How does inhibition of methanogens affect microbial community and oxygen penetration in *Reticulitermes flavipes* hindguts?

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INTRODUCTION

Over the last decades natural methane emissions have gained much attention, partly because methane is recognized as a very potent greenhouse gas. Several sources for natural methane emissions are known, among them are woodfeeding termites. The methane released by these termites is produced by anaerobic acetoclastic methanogens populating the hindgut of the insect. Up to date the ecological function of the methanogens inside the hindgut is unclear. Methanogens are believed to form biofilm colonies on the inner wall of the hindgut. In this location methanogens may be permanently exposed to low levels of oxygen. It is hypothesized that methanogens are involved in oxygen depletion in the anaerobic environment of the hindgut.
It is important for the termite to maintain anaerobic conditions in its hindgut because only under these conditions acetate, the primary carbon and energy source for the termite, can be produced by homoacetogenic bacteria.

In this project the effect of inhibition of methanogens on the microbial population in the hindgut is investigated. The key research questions for this project were:

1. What is the effect of 2-bromoethanesulfonic acid (BES) addition on the community structure in the termite hindgut?
2. If methanogens consume oxygen, how do oxygen profiles in the termite's hindgut change when methanogens are inhibited by BES?
3. Are changes in the hindgut community structure reflected in results from fluorescence in situ hybridization (FISH) experiments using group specific probes?

Three independent experimental approaches were chosen to evaluate the effect of methanogenesis inhibition on the gut fauna and provide answers to the research questions. Using molecular biological tools changes in diversity of the bacterial hindgut community were assessed by comparing clone libraries before and after the addition of 2-bromoethanesulfonic acid (BES). Secondly oxygen profiles through the hindgut were measured amperometrically. The profiles before and after the addition of BES were compared. As a last step termite hindguts were cryosectioned and subsequently

hybridized with archeal, Cytophaga/Flavobacterium/Bacteroidetes and a general bacterial probe.

MATERIALS AND METHODS

Termites

A log of partially degraded wood was obtained from the Leadbetter's backyard in Woods Hole. The log contained numerous termites of the species *Reticulitermes flavipes*. Approximately 700 individuals were collected and added in equal numbers to 6 petri dishes. The Petri dishes served as incubation chambers for about 2 weeks. Each Petri dish was supplemented with an approximately 5 mm high stack of stapled tissue paper with an area of about one cover slip. The tissue paper stack was soaked with distilled water. 2-bromoethanesulfonic acid (BES) (5mM) was added to the distilled water of three plates. BES is a known inhibitor of methanogenesis. All plates were sealed with parafilm in order to prevent dehydration. Plates were kept in darkness inside a cardboard box. Everyday deceased individuals were removed from the plates. The original decayed log was kept as control. Even after weeks the log still contained numerous termites. The log was wrapped in cling wrap.

Quantification of inhibition of methanogens

In order to evaluate the net effect of BES addition to the termites, a methane production experiment was performed after five days of incubation. For the measurements 45 termites (approximately 160 mg biomass) were collected from the communities fed DI water + BES, DI water – BES and from the wood feeding control and put into 50 ml serum bottles. The gas phase in the bottles was exchanged with fresh air from outside (not lab air). 300 µl gas samples were taken at different time points whereof 200 µl were analyzed using GC. The experiment lasted for about three hours. The termites were discarded after the experiment.

Construction of clone library

UltraClean Fecal DNA Kit (MO BIO Laboratories Inc., Solana Beach, CA, cat. no. 12811-50) was used for hindgut DNA extraction. Approximately 30 termites per treatment were degutted. The guts were collected in 550 µl fecal bead solution and subsequently treated according to the manual for the extraction kit (MO BIO, 2004). In a 25 µl reaction (16 µl sterile water, 2.5 µl 10X buffer, 1 µl dNTP (10 mM), 1 µl Mg_2Cl (50 mM), 1 µl 8F primer (10 µM), 1 µl 1492R (10µM), 2 µl extracted DNA) bacterial rDNA was amplified. The PCR products from +BES fed and –BES fed termites were cloned using TOPO 2.1 TA cloning kit (Invitrogen, cat. no. 45-0641) and sequenced at the sequencing facility at Marine Biological Laboratory (MBL), Woods Hole, MA. Alignment of the sequences and construction of trees were done using ARB (Ludwig et al, 2004). Using the distance matrix generated by ARB the libraries for –BES and +BES were compared using the software Libshuff (Singleton et al., 2001).

