Final Report

Systems Level Analysis of the Function and Adaptive Responses of Methanogenic Consortia

Award Number: DE-SC0004485

Award Dates: 7/1/2010 to 6/30/2014

Principle Investigator:
Derek R. Lovley
Department of Microbiology
University of Massachusetts
Amherst, MA 01003
Phone: 413-545-9651
fax: 413-545-1578
email: dlovley@microbio.umass.edu

The project was highly successful. We discovered a new form of microbial syntrophy in which microorganisms directly exchange electrons via physical electrical connections. Metagenomic and metatranscriptomic studies revealed that this direct interspecies electron transfer (DIET) is an important mechanism for syntrophy in methanogenic environments and identified environmental factors that can promote DIET. These findings have important implications for carbon cycling because the methanogens that produce the most methane on earth were found to participate in DIET. The findings also provide new understanding to the important bioenergy strategy of converting organic wastes to methane. A summary of results leading to peer-reviewed publications follows.

DIET was first discovered during adaptive evolution of a co-culture of *Geobacter metallireducens* and *Geobacter sulfurreducens* to grow via syntrophic electron exchange. The co-culture was grown under conditions such that the ethanol that was added as an electron donor could only be metabolized if the two species exchanged electrons. It was assumed that this co-culture would exchange electrons with hydrogen as the electron carrier. However, preliminary results, obtained prior to our obtaining this grant, suggested that the co-culture might be directly exchanging electrons. Under this grant we further evaluated the mechanisms for electron exchange with transcriptomic, proteomic, and genetic approaches. For example, the negative impact of deleting genes to prevent interspecies hydrogen transfer, or the production of electrically conductive pili or the pili-associated cytochrome OmcS, suggested that there were direct electrical connections between the two species. This conclusion was further supported by the finding that deletion of a regulatory system that promoted OmcS production enhanced DIET. A method was developed for directly measuring the conductivity of the aggregates and these measurements demonstrated that the aggregates are electrically conductive with sufficient conductance to account for the rate of electron flow in the system. A paper summarizing these results was published in *Science*.

DIET in the *G. metallireducens/G. sulfurreducens* co-culture was further investigated with transcriptional and genetic approaches in order to determine if: 1) electrons derived from DIET provided sufficient energy to support cell growth; and 2) if there were transcriptional patterns specific to DIET that might be used for determining if DIET was taking place in more complex microbial communities. These studies, which were summarized in publications in two papers in *Applied and Environmental Microbiology* and one in *Environmental Microbiology Reports*, provided further evidence for direct electrical wiring between *G. metallireducens* and *G. sulfurreducens* through their electrically conductive pili and associated cytochromes. Gene transcriptional patterns when electrons were exchanged via DIET were clearly different than those when electrons were exchange via interspecies electron transfer with hydrogen or formate. Furthermore, a strain of *G. sulfurreducens* that was genetically modified so that electrons derived from DIET were the only energy source was able to grow effectively.

Although DIET in the *G. metallireducens/G. sulfurreducens* co-cultures provided a genetically tractable system for studying this phenomenon, the ultimate goal of our project was to determine if DIET was possible in methanogenic systems and to develop genome-scale metabolic models for this interaction to predict responses to environmental perturbations. The aggregates of the *G. metallireducens/G. sulfurreducens* co-cultures were morphologically similar to the aggregates previously demonstrated to be abundant in anaerobic wastewater digesters. Therefore, in order to evaluate whether direct electron transfer might be taking place in natural methane-producing aggregates, the methanogenic aggregates that are produced in up-flow anaerobic digesters were investigated. The aggregates from two digesters treating brewery waste were found to be as electrically conductive as the aggregates that the *Geobacter* co-culture produced. Temperature analysis of conductivity indicated the origin of conductivity within the aggregates was biological and electron transfer rate calculations demonstrated that aggregates had sufficient conductance to account for the direct electron exchange within the aggregates. The potential for methane production from ethanol greatly exceeded the combined maximum potential for methane production from acetate and formate and the capacity for methane production from hydrogen was very low. These results further suggested that a mechanism other than interspecies hydrogen or formate transfer was required to account for the metabolism of ethanol within the methanogenic aggregates. Molecular analysis of the microbial composition of the aggregates demonstrated that the aggregates were dominated by *Geobacter* as the most predominant bacteria and that almost of all of the methanogens were *Methanosaeta* species. *Methanosaeta* are unable to use hydrogen or formate as an electron donor. These results and the high conductivity of the aggregates suggested that electrons were being exchanged via DIET. A paper summarizing these results was published in *mBio*.

