

# Isolation and Characterization of a *Desulfovibrio*-Related Bacterium Grown in the Presence of Trimethylamine and in the Absence of Sulfate

Cynthia Schmidt Carr  
Rice University, Dept. of Environmental Science and Engineering  
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## ABSTRACT

A nonmethanogenic bacterium was isolated in the presence of trimethylamine from anaerobic sediments taken from a marine environment. The bacterium was grown in anaerobic, sodium sulfide reduced medium containing 2% NaCl, 10 mM trimethylamine, 10 mM bromoethanesulfonic acid, no sulfate, and was buffered with sodium bicarbonate. Isolation of the bacterium was achieved by series of serial dilutions in agar shakes, followed by growth in liquid medium. Cells were small, vibrio shaped, motile rods. HPLC analysis of medium from enrichment cultures indicated that both formate and acetate were being formed presumptively during TMA metabolism, although no fatty acid production was observed in highly enriched/purified cultures. DNA was extracted from four colonies of the secondary series of agar shakes, and the genes for 16S rRNA were amplified by polymerase chain reaction. Five hundred base pairs of the 16S rRNA genes were sequenced, and two of the four colonies were found to be phylogenetically related to the organism *Desulfovibrio acrylicus* (95% identical). The other two colonies were 93% identical to *Cytophaga salmonicolor*, a presumed contaminant in the isolation or DNA extraction process.

## OBJECTIVES

The objectives of this research were the following:

1. Isolate an anaerobic, nonmethanogenic, bacterium capable of growth on trimethylamine (TMA) as sole growth substrate.
2. Characterize the isolate morphologically through microscopic evaluation.
3. Identify the metabolic end product(s) of TMA metabolism.
4. Identify the isolate based on 16S rRNA gene sequencing.

## MATERIALS AND METHODS

### Chemicals

All chemicals used were of reagent grade quality.

### Source of Inoculum and Preparation of Primary and Secondary Enrichments

Anaerobic sediments were collected from the southeastern shore of Salt Pond, Woods Hole, MA, in an area where the water column was approximately 0.2 m deep. The sediments were black in color, and had a strong sulfide odor. An aliquot of the sediment (approximately 3 mL of sediment slurry) was transferred to 50 mL of reduced, salt water anaerobic medium (as described in the course handout, "Preparation of Media for Anaerobic Bacteria", Bernhard Schink) containing 10 mM TMA and 10 mM bromoethanesulfonic acid (BES). Once growth was observed, as indicated by the presence of cells under microscopic evaluation, the enrichment culture was used as inoculum for agar shake dilutions and for the preparation of a secondary enrichment culture. The initial enrichment was lost after some time, but the secondary enrichment was maintained throughout the project.

### Microscopy

For phase contrast microscopy, wet mount preparations of cells were made and examined under oil on both Nikon and Zeiss microscopes (1000 X). Images were recorded digitally on a Zeiss Axioplan 2 microscope with a 3 chip CCD camera attached.

### Isolation

A schematic of the isolation procedure is shown in Figure 1. Several hundred microliters of the enrichment culture were used to inoculate a primary agar shake dilution series (1° series). The agar shakes were prepared as outlined in the course handout, "Preparation of Pure Cultures by the Agar Dilution Method", B. Schink, and the series was carried out to a  $10^{-6}$  dilution. Reduced, salt water anaerobic medium containing 10 mM TMA and 10 mM BES was used as the growth medium. In all cases reported throughout this text, reduced, salt water anaerobic medium was used as the growth medium. Upon solidification of the agar, the tubes were purged with  $N_2$  gas, and stored inverted in a 30 °C incubator.

Upon the formation of colonies in the highest dilution, several new series of agar shake dilutions were prepared (2° series). Colonies ( $n = 8$ ) were aseptically removed from the 1° series and transferred to individual serum bottles containing approximately 25 mL medium. An aliquot of the liquid medium (100  $\mu$ L) was then used to inoculate the new agar shakes. Eight dilution series were prepared, four containing 10 mM TMA, two containing 10 mM dimethylamine (DMA), two containing 10 mM monomethylamine (MMA), and all containing 10 mM BES. Each series was prepared to a  $10^{-6}$  dilution, and was purged and incubated as described above. The liquid cultures were purged with  $N_2$  gas and incubated with the agar shakes.

