

Identification of Enzymes involved in Syntrophic H₂ production.

Lee R. Krumholz*, Xiangzhen Li, Peter Bradstock, Michael McInerney

University of Oklahoma

Department of Botany and Microbiology

770 Van VleetOval

Norman, OK 73019

Phone: 405-325-0437; FAX 404-325-7619

*Corresponding email: krumholz@ou.edu

Subcontractors:

Judy Wall, University of Missouri

DOE Program Officer: Richard Greene

richard.greene@science.doe.gov ; 301-903-6190

Objectives

- *Bacteria that grow together in a syntrophic interaction are thought to degrade organic compounds coupled to the transfer of H₂ from one species to another. The goals of this work are to: (a) identify the genes involved in mediating H₂ transfer between species; (b) identify and characterize the enzymes that are used for H₂ production and H₂ consumption; and (c) optimize H₂ production by H₂ producing syntrophic bacteria.*

Technical Barriers

- *The work described above should identify proteins capable of H₂ production and bacterial strains that can produce H₂ while growing with lactate. The technical challenges ahead of us, lie in developing these enzymes for biohybrid H₂ production technology. Challenges will also face us in directly implementing H₂ producing strains for industrial H₂ production.*

Abstract

This project is directed at the development of Bio-hybrid energy coupled systems. Enzymes synthesized and employed by biological systems could potentially be combined with synthetic materials to construct robust, efficient hybrid systems that are scalable to H₂ production facilities. Fundamental information on ***enzymes and mechanisms of H₂ production*** is required for development of such processes. Our goals are to fully understand at the genetic and biochemical level, the H₂-producing system of a model microorganism. Sulfate-reducing bacteria grow with reduced organic compounds such as ethanol and lactate and produce acetate and H₂ as products if grown in the presence of an H₂-consuming microorganism such as *Methanospirillum hungatei*. We believe that knowledge of these components will be critical to developing an understanding and perhaps harnessing the bacterial system of H₂ production.

Desulfovibrio desulfuricans strain G20 is being used as the model organism for this project. The initial goal of the project was to identify genes that are involved in syntrophic H₂ production. Progress to date has involved screening a library of 5760 individual gene knockout mutants of strain G20 for their ability to grow in syntrophic association with the H₂-consuming organism, *M. hungatei*. The relationship involves the growth of strain G20 on lactate where it produces H₂, which is oxidized at a rapid rate by *M. hungatei* (the methanogen). We have screened the entire library and identified mutants in 27 genes that are needed for syntrophic growth. Three of these genes appear to encode for key respiratory proteins. These 3 mutations are in genes predicted to encode for Fe-hydrogenase, cytochrome *c*₃ and a molybdopterinoxidoreductase. Further characterization of these three mutants has shown that they are deficient in the ability to grow with H₂, ethanol or formate and grow poorly with pyruvate as energy sources. However, all three mutants are still capable of H₂ production when grown with lactate. Because of the physiological similarities among these

mutants, the results suggest that these three genes may act in concert to mediate H₂ consumption and may also be involved in H₂ production.

Further studies will focus on these three mutants. We will complement the mutants to be certain that the specific gene determines that phenotype. The selected genes will be cloned and expressed in *Escherichia coli* or *Desulfovibrio* sp. and subsequently characterized from purified enzyme preparations. Finally, additional genes involved in H₂ or electron transfer will be identified with microarray technology, by quantifying mRNAs of functional genes from *D. desulfuricans* strain G20 grown with lactate either syntrophically with *M. hungateior* in pure culture. The results from the above experiments will determine the mechanisms for H₂ production from organic compounds and identify cellular components needed for the reactions. Ultimately, parts or all of these enzymes may be used for industrial production of H₂.

Progress Report and Future Directions

I. Identification of Mutants. The identities and roles of certain proteins involved in syntrophic growth and H₂ metabolism have been addressed during the first part of this study. This information was obtained by first generating a transposon mutant library. The library of 5760 mutants was tested individually for growth with the methanogen, *M. hungatei*. We identified 27 mutants that were either unable to grow syntrophically or exhibit slow syntrophic growth and present a small group of them in Table 1 along with results of a number of growth experiments.

