methanotroph genomes were calculated using the Genome-to-Genome Distance Calculator v.2.1.[50] Additionally, the average nucleotide identity (ANI) values were calculated using JSpecies v3.4.8 to confirm the uniqueness of strain JS1 and strain MJC1 genomes.[51,52]

VC Cometabolism in Axenic Cultures and Soil Slurry Microcosms. Cometabolic VC degradation was tested in axenic cultures of the new acidophilic isolates *Methylomonas sp.* strain JS1 and *Methylcystis sp.* strain MJC1 and the neutrophile *Methylosinus trichosporium* strain OB3b. The latter isolate has been demonstrated to cometabolize VC at circumneutral pH.[24] VC (10% in N_2, v/v, Deokyang Co., Ulsan, South Korea) was added to the culture bottles to achieve an aqueous concentration of 50 μM, which is representative of heavily contaminated groundwater.[19] The volume of the VC gas to be added was calculated using the dimensionless Henry's law constant of 1.08 at 25 °C.[53] For each examined methanotroph strain, culture medium was prepared with and without 10 μM $CuCl_2$. Methane displaced 6% (v/v) of the air headspace to result in a dissolved CH_4 concentration of 85 μM (based on Henry's law constant of 29.2 at 25 °C). The VC batch culture degradation experiments were performed in triplicate, and the cell density (OD_{600nm}), pH, and the concentrations of VC and CH_4 were monitored until all CH_4 had been depleted. To verify sMMO expression in the Cu-free incubations, the naphthalene assay was performed at the end of the incubation period following established procedures.[54]

Cometabolic VC degradation was also tested in Sohwangbyungsan peat slurry microcosms. Triplicate peat slurry samples were prepared by adding 10 g (wet weight) of peat to 40 mL of DNMS medium in 250 mL serum bottles adjusted to pH 5.0. CH_4 and VC were added to the sealed culture bottles to achieve aqueous phase concentrations of 85 μM (525 μmol per bottle) and 50 μM (13.3 μmol per bottle), respectively. The culture vessels were agitated (200 rpm) at 25 °C in the dark, and CH_4 and VC concentrations were monitored until CH_4 had been depleted. VC degradation was also tested in triplicate peat slurry microcosms augmented with *Methylomonas sp.* strain JS1, *Methylocystis sp.* strain MJC1, or *Methylosinus trichosporium* strain OB3b. The axenic methanotroph cultures were grown in DNMS medium with 10 μM $CuCl_2$, and 0.4 mL of culture suspensions (OD_{600nm} = 0.1) served as inocula for the microcosm incubations.

Microbial Community Analysis. To identify methanotrophs responsible for VC degradation in the Sohwangbyungsan peat microcosms, samples were collected immediately after VC degradation was complete and subjected to microbial community analysis via 16S rRNA gene amplicon sequencing. Additionally, the microbial composition of the original peat soil and control microcosms incubated with VC but without CH_4 were also analyzed. DNA was extracted from 0.25 g of soil or 0.5 mL of slurry samples using the DNeasy PowerSoil DNA Isolation kit (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer. MiSeq sequencing of the 16S rRNA gene amplicons generated with primer pair 926f/1392r primers was carried out at Cosmogentech Inc. (Seoul, South Korea).[55] The raw sequences were deposited in the NCBI SRA database (BioProject accession number: PRJNA575108). Quality screening of the raw sequences was performed using Trimmomatic v0.36, and the trimmed

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Table 1. Physiological Characterization of *Methylomonas* sp. Strain JS1 and *Methylocystis* sp. Strain MJC1 at Cu-Supplemented and Cu-Free Incubation Conditions

	copper amendment (μM)	$V_{max,app}$ for CH_4 (nmoles/min/mg protein)	*pmoA* transcription $\left(\log \frac{\#transcripts}{\#cells}\right)$	*mmoX* transcription $\left(\log \frac{\#transcripts}{\#cells}\right)$
Methylomonas sp. JS1	0	140 (7.0)[a]	1.74 (0.20)[b]	0.50 (0.22)
	10	231 (16.5)	2.41 (0.38)	−1.61 (0.09)
Methylocystis sp. MJC1	0	130 (5.4)	0.06 (0.32)	0.45 (0.43)
	10	134 (5.7)	2.14 (0.25)	−0.78 (0.23)

[a]The numbers in the parentheses are the errors associated with linear regression. [b]The numbers in the parentheses are standard deviations of biological replicates.

sequences were analyzed with the QIIME2 v2020.8 pipeline.[56,57] The reads were organized into amplicon sequence variants (ASVs) using the DADA2 plugin, and the taxonomic assignments used the Silva 138 SSU database.

