

**Isolation and identification of a chloromethane-degrading bacterium from a
strictly anaerobic mixed culture**

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INTRODUCTION

Chloromethane (CH_3Cl ; CM) is a naturally occurring compound and is the most abundant halocarbon in Earth's atmosphere. CM is a clear, colorless gas that is highly flammable (ATSDR 1998). CM contributes to stratospheric ozone depletion, and it was estimated that 15-20% of chlorine-catalyzed ozone destruction was due to CM. The only molecules that had a higher contribution to ozone destruction were man-made CFCl_3 and CF_2Cl_2 (Harper 2000). The large influx of the molecule into the stratosphere is what makes it a threat and a potent toxin for ozone depletion (Butler 2000).

Most of the CM in the atmosphere is naturally formed, while CM also has industrial sources. The primary source of CM release into the atmosphere was previously thought to come from oceans; however, a study conducted between 1983 and 1996 refuted this claim and showed that oceans contributed to only 12% of total CM emissions to the atmosphere. The same study concluded that terrestrial sources of CM played a more significant role (Harper 2000). Other leading sources of CM come from the burning of biomass, specifically due to forest fires and agricultural practices in the tropics (Harper 2000). The tropics, coastal areas and salt marshes have been found to have higher amounts of CM. Salt marshes while making up less than 0.1% of the global surface area is estimated to contribute to 10% of total CM emission (Rhew 2000).

Synthetic sources of CM exist but are no longer as prominent as they used to be. CM was previously used as a refrigerant but its use was discontinued because of its toxicity. Current uses of CM include the molecule being used as an intermediate in the manufacturing of silicone polymers, as a foam blowing agent and in pesticides. The molecule can be found as a pollutant in treatment plants in municipal waste streams as a result of formation or the incomplete removal of any CM produced (ATSDR 1998). Overall, industrial processes, such as incineration and the combustion of coal, are considered to release relatively insignificant quantities of CM (Harper 2000) thus making the natural release of CM the primary culprit.

Considering there is such an abundance of toxic CM with ozone destruction potential, it is crucial to understand the transformation, fate and longevity of CM in the environment. The major process for atmospheric CM depletion is its reaction with photochemically formed hydroxyl radicals, currently estimated at 2.8 Tg per year (Harper 2000). In addition, CM is susceptible to microbial degradation, i.e., microbes utilize CM as a carbon and energy source.

Most CM-degrading bacteria are aerobic, facultative methylotrophic Proteobacteria (Roselli 2013). CM-degrading bacteria are widespread in oxic environments, and representatives are affiliated with the genera *Aminobacter*, *Hyphomicrobium*, *Leisingera*, *Methylobacterium*, *Roseovarius* (α -Proteobacteria), *Pseudomonas* (γ -Proteobacteria) and *Acetobacterium* (Actinobacteria). Chloromethane-degrading bacterial isolates are obtained from diverse environments such as soil, activated sludge, freshwater, and seawater. Most research has therefore focused on these aerobic bacteria, and the biodegradation of CM under oxic conditions has been studied in detail. So far, only one anaerobe has been described with the capability to utilize CM in the absence of oxygen, which is *Acetobacterium dehalogenans* strain MC (Traunecker et al., 1991). Far less is understood about the degradation of CM under anoxic conditions. This has left a gap in knowledge about anaerobic chloromethane-degrading bacteria. This current study attempted to close some of this gap and focused on isolating and identifying a CM-degrading bacterium in an strictly anaerobic mixed culture.

Consortium RM was derived from fresh water sediment collected from the Rio Mameyes River in the El Yunque National Forest in Puerto Rico (Justicia-Leon et al., 2012). Dichloromethane (CH_2Cl_2 , DCM) could be utilized as the carbon source and sole electron donor by consortium RM under anoxic conditions. Acetate and methane were the major terminal products of DCM degradation. The specific DCM-degrading bacterium in consortium RM has been identified as *Candidatus Dichloromethanomonas elyunquensis* representing a new genus and species within the family *Peptococcaceae* (Kleindienst et al., 2017). CM was not an intermediate of DCM degradation by consortium RM. Interestingly, a previous study showed the concomitant degradation of DCM and CM by consortium RM when they were presented together. Apparently, CM was degraded by another group of organism in consortium RM, most likely an *Acetobacterium* sp. capable of reducing CO_2 with hydrogen acting as an electron donor (i.e., CO_2/H_2 reductive acetogenesis). H_2 -mediated mutualistic interactions between the DCM-degrader (*Candidatus Dichloromethanomonas elyunquensis*) and the CM-degrading bacterium play a key role in consortium RM to facilitate the concomitant degradation of DCM and CM (Chen et al., 2017). Thus, the aim of this current study is to isolate and identify the CM-degrading bacterium in consortium RM. The CM-degrading bacterium was isolated by applying the serial dilution to extinction technique with CM and H_2 being added as substrates. The

isolated bacterium was identified by 16S rRNA gene sequence analysis and physiological features.

