NC10 phylum

Anaerobs? Methanotrophs?

Where can I find them?

Beate Kraft

Microbial Diversity 2012

Abstract

Methylomirabilis oxyfera, the only cultured member of the NC10 phylum performs the newly discovered pathway of NO-dismutation. In this study different habitats were screened for the presence of member of this phylum and the diversity of the sequences obtained was analyzed. Furthermore enrichment cultures for nitrite reduction coupled to methane oxidation were set up. Indeed, the presence of NC10 seems to be associated with nitrite and methane rich fresh water environments.

Introduction

Recently a new nitrite reduction pathway, NO-dismutation, coupled to methane oxidation has been discovered (Ettwig et al. 2010). In NO-dismutation nitrite is oxidized to NO as in denitrification but then NO dimutated into N2 and O2 instead of being further reduced to N2O. The generated oxygen is then used for methane oxidation. The organism responsible for this process is Methylomirabils oxfera. It is the only cultured member of the candidate phylum NC10.

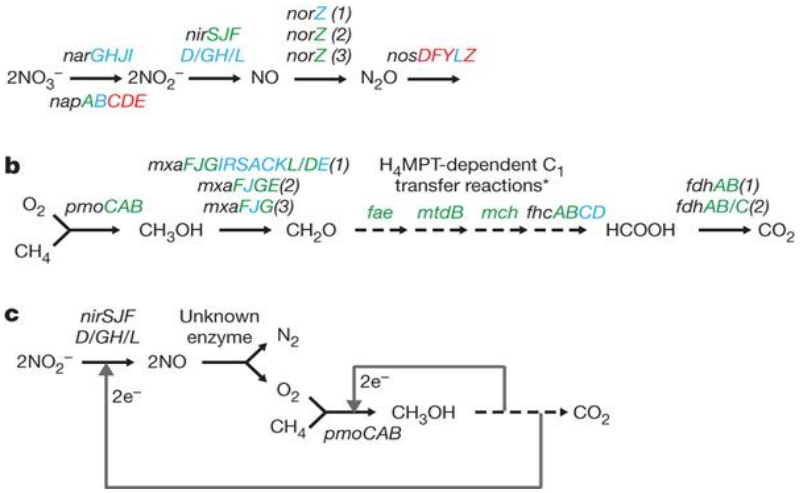

Fig 1: Pathway of NO-dismutation (from Ettwig et al. 2010)

It remains open if other members of the NC10 phylum share the same metabolism and respiratory pathway or if they are metabolically more diverse. 16S sequences that fall into the NC10 phylum have been found in mainly oxygen limited freshwater habitats such as lakes including lake sediments, rice paddy soils, wastewater sludge and ditches (Deutzmann and Schink 2011, Luesken et a.l 2011). Their occurrence apparently seem to be correlated with the presence of methane and nitrite in the habitat. This would suggest a similar respiratory pathway as in Methylomirabilis oxyfera.

The functional gene for the first step of aerobic methane oxidation, the conversion of methane to methanol, is catalyzed by the particulate methane monooxygenase. The functional gene of the alpha subunit of this enzyme (pmoA) is used as a functional marker gene for aerobic methanotrophs. PmoA genes of M. mirabilis form a distinct cluster and thus can be distinguished from other pmoA genes (Luesken et al. 2011).

The aim of the study was to screen different habitats for the presence of members of the NC10 phylum. The chosen habitats included environments with nitrite and methane being present as well as habitats where NO-dismutation would not be expected.

Samples were screened for 16S and pmoA genes belonging to the NC10 phylum. In the samples where NC10 was detected the diversity of this phylum was investigated based on 16S rDNA clone libraries using phylum specific primers. The whole corresponding microbial community of these samples was analyzed with 454 pyrosequencing. To get insight into metabolism of the detected NC10 members enrichments from the NC10 positive samples were set up with methane as electron donor and nitrite as electron acceptor.

16S rDNA 454 pyrosequencing datasets of different habitats generated in the course of 2010 were screened for the presence of the NC10 phylum.

