Attachment of Host-Associated Methanogens:
Courting the wallflowers

Jessica Blanton
Scripps Institution of Oceanography- UCSD
MBL Microbial Diversity course, 2013

Introduction

Unlike bacteria, archaea are considered to have a minor numerical presence in most metazoan microbiomes, and are widely regarded as serving a minimal functional role. With respect to direct interactions with the host organism, to date there is not a single known pathogenic member of this domain (though the presence of archaea has been correlated with disease Lepp et al. 2004).

Most known interactions between archaea and eukaryotes involve methanogenic Euryarchaeota. Well known amongst these are the methanogens that commonly colonize the gastrointestinal tracts of vertebrates and arthropods, where they are presumed take part in the fermentative processes of host digestion (reviewed in Hackstein and van Alen 2010).

In environments which have flow-through such as gastrointestinal tracts, resident microorganisms must have doubling times short enough to keep pace with the flow rate, or they must devise a way to resist washout by "holding on". For the notoriously slow growing methanogens, it is most likely that they employ strategies to attach to structures within the gut. The persistence of methanogenic archaea within many different multicellular host systems inspires interesting questions about evolution of inter-domain interactions. Because of the unique biology of the archaea, it is likely that their complement of attachment methods contains some mechanisms similar to those currently characterized for eukaryotic-bacterial interactions, as well as some that are distinctly different. Little is currently understood about how archaeal adherence takes place, but understanding the communication between archaeal and eukaryotic surface structures could offer insight to how inter-domain recognition arises.

The gastrointestinal tract of *Reticulitermes flavipes*, the eastern subterranean termite, offers a good system to begin assessing the question of methanogenic attachment in a host environment. Numerous methanogen cells are found tightly attached to the hindgut walls of these insects. These cells are easily visible under 100x magnification due to the autoflourescence of the F_{420} cofactor at 420nm (Cheeseman et al. 1972). This cofactor is a necessary and unique component of the methanogenesis pathway, allowing for identification specifically of methanogenic organisms. While the termite gut microbiome is fantastically diverse and complex (Breznak and Pankratz 1977), the insect system lacks the mucus (Bignell et al. 1980) and adaptive immunity that can complicate preliminary studies in vertebrate guts.

Thus far, three species have been cultivated from *R. flavipes* (Leadbetter and Breznak 1996; Leadbetter et al. 1998). These species are distinguishable by their morphology: *Methanobrevibacter cuticularis* is a straight rod, *Methanobrevibacter curvatus* appears as a curved rod, and *Methanobrevibacter filiformis* is identified by its long filamentous form. While all three species are found in tight association with the gut wall, the only clue to a mechanism of attachment is the presence of polar fibers on *Mbb. curvatus*. Few appendages have been characterized for the archaea in general, but they do

include functional homologs to bacterial flagella and pili, as well as novel structures such as hami and cannulae (reviewed in Jarrell et al. 2013). Additionally, genome analyses of vertebrate-associated *Methanobrevibacter rumenatium* and *Methanobrevibacter smithii* (Samuel et al. 2007; Leahy et al. 2010) have revealed candidate adhesion-like proteins, which have yet to be fully characterized.

In the face of so little information about the nature of methanogen attachment in host gastrointestinal tracts, even basic assays may provide foundational clues for further work. This report summarizes work done towards three goals over the course of three weeks to:

1. Demonstrate reproducibility of cultivation of methanogens found in *R. flavipes* hindguts.
2. Assess the detachment of methanogens from the gut wall with a panel of chemical treatments aimed to disrupt the mechanism of attachment.
3. Briefly explore of the presence of methanogens in other animal systems using molecular techniques.

Methods

Host Specimens

Reticulitermes flavipes worker termites were obtained from Gloria and Ed Leadbetter, from their yard in Woods Hole, MA. A *Geukensia demissa*, eastern mussel was collected from the Little sippewissett marsh by Microbial Diversity course participants in the first week of the class. The *Opsanus tau* fish specimen was provided by Dr. Allen Mengerink, from a tank of wild-caught fish from Massachusetts and New Jersey waters.

Enrichment setup

Anaerobic cultures were set up using three media types and BSS buffer as described by (Leadbetter and Breznak 1996). Media differed by complex nutrient load: JM2 contained 0.05% (wt/vol) of both casamino acids and yeast extract, JM3 contained 2% (vol/vol) rumen fluid, and JM4 contained 40% (vol/vol) rumen fluid. Fifty termites were washed and ground with a tissue homogenizer in 4 ml of buffer. Vials containing 40 ml of each media type were inoculated with 400 ul of termite homogenate. Serial dilutions of each media type were inoculated down to 10^{-5}. At 7 days, the new cultures were inoculated from the lowest original dilution. JM2 and JM3 cultures were transferred to JM2 media, JM4 cultures were transferred into JM3 media. All cultures were incubated at 30C.

Methane measurements and were taken with a gas chromatograph at 7 days for the initial cultures, and at 4 days for the transfers. Microscopic observations using epiflourescence at 420 nm were also made at these times.

Detachment assays

For each treatment BSS buffer was modified from the original formulation. This included Tween-80 at 0%, 0.1%, and 1%, ETDA at 0, 5 mM, 8 mM 12mM and 15 mM; NaCl at 24 mM, 124 mM, and 400 mM; and adjustments of pH to 4.0, 5.0, 6.5, 8.0, and 10 with 1M HCl or 1M Na_2CO_3.