Microelectrode measurements

Oxygen microelectrodes OX10 (tip diameter 10 µm) mounted on a motorized micromanipulator (MC-232) were used with a PA2000 picoamperage meter and a ADC-216USB data acquisition module. The data was logged using the Windows based program Profix. All equipment related to the microelectrode measurements was kindly provided by Unisense A/S, Aarhus, Denmark.

A 0.5% agarose gel with insect Ringer solution (Brune et al. 1995) was poured in a home made miniature gel chamber. The chamber consisted of two microscope slides approximately 2 mm apart from each other. A freshly prepared gut was submerged in cooled agar. One side of the gut was exposed to air.

FISH on cryosectioned hindgut

For each treatment five termites were selected and fixed in PBS (130 mM NaCl, 10 mM NaH_2PO_4, pH = 7.2) containing 2 % paraformaldehyde over night. Triton X-100 at a final concentration of 1% v/v was added to reduce the surface tension of the buffer. The fixation protocol was adapted from Thimm and Tebbe, 2003. The fixed insect was embedded in OCT compound (Tissue-Tek II; Miles, Elkhart, Ind.) and frozen at -20°C for >30 minutes. Using the cryosection facility in Lillie 208, 8 µm thick thinsections were cut and sorbed onto coated microscope slides (0.5 – 1 % gelatine, 0.05 % - 0.1 % $Cr_2K_2(SO_4)_4 \cdot X\,H_2O$). The slides were kindly provided by Alan Kuzirian from Marine Research Center. After overnight drying the OCT compound was removed by dissolution in distilled water. Samples were dehydrated by washing them in a dilution series of 25 %, 50 %, 75 % and 96 % of EtOH (adapted from Schramm et al. 1998). The dehydrated thinsections were further treated following Falk Warnecke's CARD FISH procedure handed out to the course participants (Warnecke, 2004). Minor adjustment had to be made to modify the protocol for CARD FISH on thinsections. The CARD FISH probes CF319a, EUB1 and ARCH915 were used with the dye Alexa 488.

Microscopy

Dehydrated thinsections were used directly for epifluorescence (Zeiss AxioSkop 2 mot-plus, Plan-Neofluar objectives, Zeiss MRm, AxioVision 4.2) and confocal laser scanning microscopy (CLSM, Zeiss LSM 5 PASCAL, Plan-Neofluar objectives). For the epifluorescence microscope the DAPI filter set was used to visualize autofluorescence. For CLSM excitation at 405 nm and long pass emission at 420 nm was employed. Alexa 488 was measured with the appropriate filter cubes for epifluorescence and 488 nm excitation/505 nm long pass emission for the CLSM.

RESULTS and DISCUSSION

Inhibition of methanogens

Specific methane production rates were calculated from headspace gas measurements. From Figure 1 it is obvious that the specific methane production rates for wood fed and tissue without BES fed termites are significantly different from tissue +BES fed termites. Gas production after BES addition is close zero. Therefore the prerequisite for all subsequent experiments is fulfilled.

