Degradation of 2-bromo-ethane sulfonate (BES) and 2-mercapto-ethane sulfonate (coenzyme M) by anaerobic enrichment cultures

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Abstract
The sulfonic acids coenzyme M (methanogenic cofactor) and its structural analogue 2-bromo-ethane sulfonate (BES, inhibitor of methanogenesis) are important in natural habitats where Coenzyme M is released by methanogens or in inhibition studies of anaerobic habitats, respectively. In this study, they were tested for their ability to serve as fermentation substrates or terminal electron acceptors in anaerobic mixed cultures enriched from marine sediments.

It was shown that the mechanism of BES degradation is most likely the reduction of the sulfonate moiety by sulfate reducers. Desulfovibrio-like organisms were found to be enriched in sulfate-free enrichment cultures containing BES. Growth of the cultures was clearly stimulated by BES, and sulfide accumulated. Results for Coenzyme M are less clear, but growth experiments indicate a stimulatory effect of this compound as well.
Introduction
Coenzyme M (2-mercaptop-ethane sulfonate) is a cofactor involved in the final step of methanogenesis, transferring a methyl group to methyl-coenzyme M-reductase (MCR). Being an abundant compound in methanogenic archaea (BALCH & WOLFE 1979) and chemically stable after cell lysis, it is likely to be released in significant amounts in methanogenic environments. Uptake by methanogens is described in the literature, but little is known about its degradation. In sediments with steep gradients or physical disturbances it can be expected that it frequently reaches the oxic zone, making it unavailable for methanogens but accessible for aerobic microorganisms. MCR is irreversibly inhibited by BES (2-bromo-ethane sulfonate), a non-toxic structural analogue of coenzyme M, which makes BES a widely used specific inhibitor of methanogenesis. However, after prolonged incubation methanogenesis frequently resumes on a low rate (OREMLAND & CAPONE 1988, own unpublished results, results this course). Of the two possible explanations - resistance of methanogens or degradation of BES – the latter was addressed in this project. Studying growth on Coenzyme M and BES is also of interest from a more general perspective, as relatively little is known about the anaerobic metabolism of sulfonates in general (LIE ET AL. 1998, COOK ET AL. 1999), and there is only one study reporting use of BES as terminal acceptor in a mixed culture (YE ET AL. 1999).

Possible mechanisms of degradation:
• use as terminal electron acceptor (e.g. of isethionic acid by Desulfovibrio desulfuricans, LIE ET AL. 1996), sulfonate moiety is reduced to sulfide
• dehalorespiration
• fermentation (e.g. taurine fermentation, DENER ET AL. 1997)
• use solely as a sulfur source (aerobic bacteria)

In the first three weeks of the course anaerobic chemotrophic enrichments with H₂/CO₂ and BES were set up, and two observations there supported the hypothesis of BES degradation going on:
• sulfide formation (concentrations of up to ~2 mM from 0.4 mM originally)
• high abundance of Desulfovibrio sequences in clone libraries of BES enrichments without sulfate

Figure 1: Stuctures of Coenzyme M (left) and BES (right).
Based on that and measurements of acetate being produced the following scheme of metabolic interaction in this culture was developed and tested in the experiment.

<table>
<thead>
<tr>
<th></th>
<th>Homoacetogen</th>
<th>“Sulfate-reducer”</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electron donor</td>
<td>H₂</td>
<td>acetate (and/or H₂?)</td>
</tr>
<tr>
<td>Electron acceptor</td>
<td>CO₂</td>
<td>sulfonate-group of BES</td>
</tr>
<tr>
<td>Products</td>
<td>acetate (CH₃COOH)</td>
<td>CO₂ and H₂S</td>
</tr>
</tbody>
</table>

**Material and methods**

*Sampling and primary enrichment:* Samples from anoxic sediment layers were taken on June 20th 2006 from Sippewissett salt marsh and Trunk river, Cape Cod. Anaerobic enrichments were set up in 30 ml anoxic seawater medium (M1, composition see table 1) containing BES and either 25 mM TMA (trimethylamine) or a headspace of H₂/CO₂ (80/20 %, 50 kPa). The serum bottles were incubated at 30 °C unshaken in the dark. Transfers of 0.5-1 ml to fresh identical medium were performed twice (Sippewissett) or once (Trunk river) in the first 18 days.

