Microbial biodegradation of toluene in anaerobic environments is crucial for remediating the prevalent contaminant. *bamA*, the gene encoding 6-oxocyclohex-1ene-1-carbonyl-CoA hydrolase, has been used as a molecular biomarker for anaerobic toluene oxidation however is poorly characterized among environments. No trend among habitat type was found; instead, *bamA* sequences belonged primarily to Deltaproteobacteria and formed two distinct clades. Enrichment cultures suggest that either a purple sulfur bacterium photoheterotrophically oxidized toluene or Deltaproteobacteria in the enrichment couple toluene oxidation to sulfate reduction, providing sulfide and carbon dioxide for purple sulfur bacteria.

**Introduction**

Toluene, a methylated monoaromatic hydrocarbon, is a prevalent environmental contaminant. As a constituent of petroleum and product of incomplete combustion, toluene is continually introduced to the environment through leaking underground storage tanks, oil spills, and exhaust (Staats *et al.* 2011). Natural attenuation of toxic contaminants may be the most important tool for bioremediation. Microbes are the only organisms capable of utilizing toluene as a carbon source and in the process degrade the contaminant to innocuous products such as cells, CO₂ and H₂O.

Toluene is readily degraded in aerobic environments, however oil spills often affect anoxic subsurface coastal habitats. The investigation of toluene degradation in anaerobic phototrophic environments, particularly those utilizing alternative light wavelengths, is important to the understanding of contaminant cycling. Under anaerobic conditions toluene metabolism is channeled to the benzoyl-CoA degradation pathway, where the intermediate is sequentially oxidized (Kuntze *et al.*, 2008). The de aromatized ring is cleaved by 6-oxocyclohex-1ene-1-carbonyl-CoA hydrolase, encoded by the *bamA* (Figure 1). The *bamA* gene has been used as a molecular biomarker to detect the presence of monoaromatic hydrocarbons. The understanding of *bamA* diversity among environments of various physical parameters and anthropogenic impacts is currently limited.

Figure 1. Benzoyl-CoA degradation pathway
Anaerobic toluene degradation has been described in Proteobacteria. Chemolithotrophic Deltaproteobacteria couple the oxidation of toluene with the reduction of nitrate and sulfate (Li et al., 2012). Purple nonsulfur bacteria belonging to Alpha- and Betaproteobacteria are also capable of degrading benzoate, an oxidized aromatic hydrocarbon (Imhoff et al., 2005). It would be useful to determine whether these organisms can degrade more stable aromatics such as toluene, adding to the knowledge of anthropogenic contaminant cycling in the environment. The objective of this study was to combine enrichment and functional gene diversity to characterize toluene-degrading communities and elucidate potential degradation pathways.

Methods

Sample Locations
Three sites were selected to investigate bamA diversity and/or establish enrichment cultures. Microbial mat samples and pink sand were taken from Little Sippewissett salt marsh, a relatively unimpacted location with coarse sand. Fine grained, mucky, sulfidic sediment from Wild Harbor, a site impacted by oil spills, was collected. Eel Pond, a heavily trafficked harbor, contains sandy sediment.

Enrichments
Sediment was collected from three sites: Little Sippewissett salt marsh (LS) purple sand, LS black sulfidic mud containing small berries (LS mud), Wild Harbor (WH), and Eel Pond. Sediment from Little Sippewissett salt marsh purple sand was cultured in anoxygenic phototroph media containing 1mM sulfate prepared aerobically. Serum bottles were degassed and 20mL media was sparged with N2 gas for 10 minutes immediately prior to inoculation. Original cultures (6 July 2012) received 20g sediment inoculum to demonstrate proof of concept and were fed either toluene or acetate and grown at 850nm. Transfer cultures were established at day 10 and provided 1mL inoculum, 1mM toluene (+toluene), 1mM acetate (+acetate), or no carbon substrate and were grown at 850nm, next to a window, or in the dark. A second round of enrichments (16 July 2012) were inoculated with 8g purple sand or mud containing berries and treated as described for the transfer cultures. Wild Harbor sediment was cultured in artificial seawater medium containing 28mM sulfate, degassed as described above and inoculated with 20g sediment. Enrichments were maintained at 30°C in the dark.

Toluene measurements of LS enrichments
Toluene concentration in the headspace of bottles incubated at 850nm was measured using GC/FID (180°C, 12 minute retention, Shimadzu GC/FID) on day 18 of the original enrichments and day 8 of the second set. Attempts to measure toluene throughout the entire enrichment were thwarted by a malfunctioning GC.

CARD-FISH
CARD-FISH was performed for primary pink sand enrichments grown on toluene and acetate at both 850nm and on the window. Samples were vortexed for ~5 minutes (sonication attempts destroyed cells). After shaking the bottles, 1.5mL was extracted and centrifuged to isolate a cell pellet. Supernatant was removed and CARD-FISH was performed according to Ishii et al. (2004). 200uL of a 1:5 diluted working stock was filtered in a 16mm tower. Alphaproteobacteria, betaproteobacteria, and eubacteria were targeted using the Alf968, Bet42a +
Gammaproteobacteria competitor, and EUB-III probes, respectively. Alexa488 labeled tryamide labelled the probes and DAPI was used to label all microbes on the filter. Fluorescent microscopy without staining was performed to determine autofluorescence in the samples.