Analytical Methods. The concentrations of CH_4 and VC in the headspace were measured with a GCMS-QP2010 gas chromatograph-mass spectrometer (GC−MS; Shimadzu, Kyoto, Japan) equipped with a RT-Q-BOND column (10 μm film thickness, 30 m × 0.32 mm inner diameter). Headspace samples were manually injected into the GC−MS using a 1700-series gas-tight glass syringe (Hamilton Company, Reno, NV). The detection limits for CH_4 and VC measurements were < 0.1 and < 0.01 μmol L^{-1} (gas phase concentrations at 25 °C), respectively. The protein contents of cultures were determined using the Bio-Rad protein assay kit (Hercules, CA). For each measurement, a 1-mL volume of cell suspension was centrifuged at 15,000 × g for 3 min, and the pellet was disrupted with 50 mg of 0.1 mm glass beads in a Bead Ruptor 12 (Omni International, Kennesaw, GA) for three cycles consisting of 10 min of operation at the maximum speed interrupted by 2 min intervals for sample cooling on ice. After centrifugation at 13,000 × g for 2 min, the protein content of the supernatant was measured using the protein assay kit. The linear relationships between protein concentrations and OD_{600nm} values of cell cultures were used to estimate the protein concentrations of growing methanotroph cultures.[58]

Rate Calculations and Statistical Methods. The CH_4 and VC removal rates were calculated from the progression curves of CH_4 and VC removal and the protein concentrations estimated from OD_{600nm} readings. A moving window with three consecutive time points (t_{k-1}, t_k, and t_{k+1}) was used to calculate the reaction rate at t_k. The amounts of CH_4 or VC in the reaction vessels were calculated using the headspace concentrations and their respective Henry's constants at 25 °C. The rate of removal was calculated from the slope of the linear regression line relating the time and the amount of CH_4 or VC and normalized with the protein concentration at t_k. The propagation of the error method was used to determine the uncertainty of these calculated reaction rates. One-sample *t*-tests were performed to statistically compare cell densities or substrate concentrations at two different time points. Pairwise comparisons across two different incubation conditions or organisms were performed using the two-sample *t*-tests. Statistical tests for $n > 2$ samples were performed with one-way analysis of variance and Tukey's honestly significant difference post hoc tests. All linear regression analyses and statistical tests were performed using OriginPro 2019 software package v9.6.0.172.

RESULTS

Isolation of Acidophilic Methanotrophs. *Methylomonas sp.* strain JS1 and *Methylocystis sp.* strain MJC1 were isolated from the peat soils collected from the Sohwangbyeongsan peat bog and the Moojechi peat bog, respectively. Both isolates grew with CH_4 in DNMS medium at pH 5.0. The phylogenetic analysis affiliated *Methylomonas sp.* strain JS1 with the class *Gammaproteobacteria*. The 16S rRNA gene of strain JS1 was 100% identical to *Methylomonas sp.* strain M5 previously isolated from an acidic peat bog.[36] A single-copy canonical *pmoCAB* operon and a *mmoXYBZDC* operon were identified on the strain JS1 genome. The PmoA and MmoX sequences of strain JSI shared >90% amino acid identity with the respective proteins of known methanotrophs of the class *Gammaproteobacteria* (Figure S1). A *pxmABC* operon encoding a pMMO-like monooxygenase with unknown function was also identified.[37] The genes encoding the enzymes constituting the downstream C1 metabolic pathway, which includes methanol dehydrogenases, formaldehyde dehydrogenases, and formate dehydrogenases, have also been identified in the genome (summarized in Table S3). A complete suite of genes encoding the enzymes constituting the ribulose monophosphate pathway was identified in the genome, consistent with the taxonomic grouping of strain JS1. Features of the strain JS1 genome are summarized in Table S3.

Analysis of the draft genome of *Methylocystis sp.* strain MJC1 revealed an affiliation with the class *Alphaproteobacteria*. A complete *pmoCAB* operon and an incomplete *pmo* operon truncated at 348 bp upstream of the 5′ end of the *pmoA* gene, as well as two separated *pmoC* genes, were identified. One of the separated *pmoC* genes was truncated at the 3′ end, suggesting that this copy is likely to form another *pmoCAB* operon with the truncated *pmo* operon. Similar to *Methylomonas sp.* strain JS1, strain MJC1 possessed a complete *mmoXYBZDC* operon. The predicted PmoA and MmoX sequences both share >97% amino acid identity with the respective proteins of previously characterized *Methylocystis* strains, consistent with the phylogenetic affiliation of the strain based on 16S rRNA gene sequence analysis (Figure S1). The genes encoding the downstream C1 metabolic pathway and the complete serine pathway, a characteristic feature of methanotrophs affiliated with the class *Alphaproteobacteria*, were also identified (Table S4). Features of the strain MJC1 genome are summarized in Table S4.