**Table 1:** Anoxic seawater-base media used in the study. All media were prepared using anaerobic techniques. For description of those and composition of trace element and vitamin solutions see Microbial Diversity Course protocol collection.
**Growth experiments**

*Respiration vs. fermentation experiment (RF experiment)*: To test BES and Coenzyme M as possible substrates for fermentation or terminal electron acceptors 100-200 µl of 5 turbid enrichment cultures were used to inoculate Balch tubes with 5 ml medium M2 either without addition or with 20 mM BES or Coenzyme M, respectively (see table 2). This medium (M2) was only used because the medium M3 designed for this type of enrichment cultures precipitated irreversibly in the first batch of autoclaved tubes (see also methodological remarks). The headspace consisted of N₂/CO₂ (for fermentation) or H₂/CO₂ (for respiration, both 80/20 %, 50 kPa). Growth was monitored by visual inspection and, if turbidity occurred, measurement of the optical density at 600 nm wavelength on a spectrophotometer (whole culture in Balch tube against sterile medium).

**Lactate experiment**: To test whether the growth of sulfate reducers using BES as terminal electron acceptor can be stimulated by lactate, an electron donor used by most sulfate-reducing bacteria, a set of incubations in medium M3 with either H₂, lactate or no electron donor and BES concentrations of 0, 1, 2, 6 or 10 mM was set up with the 31 days old enrichment cultures from Sippewissett and Trunk river. Growth was monitored by visual inspection and measurement of OD600 as described above.

**Acetate experiments**: To test whether the sulfonate degrading part of the microbial community is limited by acetate two different experiments were set up: The only two cultures showing turbidity after 7 days in the RF experiment (table 2), Sippewissett with BES and Trunk river with CoM, were inoculated (0.5 ml) into Balch tubes with a H₂/CO₂ atmosphere (80/20 %, 50 kPa) and 5 ml sterile medium M3, with or without addition of 5 mM acetate and incubated on a shaker at 27 °C (acetate experiment I). Alternatively, 0.5 ml of a 37 d old BES/H₂ enrichment culture from Sippewissett (5 transfers) was inoculated into medium M1 with a H₂/CO₂ atmosphere (80/20 %, 50 kPa) +/- 5 mM acetate and either no electron acceptor (besides CO₂) or BES (20 mM) or sulfate (28 mM) (acetate experiment II, figure 4).

**Community analysis**

*DNA isolation*: DNA was isolated with the Promega Wizard Genomic DNA purification kit following the instructions of the supplier for bacterial cultures, but adding two freezing (-80 °C) and thawing (37 °C) cycles of 5 min in the beginning. Samples:

- enrichment culture from Sippewissett incubated with BES and H₂/CO₂ after 26 days and 2 transfers (15 ml)
- enrichment culture from Sippewissett incubated with BES and H₂/CO₂ after 32 days and 3 transfers (2 ml)
- enrichment culture from Trunk river incubated with BES and H₂/CO₂ after 32 days and 1 transfer (2 ml)
- enrichment culture from Sippewissett incubated with BES and H₂/CO₂ for 32 days, three times transferred and in "Lactate growth experiment" incubated with 10 mM lactate and without BES over night yielding a turbid culture (1 ml)

Success of DNA isolation was confirmed by gelelectrophoresis.
**PCR:** Bacterial 16SrRNA genes were amplified from different dilutions of DNA isolated from the first sample (enrichment culture Sippewissett, 26 d) with general eubacterial primers 2F and 1492R. For the other DNA isolates universal archaeal and bacterial primers U519F and U1406R (reviewed in BAKER ET AL. 2003) were used. PCR was performed according to the course protocol with an annealing temperature of 46 °C and 30 cycles.

**Cloning, sequencing and sequence analysis:** All reactions yielded distinct bands of the correct size on agarose gels and were subsequently transformed into competent *E. coli* cells with the Invitrogen TOPO TA cloning kit following the supplier’s instructions. Clones with plasmid insertions indicated by white colony morphology were sequenced by the MBL sequencing facility and sequences were manually checked for quality with 4peaks before importing them into ARB. Sequences were aligned by ARB’s build-in aligner against close relatives from the database, and the resulting alignments were manually checked. Phylogenetic trees including close relatives of the clones were calculated in ARB with the Maximum-likelihood method AxML and a Lanemask filter.

**Fatty acid HPLC**
Centrifuged samples acidified with 0,1 vol 5N H₂SO₄ were analyzed for fatty acids on a Shimadzu HPLC equipped with an Aminex HPX-87H column and a UV detector according to the course protocols.