MATERIALS AND METHODS

Medium preparation

Anaerobic basal mineral salts medium was prepared following an established protocol (Löffler et al., 2005). The medium was buffered with 30 mM bicarbonate and reduced with 0.2 mM sodium sulfide and 0.2 mM L-cysteine. The medium was used to cultivate consortium RM and grow the CM-degrading bacterium.

Lysogeny Broth (LB) medium was prepared as follows: 10 g tryptone, 5 g yeast extract, and 10 g NaCl were weighed and dissolved in 1 L Milli-Q water, and subsequently autoclaved at 121 °C for 20 min. Agar (15 g. w/w) was added for the preparation of solid medium (i.e., LB plates). LB medium was used to grow *E.coli*, which was used as cloning host. Ampicillin was added at a final concentration of 100 µg/mL to the LB medium after autoclaving. The ampicillin stock solution (100 mg/mL) was filter-sterilized (0.22 µm) before addition to the LB medium.

Dilution to extinction

In order to obtain a pure culture of CM-degrading bacterium, the serial dilution to extinction technique in liquid medium was applied. Twelve empty serum vials (volume = 20 ml) were flushed with N₂, closed with butyl rubber stoppers, sealed with aluminum crimp caps, and autoclaved. A volume of 9 mL mineral salts medium was injected to each serum vial using a N₂-flushed 10-ml plastic syringe. CM-growing culture RM was used as an inoculum for enriching and isolating the CM-degrading bacterium. From the inoculum, 1 mL was injected to the first serum vial and mixed thoroughly, from which 1 mL culture was transferred to the next vial and so on. In total, 12 dilution serials were made, i.e., the culture was diluted to 10⁻¹, 10⁻², 10⁻³, ... 10⁻¹² (**Figure 1**). A volume of 0.1 mL CM and 2 mL H₂ was added to each vial as a growth substrate. Growth of cultures was monitored by measuring the consumption of CM using gas chromatography (GC). After performing subsequent serial dilution to extinction several times, a pure culture was obtained that can degrade CM in the presence of H₂ under anoxic conditions.

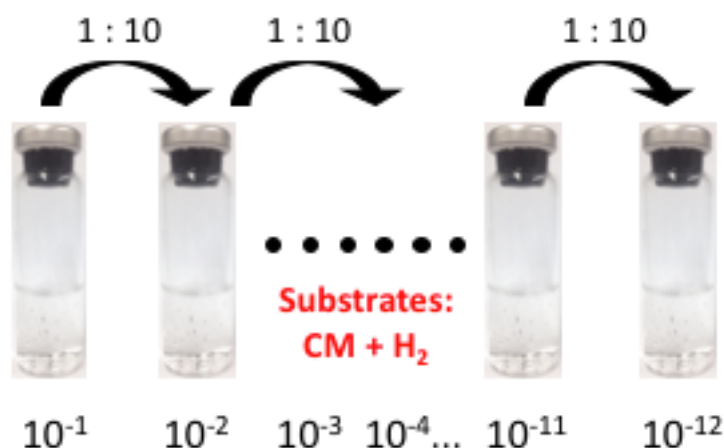


Figure 1: Scheme of serial dilutions to extinction in anaerobic liquid medium. Serial dilutions to extinction is a method used to isolate a single bacterial strain from mixed cultures. In this experiment, CM and H₂ were added as substrates to isolate the bacteria responsible for CM degradation in mixed culture RM. 12 dilution serials were made in total.

DNA isolation and PCR

The isolated CM-degrading bacterium, designated strain CMI, was cultivated in anoxic mineral salts medium. After CM was completely degraded, a volume of 5 mL culture suspension was collected and filtered onto a 0.22 μ m Durapore membrane (Millipore, Cork, Ireland). DNA was extracted using the PowerSoil DNA isolation kit (MO BIO, Carlsbad, CA) following the manufacturer's protocol. The isolated genomic DNA of strain CMI was stored at -20 °C for further analysis.

PCR was performed to amplify the 16S rRNA gene using the general bacterial primers 8F and 1541R. A master mix consisting of 10X PCR buffer, 25mM MgCl₂, 10 mM dNTP mix, DNA Taq polymerase and water was added to the DNA samples. The negative control consisted of an additional amount of water instead of DNA. The PCR program consisted of three stages. The first stage consisted of 1 cycle that ran for 2:10 minutes at 94°C. The second stage consisted of 30 cycles that ran for 30 seconds, 45 seconds, and 2:10 minutes at 94°C, 55°C, 72°C respectively. The last stage consisted of 1 cycle that ran for 6 minutes at 72°C. The final PCR product was then kept at 4°C. After PCR amplification, agarose gel electrophoresis (1%, wt/v) was conducted to examine the 16S rRNA gene amplicons.