Material and methods

Sampling and sample processing:

Sediment and water samples were collected from Little Sippewissett Marsh, Trunk River and Cedar swamp. In Cedar swamp samples were taken from 5 different depths:
S1: surface water
S2: water from 25 cm depth
S3: water from 50 cm depth (directly over the sediment)
S4: sediment surface
S5: deeper sediment
Samples for measurement of methane concentrations were taken in serum bottles with gas tight rubber stoppers. Samples for DNA extraction were stored at -20 °C until further processing. Pore water was extracted by centrifugation and directly analysed.

Nutrient analysis:

Samples for nutrient analyses were filtered (0.45 µm).
Nitrate and nitrite were measured with an ICS 2100 Ion Chromatograph (Dionex).
Ammonium was measured photometrically according to Solorzano et al. (1969).
Methane of environmental samples and enrichment cultures were measured by injecting 100 µl of headspace from the serum bottles into a gas chromatograph (GC 2014, Shimadzu) using a FID detector.

DNA extraction, clone libraries and 16S 454 sequencing

DNA was extracted from 0.25 g of sediment or water samples pellet the with the MoBio Power Soil DNA Isolation Kit. DNA concentrations were quantified with a NanoDrop (Thermo Scientific). PCR with primers specific for the Phylum NC10 (202 F deg 5'-RACCAAAGGRGGCGAGCG-3' and 1043R deg (NC10-1043Rdeg, 5'-TCTCCRCGYTCCCTTGCG-3', Deutzmann and Schink 2011) was performed for all samples.
Clone libraries were constructed for the samples that yielded amplification (Cedar Swamp S2, S3, S4, S5) using the TOPO2 TA Cloning Kit (Invitrogen).

The primers used for PCR of the functional gene pmoA were F189_b (Luesken et al. 2011) and 638R as well as 720 R (Deutzmann et al. 2011). Annealing temperatures from 42°C to 58°C were tested and the number of PCR cycles was varied between 30x and 40x.
454 16S pyrosequencing was performed for samples (Cedar Swamp S1, S2, S3, S4) using the universal primers 515F and 907R.
The phylogenetic tree of the cloned sequences was obtained using arb after aligning them with the SILVA-SINA aligner (http://www.arb-silva.de/aligner/).
454 data from Cedar Swamp and from the 2010 course were screened performing BLASTdatabase searches against a set of published NC10 16S sequences.
Positive hits for the NC10 phylum were aligned using ssu-align. A tree was constructed with fasttree and rooted with figtree. The tree was further processed using iTOL.

Enrichments:

For samples S2, S3, S4, S5 3 ml of sediment or water samples were transferred to serum bottles containing 27 ml of anoxic fresh water medium (1mM nitrite, 17.1 mM NaCl, 1.97 mM MgCl2, 0.68 mM CaCl2, 6.7 mM KCl, 1 mM NaSO4, 1 mM Na2HPO4, 50 mM MOPS and 1ml/L HCl-dissolved trace element solution. 2 further 1:10 dilution were performed. The headspace contained 20% methane and 80% N2/CO2 (95/5 vv).

Results

Trunk River, Little Sippewissett Salt Marsh and Cedar Swamp were chosen as sampling sites in order to screen for the presence of the NC10 phylum and the potential for nitrite reduction being coupled to methanotrophy. In Trunk River methane was detected in the sediment as well as in the overlying water while nitrite was only present in the water. In Sippewissett Salt Marsh nitrite was not detected and methane was not measured (Table 1).

Table 1: Nutrient concentration of Sippewissett Salt Marsh and Trunk River).

	CH4 (mM)	**Nitrite (uM)**	**Nitrate (uM)**	**Ammonium (mM)**

Sippewissett (water)	n.d.	-	-	92
Trunk river (water)	0.28	35	4	1.4
Trunk river (sediment)	0.68	-	-	98

In all of the Cedar swamp samples nitrite and methane were detected. Methane strongly increased in the sediment (19 – 28 mM) compared to the water column (0.5 -1.5 mM) likewise ammonium (Fig. 3).

