Final Technical Report

Genetics and Molecular Biology of Hydrogen Metabolism in Sulfate-Reducing Bacteria

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Judy D. Wall PI

Abstract. The degradation of our environment and the depletion of fossil fuels make the exploration of alternative fuels evermore imperative. Among the alternatives is biohydrogen which has high energy content by weight and produces only water when combusted. Considerable effort is being expended to develop photosynthetic systems -- algae, cyanobacteria, and anaerobic phototrophs -- for sustainable H_2 production. While promising, this approach also has hurdles such as the harvesting of light in densely pigmented cultures that requires costly constant mixing and large areas for exposure to sunlight. Little attention is given to fermentative H_2 generation. Thus understanding the microbial pathways to H_2 evolution and metabolic processes competing for electrons is an essential foundation that may expand the variety of fuels that can be generated or provide alternative substrates for fine chemical production. We studied a widely found soil anaerobe of the class *Deltaproteobacteria*, a sulfate-reducing bacterium to determine the electron pathways used during the oxidation of substrates and the potential for hydrogen production.

In keeping with the overall goals of Energy Biosciences, we proposed to study the energy transduction systems of the anaerobic sulfate-reducing bacterium of the genus *Desulfovibrio*, strain G20, by genetic and physiological approaches. To contribute to the platform of understanding of energy-related technologies, we wanted to explore the respiratory electron transport pathways - their assembly, regulation, and redundancy - of this environmental isolate. Improvement of the tools needed for facile creation of deletions and their analysis (Aim 1) was critical for preparing knock out mutations of the various transmembrane complexes providing the electron carriers from periplasm to cytoplasm and reverse (Aim 2). The G20 strain was chosen for its rather robust pyruvate fermentation and fumarate disproportionation that provide growth modes independent of sulfate reduction. However, G20 proved more difficult than anticipated for genetic development and our attention turned to *Desulfovibrio vulgaris* Hildenborough. There we developed a markerless deletion system for the construction of in-frame deletions that did not result in a residual antibiotic marker on the chromosome. We found surprisingly that essentially all the transmembrane complexes were individually non-essential when grown on lactate/sulfate or sulfite medium with the exception of DsrMKJOP that appears to be needed regardless of growth mode. In addition, all hydrogenase isozymes (seven in DvH) are dispensable individually as are all formate dehydrogenases.

Aim1. We proposed to improve the genetic accessibility of G20 so that gene deletions, complementation and regulatory experiments could be readily carried out. Electrotransformation of G20 with plasmids prepared from *Escherichia coli* resulted in detectable plasmid integration but not the generation of deletions through marker exchange, a process that requires two recombinational events. Unfortunately, we were unable to improve the transformation efficiencies and we were unable to delete the genes for restriction endonucleases. During these studies, an archived library of transposon mutants of >15,000 was generated for G20 by another

lab (Kuehl et al., 2014). We were able to obtain transposon mutants for some analyses but not multiple mutations in a single strain needed for testing compensatory systems. We therefore pursued an analysis of DvH where we were able to obtain a counterselectable marker for marker eviction and the creation of unmarked, in-frame deletion mutants.

For the creation of unmarked, inframe deletions, a procedure adapted from that developed for *Bacillus subtilis* (Fabret et al., 2002) and *Methanosarcina acetivorans* C2A (Pritchett et al., 2004) was explored. DvH strains and vectors for this procedure were constructed and tested (Keller et al., 2009). This is a two-step integration-segregation procedure that uses a plasmid that cannot replicate in the host bacterium. The plasmid carries DNA regions from upstream and downstream of the gene or bases to be deleted, a selectable antibiotic resistance to identify the integration and production of a merodiploid, and a counterselectable marker. Removal of the antibiotic selection allows resolution of the merodiploid by homologous recombination between the mutant and wild-type sequences and removal of the plasmid sequences. The enrichment of the desired mutants is facilitated by selection against the counterselectable marker, and its identification is by PCR analysis.

For *Desulfovibrio* constructs, we explored the counterselectable marker, the *upp* gene encoding the pyrimidine salvage enzyme, Uracil Phosphoribosyltransferase. This enzyme confers sensitivity to the toxic analog 5-fluorouracil (5FU). Mutants deleted for *upp* are resistant to 5FU and become sensitive following introduction of the *upp* gene on a plasmid. The presence of an expressed *upp* gene in the mutagenic plasmid allows enrichment for plasmid loss by resistance to 5FU. We have demonstrated these features with DvH (Keller et al., 2009).