16S rRNA gene clone library

The freshly amplified 16S rRNA gene PCR product was cloned and then transformed into *E. coli* cells using the TOPO TA clone kit (Invitrogen, Carlsbad, CA). The cloning reaction was prepared using 4 µL of fresh PCR product, 1 µL of salt solution, and 1 µL of the TOPO vector. The reaction was incubated for 30 minutes at room temperature and then placed on ice. Chemically competent *E. coli* were then added to the reaction mixture and then incubated on ice for 30 minutes. The cells were then heat shocked for 30 seconds in a 42°C water bath without shaking. They were then immediately transferred to ice. Room temperature S.O.C. medium (250 µl) was added to the tubes. S.O.C. medium is a glucose rich medium and was used because it has been shown to increase transformation efficiencies (Sun et al. 2009). The tubes were then placed on a 37°C incubator for 1 hour.

The transformed *E.coli* cells were then plated onto LB/Amp agar plates with β-galactosidase and X-gal and incubated at 37°C. Ten white colonies were selected and transferred to falcon tubes with LB/Amp broth and incubated at 37°C with shaking. Only seven tubes showed signs of *E.coli* growth. Plasmid DNA of the positive clones was then extracted from the seven tubes (Zyppy™ Plasmid Miniprep Kit Catalog No. D4036 Lot No. ZRC185027). The concentration and purity of the extracted DNA was determined using a Nanodrop 2000 (Thermo Scientific, Waltham, MA). Two samples presented with protein impurities so DNA purification was performed (DNA Clean & Concentrator™ Catalog No. D4003 Lot No. ZRC200596) and concentration was measured again. PCR was performed again and the samples were analyzed with a gel. The PCR products were purified (DNA Clean & Concentrator™ Catalog No. D4003 Lot No. ZRC200596) because two samples did not display any DNA bands and concentration was checked again via nanodrop and analyzed with a gel. The samples were then sent for Sanger sequencing and the DNA sequences were analyzed .

RESULTS AND DISCUSSION

Isolating the CM-degrading bacterium

After performing serial dilution to extinction several times, a pure culture, which can degrade CM under strictly anoxic conditions with the presence of H₂, was obtained from the 10⁻¹² tube. The CM-degrading isolate, designated strain CMI, was cultivated in reduced mineral salts

medium with the amendment of CM and H₂ as substrates. To ensure that strain CMI was indeed responsible for CM degradation in the mixed culture, CM concentration was monitored over an incubation period. **Figure 2** shows the amount of CM (μmoles per bottle) found in the pure culture over a period of 40 days. Both lines show an overall decrease in CM amount in cultures of strain CMI.

The first line on the left represents a bottle that started out with 27 μmoles of CM and by Day 20 ended up with 0 μmoles. Because CM was degraded by Day 20, the second line represents an external replenishing of CM substrate at ~35 μmoles. Unlike the first line, H₂ was not initially added on Day 20, it was added at ~Day 10. After replenishing H₂, CM degradation occurred at higher rate, and it took just over 20 days for CM to be completely degraded. The CM degradation graph shows that the serial dilutions were successful because the bacterium that was isolated from the mixed culture was responsible for the CM degradation in the bottles. During the degradation of CM, obvious turbidity occurred in culture bottles, indicative of growth of strain CMI.