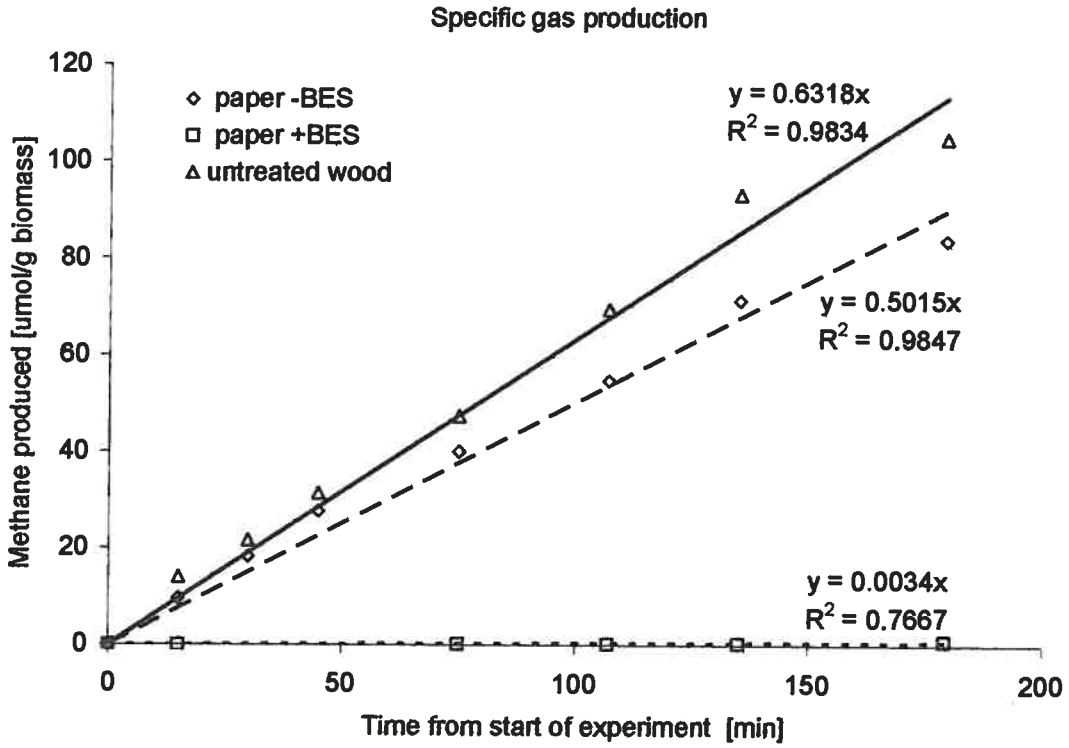

Figure 1 Specific gas production by three treatments of *R. flavipes* (approximately 160 mg biomass). Straight lines represent linear regressions.

Construction of clone library

The tree shown in Figure 1 resulted from the analysis of the cloned 16S rRNA sequences obtained from the DNA extracts. 92 sequences out of 96 – BES clones that were picked could be aligned. For + BES 85 sequences out of 96 could be successfully aligned.

A large number of both +BES and –BES clones appear in a group that was previously described by Ohkuma et al, 1996[1]. Ohkuma et al. isolated their rDNA templates from *Reticulitermes speratus*. The second largest group in the tree are clones of Bacteroidales. 23 of 29 clone sequences in this group originated from the + BES treatment, the remaining 6 were from the – BES treatment. For β proteobacteria 12 out of 16 clone sequences were from the –BES treatment whereas 4 sequences were extracted from + BES termites. All other sequences for + BES and – BES were approximately evenly distributed among the remaining major groups.

[1] At least I think it is this paper. I only found in ARB the date when the sequences were entered into the database. The date when this paper was issued is closest to the date specified in the sequence database in ARB.

The changes in the community are reflected in the results calculated with Libshuff. The comparison of the clone libraries revealed that with an alpha probability of 0.05 of type I error, it can be concluded that the +BES community is a subset of the –BES community ($\Delta C = 0.081$, $p = 0.147$). At the same time it is likely that - BES is different from + BES ($\Delta C = 0.593$, $p = 0.001$). This result is sketched in Figure 3. In order to substantiate the results it may be necessary to increase the number of termite guts to be extracted.