The possibility that *Methanosaeta* species might be capable of forging biological electrical connections was further investigated. Examination of the available *Methanosaeta* genomes revealed that, despite their inability to use hydrogen or formate as electron donors they posses genes for the complete pathway for the conversion of carbon dioxide to methane. This suggested that if *Methanosaeta* species had a

mechanism to accept electrons via DIET those electrons could be used for the reduction of carbon dioxide to methane. Metatranscriptomic analysis of gene expression of the microbial community in an anaerobic digester demonstrated that the genes for carbon dioxide reduction were among the most highly expressed genes of *Methanosaeta* in the digester. This provided strong evidence that *Methanosaeta* was accepting electrons via DIET to reduce carbon dioxide to methane.

Although the metatranscriptomic data was compelling, it was important to investigate the proposed DIET pathway under defined conditions. Therefore, the ability of *Methanosaeta haurindaceae* to grow in co-culture with *G. metallireducens* with ethanol as the electron donor was evaluated. The co-culture stoichiometrically converted ethanol to methane. Interspecies hydrogen or formate transfer between the two strains was impossible because *G. metallireducens* is not capable of hydrogen or formate production from ethanol and because *M. haurindaceae* is not able to use hydrogen or formate as an electron donor. Further evidence for electron exchange via DIET was the finding that co-cultures could not be established with a strain of *G. metallireducens* in which the gene for PilA, the structural protein for the electrically conductive pili, was deleted. *M. haurindaceae* in the co-culture converted 14C-carbon dioxide to 14C-methane at rates that accounted for the electrons released from *G. metallireducens* during ethanol metabolism. Metatranscriptomic analysis of the co-culture revealed that *M. haurindaceae* was highly expressing genes for the enzymes required for the reduction of carbon dioxide to methane.

Methanosaeta species are responsible for more methane production on earth than any other group of methanogens. Prior to our studies, it was considered that *Methanosaeta* species were restricted to acetate as a substrate for methane production. Therefore, our finding that *Methanosaeta* species also have the ability to reduce carbon dioxide to methane are of substantial importance in understanding the global production of methane, a potent greenhouse gas. Just as importantly, our study was the first report of a methanogen capable of directly accepting electrons from another organism. These results were reported in the high impact journal (impact factor > 15) *Energy and Environmental Science*.

Analysis of a microbially complex enrichment converting the hydrocarbon hexadecane to methane with metagenomic and metatranscriptomic approaches suggested substantial electron flow for methane production via DIET. A paper summarizing these results was published in *ISME Journal*.

Further investigation of the diversity of methanogens capable of participating in DIET demonstrated that *Methanosarcina barkeri* grew syntrophically with *G. metallireducens*. A number of other methanogens did not. These results demonstrate that although there are methanogens that specialize in utilizing hydrogen or formate as electron donors, the major acetate-utilizing methanogens, which are the predominant methane producers in soils and sediments as well as methanogenic digesters, are capable of DIET. These results were published in *Applied and Environmental Microbiology*.

It was found that a variety of conductive materials that are either natural constituents of soils and sediments or are agricultural additives, or added to stabilize anaerobic conversion of wastes to methane promote DIET by substituting either for the conductive pili or pili-associated cytochromes. These conductive materials include magnetite, which is produced during microbial Fe(III) reduction; biochar, which is added to soils as a stabilizer; and activated carbon, a common additive to anaerobic digesters. Studies with appropriate gene deletion mutants confirmed the role of these materials in DIET. Papers summarizing these results were published in *Energy and Environmental Science*, *Environmental Microbiology*, *Scientific Reports*, and *Bioresource Technology*.

