THE ROLE OF REX IN REGULATION
OF SULFATE REDUCTION IN
DESULFOVIBRIO VULGARIS HILDENBOROUGH

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Doctor of Philosophy

by
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The undersigned, appointed by the dean of the Graduate School, have examined the
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THE ROLE OF REX IN REGULATION
OF SULFATE REDUCTION IN
DESULFOVIBRIO VULGARIS HILDENBOROUGH

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To my wife,

Christina Christensen

“May we grow old and grey together...
just not too soon.”

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In my youth I participated in many physically demanding activities including Hurdles in Track and Field, Cross Country and Epic Mud runs. The goal was always simple, make it to the end, improve every time, do not get hurt, and have fun. I approached earning my Doctorate in Biochemistry in a similar manner. That is to say, I knew that the next several years would be physically, as well as mentally, challenging for me and that I would need support along the way.

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LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
Aa	Amino acid
Ap	Ampicillin
bp	Base pairs
cDNA	copy DNA
Cq (or Ct)	Critical Threshold (qPCR)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DvH	<i>Desulfovibrio vulgaris</i> Hildenborough
EMSA	Electrophoretic mobility shift assay
FPA	Fluorescent polarization assay
G418	Geneticin®
gDNA	Genomic DNA
Km	Kanamycin
MED	Marker-exchange deletion
MLD	Marker-less deletion
NAD ⁺	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NTC	No Template Control
pdb	Protein data bank
Rex ^{DvH}	Rex protein homolog in DvH
RFU	Relative fluorescence units
RNA	Ribonucleic acid
RNAP	RNA polymerase
Sp	Spectinomycin
SRB	Sulfate-reducing bacteria
SRM	Sulfate-reducing microbe
T _m	Melting Temperature
TSS	Transcript start site

Media components:

MO:	basal salt (standard medium)
L:	lactate (60 mM)
P:	pyruvate (60 mM)
S4:	sulfate, SO ₄ ²⁻ (30 mM)
S3:	sulfite, SO ₃ ²⁻ (40 mM)
T3:	thiosulfate S ₂ O ₃ ²⁻ (30 mM)
Pyr:	pyruvate fermentation medium (P, 60 mM)
Y:	yeast extract

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ABSTRACT

Although the enzymes for dissimilatory sulfate reduction by microbes have been studied, the mechanism for transcriptional regulation of the encoding genes remain unknown. In the work presented here, the model sulfate-reducing microbe *Desulfovibrio vulgaris* Hildenborough (DvH) was used to determine the role of Rex in sulfate reduction regulation. A deletion of the putative *rex* gene was made in DvH. the Rex mutant was assayed for growth with different combinations of electron donors and acceptors. Growth of the Rex mutant was less efficient on thiosulfate-containing medium. Here we propose a new model for thiosulfate reduction in SRB and examine a putative thiosulfate reductase. Additionally, transcript expression studies focused on *sat*, encoding sulfate adenylyl transferase, showed increased levels in the Rex mutant relative to the parental strain confirming Rex to be a repressor of *sat*. The putative Rex-binding site upstream of *sat* was also confirmed. We established *in vitro* that the presence of elevated NADH disrupted the interaction between Rex and DNA. these data support the role of Rex as a transcription repressor for *sat* that senses the redox status of the cell through NADH/NAD⁺.

Chapter 1: Introduction

Section 1. 1. Overview

Sulfate-reducing microbes (SRM) are challenged with a range of environmental stresses, such as nutrient availability or O₂ levels, and therefore need to be able to respond to these changes. One potential mechanism would be through a sensory regulatory pathway that can switch between growth modes (e.g. respiration and fermentation). Such a change in growth mode may be detected by a sensory protein that transduces the signal to alter gene expression and, subsequently, alter the functional activity of the cell. Many levels of regulation are involved in the complex process of energy conversion. This study focused on characterizing the recently annotated redox regulator, Rex, in the model anaerobic SRM *Desulfovibrio vulgaris* Hildenborough (DvH). The protein Rex senses changes in the NADH/NAD⁺ ratio in response to limitations in oxidative phosphorylation processes, and it controls the genes that code for the proteins responsible for these processes. Therefore, Rex_{DvH} is predicted to control sulfate reduction in SRM. This chapter focuses on the role of SRM in the environment, on gene regulation and on the putative regulator Rex.

Section 1. 2. Microorganisms capable of reducing sulfate

Bacteria have developed the capabilities to couple reductive processes to energy conversion through oxidative phosphorylation, with each substrate used providing a specific amount of energy. Examples of reductive processes include the following: CO₂ reduction to methane, sulfate reduction to sulfide, fumarate reduction to succinate, nitrate reduction to nitrite, nitrite reduction to N₂, and O₂ reduction to H₂O (Thauer et al. 1977). The energy gained from the anaerobic reduction of sulfate to sulfide ($\Delta G^{\circ} = -18.8$ kJ/mol) is low as compared to the reduction of other substrates, for example, nitrite reduction to nitrogen ($\Delta G^{\circ} = -132.6$ kJ/mol) (Thauer et al. 1977). SRM are anaerobic microorganisms (archaea and

bacteria) that are nearly ubiquitous in anoxic environments and use sulfate as a terminal electron acceptor for the degradation of organic compounds and energy conversion (Muyzer et al. 2008). SRM were first reported in 1895 (Beijerinck 1895); however, it was not until much later that their importance was realized. Sulfate reducers have been separated into two different groups based on their ability to oxidize organic compounds completely to carbon dioxide or incompletely to acetate (Muyzer et al. 2008). In either case, sulfate is dissimilated, or used for energy conversion and growth. This process is separate from the reduction of sulfate for assimilation of sulfur into macromolecules such as proteins. SRM are abundant in nature and considered to be important microbes in anaerobic carbon and sulfur cycles (Pfennig et al. 1982) and account for about half of the degradation of organic material in anoxic environments (Jorgensen 1977)

Although SRM are primarily found in sulfate-rich anoxic environments (Cypionka 2000), they are also abundant in habitats depleted of sulfate because they can use electron acceptors other than sulfate (e.g. sulfite or thiosulfate) (Thauer et al. 2007; Muyzer et al. 2008). The environments are as diverse as the substrates that they can utilize, such as sugars (Sass et al. 2002), amino acids (Baena et al. 1998) and one-carbon compounds (e.g. carbon monoxide (Parshina et al. 2005)). These microbes are associated with wetlands, wastewater treatment, environmental nutrient cycles, food spoilage, geochemical transformation, fuel production, animals, bioremediation and biocorrosion (Barton 1995).

It has been estimated that the annual cost of corrosion world-wide is \$2.2 trillion and is approximately 3% of the world's Gross Domestic Product (GDP) (Hays 2010). In 2001, corrosion was estimated to cost the U.S. more than \$600 billion, an equivalent of each citizen having to pay \$2000 each year (Bushman 2001). There have been advances made for corrosion control, including material selection, coatings, inhibitors, and cathodic protection

(Davis 2000); however, these all have their costs as well. The anaerobic process of microbially influenced corrosion is estimated to account for 15% of the total cost of ferrous metal and concrete/stonework corrosion (Enning et al. 2013). For the U.S. energy industry, this amounts to approximately \$100 billion yearly (Enning et al. 2013). Most notably, the metabolic processes of the SRM have been implicated in this observed corrosion and are, therefore, meaningful to study (Lee et al. 1995; Hamilton 2003).

Section 1. 3. Dissimilatory sulfate reduction

The process of dissimilatory sulfate reduction is carried out by a well-conserved biochemical pathway (Figure 1-1), which is part of a larger energy metabolism pathway of SRM (**Error! Reference source not found.**) (Peck et al. 1994; Barton et al. 2007; Thauer et al. 2007; Dahl et al. 2008; Muyzer et al. 2008; Pereira et al. 2011).

Figure 1-1: Simplified schematic of sulfate reduction. Sat, sulfate adenylyl transferase; ApsBA, APS reductase; DsrAB, dissimilatory sulfite reductase; QmoABC, quinone-interacting membrane bound oxidoreductase; DsrMKJOP, membrane portion of dissimilatory sulfite reductase; SO_4^{2-} , sulfate; APS, adenosine phosphosulfate; SO_3^{2-} , sulfite; S^{2-} , sulfide; OM, outer membrane, P, periplasm; PM, periplasmic membrane; C, cytoplasm

Figure 1-2: Main energy metabolism proteins in SRM.

Cytochrome c3 (TpI-c3), quinone reductase (QrcABCD), quinone-interacting membrane-bound oxidoreductase (QmoABC), APS reductase (ApsBA), sulfate adenylyl transferase (Sat), pyrophosphatase (PpaC), adenylate kinase (Adk), dissimilatory sulfite reductase (DsrAB, DsrMKJOP and DsrC), lactate dehydrogenase (Ldh), Lactate permeases (DVU2110, DVU2285, DVU2451, DVU2683, DVU3026, DVU3284), pyruvate oxidoreductase (Por), aldehyde:ferredoxin oxidoreductase (Aor), alcohol dehydrogenase (Adh), phosphate acetyltransferase (Pta), acetate kinase (Ack), pyruvate:formate lyase (Pfl), heterodisulfide reductase (Hdr-Flox), decaheme cytochrome c (DhcA) NADH:quinone oxidoreductase (RnfCDEAB), thiosulfate reductase (PhsAB), cytoplasmic hydrogenases (Hyn-1, Hyn-2, Hys, Hyd), periplasmic hydrogenases (CooMKLXUHAF, EchABCDEF), formate dehydrogenases (FdhAB, FdoH-FdnG-2, FdhABE-c3), high molecular weight cytochrome (HmcABCDEF), malate enzyme (ME), Fumarate reductase (FrdCAB), NADH:oxidoreductase (Nox), sulfate (SO_4^{2-}), adenylyl phosphosulfate (APS), sulfite (HSO_3^-), sulfide (HS^-).

The enzymatic equations and the free energy ($\Delta G^{o'}$) associated with these processes are shown below (Thauer 1989):

In brief, sulfate (SO_4^{2-}) is taken up by the cell through an active transport system and is activated by sulfate adenylyl transferase (Sat, encoded by *sat*) to adenosine phosphosulfate (APS) and inorganic pyrophosphate (PP_i). APS is then reduced to sulfite (SO_3^{2-}) and AMP by APS reductase (ApsBA, encoded by *apsBA*) as seen in equations [1] and [2], respectively. High specific activities of inorganic pyrophosphatase (PpaC, encoded by *ppaC*) and adenylate kinase (Adk, encoded by *adk*), equations [3] and [4], respectively, allow for this process to proceed in the forward direction. The reduction of sulfate requires an input of energy, as indicated by a positive free energy in equation [1]. Two molecules of ATP are required for each sulfate reduced, as seen when equations [1] to [4] are combined to yield equation [5].

As shown in equation [6], sulfite is further reduced to hydrogen sulfide (H_2S) by dissimilatory sulfite reductase (DsrAB, encoded by *dsrAB*). This is an exergonic reaction with enough free energy to drive the synthesis of two to three molecules of ATP, and thus overcome the initial energy demands.

Section 1. 4. **Redox control**

Significant alterations occur within a cell during the switch between respiration (i.e. oxidative phosphorylation) and fermentation (i.e. substrate-level phosphorylation) processes. Aerobes, when grown in the presence of oxygen, utilize many global regulators for controlling growth through changes in gene expression (Bauer et al. 1999). For example, transcription factors SoxR (Greenberg et al. 1990) or OxyR (Altuvia et al. 1994) regulate oxygen defense proteins, while other proteins (e.g. ArcB (Iuchi et al. 1988) and FNR (Sawers et al. 1988)) control the switch between growth modes (i.e. aerobic/respiration versus anaerobic/fermentation). Each of these “redox regulators” have specific sensitivities to oxygen and through these sensitivities provide for a fine-tuning mechanism to sense and respond to the presence (or absence) of oxygen or cellular redox state. Different mechanisms have evolved to sense the redox state of a cell, including iron-sulfur centers (e.g. SoxR or FNR), heme (e.g. FixL), flavin (e.g. NifL), disulfide bond formation (e.g. OxyR), and many others currently uncharacterized (e.g. RegB) (Bauer et al. 1999). More recently, pyridine nucleotides (specifically the ratio NADH/NAD^+), sensed by the transcriptional repressor Rex, have been shown to control for redox as well (Brekasis et al. 2003). In DvH, Rex has been identified (Ravcheev et al. 2012), although other proteins have also been examined for their role in sensing the environmental redox state including two methyl-accepting chemotaxis proteins (DcrA (Fu et al. 1994); DcrH (Xiong et al. 2000)). The validation of the role of pyridine nucleotides as a redox signal for Rex will be part of the work presented in this thesis.

Section 1. 5. Regulation of sulfate reduction in SRM

The process of dissimilatory sulfate reduction has been studied extensively. Although the enzymes for sulfate reduction have been well studied (Peck et al. 1994; Thauer et al. 2007; Dahl et al. 2008), and their crystal structures solved (Ullrich et al. 2001; Fritz et al. 2002; Oliveira et al. 2008; Schiffer et al. 2008), the mechanism for transcriptional regulation of the genes that code for these proteins remains unknown. In numerous SRM over 100 transcriptional regulators have been annotated within their genomes, with many regulators predicted to have the potential to regulate sulfate reduction, including: Rex (Ravcheev et al. 2012), HcpR (Rodionov et al. 2004), LysX and other DNA-binding proteins several of which are hypothetical proteins (Turkarslan et al. 2013).

Examination of mRNA transcription levels for several SRM reveal that many genes that code for proteins responsible for sulfate reduction are differentially expressed depending on the available nutrients (www.microbesonline.org) (Dehal et al. 2010). For example, *sat* and *apsB4*, which code for enzymes responsible for reducing sulfate to sulfite, have been shown to be altered in expression depending on the available electron acceptor (Wall et al. 2008; Zane et al. 2010). The work presented here will focus on the regulation of genes that code for proteins responsible for sulfate reduction.

Section 1. 6. Gene transcription regulation in bacteria

Transcription in bacteria is due to the RNA polymerase (RNAP) (Figure 1-3). The core enzyme, composed of $\beta\beta'\alpha_2\omega$ subunits, is capable of DNA-dependent RNA synthesis. However, transcription initiation at a specific promoter requires an additional subunit, σ . Therefore, the $\beta\beta'\alpha_2\omega\sigma$ holoenzyme is required for transcription initiation (Burgess et al. 1969). The principal or housekeeping σ factor is the σ^{70} and is responsible for most

transcription (Paget et al. 2003). It has been reported that on average there are several thousand RNAP core molecules, and greater than 1.3-fold more σ^{70} per cell (Grigorova et al. 2006). The number of holoenzymes per cell is in excess of the number of growing chains, and thus, there is a pool of polymerases not synthesizing RNA.

Figure 1-3: RNA polymerase holoenzyme ($\beta\beta'\alpha_2\omega\sigma$) positioning at a promoter. Transcript start site (TSS) marked by bent arrow. NTD, N-terminal domain; CTD, C-terminal domain. UP, upstream. Sigma subunit (σ) shown as four domains (σ^4 , σ^3 , σ^2 , σ^1). Figure adapted from Lee et al. (2012).

Transcript initiation occurs in three steps. First, the σ factor of the holoenzyme must recognize a promoter, although σ may interact with DNA independent of the rest of the holoenzyme, and it does so through its interactions with elements located upstream of a transcript start site (TSS). Secondly, RNAP forms an open complex, which allows for the template strand to be inserted into the active site of RNAP. Thirdly, there is transcription of the complementary RNA strand (Murakami et al. 2003). The efficiency of transcription is dependent on each of these steps, with the rate-limiting step determining the overall expression level. Typically, the initial binding of the RNAP holoenzyme to the promoter or the transition to the open state is the rate-limiting step (Rojo 1999).

The principal promoter elements recognized by RNAP include the UP (upstream) element, the -35 element, the extended -10 element, and the -10 element (Figure 1-3), although not all elements may be present for a given promoter. Each region is recognized by one of the four domains of the σ -subunit, and there is additional promoter recognition by the C-terminal domains of the two α -subunits (α CTDs) (Ebright et al. 1995; Gruber et al. 2003). An important component of the α subunit is the flexible unstructured linker between the N- and C-terminal domains that allows for regulation but has no role in the catalysis of RNA synthesis (Gourse et al. 2000). In most cases, the initial interaction of RNAP involves the UP and -35 elements with the downstream elements involved later.

Bacteria have developed numerous mechanisms to control gene expression ranging from initial promoter recognition through degradation of the protein. Genes can be turned on or off by alterations in RNAP, by DNA rearrangements, by a regulatory protein(s) or short RNA(s) affecting transcription initiation, or by modulation of the necessary components for initiation, elongation or termination (Rojo 1999). Additional regulation of the resulting transcript further controls the overall activity of a gene, including: mRNA and protein stability, translation efficiency, and protein turnover (Rojo 1999). A comprehensive list of bacterial transcriptional regulation is presented elsewhere (Lee et al. 2012).

Generally, repressors operate by binding to a specific promoter sequence in a way that would impede RNAP from binding or activating transcription. Multiple mechanisms for repressors have been observed. **1)** Overlapping binding sites for the repressor and RNAP. For example, Rex binds to the *cydP1* promoter (positions -6 to +26) and occludes binding of RNAP in *Streptomyces coelicolor* A3(2) (Brekasis et al. 2003). Another example is the well-studied LacI repressor, which binds to operator o_1 in the absence of lactose (Schlax et al. 1995). **2)** Selection or availability of σ factors to the core enzyme in the holoenzyme. An

example of this is the anti- σ factor, FlgM, which controls the accessibility of σ^{28} during flagellum development in *Salmonella enterica serovar typhimurium* (Chadsey et al. 1998). **3)**

Repressor binding site is located outside of RNAP binding. For example, CytR is a repressor that prevents the activation of *deoCABD* by blocking CRP activation in *Escherichia coli* (Valentin-Hansen et al. 1996). **4)** Mechanisms to control gene expression through other means may include: blocking the transition for the closed to open complex or inhibiting promoter clearance. An example of the former would include MerR, which locks RNAP in the closed complex at an operon that codes for proteins responsible for Hg^{2+} metabolism until a signal, Hg^{2+} , is present (Summers 1992).

Alternatively, activation typically occurs either by stabilizing the RNAP-promoter complex or by accelerating the switch to the open complex (Roy et al. 1998). There are multiple ways that an additional factor could influence these parameters. An activator may alter the conformation of the DNA to improve the quality of the promoter, or interact with RNAP to compensate for the poor quality of the promoter, or do both. Examples of activators that improve the interaction between DNA and RNAP include MerR and BmrR, which twist the DNA in order to reposition the -10 site and the TSS in the proper orientation with RNAP to allow transcription to proceed (Heldwein et al. 2001; Brown et al. 2003). Examples of activators that accelerate the switch to the open complex include CRP and FNR, which interact with the UP element and subsequently with αCTD to facilitate increased transcription (Wing et al. 1995; Busby et al. 1999). In this case, these activators act as “molecular velcro” by recruiting RNAP to the specific site within the DNA.

Section 1. 7. *Desulfovibrio vulgaris* Hildenborough (DvH)

To study gene regulation, and particularly those genes that code for proteins responsible for sulfate reduction, DvH (a model SRM) was used (Figure 1-4). DvH is a model organism for studying sulfate reduction as it is genetically accessible (Keller et al. 2009; Keller et al. 2011; Sim et al. 2013) with a genome that has been sequenced (Heidelberg et al. 2004) and more recently reannotated (Price et al. 2011). DvH has a chromosome consisting of 3,570,858 bp with 3,243 protein-coding genes; it also contains a plasmid with 202,301 bp with 158 protein-coding genes. Both the chromosome and the plasmid are GC-rich (>63%).

Figure 1-4: Scanning Electron Microscopy (SEM) of a single DvH cell.

Image provided by SR Fels with permission.

From the genome sequence of DvH, over 150 transcriptional regulators have been predicted (<http://networks.systemsbiology.net/dvh/search/advanced>) (Turkarslan et al. 2013). Interestingly, this anaerobe lacks most of the aerobic global regulators mentioned previously, although DvH does have FNR and Rex (Rex is confirmed by the work presented in this thesis). In DvH, four CRP/FNR-type global transcription regulators were annotated (Heidelberg et al. 2004) and recently characterized (Zhou et al. 2012). Each of the four homologs was determined to have distinct roles for DvH, including responding to stress caused by NaCl, chromate, nitrite or air. In DvH, Rex is predicted to regulate sulfate reduction (Ravcheev et al. 2012) and is the focus of the work presented here.

In DvH, genes that code for proteins responsible for sulfate reduction are differentially expressed depending on the available nutrients (<http://www.microbesonline.org/>) (Wall et al. 2008). For instance, *apsBA* expression was decreased in medium containing sulfite compared to expression in sulfate medium (Zane et al. 2010). Therefore, several of the predicted regulators, including Rex (Ravcheev et al. 2012), have the potential to be responsible for the observed expression changes.

DvH is a Gram-negative δ -proteobacterium that incompletely oxidizes organic compounds including lactate, pyruvate and formate (Heidelberg et al. 2004) to acetate. Although, DvH may also oxidize H_2 , when an additional carbon sources is available. Additionally, DvH is capable of utilizing several electron acceptors for energy conversion, including sulfate, sulfite and thiosulfate (Barton et al. 2007). Furthermore, DvH has been associated with heavy metal reduction, including uranium reduction (Lovley 1993; Wall et al. 2006), and has been used for the purpose of bioremediation of toxic metals in the environment (Goulhen et al. 2006). Because DvH can colonize concrete and ferrous piping used in the oil industry, which leads to corrosion and “souring” of the oil, this bacterium poses a substantial problem for the industrialized world (Beech et al. 2007). Therefore, by understanding better DvH and its metabolism, we can begin to control some of the environmental impacts and economic costs related to sulfate reduction.

Section 1. 8. The transcriptional redox regulator Rex

Comparative genome analyses have predicted the presence of Rex in a wide range of microbes, including Gram-negative and Gram-positive, aerobes and anaerobes. To date, Rex proteins have been studied experimentally in aerobes: *Thermus aquaticus* (Du et al. 1999; Sickmier et al. 2005; McLaughlin et al. 2010), *Streptomyces coelicolor* (Brekasis et al. 2003) *Bacillus*

subtilis (Schau et al. 2004; Larsson et al. 2005; Gyan et al. 2006; Wang et al. 2008; McLaughlin et al. 2010; Wang et al. 2011), *Staphylococcus aureus* (Pagels et al. 2010), *Streptococcus mutans* (Bitoun et al. 2011; Bitoun et al. 2012) and *Enterococcus faecalis* (Vesic et al. 2013); and the anaerobes *Thermatoga maritima* (Ravcheev et al. 2012), *Clostridium acetobutylicum* (Wietzke et al. 2012) and *Desulfovibrio alaskensis* G20 (Kuehl et al. 2014). This work will add to our understanding of the role of Rex in SRM.

Functional Rex proteins contain an N-terminal DNA-binding domain (winged helix), a dimerization domain (three-dimensional domain swap), and a C-terminal Rossman fold (Sickmier et al. 2005; McLaughlin et al. 2010) (Figure 1-5). The latter apparently functions to bind pyridine nucleotides (NADH and NAD⁺). The Rossman fold typically includes a Gly-X-Gly-X-X-Gly sequence that is part of a “P-loop”. It also contains a key acidic residue (Asp112 in *T. aquaticus*) that discriminates NAD(H) from their phosphorylated forms.

The consensus binding sequence for Rex, TTTGTGAAATATTTCACAAA, has been compiled from comparisons of over 100 genomes (Ravcheev et al. 2012). The sequence logo of the Rex DNA-binding site, specific to *Desulfovibrionales*, has been determined (Figure 1-6) (Novichkov et al. 2012). The sequence contains an inverted repeat (“GTG” and “CAC”, underlined), which is well-conserved as represented by bigger letters at positions 3-5 and 14-16. Rex functions as a homodimer, and the inverted repeat is the minimum sequence for Rex binding, as observed in *S. coelicolor* (Brekasis et al. 2003).

Figure 1-5: Crystal structure of Rex bound to DNA from *T. thermophiles* HB27. Adapted from McLaughlin et al. (2010) (pdb:3IKT). Rex monomers shown in blue (surface representation) and purple (new-cartoon representation); DNA helix in grey and orange (surface representation); and NAD⁺ in cyan (stick representation). Modelling done by Christensen GA with Visual Molecular Dynamics (VMD) software.

Figure 1-6: Sequence logo of the Rex DNA-binding site in *Desulfovibrionales*. Nine Rex regulators were analyzed from proteobacteria lineage. Figure adapted from Ravcheev et al. (2012).

Targets for Rex (i.e. the Rex regulon) typically include genes that code for proteins involved in NADH oxidation, and Rex acts as a transcriptional repressor when the NADH/NAD⁺ ratio is low (Gyan et al. 2006; Ravcheev et al. 2012). Rex from *T. aquaticus*, expressed in *E. coli*, was crystallized in the presence of NADH and NAD⁺, respectively, and the structures were solved (Sickmier et al. 2005; McLaughlin et al. 2010). From these *in vitro* assays it was clear that Rex underwent structural changes that modulated the binding activity between Rex and a consensus DNA sequence. Specifically, when Rex bound NADH, the resulting conformation of Rex was no longer able to interact within the major groove of DNA, and therefore, would no longer repress (McLaughlin et al. 2010) (Figure 1-7). Specific amino acids (R16, Y98 and D188 in *T. aquaticus*) have been shown to be responsible for this switch (McLaughlin et al. 2010) (Figure 1-8). By exchanging interactions between these select amino acids, which is modulated by the bound pyridine nucleotide (NADH or NAD⁺), a global reorientation occurs. These results were consistent with *in vitro* protein-DNA interaction assays performed on an upstream DNA sequence of *adhE2* in *Clostridium acetobutylicum*. In these assays, increased expression of *adhE2* was observed in a strain deleted for *rex* (Wietzke et al. 2012).

Figure 1-7: Binding activity of Rex is regulated by NAD(H). Rex homodimer binds to promoter sequence and represses downstream gene. Upon binding by NADH, which displaces NAD⁺, a conformational change occurs that prevents interaction between Rex and the DNA, allowing expression of the downstream gene.

Figure 1-8: Simplified schematic of Rex conformational switch. Adapted from McLaughlin et al. (2010). Monomers of the Rex homodimer are shown in purple and blue. Amino acids (1-letter designation) thought to be responsible for the shift in conformation are shown, with the bond linking the amino acids represented by a black line. (LEFT) Rex bound in the presence of NAD⁺ or (RIGHT) Rex unbound in the presence of NADH to DNA.

Examination of expression data in a SRM closely related to DvH, *D. alaskensis* G20, revealed increased transcript expression for *sat* in a strain deleted for *rex*; however, minimal differences were observed for *apsBA* and *dsrABD* (Kuehl et al. 2014). The increase in expression for *sat*, which codes for the first enzyme in the process of sulfate reduction, but not *apsBA* and *dsrABD*, provides support for the role of Rex as a transcriptional repressor for early steps in sulfate reduction. Interestingly, *dhcA-rnfCDGEABF*, which is predicted to be within the Rex regulon (Ravcheev et al. 2012), was reported to be decreased in the Rex mutant (Kuehl et al. 2014), although less than twofold. This was interpreted by the authors to mean that Rex may act not only as a repressor, but as an activator as well, although the authors also point out that this operon is predicted to be under complex regulation by other regulators as well. Therefore, these characteristics for Rex were considered in the examination of Rex in DvH.

Section 1. 9. Regulation of sulfate reduction genes by Rex_{DvH}

In DvH, DVU0916 (Rex_{DvH}) was predicted to code for a Rex protein and was hypothesized to have a role in the reduction of sulfate (Ravcheev et al. 2012). It was proposed that Rex_{DvH} regulated over 50 genes (from 14 separate operons) that code for proteins within the energy conversion pathway (Table 1-1), and the following in particular: *sat*, *apsBA*, *dsrABD*, *ppaC* and *adk*, all of which code for proteins that function within the cytoplasm to reduce sulfate to sulfide. Additionally, Rex_{DvH} was also predicted to regulate genes that code for many proteins involved in electron transfer within the periplasmic membrane, including the quinone reductase (*grcABCD*) and dissimilatory sulfite reductase (*dsrMKJOP*).

Table 1-1: Predicted targets and Rex_{DvH}-binding sites for Rex_{DvH}

Gene*	ID	Predicted Rex _{DvH} -Binding Sites
<i>atpI</i>	DVU0920	CTTGTGAACGATTGCACGAA
<i>grcA</i>	DORF6830	TTCGTGAAATATTTTCACCTT
<i>cooM</i>	DVU2286	ATTGGGAATCGATTTCACAAA
<i>dsrM</i>	DVU1292	TATGTGAAAAAATCATTTT
<i>dhcA</i>	DVU2791	CTTGTGAAATAATGTTCTTT
<i>adk</i>	DVU1932	CTCGTGAAATTAATGACAAG
<i>sat</i>	DVU1295	TTTGTAAATTTTTTCACAAG
<i>apsB</i>	DVU0846	ATTGTTAATTCATCACAAG
<i>ppaC</i>	DVU1636	ATTGTGCTATTTGGCACAAA
<i>lysB</i>	DVU1917	CGAGCTATATTTTCACAAA
<i>atpF1</i>	DVU0780	TTTGAGCTTTAATTCACAAC
<i>dsrA</i>	DVU0402	TTTGTCCAAAAAATCACGAG
<i>hupB</i>	DVU1795	TTTGGGAAAAAAGCGCAAG
<i>Null</i>	DVU2058	CTTGTGGTGTTTTACACAAA

* Only the first gene in the operon is displayed. Target list predicted from Ravcheev et al. (2012).

The properties described for Rex (i.e. a regulon containing genes that code for proteins responsible for energy conversion and a regulator that responds to the NADH/NAD⁺ ratio) would be those expected for a regulator of genes that code for proteins that function in sulfate reduction for SRM, as dissimilatory sulfate reduction is the major pathway for energy conversion. Therefore, a Rex homolog in DvH is a reasonable candidate regulator. By differentially binding NAD⁺ and NADH and de-repressing genes that code for proteins that oxidize NADH when sulfate reduction is slowed (i.e. NADH levels accumulate), Rex may maintain NADH/NAD⁺ levels by altering gene expression for those proteins that can restore steady-state respiration rates (Gyan et al. 2006). Using this paradigm, we proposed that Rex might be involved in the switch between respiration and fermentation in SRM and would be necessary for adaptation to fluctuating electron acceptor concentrations within the environment. Additionally, Rex may be responsible for directing electron flow between the cytoplasm and periplasm.

Section 1. 10. Project Aims

Bioinformatically confirm Rex in DvH:

DVU0916 was recently annotated as the redox sensor Rex based on amino acid sequence identity to known Rex proteins and consensus Rex-binding sites identified upstream of many genes that code for proteins responsible for sulfate reduction processes in DvH (Ravcheev et al. 2012). The amino acid sequence was therefore further analyzed through modelling techniques to justify pursuing the role of this regulator in sulfate reduction. This work is presented in Chapter 2, and the aims for this section are as follows:

- Compare protein sequence of putative Rex_{DvH} to characterized Rex proteins.
- Model (“thread”) Rex_{DvH} to characterized Rex proteins in multiple conformations.
- Distinguish Rex_{DvH} from other transcription regulators and pyridine nucleotide-dependent enzymes.
- Assess model quality (QMEAN4 and Global Model Quality Estimation [GMQE]) based on structural descriptors.