**PCR, Clone Libraries, and qPCR**

DNA was extracted using the MolBio Power Soil kit. 0.25g sediment from Eel Pond, Wild Harbor, and Little Sippewissett homogenized mat were used as reference environmental samples. DNA extraction of enrichment cultures required the consolidation of biomass. A 1.5mL sample was taken from the serum bottle, placed in an Eppendorf tube and centrifuged at 10,000 xg for 1 minute. 1mL supernatant was removed and the cell pellet was resuspended in the remaining 0.5mL, which was then placed in the MolBio 2mL beat beating tubes and extracted as usual.

PCR conditions of the bamA gene were applied as per Staats et al. (2011). bamA amplification of Eel Pond, WH, and Little Sippewissett microbial mat sediment, WH +toluene enrichments, LS pink sand + toluene enrichments, and Florence Shobutz’s +toluene mason jar was performed using the primer pair oah_f (GCAGTACAAYTCCTACACSACYGABATGGT) and oah_r (CRTGCTTSGGRCVGGCCTGVCCGAA). Amplified sequences were cloned into E. coli using TOPO vector and submitted for Sanger sequencing in 96 well plates. A phylogenetic tree was constructed in FastTree, which uses Neighbor Joining and heuristics (Price et al., 2009). The tree was colored in iTOL (Letunic and Bork 2006). Representative taxa from clusters within the tree were identified using BLAST (Altschul et al. 1997). qPCR was also performed to determine the copy number of the genes in environmental samples and enrichment cultures.

**Results**

**LS original enrichments: pink sand**

Differences in the acetate and toluene fed bottles were apparent after 4 days. Enrichments fed acetate were more turbid with a slight green/brown tinged supernatant, while the toluene fed supernatant was clear pink (Figure 2). Both conditions contained purple floc at the sediment-water interface.

![Figure 2. LS pink sand original enrichments](image)

**LS original enrichment transfers**

Primary transfer of acetate fed enrichments resulted in turbid, slight brown colored supernatant with poorly associated floc settled on the bottom (Figure 3). Toluene transfers, contrastingly, contained a clear supernatant with large flocculated biomass settled on the bottom of the serum...
bottle. Cultures lacking an added carbon source were clear with small green/brown settled biomass.

Figure 3. Transfer cultures

**LS second round enrichments**
Both the pink sand and berry mud enrichments provided toluene formed pink biofilm sheets on the sediment after 4 days (Figure 4). The sheets themselves were too opaque to view under 1000x, but their associated microbes contained shiny white granules indicative of purple sulfur bacteria. Serum bottles containing acetate lacked these sheets and appeared green/brown and turbid, as in the original enrichments.

Figure 4. Biofilm sheets in the serum bottle, 10x, and 1000x.

**Toluene measurements**
Because there was no toluene standard, relative peak area was compared among enrichments to estimate toluene uptake. By both day 8 and 18 there were toluene peaks in the pink sand enrichments +toluene grown at 850nm, however there was no peak in the +toluene enrichment grown in the dark. There was also no peak present in the mud with berries +toluene grown at 850nm or in the dark.
**CARD-FISH**
The DAPI signal was too weak to visualize cells, preventing the calculation of a total cell count. CARD-FISH for the three groups resulted in visualization of cells, however autofluorescence present among samples artificially inflated the number of target taxa and compromised the integrity of cell numbers. Calculations were therefore abandoned and CARD-FISH was used solely to visualize cells (Figure 5).

![Image of CARD-FISH labeled bacterium](image)

**Figure 5.** CARD-FISH Eub I-III labeled bacterium

**Functional Gene Phylogeny**
*bamA* was amplified from environmental sediment in Eel Pond, Wild Harbor, and a Little Sippiwissette microbial mat, as well as in WH +toluene enrichments. Additionally, *bamA* was amplified from Florence Shobutz’s +tol LS microbial mat enrichment. Course time restraints resulted in the sequencing of 48 out of 96 wells, so the shared plate containing the pink sand enrichment and Flo’s LS microbial mat +toluene *bamA* was not sequenced. The other 4 samples were successfully sequenced and confirmed to be *bamA* through blastn and blastx (Altschul *et al.* 1997). The phylogenetic tree of the three environmental samples and WH enrichment culture depict two distinct clades within the *bamA* sequences (Figure 6). qPCR was unsuccessful.
Discussion

*Toluene Oxidation Pathways*

Toluene degradation is a slow process and there was not sufficient sulfide initially supplied to the culture to stoichiometrically oxidize all toluene, so complete uptake of toluene was not expected. Therefore the toluene peaks present in the +toluene pink sand enrichments do not mean there was no toluene uptake. The lack of a standard, initial toluene concentration measurement, and intermediate timepoints make it impossible to quantify uptake.

