TMA Oxidation in Acetogenesis and Sulfate Reduction

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Abstract

This project was based upon class anaerobic chemotrophic enrichments with trimethylamine (TMA) as the sole electron donor. In an enrichment from Sippiwisset saltmarsh sediment, which contained BES to inhibit methanogens, acetate was produced at low levels suggesting an acetogen was utilizing the TMA for either methylotrophic growth or simply as an electron donor. Yet there has only been one reported homoacetogen that can utilize TMA. Also in enrichments from Trunk River and Sippiwisset saltmarsh, sulfide was generated when provided with TMA as the sole electron donor and sulfate as the sole electron accept. No organism has been reported to reduce sulfate with TMA as the electron donor. These enrichments were characterized via GC, HPLC, microscopy, FISH, and clone libraries. The clone library for the TMA/BES enrichment revealed that the enrichment probably selected for an organism(s) capable of fermenting BES to acetate. A comparison of the clone libraries of the TMA/SO_4 enrichments revealed a common and abundant clone most closely related to the thiosulfate reducing Firmicute, *Fusibacter paucivorans*. *F. paucivorans* couples fermentation to thiosulfate and sulfur reduction and is inhibited by H_2. This clone may be a candidate for coupling TMA oxidation/fermentation with sulfur reduction. Additionally, an attempt was made to determine if organisms at Trunk River were chemotaxic towards methylamines, including TMA.

Introduction

Trimethylamine (TMA) is a breakdown product of a number of biological important compounds to include the osmoregulatory compounds trimethylamine-N-oxide and glycine betaine as well as choline. The compound can be degraded anaerobically via classic methylotrophs like hypomicrobia (Hooper and DiSpirito, 1985) and via certain methylotrophic methanogens, particular of the *Methanosarcinales* group (Sowers and Ferry, 1983; Ferguson, and Krzycki, 1997). Under most environmental conditions, sulfur reducing bacteria out-compete methanogens for an electron donor, H_2 and/or acetate. However, the presence of TMA may allow the coexistence of sulfur reducing bacteria and methanogens (King, 1984). No sulfur reducing bacteria have been shown to utilize TMA as an electron donor. Methanogens also compete with homoacetogens for H_2 and currently only one homoacetogen has been shown to utilize TMA.

The goal of this project was to address three questions: 1) are bacteria chemotaxic behavior toward TMA; 2) are homoacetogens capable of utilizing TMA for either methylotrophic growth or simply as an e- donor; 3) are sulfur reducing bacteria capable of utilizing TMA as an e- donor. These questions were predicated upon class enrichment experiments. Group 1 (Trunk River sediment sample) and group 3 (Sippiwisset saltmarsh sediment) both exhibited activity in two key enrichments further described herein. Briefly, this project follows the activity of an enrichment from Sippiwisset saltmarsh, which contained TMA and bromoethenesulfonate (BES) to exclude methanogens, and enrichments from both locales that contained TMA and SO_4. Additionally, an attempt was made to determine if organisms at Trunk River were capable of a chemotaxic response toward methylamines, including TMA.

Materials & Methods

Field Deployable Chemotaxis Chamber. A chemotaxic chamber was constructed utilizing a plastic multiple capillary tube holder (30 tubes total) already available in the Microbial Diversity lab using the guidelines described by Overmann, 2005. Round capillary tubes were secured to the holder with modeler's clay. Every other capillary tube was filled with filter-sterilized Trunk River water, which acted as a negative control and to further separate the tested potential chemotaxic substances. Five each of remaining tubes were filled with either 25mM TMA, tetramethylamine (QMA), dimethylamine (DMA) or monomethylamine (MMA) in sterile Trunk River water. No positive control was used. Construction of the chamber was under anaerobic conditions. The chemotaxis chamber was removed from the anaerobic chamber in a sealed plastic tub. Evaporative loss in the capillary tubes was controlled by topping off the tubes immediately before deployment. The chamber was secured with fishing line attached to a float made out of a swimming noodle. It was deployed in a hole made by my foot with the tubes facing up toward the surface in a zone that was dark with decaying matter for three hours. The clay interface ends of the tubes were broken off and the contents of the capillary tubes were dispensed into eppendorf tubes. 100 μL was removed and resuspended in 900 μL filter-sterilized Trunk River water. The solution was filtered onto 0.2 μicron filter and air-dried. Vectashield with DAPI was placed directly onto the filters and they were immediately examined.