Determine the role of Rex_{DvH} on *sat* expression:

Rex is hypothesized to control, in part, the process of sulfate reduction in DvH. Sulfate adenylyl transferase (encoded by *sat*), which codes for the first enzyme in the sulfate reduction pathway, was analyzed for its regulation by Rex_{DvH}. This work is presented in Chapter 3, and the aims for this section are as follows:

- Construct a strain deleted for *rex* as well as several *sat* promoter mutation strains.
- Compare growth kinetics of constructed mutants to a parental strain.
- Restore phenotype of *rex* deletion strain by complementation.

- Construct and purify protein from an inducible overexpression strain for Rex_{DvH}.
- Confirm interaction between Rex_{DvH} and *sat* promoter with *in vitro* assays.
- Prove NADH, and no other pyridine nucleotide, modulates Rex_{DvH} activity.
- Confirm interaction between Rex_{DvH} and its predicted targets with a more high-throughput *in vitro* assay.
- Determine the transcription start site(s) for *sat* with *in vitro* assays.
- Elucidate *sat* expression among strains and growth conditions.
- Prove Rex_{DvH} represses *sat*.

Further characterize the function of Rex during growth:

The role of Rex has been shown to be important for sensing changes in the rate of respiration through the ratio NADH/NAD⁺, and controlling for gene expression to recycle the excess NADH that builds up during decreased rates of respiration (Sickmier et al. 2005). The Rex mutant was therefore examined for phenotypic differences in growth compared to a parental strain. In addition, expression differences for several genes (and proteins) responsible for sulfate reduction processes were examined. This work is presented in Chapter 4, and the aims for this section are as follows:

- Determine whole-genome gene fitness differences between the parental and Rex mutant strains for cultures respiring sulfate with lactate.
- Assess growth kinetics for the Rex mutant by pairing different electron donors/acceptors.
- Measure transcript (and protein) levels for select sulfate reduction genes (enzymes).
- Propose a model for the role of Rex in sulfate reduction, with consideration to the Rex mutant being impaired for growth when thiosulfate is the sole electron acceptor.

Chapter 2: Bioinformatic determination of Rex_{DvH}

Section 2. 1. Overview

The process of sulfate reduction is well documented, but the regulation of the genes that code for the proteins responsible for these functions is not well known. The protein encoded by DVU0916 is predicted, based on comparative genomic analyses, to be a transcriptional repressor, Rex (Ravcheev et al. 2012), which may control part of the process of sulfate reduction. Therefore, additional computational techniques were applied to the protein sequence of DVU0916 to determine if experimental characterization of this protein would be justifiable.

Section 2. 2. Sequence comparison

To examine the possible role of the protein encoded by DVU0916, the protein sequence was first compared to other known Rex proteins experimentally studied (Brekasis et al. 2003; Sickmier et al. 2005; Gyan et al. 2006; Ravcheev et al. 2012). DVU0916 shares greater than 30% protein identity with the most well-studied Rex homologs to date, including *T. aquaticus* (TT_C1293, 42% identity), *S. coelicolor* (SCO3320, 33% identity) and *B. subtilis* (BSU05970, 33% identity) (Table 2-1).

Table 2-1: Protein sequence identity among Rex homologs

	<i>T. aquaticus</i>	<i>S. coelicolor</i>	<i>B. subtilis</i>	DvH
<i>T. aquaticus</i>	ID	34.8%	33.7%	46.0%
<i>S. coelicolor</i>		ID	30.5%	33.3%
<i>B. subtilis</i>			ID	32.5%
DvH				ID

DVU0916 protein sequence was aligned to the Rex proteins discussed above (Figure 2-1). Examination of the aligned sequence revealed that the most important features, including the DNA recognition helix ($\alpha 3$), the conserved aspartate that is responsible for distinguishing NADH from NADPH, and the Gly-X-Gly-X-X-Gly sequence of the P-loop

that is common for pyridine nucleotide binding, are well conserved among the sequences, including DVU0916. Interestingly, two of three highly conserved sites within the protein thought to be responsible for the observed transition between a DNA-bound and unbound form (McLaughlin et al. 2010) were different for DvH. In the strains observed by McLaughlin et al. (2010), the amino acid (Aa) sequence is “RYD” (R16, Y98, D188, numbered for *T. aquaticus*); however, in DvH it is “QHD” (Q22, H104, D196, numbered for DvH). In fact, most sequenced strains of *Desulfovibrio* have the “QHD” triad (Figure 2-2). Therefore, DVU0916 would appear to be a Rex homolog, but the mechanism for its activity may be slightly different.

Figure 2-1: Protein sequence alignment of Rex proteins. Sequence identity (black) or similarity (grey) highlighted. Secondary structures are marked with rectangle (alpha helix) or arrow (beta sheet). The DNA-binding domain is in blue, with the ligand-binding domain (Rossmann fold) in purple. The conserved aspartate residue (D), which distinguishes NAD(H) from NADP(H), is shown with a red down-facing arrow. Figure adapted from Sickmier et al. (2005).

Figure 2-2: Sequence alignment of Rex homologs throughout *Desulfovibrionales*. Amino acid sequence alignment of the thirteen strains, with identity (black) or similarity (grey) highlighted. The position of the Aa's (1-letter designation, "RYD") that are significant for the conformational shift is marked.

Section 2. 3. **Modelling**

To further assess the likelihood of DVU0916 being a Rex protein, additional computational analyses, including protein structure homology-modelling, were performed. To that end, DVU0916 protein sequence was analyzed for its ability to fold (i.e. “thread”) to previously solved structures by SWISS-MODEL (Arnold et al. 2006; Guex et al. 2009; Kiefer et al. 2009; Biasini et al. 2014) (Table 2-2). The quality of the model was assessed by two scores: QMEAN4 and the Global Model Quality Estimation (GMQE). In brief, QMEAN4 is a composite score of four structural descriptors (C β interaction, all-atom pair-wise, solvation, and torsion angle energies) that are used to compare to scores calculated from a series of X-ray structures within the database, with acceptable values greater than -3.0. The GMQE is a quality estimation that includes the target-template alignment, and is expressed as a value ranging from 0-1, with higher numbers reflecting greater reliability and acceptable values greater than 0.75. Pairwise protein sequence alignments are performed by LALIGN server with BLOSUM50 algorithm (Goujon et al. 2010; McWilliam et al. 2013).

Section 2. 3. 1. Automated modelling

The protein sequence of DVU0916 was first automated to the SWISS-MODEL database to identify the “best-fit” template (Table 2-2). The resulting template was TTHA1657, an AT-rich DNA binding protein from *T. thermophilus* HB8 (pdb 2DT5, (Nakamura et al. 2007)) that shared 46% sequence identity across 201 Aa (QMEAN4=-1.84; GMQE=0.78). This protein, since its initial crystallization, has also been determined to be a Rex protein as part of a separate study (Ravcheev et al. 2012). Automated alignments are considered sufficiently reliable when the protein sequence identity between the template and target share >50% sequence identity, and as the two being examined share ~45% they should be scrutinized

more carefully. However, the QMEAN4 and GMQE scores are within the acceptable range. The fact that the best-fit model determined via automated modelling was a DNA-binding protein is strong evidence for the role of DVU0916 as a transcription factor. Interestingly, TTHA1657 crystallized with the ligand NAD⁺, and the molecules NAD⁺ and NADH are known to modulate the activity of Rex proteins. However, based on the model, part of the pyridine nucleotide binding-domain in DVU0916 is not conserved between the two sequences, specifically, β 5 (Figure 2-3). Therefore, DVU0916 was considered a potential transcription regulator, but may not sense pyridine nucleotides, and would need to be analyzed further.

Figure 2-3: DVU0916 protein sequence auto-aligned to pdb:2DT5.

Protein sequence alignment shown on left, with the modelled structure on the right. DVU0916 is threaded to each monomer, chains A and B of pdb:2DT5.

Estimated per-residue inaccuracies were visualized with a color gradient, with blue being more reliable and red being less reliable.

Table 2-2: Model quality assessment for DVU0916

Alignment	C β interaction energy	All-atom pairwise energy	Solvation energy	Torsion angle energy	QMEAN4	GMQE	Protein Sequence Identity	
Automated								
DVU0916 automated to pdb: 2DT5	0.20	-1.22	-2.19	-1.13	-1.84	0.78	P	46% across 201 Aa
Aligned								
DVU0916 to pdb:3IKT	0.30	-0.70	-2.52	-0.83	-1.72	0.77	P	46% across 201 Aa
DVU0916 to pdb:3IKT, TF domain	0.39	-0.20	-1.00	0.37	-0.03	0.78	P	50% across 66 Aa
DVU0916 to pdb:3IKT, ligand domain	-1.41	-2.17	-2.14	-1.98	-2.87	0.77	P	46% across 127 Aa
DVU0916 to pdb:1XCB	-0.83	-2.18	-4.02	-3.24	-4.68	0.78	F	46% across 201 Aa
DVU0916 to pdb:1XCB, TF domain	0.14	-0.48	-1.43	-1.58	-1.79	0.79	P	50% across 66 Aa
DVU0916 to pdb:1XCB, ligand domain	-2.48	-2.97	-3.57	-3.40	-4.92	0.77	F	46% across 127 Aa
DVU0916 to pdb:2ZCW, TF domain	-1.47	-0.98	-1.16	-3.72	-4.00	0.72	F	37% across 70 Aa
DVU0916 to pdb:3OIO, TF domain	-1.26	-1.62	-3.16	-3.94	-4.99	0.60	F	25% across 76 Aa
DVU0916 to pdb:WVO, TF domain	-1.69	-1.06	-2.42	-1.69	-3.10	0.44	F	23% across 56 Aa
DVU0916 to pdb:1BIA, TF domain	-1.08	-1.29	-1.40	-0.42	-1.30	0.40	F	28% across 48 Aa
DVU0916 to pdb:1GCU, ligand domain	-1.89	-2.45	-5.23	-2.25	-5.01	0.62	F	23% across 102 Aa
DVU0916 to pdb:1DRW, ligand domain	-4.62	-4.29	-4.51	-6.80	-9.01	0.64	F	31% across 121 Aa
DVU0916 to pdb:2OHX, ligand domain	-5.52	-4.36	-3.79	-3.51	-6.26	0.71	F	41% across 128 Aa
DVU0916 to pdb:1J8F, ligand domain	-4.18	-3.23	-4.37	-6.62	-8.96	0.71	F	39% across 87 Aa

P and F denote Pass or Fail, respectively, for the model generated.

Section 2. 3. 2. Aligned modelling

In order to compare how well DVU0916 protein resembled a characterized Rex protein, DVU0916 protein sequence was modelled to two unique conformations of the Rex protein from *T. aquaticus*. First, DVU0916 was compared to Rex co-crystallized with NAD⁺ in a conformation that allowed for interaction with DNA (pdb:3IKT) (McLaughlin et al. 2010)), and second, to Rex co-crystallized with NADH in a form that was unable to interact with DNA (pdb:1XCB) (Sickmier et al. 2005)) (Table 2-2). DVU0916 shared 46% protein identity across 201 Aa (6e-52) with Rex from *T. aquaticus*. Additionally, the amino terminal (N-term) DNA-binding (residues 1-81) and carboxy terminal (C-term) dimerization and pyridine dinucleotide (ligand)-binding domains (residues 82-214) of DVU0916 were aligned separately, in order to better refine the modelling for each domain (Table 2-2). Interestingly, DVU0916 modelled best to the DNA-bound form (pdb:3IKT), and less well to the unbound form (pdb:1XCB) (Figure 2-4).

Figure 2-4: DVU0916 protein sequence aligned to Rex from *T. aquaticus*. Protein sequence of DVU0916 threaded to each monomer of (left) DNA-bound (pdb: 3IKT) (McLaughlin et al. 2010) or (right) DNA unbound (pdb: 1XCB) (Sickmier et al. 2005) conformation of Rex. Estimated per-residue inaccuracies were visualized with a color gradient, with blue being more reliable and red being less reliable.

Examination of the quality scores for the individual domains revealed that the DNA-binding domain for each of the forms was satisfactory, but the C-terminal NAD(H)-binding domain for DVU0916 modelled to pdb:1XCB was the region that led to an overall poor quality score (Table 2-2). This was interpreted to mean that DVU0916 may interact with a ligand other than NADH, or that the conformational change mechanism may be slightly different for DVU0916 than for previously characterized Rex proteins. Further examination of the individual descriptors (i.e. C β interaction, all-atom pairwise, solvation, and torsion angle energies) used to assess quality revealed that the solvation and torsion angle energies caused the poor quality score for DVU0916 modelled to the DNA-unbound form in the presence of NADH. These results were interpreted to mean that DVU0916 may not fold in a similar manner as known Rex proteins, but that it is likely a transcription factor nonetheless.

In order to confirm DVU0916 as a Rex protein and not another regulator, the DVU0916 protein sequence was also modelled to other known transcription factors and NAD(H)-dependent enzymes. The list of proteins to examine was based on protein similarity to DVU0916, with a cut-off of 20% protein identity across 50 amino acids (Table 2-2). The shortened list included the transcription regulators: SdrP, a CRP/FNR-family regulator from *T. thermophilus* (pdb:2ZCW) (Agari et al. 2008); an AraC-type DNA-binding domain from *Chromobacterium violaceum* (pdb:3OIO) (in press), HET-S, a prion inhibitor from *Podospora anserina* (pdb:2WVO) (Greenwald et al. 2010), and a repressor of the biotin biosynthesis operon, BirA from *E. coli* (pdb:1BIA) (Wilson et al. 1992). Additionally, NAD(H)-dependent enzymes were also examined: BVR, biliverdin reductase from *Rattus norvegicus* (pdb:1GCU) (Kikuchi et al. 2001), dihydrodipicolinate reductase from *E. coli* (pdb:1DRW) (Reddy et al. 1996), LADH, liver alcohol dehydrogenase from *Equus caballus*

(pdb:2OHX) (Al-Karadaghi et al. 1994), and SirT2 histone deacetylase from *Homo sapiens* (pdb:1J8F) (Finnin et al. 2001). Each of the additional proteins examined failed the QMEAN4 cutoff requirements. Therefore, since DVU0916 fulfilled the QMEAN4 requirements for modelling to Rex, and to no other related functional proteins, i.e. transcription regulators or NAD(H)-dependent enzymes, DVU0916 was putatively considered to be Rex.

Section 2. 4. Discussion

The protein sequence for DVU0916 was compared to a database of solved protein structures, including the redox sensor Rex. DVU0916 shared the greatest identity (>30%) with previously characterized Rex proteins, and poorly (<10%) with most others, including the transcription repressor, LacI, involved in lactose metabolism. The high sequence identity for DVU0916 compared to Rex would suggest that the two are homologous (Rost 1999). Furthermore, all domains significant for Rex functionality were observed in the DVU0916 protein sequence, including: the DNA-recognition helix ($\alpha 3$), the P-loop important in the Rossman fold, and the conserved aspartate residue (Sickmier et al. 2005; Ravcheev et al. 2012; Wietzke et al. 2012) (Figure 2-1). Therefore, DVU0916 protein was predicted to interact with a similar DNA sequence as other characterized Rex proteins.

However, two differences were observed between DVU0916 protein and previously characterized Rex proteins. First, based on homology modelling, the unstructured wing domain (Gly-X-X-Gly-X-X-Gly-X-Gly) that follows the DNA-recognition helix ($\alpha 3$), which is essential for the interaction between Rex and the DNA, (Sickmier et al. 2005), was flagged as being “less reliable” (Figure 2-4). Note that this is separate from the Gly-rich sequence in the Rossman fold, which is conserved. Interestingly, the winged domain in the DNA-unbound form (DVU0916 modelled to pdb:1XCB) is far less reliable than in the DNA-

bound form (DVU0916 modelled to pdb:3IKT). Further examination of DVU0916 protein sequence within this region does indicate that it is variable; however, the overall motif (i.e. glycine-rich) is maintained. Additionally, this region is an unstructured flexible region, and the variability in sequence may have no effect on the activity of the protein. Regardless, as described in the next chapter, DVU0916 protein is capable of interacting with DNA and in a similar manner as for other characterized Rex proteins. The second difference observed for DVU0916 was in the Aa sequence, within the C-terminal domain, thought to be responsible for the conformational switch that occurs (McLaughlin et al. 2010). Among the thirteen sequenced *Desulfovibrio* strains, none of them contained the expected “RYD” sequence, but instead the sequence was “QHD” for twelve of the thirteen examined (Figure 2-2). This series of amino acids act as a switch, that alters protein structure in response to a particular signal, NAD(H) (McLaughlin et al. 2010). Because of these differences in sequence and mediocre quality scores for DVU0916 protein modelled to the NADH domain (Figure 2-4), we initially thought that DVU0916 protein may respond to a signal different from NAD(H). However, as described in the next chapter, NADH (and no other pyridine nucleotide that was examined) prevented DVU0916 protein interaction with DNA. Therefore, it appeared that alterations in protein structure occurred in the presence of NADH in DVU0916 protein, and these differences in sequence are likely conservative substitutions that do not affect the overall function.

After examination of DVU0916 protein sequence and modelling DVU0916 to known Rex proteins, DVU0916 protein is likely to be Rex, or at least a transcription factor. Hence, this protein is worth consideration for experimental studies and will be referred to as Rex_{DvH}. In the following chapters, Rex_{DvH} was characterized for its role as a redox regulator of sulfate reduction in DvH.

Chapter 3: Rex_{DVH} is a repressor of *sat* and its activity is modulated by NADH

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**Rex (encoded by DVU0916) in *Desulfovibrio vulgaris* Hildenborough is a repressor
of sulfate adenylyl transferase and is regulated by NADH**

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Section 3. 1. Overview

Rex is known to control gene expression for energy conversion processes. In DvH, DVU0916 encodes a potential Rex protein, Rex_{DvH}, which is predicted to control for the process of sulfate reduction (Ravcheev et al. 2012). In particular, Rex_{DvH} may be a trigger for the switch between substrate level and oxidative phosphorylation processes. In a closely related *Desulfovibrionales*, *D. alaskensis* G20, it was observed that in a strain deleted for *rex* the expression of *sat*—encoding the enzyme that activates sulfate for the first step in its reduction— was increased (Kuehl et al. 2014). Therefore, the focus of this chapter will be on the characterization of Rex_{DvH}, with an emphasis on how Rex_{DvH} controls *sat* expression. To accomplish this, growth studies comparing a Rex mutant to its parental strain cultured in media that allowed for respiration or fermentation were conducted, *sat* expression between the strains was analyzed, the transcription start site(s) for *sat* were determined, and the role of NAD(H) on Rex_{DvH}-binding to the *sat* promoter was examined. All results were consistent with the conclusion that Rex_{DvH} is a redox-responsive transcriptional repressor for *sat*, and is regulated by NADH.

Section 3. 2. *rex* appears to be monocistronic

DVU0916, predicted to encode *rex*, is annotated to be in a six gene operon (www.microbesonline.org) and this operon is also predicted to be controlled by Rex, potentially through feedback inhibition (Figure 3-1) (Ravcheev et al. 2012). This operon is on the negative strand. A predicted Rex_{DvH}-binding site is identified upstream of DVU0920 (Ravcheev et al. 2012). Recently, transcription start sites have been identified upstream of DVU0920, DVU0917 and *rex* within this operon (Price et al. 2011), which was interpreted to mean that this operon is actually three separate operons. Therefore *rex* is only expressed with

the downstream gene, DVU0915, which codes for a hypothetical protein. Therefore, with *rex* not being co-transcribed with the upstream operon, the expression of DVU0920-18 is presumed to be controlled by Rex_{DvH}, but not DVU0917 nor *rex*. Interestingly, Rex from both *B. subtilis* and *S. aureus* do not appear to have a Rex-binding site upstream of *rex* either.

Figure 3-1: RT-PCR confirms *rex* to be monocistronic. TOP) operon layout for *rex* (DVU0916) and the surrounding genes. TSS, transcription start site. PCR fragments for RT-PCR, B-F are labelled. The expected product sizes are: DVU0917 (222 bp, upstream gene, *atpE*), DVU0916 (407 bp, *rex*), DVU0915 (533 bp, downstream gene, hypothetical), DVU0917-16 (947 bp), DVU0916-0915 (1021 bp) and DVU0917-15 (1561 bp). Color coded based on new operon layout. BOTTOM) Agarose gels of RT-PCR products. M; 1kb PLUSTM DNA ladder (Gold Biotechnology®); 1, gDNA template; 2, water; 3, no reverse transcriptase control; 4, cDNA template.

To determine whether *rex* was expressed with the upstream and/or downstream genes DVU0917 and DVU0915 respectively, RT-PCR was performed on this region from a wild-type DvH culture grown by sulfate respiration with lactate (Figure 3-1). Primers were designed for each of the three genes (DVU0917, DVU0916 and DVU0915), as well as across *rex* and both of the two surrounding genes separately (DVU0917-DVU0916 and DVU0916-DVU0915). The results of the RT-PCR (50 cycles of a 100 ng cDNA template) revealed that *rex* was not transcribed with the upstream gene, as determined by bands for the single products but not the double, and no product was detected for the downstream gene DVU0915. Therefore, *rex* appears to be monocistronic. However, a more sensitive transcript analysis, qRT-PCR (data not shown), of DVU0915 revealed that DVU0915 is a gene, but expressed at a very low level. Additionally, DVU0915 is expressed approximately 15-fold lower than DVU0916. This could be explained by the 33 bp within the intergenic region between *rex* and DVU0915. Examination of this region did reveal potential hairpins as well as a ribosomal binding site that may influence the overall expression of DVU0915. Therefore, additional regulatory elements, which may be influenced by growth mode (i.e. respiration versus fermentation), in addition to the previously identified transcriptional start sites determine the overall operon structure for this region.

Section 3. 3. Deletion of *rex* increased *sat* expression

To examine the role of Rex_{DvH} , a marker-exchange deletion (MED) of *rex* and a complemented deletion strain were constructed (Korte et al. 2014). These two strains, in addition to the parental strain, were grown by sulfate respiration in MOLS4 or pyruvate fermentation in MOYPyr. The former was expected to differ from the latter by changes in the ratio of NADH/NAD^+ proposed to be a signal for Rex regulation. It was assumed that lacking an inorganic terminal electron acceptor, fermenting cultures would exhibit an

increase in NADH (Williamson et al. 1967). Examination of the growth in either medium revealed no significant differences among the three strains (Figure 3-2) (Table 3-1).

Figure 3-2: Growth studies with Rex marker-exchange deletion. Growth

of a parental DvH (JW710, black diamonds), Rex_{DvH} marker-exchange deletion (JW3311, purple squares) and complemented Rex (JW3311(pMO3313), grey triangle) strains by sulfate respiration with 60 mM lactate and 30 mM sulfate or pyruvate fermentation with 60 mM pyruvate supplemented with 0.1% (wt/vol) yeast extract and 0.5 mM cysteine.

In parental cells, qRT-PCR of *sat* expression (Table 3-1) (Table A-1) revealed that *sat* transcription had a sevenfold increase in fermenting cultures compared with cultures respiring sulfate. In the Rex_{DvH} mutant, *sat* expression was minimally increased when growing fermentatively but was already elevenfold increased from the parental strain while respiring sulfate, consistent with a repressor role for Rex. When this strain was complemented with a plasmid copy of *rex* that was transcribed from a constitutive promoter, partially restored (decreased) levels of *sat* transcription were observed. To eliminate the possibility that growth modes were affecting the expression of *rex*, transcription of that gene was examined and found to be unchanged in the parental strain and was undetected in the Rex_{DvH} mutant as expected. Therefore, these studies provided support that Rex_{DvH} is a transcriptional repressor for *sat*.

Table 3-1: Growth and transcript analysis of Rex marker-exchange deletion

Growth kinetics					Transcript ^a	
Strain	Name	Media	OD600 collected	Generation time (h)	<i>sat</i>	<i>rex</i>
JW710	Parental	MOLS4	0.25	6.5	1.0 ± 0.2	1.0 ± 0.0
JW3311	Δrex	MOLS4	0.22	5.8	11.2 ± 2.3	N.D.
JW3311 (pMO3313)	Complement of <i>rex</i>	MOLS4	0.22	6.2	5.9 ± 0.8	10.9 ± 0.0
JW710	Parental	MOYPyr	0.18	52	7.3 ± 1.2	1.7 ± 0.0
JW3311	Δrex	MOYPyr	0.17	47	13.6 ± 3.7	N.D.
JW3311 (pMO3313)	Complement of <i>rex</i>	MOYPyr	0.18	38	6.4 ± 0.6	24.9 ± 0.2

OD600 was monitored through growth and samples were collected for analysis by qRT-PCR at early-exponential. Approximately 100 ng of TURBO™ DNase-treated RNA was converted to cDNA and 1 μ L of cDNA (5 ng of RNA) was used per qRT-PCR. Each gene was assessed individually and normalized to reference genes *rplS* and *rpmC*. The efficiency for each gene was assessed: *rplS* = 92.6%, *rpmC* = 95.5%, *sat* = 91.3% and *rex* = 90.2%. Error determined as standard error of the mean. ^a Samples normalized to JW710 MOLS4. N.D. Not Detected.

Section 3. 4. Rex_{DvH} binds to the Rex_{DvH}-binding site upstream of *sat*

As the expression levels for *sat* were increased by the deletion of *rex*, we sought to determine whether the regulation was direct or indirect. There is a putative Rex binding site located at position -150 to -131 (T^{*}TTGTAAATT^{*}T^{*}TTTCACAAG) relative to the translational start codon for *sat* (Ravcheev et al. 2012). Therefore, Rex_{DvH} was purified for protein-DNA interaction studies (Figure 7-4). Four dsDNA fragments were examined for interaction with Rex_{DvH} (Figure 3-3): one upstream and one downstream of the predicted Rex binding site, and two of different sizes that contained the motif. The two fragments that contained the Rex-binding site (fragments C and D in (Figure 3-3) shifted in electrophoretic mobility when the putative Rex_{DvH} protein was present, while the other two fragments did not. These

results confirm a direct interaction between Rex_{DvH} protein and the putative Rex_{DvH}-binding site upstream of *sat*.

Figure 3-3: Rex_{DvH} interacts *in vitro* with *sat* promoter, shown by EMSA.

A) schematic representation of the *sat* promoter region approximately to scale.

The predicted Rex_{DvH}-binding site is annotated as a yellow oval. Fragments (A,

B, C, D) used in EMSA are shown with their position noted. Fragments A

(121 bp), B (130 bp), and D (271 bp) were PCR amplified, while fragment C

(40 bp) was generated by annealing two oligonucleotides. B) Native

polyacrylamide gel of individual DNA fragments (A, B, C, D; 0.1 nM, 1 nM

stock prior to column purification) without (-) or with (+) Rex_{DvH} (500 nM).

Equal concentration of DNA was labeled and then passed over a column to

separate the fragments from the rest of the components, i.e unlabeled

nucleotides. Fragment C, the smallest fragment is below the size cut-off of the

column (~100 bp) and so only a small amount of this fragment is actually

recovered compared with the other three larger fragments. Each fragment was

eluted in the same volume of buffer and so the concentration of this smaller

fragment is considerably lower than the rest. Therefore the band intensity for

fragment C is less than the others. The lowest band common in all lanes is the

dye front.

Section 3. 5. Transcript start sites for *sat*

With a physical interaction observed *in vitro* between Rex_{DvH} and the Rex_{DvH}-binding site upstream of *sat*, determination of the relative proximity of this motif to the transcript start site (TSS) for *sat* might provide a logical mechanism for regulation. This analysis could provide evidence that Rex_{DvH} repressed by occluding the polymerase from interaction with the promoter region of *sat*. Therefore, RNA samples were isolated from parental and Rex_{DvH} strains grown by sulfate respiration or pyruvate fermentation and assessed for the TSS of *sat*. To determine the 5'-end of the transcript, the Rapid Amplification of cDNA Ends (RACE) technique was applied (Figure 3-4). Examination of the TSS for the parental strain revealed two unique sites, one that was identified from cells growing by sulfate respiration at -122 bp relative to the assumed translational start codon and one from pyruvate-fermenting cells at -64 bp. A previous study performed by 5'-RNAseq analysis also identified position -122 bp as the TSS of *sat* for DvH grown by sulfate respiration (Price et al. 2011). The Rex_{DvH}-binding site (-150 to -131 bp) is less than 10 bp upstream of the 5'-end of the mRNA and therefore supports the occlusion of RNA polymerase binding for repression by Rex_{DvH} during respiration. Furthermore, a potential -35 site of a σ^{70} promoter was identified in close proximity to this region (Figure 3-4). Interestingly, examination of the TSS for the Rex_{DvH} mutant growing either by respiration or by fermentation revealed the same sites as those identified for the parental strain. Because two sites were observed and deletion of *rex* did not reveal a change, these results suggest that factors in addition to Rex_{DvH} are involved in determining the TSS.

Figure 3-4: Transcript start sites for *sat*. A) Schematic representation of the promoter region for *sat* with the position of the transcript start sites (TSSs) identified via 5'-RACE. Position of the Rex_{DvH}-binding site is highlighted in yellow. B) Chromatogram sequence reads of PCR2 fragments of the promoter region upstream of *sat* from a parental and Rex_{DvH} mutant strain grown by respiration in MOLS4 or pyruvate fermentation in MOYPyr. Arrow denotes the position of the poly-A tail added to the end of the sequence. The 5'-end of the sequence is the first base upstream of this poly-A tail. The reads are oriented in the reverse direction based on primer design. Color code for the nucleotide bases: black, G; blue, C; red, T; green, A. C). The Rex_{DvH}-binding site (highlighted in yellow) and the surrounding region is shown for the promoter sequence of *sat* of *Desulfovibrio* strains, with the predicted -35 site displayed ("TTGACA", highlighted in green). TSS (respiration) for DvH is highlighted in blue.

Section 3. 6. Effect of NADH concentrations on Rex_{DvH} function

Rex proteins contain a pyridine nucleotide binding domain that has been shown in other bacteria to interact with NAD⁺ or NADH and influence regulation (Sickmier et al. 2005; Pagels et al. 2010). To determine the role of pyridine nucleotide interaction with Rex_{DvH}, DNA-binding assays were performed with purified Rex_{DvH} protein in the presence of NAD⁺, NADH, NADP⁺ or NADPH at 0.1 or 1.0 mM concentration (Figure 3-5) and a DNA fragment containing the Rex_{DvH}-binding site (fragment C, Figure 3-3)). Addition of NAD⁺ appeared to have little effect on binding at either concentration compared with Rex_{DvH} and DNA alone; whereas, NADH disrupted the interaction at both concentrations tested. NADP⁺ did not appear to have any effect on the binding event, and NADPH disrupted binding only at high, presumably non-physiological, concentrations.

Figure 3-5: NADH disrupts interaction between Rex_{DvH} and the Rex_{DvH}-binding site. Electrophoretic assay demonstrating the effect of pyridine nucleotide on Rex_{DvH} binding. Fragment C (40 bp, including Rex_{DvH}-binding site upstream of *sat*) at 0.1 nM (1 nM prior to column purification) was incubated with Rex_{DvH} (500 nM) in the presence of a low or high concentration of specified pyridine nucleotide. Location of the DNA or protein-DNA complex is noted by arrows.