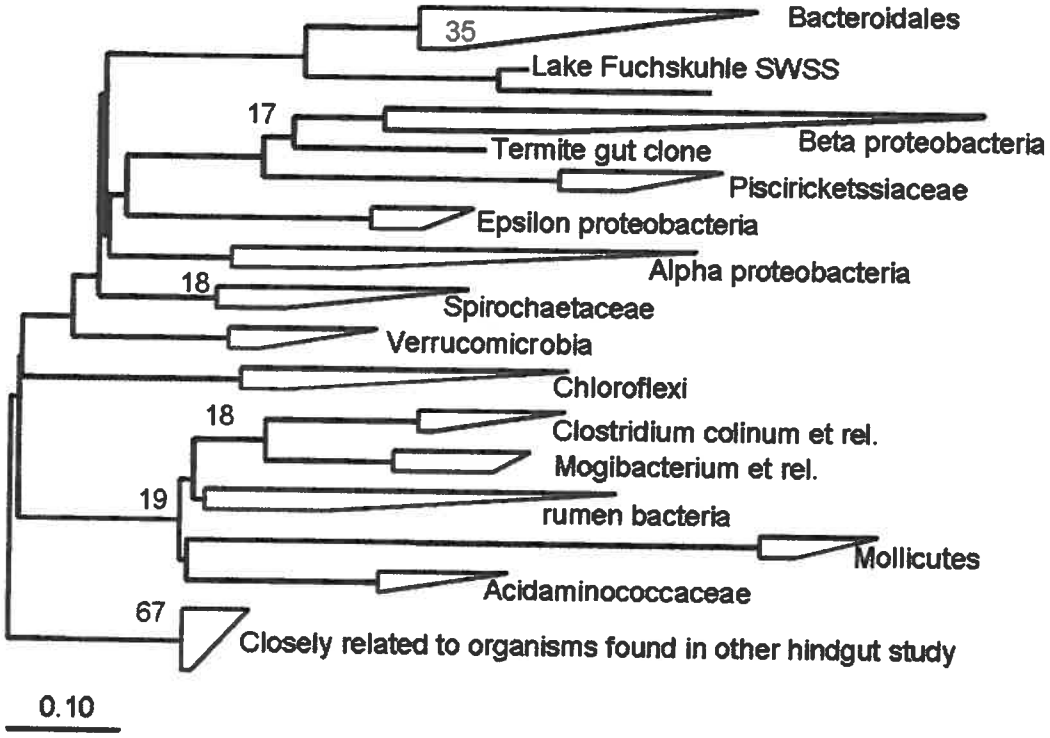

Figure 2 Combined tree of –BES and +BES sequences. Blue numbers do unfortunately not indicate the number of clones in the group but also include a small number of reference species.

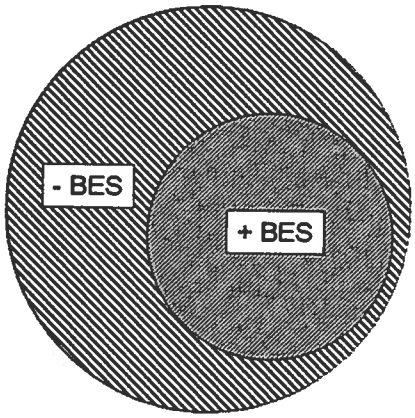

Figure 3 Sketch of Libshuff results for the comparison of the two clone libraries +BES and – BES.

Microelectrode measurements

In Figure 4 the approximate locations for oxygen profile measurements are indicated. The numbers in this figure correspond to the profile numbers in Figure 5.

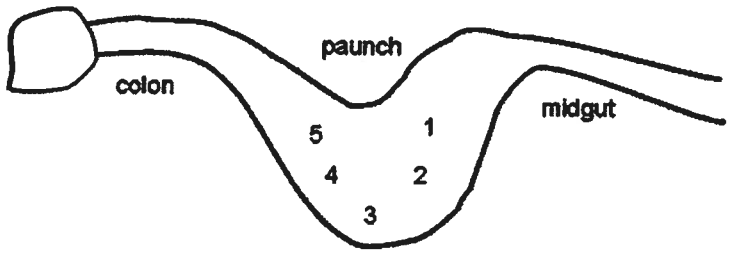

Figure 4 Termite hindgut. Approximate locations where profiles in Figure 5 were taken.