Isolated Bacteria from Pure Culture CM degradation

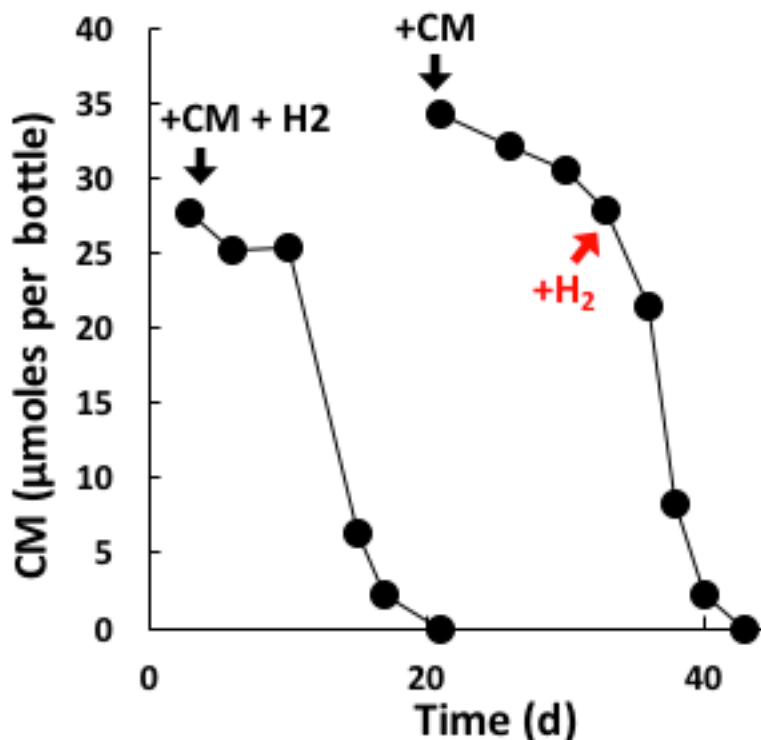


Figure 2 CM Degradation by strain CMI. CM was degraded over a 20-day incubation period. This graph allows us to show that the bacterium isolated (i.e., strain CMI) via serial dilutions was indeed the bacterium responsible for CM degradation in the original mixed culture.

Evidence for culture purity

Microscopy

The isolate was grown with CM and H₂ as substrates and phase contrast microscopic images of the bacterium were obtained (**Figure 3**). Microscopy revealed a uniform cell morphology and only short rods with an length of 2-3 μm. Also observed were cell pairs with two cells linked together near the cell termini, presumably an indication of active cell division. At the late growth phase (i.e., after all CM had been consumed) spores were observed.

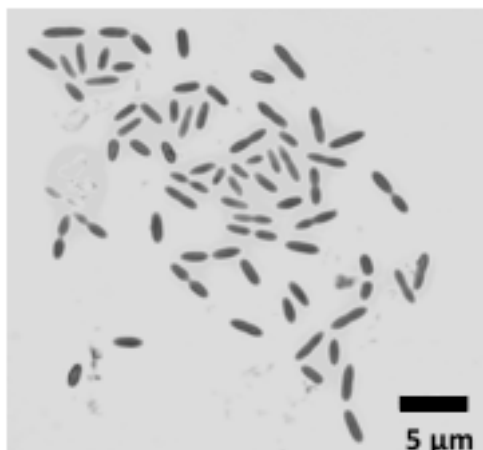


Figure 3 Microscopic Image of the CM-Degrading Bacterium. A microscopic image was taken of the isolated bacterium strain CMI. The culture displayed a uniform cell morphology and only short rods, sometimes occurring in pairs of two cells connected near the cell termini, were observed.

PCR amplification of the 16S rRNA gene and Phylogenetic Analysis

Following growth of strain CMI with CM and H₂, DNA was extracted from two culture samples and PCR was performed using degenerate primers targeting general 16S rRNA gene of bacteria in order to amplify the 16S rRNA gene of strain CMI. **Figure 4** shows the gel electrophoresis performed in order to examine the amplification of 16S rRNA genes. The left lane shows the 1 kb plus DNA ladder. Lanes A and B show bands with a size of around 1,650 bp, which matches with the expected amplicon size of a the full length 16S rRNA gene. Lane C represents the negative control without DNA template during PCR amplification. PCR was a critical step in this experiment because amplifying the 16S rRNA gene was required in order to transform the gene into *E.coli* to identify the bacteria. This image confirms that DNA extraction was successful for samples A and B as well as the PCR amplification. Why the negative control presented with a slight band is uncertain but it could be attributed to contamination while samples were being loaded.

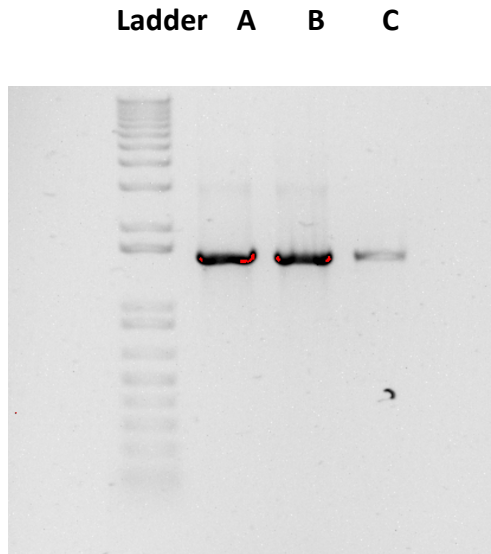


Figure 4. Gel image of 16S rRNA gene amplicons obtained with template DNA extracted from axenic strain CMI cultures. The left lane shows 1 kb Plus DNA ladder. Lane A and B are replicates of the amplicons generated with genomic DNA extracted from strain VMI cultures. Lane C represents the negative control without template DNA. [well, then there should be no band]