Termite hindguts were dissected from the insect, splayed open longitudinally, and washed in 1 ml of BSS buffer. Two or three guts were added to 1ml of each treatment buffer, and shaken at 150 rpm for 30 min, at room temperature. Guts were then washed with the corresponding treatment buffer and wet mounted on slides using immersion oil.

Microscopic counts and analysis

Methanogens on each gut wall were imaged using epifluorescence microscopy at 420 nm on a Zeiss AxioScope A.1. Areas of each gut were outlined using the resident ZEN software, and pictures were saved for cell counting. Care was taken to image randomly, but within areas where the image of the gut wall was only 1 layer thick. Cell counts were done by eye, with an average of area of 3706 um^2 and 914 cells counted across all treatments.

Totals for each gut were summed, and the average cell count per 100 um^2 was calculated. To compare the effects of the treatments, a one-way ANOVA for each was performed using R (R Development Core Team 2010).

DNA extraction

Genomic DNA was obtained from 6 samples prepped in this study. The first sample was the termite homogenate inoculum- pelleted down by centrifugation. The second was five termite hind guts dissected out of the animal, pooled together, and washed in 1 ml BSS buffer. For both the eastern mussel and the oyster toadfish, the digestive tract was dissected out, and the outside was sterilized with 70% ethanol followed by rinsing with sterile water. The toadfish samples were further processed by separating the lumenal and epithelial portions. A toadfish mucus sample was also obtained from the dorsal area outside of the fish. The mussel and toadfish samples were homogenized in sterile PBS buffer by vortexing and drawing through successively smaller needles (down to 23-gauge). For a secondary positive control, a seventh genomic DNA sample of methanogen-containing sediment was obtained from a classmate, Ederson Jesus. All DNA extractions were performed using the PowerSoil kit (MoBio, CA), as per manufacturer's instructions, with the addition of a 65C lysation step.

PCR amplification

All genomic DNA samples were subjected to PCR with primers for the bacterial and archaeal 16S genes, as well as two sets of primers for the mcrA genes (Table 1) (DeLong 1992; Turner et al. 1999; Watanabe et al. 2001; Luton et al. 2002; Steinberg and Regan 2008). Each reaction was performed as follows: initial denaturing at 95C for 2min; followed by 30 cycles of 45s at 95C, 45s at the annealing temperature listed in Table 1, 1:30 min at 72C; 10 min at 72C. All products were verified by gel

electrophoresis for presence of a band of expected length. For any reactions that did not have a visible band, PCR was repeated a second time, using 5 µl of the first reaction as template. Additionally, Bacterial and Archaeal 16S genes from the termite inoculum and the mussel gut were amplified using pyrotag primers.

454 Sequence analysis

Using the Qiime program (Caporaso et al. 2010), sequences were de-multiplexed, cleaned of chimeras, clustered at 100% similarity, annotated, and counted. The Ribosomal Database Project (Wang et al. 2007) was used as the source of the classification annotations.

Results and Discussion

Cultivation

Enrichments of termite gut methanogens varied in their production of methane according to the media composition (Figure 1). Briefly, rumen fluid appeared to contribute greatly for these enrichments to perform methanogenesis. Visualization of the enrichments revealed only straight rod and filamentous morphotypes (Figure 2), suggesting that *Mbb. curvatus* is a more fastidious organism, or perhaps is significantly slower growing. Future suggestions would of course include lower dilutions and longer incubation, as the published isolates were obtained after 8 weeks (Leadbetter and Breznak 1996; Leadbetter et al. 1998).

Microscopic examination of gut epithelium

Visual assessment of the termite gut walls indicated that all three known morphotypes remain attached after washing (Figure 3). The use of autofluorescence at 420 nm in the presented here allowed not only for visualization of the methanogens, but the gut wall as well (Figure 4). This was important, as the best cell counts were obtained in areas where the field of view was only one layer thick.

It is important to note that these assays were counted from frames of gut images that were taken as objectively as *humanly* possible. As seen in Figure 5, there are many places to observe different levels of attachment/detachment. It is recommended that these types of assays be repeated with many more replicates and more gut area observed than there was time for during this study.

Detachment of methanogens from gut wall

Each treatment varied in ability to remove methanogenic cells from the gut wall. The results outline some basic directions for future work on archaeal-epithelial adhesion.

Tween-80, a mild, non-ionic detergent, showed no significant effect between the 0, 0.1%, and 1% concentrations ($p > 0.05$, Figure 6a). This indicates, for example, that the adherence to the gut wall is unlikely to be significantly mediated by hydrophobic bonds.

Increasing ion concentrations of Na^+ and Cl^- and alkalinity showed no significant effect on methanogen attachment (both $p > 0.05$; Figure 6d and 6e, respectively). However, the assay for acidity

revealed a significant effect on attachment, with decreasing cell density occurring with increased acidity ($F = 15.3$, $p = 0.011$; Figure 6c). A possible explanation for these results is that acidic solutions may cause deformation of possible attachment proteins, but that in general disruptions of charge-charge interactions are not strong enough to register in these assays.

Interestingly, the increase of EDTA, a divalent cation chelator, also corresponded with a decrease in methanogenic cell density on the washed gut epithelium ($F = 20.7$, $p = 0.00066$; Figure 6b). This raises some very interesting possibilities for future work, as calcium-dependent adhesins (cadherins) are found not only in eukaryotes, but also found in bacteria and indicated to be present in archaea (Fraiberg et al. 2010).