**Results and discussion**

The clone libraries constructed from the 26 or 32 days old enrichment cultures from Sippewissett (figure 5) and Trunk river (figure 6) show very clear enrichment for few phylogenetic groups compared to the originally very diverse community of the raw sediment (course results, unpublished). This is also reflected by a reduced number of different cell morphologies as revealed by microscopic observations (figure 7). In both enrichments δ–proteobacteria are dominant, and sequences related to acetogenic spirochaetes or clostridia occur as well, consistent with the expectations to find a consortium of acetogens and sulfonate reducers as outlined in the introduction. Although primers covering archaea and bacteria were used, only bacterial sequences were retrieved, indicating that methanogens have been successfully suppressed by the BES in the medium. The presence of some *Desulfospirillum*-like ε-Proteobacteria in the enrichment from Trunk river cannot be easily explained, as they are closely related to microaerophilic sulfur oxidizers (FINSTER ET AL. 2005).

The respiration/fermentation experiment showed no growth without hydrogen and either CoM or BES (table 2), thus indicating that these substances were not fermented, but used as terminal electron acceptors. Not all inocula yielded growth during 14 days of observation. Although homoacetogenesis is favorable under the prevailing conditions, and acetogens were present in the enrichment cultures used
to inoculate, this process alone was apparently too slow to be noticed as turbidity in the 12 days duration of the experiment.

Table 2: Growth conditions and success in the respiration/fermentation experiment. Indicated is the number of days after which growth occurred and the increase in OD 600.

<table>
<thead>
<tr>
<th>Enrichment source, type and # of transfers</th>
<th>without H₂</th>
<th>with H₂</th>
<th>BES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>blank</td>
<td>CoM</td>
<td>BES</td>
</tr>
<tr>
<td>Sippewissett, H₂, 3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sippewissett, TMA, 3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trunk river, H₂, 2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trunk river, TMA, 2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trunk river, TMA, 1, 30 d</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

HPLC analysis, however, showed significant formation of acetate (7 mM) and formate (17 mM) in the turbid H₂+BES culture from Sippewissett. Formate is the first intermediate in the metabolic pathway of homoacetogens, but its formation does not result in energy conservation. Formate accumulation surprises therefore, but can be explained by huge partial pressure of H₂, which has been found to be linearly correlated to formate production by homoacetogens (PETERS ET AL. 1999). This also
explains the pattern of formate and acetate concentration in cultures of different age after inoculation (figure 3): Formate is formed in the young enrichment cultures with high H$_2$ partial pressure (40 kPa) and is later, when hydrogen gets depleted, used by homoacetogens and potentially sulfonate reducers as an electron donor. *Desulfovibrio ferrireducens*, the closest well-characterized relative to the *Desulfovibrios* enriched for in this study have been shown to grow on hydrogen and formate as electron donors (VANDIEKEN ET AL. 2006). Still it is possible, that acetate or other organic acids are required as a carbon source.

The lactate experiment failed in stimulating the sulfonate reducing part of the community, instead the lactate addition stimulated the fast growth of Clostridia-like Firmicutes fermenting the lactate to acetate (8.4 and 9.7 mM measured in two of the Sippewissett lactate cultures by HPLC) and propionate (5.2 and 4 mM, respectively). From a clone library 12 clones were picked, but only 5 of them sequenced by the sequencing lab, all of which were identical and most closely related to *Sedimentibacter hongkongensis*.

Table 3: Growth occuring in the lactate experiment.

<table>
<thead>
<tr>
<th>BES-concentration [mM]</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>6</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sippewissett lactate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$_2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no e- donor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trunk river lactate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H$_2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no e- donor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BES-concentration [mM]</th>
<th>20-40</th>
<th>40-60</th>
<th>70-90</th>
<th>100-130</th>
<th>&gt;130</th>
</tr>
</thead>
<tbody>
<tr>
<td>hours until exponential growth phase started and an OD 600 of &gt;0.15 was obtained</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The fact that visible growth occurred in this experiment even in the absence of BES and after relatively short time can be explained by the 10 times larger inoculum: 1 ml in 4 ml medium instead of only 0.1 ml in the respiration/fermentation experiment.

Acetate cannot be fermented and was therefore used in a similar approach. The results here are, however, contradictory. In experiment I growth occurred to a similar extent in all cultures inoculated with the Sippewissett H₂+BES enrichment, but in none of the Trunk river CoM enrichments. The latter is not surprising, as the inoculum itself had shown only puny growth (figure 2) on CoM and none on BES. The acetate experiment II (figure 4), in contrast, is in accordance with the expectations: 4 days after inoculation no or nearly no growth is observed in cultures without terminal electron acceptor other than CO₂, because homoacetogens grow slowly and methanogens have probably been close to extinction after >30 d and 4 transfers in BES medium, whereas the supposed sulfonate reducers, *Desulfovibrio*-like sulfate reducing bacteria, thrive on BES as electron acceptor, and even better on their ‘natural’ one, sulfate.