Enrichment Cultures. All cultures were in sealed serum bottles containing basal anaerobic modular seawater medium with 25 TMA. The headspace contained 80/20 H_2/CO_2, and 4 mM

cysteine and 4 mM Na_2S were the reductants. To determine is homoacetogens can utilize TMA for methylotrophic growth, 50mM bromoethanesulfonic acid was added to the medium to inhibit methanogens. To determine if sulfur-reducing bacteria can utilize TMA, 28 mM Na_2SO_4 was added. The enrichment cultures were monitored for growth via phase contrast and DIC microscopy. The presence of methanogens with confirmed using F_{420} fluorescence under a GFP filter. Subsequent enrichment cultures were generated from the TMA and SO_4 enrichments. TMA media containing either SO_4 and BES; BES; BES and crotonate; crotonate; or solely TMA were inoculated with 500 μL of saltmarsh or river water inoculum.

Fluorescent in situ hybridization. Multi-well glass slides were provided by Sheri Simmons. To each well, 5 μL of a 0.075% gelatin and 0.01% chromium potassium sulfate dodecahydrate solution was added. After the gelatin hardened, the wells were either inoculated with a saltmarsh enrichment of TMA only (3 wells), a saltmarsh TMA/BES enrichment (6 wells), and a saltmarsh TMA/SO_4 (6 wells) in a dilution series of 2 μL, 2.5 μL, and 3 μL. The slide was hybridized with Eubacterial Probe Eub338 labeled with FITC and Archaeal probe Arc915 labeled with Cy3. One well of the BES inoculum was hybridized with only NON338, a nonsense probe, while one well of the TMA/SO_4 enrichment received no hybridization treatment. The hybridization stringency was 35% for all treatments. The hybridization treatment was for two hours at 48C. Afterward the cells were rinsed with ddH_2O followed by EtOH and after drying it was placed at 4C. Twelve hours later, the slide was treated with a Citiflour DAPI stain.

Analyzing Substrate Depletion and End Product Formation. The concentration of methane, and H_2, CO_2 were monitored utilizing a Shimadzu GC-14A gas chromatograph equipped with FID and TCD. The concentration of acetate and cromate were monitored via Shimadzu LC-2010C equipped with UV and RID detectors. Attempts were made to monitor the concentration of TMA, QMA, DMA, and MMA on both the Shimadzu HPLC with a 5 mM H_2SO_4 running solution and Waters 1525 HPLC Pump with a running solution of 0.05 ammonium acetate in 5% methanol (Marzo *et al*., 1990). This reference provided more preferable GC and HPLC techniques and protocols not available in this class. Both HPLC's were unable to detect these compounds. Sulfide was measured via Cline method using the class' reagents.

Clone Libraries and Phylogeny. DNA isolation, PCR, and clone libraries were conducted on the secondary enrichments of Sippiwisset saltmarsh in TMA/BES, and both the saltmarsh and Trunk River in TMA/SO_4. The TMA/BES enrichment had low biomass thus 15 mL was first collected on filter paper and then the DNA was isolated using the MoBio Soil DNA kit. DNA was isolated from the pellet of 10mL the other enrichments via Promega Wizard Genomic DNA Purification Kit. The bacterial primers used in all three enrichments were 8F and 1492R. Only the TMA/SO_4 enrichments were examined for archaea. The primers used were 519F and 1392R. Cloning was conducted using TOPO Cloning Kit. Sequence data was read manually in 4 Peaks and was analyzed with ARB software. Phylogenetic trees were built utilizing parsimony with a lane mask filter and column exclusion filter of 442 and 2300 to exclude hypervariable unaligned sections of the sequences. Neighbor joining was subsequently utilized to access specific phylogenic relationships.