Section 3. 7. Essential nucleotides of the Rex-binding motif

To determine the key bp(s) recognized by Rex_{DvH} for binding, a number of mutations within the motif upstream of *sat* were altered and binding studies were performed (Table 3-2). The strategy for the base alterations was to make transitional mutations (i.e. purine to purine (A $\leftrightarrow$ G) or pyrimidine to pyrimidine (C $\leftrightarrow$ T)) for the most conserved bases of the predicted Rex_{DvH}-binding sites in DvH (see sequence logo, Table 3-2). From the JW9293 deletion strain (ΔP_{sat}) lacking the Rex motif, five additional strains were constructed: 1) a restored promoter sequence with the exact promoter region upstream of *sat* as the parental strain (restored P_{*sat*}), 2) a promoter with the -147 conserved “G” residue altered to an “A” (G -147 A), 3) a promoter with the distal inverted repeat “GTA” altered to “ACG” (-147 to -145) (IR1), 4) a promoter with the well-conserved proximal inverted repeat “CAC” altered to “TGT” (-135 to -133) (IR2), and 5) a promoter with mutations to both inverted repeat sequences in a single strain (IR1and2).

The mutants along with the restored promoter and parental strain were grown by sulfate respiration and pyruvate fermentation (Figure 3-6). Growth was similar for all strains with the exception of that deleted for the promoter (ΔP_{sat}), which was unable to grow by sulfate respiration and consistently grew slightly more efficiently by pyruvate fermentation.

Table 3-2: Alteration to the Rex_{DvH}-binding site within the *sat* promoter

Strain	Name	<i>sat</i> promoter sequence		EMSA Fragment	Estimated K _d (nM)

JW710	Parental	CGCTTGACATTTTGTAAATTTTTCACAAGACGGAATCAA...	(120 bps)...ATG	C	~100
JW9293	Δ <i>P_{sat}</i>	CGCTTGACAT (Δ <i>P_{sat}</i> -150 to -1)...	(1573 bp)...ATG	N.A.	N.A.
JW9312	Restored <i>P_{sat}</i>	CGCTTGACATTTTGTAAATTTTTCACAAGACGGAATCAA...	(120 bps)...ATG	N.A.	N.A.
JW9314	G -147 A	CGCTTGACATTTTATAAATTTTTCACAAGACGGAATCAA...	(120 bps)...ATG	C ^I	~500
JW9316	IR1	CGCTTGACATTTTACGAATTTTTCACAAGACGGAATCAA...	(120 bps)...ATG	C ^{II}	>2000
JW9318	IR2	CGCTTGACATTTTGTAAATTTTTCGTAAAGACGGAATCAA...	(120 bps)...ATG	C ^{III}	>2000
JW9320	IR1and2	CGCTTGACATTTTACGAATTTTTCGTAAAGACGGAATCAA...	(120 bps)...ATG	C ^{IV}	>2000

List of the strains and the alterations made to the Rex_{DvH}-binding site upstream of *sat*, with a sequence logo of the predicted Rex_{DvH}-binding site shown above. The Rex_{DvH}-binding site is underlined with the alterations made shown in red. Alignment of sequences are shown relative to the assumed translational start codon for *sat*. Fragments used for EMSA are displayed along with the estimated K_d (nM) for each 40-bp fragment. N.A. denotes not assessed. Strains: parental (JW710), *sat* promoter exchange deletion (ΔP_{sat}), restored promoter (P_{sat}), conserved “G-147” altered to “A” (G -147 A), distal inverted repeat “GTA” altered to “ACG” (IR1), proximal inverted repeat “CAC” altered to “TGT” (IR2), and alterations to both inverted repeat sites (IR1and2).

Figure 3-6: Growth kinetics of parental and *sat* promoter mutants.

Growth of three replicates of mutants and parental strain by A) sulfate respiration or B) pyruvate fermentation.

It was predicted that the modifications to the Rex_{DvH} -binding site might prevent Rex_{DvH} repression of *sat* and therefore increase expression of *sat*. Therefore, samples early in exponential growth were analyzed for *sat* and *rex* expression (Table 3-3) (Table A-2). All strains with sequence changes in the Rex_{DvH} -binding site had increased *sat* expression levels relative to the parental and restored strains.

Table 3-3: Transcript analysis of parental and Rex_{DvH} -binding site alteration strains

Strain	Name	Media	Transcript ^a	
			<i>sat</i>	<i>rex</i>
JW710	Parental	MOLS4	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1
JW9293	ΔP_{sat}	MOLS4	---	---
JW9312	Restored P_{sat}	MOLS4	0.9 $\pm$ 0.6	0.9 $\pm$ 0.5
JW9314	G -147 A	MOLS4	2.7 $\pm$ 0.2	1.2 $\pm$ 0.1
JW9316	IR1	MOLS4	1.8 $\pm$ 0.1	0.9 $\pm$ 0.1
JW9318	IR2	MOLS4	4.0 $\pm$ 0.3	1.0 $\pm$ 0.1
JW9320	IR1and2	MOLS4	4.6 $\pm$ 0.6	1.4 $\pm$ 0.3
Strain	Name	Media	Transcript ^b	
			<i>sat</i>	<i>rex</i>
JW710	Parental	MOYPyr	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1
JW9293	ΔP_{sat}	MOYPyr	N.D.	0.6 $\pm$ 0.1
JW9312	Restored P_{sat}	MOYPyr	1.0 $\pm$ 0.3	1.6 $\pm$ 0.7
JW9314	G -147 A	MOYPyr	2.9 $\pm$ 0.7	0.9 $\pm$ 0.2
JW9316	IR1	MOYPyr	3.7 $\pm$ 0.7	0.9 $\pm$ 0.1
JW9318	IR2	MOYPyr	2.8 $\pm$ 0.5	1.1 $\pm$ 0.1
JW9320	IR1and2	MOYPyr	2.3 $\pm$ 0.1	1.5 $\pm$ 0.1

OD600 was monitored through growth and samples were collected for analysis by qRT-PCR at early-exponential. Approximately 100 ng of TURBOTM DNase-treated RNA was converted to cDNA and 1 μ L of cDNA (5 ng of RNA) was used per qRT-PCR. Analysis of each gene was conducted separately for each medium tested. MOLS4, efficiency for genes: *rplS* = 91.8%, *rpmC* = 84.2%, *sat* = 92.5%, *rex* = 83.6%. MOYPyr, efficiency for genes: *rplS* = 90.7%, *rpmC* = 106.1%, *sat* = 97.7%, *rex* = 89.2%. Each gene was assessed individually and normalized to reference genes *rplS* and *rpmC*. JW9293 (ΔP_{sat}) was unable to be grown by sulfate respiration and therefore no data are provided. Error determined as standard error of the mean. ^a denotes normalized to sample JW710 MOLS4. ^b denotes normalized to sample JW710 MOYPyr. N.D. Not Detected.

dsDNA fragments of 40 bp containing the same alterations that were introduced into the genome were assayed for interactions with increasing Rex_{DvH} concentrations (0-2000 nM) (Figure 3-7). For three of the altered fragments tested, G -147 A, IR2 and IR1and2 (C^{I} , C^{III} and C^{IV}) no shift was observed (dissociation constant, $K_d > 2000$ nM). However, the fragment that contained the three base alteration to the distal inverted repeat sequence, IR1 (C^{II}), did shift ($K_d \sim 500$ nM) although not to the same extent as the wild-type sequence ($K_d \sim 100$ nM). This result was rather interesting because this sequence also contained the G -147 A alteration that appeared to completely disrupt the binding. As expected, no *sat* transcription was detected for the strain deleted for the *sat* promoter grown by pyruvate fermentation (Table 3-3). The expression of *rex* was also examined to verify that an unexpected decrease in Rex_{DvH} caused by a transcriptional change was not a factor for the observed differences in *sat* expression. Across the P_{sat} strains, *rex* expression was not significantly different. Therefore, alterations to the Rex_{DvH} -binding site within the promoter sequence of *sat* increased *sat* expression confirming the importance of the highly conserved base pairs in the motif.

Figure 3-7: Electrophoretic assay demonstrating Rex_{DvH} binding to consensus site. Rex_{DvH} bound to native (fragment C) and altered Rex_{DvH} -binding sites (C^{I} , C^{II} , C^{III} , C^{IV}). Rex_{DvH} was added with increasing concentration (0, 10, 100, 250, 500, 750, 1000 and 2000 nM) to each DNA fragment (0.1 nM, 1 nM prior to column purification). The estimated K_d is shown.

Section 3. 8. **Rex_{DvH} interacts *in vitro* with Rex_{DvH}-binding motif in DvH**

To explore potential Rex_{DvH} regulation of other target genes, a more high-throughput DNA-binding assay was employed, fluorescent polarization assay (FPA). Twelve operons with putative Rex motifs in their upstream regions, predicted at the time of this study (Ravcheev et al. 2012), were analyzed (Table 3-4). Exact 20-bp predicted Rex_{DvH}-binding sites were created with five “G’s” at the 5'-end to improve annealing and with four “G’s” with a “T” at the 3'-end to which the fluorophore would be attached. This approach takes advantage of the fact that the degree of polarization of a fluorophore is inversely related to its molecular rotation. Thus the change in fluorescence of a fast moving small unbound DNA fragment compared to a larger Rex_{DvH} bound-DNA fragment is evidence of protein-DNA interaction. By increasing the protein concentration over a range of values (0-1000 nM), a dissociation constant was determined for Rex_{DvH} with each 6-FAMTM labeled dsDNA fragment. Dissociation constants were determined, $K_d \sim 40-105$ nM, and were similar to previously published values for Rex, $K_d \sim 1-100$ nM (Wang et al. 2008; Ravcheev et al. 2012). The two techniques used in this study to calculate protein-DNA interaction between Rex_{DvH} and the Rex_{DvH}-binding site upstream of *sat* were similar (EMSA, $K_d \sim 100$ nM; FPA, $K_d \sim 90$ nM). In conclusion, Rex_{DvH} protein was determined to interact *in vitro* with all predicted Rex_{DvH}-binding sites and the calculated K_d values were similar.

Table 3-4: Rex_{DvH} interaction with predicted Rex_{DvH}-binding sites

Gene	ID	Predicted Rex _{DvH} -Binding Sites	"GTGAA"	"TTCAC"	K _d (nM) (40 bps, FPA)
<i>atpI</i>	DVU0920	CTT <u>GTGA</u> ACGATTGCACGAA	GTGAA	TGCAC	40
<i>qrcA</i>	DORF6830	TTC <u>GTGAA</u> ATATTTTCACCTT	GTGAA	TTCAC	55
<i>cooM</i>	DVU2286	ATT <u>GGGA</u> ATCGATTTCACAAA	GGGAA	TTCAC	55
<i>dsrM</i>	DVU1292	TATGTGAAAAAATCATTTT	GTGAA	ATCAT	55
<i>dhcA</i>	DVU2791	CTT <u>GTGAA</u> ATAATGTTCTTT	GTGAA	TGTTT	65
<i>adk</i>	DVU1932	CTC <u>GTGAA</u> ATTAATGACAAG	GTGAA	ATGAC	75
<i>sat</i>	DVU1295	TTTGTAAATTTTTTCACAAG	GTAAA	TTCAC	90
<i>apsB</i>	DVU0846	ATTGTTAATTCCATCACAAAG	GTTAA	ATCAC	90
<i>ppaC</i>	DVU1636	ATTGTGCTATTTGGCACAAA	GTGCT	GGCAC	100
<i>hysB</i>	DVU1917	CGAGCTATATATTTTCACAAA	GCTAT	TTCAC	100
<i>atpF1</i>	DVU0780	TTTGAGCTTTAATTCACAAC	GAGCT	TTCAC	100
<i>dsrA</i>	DVU0402	TTTGTCCAAAAAATCACGAG	GTCCA	ATCAC	105
<i>hupB</i>	DVU1795	TTTGGGAAAAAAGCGCAAG	GGGAA	AGCGC	*
<i>Null</i>	DVU2058	CTTGTGGTGTTTTTACACAAA	GTGGT	TACAC	*
Negative		TTATCAACTTAGTTTGATAT			>1000

List of target operons predicted to be regulated by Rex_{DvH} in DvH (i.e. the Rex regulon). The predicted Rex_{DvH}-binding sites are aligned across all sequences, with underlined region (inverted repeat) being the minimum sequence for Rex binding. Differences of the half-site from the consensus are marked in red. The calculated dissociation constant (K_d) for Rex_{DvH} and the predicted Rex_{DvH}-binding site, calculated by fluorescence polarization assay (FPA), are shown. * FPA was not performed on these sequences.

Section 3. 9. Discussion

The sulfate reduction gene *sat* has been shown to be altered in expression depending on the available electron acceptor (Wall et al. 2008). Bioinformatic predictions for the sulfate-reduction pathway, including co-expression studies of the genes as well as looking for conserved motifs upstream of these genes in multiple *Desulfovibrio* species, proposed regulatory contributions from Rex (encoded by DVU0916) (Ravcheev et al. 2012), HcpR (encoded by DVU2547) (Rodionov et al. 2004), LysX (encoded by DVU2567) and other DNA-binding proteins (encoded by DVU0057, DVU0744, DVU1142, DVU2275, DVU2690, DVU2799, and DVU2802) (Turkarslan et al. 2013). Preliminary examination of several of these potential regulators is discussed in Chapter 5; however, in this chapter, Rex_{DvH} was characterized for its role in *sat* expression.

Preliminary transcript studies examining *sat* expression of a Rex mutant in DvH had shown that the deletion of *rex* increased *sat* expression relative to a parental strain (data not shown). These findings are consistent with the role of Rex_{DvH} as a repressor of *sat*. This transcription factor has been proposed to be responsible for redox poise of the cell through the NADH/NAD⁺ ratio and to alter cellular metabolism to reestablish the pyridine nucleotide balance (Ravcheev et al. 2012). To confirm the role of Rex_{DvH}, the Rex mutant was cultured in two media proposed to alter the redox status of the cell, and *sat* transcription was measured (Table 3-1). Examination of the Rex mutant revealed increased *sat* transcripts compared to the parental strain for both growth modes. Furthermore, the differential increase in transcript levels observed in the absence of sulfate for the parental strain was not maintained in the Rex mutant. We interpreted these observations to mean that Rex_{DvH} is a transcriptional repressor for *sat* responding to redox status in the cell.

The *rex* gene encoded on a plasmid under a *npt* promoter was introduced into the Rex mutant to test restoration of *sat* repression. When this complementation construct was respiring sulfate, *sat* repression was only partially restored. However, *rex* was transcribed greater than tenfold higher than in the wild type (Table 3-1). Interestingly, for the fermenting culture the expression of *sat* was restored to wild-type levels. The increased transcription of *rex*, and therefore we assume the protein Rex_{DvH}, in the complemented strain might lead to higher than normal levels of repression of other targets of Rex_{DvH} not yet studied. This aberrant expression of *rex* may adversely affect the overall metabolism of the cell. However, a comparison of growth curves showed little difference in rate or extent of sulfate respiration by the parent, *rex* deletion and complemented strains under the conditions examined in this study.

Interestingly, there is a conserved hypothetical gene 33 bp downstream of *rex*, DVU0915, that was originally predicted to be in the same operon. However, the expression of DVU0915 is quite low and from a large battery of transcriptome data publically available for DvH (<http://www.microbesonline.org/>), these genes do not appear to be co-regulated. (Figure 3-1). Examination of the intergenic region revealed several strong hairpins that might function as transcriptional regulators consistent with separate operonal structure for the adjacent genes. Alternatively, the deletion of the DNA sequence of *rex* may have eliminated regulatory elements responsible for the proper expression of DVU0915. Preliminary studies examining DVU0915 expression in the three strains revealed that DVU0915 expression was indeed elevated in the Rex mutant and complemented strain compared with the parental strain (data not shown). Therefore, the increased abundance of DVU0915 may contribute to the inconsistencies in the expression of *sat* in the complemented strain, although no function is known for the protein encoded by this gene.

To date, it has been assumed that Rex_{DvH} blocks transcription by preventing the polymerase from binding to promoter DNA, limiting the expression of the downstream gene. Interestingly, based on the identified TSS for respiring cultures the -10 position should be within the Rex_{DvH} -binding site, specifically overlapping with the proximal inverted repeat (IR2). However, no conventional -10 site could be identified but a site similar to a classical -35 consensus (TTCACA) was apparent just upstream of the Rex_{DvH} -binding position. This was interpreted to mean that the RNA polymerase binding site of a respiring culture is within the Rex_{DvH} -binding site and therefore the proposed RNA polymerase occlusion mechanism for Rex seems plausible during respiration.

However, this RNA polymerase occlusion mechanism does not explain the second site of transcript initiation apparently functioning when DvH is grown by fermentation and even more *sat* transcript is produced. When sulfate becomes limiting and NADH is plentiful, increased Sat could possibly scavenge low sulfate as a terminal electron acceptor and/or provide reduced sulfur for cell biosynthesis. Preliminary work suggests that the levels of Sat may be linked to sulfate uptake (Zane et al., *in prep*). Therefore, it would be reasonable, when the preferred electron acceptor is limiting (e.g. sulfate), that the cell might increase the expression of a gene that encodes for a protein that may facilitate sulfate uptake. A second possibility is that there is an alternate regulator that recognizes a specific ligand (e.g. sulfate or sulfite) and the absence of this ligand is the signal for the selection of the second start site of transcription. It would seem plausible that sulfite, which at high concentrations is toxic to DvH, may be a ligand for controlling the overall expression of *sat* as Sat activates sulfate for reduction to sulfite. This latter interpretation might also explain why *sat* expression is relatively low when sulfite is the electron acceptor and much higher with sulfate.

It was predicted that the deletion of *rex* should resemble the state at which Rex should be removed from the promoter (i.e. fermentation); therefore, when the Rex_{DvH} mutant had the same TSS pattern as the parental strain it was clear that additional components are most certainly involved in addition to Rex, and that the order of addition of these other factors may be the mechanism for regulation and not just the presence or absence of Rex_{DvH}. Therefore, the mechanism proposed above, regarding another potential regulator that responds to sulfite levels, seems reasonable. Sulfite and not the protein Rex_{DvH} could be controlling the selection of the transcriptional start site. Overall, *sat* is still subject to transcriptional control by Rex_{DvH} in the fermentative condition since transcription is derepressed to even higher levels when Rex_{DvH} and pyruvate is being fermented.

Because *sat* expression was increased significantly as a result of a deletion of *rex*, we sought to examine the interaction of Rex_{DvH} with the promoter of *sat* (Table 3-2). To confirm the specific bases required for interaction, *in vitro* assays were performed on short DNA sequences of the promoter region. (Figure 3-7). All alterations constructed in the consensus binding site disrupted the interaction between Rex_{DvH} and the DNA *in vitro*. Interestingly, when a highly conserved base (G-147) was altered to an “A” there was a complete loss of detectable interaction (fragment C^I, G-147A, “GTA” altered to “ATA”); however, a triple mutation at IR1 (fragment C^{II}, “GTA” altered to “ACG”), which included the G-147A mutation, caused only a slight decrease in the interaction and not the complete loss that was observed for the single base mutation. Closer examination of the sequence revealed that the modified sequence for fragment C^{II} (“ACG”), for which a “G” is now in the third position, may still resemble the consensus site (“GTG”), but that the single base mutation does not. Therefore, a partially restored binding may occur, but only when the second half site, “CAC”, is present as in IR1 (fragment C^{II}). However, additional mutation

studies would be required to further characterize the specific contribution of each base on Rex_{DvH} binding.

Strains containing the altered promoter sequences discussed above were then constructed and grown by sulfate respiration or pyruvate fermentation and compared to the parental strain (Figure 3-6). Additionally, a strain that was deleted for 150 bp upstream of *sat* (ΔP_{sat}) was also assayed. As expected, *sat* expression increased for any mutation that limited Rex_{DvH} binding to the promoter region but was eliminated in the promoter deletion strain (Table 3-3). Interestingly, ΔP_{sat} grew to a higher cell density while fermenting pyruvate. This phenomenon has been observed for strains deleted for genes in the sulfate reduction step of the respiratory pathway in DvH (e.g. quinone-interacting membrane-bound oxidoreductase, *qmoABCD* [data not shown] or a tetraheme cytochrome *TpIc₃*, *gycA* (Keller et al. 2014)). The consistent increase in growth on pyruvate may result from a block in sulfate reduction that prevents flux through the activation step, functioning in the parental strain, that requires two ATP equivalents. Although 0.5 mM cysteine is the sulfur source during our growth experiments, it should be noted that nickel (2.3 μM) is added as a trace element with sulfate as a counterion in our preparation.

In a recent study (Ravcheev et al. 2012), Rex_{DvH} was predicted to interact with the promoter region for 12 operons, including many that encode proteins functioning in other steps of sulfate reduction in DvH (Table 3-4). Interestingly, most of the targets predicted to be regulated by Rex_{DvH} are not involved with NADH oxidation directly (except the Rnf complex) as they are for other bacteria (Ravcheev et al. 2012).

As this study examined only a subset of potential electron donors and acceptors for Rex_{DvH} regulation of *sat*, the effect of other substrates (e.g. pyruvate, formate, H_2 or sulfite, thiosulfate) might reveal additional features of Rex regulation. In fact, we recently reported

that the Rex_{DvH} mutant was inhibited for growth with thiosulfate as the terminal electron (Korte et al. 2014), an observation that deserves examination. Additionally, Rex_{DvH} has been predicted to regulate over 10 operons containing over 50 genes (Ravcheev et al. 2012). Recently, one of those operons, *dhcA-rnfCDGEABF*, has been shown to decrease in a strain deleted for *rex* in *D. alaskensis* G20 (Kuehl et al. 2014), suggesting Rex functions as an activator as well. Finally, over 150 potential regulators have been predicted in DvH, and should be considered either for their interaction with Rex_{DvH} or for their role in regulating genes that encode for sulfate reduction. Interestingly, the binding site for Rex_{DvH} is similar to several known FNR-family transcription factors that might compete with Rex_{DvH} for binding. Much additional work is needed to identify the transcriptional regulators that signal cellular nutrient and energy status that are integrated at the level of control of sulfate reduction. Several of these additional questions, including the examination of additional targets controlled by Rex_{DvH} , are the focus of the following chapter.

Chapter 4: Rex_{DvH} alters the electron flow between internal and external reduction processes in DvH

This chapter is currently *in prep*, for submission to *Applied and Environmental Microbiology*.

Rex_{DvH} alters the electron flow between internal and external reduction processes in *Desulfovibrio vulgaris* Hildenborough.

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Section 4. 1. Overview

Rex is a redox sensing protein that regulates the expression of genes that code for proteins responsible for energy conversion. Recently, a Rex regulator was confirmed in *Desulfovibrio vulgaris* Hildenborough (DvH) through bioinformatic techniques and many previously reported characteristics of Rex were confirmed experimentally in DvH. The work presented here examines the growth characteristics of a Rex mutant of DvH. First, whole-genome gene fitness was determined for a Rex mutant grown on minimal lactate/sulfate medium and compared to fitness data from the parental strain. A small number of genes differed in their contribution to the fitness of the strain deleted for *rex*. Additionally, growth kinetics of the Rex mutant in defined medium with several substrates compared with the parental strain revealed that the Rex mutant was impaired for growth in thiosulfate-containing medium. A strain deleted for one subunit of an annotated thiosulfate reductase, *phsA* was examined to determine its role in the process of thiosulfate reduction and any connection to Rex. A PhsA transposon insertion mutant grew on thiosulfate, suggesting that another thiosulfate reductase may be present. The growth phenotypes of the PhsA and Rex mutants were clearly different. Transcript expression for genes annotated as coding for proteins responsible for sulfate, sulfite, and thiosulfate reduction were determined. The genes that code for proteins responsible for the initial steps of sulfate reduction, *sat* and *apsA* were repressed by Rex, but the genes involved in sulfite or thiosulfate reduction (*dsrA* and *phsA*, respectively) were not. Although Rex has been shown to be a transcriptional repressor for sulfate reducing functions, it does not directly repress genes for thiosulfate reduction. Here, a model for Rex in thiosulfate reduction is proposed, suggesting that Rex controls genes responsible for the flow of electrons between the cytoplasm and the periplasm.

Section 4. 2. **Whole-genome gene fitness of a Rex_{DvH} mutant grown by lactate with sulfate respiration**

We sought to test for genes that might be changed in importance for growth of a Rex mutant when compared with the parental strain when grown in lactate/sulfate medium. A transposon mutant fitness profiling assay, Transposon Liquid Enrichment sequencing (TnLE-seq) was performed (Fels et al. 2013; Korte et al. 2014). This procedure generated random transposon mutations within the Rex mutant and parental strain for comparison. To that end, the two strains were grown in MOLS4 medium until the culture reached OD₆₀₀~0.4 and then deep sequencing was done to determine the abundance and location of each mutation (Figure 4-1) <http://desulfovibriomaps.biochem.missouri.edu/fitness/> (Figure 4-2). Gene fitness is shown by log₂R format, where R is the ratio of the number of insertions in a gene in the Rex mutant compared to the insertions in that gene in the parental strain. A positive R correlates to a fitness advantage and a negative R to a fitness defect. Of the >3000 genes within DvH, 638 were considered to be essential with fitness < -2.2, while in the Rex mutant 631 genes were considered essential. For both strains, as expected a gene that coded for an enzyme necessary for normal growth (e.g. translation, replication, sulfate reduction) had a high gene-fitness score, as observed by a mutant strain being less abundant in the population. A selected list of individual genes whose fitness was altered greater than twofold in the Rex mutant or were the focus of this study are presented in Table 4-1. The majority of the genes required for sulfate reduction were determined to be essential, including *sat*, *apsBA*, *qmoABCD*, *dsrABD* and *dsrC*. Minor fitness differences were observed on both the chromosome and native plasmid (pDV1). Fitness values for genes within operons typically clustered together.

Figure 4-1: Minimal difference in gene fitness between the Rex mutant and parental strain. Whole-genome gene fitness comparison of parental DvH (JW710, x-axis) and Rex mutant (JW3319, y-axis) cultures grown in defined lactate respiring medium with sulfate (MOLS4). Data are presented in Log₂R format, where R is the ratio of the number of insertions in a gene in the Rex mutant compared to the insertions in that gene in the parental strain. A positive R correlates to a fitness advantage and a negative R to a fitness defect. Refer to <http://desulfovibriomaps.biochem.missouri.edu/mutants/> and Table 4-1 for specific values. Genes different than the linear regression ($y = x$) are highlighted, color scheme highlights separate quadrants of fitness response between the parental and Rex mutant strains. Red and green (below the line) refer to genes when deleted in the Rex mutant compared to the parental are decreased in fitness (i.e. become more necessary), while yellow and blue (above the line) refer to genes when deleted had improved fitness in the Rex mutant compared with the parental (i.e. become less necessary).

Figure 4-2: Minimal difference in gene fitness. Whole-genome gene fitness comparison of parental DvH (JW710, x-axis) and Rex mutant (JW3319, y-axis) cultures grown in defined lactate respiring medium with sulfate (MOLS4). Axes are shown in standard non-log format. Parallel lines represent P value cutoffs for $P \leq 10^{-5}$. Axes are shown in $\log_{10}$ format. Outliers, in red, are DVU1028, DVU1672 and DVU3359. Image courtesy of Sam Fels.

Table 4-1: Gene fitness for parental strain and Rex mutant grown by sulfate respiration

DVU#	Gene Product Description	Gene Name	Fitness (Log ₂)		
			Parental	Rex mutant	Difference*
DVUA0072	Glycosyl transferase		-5.7	-4.9	0.8
DVUA0073	Asparagine synthase		-2.7	-0.8	1.9
DVUA0074	Sulfotransferase family protein		-0.8	-0.3	0.5
DVUA0075	Radical SAM domain protein		-2.3	-0.8	1.5
DVUA0076	ABC transporter, ATP-binding		-2.7	-0.8	1.9
DVUA0077	ABC transporter, permease protein		-2.8	-0.9	1.9
DVU0774	ATP synthase, F1 epsilon subunit	<i>atpC</i>	-3	-2.6	0.4
DVU0775	ATP synthase, F1 beta subunit	<i>atpD</i>	-2.8	-1.2	1.6
DVU0776	ATP synthase, F1 gamma subunit	<i>atpG</i>	-2.1	-0.9	1.2
DVU0777	ATP synthase, F1 alpha subunit	<i>atpA</i>	-1.8	-0.4	1.4
DVU0778	ATP synthase, F1 delta subunit	<i>atpH</i>	-2.6	-1.4	1.2
DVU0779	ATP synthase F0, B subunit	<i>atpF2</i>	-2.7	-1.4	1.3
DVU0780	ATP synthase F0, B subunit	<i>atpF1</i>	-1.9	-0.5	1.4
DVU0172	Thiosulfate reductase	<i>phsB</i>	0.9	0.1	-0.8
DVU0173	Thiosulfate reductase	<i>phsA</i>	0.5	-0.3	-0.8
DVU0269	Transcriptional regulator, Rrf2	<i>rrf2</i>	-0.7	-2.0	-1.3
DVU0402	Dissimilatory sulfite reductase	<i>dsrA</i>	-10.8	-9.6	1.2
DVU0403	Dissimilatory sulfite reductase	<i>dsrB</i>	N.D.	-8.4	N.A.
DVU0404	Dissimilatory sulfite reductase	<i>dsrD</i>	N.D.	-6.9	N.A.
DVU0531	High-molecular weight cytochrome c	<i>bmcF</i>	0.3	0.4	0.1
DVU0532	High-molecular weight protein	<i>bmcE</i>	0.6	0.1	-0.5
DVU0533	High-molecular weight protein	<i>bmcD</i>	0.3	0.3	0.0
DVU0534	High-molecular weight protein	<i>bmcC</i>	0.5	0.2	-0.3
DVU0535	High-molecular weight protein	<i>bmcB</i>	0.2	-0.1	-0.3
DVU0536	High-molecular weight protein	<i>bmcA</i>	0.6	0.4	-0.2
DVU0692	Quinone reductase	<i>qrcD</i>	1.0	-0.1	-1.1
DVU0693	Quinone reductase	<i>qrcC</i>	1.2	0.4	-0.8
DVU0694	Quinone reductase	<i>qrcB</i>	1.1	0.3	-0.8
DORF6830	Quinone reductase	<i>qrcA</i>	1.2	0.5	-0.7
DVU0846	Adenylyl-sulfate reductase, beta subunit	<i>apsB</i>	N.D.	-7.4	N.A.
DVU0847	Adenylyl-sulfate reductase, alpha subunit	<i>apsA</i>	-7.4	-8.2	-0.8
DVU0848	Quinone-interacting membrane-bound oxidoreductase	<i>qmoA</i>	-9.8	-7.6	1.6
DVU0849	Quinone-interacting membrane-bound oxidoreductase	<i>qmoB</i>	-8.5	-7.1	1.4
DVU0850	Quinone-interacting membrane-bound oxidoreductase	<i>qmoC</i>	-7.7	-7.7	0.0
DVU0851	Hypothetical protein	<i>qmoD</i>	-4.2	-9.0	-4.8

DVU#	Gene Product Description	Gene Name	Fitness (Log ₂)		
			Parental	Rex mutant	Difference*
DVU0917	↑ ATP synthase F0, C subunit	<i>atpE</i>	-6.0	-5.5	0.5
DVU0918	ATP synthase F0, A subunit	<i>atpB</i>	-6.3	-4.5	1.8
DVU0919	↑ Hypothetical protein	<i>atpI2</i>	-2.7	-3.4	-0.4
DVU0920	ATP synthase protein I	<i>atpI</i>	-1.5	-3.9	-2.4
DVU1028	Cytidylate kinase		-1.0	-3.8	-2.8
DVU1046	Hypothetical protein		-4.0	N.D.	N.A.
DVU1295	Sulfate adenylyltransferase	<i>sat</i>	-9.9	-7.0	2.9
DVU1297	Hypothetical protein		-0.8	0.7	1.5
DVU1336	ATP-dependent Clp protease	<i>clpX</i>	-0.9	-2.1	-1.2
DVU1393	Hypothetical protein		-2.1	-0.8	1.3
DVU1427	Response regulator		-1.9	-3.4	-1.5
DVU1672	Conserved hypothetical protein		-5.5	-1.6	3.9
DVU2008	Hypothetical protein		N.D.	-4.3	N.A.
DVU2134	Hypothetical protein		-3.8	N.D.	N.A.
DVU2259	Conserved hypothetical protein		-2.6	-1.1	1.5
DVU2377	Hypothetical protein		-0.1	1	1.1
DVU2776	Dissimilatory sulfite reductase, gamma subunit	<i>dsrC</i>	N.D.	N.D.	N.A.
DVU2870	Conserved hypothetical protein		-0.2	-1.1	-0.9
DVU3122	Hypothetical protein		-1.5	-5.5	-4.0
DVU3325	Hypothetical protein		-0.4	-1.5	-1.1
DVU3353	Phosphopantothencysteine decarboxylase	<i>coaBC</i>	-5.7	-0.6	5.1
DVU3356	NAD-dependent epimerase		-0.5	0.8	1.3
DVU3359	Hypothetical protein		-2.5	0.4	2.9
DVU3143	Iron-sulfur cluster-binding protein		0.8	0.6	-0.2
DVU3144	↓ Cytochrome c family protein		0.4	0.2	-0.2
DVU3145	↓ Hydrogenase, b-type cytochrome		1.0	0.9	-0.1
DORF25031	Hypothetical protein		-0.2	0.9	1.1

N.D. not detected. N.A. Not applicable. Arrow indicates orientation of operon. Gene fitness is shown by log₂R format, where R is the ratio of the number of insertions in a gene in the Rex mutant compared to the insertions in that gene in the parental strain. A positive R correlates to a fitness advantage and a negative R to a fitness defect. *For a gene to be considered different between the Rex mutant and parental strain a fitness difference greater than twofold was required and the gene must be defined as non-essential (Log₂R > 2.0).