Further investigations into the effects of low pH and calcium (or other divalent cations) dependence on archaeal attachment to gut walls may uncover fundamental aspects of this inter-domain relationship. Two very relevant directions would be to attachment by enzymatic digestion of surface proteins with low levels of trypsin, and to try other classic dispersement methods such as sonication.

One complicating aspect of these assays was not addressed fully in this work: the damage potentially caused by the treatments to the archaeal and eukaryotic cells themselves. Any treatment that seriously damages the cell membrane of the epithelial cell may give a false signal for detachment of the archaea. Confirmation of the results found here by scanning electron microscopy would provide much more support for the conclusions, and is highly recommended for similar future work.

In order to truly assess the question of methanogen attachment in host environments, it is important to have publically available isolates for testing. However, beyond having the organism "in hand", it would be even more useful to have a genetic system by which to test putative mechanisms with. While genetic systems do exist for some methanogens (Atomi et al. 2012), there unfortunately are none for what may be the most prevalent group of host-associated methanogens: the *Methanobrevibacter*.

Uncovering methanogens in other host systems by molecular techniques

A final aspect of this project involved using molecular screens to test for the possible presence of methanogens in other host organisms. A reconditioning reaction was required to obtain a band for all mussel and toadfish, in contrast to the clear band obtained in the first PCR reaction for all termite samples (Table 2). Samples from the reconditioning step were visualized by gel electrophoresis, revealing that most samples amplified bands of incorrect size, indicating that a lot of mispriming in these reactions was brought up in intensity by the second PCR (Figure 7). This mispriming brings into question whether these sample truly produce methanogenic signals for the 16S or mcrA genes or not.

However, analysis of the 16S gene sequence data for the mussel and termite inoculum show that while amplicons amplified easily in the first reaction for the inoculum, it was the mussel that showed the

greatest amount of archaeal sequences (1.2% in the mussel sample, 0.014% in the termite inoculum) [1]. These results indicate that while the PCR signal obtained in this study is very noisy, it is likely that there is real archaeal signal present as well. This possibility exists for all samples but the toadfish lumen and mucus samples, as there is no visible band even at the target size. Methanogens in fish are widely understudied, despite speculation that they play a role in unusually high methane concentrations of certain marine water columns (van der Maarel et al. 1999). The presence of methanogens in the epithelial-associated portion would be consistent with the idea that these organisms use attachment to host substrate as a mechanism for surviving washout from the gut. Sequencing the amplicons obtained from toadfish epithelial samples may reveal novel methanogen-metazoan relationships.

Acknowledgements

I am so very grateful for the wonderful experiences from this summer, all my thanks goes out to course directors Dan Buckley and Steve Zinder for putting together this year's inspiring course. To the course and teaching assistants: words cannot say enough about your help and your patience.

For assistance with my min-project, thank you to Suzanna Brauer for her advice, and excitement for my work with methanogen cultivation; Chuck Pepe-Ranney for walking us through analysis of the 454 sequence data; Emily Cardarelli for her help with the statistical tests; and Ederson Jesus for use of his DNA sample. Lastly I want to thank my advisor, Eric Allen for all of his enthusiasm for my work and for supporting my attendance.

This project was also made possible by generous funding from the American Society for Cell Biology and the National Science Foundation.

[1] Samples for 454 sequencing were sent prior to toadfish gut sampling

Name	Use in this study	Gene Target	Primer Sequence 5'-3'	Product length (bp)	Annealing temp used (°C)	Primer references
BAC8F	Bacterial presence screen	Bacterial 16S rRNA gene	AGAGTTTGATCCTGGCTCAG	~1465	50	Turner, 1999
BAC1492R			GGTTACCTTGTTACGACTT			Turner, 1999
ARC21F	Archaeal presence screen	Archaeal 16S rRNA gene	TTCCGGTTGATCCYGCCRG	~937	55	DeLong, 1992
ARC958R			TCCGGCGTTGAMTCCAATT			DeLong, 1992
mcrAF	Methanogen presence screen *mcr*A-Set1	methyl coenzyme M reductase	GGTGGTGTMGGATTCACACARTAYGCW ACAGC	~490	55	Luton 2001
mcrAR			TTCATTGCRTAGTTWGGRTAGTT			Luton 2001
mlasF	Methanogen presence screen *mcr*A-Set2	methyl coenzyme M reductase	GGTGGTGTMGGDTTCACMCARTA	~490	60	Innis et al. (1999)
mcrA-revR			CGTTCATBGCGTAGTTVGGRTAGT			Steinberg & Regan (2008)
PYRO515F	Pyrosequencing: Archaeal Taxonomic identifier	Bacterial 16S rRNA gene	*CGTATCGCCTCCCTCGCGCCATCAG|barcode*|GA|* GTGYCAGCMGCCGCGGTAA	~392	58	Turner, 1999 (16S complimentary region only)
PYRO907R			*CTATGCGCCTTGCCAGCCCGCTCAG|GG|* CCGYCAATTCMTTTRAGTTT			Wantanabe, 2001 (16S complimentary region only)

Table 1

Primer sets. Multiple primer sets were used to assess broad aspects of microbial community composition and presence of methanogens in different samples. Letter F and R at end of each primer name indicates priming direction.