Results

Chemotaxis toward methylamines. All cell counts were much lower than expected with generally less than 10 cells per grid at 100x and there was no consistent morphology present in any of the capillary tubes or across treatments. Cells were observed floating off the filter in the Vectashield mounting medium. Additionally, dirt had accumulated in some of the capillary tubes. The dirt fluoresced brightly and an increased number of cells were observed near dirt particles. Cells had also accumulated around the edges of the filter possibly do to the no vacuum filtration technique.

Enrichment Cultures. Turbid growth occurred in both the Trunk River and Sippiwisset Saltmarsh TMA/SO_4 enrichments. Microscopy revealed a wide variety of morphologies in the Sippiwisset saltmarsh enrichment. In particular, the culture was dominated by irregular shaped cocci, confirmed to be methanogens via F_{420} fluorescence, and several morphologies of spirilla. Over successive transferred the number of Spirochete-like morphotypes increased. Other morphotypes included half moon-like morphs, long filaments and a very large rod with an off-center "vacuole". Like the spirochete-like morphotype, the "vacuolated" organism increased in number upon successive transfers of the culture. Aggregates of multiple morphotype were also observed. Typically, the clumps consisted of a non-descriptive rod, a cocci, and occasionally a spirilla. Often, the aggregates were surrounded by Cytophaga-like gliding rods. Examination of a slide was left out over night revealed a number of spores and the organism with the central vacuole appeared to lose this "vacuole". With the exception of a very small (< 1 μm) bacterium, all morphotypes were dead upon long-term exposure to oxygen. Another observation was the culture became less dense over time; however, transferred cultures were extremely turbid in 48 hours. The Trunk River enrichment was also dominated by a cocci but was larger than the saltmarsh enrichment. It also contained the "vacuolated" rod, spirilla and spirochete-like morphotypes observed in saltmarsh enrichments. In general, the Trunk River enrichment appeared less diverse than the salt marsh enrichment.

The secondary enrichments TMA/ SO_4 reach turbid conditions within 2 days. Within 8 days, the saltmarsh inoculum in crotonate and TMA reached moderately turbid conditions. No other enrichments became turbid within 10 days. After eight days of growth sulfide was measured in the enrichments (data not shown). The assay appeared very inaccurate based on the standard curve and previous experience. There slightly more sulfide in the crotonate and SO_4 enrichments than the other enrichments. Additionally, all serum vials had FeS precipitating out after inoculum. This undoubtedly, impacted the sulfide assay.

The saltmarsh enrichment in TMA and BES never reached a very turbid condition. The initial enrichment was faintly turbid and the secondary enrichment was not turbid at all. However, there were flocs of cells on the bottom of the serum bottle. The flocs were tightly bound composed of an unidentifiable morphotype. Other organisms observed were spirilla, motile rods, large cocci with a pointy motile end, curved rods, and a extremely large filamentous rod with a diameter > 5 μm.

FISH The eubacterial probe successful bound to many organisms however, there was no significant staining via the archaeal probe. Neither probe was tested on a positive control.

Additionally, the software for the microscope was malfunctioning and it was difficult to generate a decent image. Some organisms, like the large filamentous rod in the BES/TMA enrichment also auto-fluoresced. This organism as well as most morphotype hybridized with the eubacterial probe.

Substrate Depletion and End Product Formation. Both of the TMA and SO_4 enrichments had significant production of sulfide; however, I have no confidence in the concentration of sulfide indicated by the Cline method (Table 1). However, comparison of these enrichments to concentrations of sulfide indicated in other enrichments suggests that the concentration in these enrichments were quite high (i.e. greater than concentration in the primary enrichments with H_2 and TMA). What may be considered an additional indicators of presence of high levels of sulfide was that culture had very strong sulfidic smell and that upon destruction of the primary and secondary enrichments with bleach, the bottles turned yellow presumably due to the oxidation of the sulfide to elemental sulfur. No other enrichments, including other class enrichments, exhibited this behavior. Acetate was generated in all three enrichments at low levels but increased over time in the 1st transfer of the BES enrichment. The BES/TMA also had the distinct smell of dimethyl sulfoxide.