Many of the fitness differences observed for genes between the two strains included hypotheticals (Table 4-1). However, genes that were annotated and had decreased fitness (were more important for growth and/or survival) in the Rex mutant compared to the parental strain included: the quinone reductase that is essential for the transfer of electrons from the periplasm to the cytoplasm to drive sulfate reduction (*qrABD*, DORF6830-DVU0694-2); a putative transcriptional regulator (*rrf2*, DVU0269) thought to play a role in redox signaling; a response regulator (*cheY*-like, DVU1427); an ATP-dependent Clp protease (*clpX*, DVU1336) responsible for protein degradation; and cytidylate kinase (*cmk*, DVU1028). Those genes that had increased fitness in the Rex mutant compared to the parental strain when deleted included: a putative ABC transporter (DVUA0072-77); the ATP synthase (*atpF1F2HAGDC*, DVU0780-774); a phosphopantothienoylcysteine decarboxylase (*coaBC*, DVU3353), and a NAD-dependent epimerase (DVU3356). However, most of these still have a negative fitness-value. The fitness values for genes predicted to be controlled by Rex are shown in Table 4-2. Only two operons, *atpF1F2HAGDC* and *qrABCD*, when deleted in the Rex mutant appeared to increase and decrease the fitness, respectively.

Table 4-2: Gene fitness for predicted Rex_{DvH} regulon

DVU#	Gene Product Description	Gene Name	Fitness (Log ₂)		
			Parental	Rex Mutant	Difference
DVU0402	Dissimilatory sulfite reductase	<i>dsrA</i>	-10.8	-9.6	1.2
DVU0403	Dissimilatory sulfite reductase	<i>dsrB</i>	N.D.	-8.4	N.A.
DVU0404	Dissimilatory sulfite reductase	<i>dsrD</i>	N.D.	-6.9	N.A.
DVU0692	Quinone reductase	<i>qrcD</i>	1.0	0.0	-1.0
DVU0693	Quinone reductase	<i>qrcC</i>	1.2	0.4	-0.8
DVU0694	Quinone reductase	<i>qrcB</i>	1.1	0.3	-0.8
DORF6830	Quinone reductase	<i>qrcA</i>	1.2	0.5	-0.7
DVU0774	ATP synthase, F1 epsilon subunit	<i>atpC</i>	-3.0	-2.6	0.4
DVU0775	ATP synthase, F1 beta subunit	<i>atpD</i>	-2.8	-1.2	1.6
DVU0776	ATP synthase, F1 gamma subunit	<i>atpG</i>	-2.1	-0.9	1.1
DVU0777	ATP synthase, F1 alpha subunit	<i>atpA</i>	-1.8	-0.4	1.4
DVU0778	ATP synthase, F1 delta subunit	<i>atpH</i>	-2.6	-1.4	1.2
DVU0779	ATP synthase F0, B subunit	<i>atpF2</i>	-2.7	-1.4	1.3
DVU0780	ATP synthase F0, B subunit	<i>atpF1</i>	-1.9	-0.5	1.4
DVU0846	Adenylyl-sulfate reductase	<i>apsB</i>	N.D.	-7.4	N.A.
DVU0847	Adenylyl-sulfate reductase	<i>apsA</i>	-7.4	-8.2	-0.8
DVU0848	Quinone-interacting membrane-bound oxidoreductase	<i>qmoA</i>	-9.8	-7.6	1.6
DVU0849	Quinone-interacting membrane-bound oxidoreductase	<i>qmoB</i>	-8.5	-7.1	1.4
DVU0850	Quinone-interacting membrane-bound oxidoreductase	<i>qmoC</i>	-7.7	-7.7	0.0
DVU0851	Hypothetical protein	<i>qmoD</i>	-4.2	-9.0	-4.8
#DVU0916	AT-rich DNA-binding	<i>rex</i>	-5.3	-6.5	-1.2
DVU0917	ATP synthase F0, C subunit	<i>atpE</i>	-6.0	-5.5	0.5
DVU0918	ATP synthase F0, A subunit	<i>atpB</i>	-6.3	-4.5	1.8
DVU0919	Hypothetical protein	<i>atpI2</i>	-2.7	-3.4	-0.4
DVU0920	ATP synthase protein I	<i>atpI</i>	-1.5	-3.9	-2.4
DVU1286	Integral membrane protein	<i>dsrP</i>	-11.0	-7.4	3.6
DVU1287	Periplasmic (Tat), binds 2[4Fe-4S]	<i>dsrO</i>	-9.9	-6.2	3.7
DVU1288	Periplasmic (Sec) triheme cytochrome c	<i>dsrJ</i>	-5.7	-5.2	0.5
DVU1289	Cytoplasmic, binds 2 [4Fe-4S]	<i>dsrK</i>	-7.3	-7.0	0.3
DVU1290	Inner membrane protein binds 2 heme b	<i>dsrM</i>	-8.8	-9.6	-0.8
DVU1291	Hypothetical protein		-5.2	-4.4	0.8
DVU1292	Hypothetical protein		-1.4	-1.5	-0.1
DVU1295	Sulfate adenylyltransferase	<i>sat</i>	-9.9	-7.0	2.9
DVU1636	Inorganic pyrophosphatase	<i>ppaC</i>	-7.8	-4.8	3.0
DVU1795	DNA-binding protein HU	<i>hup-3</i>	-0.7	0.0	0.7
DVU1917	Periplasmic [NiFeSe] hydrogenase	<i>hysB</i>	-0.2	0.0	0.2
DVU1918	Periplasmic [NiFeSe] hydrogenase	<i>hysA</i>	-0.3	-0.1	0.2
DVU1932	Adenylate kinase	<i>adk</i>	-9.2	-7.0	2.2
DVU2058	HDIG domain protein	<i>null</i>	0.5	0.5	0.0

DVU#	Gene Product Description	Gene Name	Fitness (Log ₂)		
			Parental	Rex Mutant	Difference
DVU2059	Glycosyl transferase, group 2 family protein		0.6	0.1	-0.5
DVU2286	Hydrogenase	<i>cooM</i>	-2.4	-2.0	0.4
DVU2287	Hydrogenase	<i>cooK</i>	-3.0	-2.5	0.5
DVU2288	Hydrogenase	<i>cooL</i>	-3.4	-3.5	-0.1
DVU2289	Hydrogenase	<i>cooX</i>	-2.7	-2.2	0.5
DVU2290	Hydrogenase	<i>cooU</i>	-2.9	-2.9	0.0
DVU2291	Carbon monoxide-induced hydrogenase	<i>cooH</i>	-3.4	-2.9	0.5
DVU2292	Hydrogenase nickel insertion protein HypA	<i>hypA</i>	-1.8	-1.7	0.1
DVU2293	Iron-sulfur protein	<i>cooF</i>	-2.9	-2.6	0.3
DVU2791	Decaheme cytochrome c	<i>dhcA</i>	0.5	0.6	0.1
DVU2792	NADH:quinone oxidoreductase	<i>rnjC</i>	0.4	0.5	0.1
DVU2793	Electron transport complex protein	<i>rnjD</i>	0.5	0.5	0.0
DVU2794	NADH:quinone oxidoreductase	<i>rnjG</i>	0.5	0.7	0.2
DVU2795	NADH:quinone oxidoreductase	<i>rnjE</i>	0.3	0.6	0.3
DVU2796	NADH:quinone oxidoreductase	<i>rnjA</i>	0.6	0.8	0.2
DVU2797	NADH:quinone oxidoreductase	<i>rnjB</i>	0.7	0.8	0.1
DVU2798	ApbE family protein		0.4	0.5	0.1

Rex regulon predictions from (Novichkov et al. 2012; Ravcheev et al. 2012; Christensen et al. 2014). N.D. not detected. N.A. Not applicable. #The presence of signal in *rex* in the Rex mutant is due to “bar-code bleed” (Kircher et al. 2010). Arrow indicates orientation of operon. *For a gene to be considered different between the Rex mutant and parental strain a fitness difference greater than twofold was required and the gene must be defined as non-essential (Log₂R >2.0).

Section 4. 3. **The Rex mutant is impaired for growth with thiosulfate as the sole electron acceptor**

A strain deleted for the *rex* gene has been shown to be minimally affected in growth when cultured by sulfate respiration with lactate in MOLS4 or by pyruvate fermentation in MOYPyr compared to the parental strain (Christensen et al. 2014). Similarly, the gene fitness profile for the Rex mutant while respiring sulfate with lactate was not significantly different from that of the parental strain. However, it was reported that the deletion of *rex* impairs growth with thiosulfate as the sole electron acceptor (Korte et al. 2014). To further characterize the role of *rex*, growth rates and extents of the Rex marker-less deletion mutant were determined on various substrates and compared to its parental strain (Figure 4-3) (Table 4-3). The cells were grown by respiration with lactate or pyruvate as the electron donor and sulfate, sulfite, or thiosulfate as the electron acceptor or by pyruvate fermentation. Cells grown by fermentation were expected to differ in their NADH/NAD⁺ ratio compared with those grown by respiration (Williamson et al. 1967).

Figure 4-3: Deletion of *rex*, and to an extent *phsA*, impair growth in thiosulfate. Two separate growth studies of (LEFT) parental DvH (JW710, black) and Rex mutant (JW3319, purple) or (RIGHT) wild-type DvH (DvH, black) and PhsA transposon insertion mutant (GZ8929, yellow) by lactate (60 mM) or pyruvate (60 mM) respiration with sulfate (SO₄²⁻, 30 mM), sulfite (SO₃²⁻, 40 mM) or thiosulfate (S₂O₃²⁻, 30 mM); or pyruvate fermentation (Pyr, 60 mM) supplemented with 0.1% (wt/vol) yeast extract and 0.5 mM cysteine. Error bars represent standard deviation among biological replicates (n=3).

Rex Mutant

PhsA Mutant

Rex Mutant

PhsA Mutant

Table 4-3: Sample collection and growth kinetics

Name	Media	Growth kinetics ^a			Total Protein ^b	
		OD600 collected ^c	Doubling time (h)	OD600 _{max}	µg/ml	µg/ml/OD600
JW710	MOLS4	0.29 ± 0.05	7.1 ± 0.2	0.91 ± 0.00	218 ± 21	240 ± 22
Rex mutant	MOLS4	0.24 ± 0.04	8.1 ± 0.5	0.93 ± 0.00	203 ± 12	227 ± 17
JW710	MOLS3	0.26 ± 0.04	4.2 ± 0.4	1.20 ± 0.03	294 ± 12	272 ± 10
Rex mutant	MOLS3	0.25 ± 0.09	5.3 ± 0.4	1.30 ± 0.03	300 ± 20	235 ± 22
JW710	MOLT3	0.30 ± 0.04	11.8 ± 0.4	0.67 ± 0.02	130 ± 14	207 ± 22
Rex mutant	MOLT3	0.09 ± 0.00	34.7 ± 13.5	0.10 ± 0.01	25 ± 3	245 ± 31
JW710	MOPS4	0.30 ± 0.07	9.1 ± 1.8	0.98 ± 0.02	303 ± 51	317 ± 53
Rex mutant	MOPS4	0.27 ± 0.07	8.4 ± 1.0	0.98 ± 0.01	253 ± 11	266 ± 11
JW710	MOPS3	0.35 ± 0.05	6.1 ± 0.1	1.20 ± 0.02	346 ± 7	303 ± 7
Rex mutant	MOPS3	0.36 ± 0.07	7.1 ± 0.0	1.10 ± 0.03	300 ± 16	283 ± 23
JW710	MOPT3	0.26 ± 0.03	7.7 ± 0.9	0.83 ± 0.01	196 ± 11	254 ± 10
Rex mutant	MOPT3	0.34 ± 0.02	13.0 ± 0.5	0.75 ± 0.01	169 ± 19	231 ± 24
JW710	MOYPyr	0.16 ± 0.01	40.8 ± 1.6	0.31 ± 0.01	90 ± 4	295 ± 7
Rex mutant	MOYPyr	0.19 ± 0.05	40.5 ± 5.6	0.31 ± 0.03	93 ± 2	307 ± 19
DvH	MOLS4	N.A.	6.2 ± 0.1	0.97 ± 0.03	182 ± 12	194 ± 16
PhsA mutant	MOLS4	N.A.	5.2 ± 0.4	1.00 ± 0.01	176 ± 11	190 ± 13
DvH	MOLS3	N.A.	5.7 ± 0.3	1.21 ± 0.03	236 ± 12	223 ± 18
PhsA mutant	MOLS3	N.A.	6.0 ± 0.0	1.26 ± 0.03	214 ± 7	210 ± 11
DvH	MOLT3	N.A.	5.8 ± 0.2	0.69 ± 0.09	122 ± 15	175 ± 10
PhsA mutant	MOLT3	N.A.	5.4 ± 0.6	0.64 ± 0.04	110 ± 8	172 ± 11
DvH	MOPS4	N.A.	6.2 ± 0.4	1.06 ± 0.04	240 ± 13	237 ± 14
PhsA mutant	MOPS4	N.A.	6.6 ± 1.3	1.00 ± 0.06	223 ± 7	220 ± 6
DvH	MOPS3	N.A.	7.8 ± 0.9	1.19 ± 0.02	241 ± 11	228 ± 14
PhsA mutant	MOPS3	N.A.	5.8 ± 0.3	1.22 ± 0.02	226 ± 19	218 ± 17
DvH	MOPT3	N.A.	3.3 ± 0.2	0.92 ± 0.01	132 ± 10	150 ± 12
PhsA mutant	MOPT3	N.A.	3.3 ± 0.3	0.86 ± 0.02	125 ± 9	142 ± 6
DvH	MOYPyr	N.A.	22.7 ± 0.2	0.46 ± 0.02	65 ± 16	200 ± 19
PhsA mutant	MOYPyr	N.A.	23.9 ± 1.8	0.46 ± 0.04	69 ± 10	196 ± 11

^a Error determined as standard deviation of biological replicates (n=3). ^b Total protein concentration determined for samples at OD600_{max} by Bradford assay (Bradford 1976), error determined as standard deviation of biological replicates (n=3). ^c 5-ml samples collected for qRT-PCR.* Because of low yield (no/minimal growth) for the Rex mutant on LT3, twice as much sample was required for analysis. Therefore, 2 5-ml aliquots were pooled and used to determine transcript (OD600 ~0.1) in triplicate. N.A. Not assayed.

These growth studies allowed several properties of Rex_{DvH} to be deduced: **1)** growth for most cultures was observed immediately after subculture (time points 0~10 h), resulting from carryover in the inoculum (2% vol/vol). **2)** The Rex mutant grew similarly to the parental strain in sulfate-containing medium regardless of the electron donor, although the strains grown with pyruvate compared with lactate were different from each other, i.e. lactate-grown cells had a diauxic growth pattern. **3)** The Rex mutant appeared to grow at a slower rate and had a longer lag period in sulfite-containing medium compared to the parental strain, regardless of the electron donor. Additionally, the ratio for either strain between the two media were quite similar (ratio = LS3/PS3 of the doubling times; parental: 4.2/6.1 ~0.7; Rex mutant: 5.3/7.1 ~ 0.7). This was interpreted to mean that sulfite, and not the electron donor, was affecting the growth of the Rex mutant. **4)** The Rex mutant in comparison to the parental strain grew at a slower rate when respiring thiosulfate with pyruvate, and did not grow with lactate as the electron donor. The maximum optical density (OD_{600max}) for the strains grown with thiosulfate was less than with sulfate or sulfite. **5)** The Rex mutant grew similarly as the parental strain when fermenting pyruvate.

Examination of the total protein as determined by Bradford assay (Bradford 1976) at the completion of the experiment (i.e. OD_{600max}) revealed two results: **1)** respiring cultures grown with pyruvate as the electron donor had a higher total protein per OD₆₀₀ as compared with lactate, and **2)** cultures grown by pyruvate fermentation had similar total protein production as cultures respiring sulfate or sulfite with pyruvate, but not thiosulfate (Table 4-3), confirming previous observations for a similar SRB, *D. alaskensis* G20 (personal communication with Barbara J. Rapp-Giles). Together, these findings support the importance of Rex in energy conversion pertaining to the available electron acceptor, but likely not the electron donor, and that Rex_{DvH} is involved in the metabolism of thiosulfate.

Section 4. 4. PhsA is not the only thiosulfate reductase in DvH

Because growth of the Rex mutant by respiration (lactate or pyruvate) with thiosulfate was impaired, additional examination of thiosulfate metabolism was necessary. The genome was examined for genes that code for proteins that may have a role in the reduction of thiosulfate. This query identified several candidates, including: thiosulfate reductase (encoded by DVU0173-2, *phsAB*) (Aketagawa et al. 1985), dissimilatory sulfite reductase (encoded by DVU0402-4, *dsrABD*), (Parey et al. 2010) along with DVU2776, *dsrC* (Venceslau et al. 2014); and a three gene operon (encoded by DVU3143-45) that may code for a second thiosulfate reductase based on protein sequence comparison to PhsABC from *Salmonella. enterica*. The role of *phsA* was examined for its role in the metabolism of thiosulfate reduction.

To begin to determine the role of PhsAB in thiosulfate reduction, a PhsA transposon mutant was grown on several substrates and compared to its parental strain, wild-type DvH (Figure 4-3) (Table 4-3). Examination of the wild-type strain grown on different substrates revealed several findings. **1)** DvH grown by sulfate respiration with lactate or pyruvate grew with similar rates to each other. Again, the strains grown with lactate, but not pyruvate, had a diauxic growth pattern. These findings were similar to what was observed for JW710 presented above. This was interpreted to mean that carryover from the inoculum provided additional substrates that prevented the immediate transition to the defined medium and is a technical, and not biological, phenomenon. **2)** The growth of the wild-type strain by sulfite respiration with lactate appeared typical (i.e. minimal lag prior to entering exponential growth phase, doubling time ~5-8 h, and complete growth within 24 h); however, when DvH was grown by sulfite respiration with pyruvate the growth rate for the wild-type strain

was slower than with lactate. Furthermore, the wild-type strain did not exhibit an extended lag-phase, as was observed for JW710 discussed previously. This may be likely due to the difference between the two strains. Specifically, JW710 is deleted for uracil phosphoribosyltransferase (*upp*), a pyrimidine scavenging protein, which may be more necessary when sulfite is provide at relatively high concentrations (40 mM). **3)** The growth of the wild-type strain when respiring thiosulfate with lactate was diauxic, as was observed with sulfate, although the growth phenotype between the sulfate- and sulfite-grown cultures are different. Furthermore, the wild-type strain grown by thiosulfate respiration with pyruvate did not experience a lag phase and grew robustly. Finally, the OD600_{max}, and thus cell number, for the wild-type samples grown with lactate were less for those strains grown with thiosulfate (OD600 ~ 0.7) compared to sulfate (OD600 ~ 1.0) or to sulfite (OD600 ~ 1.2); although when pyruvate was supplied instead of lactate the max OD600 is less distributed, with all being centered around OD600 ~ 1.0.

Next, the PhsA mutant was compared to the wild-type DvH (Figure 4-3) (Table 4-3). The *phsA::TnRL-27* mutant grew similarly to that of the wild-type DvH strain for all growth conditions tested, except when grown by thiosulfate respiration with lactate. When the PhsA mutant was grown in this manner it appeared to take an additional 15 h (i.e. 30 h time-point versus the 15 h time-point) before entering into exponential phase, although the growth rates for the two strains during exponential growth were similar. Additionally, the OD600_{max} between the two strains were similar among the media conditions. Overall, the Phs mutant was impaired in growth compared to the wild-type strain only when grown by thiosulfate respiration with lactate. However, the culture was still able to metabolize thiosulfate as seen by similar rates during exponential growth and OD600_{max}. Therefore, additional enzymes capable of utilizing thiosulfate for energy conversion exist in DvH. As expected, the deletion

of *pbsAB* and *rex* together in the JW710 parental strain did prevent growth when respiring thiosulfate with lactate (data not shown), as either deletion of *pbsA* (in DvH) or *rex* (in JW710) alone had impaired growth. Therefore, a target of Rex, in addition to PbsA, is essential for thiosulfate metabolism.

Section 4. 5. Rex controls, directly or indirectly, the expression of *sat*, *apsA* and *dsrC*

To determine the role of Re_{DvH} as a transcriptional regulator, the *rex* marker-less deletion and parental strain were grown by respiration or fermentation as described above and transcripts were assessed by qRT-PCR of early-exponential cultures (Table 4-4). Additional transcript analyses are presented in Table 4-5 and Table 4-6. The targets examined were *sat*, which had previously been shown to be directly regulated by Re_{DvH} (Christensen et al. 2014), and *apsA*, *dsrA*, *dsrC*, *rex*, and *pbsA*.

Table 4-4: Transcript analysis of Rex mutant in several different media

Media	Transcript					
	<i>sat</i>	<i>apsA</i>	<i>dsrA</i>	<i>rex</i>	<i>pbsA</i>	<i>dsrC</i>
MOLS4	4.1 ± 0.9	0.6 ± 0.1	0.6 ± 0.2	N.D.	0.5 ± 0.2	1.2 ± 0.2
MOLS3	31.7 ± 4.9	3.1 ± 0.3	0.9 ± 0.1	N.D.	0.2 ± 0.0	0.7 ± 0.1
MOLT3	23.6 ± 5.0	4.6 ± 0.7	0.5 ± 0.1	N.D.	1.8 ± 0.3	0.5 ± 0.2
MOPS4	10.3 ± 3.6	1.2 ± 0.3	0.8 ± 0.3	N.D.	0.5 ± 0.2	0.8 ± 0.2
MOPS3	93.7 ± 12.0	10.1 ± 1.6	0.7 ± 0.2	N.D.	1.2 ± 0.1	0.7 ± 0.1
MOPT3	49.7 ± 8.6	4.1 ± 0.2	0.4 ± 0.1	N.D.	1.1 ± 0.1	0.4 ± 0.1
MOYPyr	1.4 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	N.D.	1.5 ± 0.2	0.5 ± 0.1

Error determined as standard error of the mean among triplicates. Blue and yellow highlight refers to those transcripts that have decreased or increased by greater than twofold relative to the parental strain, JW710, respectively. N.D. not detected.

Table 4-5: Transcript analysis of Rex mutant and parental strain

Name	Media	Mean Cq ^a			
		<i>sat</i>	<i>apsA</i>	<i>dsrA</i>	<i>rex</i>
JW710	MOLS4	20.42 ± 0.13	21.70 ± 0.10	20.26 ± 0.05	21.90 ± 0.12
Rex mutant	MOLS4	18.68 ± 0.21	22.80 ± 0.18	20.97 ± 0.25	>40
JW710	MOLS3	23.06 ± 0.09	24.58 ± 0.09	19.95 ± 0.03	21.90 ± 0.08
Δ <i>rex</i>	MOLS3	18.33 ± 0.17	22.58 ± 0.09	19.90 ± 0.17	>40
JW710	MOLT3	22.26 ± 0.12	23.65 ± 0.10	19.72 ± 0.30	21.50 ± 0.21
Rex mutant	MOLT3	20.30 ± 0.24	23.84 ± 0.15	22.73 ± 0.14	>40
JW710	MOPS4	21.69 ± 0.17	22.48 ± 0.13	20.45 ± 0.27	21.57 ± 0.25
Rex mutant	MOPS4	18.55 ± 0.42	22.09 ± 0.37	20.63 ± 0.47	>40
JW710	MOPS3	23.41 ± 0.15	24.66 ± 0.04	18.73 ± 0.12	20.83 ± 0.13
Rex mutant	MOPS3	18.49 ± 0.06	22.29 ± 0.18	20.21 ± 0.32	>40
JW710	MOPT3	23.56 ± 0.17	24.17 ± 0.09	20.70 ± 0.10	22.11 ± 0.19
Rex mutant	MOPT3	18.93 ± 0.21	22.56 ± 0.05	22.39 ± 0.26	>40
JW710	MOYPyr	20.48 ± 0.45	21.51 ± 0.38	22.71 ± 0.46	24.01 ± 0.43
Rex mutant	MOYPyr	21.24 ± 0.03	23.91 ± 0.22	24.70 ± 0.04	>40

		<i>phsA</i>	<i>dsrC</i>	reference genes	
				<i>rplS</i>	<i>rpmC</i>
JW710	MOLS4	27.19 ± 0.25	17.08 ± 0.25	19.97 ± 0.32	22.62 ± 0.10
Rex mutant	MOLS4	28.07 ± 0.30	16.96 ± 0.18	20.59 ± 0.28	22.30 ± 0.39
JW710	MOLS3	25.88 ± 0.07	16.38 ± 0.10	20.10 ± 0.17	21.85 ± 0.08
Rex mutant	MOLS3	27.81 ± 0.11	16.77 ± 0.22	19.96 ± 0.05	21.41 ± 0.27
JW710	MOLT3	26.73 ± 0.11	15.60 ± 0.19	20.14 ± 0.31	22.51 ± 0.49
Rex mutant	MOLT3	28.13 ± 0.13	18.85 ± 0.38	23.55 ± 0.32	24.53 ± 0.11
JW710	MOPS4	26.93 ± 0.19	16.25 ± 0.24	19.53 ± 0.11	22.61 ± 0.03
Rex mutant	MOPS4	27.71 ± 0.39	16.50 ± 0.32	19.42 ± 0.33	22.50 ± 0.29
JW710	MOPS3	27.18 ± 0.11	15.41 ± 0.13	18.77 ± 0.06	20.41 ± 0.43
Rex mutant	MOPS3	27.92 ± 0.05	17.21 ± 0.11	19.77 ± 0.12	21.91 ± 0.32
JW710	MOPT3	27.57 ± 0.10	15.25 ± 0.19	20.70 ± 0.15	23.14 ± 0.11
Rex mutant	MOPT3	27.95 ± 0.08	17.23 ± 0.24	20.97 ± 0.07	23.99 ± 0.10
JW710	MOYPyr	28.55 ± 0.55	18.71 ± 0.43	24.17 ± 0.43	24.65 ± 0.57
Rex mutant	MOYPyr	29.23 ± 0.16	20.82 ± 0.01	25.64 ± 0.34	26.14 ± 0.15

^a Critical threshold (Cq) for specified gene, with technical error determined as standard error of the mean (n=3).

Table 4-6: Transcript analysis of parental strain, JW710, in several different media

Media	Transcript					
	<i>sat</i>	<i>apsA</i>	<i>dsrA</i>	<i>rex</i>	<i>pbsA</i>	<i>dsrC</i>
MOLS4	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.4	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.2
MOLS3	0.1 ± 0.0	0.1 ± 0.0	1.0 ± 0.1	0.8 ± 0.1	2.2 ± 0.2	1.3 ± 0.1
MOLT3	0.3 ± 0.1	0.3 ± 0.1	1.5 ± 0.5	1.4 ± 0.3	1.5 ± 0.3	2.7 ± 0.6
MOPS4	0.3 ± 0.0	0.5 ± 0.0	0.8 ± 0.2	1.1 ± 0.2	1.0 ± 0.2	1.5 ± 0.2
MOPS3	0.0 ± 0.0	0.1 ± 0.0	1.1 ± 0.2	0.8 ± 0.1	0.3 ± 0.0	1.0 ± 0.1
MOPT3	0.1 ± 0.0	0.3 ± 0.0	1.0 ± 0.1	1.2 ± 0.2	1.0 ± 0.1	4.8 ± 0.6
MOYPyr	6.4 ± 2.6	7.6 ± 2.5	1.0 ± 0.4	1.3 ± 0.5	2.4 ± 1.1	2.3 ± 0.8

Error determined as standard error of the mean. Blue and yellow highlight refers to those transcripts that have decreased or increased by at least twofold, respectively

Examination of the transcript expression of the specified genes of the parental strain revealed three general patterns (Table 4-6), whereby a twofold change was the cutoff requirement for a condition to be considered significantly different by analysis by qRT-PCR. Transcript abundance was normalized for each gene to the parental strain, JW710, grown by sulfate respiration with lactate. These data indicated a few trends: **1)** The expression of *sat* and *apsA* was decreased when respiring sulfite or thiosulfate with lactate regardless of the electron donor, confirming a previous study (Christensen et al. 2014). Additionally, JW710 cells respiring sulfate with pyruvate also had decreased expression. Furthermore, these genes increased in expression when JW710 was fermenting pyruvate. **2)** The expression of *dsrA*, *rex*, and *pbsA*, remained unchanged across all media conditions tested. Although *pbsA* transcript did appear to change in expression based on the fold-change differences for several media conditions, further examination of the critical threshold (Cq) revealed that this gene is expressed at very low levels, as evident by a high Cq (Cq >26) compared with the other genes assayed (Cq~15-25) in this study (Table 4-5). Therefore, *pbsA* is most likely

unchanged across all media conditions tested, or at least is present in low abundance in comparison to other sulfate reduction genes. **3)** The expression of *dsrC* increased in thiosulfate-containing medium, regardless of the electron donor, and while fermenting pyruvate.

The transcript expression results for each of the genes were compared between the Rex mutant and parental strain (Table 4-4). Examination of the expression data of the Rex mutant revealed unique responses for each of the genes assayed. As expected, *rex* was undetected in the strain deleted for *rex*. The expression of *sat* was increased in all respiring cultures compared with the parental strain. Additionally, *sat* was minimally increased when growing fermentatively but was already increased fourfold from the parental strain while respiring sulfate, consistent with a previous study (Christensen et al. 2014). The expression for *apsA* increased when respiring sulfite and thiosulfate with lactate or pyruvate when compared with the parental strain grown by sulfate respiration with lactate, but was unchanged with sulfate, regardless of the electron donor, and was also unchanged when grown fermentatively. The expression of *dsrA* and *pbsA* were unaffected by the deletion of *rex* for the conditions examined in this study. Lastly, the expression of *dsrC* appeared to decrease in the Rex mutant, or at least not increase, for all media, and was only significantly decreased for media that conferred a slowed rate of respiration within the cytoplasm (i.e. thiosulfate-containing or pyruvate fermentation media). Overall, the expression for *sat*, *apsA*, and *dsrC*, but not *dsrA* nor *pbsA*, were controlled (directly or indirectly) by Rex_{DvH} during growth on different substrates. Specifically, *sat* and *apsA* were shown to be repressed, while *dsrC* was activated by Rex_{DvH}. For several of these conditions protein abundance, of the encoding genes, had been assayed and determined to correlate reasonably well to the transcript data (Table 4-7).