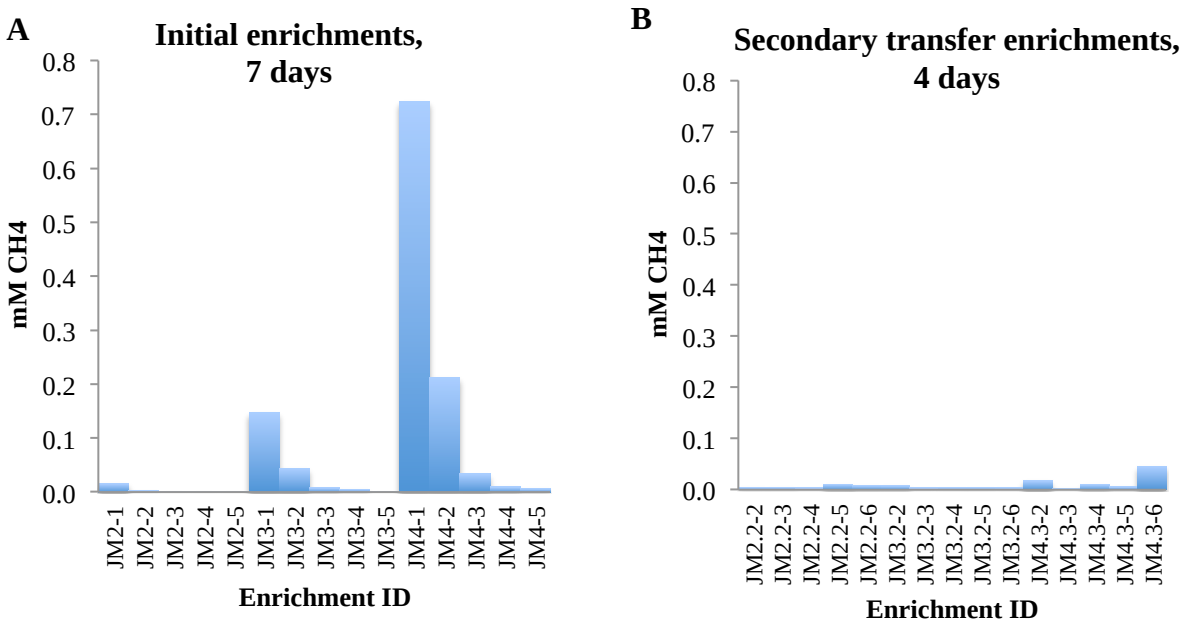

Figure 1
Methane production by initial (A) and transfer (B) enrichments. Methane production was highest in those cultures which had the most rumen fluid (JM3 and JM4). Transferred cultures produced low amounts of methane after 4 days.

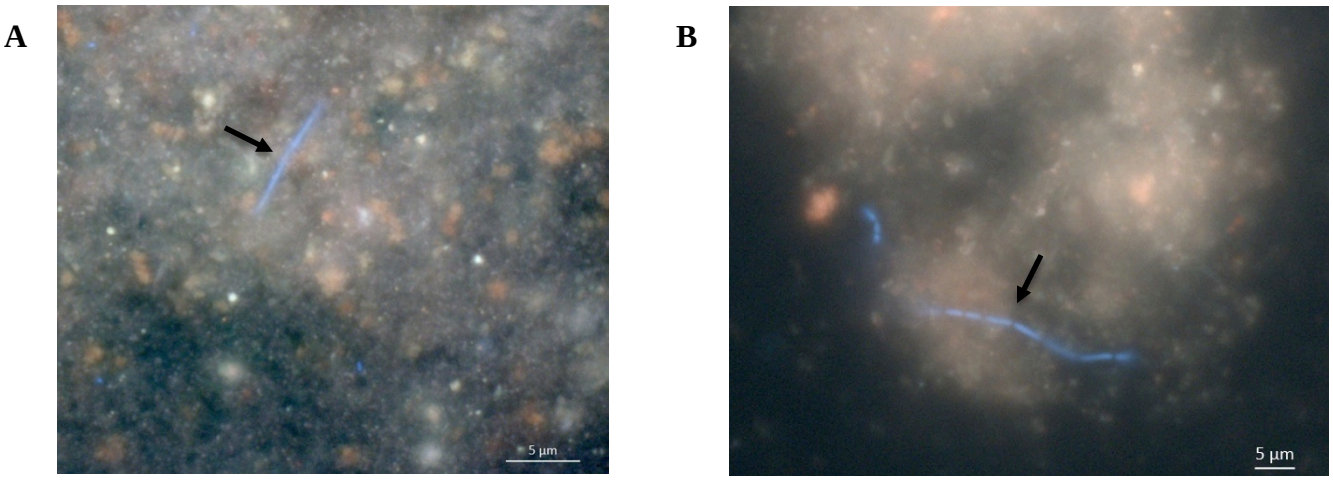

Figure 2
Morphotypes found in enrichments. Secondary enrichments were inspected by microscopy with visible light (for total cell mass) and as well as excitation at 420 nm (methanogens). Filamentous forms (A) and straight rods (B) were the only morphotypes observed. The presence of intact long chains suggest that the rod morphotype is actively growing, rather than a remainder from the initial inoculation.