Table 1. A complication some of GC, sulfide, and HPLC analysis of the Sippiwisset saltmarsh enrichments of TMA/BES and TMA/SO_4. Time constraints prevented complete analysis of the remaining data collected.

	microscopy	CH4 [mM]	H2 [mM]	CO2 [mM]	Acetate mM	H2S mM
Bottle #5 w/ BES						
6/28/06	spirilla, a few filamentous rods	0.00486	0.00432	0.49518	0.5049	<0.1
7/6/06 1st transfer	spirilla, occ. fluorescent cocci, questionable smaller rods	Enrichment discontinued				
7/6/06	spirilla, small rods, medium rods, chain-forming rods	43.2	0.00432	0.31212	0.9636	~0.064
7/28/06	large clumps	NA	NA	NA	1.298	~.22
Bottle #6 w/ SO4						
6/28/06	spirilla and cocci	1.782	0.00432	0.5265	0.3707	>6.5mM
7/6/06 1st transfer						
7/6/06	dense w/ fluorescent cocci, same spirilla as above, fat spirilla	183.6	0	0.31752	0.9383	>4.2mM
7/18/06		5.508				
7/28/06		NA	NA	NA	0.2794	>1.7mM*

Clone Libraries and Phylogeny. The clone library of the TMA/BES enrichment contained only three clonal strains. Seventeen of the 24 clones were grouped within Peptococcacae clade of the Firmicutes. The nearest isolated neighbors were *Peptococcus niger* and *Desulfonisphora thiosulfatigens*. Both organisms have been studied previously for their sulfonatase activity (Van Eldere, *1987*; Van Eldere, *1988*; Van Eldere *1991*; Denger et al., *1999*). Four of the clones were dispersed within the genus *Desulfovibrio.* The remaining three clones were a single strain within the Catabacter group of the Firmicutes. The only isolated neighbor within this group was *Catabacter hongkongensis*, which was isolated from both a patient with appendicitis and a patient with septicemia. The physiology and metabolism of this organism was not reported.

There were a number of similar organisms in the clone libraries of the TMA/SO_4 enrichments of Sippiwisset saltmarsh and Trunk River. Archaea were solely represented by the genera *Methanococcoides*. Both contained enrichments contained *M. methylutens* while the saltmarsh enrichment also contained *M. sp.* strain NaT1. These strains fall within the Methanosarcinales clade, which has been well studied for their ability to utilize TMA for methylotrophic growth including *M. methylutens* (Sowers and Ferry, 1983). Both cultures also contained a number of *Desulfovibrio* isolates. The Trunk River clone library had 11 clones mostly closely related to *D. halophilus* while the saltmarsh library had 3 clones more closely related to *D. ferrophilus* and *D. brasiliensis*. Additionally, the libraries contained clones within the Spirochetes; however, the two Trunk River clones fell within the subclade SP/WWE1 while a saltmarsh clone fell within the Leptospirales clade. Lastly, both libraries had significant representation of a strain or strains closely related to the Firmicute *Fusibacter paucivorans*. Group 1 had 11 clones while group 3 had six clones, which, while poorly aligned, appeared to be closely related to each other and to *F. paucivorans.* Interestingly, *F. paucivorans* is known to reduce thiosulfate and sulfur but not sulfate. It ferments organics to acetate, butyrate, CO_2 and H_2 (Ravot, *et al*. 1999).

The remaining clones within the Trunk River and Sippiwisset sulfate/TMA enrichments were dissimilar. The Trunk River enrichment had eight clones representing a strain of Firmicute with neighboring isolated strains of the genera *Aerococcus*, *Turibacter*, and *Gemella*. None of these organisms have been examined beyond their occasional role in pathogenicity. The Sippiwisset saltmarsh clone library had a number of clones that indicate strong organotrophic activity in the enrichment. There were 14 clones with the *Cytophaga* clade, 3 clones within the Firmicutes most closely related to the aerobe *Filibacter*, which degraded organics to acetate and butyrate, a single clone with the *Bacteriodetes*, and a single clone closely related to *Vibrio chlorea*. Additionally, there was a single clone of the same Catabacter strain seen in the Sippiwisset saltmarsh BES/TMA enrichment. Lastly, the saltmarsh enrichment had eight clones that parsimony placed within the Mollicutes and distantly related to the cell wall-less organism and parasite *Anaeroplasma*. However, neighbor joining indicated that this group was probably more closely related to *Eubacterium cylindriodes* and *Clostridium innocuum*. *E. cylindriodes* reportedly has sulfonatase activity (Lie et al., 1996).