Three of the mutations are in genes related to H₂ respiration. Dde_2933, the Molybdopterinoxidoreductase, molybdopterin-binding subunit; Dde_0082, the Iron-only hydrogenase small subunit, and Dde_3182, the cytochrome_c₃. The three mutants exhibit the same phenotype in that (a) syntrophic growth is either completely abolished or slowed; (b) no detectable growth occurs in pure culture with sulfate as the electron acceptor and either formate, H₂, or ethanol as the electron donor and (c) low levels of growth on pyruvate as the electron donor with sulfate. We hypothesize that these three proteins form an electron transfer complex in which electrons derived from formate, H₂, pyruvate or ethanol oxidation are transferred to an intermediate electron carrier or directly to the respiratory proteins involved only in sulfate reduction. These proteins may be key components responsible for H₂ cycling.

The tetrahemeperiplasmicytochrome_c₃ is perhaps the most well studied protein in *Desulfovibrio* and yet much remains to be learned regarding its function. The protein has been crystallized several times and a number of papers have been published describing its structure (see (Pattarkine et al. 2006) and included references). Molecular modeling studies have suggested that heme IV is the site of binding to other proteins during electron transfer. Studies done in cell-free preparations have shown cytochrome_c₃ to interact with and to mediate electron transfer from the Fe-hydrogenase to the transmembrane high-molecular-mass cytochrome (Hmc) (Pereira et al. 1998). A recent study showed that oxidized metals such as U(VI) can bind directly in the same region, providing additional evidence that electrons are transferred from this area (Pattarkine et al. 2006). In vivo studies of the interaction of c₃ with other proteins are lacking. As a result, the true interactions within the cell are not yet completely understood. Future studies will address the question of which electron transfer proteins interact with cytochrome c₃, through studies with mutants and through combined genetic and biochemical analyses.

The Fe-hydrogenase (Hyd) was first purified in 1984 (Huynh et al. 1984) and was shown to contain 2 ferredoxin type (4Fe-4S) clusters, but no nickel nor heme iron. This hydrogenase exhibits high activity, but a relatively low affinity for H₂ (Fauque et al. 1988) compared to other *Desulfovibrio* hydrogenases. Mutants lacking the Fe-hydrogenase (*hyd*) accumulated H₂ with lactate or formate as the electron donor and grow poorly with H₂ as electron donor (Table 1) (Pohorelic et al. 2002; Luo et al. 2007). Studies in our lab (Luo et al. 2007) have shown that this enzyme is required for growth in aquifer sediments and for growth under syntrophic conditions (Table 1). The fact that Fe-hydrogenase mutants produce H₂ yet are unable to use H₂ effectively, indicates that the Fe-hydrogenase acts as an uptake hydrogenase for *Desulfovibrio*. However, recent experiments suggest that the Fe-hydrogenase is most important when H₂ is present at high concentrations or when growing with lactate (Caffrey et al. 2007). Future experiments will address the role of this enzyme in syntrophic as well as in respiratory metabolism.

Table 1. Mutated genes identified to be involved in syntrophic growth. The table shows whether the mutant grows in pure culture with different electron donors, lactate, H₂, formate, pyruvate, and ethanol in the presence of sulfate. The ability of the mutants to grow syntrophically on lactate with *M. hungatei* and to produce H₂ during incubation with lactate or during growth on lactate-sulfate (H₂ evolution) is given.

Locus	Predicted protein	Growth						
		Syntro- phy	Lact -ate	H ₂	For mat	Pyr uva	Et- OH	H ₂ e vol
	Parent strain	+	+	+	+	+	+	+
Dde_2933	Molybdopterinoxidoreduc tase, molybdopter- binding subunit	-	+	-	-	±	-	+
Dde_3245	iron-sulfur cluster- binding protein	±	±	+	+	ND	ND	+
Dde_0082	Fe-hydrogenase small subunit	±	+	-	-	±	-	+
Dde_3775	ferredoxin I	±	±	±	±	ND	ND	+
Dde_0364	hydrogenaseexpression/fo rmation proteinHypD	±	+	+	+	ND	ND	+
Dde_0555	(NiFe) hydrogenasematuration proteinHypF	±	+	+	+	ND	ND	+
Dde_3182	cytochrome c3	-	+	-	-	±	-	+

+grow normally as parent strain or positive. ±grow slower than parent strain.- no detectable growth or negativewithin a10-d incubation at 37 °C. ND Not determined.