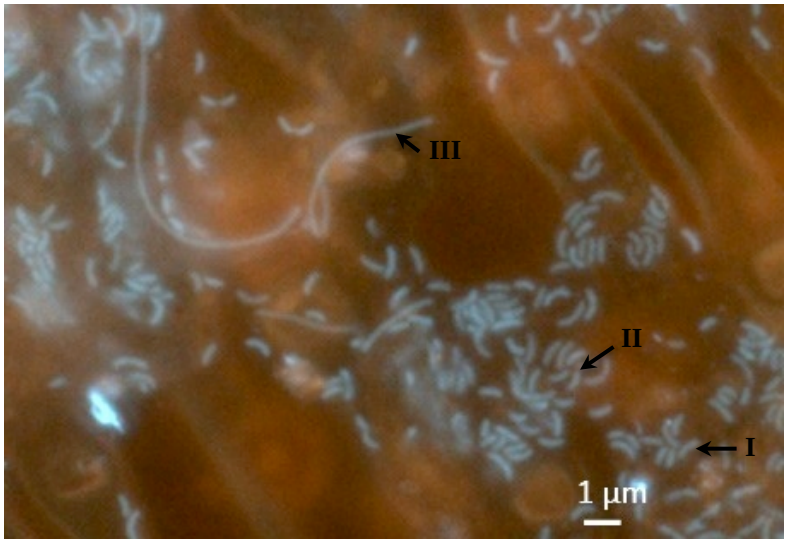

Figure 3
Observed methanogen morphotypes in cell count assays. *R. flavipes* hindgut washed with BSS buffer and imaged using epiflourescence at 420nm. I) *Mbb. cuticularis* – like (straight rods), II) *Mbb. curvatus* – like (curved rods), III) *Mbb. filiformis* – like (filamentous).

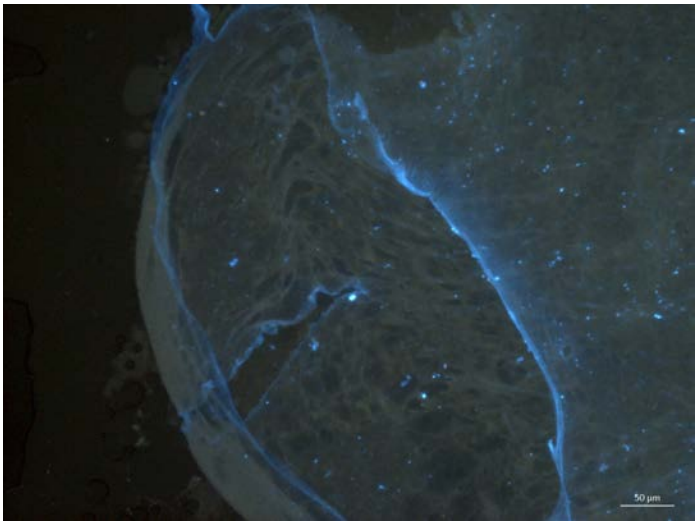

Figure 4
Autoflorescence of washed termite gut. Layers of the termite gut tissue were clearly visible at 420 nm wavelength.