Table 4-7: Protein analysis of parental strain and Rex mutant in several different

Strain	Media	OD600 collected ^a	Protein ^b					
			Sat		ApsA		DsrA	
			Peak Area (Avg.)	Peak Area (STD)	Peak Area (Avg.)	Peak Area (STD)	Peak Area (Avg.)	Peak Area (STD)
Parental	MOLS4	0.26 ± 0.07	125,602	45,644	141,372	54,081	53,401	17,584
Rex mutant	MOLS4	0.22 ± 0.04	474,204	24,911	106,628	7,287	44,401	1,580
Parental	MOLS3	0.25 ± 0.07	19,140	1,686	12,532	240	91,475	7,926
Rex mutant	MOLS3	0.24 ± 0.04	540,059	79,382	81,488	11,894	56,246	10,777
Parental	MOLT3	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Rex mutant	MOLT3	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Parental	MOPS4	0.22 ± 0.06	32,022	16,538	46,806	29,379	29,001	24,463
Rex mutant	MOPS4	0.25 ± 0.01	368,808	14,558	94,105	10,649	27,573	1,867
Parental	MOPS3	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Rex mutant	MOPS3	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Parental	MOPT3	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Rex mutant	MOPT3	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Parental	MOYPyr	0.13 ± 0.00	173,296	80,451	96,077	31,332	48,925	28,500
Rex mutant	MOYPyr	0.12 ± 0.00	469,933	375,822	52,569	36,988	35,319	29,186

Strain	Media	OD600 collected ^a	Rex		DsrC		RnfC	
			Peak Area (Avg.)	Peak Area (STD)	Peak Area (Avg.)	Peak Area (STD)	Peak Area (Avg.)	Peak Area (STD)
Parental	MOLS4	0.26 ± 0.07	1,776	611	78,178	29,978	2,465	704
Rex mutant	MOLS4	0.22 ± 0.04	46	40	38,534	2,090	743	22
Parental	MOLS3	0.25 ± 0.07	1,581	298	97,123	651	3,213	223
Rex mutant	MOLS3	0.24 ± 0.04	53	27	49,066	6,630	288	30
Parental	MOLT3	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Rex mutant	MOLT3	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Parental	MOPS4	0.22 ± 0.06	752	525	49,387	37,637	1,757	1,329
Rex mutant	MOPS4	0.25 ± 0.01	35	22	34,630	2,291	584	62
Parental	MOPS3	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Rex mutant	MOPS3	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Parental	MOPT3	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Rex mutant	MOPT3	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
Parental	MOYPyr	0.13 ± 0.00	1,226	589	40,428	24,207	443	243
Rex mutant	MOYPyr	0.12 ± 0.00	30	14	22,747	15,441	269	250

^a Error determined as standard deviation of biological replicates (n=3). PhsA was not determined. ^bProtein determined by selected-reaction monitoring, as described previously by Redding-Johanson et al. (2011). N.A. not assessed. STD standard deviation.

Section 4. 6. Discussion

In DvH, many of the genes that code for proteins responsible for dissimilatory sulfate reduction are known, and Rex_{DvH} is predicted to regulate several of these genes (Ravcheev et al. 2012). We previously characterized Rex_{DvH} for its role as a repressor of *sat* (Christensen et al. 2014). Here we confirm that Rex_{DvH} controls early steps in the process of sulfate reduction, an observation that was recently reported for Rex in a closely related sulfate reducer, *D. alaskensis* G20 (Kuehl et al. 2014). Specifically, Rex altered the expression of genes that code for proteins responsible for the activation and reduction of sulfate to sulfite (i.e. *sat* and *apsA*). Furthermore, the strain deleted for *rex* was impaired for growth in thiosulfate-containing medium.

To gain insight into what genes were necessary for sulfate reduction and how Rex may be involved, a whole-genome gene fitness study was performed on a parental DvH strain grown by sulfate respiration with lactate and compared to a strain deleted for *rex* (Table 4-1). Interestingly, the fitness value for only a small number of genes was different between the two strains and the differences observed were minor (typically less than twofold), which is in contrast to previous fitness studies that examined DvH under different stresses (e.g. minimal vs rich media (Fels et al. 2013) or nitrate stress (Korte et al. 2014)). This was interpreted to mean that the deletion of *rex* was not detrimental to growth by sulfate respiration with lactate. This result was expected as the deletion of *rex* had been shown to increase the expression of *sat* (Christensen et al. 2014) and presumably the capacity to reduce sulfate.

Further examination of the gene fitness results revealed that the quinone reductase operon (*qrvABCD*) had decreased fitness (i.e. became more negative) in the Rex mutant compared with the parental strain; although, these genes were considered to be non-essential

regardless of the strain (Table 4-1). QrcABCD is important for the proper metabolism of sulfate reduction with lactate in SRB as the deletion of *qrcABCD* appeared to be delayed for growth and have slightly lower growth rates compared to the parental strain (data not shown). Specifically, QrcABCD transfers electrons from the periplasm to the cytoplasm for sulfate reduction with the quinone-interacting membrane-bound oxidoreductase (QmoABC) as an intermediate (Keller et al. 2014) (Figure 4-4). However, electrons also feed into QmoABC via lactate oxidation through lactate dehydrogenase, thereby bypassing QrcABCD and allowing for sulfate reduction to proceed (Keller et al. 2014). For SRB, the deletion of *rex* increased expression of *qrcABCD* along with many of the genes that code for proteins responsible for the activation of sulfate to APS and the reduction of APS to sulfite, including *sat*, *qmoABCD*, and *apsBA*. (Christensen et al. 2014; Kuehl et al. 2014). Therefore, when either of the genes in the *qrcABCD* operon are also deleted in the Rex mutant strain the electrons are unable to be transferred from the periplasm to APS reductase. However, electrons can still feed into sulfate reduction via lactate oxidation. The reason for the *qrcABCD* deletion being more detrimental to the Rex mutant as compared to the parental strain is most likely due to the upregulation of *sat*, which would squander the ATP. Consequently, by increasing the sulfate activation enzyme, Sat, which is reversible, but not the enzyme responsible for transferring electrons generated within the periplasm to APS reduction, the cell is unable to reduce APS and instead APS converts back to sulfate and consumes an ATP molecule.

OM

OM

Figure 4-4: Sulfate reduction in DvH. Gene expression results for (TOP) DvH grown by thiosulfate respiration with lactate as compared to sulfate respiration with lactate presented in a new way from www.microbesonline.org (Dehal et al. 2010) and (BOTTOM) Rex mutant grown by respiration with sulfate as compared to parental strain (Kuehl et al. 2014). Thickness of arrows denote the presumed flux as determined by gene expression. Changes in gene expression by at least twofold between DvH grown by thiosulfate respiration compared to sulfate respiration with lactate are color coded: yellow (increase), blue (decrease), grey (unchanged). TpI-c₃, cytochrome c₃; QrcABCD, Quinone reductase complex; MQ, menaquinone; QmoABC, quinone-interacting membrane-bound oxidoreductase; ApsBA, adenylylsulfate reductase; DsrMKJOP, membrane portion of dissimilatory sulfite reductase; DsrAB and DsrC, dissimilatory sulfite reductase; Sat, sulfate adenylyltransferase; Ldh, lactate dehydrogenase; Por, pyruvate oxidoreductase; Pta, phosphate acetyltransferase; Ack, acetate kinase; Fd, ferredoxin; PhsAB, thiosulfate reductase; DhcA, decaheme cytochrome c; RrfCDEAB, NADH:quinone oxidoreductase; SO₄²⁻, sulfate; APS, adenosine phosphosulfate; SO₃²⁻, sulfite; S²⁻, sulfide; S₂O₃²⁻, thiosulfate; OM, outer membrane; P, periplasmic space; PM, periplasmic membrane; C, cytoplasm. Thiosulfate reductase is predicted to function within the periplasm in DvH as it does for *Archaeoglobus fulgidus* (Hocking et al. 2014) and *S. enterica* (Stoffels et al. 2012), although no transmembrane component is currently known to be associated with PhsAB in DvH.

In contrast to the *qrzABCD* operon, the deletion of any gene within the ATP synthase operon, *atpF1F2HAGDC*, increased the fitness (i.e. became more positive) in the Rex mutant compared to the parental strain, except *atpC*, although several fitness values were close to the cutoff of being considered essential (i.e. $\text{Log}_2 = -1.7$ -2.2) (Table 4-2). The *atpEB* operon, which encode F_o subunits A and C however, were still essential. Furthermore, transcript analyses of *atpF1F2HAGDC*, as performed by microarray, for DvH grown by sulfite or thiosulfate respiration with lactate were decreased in expression compared to when respiring sulfate with lactate (Dehal et al. 2010) (Table 4-8). The decreased gene expression while respiring most substrates was interpreted to mean that this operon is only essential at high concentrations when respiring sulfate with lactate. The deletion of *rex* in SRB (Table 4-4) has shown that genes that code for proteins necessary for the reduction of sulfate to sulfite and the synthesis of ATP (e.g. *sat*, *ppaC*, *adk*, *atpF1F2HAGDC*) are increased in expression. We interpret these findings to mean that genes that code for proteins responsible for ATP synthesis are overexpressed in the Rex mutant and that their increased abundance may disrupt sulfate reduction.

Table 4-8: DvH Transcript analysis as determined by Microarray

DVU	name	transcript*	Experiment#				
			389	1301	388	1737	7241
			LS3	LT3	PS4	PT3	Pyr
0172	↑ <i>phsB</i>	-13.63	-0.7	-1.7	0.1	-0.5	-0.6
0173	↑ <i>phsA</i>	-13.90	-0.3	-0.6	0.0	-0.4	N.A.
0384	<i>flr</i>	-13.15	-0.4	-0.7	-0.6	-0.4	0.7
0402	↓ <i>dsrA</i>	-9.33	-0.8	-1.3	0.2	0.2	0.9
0403	↓ <i>dsrB</i>	-9.46	-0.9	-1.2	-0.1	-0.7	0.9
0404	↓ <i>dsrD</i>	-8.76	0.3	0.0	0.7	0.7	0.1
0414	<i>tme</i>	-13.70	-0.8	-2.0	0.4	-0.9	0.4
0429	↑ <i>echF</i>	-14.95	-0.5	-0.3	0.4	0.9	1.7
0430	↑ <i>echE</i>	-9.46	N.A.	N.A.	N.A.	N.A.	N.A.
0431	↑ <i>echD</i>	-14.79	-0.5	-1.7	0.5	0.6	2.0
0432	↑ <i>echC</i>	-14.48	0.1	-1.8	0.7	0.3	2.0
0433	↑ <i>echB</i>	-14.98	-0.5	-1.1	0.4	0.4	2.4
0434	↑ <i>echA</i>	-13.99	-0.6	-0.5	0.2	-0.3	
0531	↑ <i>bmcF</i>	-15.55	0.1	0.8	0.6	1.2	3.6
0532	↑ <i>bmcE</i>	-15.19	-0.2	-0.2	0.3	0.5	3.1
0533	↑ <i>bmcD</i>	-15.62	-0.5	-0.6	0.2	0.3	2.4
0534	↑ <i>bmcC</i>	-14.72	-0.3	-1.5	0.4	-0.4	N.A.
0535	↑ <i>bmcB</i>	-14.86	-0.2	1.1	0.4	1.4	N.A.
0536	↑ <i>bmcA</i>	-15.09	0.1	0.7	0.4	1.9	N.A.
0587	↑ <i>fdbA</i>	-13.36	N.A.	N.A.	N.A.	N.A.	N.A.
0588	↑ <i>fdbB</i>	-13.92	1.1	0.5	1.6	1.8	-0.7
0600	<i>ldb</i>	-14.97	0.0	-0.3	0.7	-0.3	-0.3
0687	<i>aor-2</i>	-14.21	-0.5	-0.4	-0.1	-1.4	N.A.
0692	↑ <i>qrcD</i>	-12.54	-0.7	-0.9	-0.7	-0.9	1.1
0693	↑ <i>qrcC</i>	-12.55	-0.8	-1.5	-0.9	-0.3	2.1
0694	↑ <i>qrcB</i>	-12.59	-1.2	-2.2	-1.2	-0.1	1.0
DORF6830	↑ <i>qrcA</i>	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
0774	↑ <i>atpC</i>	-11.90	-0.9	-0.5	-0.8	-1.2	0.2
0775	↑ <i>atpD</i>	-12.14	-1.5	-1.9	-1.2	-1.1	0.3
0776	↑ <i>atpG</i>	-11.58	-1.3	-2.3	-0.8	-1.2	0.9
0777	↑ <i>atpA</i>	-11.33	-1.7	-3.1	-1.5	-1.0	0.8
0778	↑ <i>atpH</i>	-12.57	-1.2	-2.0	-0.9	-0.8	0.9
0779	↑ <i>atpF1</i>	-12.11	-1.4	-2.2	-1.0	-0.8	0.4
0780	↑ <i>atpF1</i>	-10.61	-0.1	-0.5	-0.7	-0.8	0.3
0819	<i>isf-1</i>	-13.09	-1.8	-1.2	-0.5	-0.3	1.3
0846	↓ <i>apsB</i>	-8.75	-1.4	-1.6	-0.4	-1.4	1.5
0847	↓ <i>apsA</i>	-8.93	-2.0	-2.4	-1.0	-1.6	1.2
0848	↓ <i>qmoA</i>	-11.01	-1.4	-2.3	-1.3	-3.3	0.5
0849	↓ <i>qmoB</i>	-12.03	-1.6	-1.8	-0.8	-2.1	1.0
0850	↓ <i>qmoC</i>	-12.82	-1.8	-2.7	-0.9	-1.0	1.3
0851	↓ <i>qmoD</i>	-12.10	-0.6	-0.6	-0.6	-1.4	1.1

DVU	name	transcript*	LS3	LT3	PS4	PT3	Pyr
1165	<i>ndb</i>	-14.97	-0.3	-0.3	0.2	-0.2	-0.1
1179	<i>aor</i>	-10.61	-0.6	-0.6	-0.2	-0.7	N.A.
1286	↑ <i>dsrP</i>	-11.59	N.A.	N.A.	N.A.	N.A.	0.9
1287	<i>dsrO</i>	-12.22	-0.8	-1.5	-0.3	0.3	1.1
1288	<i>dsrJ</i>	-12.23	N.A.	N.A.	N.A.	N.A.	1.0
1289	<i>dsrK</i>	-12.61	-1.1	-1.9	-0.7	-0.2	1.3
1290	↑ <i>dsrM</i>	-12.28	N.A.	N.A.	N.A.	N.A.	N.A.
1295	<i>sat</i>	-9.21	-1.4	-2.0	-2.2	-2.5	0.4
1636	<i>ppaC</i>	-11.13	-0.6	-1.2	-1.0	-1.1	1.1
1769	↓ <i>hydA</i>	-13.71	-0.7	-0.5	-0.4	0.1	0.6
1770	↓ <i>hydB</i>	-13.94	-0.3	-0.6	-0.3	0.1	N.A.
1917	↓ <i>hysB</i>	-11.77	-1.0	-2.0	-0.4	-0.5	-0.1
1918	↓ <i>hysA</i>	-11.22	N.A.	N.A.	N.A.	N.A.	N.A.
1921	↓ <i>hynB-1</i>	-12.91	-1.2	-2.0	-0.5	-2.2	1.1
1922	↓ <i>hynA-1</i>	-13.26	-1.5	-2.3	-0.6	-2.4	1.0
1932	<i>adk</i>	-9.25	-1.0	-0.3	-1.2	-1.6	0.4
1974	<i>ndb-like</i>	-13.43	0.7	1.0	1.2	-0.2	N.A.
2110	<i>b2975</i>	-14.50	0.8	0.0	-1.3	-2.3	N.A.
2271	↑ <i>pflA</i>	-15.41	-0.2	0.2	-0.4	0.9	0.9
2272	↑ <i>pflD</i>	-15.33	-1.0	-0.6	-1.2	0.6	N.A.
2285		-13.52	0.1	-0.9	-1.0	-0.1	-0.2
2286	↑ <i>cooM</i>	-12.35	-0.4	-1.4	-1.5	-1.6	-0.2
2287	<i>cooK</i>	-12.13	N.A.	N.A.	N.A.	N.A.	-0.3
2288	<i>cooL</i>	-11.96	-0.2	-1.8	-1.6	-1.5	-0.4
2289	<i>cooX</i>	-11.92	-0.6	-1.6	-1.5	-0.9	-0.4
2290	<i>cooU</i>	-11.55	-0.3	-1.6	-1.5	-1.4	-0.5
2291	<i>cooH</i>	-11.83	-0.1	-0.9	-1.5	-1.3	-0.9
2292	↓ <i>cooA</i>	-10.83	-0.3	-1.2	-1.0	-1.6	-0.3
2293	↓ <i>cooF</i>	-10.95	-0.3	-0.7	-0.8	-1.1	-0.7
2397	↑	-12.50	0.1	-1.7	0.5	0.0	0.4
2398		-12.24	-1.0	-2.1	0.2	-0.6	0.5
2399		-11.82	-1.2	-1.9	-0.5	-1.0	0.3
2400		-11.50	-0.9	-1.7	-0.5	-1.1	1.1
2401		-12.08	-0.6	-1.7	0.0	-0.6	0.2
2402	<i>bdrA</i>	-12.04	-1.2	1.7	-0.1	-1.3	0.6
2403	<i>bdrB</i>	-11.51	-1.1	-1.8	-0.2	-1.2	-0.4
2404	↑ <i>bdrC</i>	-11.17	-0.9	-1.7	0.2	-1.2	-0.6
2405	<i>adh</i>	-9.08	-0.4	-1.3	0.8	0.6	N.A.
2451		-12.96	0.0	-0.5	-4.0	-2.4	-0.7
2481	↑ <i>cfdB</i>	-13.00	-0.3	-1.2	0.2	-0.9	0.0
2482	↑ <i>cfdB</i>	-13.65	N.A.	N.A.	N.A.	N.A.	N.A.
DVU	name	transcript*	LS3	LT3	PS4	PT3	Pyr

2525	↓	<i>hynB-2</i>	-14.61	-1.2	-0.8	-0.5	-0.5	1.4
2526	↓	<i>hynA-2</i>	-14.09	-1.0	-0.1	-0.4	-0.8	0.6
2673	↓	<i>glpD</i>	-13.32	-0.1	-0.5	-0.2	0.3	0.5
2674	↓	<i>sdbB</i>	-13.92	-0.2	-1.4	0.0	-0.1	0.1
2683			-13.18	-0.9	-0.3	-1.4	-0.1	-0.7
2776		<i>dsrC</i>	-10.26	0.1	-0.4	-0.2	-0.3	N.A.
2783	↑		-13.49	0.8	0.4	-0.5	-1.4	-0.6
2784	↑	<i>lldD</i>	-13.58	0.5	-0.2	-0.6	-1.2	-0.9
2791	↓	<i>dbcA</i>	-12.62	0.2	-0.5	-0.3	-0.9	0.7
2792	↓	<i>rnfC</i>	-13.44	-0.8	-1.8	-0.2	-0.9	1.0
2793	↓	<i>rnfD</i>	-14.08	-0.3	-1.7	0.0	-0.6	0.9
2794	↓	<i>rnfG</i>	-13.70	-0.5	-1.2	-0.5	-0.7	0.8
2795	↓	<i>rnfR</i>	-14.68	-0.6	-1.7	-0.5	-0.8	0.4
2769	↓	<i>rnfA</i>	-14.40	-0.5	-1.4	-0.3	-0.2	0.6
2797	↓	<i>rnfB</i>	-13.60	-0.3	-0.9	0.0	-0.5	1.2
2798	↓	<i>apbE</i>	-13.29	0.0	-0.2	0.3	-0.3	0.6
2809	↑	<i>fdbC₃</i>	-15.16	0.0	0.2	0.2	1.2	-1.5
2810	↑	<i>fdbE</i>	-15.35	-0.2	0.3	0.0	0.7	-1.2
2811	↑	<i>fdbB</i>	-15.60	0.2	0.7	0.2	0.7	-2.0
2812	↑	<i>fdbA</i>	-15.40	N.A.	N.A.	N.A.	N.A.	-2.7
3025	↓	<i>por</i>	-10.82	-0.8	-2.1	-1.4	-1.3	0.5
3026	↓	<i>lldP</i>	-12.91	-1.4	-1.9	-2.9	-2.3	0.2
3027	↓	<i>glcD</i>	-11.66	-1.3	-2.4	-1.4	-1.4	0.8
3028	↓	<i>glpC</i>	-12.21	-1.2	-1.4	-1.4	-1.6	-0.3
3029	↓	<i>pta</i>	-11.85	-0.9	-2.9	-1.3	-1.7	-0.1
3030	↓	<i>ackA</i>	-11.86	-1.1	-2.7	-1.8	-1.3	0.3
3031	↓	<i>pta-like</i>	-11.76	-0.9	-2.6	-1.9	-2.2	0.0
3032	↓		-12.13	-1.6	-2.5	-1.4	-1.2	0.4
3033	↓		-12.18	-1.4	-2.1	-1.1	-0.7	N.A.
3035	↓		-13.35	-0.4	-0.7	-0.3	-1.1	0.0
3143	↓	<i>phsB</i>	-15.98	-1.2	-0.4	-0.7	2.9	2.2
3144	↓	<i>phsA</i>	-15.38	-0.7	-1.4	0.4	3.4	1.2
3145	↓	<i>PhsC</i>	-15.40	0.1	0.1	0.1	2.7	1.2
3171		TpI-c3		-0.3	-1.3	0.10	-1.4	1.3
3212		<i>nax</i>	-12.97	-1.6	-1.3	0.1	-0.5	0.2
3261	↓	<i>frdC</i>	-14.00	0.3	0.1	-0.5	-0.6	-0.6
3262	↓	<i>frdA</i>	-14.60	-0.2	-1.0	-0.4	-0.6	-0.5
3263	↓	<i>frdB</i>	-14.26	0.3	-0.5	-0.3	-0.6	0.3
3284		<i>b2975</i>	-15.33	0.3	1.9	-0.4	1.1	-0.3

#Microarray experiments previously performed on DvH cultures www.microbesonline.org (Dehal et al. 2010). Each sample was normalized to the culture grown in defined lactate-sulfate medium (LS4D) and presented in Log₂ format. Experiment number: 389, 60 mM Lactate with 40 mM sulfite; 1301, 60 mM lactate with 30 mM thiosulfate; 388, 120 mM pyruvate with 30 mM sulfate; 1737, 120 mM pyruvate with 30 mM thiosulfate; and 7241, 120 mM pyruvate (fermentation). Blue and yellow highlight refers to those transcripts that have decreased or

increased by greater than twofold, respectively.*Microarray study (Wall et al. 2008) for DvH grown by sulfate respiration with lactate in LS4D medium, log₂R format, average = 13.7. mRNA transcript is normalized to gDNA. N.A. Not applicable.

Because the Rex mutant strain is able to grow when any gene in the *atpF1F2HAGDC* operon was also deleted, and these genes were considered to be non-essential based on fitness scores (at least in the Rex mutant), other mechanisms for ATP generation must be compensating for the loss of ATP synthase in these strains. For *D. alaskensis* G20, a strain deleted for *rex* has been shown to have increased expression in the genes needed for formate production and metabolism (e.g. *pfl* and *fdhABE-c3*), as well as hydrogen production at the periplasm linked to proton translocation (e.g. *echABCDEF*), and a high molecular weight membrane-bound electron carrier predicted to transfer electrons across the membrane (e.g. *bmcABCDEF*) (Kuehl et al. 2014). Interestingly, genes that code for proteins responsible for substrate level phosphorylation via pyruvate oxidation to acetate (e.g. *por*, *pta*, and *ack*) remained unchanged. Genes that code for proteins responsible for other processes were decreased in expression, including periplasmic hydrogenase (e.g. *hys*); and the membrane-bound Rnf complex (*dhcA-rnfCDEAB*) that is associated with proton translocation and electron flow as well as NAD⁺ reduction. These findings were interpreted to mean that formate production (via pyruvate formate lyase, Pfl) and not acetyl-CoA production (via pyruvate oxidoreductase, Por) from pyruvate allowed the electrons to move directly to the periplasm (as formate) to be transferred to TpI-c₃ and subsequently to QrcABCD for the reduction of sulfate. Two protons are also released when formate is oxidized which contribute to the proton gradient and subsequently to ATP synthesis. Therefore, in the strain deleted for *rex*, other mechanisms for ATP generation increased which may compensate and increase gene fitness when *atpF1F2HAGDC* is deleted in the Rex mutant compared with the parental strain.

Rex proteins have been shown to respond to changes in the redox state of the cell through the NADH/NAD⁺ ratio, and adjust gene expression in order to maintain this ratio (Gyan et al. 2006). Typically, Rex proteins repress genes that code for proteins that oxidize NADH. For example, when cells are grown by respiration, Rex represses such genes as lactate dehydrogenase (*ldh*) and acetaldehyde dehydrogenase (*adhE2*) as observed in *Clostridium acetobutylicum* (Wietzke et al. 2012) and *Staphylococcus aureus* (Pagels et al. 2010), and NADH dehydrogenase (*nuo* or *ndh*) as shown in *Thermotoga maritima* (Ravcheev et al. 2012), *Streptomyces coelicolor* A3(2) (Brekasis et al. 2003), and *Bacillus subtilis* (Gyan et al. 2006). NADH is presumed to increase when respiration is slowed or halted (Williamson et al. 1967). As expected, genes that code for proteins responsible for NADH oxidation were increased under fermentation conditions, as observed for *adhE*, *adhI* and *ldh* in *Staphylococcus aureus* (Pagels et al. 2010). Therefore, it was concluded that Rex represses genes that code for proteins that oxidize NADH, and this repression is removed upon increasing NADH concentration.

In SRB, slowed respiration rates would occur when a terminal electron acceptor (i.e. sulfate, sulfite or thiosulfate) is not readily available, and the relative concentration of NADH is presumed to increase when fermenting pyruvate (Meyer et al. 2014). Therefore, we hypothesized that when a cell lacked a terminal electron acceptor, the cell would increase expression for genes that code for proteins responsible for oxidizing NADH as discussed above. Examination of the transcript expression results for DvH grown by pyruvate fermentation revealed genes that code for proteins responsible for sulfate reduction were increased, including: *qrcABCD*, *qmoABCD*, *apsBA*, *sat*, *ppaC*, *adk*, but not enzymes responsible for the oxidation of NADH (e.g. *nox* and *ndh*), although *dhcA-rnfCDGRAB* did appear to increase slightly (Table 4-8) (Dehal et al. 2010) (Table 4-6). Interestingly, the genes

predicted to be regulated by Rex in SRB typically code for proteins in sulfate reduction (Ravcheev et al. 2012). One of these, DhcA-RnfCDEAB, is known to play a role in NADH oxidation. The encoding operon has also been experimentally confirmed to be activated and not repressed by Rex in *D. alaskensis* G20 by microarray (Kuehl et al. 2014). Therefore, in SRB, it appeared that Rex increased respiration processes, potentially to scavenge nutrients from the environment, as opposed to oxidizing NADH to maintain steady-state NAD^+ concentrations. To elaborate on this hypothesis, the overexpression of *sat* in DvH had shown increased sulfate uptake for a culture starved for a terminal electron acceptor, and conversely the deletion of *sat* decreased sulfate uptake (personal communication with Grant Zane). The deletion of *rex* also had increased sulfate uptake. As *sat* is de-repressed during fermentation by Rex, as well as increased in expression when *rex* is deleted, it is reasonable to presume that Rex may control sulfate uptake through the expression of *sat*. The exact mechanism for how Sat increases sulfate uptake is not currently known.

To understand better the role that Rex may have on controlling sulfate reduction, a strain deleted for *rex* was grown on different substrates and compared to a parental strain (Table 4-2). As Rex has been shown to respond to the NADH/NAD^+ ratio, the deletion of *rex* should prevent the cell from responding to changes in this ratio. In fact, the deletion of Rex should resemble the state at which Rex is de-repressed, which would be similar to when starved for an electron donor, i.e. pyruvate fermentation. Under these circumstances, proteins involved in sulfate reduction are presumed to be increased as the expression of the genes that code for these proteins is increased. The deletion of *rex* did not affect growth by respiration with sulfate nor by pyruvate fermentation compared to the parental strain. However, the deletion of *rex* did appear to delay growth when sulfite was provided as the electron donor. Nevertheless, the rates between the Rex mutant and parental strain were

similar. The parental strain, JW710, is deleted for uracil phosphoribosyltransferase (*upp*), a pyrimidine salvaging enzyme. To our knowledge, the deletion of this gene is not known to be a stress for the cell. Examination of the wild-type strain, DvH, in this study did reveal that the rates were similar to JW710. However, the wild-type strain did not experience an initial lag that had been observed for JW710. Furthermore, high concentrations of sulfite is a known stress for DvH (Badziong et al. 1978; Pereira et al. 2008), and the concentration used in this study (40 mM) may have exceeded the toxic levels, and only once sulfite levels had decreased (i.e. detoxified), or cell numbers were increased, was sulfite efficiently coupled to energy conversion for growth. Therefore, the deletion of *upp*, in addition to the sulfite toxicity, may be the reason for the longer lag prior to exponential growth for the parental strain and even more so with the Rex mutant than the wild-type DvH strain. Finally, the deletion of *rex* decreased the rate of growth when respiring thiosulfate, regardless of the electron donor. Therefore, from growth experiments, the deletion of *rex* appeared to disrupt the metabolism of thiosulfate, but not any other electron acceptor. Additionally, the provided electron donor, lactate or pyruvate, did not appear to affect the Rex mutant compared to the parental strain.

If the role of Rex was specific to respiration versus fermentation, as previously alluded to, then there should not have been as drastic (or any) difference in growth between the parental and Rex mutant strain when grown by respiration with sulfate-, sulfite-, or thiosulfate-containing media, unless there was something different between the reduction of sulfate and sulfite and the metabolism of thiosulfate. Interestingly, sulfate and sulfite are reduced within the cytoplasm, whereas thiosulfate is presumed to be reduced in the periplasm (Stoffels et al. 2012). Bacteria have been shown to metabolize thiosulfate by at least two distinct processes. Firstly, thiosulfate can be reduced to sulfite and sulfide by a two-

electron reduction reaction, as was shown *in vitro* for a 16 kDa enzyme in DvH (Findley et al. 1970; Haschke et al. 1971). Or secondly, it could be disproportionated to sulfate and sulfide in the presence of water (Ravot et al. 1995); however, no enzyme capable of the latter process has been identified in DvH (Finster et al. 1998; Heidelberg et al. 2004). The deletion of *qmoABC* is impaired for growth only on sulfate, and not sulfite or thiosulfate, which was interpreted to mean that thiosulfate is converted to sulfite and not sulfate in DvH (Zane et al. 2010). The hypothesis was presented that Rex_{DvH} may be necessary for altering the electron flow between cytoplasmically and periplasmically located reduction processes and not specifically to increasing sulfate reduction rates.