Although 16S rRNA gene and translated PmoA sequences of the new isolates were virtually identical to previously described sequences, genome-wide comparisons with 27 methanotroph genomes indicated that both *Methylomonas sp.* strain JS1 and *Methylocystis sp.* strain MJC1 represent novel species. The dDDH and ANI values calculated between the two genomes

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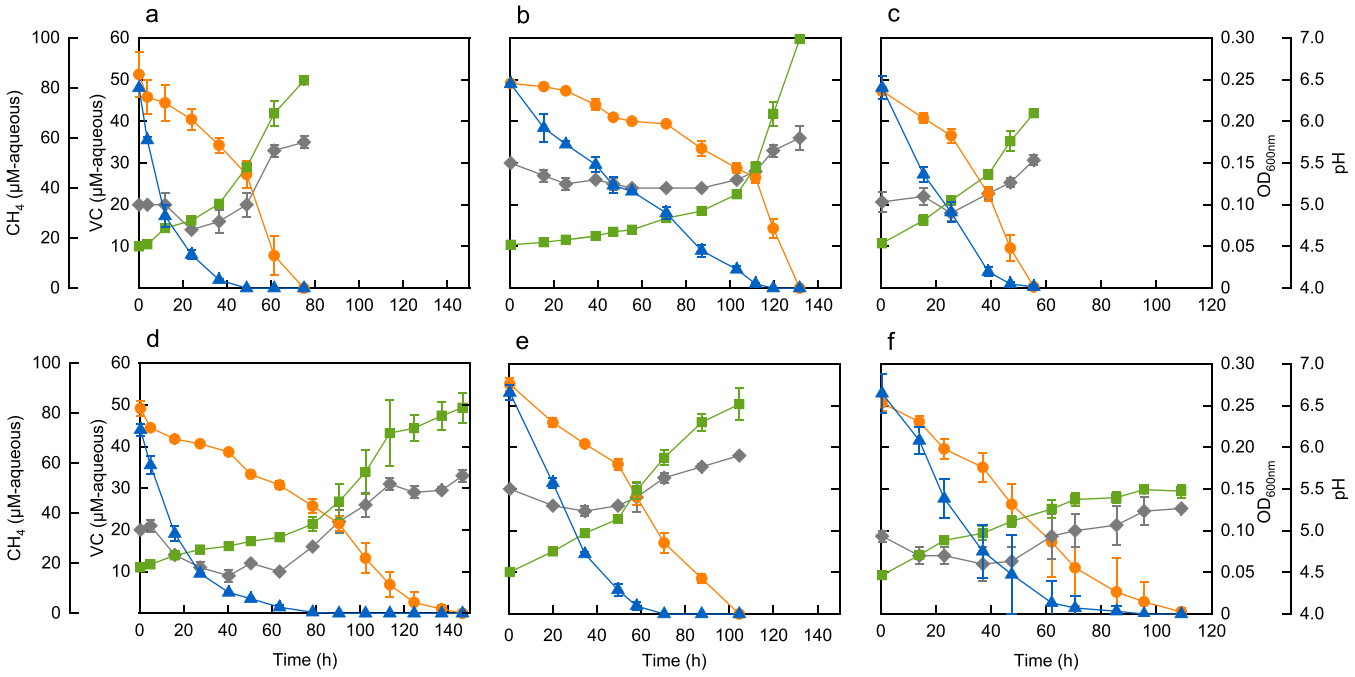

Figure 1. CH_4 consumption and cometabolic VC degradation in axenic cultures of *Methylomonas* sp. strain JS1 (a, d), *Methylocystis* sp. strain MJC1 (b, e), and *Methylosinus trichosporium* strain OB3b (c, f). Incubations with 10 μM Cu (a-c) and 0 μM Cu (d-f) were examined to assess the effect of sMMO activity on VC degradation in the methanotroph cultures. Dissolved concentrations of CH_4 (orange circle) and VC (blue triangle), OD_{600nm} (green square), and pH (gray rhombus) were monitored until CH_4 was depleted. The average values of biological triplicates are presented with the error bars representing their standard deviations.

and other methanotroph genomes were below the single-species thresholds, that is, 70% for dDDH and 95% for ANI (Figures S2 and S3).

Strain JS1 exhibited exponential growth in pH 5.0 medium with and without Cu supplementation, and statistically indistinguishable specific growth rates of 0.079 ± 0.009 h^{-1} and 0.070 ± 0.013 h^{-1}, respectively, were calculated. The culture reached maximum cell densities at pH 5.0 (SI Figure S4). Strain MJC1 also grew at pH 5.0 but exhibited the highest growth rates and maximum cell densities at pH 5.5 and pH 6.0. With Cu provided, specific growth rates of 0.063 ± 0.012 h^{-1} and 0.059 ± 0.005 h^{-1} were determined at pH 5.5 and 6.0, respectively ($p > 0.05$). In medium without Cu amendment, higher specific growth rates of 0.094 ± 0.005 h^{-1} and 0.091 ± 0.001 h^{-1} were determined at pH 5.5 and 6.0, respectively ($p > 0.05$). Neither organism grew at pH 4.5, and their growth was substantially slower at pH 7.0, indicating that both isolates were moderately acidophilic.