In an attempt to obtain a pure culture, a tertiary (3° series) set of agar shake dilutions was prepared from the 2° series. One colony was removed from a  $10^{-6}$  agar shake containing TMA, DMA, and MMA. Three new agar shake dilution series were prepared, as outlined above, with

one of the colonies. In this case, all three series contained 10 mM TMA and 10 mM BES. Prior to inoculation of the agar shakes, the colonies were diluted in 2 mL medium and observed under the microscope (1000 X, phase contrast). Afterwards, an aliquot of this medium (100  $\mu$ L) was used as inoculum in the agar shakes, and the rest was used to inoculate a parallel liquid enrichment culture (50 mL medium containing 5 mM TMA and 10 mM BES). The liquid enrichment cultures were incubated with the agar shakes at 30 °C.

Colonies were removed from the 3<sup>o</sup> series after growth appeared, and were transferred to 250  $\mu$ L liquid medium. The diluted colonies were examined microscopically to determine morphology and purity, and then added to 50 mL medium and incubated at 30 °C. These highly enriched/purified cultures were maintained for future research (i.e., determination of TMA metabolic pathway).

#### HPLC Analysis of TMA Metabolites

Fatty acids were detected and quantified in liquid medium using a Waters HPLC equipped with an UV detector (wavelength of 210 nm). The oven was operated isothermally at 60 °C. A Shodex column, Ionpak KC-811, was used for separation, and the eluent was 0.1% H<sub>3</sub>PO<sub>4</sub> (flow rate of 1 ml/min). Samples were prepared by transferring 400  $\mu$ L of medium into sterile 1.5 mL eppendorf tubes. The tubes were centrifuged for 10 minutes at 13,000 g. The supernatant was transferred to clean HPLC vial inserts for analysis. Formate and acetate standards (5 mM) were prepared and analyzed with the samples.

#### 16S rRNA Gene Sequencing

One lens-shaped colony from a 10<sup>-6</sup> dilution of one of the TMA, DMA, and MMA fed 2<sup>o</sup> series was removed aseptically, and transferred into 100  $\mu$ L of sterile deionized water. The DNA from each colony was extracted as outlined in the course handout, "DNA Lysis Procedures".

Ribosomal RNA (16S) genes were amplified from cellular DNA using universal primers in a polymerase chain reaction (PCR). Both undiluted DNA extract, and 1/10, 1/100, and 1/1000 dilutions were used for PCR amplification. Details of the PCR reaction can be found in the course handout, "PCR Protocol". PCR products were evaluated by gel electrophoresis to determine whether the amplification was successful. PCR products containing the 16S rRNA amplicon were sent to the Forsyth Dental Center where 500 base pairs of the 16S rRNA gene were sequenced. The closest phylogenetic match of the isolates was determined by BLAST search with the assistance of Bruce J. Paster, Ph.D., course instructor.

## RESULTS AND DISCUSSION

### *Isolation and Cell Characterization*

When colonies formed in the 2° series of agar shakes, individual colonies were removed from the highest dilution series and observed under the microscope (1000 X, phase contrast). In all cases, the cells were small, rod shaped and appeared to be in pure culture (i.e., only one morphology was observed). Cells from the TMA and DMA fed cultures appeared identical (motile vibrios), but this was not the case with the MMA fed culture. Rod shaped cells in the MMA fed culture were nonmotile, and were straight, not curved. Each of these three colonies was used to inoculate the 3° series of agar shake dilutions, as well as a parallel liquid enrichment culture.

After an incubation period of seven days, the corresponding parallel liquid enrichment cultures for the 2° series were examined microscopically (1000 X, phase contrast). The liquid culture containing the TMA 10<sup>-6</sup> inoculum was the only culture in which a large amount of

growth had occurred. In this case, the cells were motile, vibrio shaped rods, as seen previously in the corresponding agar shake.

Colonies were also removed from the 3<sup>o</sup> series of agar shakes and examined microscopically before being used as an inoculum in the final transfer to liquid. No colonies were removed or transferred from the agar shake dilution series inoculated with the MMA 10<sup>-6</sup> dilution because the tubes contained a significant amount of gas production, and the colonies were round and spindly rather than lens shaped. A total of five colonies were removed, two from the DMA 10<sup>-6</sup> inoculated series, and three from the TMA 10<sup>-6</sup> inoculated series. Under microscopic evaluation, all of the colonies contained motile vibrios and appeared to be in pure culture. Micrographs of the bacteria (1000 X, phase contrast) are shown in Fig. 2. In one case, it appears that a cell has a bundle of flagella located at one end (last micrograph). The colonies were transferred to sterile, liquid medium for cultivation and maintenance of the highly enriched/purified culture.