16S rRNA Gene Clone Library

The 16S rRNA gene amplicons obtained in PCR with strain CMI template DNA were transformed into *E. coli* cells using the TOPO[®] TA cloning kit according to the manufactures' protocol. Transformed *E.coli* cells were spread on LB/Amp plates to select clones with inserted 16S rRNA genes. All LB/Amp plates that were spread with the transformed *E.coli* displayed white colonies. **Figure 5** shows images of two of the plates that displayed positive clones. These white colonies were evidence that *lacZ*, the gene responsible for the production of β -galactosidase, was interrupted. When *lacZ* is fully operational and in the presence of X-gal, lactose is broken down into glucose and galactose and blue colonies are produced (Leonard et al 2015). The TOPO[®] kit used a vector that cut at the *lac* operon and subsequently the 16S rRNA gene was inserted at the restriction site. A successful insertion of the 16S rRNA gene meant that no blue colonies would be produced but instead white ones would be. **Figure 6** shows the restriction site on the *E. coli* plasmid where the 16S rRNA gene fragment was inserted. Positive clones were picked with sterile tooth picks and transferred to LB/Amp plates. *E. coli* transformants that grew on the LB/Amp plates were picked and transferred to liquid LB/Amp medium in 50-mL plastic (i.e.,Falcon) tubes.

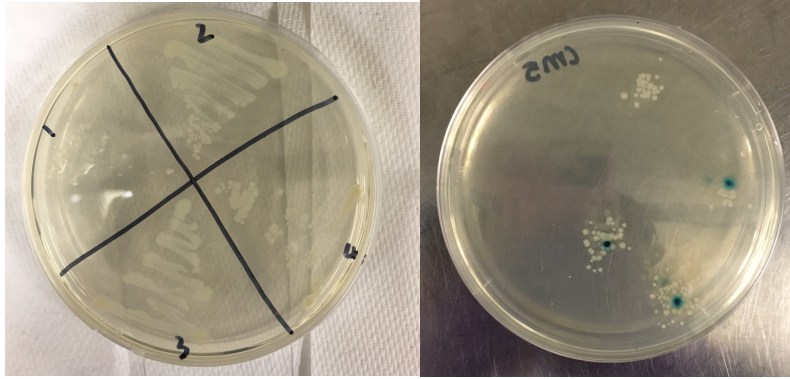
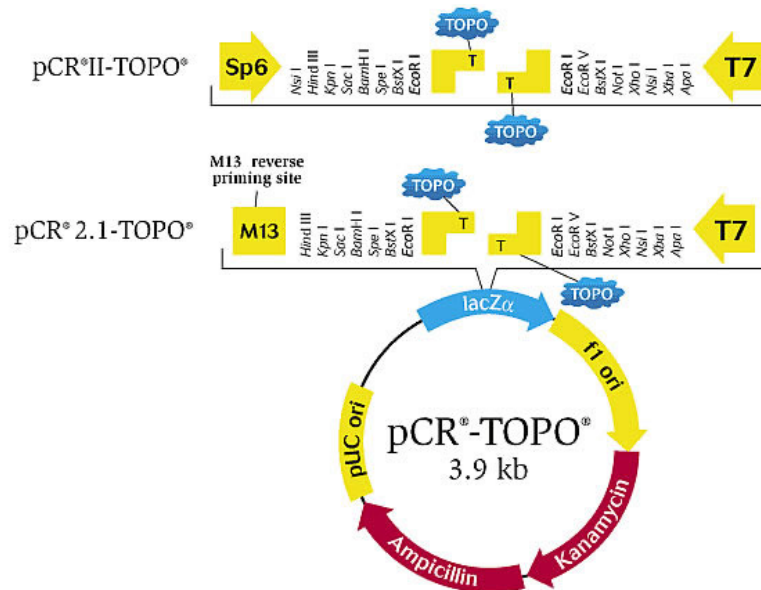


Figure 5 Plates that Displayed Positive Clones. These plates display the successfully transformed *E. coli* cells in which *lacZ* gene function was disrupted upon insertion of 16Sr RNA from the unknown bacteria that resulted in X-gal not being able to be cleaved, resulting in white colonies.



The pCR®-TOPO® vectors

TOPO® TA Cloning® Kits contain the popular pCR™2.1 TOPO® TA vector. TOPO® TA Cloning® Kits with Dual Promoter contains pCR™II TOPO® TA vector that allows subsequent in-vitro transcription following cloning.

Figure 6 Restriction Site Using TOPO® Vector.*[upper vs lower case – be consistent]* The 16S rRNA gene from our unidentified bacterium was inserted at the restriction site created by the TOPO® vector. *The restriction site interrupted the function of the lac operon, disallowing E.coli from being able to break down X-gal and produce blue colonies.*

After incubation overnight, plasmid DNA was extracted from positive clones. **Table 1** shows the DNA concentration measured with the Nanodrop 2000. Concentration values for all clones were relatively high. However, the values most important are the DNA-to-RNA ratio and nucleic acid-to-protein ratios, 260/280 and 260/230 respectively. An acceptable A260/A280 value is ~1.8. The 260/280 values remained consistent for almost all clones with the lowest value belonging to clone 18. An acceptable A260/A230 value is ~2.0-2.2. The A260/230 value was lowest for clone 3, which was significantly lower than other values. Because of impurities, DNA

purification had to be performed for clones 3 and 17 and DNA concentration was once again measured. Concentration values after purification can also be seen in Table 1. Retrospectively, clone 18 should have been purified as well because of its lower DNA concentration and relatively lower ratio values.

Figure 7 shows the gel image after PCR was performed with plasmid DNA extracted from the different clones. The ladder used was 1kb Plus DNA ladder. There are strong bands around ~1,650 bp for clones 3,5,17,19 and 2 indicating that the PCR successfully amplified the cloned 16S rRNA genes. Clones 4 and 18 did not display bands so the PCR products were purified and DNA concentrations were measured again. **Table 2** displays the DNA concentrations obtained after purification of PCR amplicons. Clones 3 and 18 had the lowest DNA concentration values as well as the lowest 260/230 ratios. Gel electrophoresis indicated that bands were still not visible for clones 4 and 18, similar to Figure 7. Because no bands were displayed, these DNA samples were not sequenced.

Table 1 Plasmid DNA Concentration Before and After Purification. DNA concentration was measured in ng/μl. The A260 values represent the absorbance of the nucleic acid while A280 represents the absorbance of any protein contamination. The 260/280 value represents the ratio of nucleic acid and proteins. The 230 value comes from any other possible contamination so the 260/240 ratio is that of nucleic acid over any other contamination.

Clone	Concentration (ng/μl)	A260	A280	260/280	260/230
3	234.8	4.696	2.5	1.88	0.27
4	102.9	2.059	1.156	1.78	1.98
5	287.5	5.75	3.071	1.87	2.2
17	119.1	2.381	1.361	1.75	1.43
18	56.1	1.123	0.661	1.7	1.23
19	168.6	3.371	1.826	1.85	2.04
22	470.5	9.41	5.064	1.86	1.9
After Purification					
3	3.6	0.071	- 0.075	-0.95	2.36
17	1.2	0.023	- 0.063	-0.37	-1.32

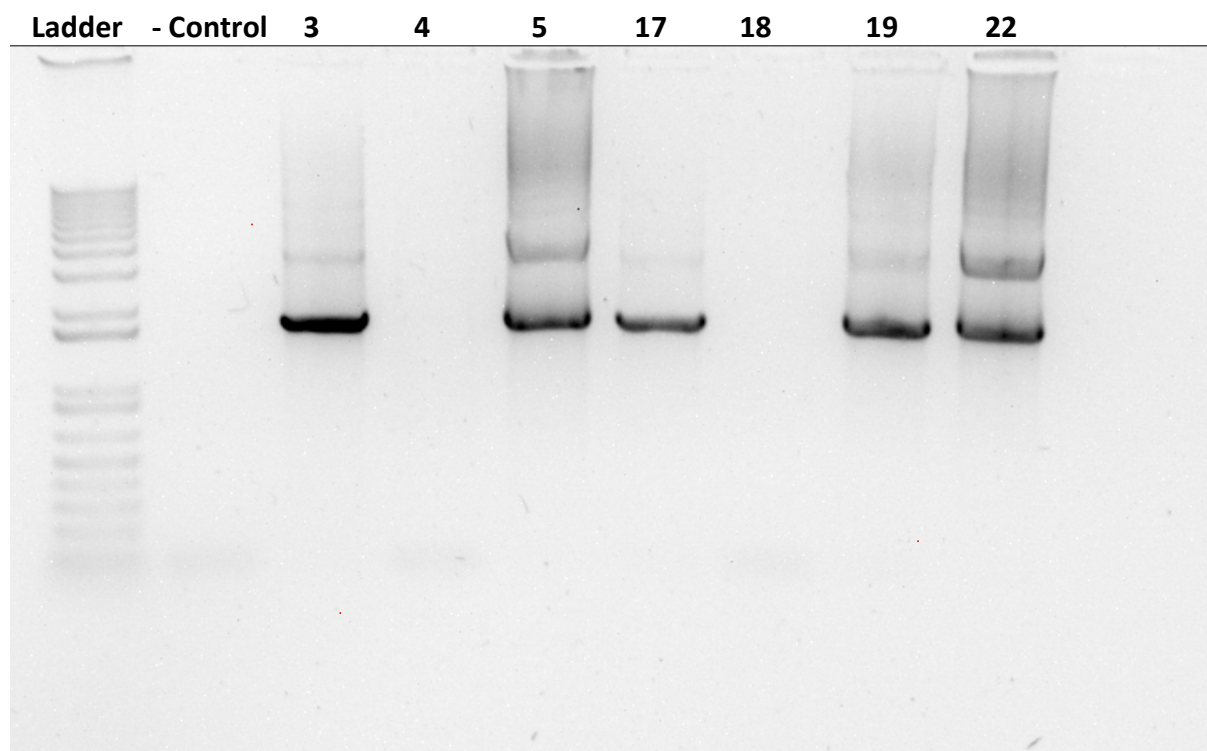


Figure 7 Gel image showing plasmid DNA extracted from clones. Each lane represents the clones whose plasmid was extracted and then ran through the PCR. The first lane represents DNA ladder and the second land represents the negative control.

