

Linking Phylogeny and Function of Methanotrophic Archaeal-Bacterial Consortia in Deep-Sea Methane Seeps using *in situ* Targeted Metagenomics, Molecular Ecology, and Stable Isotope Tracer Experiments

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Project Goals: We are exploring the biogeochemical functions and ecological relationships between ANaerobic MEthane oxidizing Archaea (ANME) and Sulfate Reducing Bacteria (SRB) and associated prokaryotes. We have pursued the following approaches: 1) Magneto-FISH to isolate the ANME-SRB consortia, 2) molecular investigation of phylogeny and function of the ANME-SRB metagenomes and the *in situ* community, 3) stable isotope tracer experiments plus single-cell FISH-nanoSIMS analysis to link organisms with function, and 4) bulk geochemical measurements to observe biogeochemical significance. Targeting ANME-SRB metagenomes has allowed comparison with other metagenomes and supported the reverse-methanogenesis hypothesis for methane oxidation. *In situ* measurements and stable isotope tracer experiments enabled testing the functionality of genes identified by metagenomic analysis within the ANME-SRB consortia, and revealed an unexpected role for these organisms in the nitrogen cycle. Although valuable in isolation, combining these techniques yields new insights into these uncultured methane-based syntrophic partnerships .

Targeting the uncultivable ANME-2c and their bacterial consortia in methane seep sediment from Eel River Basin, we have built on the Magneto-FISH technique pioneered by our lab group (Pernthaler et al 2008). We have generated and analyzed more than 10 times the sequence from our initial effort and, using the Community cyberinfrastructure for Advanced Microbial Ecology Research and Analysis (CAMERA) pipeline, identified a greater number of ORFs and gene/protein families. Due to the unique environment of the methane seep, the proportion of sequence assigned to COG or pfam database sequences has remained low (e.g. ~8% currently compared to 4% previously); also, when compared to the NCBI RefSeq database only 50-70% of ORFs in assembled contigs were similar to any protein sequences of characterized organisms. Similar to what we found in earlier targeted metagenomes, there was a wide variety of ANME-2c-associated bacteria, dominated by δ - and γ - *Proteobacteria*. δ -*Proteobacteria*-derived sequences were primarily affiliated with *Desulfobacterales*, *Desulfuromonadales*, and *Syntrophobacterales*. Many different γ -*Proteobacteria* were present with no single group predominating. Archaeal sequences made up a smaller percentage of the metagenome and were mostly from the *Methanosarcinales*, *Methanomicrobiales*, and *Methanocellales* and other methanogenic groups. Using 16S rRNA gene quantitative PCR, we were able to confirm our ability to enrich for specific syntrophic partners with Magneto-FISH,

with genomic DNA extracts from the *Desulfobulbus*-targeted magneto-FISH revealing a greater proportion of *Desulfobulbus* 16S rRNA genes compared with the original sediment.

To generate better contig assembly and to overcome limitations in annotation we have combined our individual ANME-2c targeted metagenome libraries from a single sample in the Eel River Basin into a single contig assembly. This combined metagenome was compared to publicly available metagenomes from methane seep sediments within the Integrated Microbial Genome (IMG) database and to recently published genomes of ANME-1, *Desulfosarcina*, and *Desulfobulbus*. Recruiting our contigs to previously published methane seep fosmid libraries, revealed up to 30% coverage of ANME-2 and SRB fosmids and little to no recruitment to taxonomically identified ANME-1 fosmids. All of the genes necessary for the reverse methanogenesis pathway are present in the ANME-2c enriched metagenome, confirming earlier findings. Genes for carbon fixation, sulfate reduction, nitrate reduction, and nitrogen fixation are also present. Grouping the reads and contigs using sequence-based categorization (e.g. tetranucleotide correlation and self organizing map algorithms) will increase confidence in these characterizations. Further association of identity and function using 16S rRNA genes and other housekeeping genes (e.g. RecA/RadA, RecG, leuS etc.) correlated to functional genes involved in sulfate reduction (*aprBA*, *dsrAB*), nitrogen metabolism (*nifHDK*, *nirK*, *napG*, etc.) and methanotrophy (e.g. *mcrA*) in the ANME-2c enriched metagenome and in other methane seep habitats. Combining these techniques should provide phylogenetic characterization that is less dependent on databases from cultivated organisms.

We leveraged information from the ANME-2c metagenome to show ANME archaea are involved in N₂ fixation at methane seeps. We initially identified the potential for ANME related *nif* genes in the metagenome (Pernthaler et al. 2008) and bolstered this with a survey of the *nifH* genes in the ANME-2c enriched sample and the microbial community at the Eel River Basin (ERB) methane seep. We showed direct incorporation of ¹⁵N₂ into ANME-2 archaea collected at the ERB using FISH-nanoSIMS (Dekas et al. 2009). We have expanded our study to other methane seeps (Costa Rica; Hydrate Ridge, USA; Monterey Canyon, USA) and to potential bacterial diazotrophs. Nitrogen fixation rates are highly spatially variable at methane seeps, both laterally and with sediment depth, with the highest rates observed within the methane-sulfate transition zone. Quantitative comparison between the EA-irMS bulk rates and the NanoSIMS single-cell rates of nitrogen fixation suggest that the ANME are responsible for the majority (~80%) of seep nitrogen fixation, but that the remainder is fixed by other diazotrophic organisms, likely including free-living sulfate reducing bacteria.

Publications:

1. Pernthaler, A., Dekas, A. E., Brown, C. T., Goffredi, S. K., Embaye, T., Orphan, V.J. (2008) Diverse syntrophic partnerships from deep-sea methane vents revealed by direct cell capture and metagenomics. *PNAS* 105(19) 7052-7057
2. Dekas, A.E., Poretsky, R.S., and Orphan, V.J. (2009) Deep-Sea Archaea Fix and Share Nitrogen in Methane-Consuming Microbial Consortia. *Science* 326: 422-426

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