Anaerobic oxidation of trimethylamine by marine phototrophs in Sippewisset marsh

Sander Hogewoning
Delft University of Technology, Department of Environmental Biotechnology
Julianalaan 67, 2628BT Delft, The Netherlands

Abstract
Trimethylamine (TMA) is commonly found in marine environments as a result of decaying organic matter. It has been shown by Dawn Ward (microbial diversity course 2000) that a purple non-sulfur bacterium closely related to *Rhodovulum sulfidophilum* was capable of phototrophic growth with TMA as the sole electron donor. Agar shake tube isolates showed peculiar growth where different colony’s seem to depend on each other. Clone library studies showed that the organisms involved in this possible syntrophy were closely related to *Rhodovulum sulfidophilum* and *Thiorhodococcus drewsi*.

Introduction
Trimethylamine (TMA) is commonly found in marine environments as a result of decaying organic matter. It has been shown by Dawn Ward (microbial diversity course 2000) that a purple non-sulfur bacterium closely related to *Rhodovulum sulfidophilum* was capable of phototrophic growth with TMA as the sole electron donor.

It is possible that TMA is oxidized according to the following three equations:

(1) \((\text{CH}_3)_3\text{N} + \text{H}_2\text{O} \xrightarrow{\text{CH}_3\text{N}_2\text{O} + 2[\text{H}]} \)

(2) \(4(\text{CH}_3)_3\text{N} + 6\text{H}_2\text{O} \xrightarrow{9\text{CH}_4 + 3\text{CO}_2 + 4\text{NH}_3} \)

(3) \((\text{CH}_3)_3\text{N} + \text{H}_2\text{O} + \text{FAD} \xrightarrow{(\text{CH}_3)_2\text{NH} + \text{CH}_2\text{O} + \text{FADH}} \)

Equation 1 describes the oxidation of TMA to TMAO. The enzyme responsible for this reaction, TMA-oxidase/TMAO reductase, can be found in several bacteria. Phototrophic purple bacteria are able to use TMAO as an electron acceptor anaerobic metabolism in darkness. Equation 2 describes the use of TMA by methanogens and it is very unlikely to occur in the phototroph enrichments. Equation 3 describes the conversion of TMA to dimethylamine (DMA) and formate by methylotrophic bacteria.

During the microbial diversity course several enrichments were set up. The enrichment for phototrophs was performed in pfennig bottles with TMA, thiosulfate and succinate as electron donors, at different light regimes ranging from 475 nm to >1000nm. The pfennig bottle at 880nm with TMA as electron donor turned black and was used for further study.
**Material and Methods**

**Isolation and cultivation**

Samples were taken from an anaerobic microbial mat and from purple deposits at Sippewisset marsh, Woods Hole (Mass., USA). Samples were kept in closed glass bottles and used for inoculation within 48 hours. Enrichment cultures were set up in seawater based media with different electron donors.

The enrichments were carried out at room temperature in Pfennig bottles at 880nm. When turbidity was observed 1 ml of the primary enrichment culture was transferred to fresh medium. The seawater based TMA enrichment medium contained (per liter of distilled water): 20g NaCl, 3g MgCl\(_2\)\(\cdot\)6H\(_2\)O, 0.15g CaCl\(_2\)\(\cdot\)2H\(_2\)O and 0.5g KCl, 10ml 0.5M NH\(_4\)Cl solution, 10ml 150mM potassium phosphate, 0.25ml 1M sodium sulphate, 5ml 1M MOPS buffer, 1ml HCl dissolved trace elements, 2g BES, 0.1ml 12 vitamin solution, 0.1ml Vitamin B12 solution, 2ml 5M TMA, 1ml 1M bicarbonate 12.5mg DCMU.

Isolates were obtained by plating 100µl on agar plates. The agar plates contained seawater base medium supplemented with (per liter) 10ml 1M sodium succinate, 10ml 1M sodium thiosulphate, 10ml 1M acetate and 1.5% washed Difco agar (TMA was omitted from the agar plates, due to the awful smell). The plates were incubated in anaerobic jars in front of a light bulb. Isolated colony’s were restreaked on agar plates to obtain pure cultures.

Shake tubes were prepared by adding seawater based medium (supplemented with TMA) to molten agar. A 10-fold serial dilution of \(10^1\) to \(10^4\) was incubated at room temperature in front of a light bulb.

Colony’s on agar plates were characterized by light microscopy and by colony PCR. General bacterial primers 8F and 1492B were used to obtain 16S rRNA gene sequences.

A clone library was prepared by isolating DNA from several colony’s taken from a shake tube. After PCR with general bacterial primers 8F and 1492R, the PCR product was ligated in to plasmids and transformed in to competent *Escherichia coli* cells. 46 colony’s were picked for sequencing. The sequences were analyzed with ARB.

**Results**

**Isolation and cultivation**

After 14 days the TMA enrichment culture showed a black colour. Microscopic examination showed that the enrichment culture was dominated by small short rods (fig. 1).