Table 2 Plasmid DNA Concentration After Purification. DNA concentration was measured in ng/μl. The A260 values represent the absorbance of the nucleic acid while A280 represents the absorbance of any protein contamination. The 260/280 value represents the ratio of nucleic acid and proteins. The 230 value comes from any other possible contamination so the 260/240 ratio is that of nucleic acid over any other contamination.

Clone	Concentration (ng/μl)	A260	A280	260/280	260/230
3	98.6	1.973	1.088	1.81	2.09
4	13.7	0.274	0.139	1.98	1.7
5	135.6	2.712	1.477	1.84	2.13
17	81.7	1.634	0.889	1.84	1.99
18	7.8	0.156	0.073	2.14	1.75
19	95.4	1.907	1.037	1.84	2.24
22	127.6	2.552	1.388	1.84	2.06

Phylogenetic Analysis

After Sanger sequencing, 16S rRNA gene sequences were obtained. The sequences were trimmed on both 3' and 5' ends, and were assembled by using Geneious software. The sequences were analyzed using NCBI Nucleotide BLAST (<https://www.ncbi.nlm.nih.gov>) to identify

bacteria with the most closely related 16S rRNA gene sequences. The obtained 16S rRNA gene sequences were uploaded to the RDP database (<https://rdp.cme.msu.edu>) to find closely related genes. 16S rRNA genes of clones and related genes were collected and a phylogenetic tree was constructed using the tools provided on the RDP website. **Figure 7** shows the phylogenetic tree constructed. According to the analysis, the 16S rRNA genes of strain CMI affiliated with the species *Acetobacterium*, suggests that the new isolate belongs to the species *Acetobacterium*.

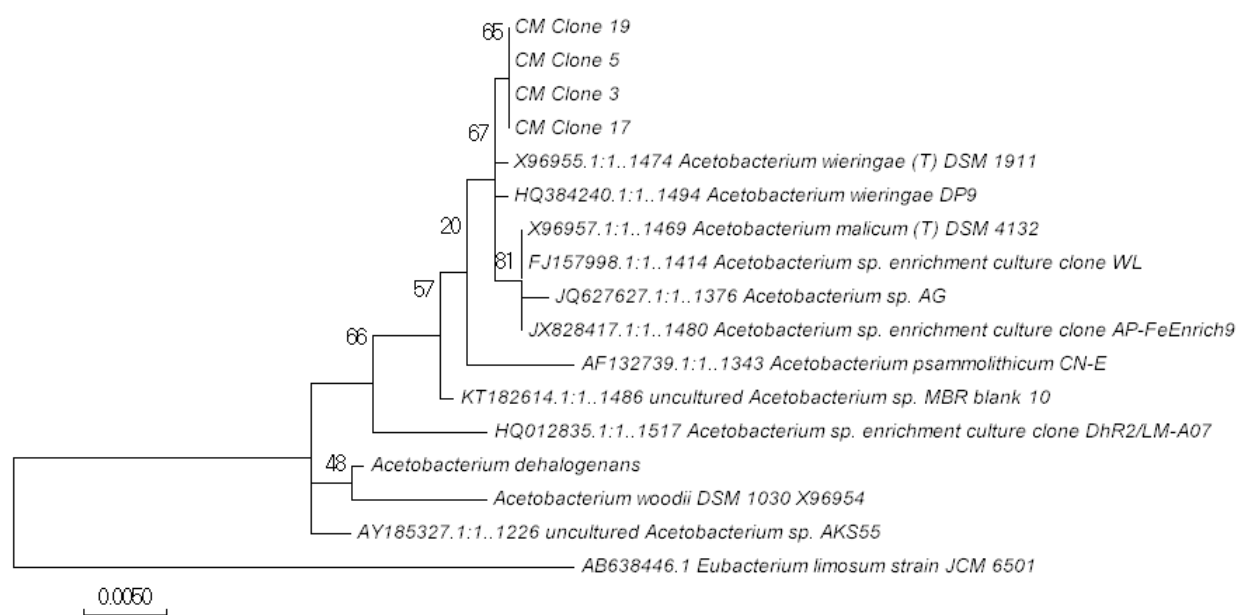


Figure 7 Phylogenetic Tree of Clones. The phylogenetic tree constructed placed the clones closest to the genus of *Acetobacterium*, suggesting strain CMI is an *Acetobacterium* sp.