A key component of this project was to develop multi-species genome-scale metabolic models that could predict the interactions between methanogens and their syntrophic partners under different environmental conditions. It was considered particularly challenging to develop a model for DIET. Therefore, we began with a DIET model for the *G. metallireducens/G. sulfurreducens* co-culture because the genome-scale models for these organisms were significantly more advanced than the models for methanogens, especially those capable of DIET. In fact, there was no genome-scale model for *Methanosaeta* species.

The genome-scale model of *G. metallireducens* was substantially improved and published in *PLOS Computational Biology*. Then the first-ever modeling framework for coupling energy conservation between two species via DIET was constructed. Genome-scale transcriptional analysis of the co-culture was carried out under various conditions, including studies with a strain of *G. sulfurreducens* that prevented it from metabolizing the acetate also released during ethanol metabolism. Furthermore, predicted metabolism in *G. metallireducens/G. sulfurreducens* exchanging electrons via DIET was compared with predicted metabolism in co-cultures of *Pelobacter carbinolicus* and *G. sulfurreducens* in which electrons are exchanged via interspecies hydrogen transfer. The modeling predicted that DIET is energetically more advantageous than interspecies hydrogen transfer for the electron-donating partner. The benefits to the electron-accepting partner are less pronounced and can strongly be influenced by environmental conditions. The *G. metallireducens/G. sulfurreducens* model also correctly predicted the relative abundance of the two organisms under different growth conditions. The results of the modeling and genome resequencing of the co-culture suggested that, in syntrophic associations, the accepting partner streamlines its metabolic capabilities to enhance the most efficient mode of electron transfer. A paper summarizing these results was published in *Nature Communications*.

Considerable progress was made in generating high-quality genome-scale reconstructions for three methanogens: *Methanosarcina barkeri*, *Methanococcus maripaludis* and *Methanospirillum hungatei*. It is expected that these models will be helpful in future comparative modeling of DIET and interspecies hydrogen transfer in the future.

A number of invited review and opinion articles were published in high impact journals.

The multi-omic approach developed in these studies should be useful for elucidating the mechanisms for electron flow in methanogenic communities in soils and sediments that make substantial contributions to atmospheric methane. Genome-scale models of these communities should make it feasible to make effective predictions on the response of these communities to environmental perturbations such as climate change.

Publications

Summers, Z. M., H. Fogarty, C. Leang, A. E. Franks, N. S. Malvankar, and D. R. Lovley. 2010. Direct exchange of electrons within aggregates of an evolved syntrophic co-culture of anaerobic bacteria. Science 330:1413-1415.

Lovley, D. R. 2011. Reach out and touch someone: potential impact of DIET (direct interspecies energy transfer) on anaerobic biogeochemistry, bioremediation, and bioenergy. Rev. Environ. Sci. Biotechnol. 10:101-105.

Morita, M., N. S. Malvankar, A. E. Franks, Z. M. Summers, L. Giloteaux, A. E. Rotaru, C. Rotaru, and D. R. Lovley. 2011. Potential for direct interspecies electron transfer in methanogenic wastewater digester aggregates. mBio 2:e00159-11.

Lovley, D. R. 2011. Live wires: direct extracellular electron exchange for bioenergy and the bioremediation of energy-related contamination. Energy Environ. Sci. 4:4896-4906.

Lovley, D. R., T. Ueki, T. Zhang, N. S. Malvankar, P. M. Shrestha, K. Flanagan, M. Aklujkar, J. E. Butler, L. Giloteaux, A.-E. Rotaru, D. E. Holmes, A. E. Franks, R. Orellana, C. Risso, and K. P. Nevin. 2011. Geobacter: the microbe electric's physiology, ecology, and practical applications. Adv. Microb. Physiol. 59:1-100.

Rotaru, A.-E., P. M. Shrestha, A. Liu, T. Ueki, K. P. Nevin, and D. R. Lovley. 2012. Interspecies electron transfer via H2 and formate rather than direct electrical connections in co-cultures of Pelobacter carbinolicus and Geobacter sulfurreducens. Appl. Environ. Microbiol. 78:7645-7651.