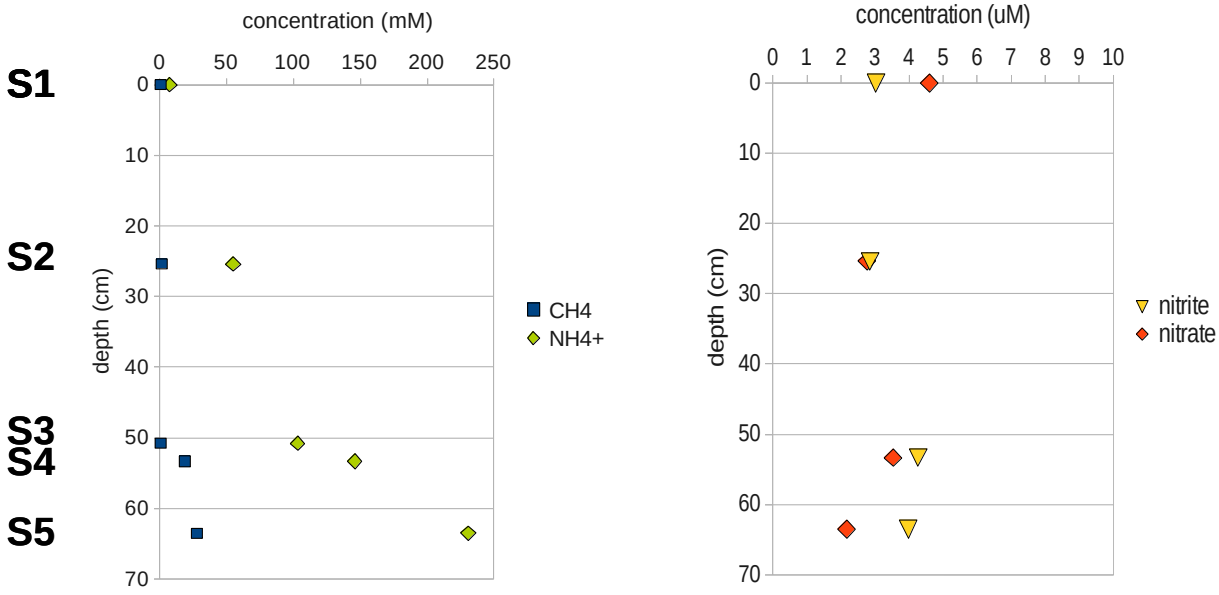

Fig 2. Nutrient profiles of Cedar Swamp.

Screening for the presence of the NC10 phylum

PCR with specific primer for the NC10 phylum only resulted in amplified products for samples from Cedar Swamp. Here, a PCR product was obtained for all depths sampled except for the surface water.

Table 2: PCR amplification indicating the presence of 16S sequences belonging to the NC10 phylum.

Sample	PCR
Sippewissett (water)	-
Sippewissett (sediment surface)	-
Trunk river (water)	-
Trunk river (sediment surface)	-
Trunk river (deeper sediment)	-
Cedar Swamp S1 (surface water)	-
Cedar Swamp S2 (medium water)	+
Cedar Swamp S3 (bottom water)	+
Cedar Swamp S4 (sediment surface)	+
Cedar Swamp S5 (deeper sediment)	+

PCR products were clones. In total 270 sequences were obtained. All of them except for 2 sequences clustered within the NC10 phylum. All NC10 sequences had a similarity of more then 99% to each other and to the 16S sequences from NO-dismutating NC10 enrichments. No differentiated clustering between different samples of Cedar Swamp was observed (Fig 3).