The RT-qPCR analysis confirmed that Cu availability affected the expression of *pmoA* and *mmoX* in the new isolates (Table 1). In strain JS1 cultures grown at pH 5.0 in the presence of 10 μM Cu, the average *pmoA* transcript copy number per cell was 4.7-fold higher and the *mmoX* transcript copy number per cell was 135-fold lower compared to the replicate cultures grown without Cu. The RT-qPCR results suggested near-complete repression of *mmoX* transcription by Cu, while *pmoA* was still expressed at substantial levels in cultures without Cu. Similar observations were made in *Methylocystis sp.* strain MJC1 cultures grown at pH 5.5 with and without Cu; however, the *pmoA* transcript copy number per cell was 2 orders of magnitude lower in cultures grown without Cu supplement compared to the cells grown with 10 μM Cu, indicating a strong effect of Cu on *pmoA* expression in strain MJC1. The maximum CH_4 consumption rates ($V_{max,app}$) observed in chloramphenicol-treated resting cell assays of strain JS1 (pH 5.0) and strain MJC1 (pH 5.5) were 231 ± 17 and 134 ± 6 nmol min^{-1} (mg protein)$^{-1}$, respectively, under the Cu-supplemented condition and 140 ± 7 and 130 ± 5 nmol min^{-1} (mg protein)$^{-1}$, respectively, under Cu-free condition (Table 1, Figure S5). Copper availability did not significantly affect $V_{max,app}$ in strain MJC1; however, the $V_{max,app}$ observed in strain JS1 cultures was substantially (1.7-fold) higher with Cu than without.

Cometabolic VC Degradation by the New Methanotroph Isolates at Acidic pH. The two acidophilic methanotroph isolates and the neutrophilic model methanotroph *Methylosinus trichosporium* strain OB3b were all capable of degrading VC to below the detection limit under moderately acidic incubation conditions, that is, pH of 5.0 or 5.5 (Figure 1, Figure S6). During exponential growth of *Methylomonas sp.* strain JS1 with 85 μM CH_4, 50 μM VC was degraded to below the detection limit under both Cu-supplemented and Cu-free incubation conditions. Negative naphthalene assay confirmed inactivity of sMMO in the Cu-supplemented cultures; however, it needs to be noted that inactivity of pMMO cannot be verified in the Cu-free cultures due to the lack of reliable experimental method for separately assessing pMMO activity. The specific growth rate during the exponential phase was slightly higher with Cu (0.026 ± 0.002 h^{-1}) than without (0.020 ± 0.003 h^{-1}), and the CH_4 consumption rate was higher under the Cu-supplemented condition throughout the growth period (Figure 1a,b, Figure S6d). The Cu-free culture needed more time (90.5 h) than the Cu-supplemented culture (49 h) for complete removal of VC, apparently due to the slower growth without Cu supplementation. Notably, the maximum observed biomass-normalized VC degradation rates were significantly higher in the Cu-supplemented cultures with

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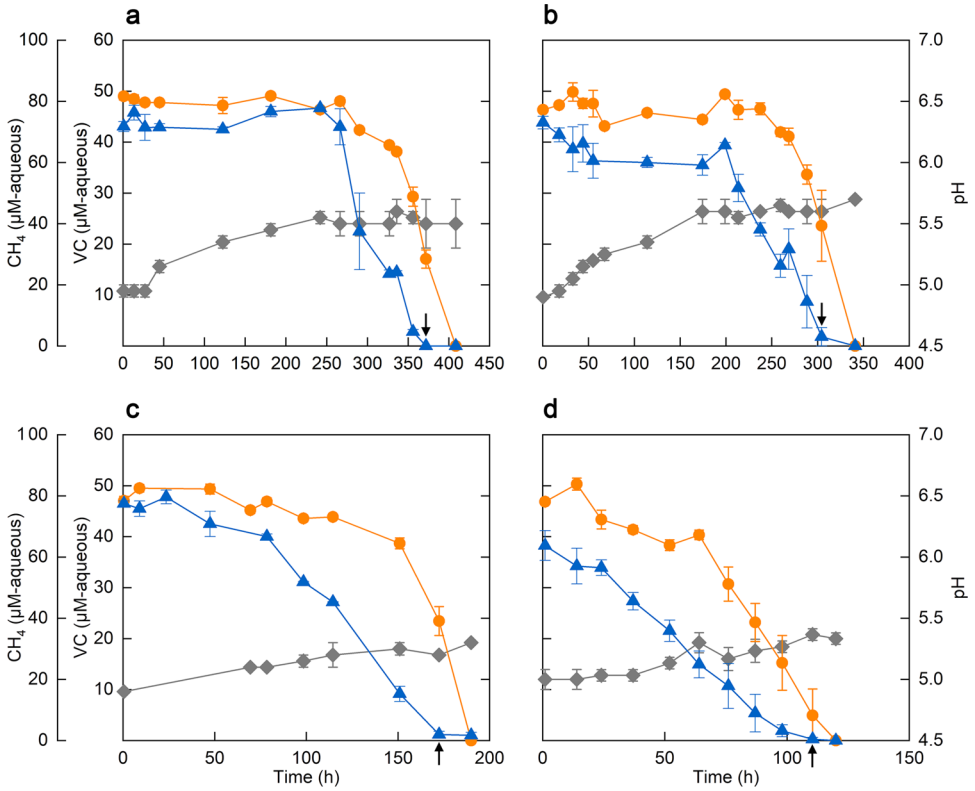

Figure 2. Monitoring of methanotrophic VC degradation in microcosms established with Sohwangbyungsan peat bog material and incubated with 85 μM CH_4 with (a) no added methanotroph inoculum, (b) bioaugmented *Methylomonas* sp. strain JS1, (c) bioaugmented *Methylocystis* sp. strain MJC1, and (d) bioaugmented *Methylosinus trichosporium* strain OB3b. Dissolved concentrations of CH_4 (orange circle) and VC (blue triangle), as well as pH (gray rhombus) were monitored until CH_4 was depleted. The black arrows indicate the time of sample collection for the microbial community analyses. The average values of biological triplicates are presented with the error bars representing their standard deviations.

diminished sMMO expression and activity compared to the Cu-free cultures ($p < 0.05$; Figure S6a).