Zengler, K., and B. O. Palsson. 2012. A road map for the development of community systems (CoSy) biology. Nature Rev. Microbiol. 10:366-372.

Liu, F., A.-E. Rotaru, P. M. Shrestha, N. S. Malvankar, K. P. Nevin, and D. R. Lovley. 2012. Promoting direct interspecies electron transfer with activated carbon. Energy Environ. Sci. 5:8982-8989.

Lewis, N. E., H. Nagarajan, and B. O. Palsson. 2012. Constraining the metabolic genotype-phenotype relationship using a phylogeny of in silico methods. Nature Rev. Microbiol. 10:291-305.

Qiu, Y., H. Nagarajan, M. Embree, W. Shieu, E. Abate, K. Juarez, B.-K. Cho, J. G. Elkins, K. P. Nevin, C. L. Barrett, D. R. Lovley, B. O. Palsson, and K. Zengler. 2013. Characterizing the interplay between multiple levels of organization within bacterial sigma factor regulatory networks. Nature Comm. 4:1755.

Shrestha, P. M., A.-E. Rotaru, M. Aklujkar, F. Liu, M. Shrestha, Z. M. Summers, N. S. Malvankar, D. C. Flores, and D. R. Lovley. 2013. Syntrophic growth with direct Interspecies electron transfer as the primary mechanism for energy exchange. Environ. Microbio. Rep. 5:904-910.

Shrestha, P. M., A.-E. Rotaru, Z. M. Summers, M. Shrestha, F. Liu, and D. R. Lovley. 2013. Transcriptomic and genetic analysis of direct interspecies electron transfer. Appl. Environ. Microbiol. 79:2397-2404.

Nagarajan, H., M. Embree, A.-E. Rotaru, P. M. Shrestha, A. M. Feist, B. O. Palsson, D. R. Lovley, and K. Zengler. 2013. Multi-omic characterization and modeling of interspecies electron transfer mechanisms and microbial community dynamics of syntrophic associations. Nature Comm. 4:2809.

Rotaru, A.-E., P. M. Shrestha, F. Liu, M. Shrestha, D. Shrestha, M. Embree, K. Zengler, C. Wardman, K. P. Nevin, and D. R. Lovley. 2014. A new model for electron fow during anaerobic digestion: direct interspecies electron transfer to Methanosaeta for the reduction of carbon dioxide to methane. Energy Environ. Sci. 7:408-415.

Chen, S., A.-E. Rotaru, F. Liu, J. Philips, T. L. Woodard, K. P. Nevin, and D. R. Lovley. 2014. Carbon cloth stimulates direct interspecies electron transfer in syntrophic co-cultures. Bioresource Tech. 173:82-86.

Rotaru, A.-E., P. M. Shrestha, F. Liu, K. P. Nevin, and D. R. Lovley. 2014. Direct interspecies electron transfer during syntrophic growth of Geobacter metallireducens and Methanosarcina barkeri on ethanol. Appl. Environ. Microbiol. 80:4599-4605.

Embree, M., Y. Qiu, W. Shieu, H. Nagarajan, R. O'Neil, D. Lovley, and K. Zengler. 2014. The iron stimulon and fur regulon of Geobacter sulfurreducens and their role in energy metabolism. Appl. Environ. Microbiol. 80:2918-27.

Chen, S., A.-E. Rotaru, P. M. Shrestha, N. S. Malvankar, F. Liu, W. Fan, K. P. Nevin, and D. R. Lovley. 2014. Promoting interspecies electron transfer with biochar. Scientific Reports 4:5019.

Liu, F., A.-E. Rotaru, P. M. Shrestha, N. S. Malvankar, K. P. Nevin, and D. R. Lovley. 2015. Magnetite compensates for the lack of a pilin-assoicated c-type cytochrome in extracellular electron exchange. Environ Microbiol (in press) doi:10.1111/1462-2920.12485.