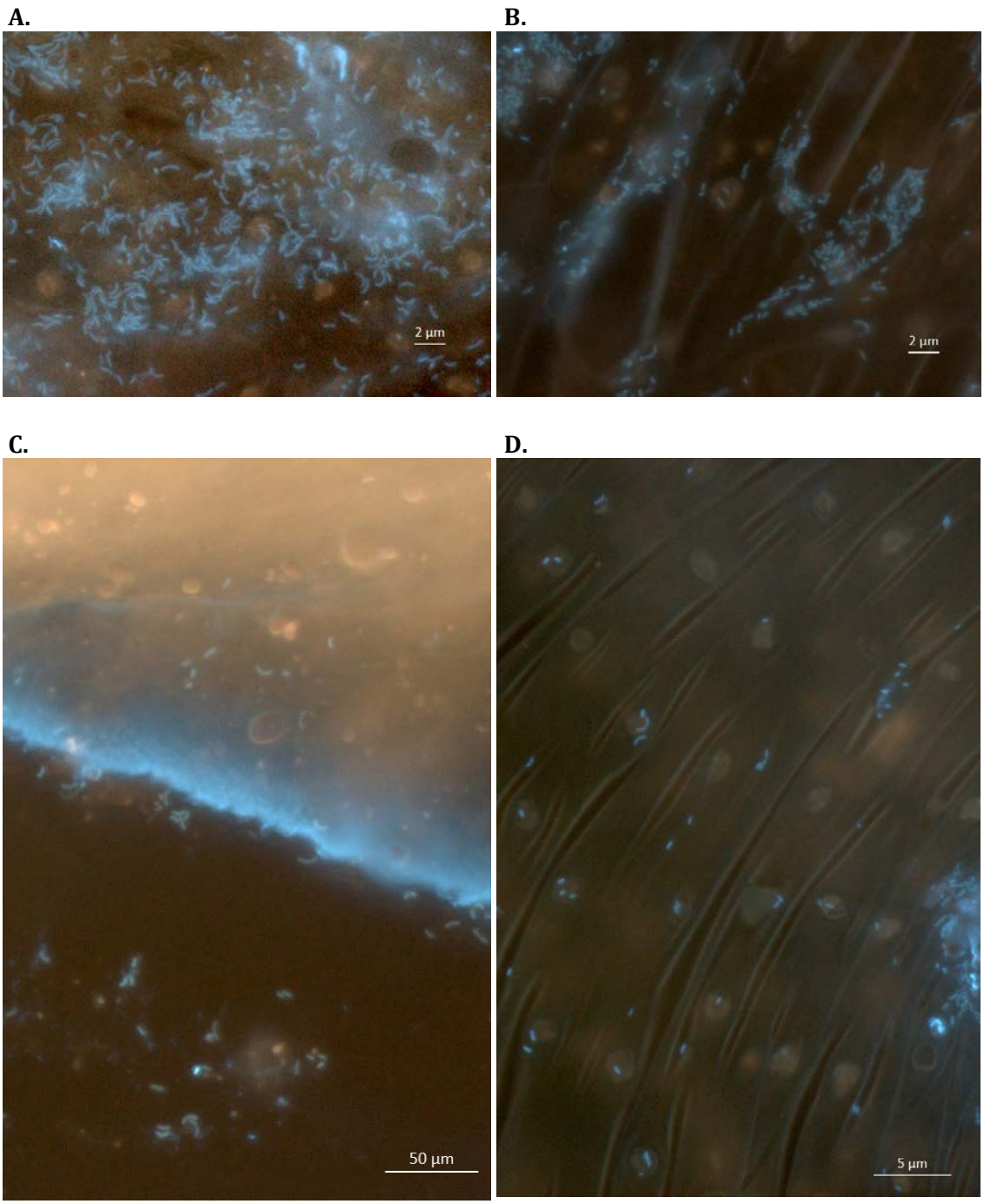

Figure 5
Diversity of gut surface images. No-Treatment washed gut (A), heterogeneity in the field of view for a sample treated with 400 mM NaCl (B), Unattached cells seen free in mostly cleared gut treated with pH 5.0 buffer (C), visible monolayer with few methanogen cells of a gut treated with pH 8.0 buffer (D).

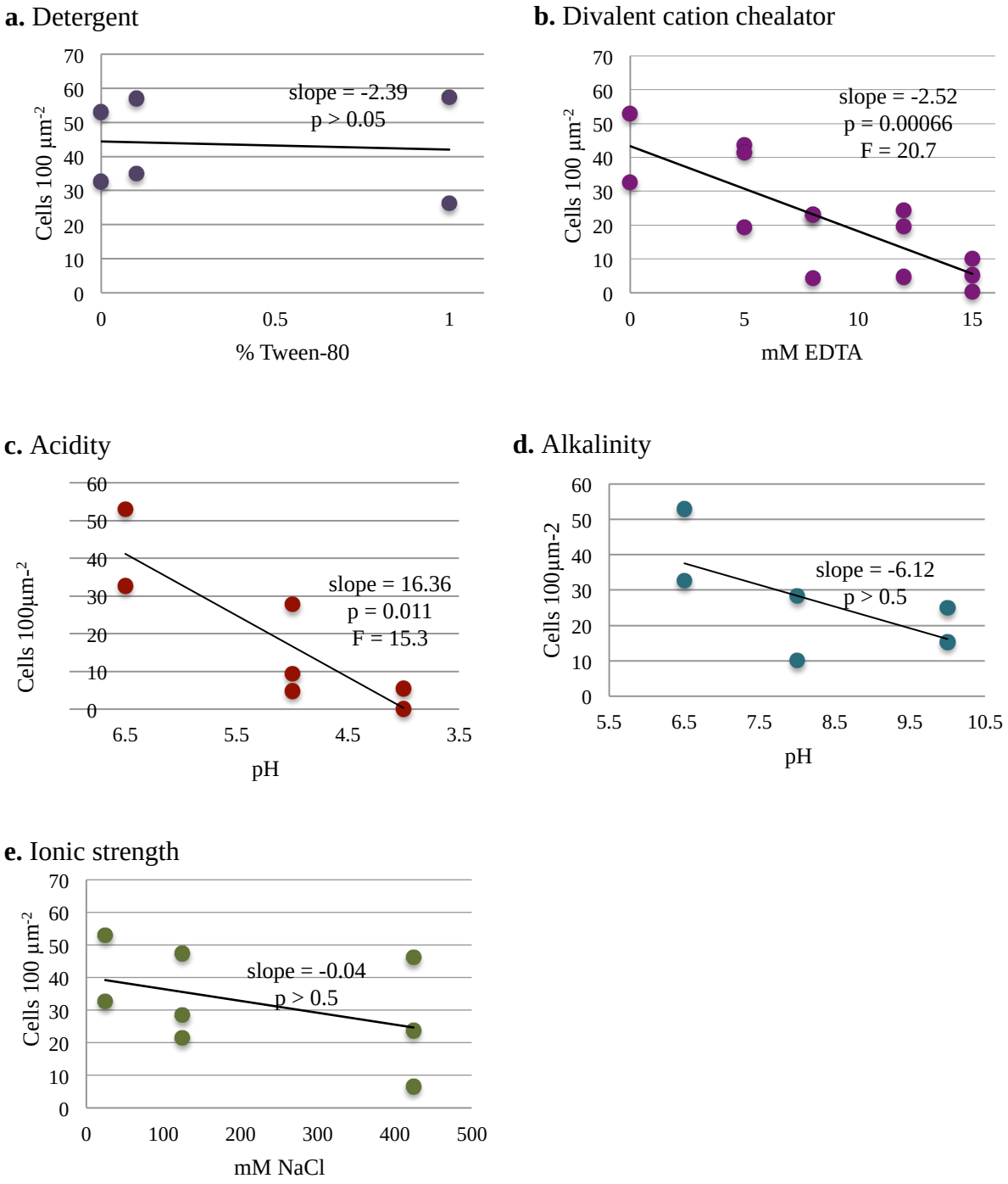

Figure 6
Detachment assays. Methanogen cell counts for each gut replicate are plotted according to treatment. P-values from the ANOVA analysis are reported as greater than 0.05 or, if lower, the value itself along with the F value.

