New thoughts on deep sedimentary sulfur cycling (the deep "cryptic S cycle")

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The sulfur cycle in marine sediments exerts a major control on the redox state of the ocean and atmosphere. The overall driver in the sulfur cycle is the microbial mediated sulfate reduction to sulfide (SR). We broadly divide sedimentary SR into two categories: organoclastic sulfate reduction (OSR), which acts as the terminal electron acceptor during the degradation of buried organic matter; and methanotrophic sulfate reduction (MSR), better known as the sulfate-dependent anaerobic oxidation of methane (AOM). When integrated over depth, the vast majority of sulfate reducing activity occurs within the surface sediments (e.g. 0 – 30 cm below surface), whereas low rates of OSR and a distinct a distinct layer of MSR (where the sulfate and methane gradients overlap) characterize deeper sedimentary sulfur cycling. In near-surface sediments a vigorous oxidative sulfur cycle operates, and only a small fraction of the sulfide produced via OSR becomes permanently buried as pyrite (FeS_2).

Paradoxically, the deep, reduced, sulfidic zone of marine sediments is often characterized by the presence of zero-valent sulfur compounds (S^0), e.g. elemental sulfur and polysulfides *[1,2,3]*. Fluxes of oxidized iron and manganese have been suggested as the source of oxidizing power in these deep, anoxic and sulfidic sediment systems *[1,2]*. These intermediates may help drive a deep "cryptic sulfur cycle" below the methane transition zone. MSR may provide another source of sulfur intermediates for deep sulfur cycling.

Milucka et al. *[4]* offer a new view on the associated microbially-mediated zerovalent sulfur transformations in sediments associated with sulfate dependent AOM (or MSR). MSR is thought to be mediated by a consortium of methanotrophic *Archaea* (ANME) and sulfate-reducing *Deltaproteobacteria*. Milucka et al., however, showed that zero-valent sulfur compounds can form during MSR. They conclude that the S^0 is a product of a novel pathway for sulfate reduction performed by the ANME, thus MSR may not be an obligately syntrophic process. Furthermore, it was shown that the produced S^0 in the form of hydrodisulfide can serve as a substrate for disproportionation by the *Deltaproteobacteria* associated with the ANME, and that this disproportionation proceeds under sulfidic conditions.

These observations may have significant implications for role of sulfur intermediates in our understanding of the biogeochemical carbon and sulfur cycle in modern and past environments. Archaeal sulfate reduction to elemental sulfur may provide a source of sulfur intermediates in otherwise sulfidic environments, and disproportionation may be more widely spread than originally thought.

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Insights into the bioenergetics of dissimilatory sulfate reduction – a biochemical approach

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The dissimilation of sulfur compounds is likely to have been one of the first energy metabolisms in the early Earth. However, many questions remain about how sulfur-metabolizing organisms obtain energy for growth from reducing, oxidizing or disproportionating sulfur compounds. In the case of sulfate reduction, the terminal reductases involved (APS reductase, AprAB, and dissimilatory sulfite reductase, DsrAB) have long been recognized, but how these two reactions are coupled to energy conservation is still not clear. DsrAB, in particular, is a key enzyme in dissimilatory sulfur metabolism, being present not only sulfate/thiosulfate/sulfite reducing organisms, but also in sulfur-oxidizers (where it is thought to operate in reverse) and sulfur disproportionators. The mechanism of sulfite reduction by DsrAB has long been the subject of controversy due to the *in vitro* production of thiosulfate and trithionate, in contrast to the closely-related assimilatory enzyme that produces only sulfide. It is not clear if the physiological reduction of sulfite involves its six-electron reduction to sulfide or the production of thiosulfate and trithionate as intermediates in a stepwise two-electron process.