Methylocystis sp. strain MJC1 initially consumed CH_4 and VC at pH 5.0 under both Cu-supplemented and Cu-free conditions; however, the viability of strain MJC1 was reduced after the pH dropped below 4.7, presumably due to release of protons during dechlorination (Figure S7). When strain MJC1 was grown at an initial pH of 5.5, a pH above 5.0 was maintained throughout the incubation period, and 50 μM VC was cometabolized to below the detection limit under both Cu-free and Cu-supplemented conditions (Figure 1b,e). The lag time was shorter in the Cu-free cultures than in the Cu-supplemented cultures; however, the maximum growth rate of strain MJC1 was significantly higher with Cu supplementation compared to cultures without Cu (i.e., 0.035 ± 0.001 h^{-1} versus 0.018 ± 0.001 h^{-1}). Complete VC degradation required longer time periods in the Cu-supplemented cultures compared to the Cu-free cultures. The maximum observed VC degradation rate was significantly higher in the Cu-free cultures with sMMO activity (38.2 ± 1.4 nmol min^{-1} mg $protein^{-1}$) than in the Cu-supplemented Cu culture without (26.0 ± 1.2 nmol min^{-1} mg $protein^{-1}$; Figure S6b).

The well-studied neutrophilic methanotroph *M. trichosporium* strain OB3b grew poorly at pH 5.0 (Figure 1c,f). Exponential growth was not observed in medium without Cu, and the specific growth rate of 0.023 ± 0.001 h^{-1} observed with Cu supplement was significantly lower than the rates measured in the two acidophilic methanotroph cultures ($p < 0.05$). At pH 5.0, strain OB3b produced fewer cells under both Cu-supplemented and Cu-free conditions, with maximum OD_{600nm} values of 0.210 ± 0.003 and 0.147 ± 0.008, respectively. Nevertheless, strain OB3b completely oxidized CH_4 and VC within 109 and 56 h in cultures with 10 μM Cu and without Cu amendment, respectively (Figure 1c,f). Prior work with strain OB3b estimated a $V_{max, app}$ at pH 7.0 to reach 2100 and 42 nmol VC min^{-1} mg $protein^{-1}$ for Cu-free and Cu-supplemented (20 μM) conditions, respectively.[24] In contrast, the maximum observed VC degradation rates in strain OB3b cultures maintained in pH 5.0 medium with and without Cu amendment were statistically indistinguishable ($p > 0.05$; Figure S6c). None of the three examined strains were capable of utilizing VC as the sole carbon and energy source, as neither cell growth nor VC degradation occurred without CH_4 (data not shown).

Cometabolic Degradation of VC in Peat Soil Slurry Microcosms. VC degradation occurred in acidic (pH 5.0) peat slurry microcosms following the addition of CH_4 (i.e., biostimulation) but not in control microcosm without CH_4 addition (Figure 2, Figure S8). The peat contained 1.12 ± 0.01 mg bioavailable Cu per kg (wet weight), and thus, the dissolved Cu concentration in the aqueous phase of the soil slurry was estimated to be 4.45 ± 0.03 μM. The naphthalene assay failed to demonstrate sMMO activity in all examined microcosms, consistent with the presence of Cu in the peat (data not shown). Consumption of CH_4 and VC was observed in all four sets of the slurry incubations, albeit with different lag times before VC consumption commenced. Biostimulation with CH_4 was sufficient to induce methanotrophic VC degradation, but only after the acclimation period of approximately 250 days. Bioaugmentation of microcosms