The lack of a toluene peak in the LS mud containing berries can be the result of either toluene degradation or sorption to the organic mud matrix. Because the GC was not measuring toluene in the beginning of the experiment, a control (sterile culture +toluene) to determine abiotic uptake was not established. Toluene measurements are thus inconclusive and generally uninformative.
Distinct morphological differences were apparent between +toluene and +acetate enrichments of LS pink sand and mud. Secondary transfers receiving toluene, acetate, or no carbon substrate displayed the same pattern. A comparison of enrichments receiving either toluene or no carbon provides evidence for the use of toluene as a carbon substrate, and the variation in morphologies between +toluene and +acetate enrichments indicate that different biochemical pathways are utilized.

Toluene may be oxidized through two main pathways: anoxygenic photoheterotrophy or sulfate reduction (Figure 7). The utilization of toluene by anoxygenic phototrophs has been demonstrated in purple nonsulfur bacteria, however purple bacteria in the +toluene enrichment contain white granules indicative of elemental sulfur. Purple photoheterotrophic sulfur bacteria, such as Chromatiaceae, grow on simple carbon compounds (Butow and Burgstein-Ben Dan 1991). It is therefore possible but not likely that purple sulfur bacteria are solely responsible for initiating carbon cycling in +toluene enrichments. Sulfate reduction would likely be performed by Deltaproteobacteria, involving the oxidation of toluene and reduction of $\text{SO}_4^{2-}$ to $\text{HS}^-$.

Toluene may be partially oxidized to 2-3 carbon compounds, such as acetate, or fully oxidized to $\text{CO}_2$. Both $\text{CO}_2 + \text{HS}^-$ produced during the complete sulfidogenic oxidation of toluene can be used by photoautotrophs. The prevalence of both $\text{HS}^-$ and $\text{CO}_2$ could specifically select for purple sulfur bacteria. Elemental sulfur produced by these bacteria can then be abiotically or biotically oxidized back to sulfate. This sulfur cycle within the enrichment could compensate for low (1mM) sulfate concentrations in the media. The relatively slow oxidation of toluene with sulfate reduction could maintain favorable thermodynamics within the system; sulfate wouldn’t be sequestered as quickly as if the carbon source were more easily degradable. The contrasting appearance between +toluene and +acetate enrichments implies differential carbon utilization in the two conditions. If toluene were only partially oxidized, the enrichments would ultimately contain comparable substrates and thus display the +acetate phenotype. Because the +toluene and +acetate enrichments contain contrasting pigments, turbidity, and biofilm growth, it is possible that sulfate reducers are fully oxidizing toluene to $\text{CO}_2$, which is then used as a carbon source by purple sulfur bacteria.
Figure 7. Possible pathways of toluene degradation

bamA Diversity
The phylogenetic tree of bamA sequences clearly depicts a lack of clustering by environment despite strong differences in physical parameters (ecosystem, sediment morphology, tides) and extent of hydrocarbon exposure. The introduction of toluene also failed to impact diversity. bamA sequence diversity does not appear to be dictated by environment or toluene addition; instead, sequences from different conditions cluster together. The two distinct clades present in the tree of bamA sequences imply that there is a distinct difference among sequences within the same environments. Further analysis of conserved and variable regions within the gene may clarify the cause divergence in these clades. Blastn and blastx analysis of representative sequences reveal that the two distinct clades share many genera (Table 1). Both are dominated by Deltaproteobacteria, with the exception of several sequences of Alphaproteobacteria.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Representative Genus</th>
<th>Phylum</th>
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<td>Rhodomicrobium</td>
<td>Alpha</td>
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</table>
Table 1. Representative taxa of bamA sequences according to cluster

Known toluene degrading purple nonsulfur bacteria fall in Alpha- and Betaproteobacteria. The discovery of alphaproteobacteria from Eel Pond bamA sequences reveals a possibility for toluene degrading photoheterotrophic purple nonsulfur bacteria. The presence of Deltaproteobacteria containing bamA supports the potential for toluene degradation by sulfate reduction, which would promote the growth of purple sulfur bacteria.

Future Directions

Identifying active members in the +toluene and +acetate enrichments would provide evidence for the biochemical pathway. To do so, it would be instructive to perform high-throughput sequencing of 16S rRNA to determine community members and track shifts in enrichment cultures. CARD-FISH with Delta- and Gammaproteobacteria probes would provide information on the quantity of bacteria capable of toluene degradation. Successful bamA sequence analysis reveals potential for toluene degradation in these environments and enrichments, however transcript analysis would confirm active toluene degradation. A bamA clone library constructed from cDNA of extracted RNA combined with qPCR would confirm toluene degradation and gene copy, allowing for comparison among environments and between the environment and enrichments.

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