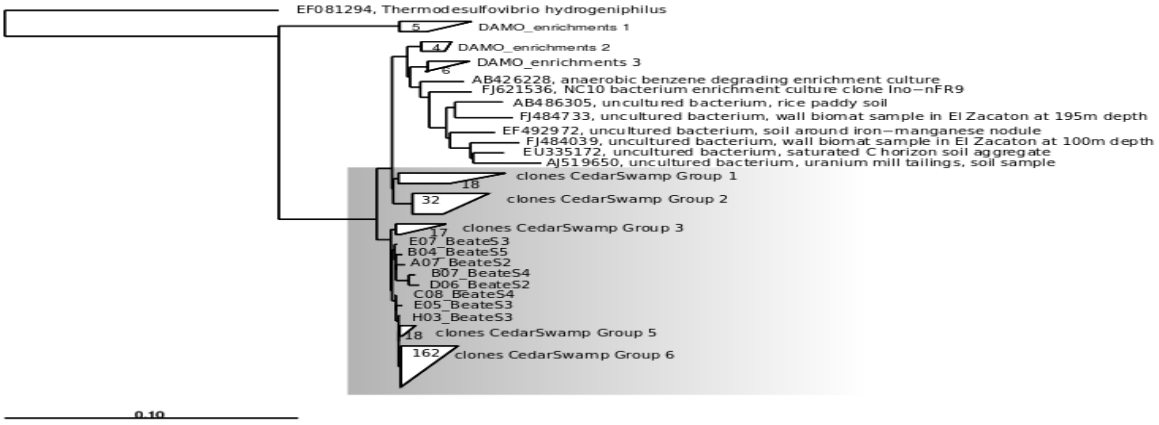

Fig. 3: 16S tree of NC10 clone libraries.

Screening 16S rDNA 454-libraries

The 454 pyrosequencing data sets from the 2010 course were searched for sequences that belong to the NC10 phylum. Members of this phylum could not be found in any of the marine samples, which included Sippewissett, Eal pond and Stony Beach. The School street marsh and the MRC biofilm libraries neither contained NC10. The only library which contained NC10 16S rDNA sequences belonged to Cedar Swamp. 37 sequences that were closely

related (more then 97% similarity) to Methylomirabilis oxyfera were obtained out of 16311 total sequences (Table 3).

All 4 libraries from 2012 (S1, S2, S2 and S4) contained sequences that belonged to the NC10 phylum. Remarkably, NC10-related sequences were also present in S1. This stands in contrast to the results obtained for the PCR with specific primers.

Table 3: Overview of 454 libraries from 2010 screened for sequences closely related to NC10.

Origin of dataset	**Presence of NC10 phylum**
Sippewissett	-
School Street Marsh	-
Eal pond	-
Stony beach	-
MRC biofilm	-
Cedar Swamp	37 / 16311

Phylogenetic analysis of NC10 16S rDNA sequences from 454 libraries

NC10 16S rDNA Sequences of all 454-libraries of Cedar Swamp from 2012 (S1, S2, S3, S4) did not show any clustering based on sampling depth. Sequences from 2010 clustered slightly bigger units but were also dispersed over the whole tree. Sequences were generally very similar to each other (more than 99%).

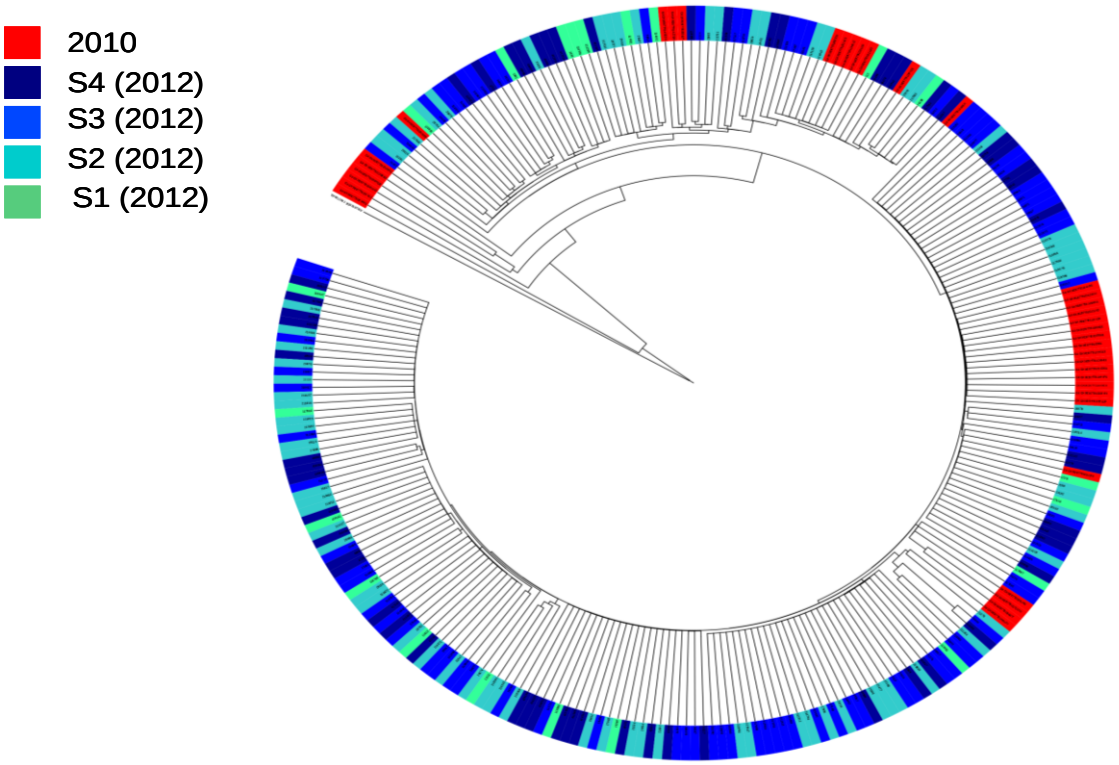

Fig. 4: Phylogenetic clustering of NC10 16S rDNA sequences from 454 libraries

Screening for pmoA

PCR for Methylomirabilis oxyfera related pmoA genes using specific primers did not result in any positive product for none of the samples. The PCR conditions could not be optimized to yield products. Analysis of pmoA sequences available at NCBI revealed that the reverse primer 720 R was designed based on only very few sequences. The reverse primer 638R had at least 2 mismatches to the target sequences.

Comparison of community composition of Cedar swamp samples from different depth by 454 sequencing.

The community composition of samples S2, S3 and S4 was very similar. Only sample S1 differed. This was mainly due to high percentage (42%) of Betaproteobacteria affiliating with Albidiferax, an iron-reducer (Figure 5). The relative abundance Methanomicrobia, Spirochaetes and Deltaproteobacteria increased with depth. The relative abundance of Gammaproteobacteria and Holophagae stayed constant in all samples.

The bacterial communities of sample S2 and S3 clustered most closely together. The most distinct sample was S1. The same pattern was observed for clustering of the archeal communities.

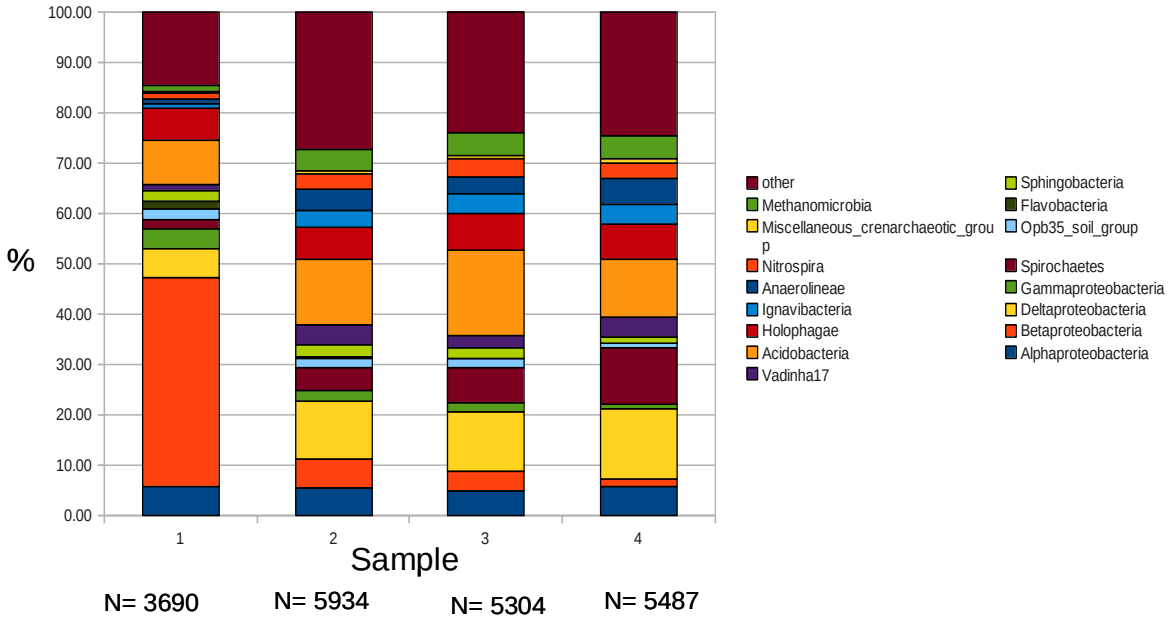

Fig. 5: Community composition of samples from different depths of Cedar Swamp.