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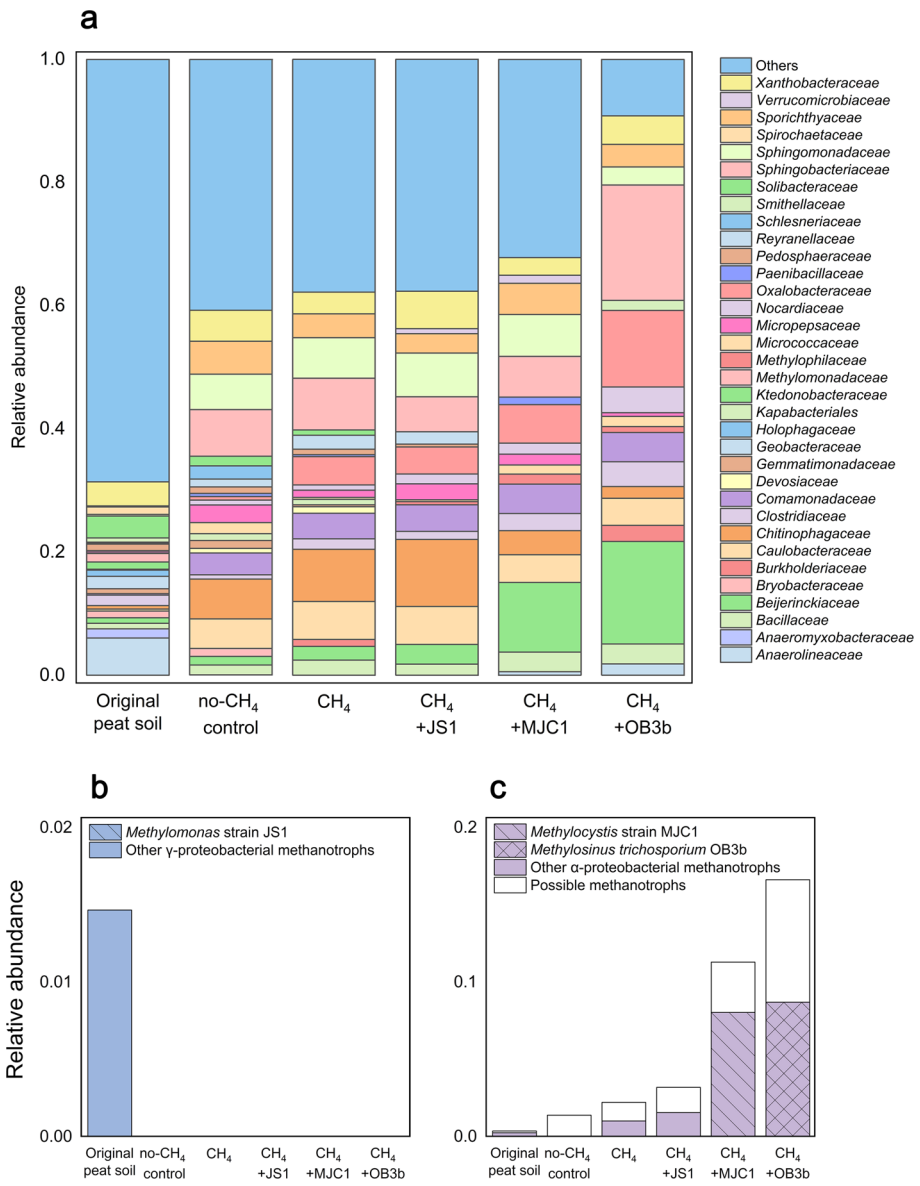

Figure 3. (a) The family-level microbial community compositions of the original Sohwangbyungsan peatbog soil and the soil slurry microcosms incubated with and without 85 μM CH_4 in presence of 50 μM VC. The strain names (JS1, MJC1, and OB3b) indicate the soil slurry microcosms augmented with the respective axenic methanotrophic cultures before incubation. The group labeled as 'others' include sequences representing families with relative abundances below 1% and sequences without taxonomy assignment at the family level. The relative abundances of the sequences representing methanotrophs of the classes (b) *Gammaproteobacteria* and (c) *Alphaproteobacteria* are presented. The meshed or hatched portions of the bars indicate the relative abundances of the augmented strains, and the empty portion represent *Beijerinckiaceae* sequences not annotated as methanotrophs, but share >95% 16S rRNA gene sequence identity with confirmed methanotrophic genera.

with *Methylomonas sp.* strain JS1 resulted in a slightly reduced lag times of ~200 h before the onset of CH_4 oxidation; however, the maximum VC consumption rate of 67.1 ± 10.2 μmol L^{-1} d^{-1} observed in microcosms augmented with strain JS1 was statistically indistinct from the rate measured in the CH_4-only microcosms, 63.3 ± 11.0 μmol L^{-1} d^{-1}. Bioaugmentation of strain OB3b or strain MJC1 to the soil slurry resulted in substantially reduced lag times (Figure 2c,d). For unknown reasons, however, 1.03 ± 0.57 μM VC remained in the microcosm bioaugmented with strain MJC1, while all other CH_4-fed microcosms resulted in VC removal below the detection limit. The pronounced effect of bioaugmentation occurred in the microcosms augmented with strain OB3b, resulting in a significant drop ($p < 0.05$) in VC concentrations from 38.3 ± 2.9 to 33.9 ± 1.5 μM and CH_4 concentration from 78.1 ± 0.8 to 72.3 ± 3.1 μM within 14 h following inoculation (Figure 2d). The maximum observed VC consumption rates of the slurries bioaugmented with strain OB3b and strain MJC1, 68.5 ± 1.8 and 66.7 ± 2.8 μmol L^{-1} d^{-1}, respectively, were not statistically different from that measured in the biostimulation-only microcosms ($p > 0.05$). No significant VC loss was observed during 943 h of incubation in the triplicate control microcosms without CH_4 amendment ($p > 0.05$; SI Figure S8). Of note, pH changes independent of CH_4 and/or VC oxidation were observed in live microcosms but not in the autoclaved controls, presumably due to microbial metabolism of organics associated with the peat.