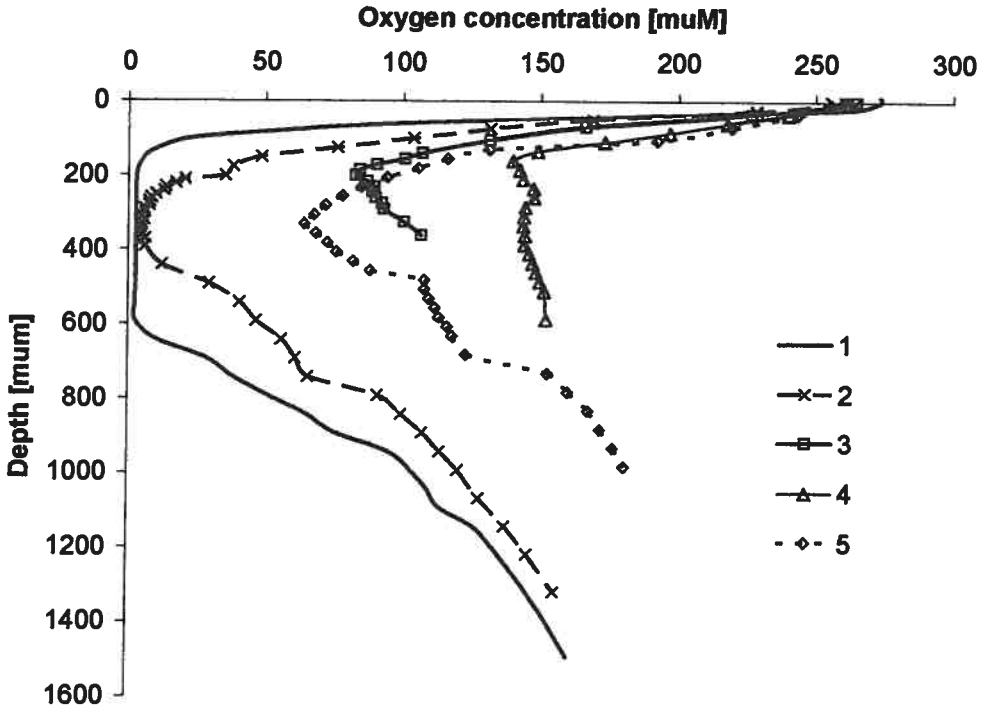

Figure 5 Oxygen profiles through different location of the hindgut of a + BES termite.

The most dramatic profile (number 1) plotted in Figure 5 indicates anoxic conditions in the center of the hindgut. At locations 2 – 5 truly anoxic conditions are never reached. This difference is caused by the geometry of the hindgut. When taking repeated measurements in one hindgut, it is unavoidable that the point of insertion of the electrode into the hindgut is changed. The dependence of oxygen profiles on the location in the gut is depicted in a conceptual model in Figure 6. This model explains how very different profiles can be expected for different locations. Therefore it is important to be able to

precisely control where the electrode is inserted into the hindgut, because changes in location have drastically changed oxygen profiles as a consequence.

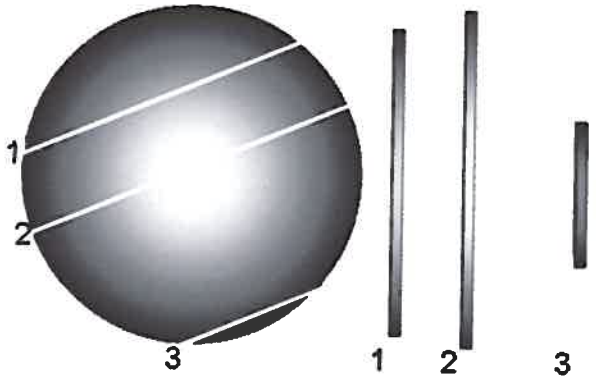

Figure 6 Conceptual model for different oxygen profiles at different hindgut locations: The circle represents a cross section through a hindgut, bars on the right show gray scale profiles taken from locations 1, 2 and 3. The darker the gray scale, the more oxygen is present.

Autofluorescence and FISH on cryosectioned hindgut

Below a number of pictures are presented that document the partial successes and problems with autofluorescence and CARD FISH.