Individual marker-less deletions have been generated for each of the seven of the hydrogenases of *D. vulgaris*. Table 1 shows part of the progress made in making multiple deletions of the hydrogenase isozymes. When the mutant lacking six hydrogenases was tested for growth on hydrogen, it grew after a lag. This result confirmed the functioning of a seventh hydrogenase isozyme in this strain that is now the focus of an additional deletion in the background of the existing six

Multiple Hydrogenase Mutants

Strain	Deletions	Description
JW2089	Δ*hysBA*, Δ*hynBA-1*, Δ*hynBA-2*	[NiFeSe], $[NiFe]_1$, $[NiFe]_2$ periplasmic Hases deleted
JW5009	Δ*hydAB*, Δ*hysBA*, Δ*hynBA-1*, Δ*hynBA-2*	[FeFe], [NiFeSe], $[NiFe]_1$, $[NiFe]_2$ periplasmic Hases deleted
JW5063	Δ*hydAB*, Δ*hysBA*, Δ*hynBA-1*, Δ*hynBA-2*, Δ*coo*[1]	Only possesses Ech [NiFe] cytoplasmic Hase
JW5065	Δ*hydAB*, Δ*hysBA*, Δ*hynBA-1*, Δ*hynBA-2*, Δ*ech*[2]	Only possesses Coo [NiFe] cytoplasmic Hase
JW5076	Δ*hydAB*, Δ*hysBA*, Δ*hynBA-1*, Δ*hynBA-2*, Δ*coo*[1], Δ*ech*[2]	6 Hases deleted

[1] *coo* represents the operon *cooMKLXUHhypAcooF*
[2] *ech* represents the operon *echABCDEF*

deletions. In addition, the six hydrogenases are being returned as single isozymes to the mutant lacking all seven enzymes. This work is being prepared for publication.

Aim 2. Electron transport complex analyses. We approached an elucidation of the functions of transmembrane complexes in sulfate respiration by deletion analyses. Quite a number and variety of transmembrane complexes have been identified or predicted from the genome sequences of both sulfate reducers and sulfur or thiosulfate oxidizers (Matais et al., 2005;Pereira et al., 2011). As our first priority, we proposed to delete the predicted operons for the Qmo and Dsr complexes. These two complexes are proposed to deliver electrons specifically for the reduction of sulfate and sulfite, respectively. Thus, if true, they are fundamental to the feature of energy transduction that makes these bacteria sulfate reducers. Qmo deletions were made and found to be essential for sulfate reduction but no deletions were obtained for DsrMKJOP deletions when cells were grown out by pyruvate fermentation.

Qmo complex: Quinone-interacting membrane-bound oxidoreductase complex (Fig.1A) is composed of three proteins encoded by *qmoABC* (Pires et al., 2003) located immediately downstream of the *apsBA* genes (Dde1109-1110) that encode the APS reductase. ApsBA reduces activated sulfate to sulfite. In both G20 and DvH, *qmo* genes have been predicted to be transcribed with *apsBA*. We have created a marker exchange deletion in DvH of *qmoABC* and a predicted hypothetical gene (HP) (Zane et al., 2010). This DvH mutant does not grow with sulfate as a terminal electron acceptor but grows as well as the parental strain with sulfite or thiosulfate. Remarkably no compensation or suppression has been observed. Complementation by single copy integration of *qmoABC* HP into the chromosome restored the ability of DvH to grow with sulfate.

DsrMKJOP complex: The dissimilatory sulfite reductase genes, *dsrABD* (Dde0526-0528), encode a soluble complex thought to interact with a transmembrane complex coded for by the operon, *dsrMKJOP* (Dde2271-2275; Fig.1B). We hypothesize that deletions of the structural genes for sulfite reduction as well as the predicted interacting membrane complex should result in mutants with similar phenotypes. There should be no growth possible on sulfate, sulfite or thiosulfate but pyruvate fermentation or fumarate dismutation should function as in wild-type. If our model were correct, it would not be possible to isolate these mutants without a reasonable fermentation growth mode. It was subsequently found that we could not delete *dsrMKJOP* either because our growth on pyruvate was insufficient or because the complex is essential for some unknown reason.

Further analysis of a transposon library of >12,000 unique mutants in DvH showed that, during growth on lactate/sulfate, the transmembrane complexes Rnf, TMC, Hmc or Ohc were individually not essential.

From our studies of the G20 plasmid insertion mutant in *cycA,* the gene for the tetraheme Type I cytochrome c_3 (TpIc_3) (Rapp-Giles et al., 2000, AEM 66:671-677), we have established that electrons from pyruvate, H_2, or formate do not reach sulfate. When relative protein abundances from proteomic data (obtained in collaboration with PNNL) of G20 were compared with those from the CycA mutant growing by sulfate respiration with lactate oxidation or by pyruvate fermentation, we found that the enzymes for fumarate reduction were abundant in G20

but were undetected in the mutant (Fig. 1). Consistent with this finding, the CycA mutant was unable to grow by fumarate disproportionation. Also, the pathway for ATP formation by substrate-level phosphorylation was not increased when cells were asked to grow by pyruvate fermentation. Interestingly, formate and CO formation would appear to serve as electron sinks when the CycA mutant was unable to transfer electrons through the transport system to sulfate. These findings led to a new model of electron flow in G20 (Keller et al., 2014).