To begin to address how the electrons are transferred to a thiosulfate reductase located within the periplasm (or periplasmic membrane) in DvH, and how Rex may disrupt this transfer, we first looked for genes that increased in expression in the presence of thiosulfate as compared to sulfate (Dehal et al. 2010) (Table 4-8). In these studies, genes that were increased in expression included: two of the three formate dehydrogenases (*fdhAB* and *fdhABEc*), pyruvate formate lyase (*pfl*), a high-molecular weight cytochrome c complex that is located within the periplasmic membrane (*bmcABCDEF*), a putative lactate permease (DVU3284) and DVU3143-5. In the transcript studies presented here (Table 4-6), *dsrC* was also shown to increase in thiosulfate-containing medium. Interestingly, genes that code for proteins responsible for early steps in sulfate reduction (e.g. *sat*, *apsBA*, *qmoABCD*, and *ppaC*) and substrate-level phosphorylation (e.g. *por*, *pta* and *ack*) decreased in expression. These findings were interpreted to mean that the cell was switching away from sulfate reduction (although genes that code for proteins responsible for sulfite reduction [e.g. *dsrABD*] remained unchanged) and instead to thiosulfate reduction by a currently unknown mechanism when thiosulfate is the sole electron acceptor.

Next, the expression of sulfate reduction genes was considered in the Rex mutant, in order to determine whether the electrons may be forced internally (i.e. away from thiosulfate reduction processes) in the Rex mutant strain as compared to the parental strain. The deletion of *rex* increased expression for those genes that code for proteins associated with the early steps in sulfate reduction, specifically *qmoABCD* (Kuehl et al. 2014) and *sat* (Table 4-4). Interestingly, these were the same genes that were decreased in the parental strain when grown with thiosulfate. Therefore, the deletion of *rex* most likely disrupted the proper electron flow for thiosulfate reduction to proceed. Specifically, the Rex mutant resembled a sulfate-starved state so gene expression is altered to increase sulfate reduction, which was inhibitory when thiosulfate was the sole electron acceptor.

To begin to determine the mechanism for thiosulfate reduction in DvH and how Rex disrupts this mechanism, thiosulfate metabolism was considered further. The reduction of thiosulfate is endergonic and therefore, must be coupled to a more exergonic process in order to convert energy (Stoffels et al. 2012). One possible mechanism would be to link the reduction of thiosulfate to the proton motive force, as was shown for PhsABC from *S. enterica* (Stoffels et al. 2012). This enzyme is similar to the more familiar formate dehydrogenase (FhnGHI) from *E. coli* (Jormakka et al. 2002), except, for PhsABC the proton motive force is used to drive thiosulfate reduction instead of adding to the proton gradient. Because of this, thiosulfate reduction is presumed to occur at the periplasm in DvH, and linked to the periplasmic membrane with the electrons being supplied by the menaquinone pool (Stoffels et al. 2012; Hocking et al. 2014).

In DvH, the genes annotated as *phsAB* have high protein identity with PhsAB as expected (PhsA: 32% identity, BLAST probability 3e-112, 94% coverage; PhsB 72% identity, BLAST probability 3e-29, 43% coverage); however, *phsC* has not been annotated in DvH.

Additionally, a much smaller enzyme, purified from cell extract, was shown to reduce thiosulfate, but required a hydrogenase and methyl viologen as an electron carrier to facilitate thiosulfate reduction *in vitro* (Haschke et al. 1971). Therefore, the specific membrane component (PhsC) is not currently known for DvH. The role of PhsAB was examined for its role in the reduction of thiosulfate and its connection to Rex.

Interestingly, *phsAB* is thought to be controlled by Rex in two *Desulfovibrionales*, *D. alaskensis* G20 and *D. vulgaris* str. Miyazaki F, but not DvH, based on an identified Rex-binding site upstream of the encoding operons (Ravcheev et al. 2012). However, transcript studies examining a Rex mutant in *D. alaskensis* G20 (Kuehl et al. 2014) and DvH grown by sulfate respiration with lactate compared to the parental strain showed no change (cutoff being twofold) in expression of *phsAB*, which was interpreted here to mean that Rex does not control *phsAB* expression. A transposon mutant for *phsA* was grown on different substrates (Figure 4-3). As predicted, the PhsA mutant was impaired for growth when respiring thiosulfate with lactate as compared to the wild-type DvH strain, although this strain was unaffected when pyruvate was added instead of lactate. The deletion of *phsA* did not prevent growth on thiosulfate and *phsAB* transcript is much lower in concentration (~15-100-fold less) than the genes that code for proteins responsible for sulfate reduction processes (Table 4-8). This was interpreted to mean that additional thiosulfate reductases are encoded in the genome, and the observed differences between the Rex mutant and parental strain grown by respiration with thiosulfate were a growth effect and not due to the regulation of *phsAB* by Rex.

Further examination of the genome of DvH revealed another possible thiosulfate reductase, DVU3143-5, which did increase in expression when DvH was respiring thiosulfate with pyruvate (Table 4-8). DVU3143 is annotated as an iron-sulfur cluster

binding protein that resembles PhsB (30% identity, BLAST probability $3e-16$, 35% coverage). DVU3144 is annotated as a cytochrome c family protein that poorly resembles PhsA. And DVU3145 is annotated as a b-type hydrogenase (periplasmic) that resembles PhsC (34% identity, BLAST probability $1e-21$, 96% coverage). Additionally, DVU3143 and DVU3144 appeared to have a periplasmic membrane localization signal as would be predicted for these two components (SignalP 4.1 server, data not shown) (Petersen et al. 2011). Therefore, we predict DVU3143-5 to be a periplasmic-facing membrane-bound thiosulfate reductase that is linked to the proton motive force. Characterization of DVU3143-5 is currently in progress.

In *Desulfovibrio gigas*, flavoredoxin (Flr) is required to couple the oxidation of hydrogen to thiosulfate reduction within the cytoplasm, and the expression of *flr* was shown by northern blot to increase in the presence of sulfite or thiosulfate, but not sulfate (Broco et al. 2005). Because a Flr mutant was impaired for growth on sulfite as well as thiosulfate, thiosulfate was concluded to be a product of sulfite reduction, giving credence to sulfite reduction by intermediates. Specifically, trithionate and thiosulfate were formed, each occurring by a two-electron transfer reaction (Kobayashi et al. 1969; Broco et al. 2005). Furthermore, the presence of sulfite was shown to inhibit the reduction of trithionate or thiosulfate although the mechanism for this inhibition is unknown. Once sulfite is depleted from the culture trithionate or thiosulfate were reduced as observed by depletion of these intermediates and formation of sulfide (Broco et al. 2005). In DvH, *flr* did not increase in expression regardless of the electron acceptor (Dehal et al. 2010), and Rex did not appear to control the expression of *flr* in *D. alaskensis* G20 (Kuehl et al. 2014). Additionally, the Rex^{DvH} mutant resembles an electron starved state which should be similar to the build-up of thiosulfate within the cytoplasm and be similar to a Flr mutant. However, a Flr mutant in

DvH did not appear to be impaired for growth in sulfate, sulfite, or thiosulfate as compared to the parental strain (data not shown). This was interpreted to mean that the role Flr plays in DvH may be different from what was observed in *D. gigas*.

From these findings we present a new model for thiosulfate metabolism in DvH (Figure 4-4) and the role of Rex in thiosulfate reduction. In this model, DVU3143-5 is the thiosulfate reductase that is coupled to the proton motive force, although PhsAB is still functioning in the periplasm to generate sulfite for subsequent reduction in the cytoplasm. DVU3143-5 is presumed to receive electrons from the menaquinone pool, most likely supplied by QrcABCD and is in competition with QmoABC that supplies electrons to APS reductase for sulfate reduction. To substantiate this, the deletion of *qrcABCD* impairs growth on thiosulfate, suggesting its role in thiosulfate reduction (data not shown). However, the QrcABCD mutant after an extended lag is able to grow, although at a slower rate, which was interpreted to mean that electrons may be supplied to DVU3143-5 from an alternate source as well. Ldh was proposed to provide electrons to DVU3143-5 in a similar manner as it does to QmoABC. Furthermore, DVU3145 was predicted to resemble a hydrogenase, which may allow for electrons to be transferred directly to this complex from hydrogen. Interestingly, during thiosulfate reduction, *pfl* and *fdhAB* increase in expression, which was interpreted to mean that formate is produced in greater quantity in the cytoplasm from pyruvate. Subsequently formate would be transported to the periplasm, where it is oxidized to protons and CO₂, with the electrons being transferred to TpI-c₃. This would allow for an electron circuit that is shorter and more direct, and avoid transferring electrons to ferredoxin which is associated with sulfate-reduction processes within the cytoplasm. Additionally, the hydrogenases in the periplasm are also decreased in the presence of thiosulfate, which may allow for DVU3143-5 to oxidize the hydrogen instead. Also, TpIc3 may be more likely to

target QrcABCD at the periplasmic membrane with the other hydrogenases being decreased in abundance. Therefore, in a strain deleted for *rex*, we hypothesize that the increased expression of *qmoABC* may funnel the electrons towards sulfate reduction and away from DVU3143-5, limiting thiosulfate reduction. Thus, the role of Rex was determined to maintain steady-state sulfate reduction levels, and when sulfate reduction is slowed, i.e. an increase in NADH/NAD⁺ ratio, Rex de-represses genes that code for proteins involved with funneling the electrons to sulfate reduction processes, which may also include scavenging mechanisms for sulfate, in order to restore NADH/NAD⁺ levels. Consequently, as thiosulfate reduction does not occur within the cytoplasm the Rex mutant was impaired for growth when respiring thiosulfate regardless of the electron donor.

Chapter 5: Supplementary projects and discussion

Section 5. 1. Overview

In this section, additional projects not directly discussed in earlier chapters are provided. This chapter includes: requirement of yeast extract in fermenting cultures, northern blot analysis of *apsA*, Rex mutant sensitivity to molybdate, examination of additional regulators of sulfate reduction (e.g. HcpR and LysX), and additional details of several protocols (qPCR, RACE, NADH quantification).

Section 5. 2. Yeast extract required for DvH to ferment pyruvate efficiently

DvH does not grow appreciably in pyruvate fermenting medium as observed spectrophotometrically by a low optical density ($OD_{600} < 0.1$). This low yield poses a challenge for several of the analyses to be performed in this study, where cell number and growth are essential. In our laboratory, addition of yeast extract at 0.1% (wt/vol) and/or cysteine (0.5 mM) to the media have been shown to assist in the growth of DvH by fermentation. Because the work planned was focused on respiration and fermentation processes specific to the sulfate reduction pathway, it was essential to make sure that any differences being observed were not related to the addition of yeast extract or cysteine to the fermenting medium. Therefore, JW710 (the parental strain used to construct any of the MED strains) was grown fermentatively with pyruvate, with yeast extract, with cysteine, or with all components, in triplicate. After 73 hours cultures were assessed for protein and *sat* expression (Figure 5-1). JW710 grew to a higher $OD_{600_{max}}$ and higher total protein content when both yeast extract and cysteine were added. The addition of both yeast extract and cysteine allowed for increased cell density and protein, while the addition of either one alone had slightly increased or no affect at all, respectively. Furthermore, when *sat* transcript is

normalized to total protein the abundance of *sat* is consistent across all samples. Therefore, addition of yeast extract at 1% wt/vol and cysteine at 0.5 mM should not interfere with the studies planned.

Figure 5-1: Pyruvate fermentation control experiment. JW710 was grown fermentatively for 73 hours in MO basal salts supplemented with either Pyruvate (P), Yeast Extract (Y), Cysteine (cys) or all three. A) Optical density after 73 hours was determined and samples were collected and assayed for total protein. B) *sat* transcript was determined and normalized to protein, and then normalized to JW710 MOYP + cys.

Section 5. 3. Northern blot analysis

Initial examination of transcript analysis for *apsB* was determined by northern blot. DvH was grown and mRNA isolated according to protocols discussed in the methods section (Zane et al. 2010). The cells were cultured in lactate (60 mM) respiring medium with either sulfate (30 mM) or sulfite (20 mM) as the terminal electron acceptor. Approximately 5 µg of total RNA was electrophoresed (90 min at 75V) through a 1.5% (wt/vol) agarose gel followed by transfer to a positively charged membrane (Figure 5-2). *apsB/A* operon was determined to be expressed at a higher level when DvH was grown in sulfate compared with sulfite as the terminal electron acceptor confirming previous studies (Zane et al. 2010).

Figure 5-2: Northern blot analysis of *apsA*. DvH strain probed for *apsA*-containing transcript. Determination of *apsA* expression in RNA samples isolated from DvH grown by sulfate respiration with lactate or sulfite as terminal electron acceptor. RNA was probed with a DNA fragment of *apsB*. X-ray film (LEFT), denaturing agarose gel (Middle) are shown.

Section 5. 4. Rex mutant is sensitive to molybdate

SRB, including DvH, are sensitive to high concentrations of molybdate, although trace amounts are required for growth (Biswas et al. 2009). Molybdate has a similar structure as sulfate and competes with sulfate for the active site of Sat. However, the complex formed by Sat with molybdate is unstable and an ATP molecule would be squandered. Because of the competition between sulfate and molybdate for Sat the cell cannot sustain growth (Peck 1959; 1962). Since a deletion in *rex* was predicted to increase *sat* expression, and therefore Sat, it was assumed that this strain should be sensitive to molybdate. The Rex marker-exchange deletion was assayed for molybdate sensitivity, and as expected the Rex mutant was inhibited for growth compared with the parental strain on sub-lethal concentrations of molybdate (Figure 5-3). The interpretation of these results was that energy was consumed but an electron acceptor was not generated.

Figure 5-3: Rex mutant is more sensitive to molybdate than its parental strain. Parental and Rex mutant grown in defined MOLS4 (n=3). Addition of Molybdate to a final concentration of 0 and 0.1 mM.

Section 5. 5. Other regulators predicted to control sulfate reduction

Section 5. 5. 1. Overview

From the work performed on Re_{DvH} , there are certainly other components involved in regulating the sulfate reduction pathway, several of which have been postulated (Table 5-1) (Rodionov et al. 2004; Turkarslan et al. 2013; Christensen et al. 2014). The majority of these regulators are likely to control gene expression through their interaction with DNA.

Table 5-1: Potential regulators of sulfate reduction

DVU	Name	Strain identifier	Gene Fitness* (Log ₂)
DVU0057	Transcriptional regulator, TetR family	GZ7888	0.86
DVU0744	Sigma-54 dependent transcriptional regulator	GZ12635	-0.05
DVU0916	Rex, transcriptional regulator	JW3311, JW3319	-2.86
DVU1142	Transcriptional regulator, putative	GZ4443	0.44
DVU2275	Sigma-54 dependent transcriptional regulator	GZ5190	-0.04
DVU2547	Transcriptional regulator, putative, HcpR family	JW9011	0.47
DVU2567	Regulator for lysine biosynthesis and transport	GZ0481	0.28
DVU2690	Hypothetical protein	GZ1801	0.56
DVU2799	Transcriptional regulator, MarR family	GZ12386	-0.71
DVU2802	Transcriptional regulator, GntR family	GZ12985	0.92

*Gene fitness values from DvH_MOLS4 experiment

Gene fitness across the genome for DvH grown on minimal medium containing lactate and sulfate has been determined (Fels et al. 2013). Examination of gene fitness for these predicted regulators revealed that the interruption of an individual gene correlated to a minor fitness advantage (positive) or defect (negative) compared to the parental strain (Table 5-1). The contribution of these potential regulators is less significant than what had been observed for *rex*, with a fitness defect ($\log_2 = -2.86$). Additionally, in the DvH transposon library *rex* was not obtained but all of the potential regulators mentioned above were. Therefore, if these regulators are involved with the regulation of sulfate reduction they have minor roles or are compensated elsewhere.

Section 5. 5. 2. Transcript expression results for potential regulators

Expression of sulfate reduction genes is known to decrease when sulfite is provided as the terminal electron acceptor as compared with sulfate (Wall et al. 2008; Zane et al. 2010). The mechanism for this is unknown. Recently, several regulators have been predicted to control sulfate reduction (Table 5-1), potentially by altering the expression of *sat* and *apsA*. It was interpreted that the change in abundance of the encoded proteins would alter the flux of electrons through this metabolic pathway (i.e. more transcript and so more protein would increase sulfate reduction processes). To determine the role of these potential regulators, qRT-PCR was performed on parental DvH cells, on a marker-exchange deletion of *hcrR*, and on individual transposon insertion mutants of the regulators mentioned above, in defined lactate (60 mM) medium with either sulfate (30 mM) or sulfite (20 mM) as the terminal electron acceptor (Table 5-2) (Table 5-3). Expression of *sat*, *apsA* and *dsrA* was determined. No observable difference greater than twofold was found among the majority of the strains, for the conditions assayed. Although, the hypothetical gene DVU2690 and sigma-54 transcription regulator DVU0744 did appear to have a slight loss of regulation control (i.e. *sat* did not decrease to the same degree as the parental strain with sulfite compared to sulfate). The annotated transcription regulators DVU0744, DVU2802 and DVU2275 and their role in sulfate reduction regulation are examined elsewhere (Turkarslan et al. 2013) (Serdar Turkarslan, personal communication). Overall, this preliminary study would suggest that several of these candidates seem likely to control gene expression and should be pursued in greater detail for their role in sulfate reduction regulation.

Table 5-2: Sample collection and transcript analysis of potential regulators

Strain	Name	Media	OD600 collected	Mean Cq ^a				
				<i>rplS</i>	<i>rpmC</i>	<i>sat</i>	<i>apsA</i>	<i>dsrA</i>
DvH*	wild-type	LS4	0.455	22.62 ± 0.03	23.99 ± 0.02	22.73 ± 0.02	23.62 ± 0.01	21.68 ± 0.02
		LS3	0.414	20.88 ± 0.06	23.61 ± 0.03	22.65 ± 0.06	24.71 ± 0.06	22.40 ± 0.03
GZ0481 [#]	<i>lysX</i>	LS4	0.249	21.85 ± 0.00	24.44 ± 0.03	23.51 ± 0.02	24.57 ± 0.00	22.22 ± 0.02
		LS3	0.228	20.03 ± 0.05	23.08 ± 0.04	24.65 ± 0.01	26.48 ± 0.00	21.11 ± 0.05
GZ1801*	DVU2690	LS4	0.560	21.67 ± 0.02	23.57 ± 0.01	22.25 ± 0.03	22.97 ± 0.01	21.30 ± 0.06
		LS3	0.451	20.25 ± 0.01	22.75 ± 0.04	21.53 ± 0.02	23.12 ± 0.01	20.76 ± 0.00
GZ12635*	DVU0744	LS4	0.320	21.89 ± 0.01	23.76 ± 0.02	22.83 ± 0.03	23.32 ± 0.01	21.67 ± 0.03
		LS3	0.421	20.93 ± 0.02	23.92 ± 0.07	22.95 ± 0.04	24.61 ± 0.01	22.05 ± 0.01
GZ7888*	DVU0057	LS4	0.425	22.19 ± 0.03	23.91 ± 0.03	22.64 ± 0.05	23.28 ± 0.05	21.74 ± 0.03
		LS3	0.420	20.72 ± 0.02	23.38 ± 0.03	23.74 ± 0.05	25.43 ± 0.04	21.69 ± 0.06
GZ5190*	DVU2275	LS4	0.380	21.43 ± 0.05	23.67 ± 0.03	22.21 ± 0.05	23.05 ± 0.01	21.19 ± 0.02
		LS3	0.440	20.71 ± 0.01	23.40 ± 0.06	22.70 ± 0.02	24.50 ± 0.01	21.72 ± 0.03
GZ12386*	DVU2799	LS4	0.535	21.61 ± 0.00	23.26 ± 0.04	22.15 ± 0.01	22.92 ± 0.01	21.07 ± 0.01
		LS3	0.410	20.41 ± 0.01	23.00 ± 0.02	22.61 ± 0.02	24.39 ± 0.06	21.32 ± 0.03
GZ12985*	DUV2802	LS4	0.435	21.22 ± 0.01	22.84 ± 0.04	21.87 ± 0.03	22.50 ± 0.01	20.93 ± 0.01
		LS3	0.451	20.64 ± 0.05	22.75 ± 0.01	22.43 ± 0.05	24.55 ± 0.01	20.64 ± 0.01
GZ4443*	DVU1142	LS4	0.410	21.86 ± 0.02	23.61 ± 0.01	22.63 ± 0.01	23.42 ± 0.03	21.63 ± 0.02
		LS3	0.495	20.88 ± 0.02	22.79 ± 0.04	22.76 ± 0.02	25.10 ± 0.02	20.98 ± 0.02
JW710 [#]	parental	LS4	0.257	20.49 ± 0.02	23.70 ± 0.01	21.62 ± 0.01	22.72 ± 0.03	21.84 ± 0.04
		LS3	0.243	19.31 ± 0.06	22.11 ± 0.03	24.46 ± 0.04	25.48 ± 0.02	20.16 ± 0.03
JW9011 [#]	$\Delta hcpR$	LS4	0.236	21.96 ± 0.03	24.03 ± 0.02	22.74 ± 0.00	23.90 ± 0.01	22.29 ± 0.04
		LS3	0.261	19.78 ± 0.05	22.90 ± 0.05	25.54 ± 0.01	26.66 ± 0.02	21.03 ± 0.02

Refer to Table 5-3 for descriptive legend.

Table 5-3: Transcript analysis of potential regulators

Name	Media	Transcript ^a		
		<i>sat</i>	<i>apsA</i>	<i>dsrA</i>
*wild-type	LS4	1.00 ± 0.02	1.00 ± 0.01	1.00 ± 0.01
	LS3	0.52 ± 0.02	0.24 ± 0.01	0.60 ± 0.01
# <i>lysX</i> transposon	LS4	1.00 ± 0.02	1.00 ± 0.01	1.00 ± 0.01
	LS3	0.16 ± 0.00	0.10 ± 0.00	0.71 ± 0.03
*DVU2690 transposon	LS4	1.00 ± 0.02	1.00 ± 0.01	1.00 ± 0.04
	LS3	0.78 ± 0.02	0.43 ± 0.01	0.69 ± 0.01
*DVU0744 transposon	LS4	1.00 ± 0.02	1.00 ± 0.01	1.00 ± 0.03
	LS3	0.71 ± 0.03	0.32 ± 0.01	0.59 ± 0.01
*DVU0057 transposon	LS4	1.00 ± 0.04	1.00 ± 0.04	1.00 ± 0.02
	LS3	0.24 ± 0.01	0.12 ± 0.00	0.53 ± 0.02
*DVU2275 transposon	LS4	1.00 ± 0.04	1.00 ± 0.02	1.00 ± 0.03
	LS3	0.51 ± 0.01	0.27 ± 0.01	0.50 ± 0.01
*DVU2799 transposon	LS4	1.00 ± 0.02	1.00 ± 0.02	1.00 ± 0.02
	LS3	0.45 ± 0.01	0.23 ± 0.01	0.52 ± 0.01
*DUV2802 transposon	LS4	1.00 ± 0.03	1.00 ± 0.02	1.00 ± 0.02
	LS3	0.54 ± 0.02	0.20 ± 0.00	0.61 ± 0.01
*DVU1142 transposon	LS4	1.00 ± 0.01	1.00 ± 0.02	1.00 ± 0.02
	LS3	0.50 ± 0.01	0.18 ± 0.00	0.86 ± 0.02
#parental	LS4	1.00 ± 0.01	1.00 ± 0.02	1.00 ± 0.03
	LS3	0.06 ± 0.00	0.07 ± 0.00	1.18 ± 0.03
# $\Delta hcpR$	LS4	1.00 ± 0.01	1.00 ± 0.01	1.00 ± 0.03
	LS3	0.05 ± 0.00	0.06 ± 0.00	0.75 ± 0.02

These results include two experiments that were performed at two separate times. *denotes experiment 1, while # denotes experiment 2. OD600 was monitored through growth and samples were collected for analysis at mid-exponential (experiment 1) or at early-exponential (experiment 2). Approximately 100 ng of TURBO™ DNase-treated RNA was converted to cDNA and 1 μ L of cDNA (5 ng of RNA) was used per qRT-PCR. Critical threshold (Cq) is presented for all genes tested, including two reference genes, *rplS* and *rpmC*. For experiment 1, the efficiency for each gene was determined: *rplS* = 95.2%, *rpmC* = 91.2%, *sat* = 100.5%, *apsA* = 95.0% and *dsrA* = 98.6%; and for experiment 2: *rplS* = 94.7%, *rpmC* = 89.6%, *sat* = 100.3%, *apsA* = 92.0% and *dsrA* = 89.0%. ^a Critical threshold (Cq) for specified gene, with technical error determined as standard error of the mean (n=3).

Section 5. 5. 3. qPCR primer design strategy

For every gene analyzed, two optimization steps were taken for primer design to ensure quality of transcript data. First, three forward and three reverse primers were designed with specified parameters (i.e. T_m and length). Then, each combination (e.g. Forward primer 1 with Reverse primer 1), for a total of nine, were used to amplify the target from 10 ng of gDNA template. The quality of each primer set was then determined (Figure 5-4) and two with reasonable amplification curves, including no amplification of the no-template controls (NTC) and having a single peak in the melt curve, are then used to determine efficiency (Figure 5-5). The efficiency of a primer set is determined by amplifying a dilution series of template DNA (or RNA, in the form of cDNA), typically starting from a 100 ng/ μ l stock, and solving for the curve generated from plotting Ct versus concentration. Acceptable primer sets for a 10-fold dilution series would have a slope (m) of ~ -3.4 , $R^2 > 0.990$ and efficiency 80-110%. For each gene the one primer set determined to fulfill these requirements was used. A list for all optimized primer sets is provided (Table 7-5).

Figure 5-4: Primer optimization results of a “good” and “bad” primer set. Graphs A and C are amplification curves (RFU vs Cycle) and graphs B and D are melt curves (dRFU/dT vs temperature) of two separate primers sets (F/R1 vs F2/R2), of the nine that were performed, that have been amplified from the same gDNA template. In the “bad” example, fluorescence was detected in the no template control (NTC) in graph C, and in the melt curve (D) multiple peaks (and shouldered curves) were seen as a result of the NTC amplification (i.e. primer dimers).

Figure 5-5: Primer efficiency determination for *apsA*. gDNA used as template for standard curve (100 ng-10 pg, 10-fold dilution). A) Standard curve plot for efficiency determination (Ct vs template concentration); B) melt curve (dRFU/dT vs temperature); and C) Amplification Plot (Semi-log) (RFU vs Cycle).

Section 5. 6. Additional RACE protocol and discussion

Section 5. 6. 1. Overview

In the case that the strategy described in the methods section did not work (Section 7.9) (Figure 7-6), i.e. poor sequence reads or no single fragment, a new approach was developed. The modifications to the protocol included modified primers that contained overhangs for cloning purposes via SLIC (Li et al. 2007), and additional components for stabilization of the desired product throughout many of the steps. As a proof of principle for the cloning procedure the TSS for *sat* was first determined for DvH grown by sulfate respiration with lactate. Indeed, the results for the TSS of *sat* confirmed previous results.

Section 5. 6. 2. Modifications to the TSS protocol

RNA was isolated as described elsewhere. A 5- μ l aliquot (20 ng/ μ l) of DNase-treated RNA was reverse transcribed to single stranded cDNA via iScript™ Select cDNA synthesis kit (Bio-Rad) with primer DVU1295-sat-GSP1, degraded with RNase A/T1 Mix and RNaseH (Thermo Fischer Scientific) according to manufacturers protocol. With terminal deoxynucleotidyl transferase (Thermo Fisher Scientific), an adenosine tail was added to the cDNA, followed by generation of the second DNA strand with iScript™ and primer RACE-2nd Strand. dsDNA was purified, diluted 1:1000 and amplified by PCR to generate DNA fragment PCR1, with primers: DVU1295-GSP2 and AUAP. Thirty cycles of amplification were carried out (30 sec at 94°C, 30 sec at 58°C, 1 min at 72°C) followed by a final extension for 5 min at 72°C with *Taq* DNA Polymerase (NEB). From this point on in the protocol the modifications were made. An additional PCR (PCR2) followed, which was the same protocol as PCR1, but with 1:1000 diluted PCR1 as template and nested primers (AUAP-cloning and DVU1295-GSP4-cloning). Following standard PCR protocols, an additional fragment

(backbone fragment, “SpUC”, 1885 bp) was amplified from pMO719 with primers SpecRpUC-F and SpecRpUC-R. This fragment included the spectinomycin resistance marker and pUC *ori*. The PCR2 fragments containing overhangs to the “SpUC” backbone fragment and “SpUC” backbone fragment were T4-treated and cloned via a standard SLIC protocol. A 5-, 50-, and 500- μ l aliquot of transformed cells were plated in LC-medium containing spectinomycin. Colony forming units were counted and PCR screened with primers pMO719-XbaI-dn and pBG1-2199-F for inserted segments (>200 bps). For correctly screened colonies, plasmid was purified, sequenced and mapped to the genome.

Additional measures were taken, including addition of ectoine (0.5 M) to the cDNA generation step or betaine (1M) or DMSO (5% vol/vol) throughout several of the PCR amplification steps (Shi et al. 2006), use of a more thermo-stable enzyme in the cDNA generation step (ThermoScript-RT), and modified PCR protocols (e.g. touchdown-PCR (Don et al. 1991)), which had previously been shown to improve these *in vitro* processes. An additional modification to this procedure, although not tested, could have been the use of a modified base, 7-deaza-2'-dGTP, which has been shown to alleviate some of the difficulties in polymerizing GC-rich regions (Hurt et al. 2012).

Table 5-4: Primer list for RACE experiments (cloning method)

Name	Sequence (5'-3')	bp	Source	Descriptor
DVU1295-sat-GSP1	GACGAGAGCGATTTCC	16	(Christensen et al. 2014)	Gene specific primer (GSP1) for cDNA generation of <i>sat</i> transcript. Reverse Primer.
RACE-2nd Strand	<u>GGCCACGCGTCGACTAGTAC</u> TTTTTTTTTTTTTTTTTV	39	(Christensen et al. 2014)	For generation of complementary strand of <i>sat</i> cDNA. Underlined portion contains overhang region for amplification by PCR. "V" denotes base A/G/C but not T.
AUAP	GGCCACGCGTCGACTAGTAC	20	Invitrogen 5'-RACE system	Abridged Universal Amplification Primer. For amplification of <i>sat</i> cDNA for PCR1 (with primer DVU1295-sat-GSP2) or PCR2 (with primer DVU1295-sat-GSP3).
DVU1295-sat-GSP2	CGTCGTCTTGGACACGTCA	20	(Christensen et al. 2014)	(GSP2) For amplification of <i>sat</i> cDNA with primer AUAP (PCR1).
AUAP-cloning	<u>gccttttgctggccttttgctcacat</u> GGCCAC GCGTCGACTAGTAC	46	This Study	For amplification of PCR with primer DVU1295-sat-GSP4-cloning (PCR2). Underlined portion contain overhangs with primer SpecRpUC-R for cloning if necessary.
DVU1295-sat-GSP4-cloning	<u>cgaggcatttctgtcctggctgg</u> ATTTCGA TCTGCTTGAGGCCGG	45	This Study	For amplification of PCR1 with primer AUAP-cloning (fragment PCR2). Underlined portion contains overhangs with primer SpecRpUC-F for cloning if necessary.
DVU1295-sat-GSP3	ATTTCGATCTGCTTGAGGCC GG	22	(Christensen et al. 2014)	(GSP3) For nested amplification of PCR1 fragment with primer AUAP (PCR2). For sequencing of PCR2 fragment.
SpecRpUC-F	CCAGCCAGGACAGAAATGCC TCG	23	(Parks et al. 2013)	For amplification of Sp ^r and pUC ori from pMO719 to make backbone. Used as overhang for SLIC. Forward Primer.
SpecRpUC-R	ATGTGAGCAAAAGGCCAGC AAAAGGC	26	(Parks et al. 2013)	For amplification of Sp ^r and pUC <i>ori</i> from pMO719 to make backbone. Used as overhang for SLIC. Reverse Primer.
pMO719XbaI-Dn	TGGGTTCGTGCCTTCATCCG	20	(Parks et al. 2013)	For colony PCR with primer pBG1-2199-F, and sequencing. Reverse primer.
pBG1-2199-F	GCTGAAAGCGAGAAGAGCG CAC	22	(Parks et al. 2013)	For colony PCR with primer pMO719XbaI-Dn, and Sequencing Primer. Forward Primer.