DNA Source	BAC16S	ARC16S	*mcrA* Set 1	*mcrA* Set 2
Enrichment innoculum	+	+	+	+
Washed termite guts	+	+	+	+
Mussel digestive organ	+	+	+	+
Toadfish gut lumen	+	+?	+?	+
Toadfish gut epi	+	+	+	-
Toadfish outer mucus	+	+	+?	-
Methanogen-containing sediment	+	+	+	+

= Sample required reconditioning pcr

Table 2
Animal Host PCR results. Samples that produced a visible band at approximately the correct size are marked with "+", and those which clearly did not are marked with "-". Those DNA samples that produced a band only with a reconditioning step are shaded. For three reactions, the PCR was reconditioned, and produced a band *near* the correct length; these are marked with "+?". All negative controls (H_2O) for each primer set resulted in no band.

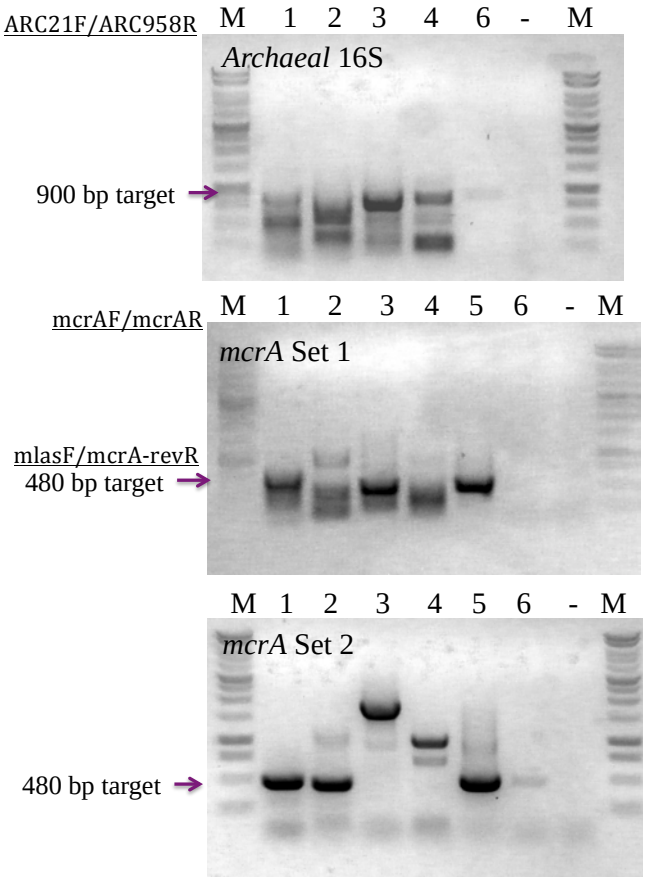

Figure 7
PCR reconditioning results. Reactions which did not produce a visible band with the first PCR were reconditioned by using 5 ul of product from the original reaction as template for a second PCR. Lane assignments are as follows: 1. Mussel digestive organ, 2. Toadfish gut epithelium, 3. Toadfish lumen, 4. Toadfish external mucus 5. Methanogen-containing sediment (reconditioned positive control) 6. Enrichment innoculum (fresh PCR positive control), last row H20 (-). DNA from the enrichment inoculum was included as a non-reconditioned positive control. Primer sets are listed to the left of each gel photo.