Environmental Relevance

Every year, the United States spends billions of dollars cleaning up sites contaminated with toxic chemicals, such as chlorinated solvents. These sites pose tremendous risks to the environment, humans, plants and animal health. Bioremediation is a way that these sites can be cleaned up naturally through the use of biological agents such as microbes (Megharaj & Naidu 2017). This experiment focused on isolating bacteria capable of degrading toxic CM under anoxic conditions. The CM-degrading isolate strain CMI might play important roles in transforming CM in anoxic environments and the new findings have implications for CM bioremediation in groundwater. Because of CM's toxicity and its contribution towards

stratospheric ozone depletion, it is important to continue research and further develop bioremediation techniques and utilize them to attempt to reverse pollution and clean the environment. A comprehensive understanding of CM generating and degrading processes is also needed so that we can understand more about the CM fluxes that lead to stratospheric ozone depletion.

REFERENCES

- ATSDR (1998)** Toxicological profile for chloromethane. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service. [WWW document]. URL <https://www.atsdr.cdc.gov/toxprofiles/tp106.pdf>.
- Butler, J.H.** (2000) Better budgets for methyl halides? *Nature* 403: 260–261.
- Chen, Gao, et al.** “Mutualistic Interaction between Dichloromethane- and Chloromethane-Degrading Bacteria in an Anaerobic Mixed Culture.” *Environmental Microbiology*, vol. 19, no. 11, Nov. 2017, pp. 4784–4796. EBSCOhost, doi:10.1111/1462-2920.13945.
- Harper, D.B.** (2000) The global chloromethane cycle: biosynthesis, biodegradation and metabolic role. *Nat Prod Rep* 17: 337–348.
- Justicia-Leon, S.D., Ritalahti, K.M., Mack, E.E., and Löffler, F.E.** (2012) Dichloromethane fermentation by a Dehalo- bacter sp. in an enrichment culture derived from pristine river sediment. *Appl Environ Microbiol* 78: 1288–1291.
- Kleindienst, S., Higgins, S.A., Tsementzi, D., Chen, G., Konstantinidis, K.T., Mack, E.E., and Löffler, F.E.** (2017) ‘Candidatus Dichloromethanomonas elyunquensis’ gen. nov., sp. nov., a dichloromethane-degrading anaerobe of the Peptococcaceae family. *Syst Appl Microbiol* 40: 150– 159.
- Leonard, Susan R., et al.** "Acquisition of the Lac Operon by Salmonella Enterica." *BMC Microbiology*, vol. 15, no. 1, Aug. 2015, pp. 1-8. EBSCOhost, doi:10.1186/s12866-015-0511-8.
- Löffler, F.E., Sanford, R.A., and Ritalahti, K.M.** (2005) Enrich- ment, cultivation, and detection of reductively dechlorinating bacteria. *Methods Enzymol* 397: 77–111.
- Matlock, Brian.** “NanoDrop Spectrophotometers.” *Assessment of Nucleic Acid Purity*, 2000, ThermoFisher, tools.thermofisher.com/content/sfs/brochures/TN52646-E-0215M-NucleicAcid.pdf.
- Megharaj, Mallavarapu and Ravi Naidu.** “Soil and Brownfield Bioremediation.” *Microbial Biotechnology*, vol. 10, no. 5, Sept. 2017, pp.1244-1249. EBSCOhost, doi:10.1111/1751-7915.12840.
- Rhew, R.C., Miller, B.R., and Weiss, R.F.** (2000) Natural methyl bromide and methyl chloride emissions from coastal salt marshes. *Nature* 403: 292–295.
- Roselli S, Nadalig T, Vuilleumier S, Bringel F.** The 380 kb pCMU01 plasmid encodes chloromethane utilization genes and redundant genes for vitamin B12- and tetrahydrofolate-dependent chloromethane metabolism in *Methylobacterium*

extorquens CM4: a proteomic and bioinformatics study. PloS ONE. 2013;8:art. e56598. doi: 10.1371/journal.pone.0056598.

Sun, Qiao-Yang, et al. “Culture of Escherichia Coli in SOC Medium Improves the Cloning Efficiency of Toxic Protein Genes.” *Analytical Biochemistry*, vol. 394, no. 1, Nov. 2009, pp. 144-146. EBSCOhost, doi:10.1016/j.ab.2009.07.023.

Traunecker, J., Preuß, A., and Diekert, G. (1991) Isolation and characterization of a methyl-chloride utilizing, strictly anaerobic bacterium. *Arch Microbiol* 156: 416–421.