Section 5. 7. **NADH quantification discussion**

The specific method for NADH quantification is described elsewhere (Section 7.11), this section is designated for the discussion of the assay. After an initial attempt (data not shown), in which NADH was undetected, there was concern that the reduced form was not recovered due to initial purification procedures. In particular, the protocol called for a freeze/thaw cycle whereby an extended period of time would elapse and be exposed to oxygen. To determine whether NADH was being oxidized within the time-frame of the experiment, purified NADH (1 mM) was added to several steps along the process and assayed for both NAD^+ and $\text{NAD}_{\text{total}}$ in order to determine when NADH was “lost”. As expected, the added NADH was only recovered in the “correct” reduced form when it was added after the desalting column step (Figure 5-6). Most likely, proteins capable of oxidizing NADH had been separated from the sample after the column purification. Therefore, this method would not allow determination of the ratio of the pyridine nucleotides from a cell as all NADH is converted to its oxidized form, NAD^+ , during the purification process.

Additionally, to determine that the media used in the studies planned would not affect the signal, standards for NAD^+ and $\text{NAD}_{\text{total}}$ at several concentrations were determined in solutions containing media components, including: MO basal salts, sulfide (0.5 mM), cysteine (0.5 mM), and thioglycolate (1.2 mM). The results revealed that that sulfide and cysteine artificially increased the signal, while MO basal salts decreased the signal and thioglycolate did not affect the assay (Figure 5-7). Therefore, additional steps would be required to properly assess pyridine nucleotide concentrations. Specifically, a different analysis altogether, and not the colorimetric assay used in this study, would be required to obtain the desired results. Therefore,

an assay that included mass spectrometry analysis (Sporty et al. 2008), tools our laboratory does not have immediate access too, may be necessary to determine NAD^+ and NADH concentrations.

Figure 5-6: Pyridine quantification determination. Eight samples were run in parallel (A-H). NADH (1 mM) was added to each of the samples along the purification process, except sample A (control). Sample G is addition of NADH right after being passed through desalting column. Sample H is sample G that had been incubated an additional 12 hours on the benchtop.

Figure 5-7: Media components disrupt pyridine nucleotide assay. Sulfide (0.5 mM), cysteine (0.5 mM), thioglycolate (1.2 mM) and MO basal salts were added to standard curves mixtures and assayed for A₄₅₀. The line represents the standard curve as determined by pure NAD⁺ without additional amenities.

Chapter 6: General discussion and concluding remarks

Section 6. 1. Overview

Bacteria have developed machinery to couple oxidative processes to energy conversion through the reduction of different terminal electron acceptors. Additionally, bacteria can undergo substrate-level phosphorylation when a terminal electron acceptor is limited. For SRM, sulfate reduction is coupled to the oxidation of many different substrates, including: H_2 , lactate, pyruvate, or formate. In many SRM, sulfate is not the only terminal electron acceptor that can be used. For example, the reduction of sulfite or thiosulfate is sufficient enough to support respiration (Muyzer et al. 2008). However, it is presumed that when thiosulfate is used for energy conversion, its reduction is coupled to the proton motive force as the reduction of thiosulfate is an endergonic reaction (Stoffels et al. 2012).

A strict anaerobe such as DvH can sustain growth in sulfate-rich and sulfate-limited environments because of its ability to use many different substrates for its respiration, explaining why DvH is nearly ubiquitous in nature. As DvH can maintain sustainable growth in many differing environments, a regulatory network that determines which substrates, and in what order, to use from the surrounding environment is critical (Barton et al. 2007). One regulator, Rex, has recently been identified to sense the rise in $NADH/NAD^+$ ratio caused by limitations in the electron acceptor (i.e. sulfate, sulfite or thiosulfate) as a result of metabolism being slowed or halted (Brekasis et al. 2003). Rex binds to a promoter region upstream of genes responsible for energy conversion processes during respiration (i.e. sulfate reduction); however, Rex dissociates when $NADH$ levels rise, a consequence of slowed metabolism, which consequently increases expression for those genes that code for sulfate reduction processes (McLaughlin et al. 2010).

The work presented here confirmed these traits for Rex_{DvH} on *sat* by *in vitro* assays (Figure 6-1). We also proposed that Rex_{DvH} controlled the electron flow internally towards

sulfate reduction processes. The reasoning for this was due to a couple of conclusions determined in this study. First, Rex repressed genes that code for proteins that function in early sulfate reduction processes centered at the periplasmic membrane and within the cytoplasm (e.g. *qmoABCD* and *sat*). Secondly, as sulfate reduction rates are slowed (i.e. limited for a terminal electron acceptor) the NADH/NAD⁺ ratio was presumed to increase, which de-repressed Rex and lead to increased expression of these targets. Thirdly, the Rex mutant is impaired for growth when thiosulfate is the sole electron acceptor. Thiosulfate is presumed to be reduced in the periplasm (and not the cytoplasm) coupled to the proton motive force, which is different in location from where sulfate and sulfite are reduced.

Figure 6-1: Mechanism of Rex_{DvH} in sulfate reduction. (LEFT)

Respiration, Rex bound to NAD⁺, which allowed it to bind to the promoter region and repressed the downstream gene. (RIGHT) Fermentation, Rex bound to NADH, which caused a conformational change preventing Rex from binding to the promoter region, and therefore, allowed expression of the downstream gene.

Additional terminal electron sensors previously studied in bacteria include ArcAB (Iuchi et al. 1988) and ResDE (Sun et al. 1996); however, these regulators are not found in DvH and seem to be specific to oxygen. The study presented here focused on a relatively new redox sensor, Rex, and specifically examined how Rex controlled the expression of several genes that code for proteins involved throughout the complete reduction of sulfate to sulfide. To that end, a Rex mutant and parental strain were grown by respiration or fermentation and analyzed for rates and extents of growth and transcript abundance of select sulfate reduction genes.

Section 6.2. Role of Rex_{DvH}

The amino acid sequence for the predicted Rex regulator was modelled to several transcription factors, to NADH-dependent enzymes, and to Rex proteins. From these studies it was concluded that this putative protein was likely to be a Rex homolog, and not some other transcription factor. In this work, all the hallmark traits for Rex proteins were confirmed for the Rex homolog in DvH, Rex_{DvH}, including: protein-DNA interaction with the Rex-binding motif, de-repression by NADH and Rex targets are genes that code for proteins responsible for energy conversion processes.

Typically, Rex targets include many genes that code for proteins responsible for the oxidation of NADH to NAD⁺, allowing for the pyridine nucleotide pool to be reestablished and for respiration to proceed in a forward direction (Gyan et al. 2006). Targets repressed by Rex in other systems included: lactate dehydrogenase (*ldh*) and acetaldehyde dehydrogenase (*adhE2*) as observed in *Clostridium acetobutylicum* (Wietzke et al. 2012) and *Staphylococcus aureus* (Pagels et al. 2010); and NADH dehydrogenase (*nuo* or *ndh*) as shown in *Thermotoga maritima* (Ravcheev et al. 2012), *Streptomyces coelicolor* A3(2) (Brekasis et al. 2003), and *Bacillus subtilis* (Gyan et al. 2006). In DvH, this would include NAD respiratory dehydrogenase (encoded by

ndh), two separate pyridine dinucleotide-disulfide oxidoreductases (encoded by *nox* and DVU1974, respectively), and L-lactate dehydrogenase (encoded by *ldh*). However, upon examination of the targets predicted to be controlled by Rex_{DvH}, none of those mentioned above were included (Ravcheev et al. 2012). From a transcriptomic study (of a closely related SRB) that compared a Rex mutant to a parental strain grown by sulfate respiration with lactate, Rex did not appear to control any of the targets capable of oxidizing NADH mentioned above (Kuehl et al. 2014). In fact, the only protein known to be associated with coupling NADH oxidation to proton pumping that has been predicted to be controlled by Rex_{DvH} is the Rnf complex, *dhcA-rnfCDGEAB-apbE* (Ravcheev et al. 2012). Interestingly, the expression for *rnfC* (the first gene in the functioning operon) appeared to be activated and not repressed by Rex in *D. alaskensis* G20 (Kuehl et al. 2014) and was confirmed in DvH by the work presented here. Therefore, the mechanism (or targets) by which Rex controls NADH levels in SRM may be different from the systems that have previously been characterized. Specifically, as we proposed here, Rex_{DvH} appears to alter the electron flow between reductive processes within the cytoplasm and periplasm. The presence of Rex therefore maintains steady-state levels of sulfate reduction. Only when this process is slowed, as the case during pyruvate fermentation, did Rex increase expression of those genes that code for proteins responsible for the initial activation and reduction of sulfate to sulfite. The role of Rex in DvH may be to indirectly oxidize NADH by increasing the rate of sulfate reduction processes. This mechanism for Rex appears to be different from other systems that increase genes that directly function in oxidizing NADH (Brekasis et al. 2003; Sickmier et al. 2005).

Examination of gene fitness across the genome for Rex mutant and parental strain grown by sulfate respiration with lactate did not reveal many significant differences. Of the

more than 3000 protein coding genes, only a few were shown to be altered in fitness greater than twofold between the two strains, and those genes considered to be essential in the Rex mutant were overlapping with those identified in the parental strain. This is quite different from previous studies that examined DvH under different stresses (e.g. nitrate (Korte et al. 2014)). Therefore, under these conditions (sulfate respiration with lactate) Rex_{DvH} did not appear to be significant. These results were not a surprise as the deletion of *rex* in DvH was predicted to facilitate sulfate reduction.

Further examination of the Rex mutant by growth kinetic studies revealed that the deletion of *rex* impaired growth in thiosulfate-containing medium (with lactate or pyruvate as electron donor) compared with the parental strain. Reduction of thiosulfate was thought to occur by thiosulfate reductase, PhsAB (Aketagawa et al. 1985) within the periplasm. In this study we also proposed DVU3143-5 as a potential thiosulfate reductase in the periplasmic membrane that is linked to the proton motive force as was characterized for PhsABC in *S. enterica* (Stoffels et al. 2012). Rex_{DvH} was not predicted to control either of these targets in DvH, and transcript analysis performed in this study for *phsA* expression concluded this finding as well. Therefore, the role of either of these thiosulfate reductases was more likely to be controlled by growth conditions or Rex_{DvH} indirectly. Further examination of DVU3143-5 is currently underway. From these findings, a model for thiosulfate reduction in DvH was proposed, which concluded that electrons were funneled through the menaquinone pool, possibly supplied by QrcABCD, and reduced thiosulfate while consuming the proton motive force at the periplasm. Additionally, the deletion of *rex* disrupted the flow of electrons to thiosulfate reductase, and instead forced the electrons to the cytoplasm for sulfate reduction, through overexpression of *qmoABC* and *sat*. Therefore,

the deletion of *rex* would limit thiosulfate reduction and thus limit growth when thiosulfate was the sole terminal electron acceptor, as was observed in this study.

The transcript studies presented in this work, although not inclusive for all Rex_{DVH} targets, revealed that Rex_{DVH} was most effective at modulating those genes that code for proteins responsible for early steps in the complete reduction of sulfate, although Rex_{DVH} was predicted to control many genes. Rex_{DVH} repressed the expression of *sat*, *apsA*, *ppaC*, and *adk*; but not *dsrA*, nor *phsA*. Interestingly, *dsrC*, not predicted to be controlled by Rex_{DVH} , appeared to be activated by Rex_{DVH} . The specifics to how Rex_{DVH} more strongly controls expression of some genes and not others is likely to be based on the position of Rex_{DVH} binding site relative to the downstream start codon, additional binding-sites and thus regulators (and their signal molecule(s)) that act within the promoter, and the conservation (i.e. strength) of the Rex_{DVH} -binding site. However, initial examination of these sequences did not reveal any correlation between strongly and weakly controlled targets for Rex_{DVH} . For example, *sat* and *dsrA* (a strongly and weakly controlled target of Rex_{DVH} , respectively) have similar Rex_{DVH} -binding motifs and position relative to the downstream start codon, similar *in vitro* calculated dissociation constants, but significantly different transcript expression responses in a strain deleted for *rex* compared to a parental strain. Therefore, the complete mechanism for Rex_{DVH} activity has only begun to be determined, with additional studies required for complete characterization for the role of Rex_{DVH} and its role in the regulation of sulfate reduction. Overall, from these studies it was clear that Rex_{DVH} was important for facilitating sulfate reduction within the cytoplasm, either by increasing sulfate uptake into the cell or by increasing gene expression for those genes that code for proteins responsible for the early steps in sulfate reduction.

Section 6. 3. Future plans...where to next?

From this work it is clear that Rex_{DvH} is involved in sulfate reduction, and specifically repressing genes (e.g. *sat*) that code for proteins responsible for early steps in the sulfate reduction pathway. It would also appear that Rex_{DvH} acts as a molecular switch that alters the flow of electrons for reductive processes between the cytoplasm and periplasm (or periplasmic membrane). Rex_{DvH} has been confirmed *in vitro* to act with predicted Rex_{DvH}-binding sites and the affinity of Rex_{DvH} for these sites have been determined. Additional experiments could be performed to determine all targets of Rex_{DvH} within the genome and not just those examined in this study. For example, Chromatin Immunoprecipitation (ChIP) would accomplish this goal and has been done successfully for Rex in *S. coelicolor* (Strain-Damerell 2010).

In regard to the Rex_{DvH}-binding site, the specific contribution of each base within the consensus is not known. Several studies have been performed to begin to address this question for other organisms (Brekasis et al. 2003; Gyan et al. 2006), and was examined for DvH in this study. Therefore, a more thorough examination of each Rex_{DvH}-binding site would be required to understand better how the sequence affects binding and, therefore, how Rex_{DvH} regulates gene transcription. The necessary analyses would include adapting the protocols performed on *sat* in this study to each of the promoters regulated by Rex_{DvH}. Addition of single base deletion or substitution within the Rex_{DvH}-binding site may also be informative.

In addition to the specific sequence, other factors play a role in gene regulation, including (Rojo 1999; Lee et al. 2012): **1)** distance between translation start codon and regulator binding site, **2)** additional regulators and therefore external signals (stress), **3)** order of addition of the components required to activate or repress transcription, **4)** DNA

accessibility (i.e. supercoiling), **5**) RNAP holoenzyme availability and subunits (i.e. σ -factor used) and **6**) stability of the macromolecules (i.e. protein or mRNA).

To begin to understand some of these factors additional *in vitro* studies were undertaken. For example, to determine the TSS for *sat*, 5'-RACE was conducted on mRNA isolated from DvH grown by sulfate respiration with lactate and by fermentation with pyruvate. A more high-throughput analysis, e.g. 5'-RNA-sequencing, had been performed previously on DvH grown by sulfate respiration (Price et al. 2011). From the work presented in this study, the TSS was dependent on the medium that the cells were actively growing. Complementary studies, i.e. EMSA, transcript and protein expression studies, of genes that code for proteins involved in sulfate reduction processes not studied here would be insightful and would further our understanding of gene regulation. The Rex mutant did not affect the TSS, a result that was unexpected; therefore, a similar analysis on the Rex mutant would also be informative.

The *in vitro* DNA-binding assays performed here have determined that Rex_{DvH} likely plays a role in the regulation of over 50 genes. However, expression studies determining the *in vivo* role of Rex_{DvH} has only been determined for a handful of these genes, including *sat*, *apsA*, and *dsrA*. Therefore, a more exhaustive analysis of the remaining genes would further characterize the role of Rex_{DvH} in sulfate respiration processes. A more high-throughput approach, including microarray of the Rex mutant in comparison to the parental strain, would facilitate this remaining question. A similar approach was performed on the closely related *Desulfovibionales*, *D. alaskensis* G20, and several targets not predicted to be controlled by Rex were identified, as well as confirming previous predictions (Kuehl et al. 2014). DvH and *D. alaskensis* G20 share ~50% of their genome and a few of the targets for Rex are not shared between the strains (e.g. *phsBA*, and *cooMKLXUHAF*). Therefore, it would be

reasonable to postulate that Rex may control targets in a similar manner (i.e. repress/activate), but the overall response to the environment may be achieved differently and related to how each strain has adapted to their environmental niches.

Finally, the Rex mutant was shown to be impaired for growth when grown by thiosulfate respiration with lactate or pyruvate compared to its parental strain. This phenomenon has not been observed for Rex, although most studies to-date have not been on SRM, except in *D. alaskensis* G20 that only examined growth by sulfate respiration with lactate (Kuehl et al. 2014). In DvH, PhsAB (Findley et al. 1970) and DsrAB (Parey et al. 2010) have been shown to reduce thiosulfate, but in this work Rex_{DvH} was shown to not control the expression of the encoding genes. Further examination and characterization of DVU3143-5 would further our understanding of the metabolism of thiosulfate reduction in DvH. Furthermore, additional work is needed to fully characterize the regulators that signal cellular nutrient and energy status that are integrated at the level of control of sulfate reduction. The work presented here was part of this effort to further characterize the process of sulfate reduction.

Chapter 7: Materials and methods

This chapter includes the methods for the construction of the Rex marker-exchange deletion that was published by *Frontiers in Microbiology*, April 2014 | doi: 10.3389/fmicb.2014.00153. It is presented here with minor revisions.

This chapter includes the methods for the *in vitro* and *in vivo* characterization of Rex_{DvH} that will be published by *Journal of Bacteriology*, January 2015 | doi: 10.1128/JB.02083-14. It is presented here with minor revisions.

Additionally, this chapter includes methods that are currently *in prep*, for submission to *Applied and Environmental Microbiology*.

Rex_{DvH} alters the electron flow between internal and external reduction processes in *Desulfovibrio vulgaris* Hildenborough.

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Section 7. 1. Protein sequence modelling

The protein sequences for Rex for *T. aquaticus*, *S. coelicolor*, *B. subtilis*, and DvH were obtained from NCBI (Thompson et al. 1994) and protein identity calculated with BioEdit (Hall 1999). DVU0916 protein sequence was modelled by SWISS-MODEL (Arnold et al. 2006; Guex et al. 2009; Kiefer et al. 2009; Biasini et al. 2014). This database includes >385,000 chains encompassing 68493 unique sequences. The sequence was first automated via sequence alignment to the database for the “best-fit” model. This was followed by alignment to two solved Rex structures (pdb:3IKT and 1XCB) as well as additional targets discussed elsewhere. For the alignment mode, DVU0916 protein sequence was first aligned to Rex from *T. aquaticus* with LAlign (Huang et al. 1991) (parameters: matrix file BL50 (15/-5), gap-open/ext: 14/-4 E(limit) 0.05)) resulting in a 45.8% identity, 201Aa overlap, BLAST probability 5.5e-47. This template-target alignment was then used to model DVU0916 to pdb 3IKT chains A and B (2.26Å crystal structure of a Rex-family repressor/DNA/NAD⁺ complex from *T. aquaticus*, (McLaughlin et al. 2010)), and pdb 1XCB chains A, B, C, D, E, F and G (2.90Å crystal structure of a Rex-family repressor/NADH complex from *T. aquaticus* (Sickmier et al. 2005)). Visual Molecular Dynamics (VMD) (Humphrey et al. 1996) and PV (protein viewer) were used to visualize the threaded structure.

Section 7. 2. Basic supplies and protocols

Section 7. 2. 1. Antibiotics and inhibitors

Inhibitors for DvH strains were as follows: Geneticin® (G418, 400 µg/ml), spectinomycin (Sp, 100 µg/ml), kanamycin (Km, 50 µg/ml), ampicillin (Ap, 100 µg/ml), 5-fluorouracil (5-FU, 40 µg/ml) or molybdate (Mo, 1 M stock) obtained from Thermo Fisher Scientific (Waltham, MA), Sigma-Aldrich (St. Louis, MO), or Gold Biotechnologies® (St. Louis, MO).

Section 7. 2. 2. Primers/Oligonucleotides

Oligonucleotides necessary for these studies were purchased from Integrated DNA Technologies (IDT, Coralville, IA). The lyophilized pellet was rehydrated to a final stock concentration of 100 µM and stored at -20°C until use. Primers are kept at 4°C if they are to be used frequently within the week. Primer concentrations and quality (ratio A_{260}/A_{280}) were calculated from Nanodrop ND-1000 spectrophotometric readings (Thermo Fisher Scientific) after initial rehydration. Primers necessary for a given project are listed in separate tables in their specified section.

Section 7. 2. 3. Sequence analysis

Sequence analysis was performed by University of Missouri DNA Core Facility. For each DNA sample to be sequence, a 16 µl reaction volume was provided. This included ~1000 ng DNA template (50-500 ng PCR or plasmid DNA), 20 pmol specified primer. For templates that were GC-rich or failed the first read, 5% (vol/vol) DMSO was added per their suggestion.

Section 7. 2. 4. Culturing techniques for DvH

The growth of all cultures was measured in Balch tubes by optical density (OD₆₀₀ nm) with a Spectronic™ GENESYS™ 20 spectrophotometer (Thermo Fisher Scientific). *E. coli* strains were grown at 37°C aerobically in 5 ml LC medium containing per liter (10 g tryptone, 5 g sodium chloride, and 5 g yeast extract), pH 7.0. DvH strains were started from freezer stocks that contained 10% (vol/vol) glycerol in growth medium and frozen at -80°C. Growth medium was generally MO medium (Zane et al. 2010) supplemented with 0.1% (wt/vol) yeast extract and 60 mM lactate with 30 mM sulfate (MOYLS4). The medium was reduced with 1.2 mM sodium thioglycolate and pH was adjusted to pH 7.2 with 12 M HCl. DvH cultures were grown in an anaerobic chamber (Coy Laboratory Product, Inc., Grass Lake, MI) overnight until stationary phase (OD₆₀₀ >1). The atmosphere of the chamber was approximately 95% N₂ and 5% H₂. A 2% (vol/vol) inoculum was used to start 5 ml cultures in defined medium (MO) containing lactate (60 mM, L) or pyruvate (60 mM, P) as the electron donor and sulfate (30 mM, S4), sulfite (40 mM, S3) or thiosulfate (30 mM, T3) as the electron acceptor or MOYPyr (60 mM pyruvate, supplemented with 0.1% (wt/vol) yeast extract and 0.5 mM cysteine). For pyruvate-fermenting cultures, cysteine was provided as a sulfur source and reductant. DvH did not grow on yeast extract alone in the absence of pyruvate. Validation of this is shown in Chapter 5.

Section 7. 2. 5. Electroporation

Standard electroporation protocols were followed with an ECM 630 electroporator (BTX) (Keller et al. 2011), with parameters: 1500V 250Ω and 25uF. Typically, 300-500 ng of template DNA was electroporated into 50 µl of a 50 ml culture that had been washed with TE buffer and resuspended into 300-500 µl (depending on density of the culture and the

number of samples to be electroporated). A 1-ml aliquot of rich medium (MOYLS4) is added to the samples and let recover overnight at 34°C within an anaerobic chamber. The next day the cells are plated (typically 10 µl, 100 µl and 880 µl) on the desired media.

Section 7. 2. 6. DNA purification

Genomic DNA (Wizard® Genomic DNA Purification Kit, Promega, Madison WI) and plasmid (GeneJET plasmid kit, Thermo Fisher Scientific) were purified according to manufacturer's protocol from a 1.5-ml aliquot of an overnight culture (OD₆₀₀>1). DNA fragments (via PCR or primer annealing) were purified according to manufacturer's protocol (Wizard® SV Gel and PCR Clean-Up System [Promega]). DNA concentrations and quality (ratio A₂₆₀/A₂₈₀) were calculated from Nanodrop ND-1000 spectrophotometric readings (Thermo Fisher Scientific).

Section 7. 2. 7. RNA purification

Samples to be analyzed for RNA content were cultured as described in the culturing DvH section. At the specified optical density, 4-ml samples were spun down anaerobically at 34 °C (10 min at 5600 x g) and resuspended in 1 ml TRI Reagent® (Sigma-Aldrich). RNA was purified based on phenol extraction and ethanol precipitation methods previously described (Chomczynski et al. 1987; 2006). RNA quality was assessed visually in an agarose denaturing gel and an A₂₆₀/A₂₈₀ ratio >1.8 was required (Figure 7-1). RNA samples were treated with TURBO™ DNase (Life Technologies, Carlsbad CA) according to the manufacturer's protocol and confirmed by PCR to be free of gDNA (Figure 7-1).

Figure 7-1: RNA quality assurance. A) RNA gel. Seven RNA samples after ethanol precipitation are electrophoresed on a 1.5% (wt/vol) denaturing agarose gel. The marker is RiboRuler High Range (Thermo Fisher Scientific). B) DNA contamination screen of seven samples after RNA isolation protocol. M) 1kb plus ladder (Fermentas), +/- with or without gDNA DvH (+/- controls). RNA (untreated): RNA samples after initial RNA purification. RNA (treated): Isolated RNA samples after DNase Treatment. Samples properly treated no longer generate a band (~500 bp).

Section 7. 2. 8. Polymerase Chain Reaction (PCR)

All DNA to be amplified by PCR followed standard protocols. For typical reactions (e.g. for screening purposes) either *Taq* DNA polymerase (NEB) or Bullseye *Taq* DNA polymerase (MIDSCI, St Louis, MO) were used. When accuracy was essential (e.g. cloning) Herculase® II Fusion DNA polymerase (Agilent, Santa Clara, CA) was used. Reaction mixes were set up according to the manufacturer's protocol, with the annealing temperature set for 1-5°C less than the T_m of the primers. If multiple bands or no product was obtained after 30 cycles, the experiment was set-up a second time with 5% (vol/vol) DMSO added.

Section 7. 3. Strains and plasmids

All strains in this study are listed in Table 7-1 and plasmids in Table 7-2. Strains designated with JW33XX were those I personally constructed. Additional strains were constructed by the Wall laboratory, specifically Grant Zane, or were purchased as annotated. Plasmids designated with pMO33X were those I personally constructed. Primers necessary for strain generation are listed in Table 7-3. Plasmids are confirmed by Sanger sequencing by DNA Core Facility at University of Missouri.

The general strategy used to generate a desired deletion strain is described in great detail elsewhere (Keller et al. 2009; Keller et al. 2011; Keller et al. 2011). In brief, a plasmid is constructed by the process of Sequence Ligation Independent Cloning (SLIC)(Li et al. 2007), which contained two flanking homologous regions to the sequence to be deleted. Additionally, this plasmid has a selectable and counter-selectable marker for isolation purposes. This sequence-confirmed plasmid is transformed into the designated DvH strain and selected on the appropriate antibiotics followed by confirming the strain by Southern blot (Zane et al. 2010).