Discussion

Chemotaxis. The deployment of the chemotaxis chamber failed to yield significant results. However, I consider the construction the chamber to be very successful design. I think that I made three mistakes. First, I did not include a positive control. Second, I left the chamber in too

long. According to Kou-san, the longest the chamber should have been deployed was 30 minutes because a liquid attractant will equilibrate quickly with the external environment. Lastly, I placed the chamber in too high of a flow regime. This may have impacted bacterial motility and increased the rate of sedimentation into the capillary tubes. Although I think that this system would work in the field, I think that it would have been better to test for chemotaxis in the lab on enrichment cultures where the environment could have been better controlled.

Enrichments and Clone Libraries. The goal of the enrichment culture of BES/TMA was to select for TMA utilizing homoacetogens. Instead, the culture may have selected for a organism capable of fermenting BES, i.e. the 17 clones mostly closely related to *P. niger* and *D. thiosulfatigens*. Both of these isolates ferment utilize taurine, $H_2CH_2CH_2(SO_2)OH$, which is chemically similar to BES, $BrCH_2CH_2(SO_2)ONa$. The end products of taurine fermentation include both acetate and thiosulfate. It is conceivable that the *Desulfovibrio* strains are dependent upon one or both of the products for respiration. It is also possible that these *Desulfovibrio* strains are also capable of growing on BES. According to Lie *et al*., 1996 some stains of *Desulfovibrio* are capable of utilizing sulfonates like taurine as terminal electron acceptors. It is interesting to note that the *Desulfovibrio* strains in the BES/TMA saltmarsh enrichment were different strains from the SO_4/TMA enrichment. However, the slow growth of these organisms in the BES/TMA suggests that these strains were limited by a substrate. Considering that acetate appeared in excess, it seems plausible that these strains were limited by a suitable oxidant. It is also interesting to note the occurrence of the *Catabacter* strain in both of the saltmarsh enrichments. This group of organisms appeared relatively uncommon and certainly nothing is known about their role in the environment.

The goal of the enrichment cultures of TMA/SO_4 was to select for TMA utilizing sulfate reducing bacteria. Clearly, the methanogens dominated the enrichments; however, it was clear the H_2S was also generated. Moreover, microscopy and clone libraries revealed a wide diversity of organisms present, many of which within groups known to utilize various sulfur species. While the *Desulfovibrio* strains in these cultures may potentially utilize TMA as an electron donor, a more attractive candidate may be the strain of bacterium mostly closely related to *Fusibacter paucivorans* even though this particular strain doesn't utilize SO_4. What makes this clone a more attractive candidate is that this clone was found in both cultures and *Fusibacter paucivorans*' uses a variety of organics for fermentation and the end products of such fermentation are butyrate, acetate, CO_2 and H_2. These end products would support both the growth of organotrophs, like Cytophaga, and sulfur-reducing bacteria like some *Desulfovibrio*. Additionally, the eight clones that aligned with *Anaeroplasma* via parsimony and with *Eubacterium cylindriodes* via neighbor-joining may also be important components if not just interesting members of this enrichment. If these organisms were cell wall-less then they were most likely parasitic of members of the enrichment. However, the more plausible scenario is that these organisms were more closely related to *Eubacterium cylindriodes*, which may suggest a role for these organisms in the sulfur utilization. Unfortunately, the secondary enrichments cultures have yet to yield significant growth when suppressing the growth of methanogens via BES. However, I intend to continue to monitor these cultures and isolate and characterize them as a side project at my home institution. It was also unfortunate that the FISH experiment failed. This was the first attempt to use the archaeal probe and we did not have access to a positive control. I suspect that the hybridization stringency was too high for this particular probe. I think

it would be ideal to continue to follow the interactions between the bacteria and archaea as well as between the bacterial strains.

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