Microbial Community Compositions of the Peat Slurry Microcosms. In the control microcosm amended with VC only, ASVs (% of the total read counts in parenthesis)

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affiliated with *Caulobacteraceae* (4.8%), *Chitinophagaceae* (6.5%), *Sphingobacteriaceae* (7.6%), *Sphingomonadaceae* (5.8%), *Sporichthyaceae* (5.3%), and *Xantobacteraceae* (5.0%) were enriched (Figure 3a). The microbial composition of this control microcosm was clearly distinct from that of the raw peat soil despite the absence of an external electron donor, which suggest that labile organic carbon compounds in the peat were metabolized. These ASVs were also abundant in all CH_4-biostimulated and bioaugmented microcosms. In fact, the microbial communities of the laboratory-incubated microcosms all clustered together in the nonmetric multidimensional scaling plot, except for the microcosm bioaugmented with *M. trichosporium* strain OB3b (Figure S9). *Methylosinus* and *Methylocystis* were the only methanotrophic taxa with >1% relative abundance in all four CH_4-enriched microcosms, and sequences representing methanotrophs of the class *Gammaproteobacteria* were not detected in any microcosm, including the microcosms bioaugmented with stain JS1 (Figure 3b,c). The total relative abundance of methanotrophs in the CH_4-fed microcosms may have been underestimated, as additional ASVs of the family *Beijerinckiaceae* assigned to the genera *Roseiarcus* and *Rhodoblastus* exhibited >95% identity to the *Methylosinus*, *Methylocystis*, or *Methylocella* genera (Figure 3c).

The microcosms augmented with *Methylocystis sp.* strain MJC1 and *M. trichosporium* strain OB3b had markedly larger proportions of sequences affiliated to *Methylocystis* and *Methylosinus* with 8.0 and 8.7% of the total microbial population than the other microcosms (Figure 3c). The ASVs attributed to the augmented strains were the only confirmed methanotroph ASVs recovered from these microcosms. Even with the ambiguous *Beijerinckia* ASVs considered as possible methanotrophs, the augmented methanotrophs would still be the majority (>50%) of the methanotroph community in both the strain MJC1- and the strain OB3b-augmented microcosms.

DISCUSSION

Cometabolic biodegradation of chlorinated ethenes via biostimulation or bioaugmentation of methanotroph activity has been extensively studied and implemented in situ since the 1980s; however, the possibility of applying methanotrophs for bioremediation of acidic groundwater aquifers has received little attention.[27,38,59,60] A recent study showed partial VC degradation in a CH_4 enrichment obtained from an acidic aquifer.[27] Significant oxidation of VC was observed in this enrichment, although no conclusive evidence for methanotrophic cometabolim was generated. Also, the enrichment cultures did not achieve complete degradation of VC.[27] In contrast, complete VC removal occurred in the pure cultures of strain JS1, strain MJC1, and strain OB3b. Further, the microcosms augmented with strain JS1 or strain OB3b, as well as the CH_4-biostimulated microcosm, observed complete VC removal. For remediation of low-pH aquifers, achieving VC removal below the MCL of 2 ppb is obviously desirable. The new findings indicate that the stimulation of acidophilic methanotrophs can achieve detoxification at low-pH VC-contaminated sites, where the reductive dechlorination process has stalled due to unfavorable conditions.[8,11]

The enzymes catalyzing CH_4 to CH_3OH oxidation and cometabolic oxidation of chlorinated ethenes in proteobacterial methanotrophs are pMMO and sMMO.[23] Copper is a key regulator of pMMO and sMMO expression in methanotrophs and has a crucial but not fully understood role in pMMO activation.[61] Consistent with previous findings, sMMO expression (RT-qPCR) and activity (naphthalene assay) were negligible in axenic cultures of the two new acidophilic methanotroph isolates when Cu was provided (Table 1). Previous observations with axenic methanotroph cultures at circumneutral pH have repeatedly suggested that sMMO exhibits higher turnover rates for non-methane substrates than pMMO.[24−26] The $V_{max,app}$ for VC oxidation measured previously in *M. trichosporium* strain OB3b cultures maintained without Cu exceeded the rates observed in cultures with Cu amendment by at least 2 orders of magnitude.[24,25] The results of the current study suggest that the kinetic data obtained with methanotroph cultures at circumneutral pH do not predict the efficacy of VC co-oxidation in methanotroph cultures growing under acidic conditions. The VC consumption rates measured for the three methanotroph pure cultures examined in this study were independent of Cu amendment, suggesting that pMMO, as well as sMMO, effectively cometaboliz VC under acidic pH conditions (Figure S6). This observation substantially expands the applicability of methanotrophs for bioremediation, as the difficulty in sustaining sMMO activity has been a major challenge under in situ conditions, where bioavailable Cu inhibit sMMO expression.[62]