Our lab has taken a biochemical approach to study several of the key proteins involved in sulfate reduction, including DsrAB and two respiratory membrane complexes specific to sulfur-metabolizing organisms, QmoABC and DsrMKJOP, which are likely involved in the electron transfer pathways with AprAB and DsrAB [1]. These complexes hint at the possibility of menaquinone involvement and chemiosmotic energy conservation, during sulfate reduction. In addition, a genomic analysis of energy metabolism genes in sulfate reducers suggested that the recently recognized process of electron bifurcation may also be involved [2], and that there are conspicuous links between sulfate reducers and methanogenic organisms [3].

In this talk I will present recent results on the role of these several proteins in sulfate reduction, with a special focus on the function of the small protein DsrC as a physiological partner of DsrAB and the DsrMKJOP complex [4], and on the involvement of electron confurcation/bifurcation processes during sulfate reduction.

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Title: Genetic tool development for environmental microbes: Examination of the physiology of *Desulfovibrio* strains.

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The biochemistry of sulphate reduction and sulphate-reducing microbes has far out-paced the development of genetics and the elucidation of regulatory aspects of sulphate metabolism. A concerted effort to bring strains of the *Desulfovibrio* genus to model status has resulted in the generation of whole genome sequences and advances in genetic tools. Among the tools successfully applied to *Desulfovibrio vulgaris* Hildenborough and *Desulfovibrio alaskensis* G20 are electroporation, whole genome mutagenesis by transposon insertions, and archived libraries of transposon mutants representing mutations in the majority of non-essential genes. A procedure for high-throughput parallel sequencing for gene fitness determinations of transposon mutants, TnLE-seq, has been developed for these strains that avoids the necessity of colony plating. Examples of the application of these tools for exploration of the metabolism of *Desulfovibrio* strains include TnLE-seq that revealed a surprising increase in fitness of nitrate-stressed cells when genes in a small cluster of poorly annotated genes were inactivated. Second, the ability to make deletions without retention of a selectable marker has allowed multiple deletions to be made in a single strain, a capacity needed to overcome compensatory phenotypes of isozymes. To date, six hydrogenases have been deleted from *D. vulgaris*, yet the mutant continues to grow on hydrogen and to produce hydrogen. Finally, the approaches developed have been used with *Desulfovibrio desulfuricans* ND132, a mercury methylating strain. Site-directed mutations of the HgcAB complex, shown to be essential for mercury methylation, have allowed the examination of structural predictions of the corrinoid protein. The constructed mutations provide data that support the prediction of unusual carbanion transfer of methyl from the corrinoid to Hg(II). These genetic advances offer approaches that may be used to bring other environmental microbes to model status rapidly.

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From functional gene studies to ecosystem functions: a case study of hidden sulfate-reducing microorganisms in wetlands

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The energy metabolism of different microbial guilds in the global sulfur cycle is based on a DsrAB-type dissimilatory (bi)sulfite reductase that either catalyzes the reduction of sulfite to sulfide during anaerobic sulfate/sulfite respiration and organosulfonate degradation, or acts as oxidative enzyme during sulfur oxidation. A recent census of the evolutionary history and environmental diversity of *dsrAB* highlighted the existence of at least thirteen, family-level lineages without cultivated representatives, which suggests that major taxa of reductive *dsrAB*-containing microorganisms have not yet been identified (1). Linking *dsrAB* sequence information directly to the ecosystem process sulfate reduction is difficult because *dsrAB* are still present and transcribed in some bacteria that apparently lost the capability for sulfate reduction. By using wetlands as an example, I will show that, in order to unambiguously identify sulfate-reducing microorganisms and reveal their role in the environment, functional gene-based diversity analyses need to be complemented by other methods and jointly integrated in defined experimental set-ups.

Wetlands are important carbon sinks but at the same time a major global source of the greenhouse gas methane. How wetland microorganisms will respond to global warming and increasing aerial sulfur pollution in the upcoming decades to centuries is one of the largest unknowns. Although regarded primarily as methanogenic environments, a hidden sulfur cycle exerts important ecosystem functions in wetlands (2). Dissimilatory sulfate reduction is thermodynamically favorable relative to methanogenic processes and often occurs in wetlands at rates comparable to marine surface sediments, despite significantly lower sulfate concentrations. The underlying interspecies resource competition thus effectively controls gross production of methane in wetlands.