Based on the results from the isolation experiments, it appears that the isolate was a motile vibrio, and that perhaps both TMA and DMA may have supported growth of this organism. The culture that initially colonized the MMA fed 2<sup>o</sup> series was not able to grow with TMA in the 3<sup>o</sup> series, but was overtaken by an apparent contaminant (based on colony appearance).

#### HPLC Analysis of TMA Metabolites

The production of fatty acids in the 2<sup>o</sup> enrichment culture and parallel liquid enrichments was monitored periodically by HPLC, and the results are presented in Table 1. Acetate and formate were detected in the 2<sup>o</sup> enrichment culture and in four of the parallel liquid cultures for the 2<sup>o</sup> series (fatty acids were not detected on day 6 in the other 2<sup>o</sup> series cultures). The 2<sup>o</sup> series

parallel liquid enrichments that contained fatty acids were examined under the microscope (1000X, phase contrast), and both motile vibrios and spirochetes were observed. It was not possible, therefore, to conclude that the motile vibrios were responsible for fatty acid production.

Table 1. Results from HPLC fatty acid analyses in liquid cultures.

Culture	Days Post Inoculation	Acetate (mM)	Formate <sup>a</sup>	Cells Present (1000X, phase contrast)
Secondary Enrichment	12	0.86	+	motile and nonmotile rods (various)
2° Series	6	0.38 - 1.48	+	motile vibrios spirochetes
3° Series	8	-	-	motile vibrios
highly enriched/purified cultures	2	-	-	motile vibrios

<sup>a</sup> Formate was not quantified.

Samples from the 3° series parallel liquid enrichments and highly enriched/purified cultures were analyzed for the presence of fatty acids on day 8 and 2, respectively, after inoculation. No acetate, formate, or other fatty acids were detected. Microscopic evaluation of the 3° cultures indicated that only motile vibrios were present. The lack of fatty acid production in these cultures may be due to the small incubation period preceding HPLC analysis. Further measurements were not possible based on time restrictions. It is also possible, however, that the isolated motile vibrios did not convert TMA to acetate, but rather utilized some unknown compound in the agar for growth.

### 16S rRNA Sequences

PCR amplification was not successful in all three colonies taken from the 2° series, and only DNA from the DMA and MMA fed 10<sup>-6</sup> dilutions were sequenced. For reasons unknown, no DNA from the TMA fed series was amplified. Amplicons were obtained from both the extracted DNA and 1/10 dilutions of the DNA extract in the DMA and MMA fed colonies, and all four products were sequenced. Results from the sequencing reaction and BLAST search are shown in Table 2.

Table 2. Closest phylogenetic match and percent identity, as determined by BLAST search, for the bacterial colonies in the DMA and MMA fed 2° series (10<sup>-6</sup>) dilutions.

DNA Extract Sample	Closest Phylogenetic Match	Percent Identity
DMA 10 <sup>-6</sup> , no dilution	<i>Desulfovibrio acrylicus</i>	95%
DMA 10 <sup>-6</sup> , 1/10 dilution	<i>Desulfovibrio acrylicus</i>	95%
MMA 10 <sup>-6</sup> , no dilution	<i>Cytophaga salmonicolor</i>	93%
MMA 10 <sup>-6</sup> , 1/10 dilution	<i>Cytophaga salmonicolor</i>	93%

These results indicate that the motile vibrios observed in the 2° series DMA and TMA fed cultures and 3° series may be closely related phylogenetically to the marine organism *Desulfovibrio acrylicus*. The *Cytophaga*-related clone was assumed to be unrelated to the TMA-utilizing bacterium since this organism was found in numerous other experiments currently being conducted in the lab. The family Desulfovibrionaceae is characterized by the ability to utilize a diverse group of electron donors including hydrogen, fatty acids, ethanol, and malate and fumarate, the last two of which are incompletely oxidized to acetate (1). Desulfovibrios can also ferment pyruvate in the absence of sulfate (1). The ability of this family to utilize TMA as energy source has not been reported.

The closest phylogenetic match, *Desulfovibrio acrylicus*, is a motile, vibrio to rod shaped bacterium that was isolated from Wadden Sea intertidal sediments (2). This organism cleaves dimethylsulfoniopropionate (DMSP) to acrylate, which is then used as a terminal electron acceptor. Terminal electron acceptors other than acrylate include thiosulfate and sulfate. Energy sources include fatty acids, some alcohols, hydrogen, and several amino acids. Acetate was observed during growth on DMSP. The relationship between this organism and the organism recovered from the Salt Pond is not understood, although their close phylogenetic relationship may indicate that metabolic processes may be similar in both organisms. However, it is still not evident whether the motile vibrio isolated in this report was utilizing TMA, and any comments on its metabolic pathway would be speculative at this point.