The results of the microcosm study demonstrated that the addition of CH_4 and air (i.e., biostimulation) was sufficient for supporting methanotrophic cometabolism of VC. The community analysis indicated that acidophilic methanotrophs of the class *Alphaproteobacteria* were responsible for VC biodegradation in microcosms established with Sohwangbyungsan peat. In support of this observation, *Methylocystis sp.* strain MJC1, capable of cometabolic VC degradation, was isolated from this peat. Of note, *Methylocystaceae*-type sequences also dominated the Sohwangbyungsan peat microcosms augmented with *Methylomonas sp.* strain JS1, suggesting that *Methylocystis spp.* may be better adapted to low-pH growth and VC cometabolism under the conditions tested. Still, the microcosm experiments demonstrated the feasibility and value of bioaugmentation to accelerate VC cometabolism. As continuous supply of CH_4 and O_2 are needed to stimulate and maintain methanotrophic activity, fast response time and efficient substrate utilization are desirable in utilizing methanotrophs for bioremediation.[62] The microcosm experiments indicated that the well-studied neutrophilic methanotroph *M. trichosporium* strain OB3b outperformed the two acidophilic methanotrophs isolated from acidic peatbogs as the inoculant for bioaugmentation, despite exhibiting the lowest growth under acidic pH conditions as axenic culture. This finding challenges the common perception that native organisms, or organisms acclimated to specific environmental conditions, would be more suitable for bioaugmentation.[63]

An interesting observation from the peat microcosm experiment was the effect of *M. trichosporium* stain OB3b augmentation on the microbial community structure of non-methanotrophs, which may be attributed to the release of labile organic exudates by methanotrophs, a phenomenon previously reported to affect the non-methanotroph community.[64,65] For example, increased export of organic acids has been observed in cultures of the methanotrophic bacterium *Methylomicrobium alkaliphilum* strain 20Z under O_2 starvation conditions. The release of CH_4-derived organic compounds may be a response of strain OB3b cells to low pH exposure, possibly due to a less stringent orchestration of carbon metabolism under stress conditions.[66] Another possibility involves the potential

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ecological role of methanobactin, a potent Cu-chelating agent synthesized and exuded by select groups of methanotrophs including *M. trichosporium* strain OB3b.[67,68] Recently, methanobactin-mediated microbial interactions have been suggested, raising the possibility that methanobactin control of Cu availability may be selected for or against certain organismal groups.[42,69,70] Whether these effects on microbial community structure are related to the observed VC cooxidation performance warrants further study.

Microbial reductive dechlorination is a cornerstone process for bioremediation at sites impacted with chlorinated ethenes.[2,71] While formation of ethene has been documented, organohalide-respiring *Dehalococcoidia* require circumneutral pH conditions, and stalled dechlorination activity has been observed under acidic pH conditions.[9,11] Economically viable remedial approaches to remove lingering VC concentrations to below the MCL at stalled reductive dechlorination sites are lacking, and methanotrophic co-oxidation of VC would be a promising strategy to achieve complete detoxification of chlorinated ethenes. Such partnering of *Dehalococcoidia* with other metabolic groups of microorganisms has proven successful in expanding applicability of the reductive dehalogenation process.[72] Further, cultivation of the three methanotroph cultures studied here resulted in increased medium pH, contrary to the general perception that CH_4 oxidation results in acidification due to the formation of CO_2.[73] This neutralizing effect of acidophilic/acidotolerant methanotrophs may be beneficial, in that it may compensate for the acidification caused by dechlorination reactions and possibly sustain *Dehalococcoidia*-reductive dechlorination activity.[16,74]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c08766.

> Phylogenetic trees of 16S rRNA gene, PmoA, and MmoX amino acid sequences, genome-wide comparisons (digital DNA-DNA hybridization and average nucleotide identity), growth curves at varying initial pH, CH_4 consumption curves, biomass-normalized VC and CH_4 consumption rates, VC degradation and pH change in the control microcosm, nonmetric multidimentional scaling analysis, the list of 16S rRNA gene, PmoA, and MmoX sequences used for construction of the phylogenetic trees, genome and draft genome statistics, a summary of genes implicated in C1 metabolism, the list of primer sets used in this study, and a detailed method for RT-qPCR (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Sukhwan Yoon − *Department of Civil and Environmental Engineering, KAIST, Daejeon 34141, South Korea;* orcid.org/0000-0002-9933-7054; Phone: +82) 10−3461−2024; Email: syoon80@kaist.ac.kr; Fax: +82) 42−350-3610

Authors

Munjeong Choi − *Department of Civil and Environmental Engineering, KAIST, Daejeon 34141, South Korea*

Taeho Yun − *Department of Civil and Environmental Engineering, KAIST, Daejeon 34141, South Korea*

Min Joon Song − *Department of Civil and Environmental Engineering, KAIST, Daejeon 34141, South Korea*

Jisun Kim − *Department of Civil and Environmental Engineering, KAIST, Daejeon 34141, South Korea*

Byoung-Hee Lee − *Microorganism Resources Division, National Institute of Biological Resources, NIBR, Incheon 22689, South Korea*

Frank E. Löffler − *Center for Environmental Biotechnology, Department of Microbiology, Department of Civil and Environmental Engineering, Department of Biosystems Engineering and Soil Science, University of Tennessee, Knoxville, Tennessee 37996, United States; Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, United States;* orcid.org/0000-0002-9797-4279

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.est.0c08766

Author Contributions

#M.C. and T.Y. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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