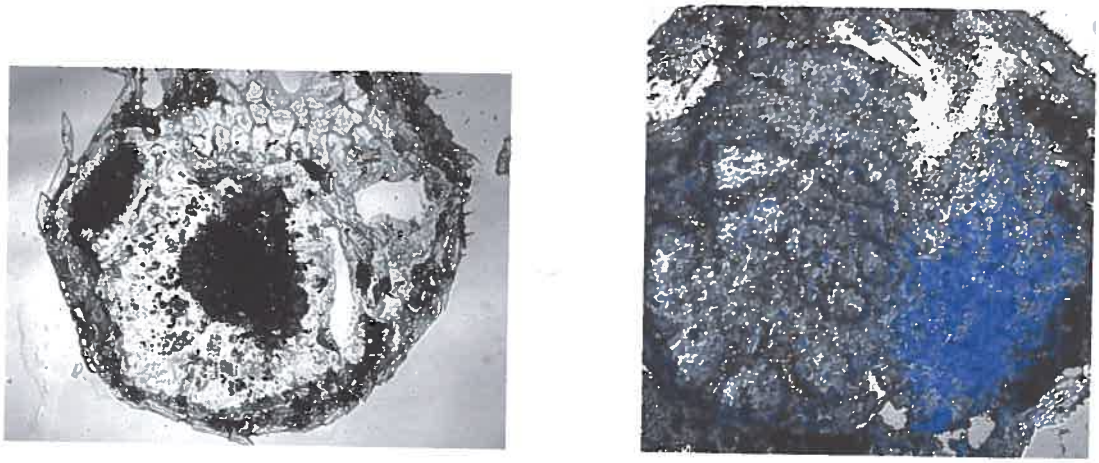

Figure 7 Brightfield DIC (epifluorescence microscope) and DIC & 405 nm excitation overlay (CLSM) of cryosectioned hindguts, unstained.

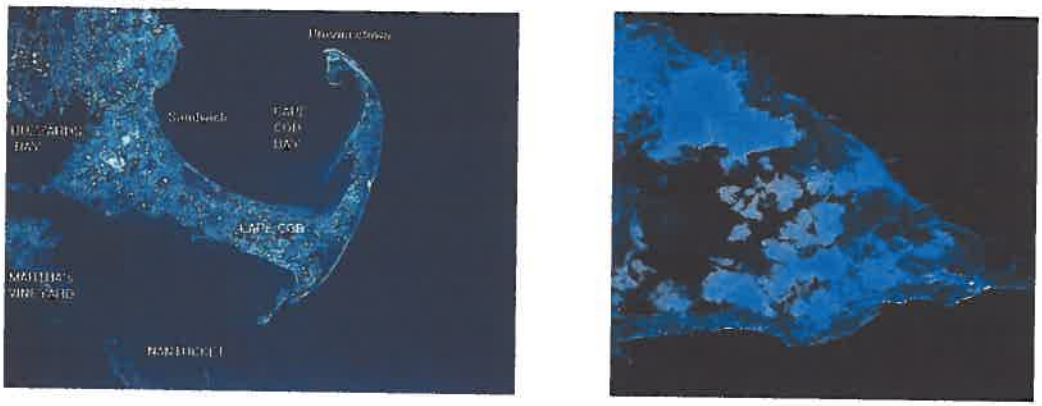

Figure 8 Satellite image of Cape Cod (left) and autofluorescence image of a (slightly stretched) thinsectioned termite part, darkfield overlay with DAPI autofluorescence, epifluorescence microscope (right).