Figure 4: Acetate experiment II after 4 days. Acetate is of minor importance for growth, sulfate seems to be preferred over BES.

Conclusions
The results of the fermentation/respiration experiment where growth occurred only in treatments with hydrogen and either BES or CoenzymeM and the acetate experiment II clearly indicate the enrichment for a culture using these substances as terminal electron acceptors. This is consistent with a clear enrichment for *Desulfovibrio* as shown in the clone libraries and microscopic observations. Unfortunately the method used for sulfide determination (see methodological remarks) did not work well, so that a clear quantification of formed sulfide could not be obtained. However, taking into account the uncertainty of the method, the accumulation of 1-3 mM sulfide within 3 days in the 5 transfer of the Sippewissett enrichment with H₂/BES, whereas BES-free cultures never exceeded 0.1 mM.
sulfide, are a good additional hint. The scheme of metabolic interactions outlined in the introduction can now be modified as follows:

<table>
<thead>
<tr>
<th></th>
<th>Homoacetogen</th>
<th>Desulfovibrio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electron donor</td>
<td>H₂ (and formate)</td>
<td>H₂(!), formate and acetate (?)</td>
</tr>
<tr>
<td>Electron acceptor</td>
<td>CO₂</td>
<td>sulfonate-group of BES (possibly CoenzymeM)</td>
</tr>
<tr>
<td>Products</td>
<td>acetate and formate</td>
<td>CO₂ and H₂S</td>
</tr>
</tbody>
</table>

**Methodological remarks**

*Medium precipitation:* The medium M3 was distributed in the anaerobic chamber to Balch tubes directly after preparation, and a lot of whitish precipitate was formed during autoclaving, which could not be redissolved by neither shaking, cooling nor pressurizing to 0.2 atm CO₂. The next day, the appearance of the medium had changed from an initially brownish colour to transparent with blackish precipitates. Autoclaving the medium afterwards yielded no or only readily redissolvable precipitate.

*Quantification of BES and CoM:* Coenzyme M is called MESNA in the medical literature and several quantification methods are described (e.g. Glowacki et al. (2005) *Talanta* **55**: 534-539), but none could be performed with the available equipment. BES cannot be measured with the sulfide assay of CLINE (see below).

*Sulfide measurement:* Sulfide was attempted to be measured with the colorimetric method of CLINE (1969). In the first attempt the calibration curve was approximately linear up to 400 µM sulfide (slope 0.0017), but duplicate measurements and 5x vs. 20x diluted samples did not result in similar reads. As pink coloration of resazurin dye in the oxic sample solution possibly interfered with the coloration of the colorimetric reaction, sample preparation in the second attempt was performed in the anaerobic chamber and cuvettes were stoppered before measuring on the photometer at 665 nm. However, calibration curves were only linear up to 100 µM, the zero read was 0.32, and duplicate samples differed by up to 0.4. This is possibly caused by aged DPDS solution.

**Acknowledgements**

All course teachers, TAs and students are acknowledged for the good atmosphere during all the summer. This work especially benefited from discussions with Jared Leadbetter, Bill Metcalf, Kou-San Ju, and help from Dave Walsh, Dion Antonopoulos and Jean Huang, thanks a lot! Beverly Flood shared all the troubles with slow growth, the terrible sulfide assay, large clone libraries, and helped me in my forgetfulness. Huge thanks also to the changing crew in the computer room for solving innumerable Apple and ARB problems and keeping me awake.
Literature


VANDIEKEN, V., C. KNOBLAUCH & B.B. JØRGENSEN (2006): *Desulfovibrio frigidus* sp. nov. and *Desulfovibrio ferrireducens* sp. nov., psychrotolerant bacteria isolated from Arctic fjord sediments (Svalbard) with the ability to reduce Fe(III). *International Journal of Systematic and Evolutionary Microbiology* 56: 681–685.

Figure 5: Maximum-likelihood phylogenetic tree of clones from Sippewissett BES/H₂ enrichment cultures after 3 (blue) and 4 (pink) transfers.
Figure 6: Maximum-likelihood phylogenetic tree of clones from Trunk river BES/H$_2$ enrichment cultures after 3 transfers.
Figure 7: Phase contrast pictures of Trunk river (left, 30 d old, 2 transfers, with Acetobacterium woodii for comparison) and Sippewissett (right, 35 d old, 3 transfers) showing clearly reduced diversity in cell morphology.

Acetobacterium woodii (Schink & Bomar, The Prokaryotes)