![Figure 1: A 100x micrograph taken from the primary TMA enrichment culture after 14 days.](image)
Isolates were obtained on plate and four distinct colony morphology’s were observed. Bright red colony’s were later identified by 16S rRNA gene PCR to be closely related to *Rhodovulum sulfidophilum* (99% similarity). Pink colony’s were identified as to be related to *Thiorhodococcus drewii* (99% similarity). Two white colony morphologies were less prevalent and identified to be related to *Neptunonas naphtovorans* (91% similarity) and *Thiomicrospira denitrificans* (91% similarity).

The agar shake tube with the lowest dilution showed peculiar growth were different colony’s seem to depend on each other. Several big white colony’s were located around a single red colony and the white colony’s became smaller when they were further located from the red colony. This kind of growth can be an indicator for syntrophy, were both colony’s are only seen in the vicinity of each other (fig. 2a). Samples were taken and studied under the light microscope (fig 2b). It is evident that the red colony consisted of large motile cocii and the white colony’s consisted of small non-motile rods.

A sample was taken from the intertwined colony’s for clone library analyses. Both colony were also plated on agar plates and isolated colony’s (red and pink) were picked for PCR. The results of the clone library and colony PCR can be seen in table 1.

**Table 1: Results of the characterization of the intertwined colony’s from the agar shake tube.**

<table>
<thead>
<tr>
<th>Clone library</th>
<th>Colony PCR</th>
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</thead>
<tbody>
<tr>
<td>17 clones 96% similar to <em>Rhodovulum sulfidophilum</em></td>
<td>Red colony from plate isolate* 96% similar to <em>Rhodovulum sulfidophilum</em></td>
</tr>
<tr>
<td>15 clones 99% similar to <em>Thiorhodococcus drewii</em></td>
<td>Pink colony from plate isolate* 99% similar to <em>Thiorhodococcus drewii</em></td>
</tr>
<tr>
<td>1 clone 99% similar to <em>Vibrio parahaemolyticus</em></td>
<td></td>
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</table>

Plates were inoculated with the colony’s obtained from the agar shake tube.
An agar shake tube inoculated with a 10-fold diluted sample was incubated at room temperature in the dark. No growth was observed after 10 days and it be concluded that energy from light is essential for the enriched cultures for growth on TMA. There was no growth observed after transfer of the primary enrichment into fresh medium.

**Discussion**

A purple non-sulfur bacterium closely related to *Rhodovulum sulfidophilum* was isolated by Dawn Ward in 2000. The oxidation of TMA by phototrophic bacteria is not well understood and it is unclear which reaction takes place. Dimethyl sulphide dehydrogenase catalyses the oxidation of dimethyl sulphide to dimethyl sulphoxide (DMSO) during photoautotrophic growth of *Rhodovulum sulfidophilum* (McDevitt et al. 2002). McDevitt et al. (2002) showed that *R. sulfidophilum* was able to grow photolithotrophically with dimethyl sulphide as electron donor. Dimethyl sulphide dehydrogenase is closely related to TMAO reductase and therefore it is likely that *R. sulfidophilum* is able to grow photolithotrophically with TMA as the electron donor.

*Thiorhodococcus drewsii* belongs to the purple sulfur bacteria. There is no sulphide or thiosulfate present in the enrichment medium and in the shake tubes. It is possible that sulfate reducing organisms are present in the primary enrichment culture but it does not explain the growth in the low dilution shake tube. Growth of the organism on agar plates was probably stimulated by the addition of thiosulphate. The cells from the agar shake tube did not show intracellular sulfur globules, unlike the same culture taken from an agar plate (grown in the presence of thiosulphate).

*T. drewsii* is well described by Zaar et al. and they determined that hydrogen sulfide (up to 11 mM), sulfur, thiosulfate, and molecular hydrogen were used as electron donors during anaerobic phototrophic growth. In the presence of CO$_2$, it is capable of assimilating C1–C5 fatty acids, alcohols, and intermediates of the tricarboxylic acid cycle. Strain AZ1 could also grow photoorganotrophically with acetate as the sole photosynthetic electron donor. Chemotrophic growth in the dark under microoxic conditions was not detected.

The basis for syntrophy between *R. sulfidophilum* and *T. Drewsii* remains unclear. Possibilities are:

1. *Rhodobacter spaeroides* (related to Rhodovulum) is capable of hydrogen production under illumination in the presence of an inert, anaerobic atmosphere (such as argon), from the breakdown of organic substrates such as malate and lactate. The culture medium should be under a nitrogen limitation (i.e. a high C/N ratio), which forces the bacteria to ‘dump’ the excess energy and reducing power through the production of hydrogen (Koku et al. 2002). *T. Drewsii* can use the molecular hydrogen as electron donor during anaerobic phototrophic growth. This is unlikely because there are no organic substrates beside TMA and the medium is not nitrogen limited.
2. *R. sulfiophilum* might demethylate TMA as described in equation (3) and the products of this reaction might be used by *T. Drewsii* for photoorganotrophic growth. This reaction remains unclear and future studies might elucidate the use of TMA by *R. sulfiophilum*.

3. The medium contained reasonable amounts of BES to select against methanogens. BES (N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid or N,N-Bis(2-hydroxyethyl)taurin) might be used as electron donor for photolithotrophic growth by any of the two organisms.

Further chemical analyses of the enrichment is needed to determine the reactions during growth. Measuring TMAO and TMA content of the medium during growth can be good indicator.

References:


