Methanotrophic bacteria, and environmental genomics/transcriptomics

Our research covers topics at the community, cellular, genomic, and molecular levels. In 2012 and 2013, we continued our work on (i) the molecular biology and ecophysiology of the type II methanotroph *Methylocystis* sp. strain SC2, (ii) soil metatranscriptomics, and (iii) microbial communities in *Sphagnum*-dominated peatlands.

Methylocystis sp. strain SC2

Strain SC2 can adapt to a wide range of methane concentrations. This is due to its ability to produce two isozymes of particulate methane monooxygenase (pMMO). These exhibit different methane oxidation kinetics and are encoded by *pmoCAB1* (low-affinity pMMO1) and *pmoCAB2* (high-affinity pMMO2). To gain insight into the underlying genetic information, the genome of strain SC2 was sequenced and found to comprise a 3.77 Mb circular chromosome and two large plasmids of 229.6 kb (pBSC2-1) and 143.5 kb (pBSC2-2) [1,2,3].

In addition to methane, nitrogen source and concentration are major determinants of methanotrophic activity. The application of ammonium fertilizers to various soils and sediments has been shown to inhibit methanotrophic activity. Long-term effects were observed particularly for atmospheric methane oxidation in various upland soils. Thus, besides methane, ammonia is a major factor determining methanotrophic activity in soil. The pMMO is evolutionarily related to the ammonia monooxygenase and methanotrophs, like ammonia oxidizers, are able to convert ammonia to hydroxylamine. Ammonia oxidizers can couple the oxidation of hydroxylamine with energy production and cellular growth, while methanotrophic bacteria cannot. Because hydroxylamine is a highly toxic intermediate, methanotrophs rely on the ability to remove it quickly by a detoxifying reaction.

We used Illumina RNA-Seq to identify strain SC2 genes that respond to standard (10 mM) and high (30 mM) NH_4^+ concentrations in the growth medium, compared to 10 mM NO_3^-. The experimental treatments were named AMS (10 mM NH_4^+), NH4 (30 mM NH_4^+), and NMS (10 mM NO_3^-). Strain SC2 cells were grown under high methane concentrations (20%, v/v) . High-quality non-rRNA reads were mapped against the concatenated sequence of the chromosome and the two plasmids of strain SC2; totaling 4,058 genes and referred to as the

genome (Figure 1). RNA-Seq expression data were presented as RPKM (Reads Per Kilobase of CDS [coding sequence] model per Million mapped reads) values.

The majority of the strain SC2 genes showed no significant differential expression (Figure 2). Based on $\log_2$ fold change values of ≥ 2 or ≤ -2, a total of 198 genes were identified as differentially expressed between the different nitrogen conditions (NMS vs. AMS, NMS vs. NH4). Among these were *pmoCAB2* and the genes (*haoAB*) encoding hydroxylamine oxidoreductase (HAO). In addition, a set of 67 genes was predicted to be differentially expressed in AMS/NH4, but not in NMS/AMS or NMS/NH4.

While the expression of *pmoCAB1* was unaffected, *pmoCAB2* was significantly downregulated ($\log_2$ fold changes of -5.0 to -6.0). Among nitrogen metabolism-related processes, genes involved in hydroxylamine detoxification (*haoAB*) were highly upregulated, while those for assimilatory nitrate/nitrite reduction, high-affinity ammonium uptake, and nitrogen regulatory protein PII were downregulated. The most likely explanation for the downregulation of *pmoCAB2* is the ability of ammonia to competitively inhibit methane oxidation by pMMO. This would lead to the production of hydroxylamine. Competitive inhibition and poisoning of strain SC2 by hydroxylamine would be of ecophysiological relevance particularly in low-methane environments, where pMMO2 but not pMMO1 is functional [Baani and Liesack (2008) PNAS 105: 10203-10208]. The upregulation of *haoAB* expression with increasing ammonium concentration also fits well into such an ecophysiological perspective, assuming that the primary role of HAO is to detoxify hydroxylamine that has been produced by the pMMO isozymes.

Thus, *pmoCAB1* and *pmoCAB2* in strain SC2 are differentially expressed but in response to two different key drivers of methanotrophic activity. While methane concentration is the factor controlling differential expression of *pmoCAB1*, it has no effect on *pmoCAB2* expression (Baani and Liesack, 2008). Conversely, *pmoCAB2* expression is affected by the nitrogen source and, in particular, the concentration of ammonia, but not the expression of *pmoCAB1*. *Methylocystis* is a major component of the methanotrophic communities in upland and hydromorphic soils. Differential expression of *pmoCAB2* thus provides some explanation as to why ammonium fertilizers have a strong inhibitory effect on atmospheric methane oxidation in such soils [4].

We also performed physiological experiments to verify that strain SC2 has diverse nitrogen metabolism capabilities. In correspondence to a full complement of 34 genes involved in N_2 fixation, strain SC2 was found to grow with atmospheric N_2 as the sole nitrogen source, preferably at low oxygen concentrations. Denitrification-mediated accumulation of 0.7 nmol $^{30}N_2$/hr/mg dry weight of cells under anoxic conditions was detected by tracer analysis. N_2 production was related to the activities of plasmid-borne nitric oxide and nitrous oxide reductases. The presence of a complete denitrification pathway in strain SC2, including the plasmid-encoded *nosRZDFYX* operon, is unique among known methanotrophs. However, the exact ecophysiological role of this pathway still needs to be elucidated. Detoxification of toxic nitrogen compounds and energy conservation under oxygen-limiting conditions are among the possible roles [3].

Soil metatranscriptomics

We explored the global transcriptome, or metatranscriptome, of soil microbial communities using the oxic surface layer and the anoxic bulk soil of flooded paddy soil as the model system. Using conventional Sanger sequencing, our first attempt to examine global gene expression allowed us to detect the active components of the microbial communities but provided limited insight into their ecosystem functions [Shrestha *et al*. (2009) Environ. Microbiol. 11: 960-970].

More recently, we developed an efficient method for extracting high quality mRNA from soil. Key steps in the isolation of total RNA are low-pH extraction (pH 5.0) and Q-Sepharose chromatography. The removal efficiency of humic acids was 94 to 98% for all soils tested. To enrich mRNA, subtractive hybridization of rRNA is most efficient. The rRNA-depleted RNA is of sufficient purity and integrity (size range of 0.3 to 4 kb) for further applications [Mettel et *al*. (2010) Appl. Environ. Microbiol. 76: 5995-6000].

The procedural steps of our current metatranscriptomic approaches involve (i) extraction of total RNA, (ii) enrichment of mRNA, (iii) cDNA synthesis and next-generation sequencing (454 pyrosequencing, Illumina HiSeq/MiSeq), (iv) preprocessing of raw reads, and (v) data analysis using either online tools (rRNA: QIIME, Mothur, Silva; mRNA: Camera, MG-Rast) or a local bioinformatic pipeline [5].

Bacterial communities in *Sphagnum*-dominated peatlands

This research is carried out in close collaboration with Dr. Svetlana N. Dedysh and her group (Laboratory of Wetland Microbiology, Winogradsky Institute of Microbiology RAS, Moscow, Russia). Northern peatlands play a key role in the global carbon and water budget. A large proportion of them consist of *Sphagnum*-dominated ombrotrophic bogs, which are characterized by low pH values of 3.5 to 5 and extremely low rates of plant debris decomposition. The bacterial diversity in these ecosystems remains poorly described. Over the last two years, we compared the bacterial community composition in the surface (0–5 cm depth) and subsurface (45–50 cm) peat layers of an acidic (pH 4.0) *Sphagnum*-dominated wetland, using 454 pyrosequencing of 16S rRNA genes [6]. The denoised sequences (37,229 reads) were affiliated with 27 bacterial phyla and corresponded to 1,269 operational taxonomic units (OTUs) determined at 97% sequence identity. OTU richness was higher in the surface layer (882 OTUs) than in the anoxic subsurface peat (483 OTUs), with only 96 OTUs common to both datasets. Abundant OTUs were affiliated with the *Acidobacteria*. They were represented by more than one-third of all sequences, in both surface and subsurface peat. Other major OTUs were affiliated with the *Alphaproteobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Planctomycetes*, *Deltaproteo-bacteria*, and *Gammaproteobacteria*.

The taxonomic patterns of the abundant OTUs were uniform across all subsamples taken from the same peat layer. In contrast, the taxonomic patterns of rare OTUs were different from those of the abundant OTUs and varied greatly among subsamples, in both surface and subsurface peat. In addition to the bacterial taxa listed above, rare OTUs were affiliated with the following groups: *Armatimonadetes*, *Bacteroidetes*, *Chlamydia*, *Chloroflexi*, *Cyanobacteria*, *Elusimicrobia*, *Fibrobacteres*, *Firmicutes*, *Gemmatimonadetes*, *Spirochaetes*, AD3, WS1, WS4, WS5, WYO, OD1, OP3, BRC1, TM6, TM7, WPS-2, and FCPU426. The rare groups represented population sizes between 10^3 and 10^5 cells per gram of wet peat. Most members of poorly studied phyla, such as the *Acidobacteria*, *Verrucomicrobia*, *Planctomycetes* and the candidate division TM6, showed a clear preference for growth in either aerobic or anaerobic conditions. For example, OTUs related to the recently described *Telmatocola sphagniphila* [7] were detected primarily in the oxic surface layer. Apparently, the bacterial communities in surface and subsurface layers of northern peatlands are highly diverse and taxonomically distinct, reflecting the different abiotic conditions that occur along the peat profile [6].

References

[1] Dam, B., Kube, M., Dam, S., Reinhardt, R., Liesack, W. (2012) Methanotroph-specific *repABC*-containing plasmids from *Methylocystis* sp. strain SC2. Appl. Environ. Microbiol. 78, 4373–4379.

[2] Dam, B., Dam, S., Kube, M., Reinhardt, R., Liesack, W. (2012) Complete genome sequence of *Methylocystis* sp. strain SC2, an aerobic methanotroph with high-affinity methane oxidation potential. J. Bacteriol. 21, 6008–6009.

[3] Dam, B., Dam, S., Blom, J., Liesack, W. (2013) Genome analysis coupled with physiological studies reveals a diverse nitrogen metabolism in *Methylocystis* sp. strain SC2. PLoS ONE 8(10): e74767. doi:10.1371/journal.pone.0074767.

[4] Dam, B., Dam, S., Kim, Y., Liesack, W. (2013) Ammonium induces differential expression of methane and nitrogen metabolism-related genes in *Methylocystis* sp. strain SC2. Environ. Microbiol., publication online ahead of print, doi: 10.1111/1462-2920.12367.

[5] Kim, Y., Wegner, C.E., Liesack, W. (2014) Soil Metatranscriptomics, p. 63-93. In P. Nannipieri, G. Pietramellara, and G. Renell (ed.), Omics in Soil Science, Caister Academic Press.

[6] Serkebaeva, Y.M., Kim, Y., Liesack, W., Dedysh S.N. (2013) Pyrosequencing-based assessment of the *Bacteria* diversity in surface and subsurface peat layers of a northern wetland, with focus on poorly studied phyla and candidate divisions. PLoS ONE 8(5): e63994. doi:10.1371/journal.pone.0063994.

[7] Kulichevskaya, I.S., Serkebaeva, Y.M., Kim, Y., Rijpstra, W.I.C., Damste, J.S.S., Liesack, W., Dedysh, S.N. (2012) *Telmatocola sphagniphila* gen. nov., sp. nov., a novel dendriform planctomycete from northern wetlands. Front. Microbiol. 3: e146. doi: 10.3389/ fmicb.2012.00146.

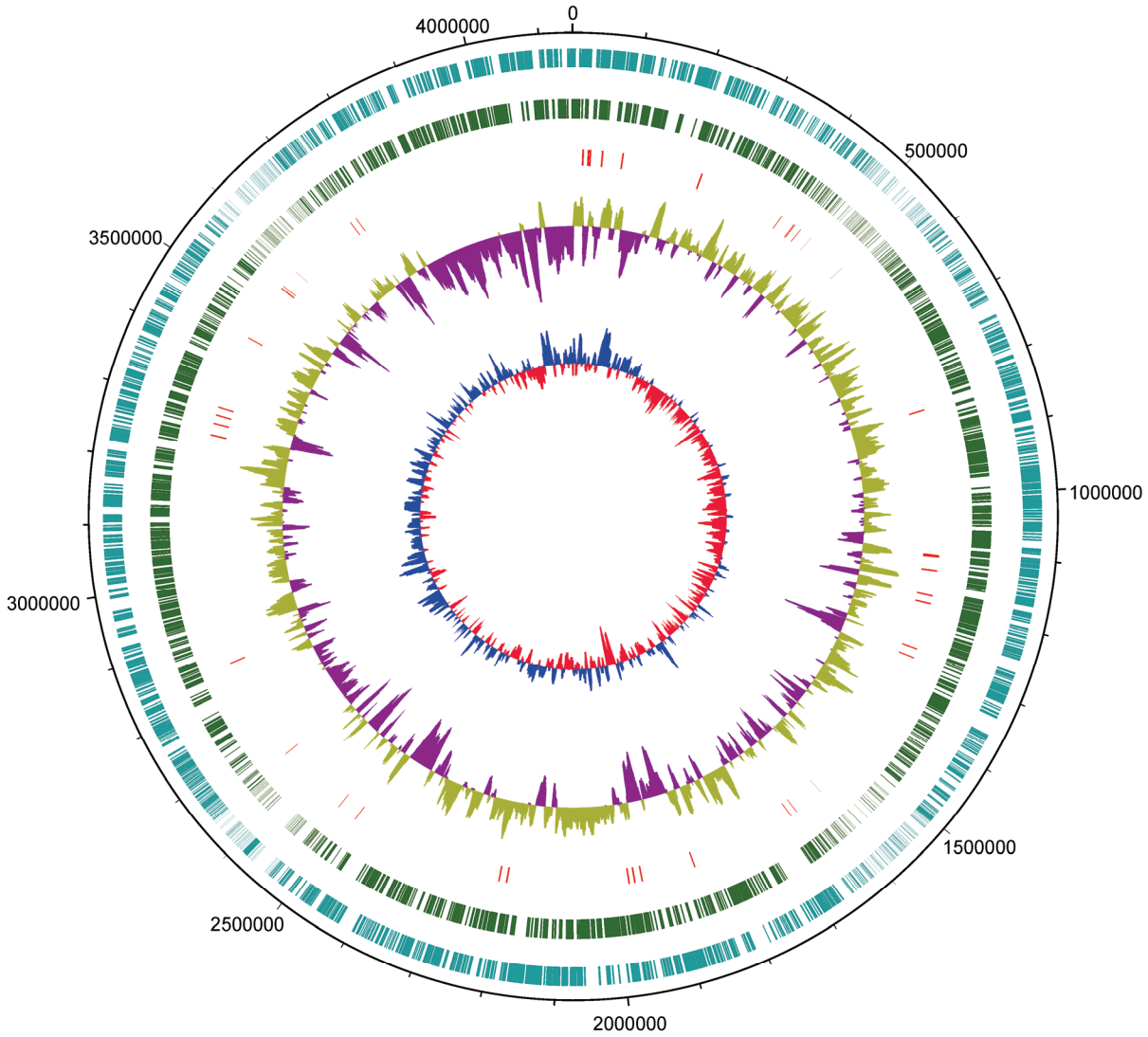

Figure 1. Whole-genome plot of *Methylocystis* sp. strain SC2. The circles represent from outside to inside: circle 1, DNA base position (bp), base 1 to 3,773,444 are for the chromosome, followed by plasmids pBSC2-1 and pBSC2-2; circle 2, protein-coding regions transcribed on the plus strand (clockwise); circle 3, protein-coding regions transcribed on the minus strand (anticlockwise); circle 4, tRNA genes; circle 5, G+C content plotted using a 10-kb window (sea green and magenta indicate values greater and less than the average G+C content, respectively); circle 6, GC skew ([G+C]/[G-C]) plotted using a 10-kb window (blue indicates values above average and red indicates values below average). The whole-genome plot was generated using DNAPlotter version 1.4 from Artemis 12.0, Sanger Institute (taken from Dam *et al*. 2013, DOI: 10.1371/journal.pone.0074767; but modified to include the plasmids pBSC2-1 and pBSC2-2 in the genome plot).

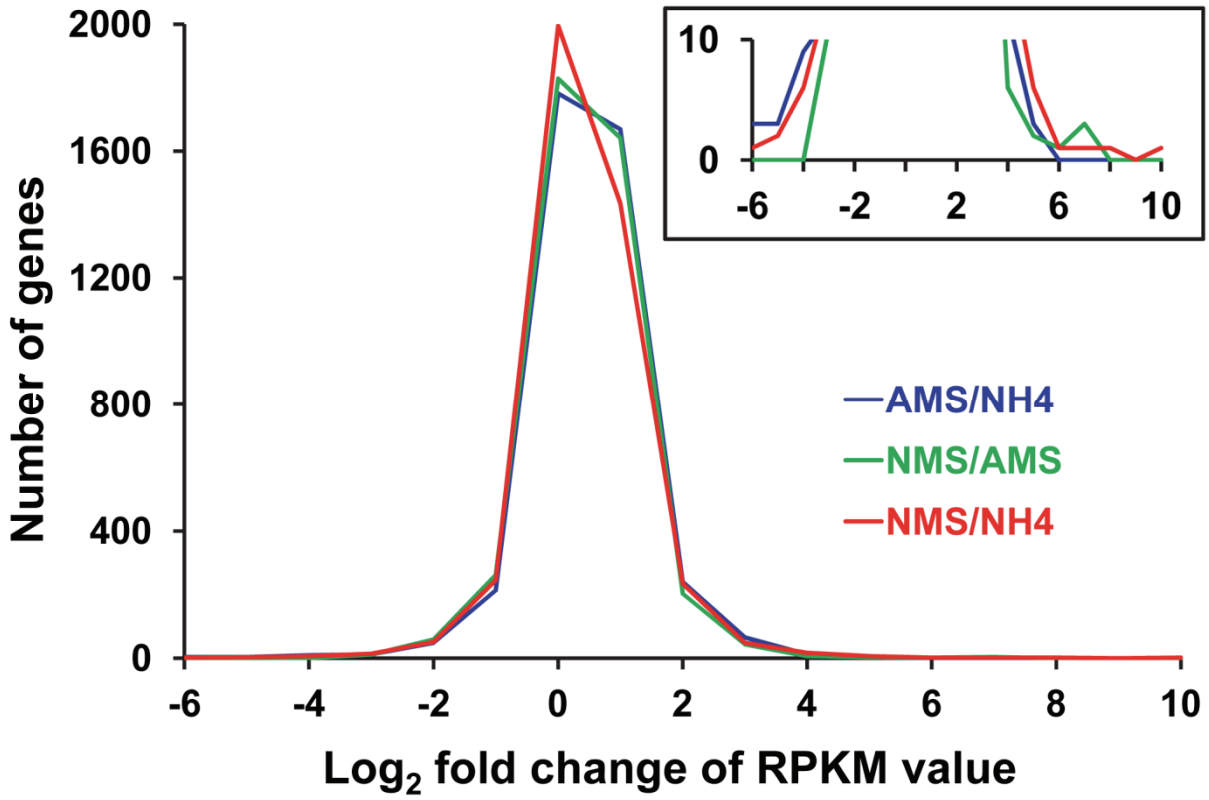

Figure 2. Differential expression of genes in strain SC2 in response to different nitrogen conditions. The histogram indicates differential expression levels of the complete set of 4,058 genes identified in the genome of strain SC2. Log_2 fold changes of RPKM values were compared for NMS/AMS (green), NMS/NH4 (red), and AMS/NH4 (blue). The inset shows the same graph with a y-axis zoomed in for the range 0 to 10 (taken from Dam *et al*. 2014, DOI: 10.1111/1462-2920.12367).

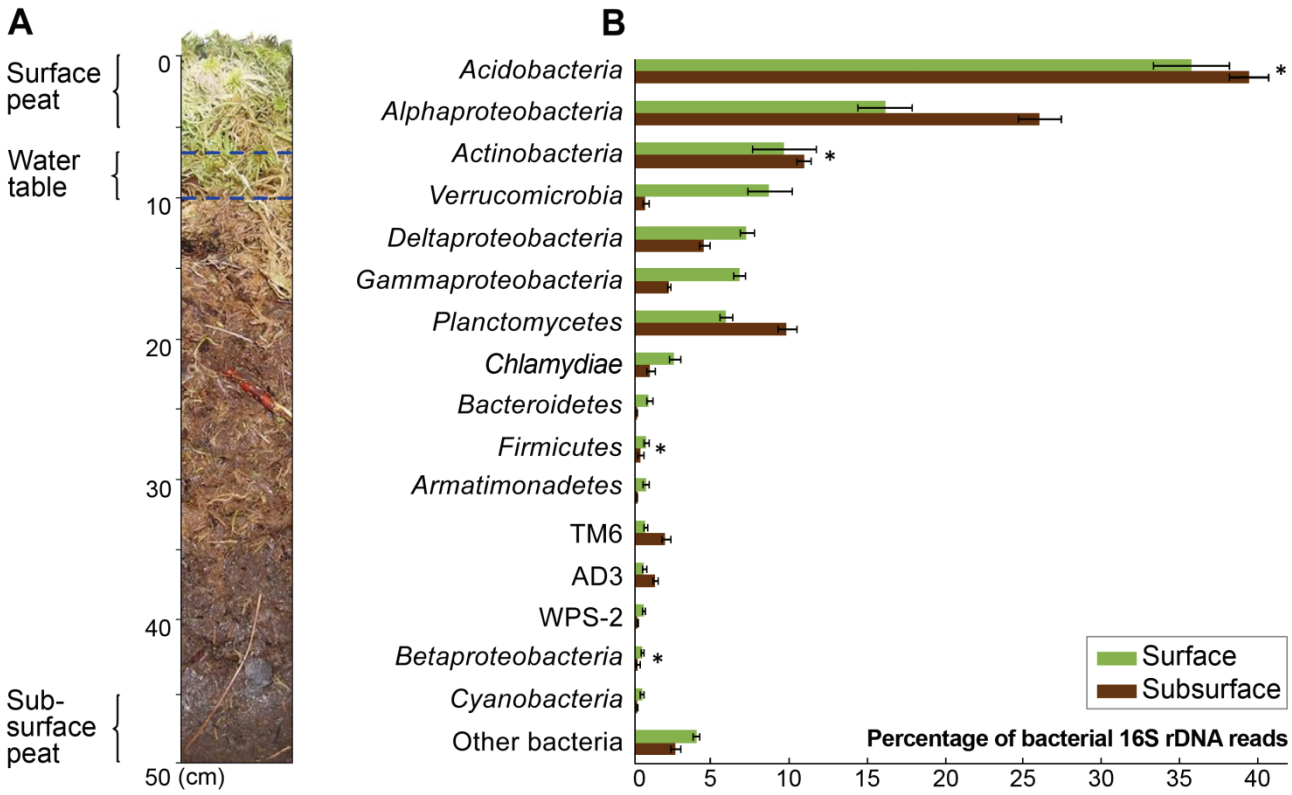

Figure 3. Bacterial community composition in surface (green bars) and subsurface (brown bars) peat. (A) Depth profile of *Sphagnum*-dominated peat bog. (B) Major taxa detected with a relative sequence abundance ≥ 0.5% are displayed. Column "other bacteria" indicates the relative sequence abundance of all the rare phyla and candidate divisions [each < 0.5%] and taxonomically unclassified sequences. The error bars indicate the standard deviation of relative sequence abundances between four subsamples. The number of 454 reads assigned to a particular taxon was significantly different between surface and subsurface peat based on two-tailed t-test ($P < 0.05$), except for those four taxa indicated by asterisk (taken from Serkebaeva *et al*. 2012, DOI: 10.1371/journal.pone.0063994).