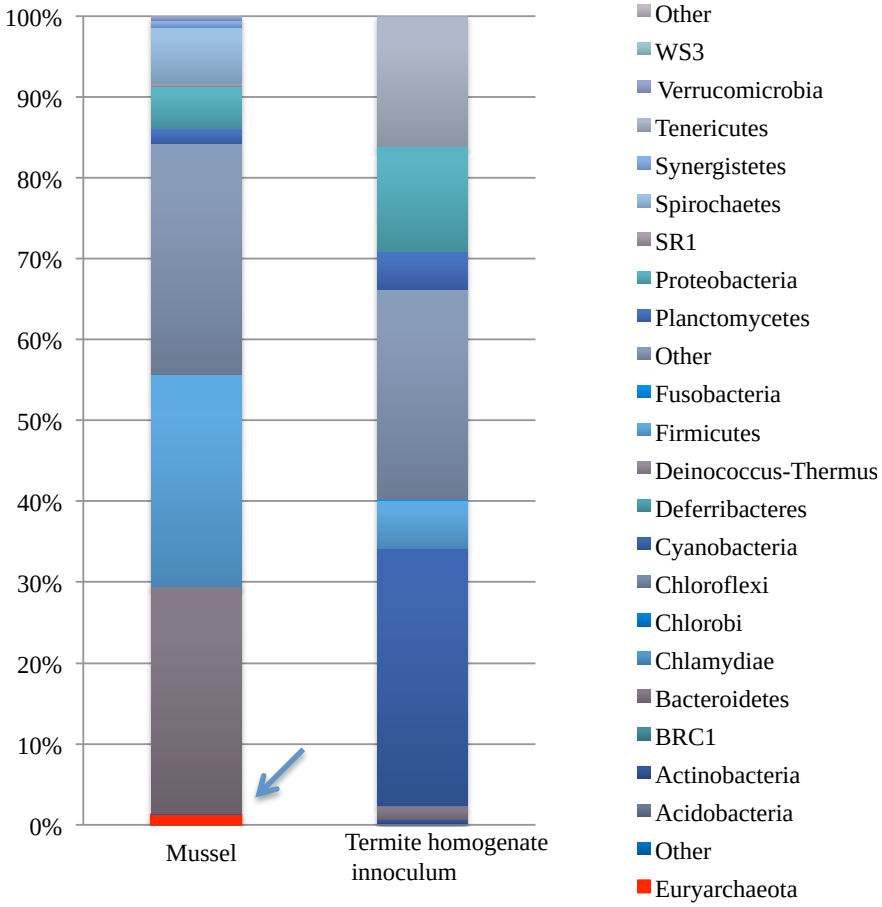

Figure 8.
Distribution of 16S diversity from 454 sequencing of mussel and termite inoculum.
Archaea are highlighted in red, and indicated by an arrow.

References

Atomi, H., T. Imanaka, et al. (2012). "Overview of the genetic tools in the Archaea." Front Microbiol **3**: 337.

Bignell, D. E., H. Oskarsson, et al. (1980). "Specialization of the Hindgut Wall for the Attachment of Symbiotic Microorganisms in a Termite Procubitermes-Aburiensis (Isoptera, Termitidae, Termitinae)." Zoomorphology **96**(1-2): 103-112.

Breznak, J. A. and H. S. Pankratz (1977). "Insitu Morphology of Gut Microbiota of Wood-Eating Termites [Reticulitermes-Flavipes (Kollar) and Coptotermes-Formosanus Shiraki)." Applied and Environmental Microbiology **33**(2): 406-426.

Caporaso, J. G., J. Kuczynski, et al. (2010). "QIIME allows analysis of high-throughput community sequencing data." Nature Methods **7**(5): 335-336.

Cheeseman, P., A. Toms-Wood, et al. (1972). "Isolation and properties of a fluorescent compound, factor 420 , from Methanobacterium strain M.o.H." J Bacteriol **112**(1): 527-531.

DeLong, E. F. (1992). "Archaea in coastal marine environments." Proc Natl Acad Sci U S A **89**(12): 5685-5689.

Fraiberg, M., I. Borovok, et al. (2010). "Discovery and characterization of cadherin domains in Saccharophagus degradans 2-40." J Bacteriol **192**(4): 1066-1074.

Hackstein, J. H. P. and T. A. van Alen (2010). Methanogens in the Gastro-Intestinal Tract of Animals. (Endo)symbiotic methanogenic archaea. Heidelberg, Springer.

Jarrell, K. F., Y. Ding, et al. (2013). "Surface Appendages of Archaea: Structure, Function, Genetics and Assembly." Life **3**: 86-117.

Leadbetter, J. R. and J. A. Breznak (1996). "Physiological ecology of Methanobrevibacter cuticularis sp nov and Methanobrevibacter curvatus sp nov, isolated from the hindgut of the termite Reticulitermes flavipes." Applied and Environmental Microbiology **62**(10): 3620-3631.

Leadbetter, J. R., L. D. Crosby, et al. (1998). "Methanobrevibacter filiformis sp. nov., a filamentous methanogen from termite hindguts." Archives of Microbiology **169**(4): 287-292.

Leahy, S. C., W. J. Kelly, et al. (2010). "The genome sequence of the rumen methanogen Methanobrevibacter ruminantium reveals new possibilities for controlling ruminant methane emissions." PLoS One **5**(1): e8926.

Lepp, P. W., M. M. Brinig, et al. (2004). "Methanogenic Archaea and human periodontal disease." Proc Natl Acad Sci U S A **101**(16): 6176-6181.

Luton, P. E., J. M. Wayne, et al. (2002). "The mcrA gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogen populations in landfill." Microbiology-Sgm **148**: 3521-3530.

R Development Core Team (2010). R: A language and environment for statistical computing. Vienna, Austria, R Foundation for Statistical Computing.

Samuel, B. S., E. E. Hansen, et al. (2007). "Genomic and metabolic adaptations of Methanobrevibacter smithii to the human gut." Proc Natl Acad Sci U S A **104**(25): 10643-10648.

Steinberg, L. M. and J. M. Regan (2008). "Phylogenetic Comparison of the Methanogenic Communities from an Acidic, Oligotrophic Fen and an Anaerobic Digester Treating Municipal Wastewater Sludge." Applied and Environmental Microbiology **74**(21): 6663-6671.

Turner, S., K. M. Pryer, et al. (1999). "Investigating deep phylogenetic relationships among cyanobacteria and plastids by small submit rRNA sequence analysis." Journal of Eukaryotic Microbiology **46**(4): 327-338.

van der Maarel, M. J. E. C., W. Sprenger, et al. (1999). "Detection of methanogenic archaea in seawater particles and the digestive tract of a marine fish species." Fems Microbiology Letters **173**(1): 189-194.

Wang, Q., G. M. Garrity, et al. (2007). "Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy." Appl Environ Microbiol **73**(16): 5261-5267.

Watanabe, K., Y. Kodama, et al. (2001). "Design and evaluation of PCR primers to amplify bacterial 16S ribosomal DNA fragments used for community fingerprinting." Journal of Microbiological Methods **44**(3): 253-262.