N= number of sequences in the library

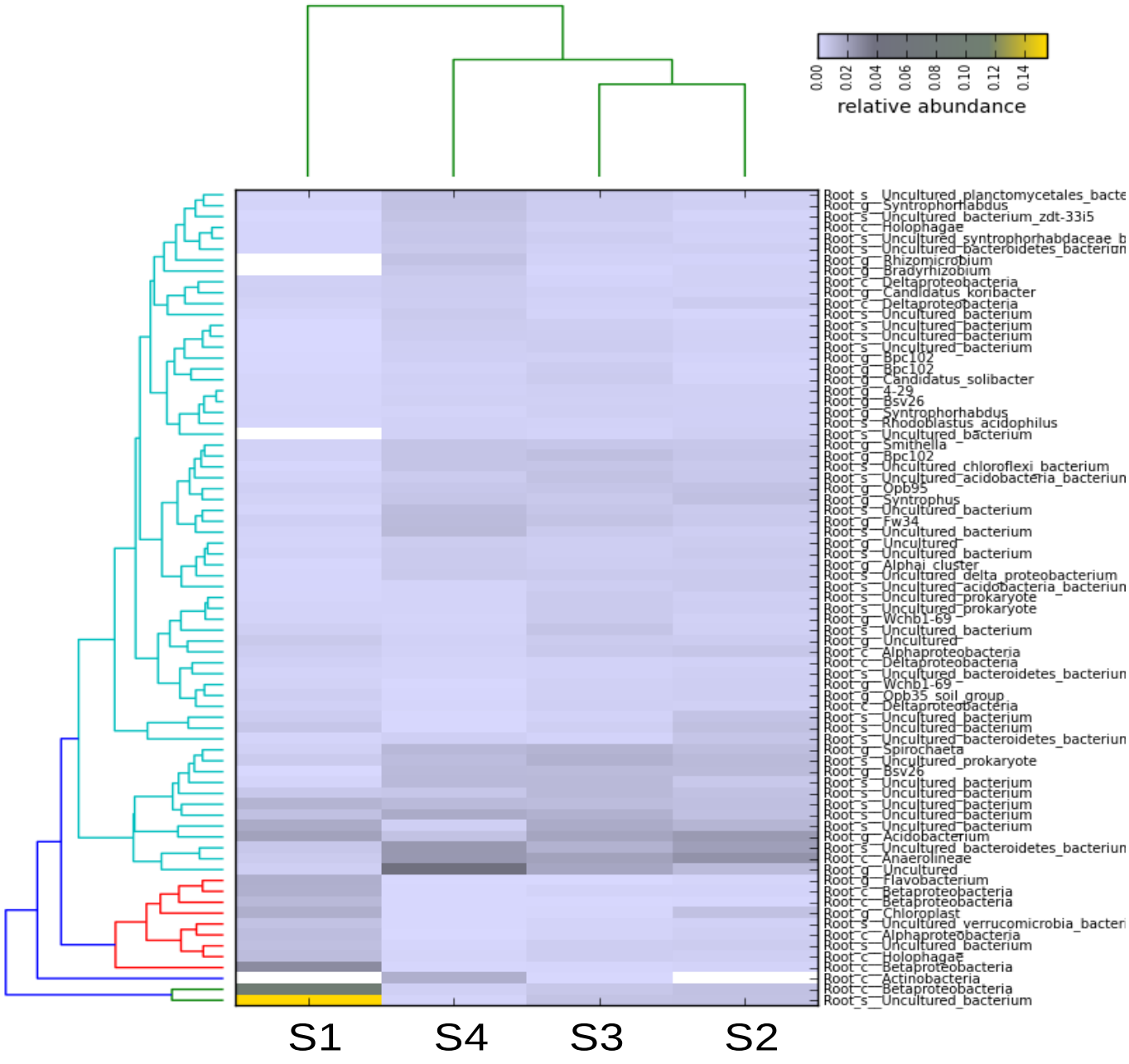

Fig. 6: Clustering by taxa and sampling site of bacterial communities from different depth of Cedar Swamp.