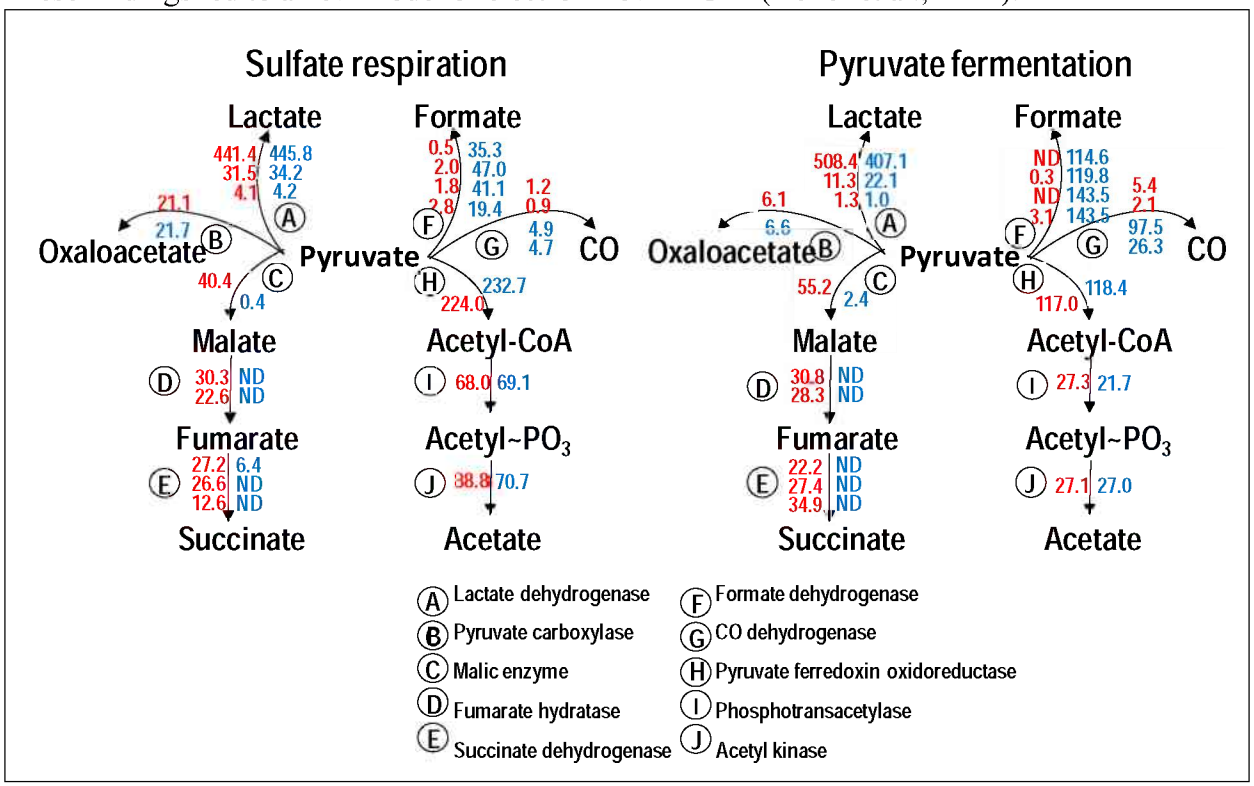

Figure 1. Relative protein abundances from proteomics data of cultures grown with lactate/sulfate (40/60 mM) or pyruvate (60 mM) alone. Numbers represent protein abundances from the most highly expressed isozymes. Multiple values for some enzymes reflect the abundance of each subunit. *D. alaskensis* G20 data are in **red** and the CycA mutant in **blue**. ND was not detected. (Keller et al., 2014)

The transmembrane complex QrcABCD has recently been identified in *Desulfovibrio* (Venceslau et al., 2010) and suggested to accept electrons from hydrogenases and formate dehydrogenases in the periplasm via TpIc_3 and function as a TpIc_3:menaquinone oxidoreductase (Venceslau et al., 2011). A G20 derived QrcB Tn5 mutant has been reported by Li et al. (2009) not to grow with H_2 or formate as electron donors and sulfate as electron acceptor; however, the mutant grows well on lactate/sulfate. A QrcA mutant of G20 obtained from the transposon library had the same phenotype as the QrcB mutant. In addition a marker exchange deletion of *qrcABCD* of *D. vulgaris* has also been shown to have this phenotype. When the Δ*qrcABCD* mutant was assayed for U(VI) reduction with H_2 as electron donor, U(IV) was generated. This result is in contrast to that for the CycA mutant that could not reduce U(VI) with electrons with H_2 (Semkiw et al., 2012 (Abstr.)).

From these results, we infer that, for sulfate reduction, electrons from H_2 oxidized by periplasmic hydrogenases are transferred to TpIc_3 cytochrome, which delivers electrons to QrcABCD, where the inner membrane subunit QrcD transfers them to the menaquinone pool. Menaquinone reduction and subsequent electron transfer to APS reductase occurs through the QmoABC complex thus allowing sulfate reduction to occur. The flow of electrons suggested is diagrammed below.

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