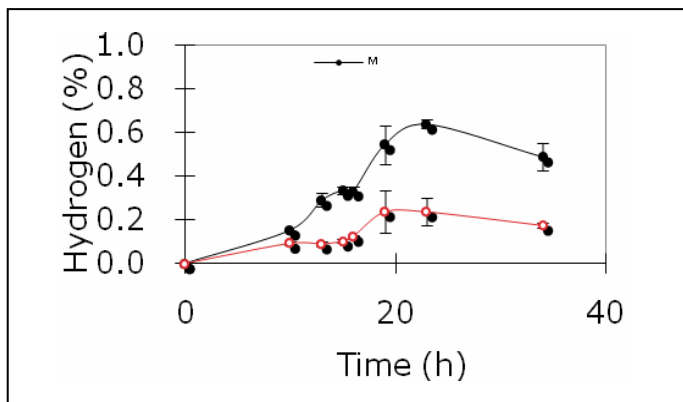
The final gene of interest is predicted to be a molybdopterinoxidoreductase. The mutated gene is predicted to encode for a molybdopterin-binding subunit typically associated with a diverse class of redox proteins. In *Bacteria*, molybdopterinoxidoreductases typically have a molybdenumcomplexed with two sulfur groups from a dinucleotide in the catalytic subunit of the complex. There are three classes of molybdopterin -containing proteins. The molybdopterinoxidoreductase of interest in strain G20 is most similar to members of the DMSO reductase family based on BLAST and COGs analysis. This family is the most diverse group and includes formatedehydrogenase, nitrate reductase, and arseniteoxidase from *Bacteria*. Oxygen atom transfer or dehydrogenation reactions are possible with this family(Hille 2002). Two well-characterized proteins from this family are the formatedehydrogenase and nitrate reductase from *E. coli*. The formatedehydrogenase transfers electrons from formate to oxidized menaquinone. The reduced menquinone is then able to donate electrons to the nitrate reductase in an energy-conserving manner. At least one of the other subunits is involved in anchoring the complex in the membrane.

The mutated gene in *Desulfovibrio* G20 is present as the second gene in a 4 gene operon. Based upon homology with orthologs in *E. coli*, it is likely that at least 3 of these genes (2,3&4) encode peptides involved in the functional oxidoreductase. The first gene has been predicted to encode a hexahemecytochromec₃ which may or may not be present in the complex. The complex in strain G20 shows some similarity to some well characterizedformatedehydrogenases and it is possible that this is its function, as the mutant cannot use formate. However, this mutant is also unable to use several other substrates and there are several other annotated formatedehydrogenases in the G20 genome. The function of this protein complex will be explored in future studies.

2. Hydrogen production and consumption. Experiments have been carried out to characterize the physiological changes associated with the mutations of interest. The three mutants, the molybdopterinoxidoreductase, the Fe-hydrogenase and the cytochromec₃ mutants, have similar physiological features. All grow poorly with pyruvate and not at all with ethanol, H₂ or formate (Table 1). Only the Fe-hydrogenase mutant will grow slowly under syntrophic conditions (lactate as the donor) while the other two mutants do not grow at all. As the mutants do not grow syntrophically and they do not use H₂, we would

predict that they would not produce H₂ either. However, this is not the case. Experiments, in which mutants and the parent strain were grown with 20 mM each of lactate and sulfate or pyruvate and sulfate show that H₂ is produced by the mutants above the level of the parent strain (fig. 1). Similar results have also been obtained showing higher levels of formate production by the mutants. The higher levels of H₂/formate production are likely driven by the lack of H₂/formate consumption by the mutants with H₂ or formate accumulating rather than being used as it is produced. These results present the opportunity to determine whether mutants in these systems can be used as biological H₂ production systems. This question will be addressed in future work.

Figure 1. H₂ production by cultures of the molybdopterinoxidoreductase mutant grown in pure culture in serum tubes with lactate and sulfate. A headspace concentration of 1% H₂ is equivalent to 1.8 mmols H₂ produced per liter of



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