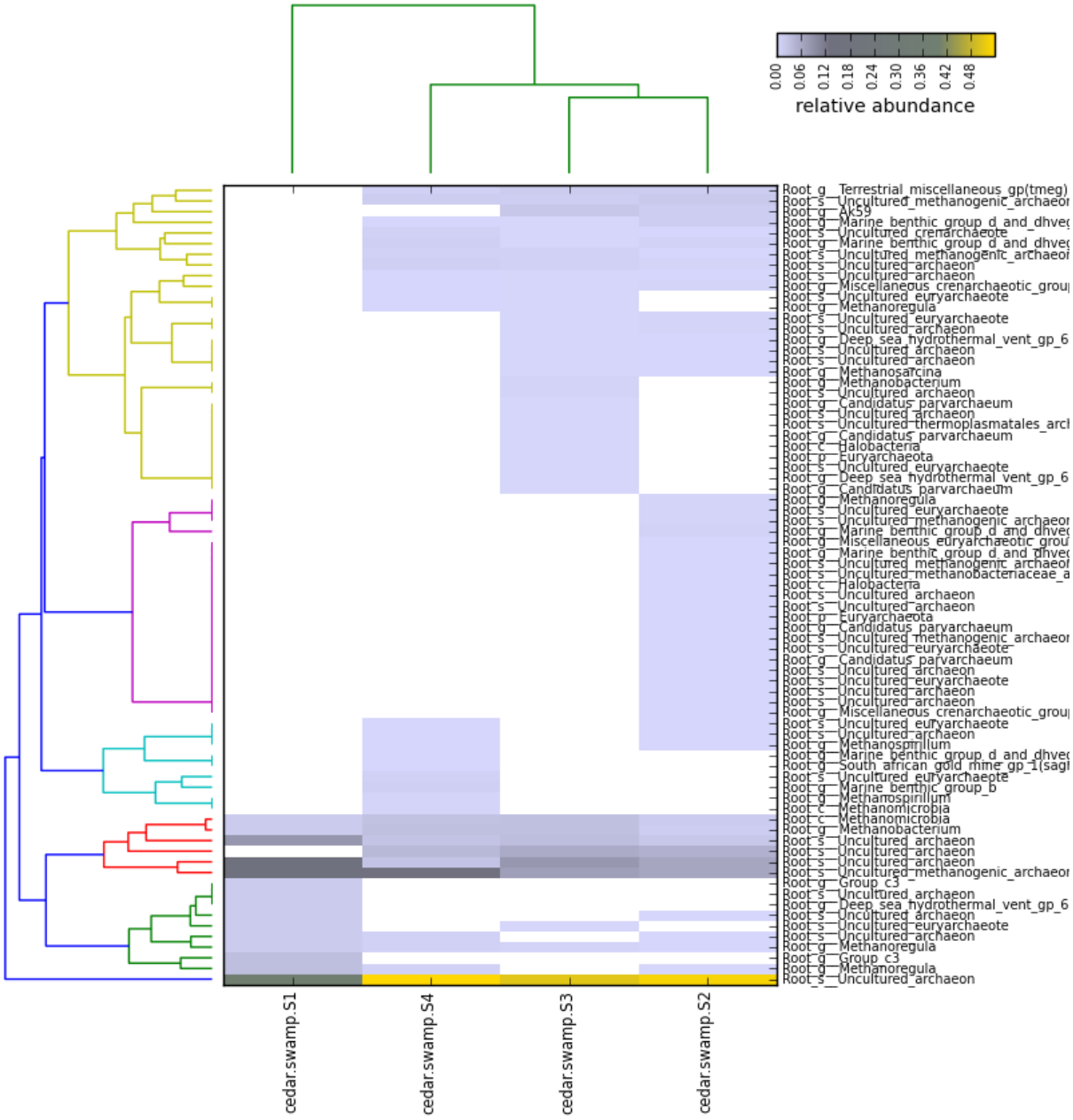

Fig. 7: Clustering by taxa and sampling site of archeal communities from different depth of Cedar Swamp.

Enrichment of methanotrophic nitrite reducing bacteria

Enrichment cultures showed consumption of approximately 1 mM of nitrite per week. Transfers consumed nitrite slower. No significant methane consumptions was observed over the time frame of 15 days.

Discussion

NC10 are consistently present in cedar swamp in all the layers sampled. The NC10 population appears to be very uniform as the obtained sequences were very similar. Furthermore, the NC10 population appears to be stable as it was present in 2010 and 2012. No indication for the metabolism of the member of this phylum in Cedar swamp could be obtained as amplification of the functional gene for methane oxidation (pmoA) failed and enrichment cultures did not lead to enrichment in the short time frame of the project. This is not surprising: Methylomirabilis oxyfera has a doubling time of 10 days. The development of primers targeting the gene for NO – dismutation would shed new light on the occurrence of this pathway and the role of the NC10 phylum.
The screening of different habitats suggests that members of the NC10 phylum seem to prefer methane-rich nitrite containing environment indicating potential for the performance of NO-dismutation.

Acknowledgements

I would like to thank Steve and Dan for letting me have this awesome experience.
Many thanks to all the TAs, especially to Chuck for being there for support all the time when needed. I thank the whole course of 2012 for this wonderful summer.

References

Deutzmann and Schink (2011): Anaerobic Oxidation of Methane in Sediments of Lake Constance, an Oligotrophic Freshwater Lake. Appl Environ Microbiol. 77(13): 4429–4436.

Ettwig K. F., et al. 2010. Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature 464:543–548.

Luesken, Zhul, van Alen, Butler, Rodriguez Diaz, Song, Op den Camp, Jetten and Ettwig (2011): pmoA Primers for Detection of Anaerobic Methanotrophs. Appl. Environ. Microbiol. 77 (11):3877-3880.

Luesken, van Alen, van der Biezen, Frijters et al. (2011): Diversity and enrichment of nitrite-dependent anaerobic methane oxidizing bacteria from wastewater sludge. Appl Microbiol Biotechnol. 92(4): 845–854.

Beate's clone libraries:

Cedar Swamp: different sampling depth
S2= medium water layer, 25 cm
S3= water directly above sediment, 50 cm
S4= sediment, surface layer
S5= sediment, deeper layer

BeateS2S3 = A-D 1-12 belongs to S2, E-H belongs to S3
BK45 = A-H 1-6 belongs to S4, A-G 7-12 belongs to S5
BK54 = A-H 1-6 belongs to S5, A-G 7-12 belongs to S4