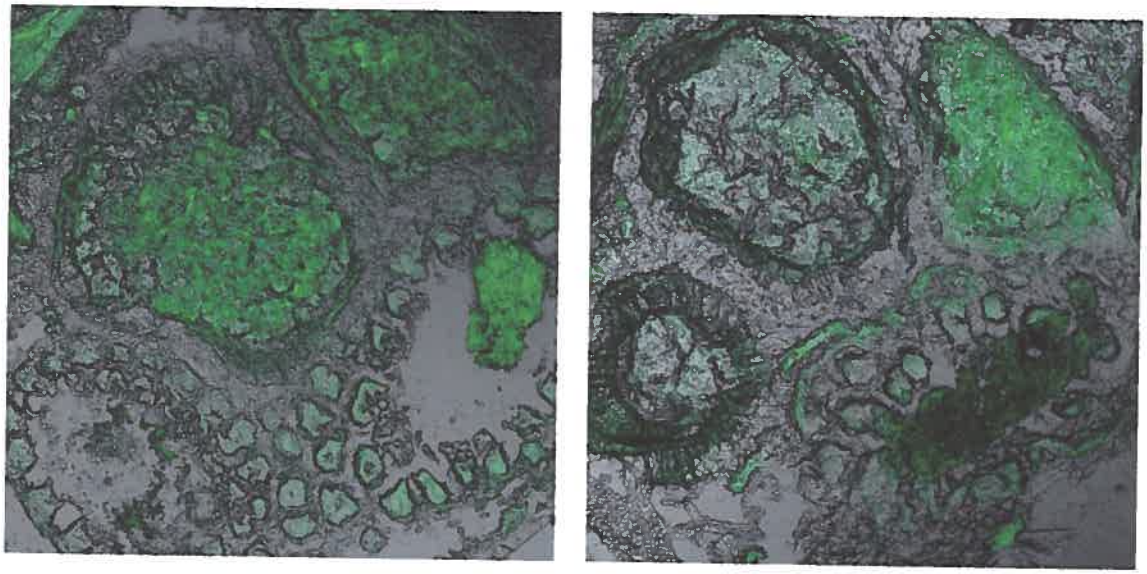

Figure 9 Two CARD FISH CLSM images taken of cryosectioned hindguts. On the left hybridized with EUB1, on the right hybridized with ARCH915.

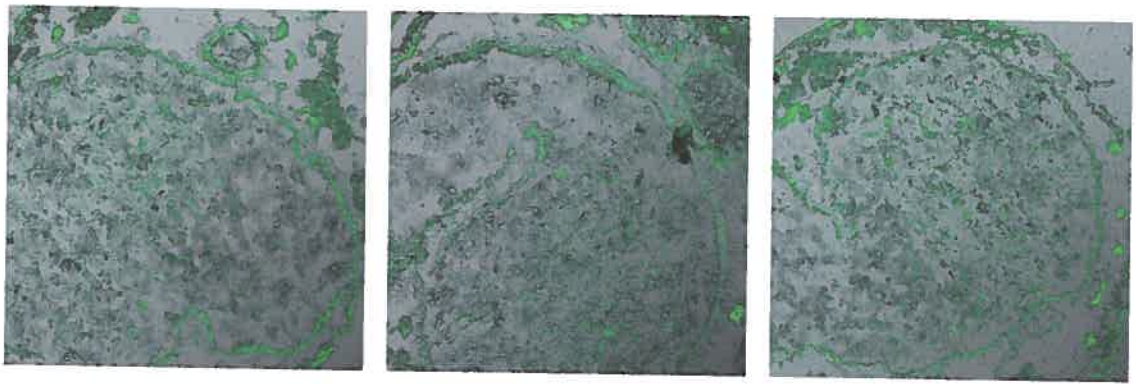

Figure 10 CARD FISH CLSM images of thinsections of termite hindguts. Far left ARCH915, middle CF319a, right EUB1.

It appears that in Figure 9 a difference between ARCH915 and EUB1 hybridization can be seen. On the other hand Figure 10 indicates that all three probes that were used in the study result in very similar fluorescence patterns. Due to the high level of autofluorescence at 488 nm excitation no meaningful results could be obtained from the CARD FISH analysis. It is not clear to me whether the autofluorescence at 420 nm is caused by methanogens or by tissue material. CARD FISH and also F420 analysis need further optimization.

CONCLUSION

DNA extraction and constructing of clone libraries worked. Based on the comparison of the two libraries, it is concluded that the community in the termite hindgut after +BES treatment is a subset of the hindgut community before BES treatment. Changes in community structure can be seen in Bacteroidales (number of clones increased after +BES treatment) and β proteobacteria (number of clones decreased after +BES treatment).

Fluorescence studies making use of F420 autofluorescence and CARD FISH could not document changes in the microbial community after +BES addition. The failure of the fluorescence approach is most likely caused by autofluorescence of hindgut tissue material.

It was possible to measure reasonable oxygen profiles through extracted hindguts. At the same time it was impossible to compare data from +BES treatment with –BES treatment since it turned out that it is absolutely crucial to know exactly at what location in the hindgut the profile was taken. Small changes in location resulted in dramatically different oxygen profiles.

ACKNOWLEDGEMENTS

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