#### SUGGESTIONS FOR FURTHER RESEARCH

- ◆ Quantify TMA consumption and fatty acid production.
- ◆ Sequence entire 16S rRNA gene and repeat Blast Search.
- ◆ Test highly enriched/purified cultures for sulfate utilization and ability to utilize other energy sources.
- ◆ Follow up on freshwater isolate.

#### ACKNOWLEDGMENTS

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## REFERENCES

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Figure 1. Schematic of procedure used for isolation of anaerobic bacteria.

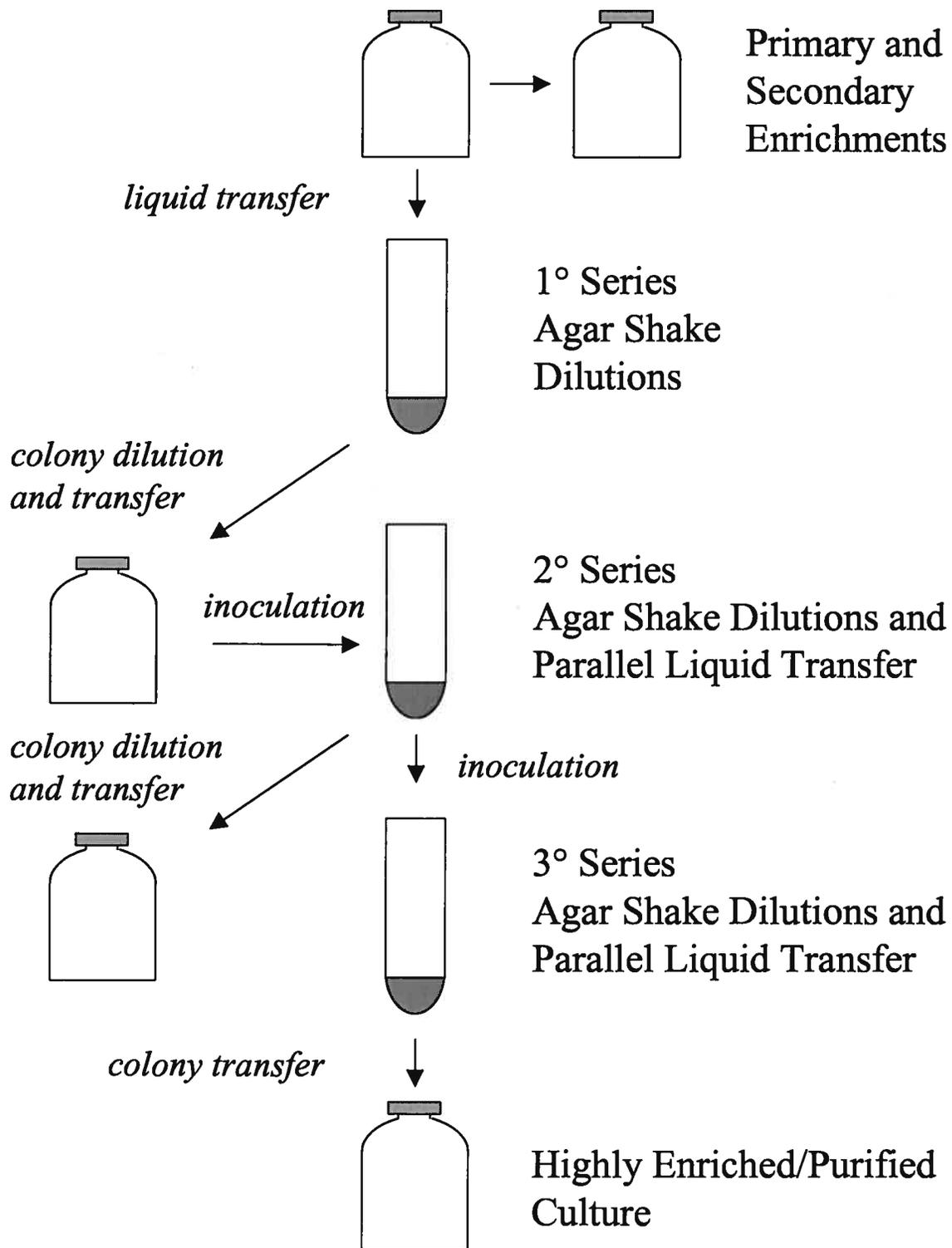


Fig. 2