Novel lineages dominate *dsrAB* diversity in wetlands (2). However, comparative 16S rRNA gene- and *dsrAB*-stable isotope probing in the presence and absence of sulfate identified *Desulfosporosinus* members, which constituted only 0.01% of the total microbial community 16S rRNA genes, as important sulfate reducers in an acidic peatland (3). In follow-up experiments, anoxic microcosms were supplemented with typical degradation intermediates of organic matter at *in situ* concentrations and incubated under methanogenic or sulfate-reducing conditions. Microbial community dynamics, interactions, and activities (as analyzed by Illumina amplicon sequencing and quantitative real-time PCR of 16S rRNA genes/cDNA, and *dsrB*, and metatranscriptomics) were correlated with substrate turnover and formation of carbon dioxide and methane. Amendment with different substrates resulted in heterogeneous turnover rates of sulfate; the strongest turnover was stimulated by butyrate, followed by propionate, lactate, formate, and the weakest with acetate. Only few microbial taxa/phylotypes responded positively to sulfate reduction. For example, *Desulfosporosinus* 16S rRNA copy numbers strongly correlated with sulfate turnover under all tested substrates. Despite significant transcriptional responses of individual populations, phylotype abundances of known sulfate reducers remained low throughout the incubations. Interestingly, two *Telmatospirillum* phylotypes (*Alphaproteobacteria*) showed a very contrasting response in the incubations with butyrate; one phylotype was most active with sulfate and the other without sulfate. In addition, sequencing of the peatland metagenome enriched by DNA-stable isotope probing allowed almost complete genomic reconstruction of the rare *Desulfosporosinus* population and confirmed functional properties of this novel acidic fen sulfate reducer, as inferred from the microcosm studies. In conclusion, this holistic approach provided new insights into the ecophysiology, possible interspecies interactions, and genomic repertoire of sulfate-reducing keystone members of the rare wetland biosphere.

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Title: Alternative lifestyles of sulfate-reducing bacteria: *Desulfovibrio* as facultative syntrophs

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In the absence of inorganic electron acceptors, the complete degradation of organic matter relies on the cooperative activities between distinct microbial groups, e.g. secondary-fermenters and methanogens that are obligately linked through metabolite exchange in syntrophic association. Virtually all methane released to the biosphere (estimated to be approximately 1 billion tons per year) is produced by methanogenic archaea living in close association with other anaerobic microorganisms. To extend understanding of the genetic and metabolic basis of this common microbial mutualism, we are characterizing different assemblies of syntrophic *Desulfovibrio* species and hydrogenotrophic methanogens. Comparative whole genome transcript analyses, complemented by physiological studies and full transposon library and individual mutant growth experiments, have established distinctive differences in the population structure, electron transfer systems, ratios of interspecies electron carriers (H_2/formate), and energy conservation mechanisms sustaining syntrophic growth of the different species. The results have also informed understanding of the genetic and metabolic basis for rapid adaptation of *Desulfovibrio* to fluctuations in electron acceptor availability in their natural environments. Most notably, a newly recognized mechanism of energy transduction via flavin-mediated electron bifurcation and electron confurcation has been shown to serve a fundamental role in adaptive capability. Complementary studies have also revealed the capacity for rapid adaptive improvement of nascent mutualisms over relatively short evolutionary time periods in the laboratory. Evolved communities grew two- to three-fold faster and were up to 40% more productive than ancestral community assemblies. Sequence analysis of the genomes of the evolved populations has identified shared mutations in metabolic systems associated with energy transformation, suggesting common adaptive solutions. The new understanding of electron transfer pathways involved in syntrophic and respiratory growth is now being used in the construction of revised whole organism metabolic and regulatory models.

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