Table 7-1: Strains

Strain	Genotype or relevant characteristics ^a	Source or reference
Strain:		
<i>E. coli</i>		
α-Select (Silver Efficiency)	<i>deoR endA1 recA1 relA1 gyrA96 hsdR17</i> (r _k ⁻ , m _k ⁺) <i>supE44 thi-1 Δ(lacZYA-argFV169) Φ80dl acZΔM15 F⁻</i>	Bioline
BL21 (DE3) competent cells	<i>E. coli</i> B F ⁻ <i>dcm ompT hsdS</i> (r _B ⁻ m _B ⁻) <i>galλ</i> (DE3)	Agilent
<i>Desulfovibrio vulgaris</i> Hildenborough		
ATCC29579	Wild-type <i>D. vulgaris</i> Hildenborough; 5-FU ^s	ATCC
JW710	WT <i>Δupp</i> ; 5-FU ^r (used as “WT” control for DvH growth kinetics in this study; parent strain for deletions; retains pDV1 present in WT)	(Keller et al. 2009)
JW3311	JW710 <i>ΔDVU0916::(npt upp)</i> ; Km ^r ; 5-FU ^s (<i>Δrex</i> marker exchange)	(Korte et al. 2014)
JW3317	JW710 <i>ΔDVU0916-0915::(npt upp)</i> ; Km ^r ; 5-FU ^s (<i>Δrex</i> and <i>ΔDVU0915</i> marker exchange)	This study
JW3319	JW710 <i>ΔDVU0916</i> ; Km ^s ; 5-FU ^r (<i>Δrex</i> marker-less deletion)	This study
JW9011	JW710 <i>ΔDVU2547::(npt upp)</i> ; Km ^r ; 5-FU ^s (<i>ΔhcrR</i> marker exchange)	(Zhou et al. 2012)
JW9293	JW710 <i>Δ₋₁₅₀₋₁P_{sat}::(npt upp)</i> ; Km ^r ; 5-FU ^s (<i>P_{sat}</i> disruption)	(Christensen et al. 2014)
JW9312	JW710; Km ^s ; 5-FU ^r (<i>sat</i> promoter restored)	(Christensen et al. 2014)
JW9314	JW9293 G ₋₁₄₇ A <i>P_{sat}</i> ; Km ^s ; 5-FU ^r (G ₋₁₄₇ A)	(Christensen et al. 2014)
JW9316	JW9293 GTA ₋₁₄₇₋₁₄₅ ACG <i>P_{sat}</i> ; Km ^s ; 5-FU ^r (IR1)	(Christensen et al. 2014)
JW9318	JW9293 CAC ₋₁₃₆₋₁₃₄ TGT <i>P_{sat}</i> ; Km ^s ; 5-FU ^r (IR2)	(Christensen et al. 2014)
JW9320	JW9293 GTA ₋₁₄₇₋₁₄₅ ACG <i>P_{sat}</i> and CAC ₋₁₃₆₋₁₃₄ TGT <i>P_{sat}</i> ; Km ^s ; 5-FU ^r (IR1and2)	(Christensen et al. 2014)
JW9011	JW710 <i>ΔDVU2547::(npt upp)</i> ; Km ^r ; 5-FU ^s (<i>ΔhcrR</i> marker exchange)	(Zhou et al. 2012)
GZ0481	Genome position 2680507::Tn5-RL27; insertion 273 bp from predicted AUG start codon within DVU2567; Km ^r (LysX mutant)	Wall Laboratory
GZ1801	Genome position 2804495::Tn5-RL27; insertion 178 bp from predicted AUG start codon within DVU0269; Km ^r (DVU2690 mutant)	Wall Laboratory
GZ4443	Genome position 1238583::Tn5-RL27; insertion 52 bp from predicted AUG start codon within DVU1142; Km ^r (DVU1142 mutant)	Wall Laboratory
GZ5190	Genome position 2364109::Tn5-RL27; insertion 118 bp from predicted AUG start codon within DVU5190; Km ^r (RocR mutant)	Wall Laboratory
GZ7888	Genome position 64424::Tn5-RL27; insertion 535 bp from predicted AUG start codon within DVU0057; Km ^r (DVU0057 mutant)	Wall Laboratory
GZ8929	Genome position 218225::Tn5-RL27; insertion 366 bp from predicted AUG start codon within <i>phsA</i> ; Km ^r (<i>phsA</i> mutant)	Wall Laboratory
GZ12386	Genome position 2900951::Tn5-RL27; insertion 102 bp from predicted AUG start codon within DVU2799; Km ^r (DVU2799 mutant)	Wall Laboratory
GZ12635	Genome position 832548::Tn5-RL27; insertion 80 bp from predicted AUG start codon within DVU0744; Km ^r (DVU0744 mutant)	Wall Laboratory
GZ12985	Genome position 2905014::Tn5-RL27; insertion 75 bp from predicted AUG start codon within DVU2802; Km ^r (DVU2802 mutant)	Wall Laboratory

^aKm, kanamycin; Sp, spectinomycin; Ap, ampicillin; 5-FU, 5-fluorouracil; superscript “r” or “s”, resistance or sensitivity

Table 7-2: Plasmids

Plasmid	Genotype or relevant characteristics ^a	Source or reference
pET14b	6XHis-tag fusion protein vector with T7 promoter	Novagen
pMO719	pCR8/GW/TOPO containing SRB replicon (pBG1); Sp ^r ; source of Sp ^r and pUC <i>ori</i> fragment; for marker exchange suicide plasmid construction	(Keller et al. 2009)
pMO746	Source of <i>upp</i> in artificial operon with <i>npt</i> and Ap ^r -pUC <i>ori</i> ; P _{<i>npt-upp</i>} ; Km ^r ; 5-FU ^s ; for marker exchange suicide plasmid construction	(Parks et al. 2013)
pMO3311	Sp ^r and pUC <i>ori</i> from pMO719 plus 1630 bp upstream and 1590 bp downstream DNA regions from DVU0916 (<i>rex</i>) flanking the artificial operon of P _{<i>npt-upp</i>} from pMO746; marker exchange deletion of <i>rex</i> Sp ^r ; Km ^r	(Korte et al. 2014)
pMO3312	pET14b plus <i>rex</i> (without start codon, 642 bp); Ap ^r ; for Rex expression in BL21 (DE3) competent cells.	(Christensen et al. 2014)
pMO3313	pMO9075 with DVU0916 (<i>rex</i>) constitutively expressed from P _{<i>npt</i>}	(Korte et al. 2014)
pMO3316	pMO9075 with DVU0916 (<i>rex</i>) and DVU0915 constitutively expressed from P _{<i>npt</i>}	This study
pMO3317	Sp ^r and pUC <i>ori</i> from pMO719 plus 1630 bp upstream of <i>rex</i> and 669 bp downstream of DVU0915 DNA regions flanking the artificial operon of P _{<i>npt-upp</i>} from pMO746; marker exchange deletion of <i>rex</i> and DVU0915 Sp ^r ; Km ^r	This study
pMO3319	Sp ^r and pUC <i>ori</i> from pMO719 plus 1630 bp upstream and 1590 bp downstream DNA region from DVU0916 (<i>rex</i>); marker-less deletion plasmid; Sp ^r	This study
pMO9075	pMO719 containing P _{<i>npt</i>} for constitutive expression of complementation constructs; pBG1 stable SRB replicon; Sp ^r	(Keller et al. 2011)
pMO9292	Sp ^r and pUC <i>ori</i> from pMO719 plus 383 bp upstream and 319 bps downstream DNA regions from P _{<i>sat</i>} (-150--1) flanking the artificial operon of P _{<i>npt-upp</i>} from pMO746; for marker exchange mutagenesis; Sp ^r and Km ^r	(Christensen et al. 2014)
pMO9311	Sp ^r and pUC <i>ori</i> from pMO719 plus 403 bp upstream and 467 bp downstream DNA regions from P _{<i>sat</i>} (-150); wild-type sequence; Sp ^r ; for site directed mutagenesis	(Christensen et al. 2014)
pMO9313	Sp ^r and pUC <i>ori</i> from pMO719 plus 403 bp upstream and 467 bp downstream DNA regions from P _{<i>sat</i>} (-150); G ₋₁₄₇ A P _{<i>sat</i>} ; Sp ^r ; site directed mutagenesis	(Christensen et al. 2014)
pMO9315	Sp ^r and pUC <i>ori</i> from pMO719 plus 403 bp upstream and 467 bp downstream DNA regions from P _{<i>sat</i>} (-150); GTA ₋₁₄₇₋₋₁₄₅ ACG P _{<i>sat</i>} ; Sp ^r ; for site directed mutagenesis	(Christensen et al. 2014)
pMO9317	Sp ^r and pUC <i>ori</i> from pMO719 plus 403 bp upstream and 467 bp downstream DNA regions from P _{<i>sat</i>} (-150); CAC ₋₁₃₆₋₋₁₃₄ TGT P _{<i>sat</i>} ; Sp ^r ; for site directed mutagenesis	(Christensen et al. 2014)
pMO9319	Sp ^r and pUC <i>ori</i> from pMO719 plus 403 bp upstream and 467 bp downstream DNA regions from P _{<i>sat</i>} (-150); GTA ₋₁₄₇₋₋₁₄₅ ACG P _{<i>sat</i>} and CAC ₋₁₃₆₋₋₁₃₄ TGT P _{<i>sat</i>} ; Sp ^r ; for site directed mutagenesis	(Christensen et al. 2014)
pRL27	Tn5-RL27 (Km ^r - <i>ori</i> R6 K) delivery vector; for transposon mutagenesis of DvH strains	(Larsen et al. 2002)

^aKm, kanamycin; Sp, spectinomycin; Ap, ampicillin; 5-FU, 5-fluorouracil; superscript “r” or “s”, resistance or sensitivity

Section 7. 3. 1. Marker-exchange deletion of *rex*

A marker-exchange deletion (MED) of *rex* (JW3311) was constructed. In brief, a plasmid (designated pMO3311) was constructed by the process of SLIC (Li et al. 2007; Keller et al. 2009; Korte et al. 2014). pMO3311 included 1630 bases upstream of the putative AUG start codon and 1590 bases downstream of *rex* from DvH amplified from gDNA; 1885 bases from pMO719 that included the spectinomycin resistance marker and pUC *ori* amplified from pMO719; and a kanamycin resistance cassette coupled with a counter-selectable marker (designated as KanR*upp*) amplified from pMO746. This plasmid was electroporated into the Δ *upp* strain, JW710, with an ECM 630 electroporator (BTX) following common protocols with parameters: 1500V, 250 Ω and 25uF (Keller et al. 2011). The cells were recovered in 1 ml MOYLS4 (supplemented with 1 mM sulfite) overnight at 34°C in a 1.5 ml microfuge tube. Recovered cells were plated on MOYLS4 + G418 and incubated for 5 days. Individual colony forming units (cfus) were replica screened on MOYLS4, MOYLS4+sp, and MOYLS4+G418. Three cfus with the correct phenotype (G418^r, Sp^s) were toothpicked into 5 ml cultures (MOYLS4+G418) and incubated overnight at 34°C. gDNA was isolated from each culture and a southern blot (Brown 1993; Zane et al. 2010) was performed with restriction enzyme *Bgl*I (NEB). *Bgl*I-digested genomic DNAs were probed with a PCR fragment upstream of the *rex* gene. A DNA band of 4129 bps showed hybridization in the wild-type sample, compared with a fragment of 2645 bps for a correctly constructed MED in JW3311 (Figure 7-2). Rex mutant was further confirmed by sensitivity to molybdate (Figure 5-3). A correctly identified construct, MED strain for *rex*, was designated JW3311. Freezer stocks were made and contain 10% (vol/vol) glycerol.

Figure 7-2: Rex mutants confirmed by Southern blot. A) DNA agarose gel of *Bgl*I-digested gDNA from parental (JW710), *rex* marker-exchange deletion (MED, JW3311) and *rex* marker-less deletion (MLD, JW3319) strains. Lanes: 1: ladder; 2, JW710; 3 *rex* MED; and 4, *rex* MLD. B) X-ray film of gDNA digested with *Bgl*I, and probed for an ~1kb fragment. Expected sizes are: JW710 4129 bp, JW3311 2488 bp and JW3319 3484 bp.

Section 7. 3. 2. Marker-less deletion of *rex*

A marker-less in frame deletion of *rex* (JW3319) was constructed. In brief, a plasmid (designated pMO3319) was constructed by the process of sequence and ligation independent cloning (Li et al. 2007; Keller et al. 2009; Korte et al. 2014). pMO3319 included 1630 bases upstream of the putative AUG start codon and 1590 bases downstream of *rex* from DvH, and 1885 bases from pMO719 that included the spectinomycin resistance marker and pUC *ori*. This plasmid was electroporated into the *rex* marker exchange strain, JW3311 (Korte et al. 2014). The cells were recovered overnight in 1 ml MOYLS4 and plated for single colonies in molten MOYLS4 + 5-FU for growth at 34°C. After five days, colony forming units (cfus) were counted and screened by replica plating for 5-FU^r, Km^s and Sp^s phenotypes. Five cfus

from the MOYLS4 plate with the correct phenotype were toothpicked into 5 ml MOYLS4 and incubated overnight at 34°C. gDNA was purified and the deletion was confirmed by Southern blot (Figure 7-2) (Zane et al. 2010) and freezer stocks to 10% (vol/vol) glycerol were made.

One final mutant, JW3317, was also constructed by the same protocol. This strain was a marker-less deletion of *rex* through the downstream gene, DVU0915. To generate this strain, a plasmid, pMO3317, was constructed to include the same upstream region as described above but included 669 bps of a homologous region downstream of DVU0915. This plasmid was then transformed into the marker-exchange strain, JW3311, and the normal strain construction protocol was followed.

Section 7. 3. 3. Complementation strategy

The strategy for complementation was to transform a plasmid containing the gene of interest back into the specified gene deleted strain (Zane et al. 2010). The gene of interest would be downstream of a constitutive promoter and, therefore, would be expressed most likely at a higher than normal level. Each plasmid was constructed by the process of SLIC (Li et al. 2007), generating the backbone from pMO9075 with primers pMO9075-SLIC-F and pMO9075-SLIC-R3. The sequence confirmed plasmid was electroporated into the desired DvH strain according to standard protocol and selected for spectinomycin resistance. Plasmid was then purified from the DvH strain and passed back through *E.coli* for amplification purposes and the sequence was confirmed.

Section 7. 3. 4. Sat promoter mutants

The protocol used to generate the strain lacking the *sat* promoter (ΔP_{sat}), JW9293 conferring Km^r and 5-FU^s, has previously been described (Korte et al. 2014). Briefly, 150 bp upstream

of *sat* (-150 to -1) was replaced with a cassette conferring resistance to kanamycin and a counter-selectable marker (*upp*, the gene encoding uracil phosphoribosyltransferase that confers 5-fluorouracil sensitivity to the 5-FU^r parental strain). To accomplish the cassette insertion, a delivery plasmid (pMO9292) was constructed that contained the selectable cassette flanked by chromosomal regions from either side of the promoter sequence to be deleted. This plasmid was electroporated into the parental strain, JW710, and selected for kanamycin resistance and screened for 5FU sensitivity. The successful recombinant was designated as JW9293. For the construction of the subsequent P_{*sat*} mutants with alterations to the Rex_{DvH}-binding site (JW9312, JW9314, JW9316, JW9318, JW9320) a similar protocol as (Parks et al. 2013) was followed. JW9293 was transformed with non-replicating plasmids with individual site-specific mutations in the 150 bp fragment flanked by DNA homologous to that on either side of the integrated cassette. These P_{*sat*} mutant or restored strains were selected as 5-FU^r and screened for kanamycin sensitivity. The accuracy of the constructs was confirmed by sequencing both strands of a PCR amplified product across the mutation site. The deletion strain and promoter mutation strains that prevented *sat* transcription were expected not to grow with sulfate as electron acceptor and therefore 20 mM sulfite was used in medium to recover these promoter mutations.

Section 7. 3. 5. Rex protein expression cell-line

A T7 promoter, amino terminal histidine tagged, *rex* expression plasmid (designated pMO3312) was constructed in *E. coli*. by the process of SLIC (Li et al. 2007) (Figure 7-3) from *rex* and the vector pET-14b. Primers used are listed in Table 7-3. The polymerase used for PCR was Herculanase II Fusion DNA Polymerase. In brief, *rex*, without the translational start codon (ATG) was amplified by PCR from DvH gDNA (primers: DVU0916-explone-F and DVU0916-explone-R, 642 bp not including the additional overhangs added for

cloning purposes). The majority of the pET-14b vector (not including 45 bases of the multi-cloning site) was amplified by PCR in two fragments. The first fragment contained the T7 promoter, ribosomal binding site, a 6x-histidine-tag, and a thrombin cut-site (primers: pET14b-1 and pET14b-22, 2387 bp), while the second fragment included the T7 terminator and an ampicillin resistance cassette (primers: pET14b-3 and pET14b-4, 2486 bp). The plasmid, pMO3312 (5268 bp), was sequenced across the insert with primers pET14b-seq-F and pET14b-seq-R and 1 ml freezer stocks (10% vol/vol glycerol) were made. A detailed description of protein expression and purification is described in a following section.

Figure 7-3: construction of pMO3312 (Rex expression plasmid).

pMO3312 was generated by the process of SLIC (Li et al. 2007). Three fragments (A, B, C) were PCR amplified, A and B from pET14b, while C (containing the *rex* gene (without the start codon)) was amplified from genomic DNA. Primers for amplification of each fragment are shown as arrows. Overhanging regions are color coded to match where they align. Multi-cloning site (MCS); AmpR refers to antibiotic resistance marker for ampicillin; RC, reverse complement primer. The figure to the right is the constructed plasmid, pMO3312, color coded based on the three fragments used to generated the construct, with the T7 promoter identified.

Table 7-3: Primer list for strains and plasmids generation

Primer Name	Sequence	Length	Source	Descriptor
General Primers				
SpecRpUC-up	GGGAAACGCCTGGTATCTTTATAG TCCT	28	(Parks et al. 2013)	For colony PCR, screen of and sequencing upstream region of deletion strains. Forward primer.
pMO719XbaI-Dn	TGGGTTCGTGCCTTCATCCG	20	(Parks et al. 2013)	For colony PCR, screen of and sequencing upstream region of deletion strains. Reverse primer.
Kan-int-Fwd-rev-comp	CTCATCCTGTCTCTTGATCAGATCT	25	(Parks et al. 2013)	Sequencing Primer. Forward Primer.
DvH-Upp gene Cterm-out	GCTGAAGCGCATCGTGGACAA	21	(Parks et al. 2013)	Sequencing Primer. Reverse Primer.
pBG1-2199-F	GCTGAAAGCGAGAAGAGCGCAC	22	(Parks et al. 2013)	Sequencing Primer. Forward Primer.
SpecRpUC-F	CCAGCCAGGACAGAAATGCCTCG	23	(Parks et al. 2013)	For amplification of Sp ^r and pUC ori from pMO719 to make MED plasmid. Used as overhang for SLIC. Forward Primer.
SpecRpUC-R	ATGTGAGCAAAAGGCCAGCAAAAG GC	26	(Parks et al. 2013)	For amplification of Sp ^r and pUC <i>ori</i> from pMO719 to make MED plasmid. Used as overhang for SLIC. Reverse Primer.
KanPromNterm	CCGGAATTGCCAGCTGGG	18	(Parks et al. 2013)	For amplification of Km ^r from pMO719 to make MED plasmid. Used as overhang for SLIC. Forward Primer.
UPPCTerm	CTTACTTGGTGCCGAATATCTTGTC GC	27	(Parks et al. 2013)	For amplification of Km ^r from pMO719 to make MED plasmid. Used as overhang for SLIC. reverse
Protein Expression Strain				
pET14b-1	tcgccacctctgacttgagc	20	(Christensen et al. 2014)	For amplification of a portion of pET14b with primer pET14b-22 to make pMO3312. Forward Primer.
pET14b-22	catatggctgccgcggcaccagg	25	(Christensen et al. 2014)	For amplification of a portion of pET14b with primer pET14b-1 to make pMO3312. Reverse Primer.
pET14b-3	tcagggataacgcaggaaagaacatg	27	(Christensen et al. 2014)	For amplification of a portion of pET14b with primer pET14b-4 to make pMO3312. Reverse Primer.
pET14b-4	gctgctgccaccgctgagcaataa	24	(Christensen et al. 2014)	For amplification of a portion of pET14b with primer pET14b-3 to make pMO3312. Forward Primer.
DVU0916-explone-F	<u>cctggtgccgcgcgcagccatg</u> ACCAACATC AAAAGCGAACACATCCC	51	(Christensen et al. 2014)	For amplification of DVU0916 (not including start codon) from gDNA with primer DVU0916-explone-R. Underlined portion used as overhang for SLIC with fragment A (primer pET14b-22). Forward Primer.
DVU0916-explone-R	<u>ttattgctcagcggtgcgcagc</u> CTATTGTTGC GCGAGAACGTGATGT	50	(Christensen et al. 2014)	For amplification of DVU0916 (stop codon) from gDNA with primer DVU0916-explone-F. Underlined portion used as overhang for SLIC with fragment B (primer pET14b-4). Reverse Primer.
pET14b-seq-F	aagtggcgagcccgatcttc	20	(Christensen et al. 2014)	Sequencing primer located within pET14b in direction of insert. Forward.

pET14b-seq-R	ctgctcgcttcgctacttgga	21	(Christensen et al. 2014)	Sequencing primer located within pET14b in direction of insert. Reverse
Rex Marker-Exchange Deletions (MED)				
DVU0916-1	GCCTTTTGCTGGCCTTTGTCTCACA TGATGCTGAGAAGTTCGGTCCGAA G	50	(Korte et al. 2014)	For amplification of DVU0916 upstream region from gDNA with DVU0916-2 primer to make pMO3311. Underlined portion used as overhang for SLIC with Spr,pUC ori fragment (SpecRpUC-R). Amplification of Southern probe for confirmation of DVU0916 deletion. Forward Primer.
DVU0916-2	GCGACAAGATATTTCGGCACCAAGT AAGCGTTCGTTAACTTCACTTTTGT CAATGCAC	57	(Korte et al. 2014)	For amplification of DVU0916 upstream from gDNA with DVU0916-1 primer to make pMO3311. Underlined portion used as overhang for SLIC with Kmr, upp fragment (UppCterm). Amplification of Southern probe for confirmation of DVU0916 deletion. Reverse Primer.
DVU0916-3	GCGCCCCAGCTGGCAATTCGGG CTGGAGCGTGAACGCCTCC	42	(Korte et al. 2014)	For amplification of DVU0916 downstream from gDNA with DVU0916-4 to make pMO3311. Underlined portion used as overhang for SLIC with Kmr, upp fragment (KanPromNterm). Forward Primer.
DVU0916-4	GTCGAGGCATTTCTGTCTGGCTG GGATTTTCATGGGCCCCGATGTATT GG	50	(Korte et al. 2014)	For amplification of DVU0916 upstream region from gDNA with DVU0916-3 primer to make pMO3311. Underlined portion used as overhang for SLIC with Spr,pUC ori fragment (SpecRpUC-F). Reverse Primer.
DVU0916-UP-int-F	CCTACGGCCAACGTCAACACCAAC	23	(Korte et al. 2014)	Sequencing primer to confirm upstream region of deletion cassette of pMO3311. Forward Primer.
DVU0916-UP-int-R	GTGGGTGTTGACGTTGGCCGTAGG	24	(Korte et al. 2014)	Sequencing primer to confirm upstream region of deletion cassette of pMO3311. Reverse Primer.
DVU0916-DWN-int-F	GGATAGCGTGACATTCCTGGACGT G	25	(Korte et al. 2014)	Sequencing primer to confirm downstream region of deletion cassette of pMO3311. Forward Primer.
DVU0916-DWN-int-R	CACGTCCGGGAATGTCACGCTATC C	25	(Korte et al. 2014)	Sequencing primer to confirm downstream region of deletion cassette of pMO3311. Reverse Primer.
Rex Marker-Less Deletions (MLD)				
DVU0916-UP-MLD-R	GGAGGCGTTACGCTCCAGCGTTC GTTAACTTCACTTTTGTCAATGCAC	49	This study	For amplification of upstream fragment of DVU0916 with primer DVU0916-1 to make pMO3319. Reverse Primer.
DVU0916-DWN-MLD-F	GTGCAATTGCAAAAAGTGAAGTTAA CGAACGCTGGAGCGTGAACGCCTC C	49	This study	For amplification of downstream fragment of DVU0916 with primer DVU0916-4 to make pMO3319. Forward Primer.
Complement				
SLIC-DVU0916-comp-F	AGGTTGGGAAGCCCTGCAATGCAG TCCCAGGAGGTACCATATGACCAA CATCAAAAGCGAACACATCC	68	(Korte et al. 2014)	For amplification of DVU0916 to make pMO3313 complementation construct. Underlined portion used as overhang for SLIC assembly with pMO9075 fragment. Forward.
SLIC-DVU0916-comp-R	GATCGTGATCCCCGCGCCATCAG ATCCTTGCTATTTGTTGCGCGAGA ACGTGATGTT	58	(Korte et al. 2014)	For amplification of DVU0916 to make pMO3313 complementation construct. Underlined portion used as overhang for SLIC assembly with pMO9075 fragment. reverse

pMO9075-SLIC-F	CAAGGATCTGATGGCGCAGGG	22	(Korte et al. 2014)	For amplification of pMO9075 fragment for SLIC to make pMO3313 complementation construct. forward
pMO9075-SLIC-R3	CTGGGACTGCATTGCAGGGCTTCC CAACCT	21	(Korte et al. 2014)	For amplification of pMO9075 fragment for SLIC to make pMO3313 complementation construct. reverse
Sat Promoter Mutant strains				
DVU1295-dProm-upF	<u>gccttttgctggccttttgctcacat</u> CCTACCCGGC AACGATGCG	45	(Christensen et al. 2014)	For amplification of upstream fragment of DVU1295 with primer DVU1295-dProm-upR to make pMO9292. DvH gDNA as template. Underlined portion used as overhang for SLIC with fragment backbone (primer SpecRpUC-R). Forward Primer.
DVU1295-dProm-upR	<u>GCGACAAGATATTCGGCACCAAGT</u> <u>AAGATGTCAAGCGGCAAAGCGCG</u>	47	(Christensen et al. 2014)	For amplification of upstream fragment of DVU1295 with primer DVU1295-dProm-upF to make pMO9292. DvH gDNA as template. Underlined portion used as overhang for SLIC with fragment kanRUPP (primer UPPCTerm). Reverse Primer.
DVU1295-dProm-dnF	<u>GCGCCCCAGCTGGCAATTCCGGAT</u> GTCCAAGCTGGTCCCCGCTC	44	(Christensen et al. 2014)	For amplification of downstream fragment (start codon of DVU1295) with primer DVU1295-dProm-dnR to make pMO9292. DvH gDNA as template. Underlined portion used as overhang for SLIC with fragment KanRUPP (primer KanPromNTerm). Forward Primer.
DVU1295-dProm-dnR	<u>cgaggcatttctgctcctgct</u> GGACGAGAGCGA TTTCCTGACC	43	(Christensen et al. 2014)	For amplification of downstream fragment with primer DVU1295-dProm-DnF to make pMO9292. DvH gDNA as template. Underlined portion used as overhang for SLIC with fragment backbone (primerSpecRpUC-F). Reverse Primer.
DVU1295-upF	ACCGTCGTACATGAGTCGGTTGAT G	25	(Christensen et al. 2014)	For amplification of <i>sat</i> promoter for confirmation of point mutations with primer DVU1295-upR. Forward Primer.
DVU1295-upR	ACCTTACATCCTCCAGATGCGTGAT G	26	(Christensen et al. 2014)	For amplification of <i>sat</i> promoter for confirmation of point mutations with primer DVU1295-upF. Reverse Primer.
sat-ROP-R	<u>CTTGTAATAAAATTTACAAA</u> atgtcaag cggcaaagcgc	39	(Christensen et al. 2014)	For amplification of upstream fragment of DVU1295 with primer DVU1295-dProm-upF to make pMO9311. Underlined portion used as overhang for SLIC with fragment downstream (primer sat-ROP-F). Reverse Primer.
sat-ROP-F	<u>TTTGTAATAATTTTTCACAAG</u> acggaatca acgcgacgcc	39	(Christensen et al. 2014)	For amplification of downstream fragment of DVU1295 with primer DVU1295-dProm-dnR to make pMO9311. Underlined portion used as overhang for SLIC with fragment upstream (primer sat-ROP-R). Forward Primer
sat-ROP-GtoA-R	<u>CTTGTAATAAAATTTATAAAA</u> atgtcaag cggcaaagcgc	39	(Christensen et al. 2014)	For amplification of upstream fragment of DVU1295 with primer DVU1295-dProm-upF to make pMO9313. Underlined portion used as overhang for SLIC with fragment downstream (primer sat-ROP-GtoA-F). Reverse Primer
sat-ROP-GtoA-F	<u>TTTATAAATTTTTCACAAG</u> acggaatca acgcgacgcc	39	(Christensen et al. 2014)	For amplification of downstream fragment of DVU1295 with primer DVU1295-dProm-dnR to make pMO9313. Underlined portion used as overhang for SLIC with fragment upstream (primer sat-ROP-GtoA-R). Forward Primer

sat-ROP-IR1-R	<u>CTTGTGAAAAAATTCGTAAA</u> atgtcaag cggcaaagcgc	39	(Christensen et al. 2014)	For amplification of upstream fragment of DVU1295 with primer DVU1295-dProm-upF to make pMO9315. Underlined portion used as overhang for SLIC with fragment downstream (primer sat-ROP-IR1-F). Reverse Primer
sat-ROP-IR1-F	<u>TTTACGAATTTTTTCACAAG</u> acggaatca acgcgacgcc	39	(Christensen et al. 2014)	For amplification of downstream fragment of DVU1295 with primer DVU1295-dProm-dnR to make pMO9315. Underlined portion used as overhang for SLIC with fragment upstream (primer sat-ROP-IR1-R). Forward Primer
sat-ROP-IR2-R	<u>CTTACAAAAAATTTACAAA</u> atgtcaagc ggcaaagcgc	39	(Christensen et al. 2014)	For amplification of upstream fragment of DVU1295 with primer DVU1295-dProm-upF to make pMO9317. Underlined portion used as overhang for SLIC with fragment downstream (primer sat-ROP-IR2-F). Reverse Primer
sat-ROP-IR2-F	<u>TTTGTAAATTTTTTGTAAG</u> acggaatc aacgcgacgcc	39	(Christensen et al. 2014)	For amplification of downstream fragment of DVU1295 with primer DVU1295-dProm-dnR to make pMO9317. Underlined portion used as overhang for SLIC with fragment upstream (primer sat-ROP-IR2-R). Forward Primer
sat-ROP-IR1and2-R	<u>CTTACAAAAAATTCGTAAA</u> atgtcaag cggcaaagcgc	39	(Christensen et al. 2014)	For amplification of upstream fragment of DVU1295 with primer DVU1295-dProm-upF to make pMO9319. Underlined portion used as overhang for SLIC with fragment downstream (primer sat-ROP-IR1and2-F). Reverse Primer
sat-ROP-IR1and2-F	<u>TTTACGAATTTTTTGTAAG</u> acggaatc aacgcgacgcc	39	(Christensen et al. 2014)	For amplification of downstream fragment of DVU1295 with primer DVU1295-dProm-dnR to make pMO9319. Underlined portion used as overhang for SLIC with fragment upstream (primer sat-ROP-IR1and2-R). Forward Primer

Section 7. 4. Rex_{DvH} protein purification

To obtain Rex_{DvH}, a 6XHis tag was added cloning *rex* into pET-14b (Novagen, Madison, WI) (Figure 7-3) and transformed into BL21(DE3) competent cells (Agilent). After induction with isopropyl-beta-D-thiogalactopyranoside (Gold Biotechnologies®) the protein was purified by the His60 Ni Gravity Column Purification Kit (Clontech, Mountain View, CA).

In detail, 50 ng of pMO3312 was transformed, according to the manufacturer's protocol, plated on LC + ampicillin (100 µg/ml) and incubated overnight at 37°C. Individual isolates were PCR screened for plasmid (primers: pET14b-seq-F and pET14b-seq-R) and one correctly identified isolate was grown in 10 ml LC + ampicillin (100 µg/ml) overnight at 37°C and confirmed for the correct sequence. From the overnight culture, 5 ml was transferred to 95 ml LC (no antibiotics) and incubated at 26°C with shaking for 2.5 hours. Half of the culture (50 ml) was removed and frozen (-20°C) to be used as a non-induced (NI) control. IPTG (Gold Biotechnologies®) at 1 mM final concentration was added to the remaining 50 ml and then incubated at 26°C with shaking for an additional 2.5 hours, induced sample (I). Next, 45 ml of NI and I culture were spun down (4°C, 4150 rpm, 12 min) and the pellet was stored on ice. His-Rex protein was purified with the His60 Ni Gravity Column Purification Kit according to the manufacturer's protocol. The cell pellets were resuspended in xTractor Buffer (2 ml per 100 mg) and supplemented with TURBO™ DNase (2 U/100 mg pellet; Ambion), 1 mM phenylmethanesulfonyl fluoride (PMSF, 0.2 M stock in isopropanol) (Sigma-Aldrich)) and a protease inhibitor (Pierce Halt™ Protease Inhibitor Cocktail, EDTA-Free) (Thermo Fisher Scientific). The samples were incubated on ice for 15 min, mixing occasionally and then centrifuged for 20 min at 10,000 x g at 4°C. The supernatant was collected and stored on ice until sample preparation. While the samples were being centrifuged two columns were equilibrated with 10 bed volumes (1 ml = 1 bed

volume) of His60 Ni Equilibration Buffer. Protein sample (4 ml) was added to the column, resuspended with the resin and rotated for 1 hr at 4°C. The columns were allowed to settle, rinsed with 10 ml His60 Ni Equilibration Buffer, followed by 10 ml His60 Wash Buffer, and eluted with 10 ml His60 Ni Elution Buffer, collecting 1-ml fractions and analyzed for protein (Noble et al. 2009) (Figure 7-4). Samples with the greatest concentration were pooled together. The pooled sample was passed through a PD-10 desalting column (GE Healthcare Bio-Sciences, Piscataway, NJ) according to the manufacturer's protocol and analyzed by Bradford (Noble et al. 2009) or Qubit® Protein Assay Kit. Several 100 µl aliquots were made and stored at -20°C. An SDS-PAGE was performed on the purified protein to ensure purity of the Rex_{DvH} monomer (~25 kDa) (Figure 7-4). Each sample was mixed with an equal volume of 2X SDS loading dye and loaded to a 4-20% wt/vol Tris-Glycine-SDS polyacrylamide gel (Bio-Rad), and electrophoresed at 200 V (50 mAmps) for 30 min in 1X Tris-Glycine-SDS Buffer (Bio-Rad). The gel was then stained for 1 hr with 25 ml coomassie blue (per 2 liters: 2 g coomassie BB G-250, 140 ml acetic acid, 1000 ml methanol, 860 ml water, filter sterilized) and destained (140 ml acetic acid, 400 ml methanol, 60 ml glycerol, 1400 ml water) overnight. The presence of NADH bound to Rex_{DvH} was checked by measuring A₃₄₀ to ensure purity of the protein from additional co-factors (Figure 7-5).

The protocol by Brekasis and Paget (Brekasis et al. 2003) was used for DNA template generation of the relevant Rex_{DvH}-binding site (>100 bp). For smaller DNA fragments (40 bp), reverse complemented primers were annealed following previous protocols (Sigma-Aldrich). These sequences were 5'-end labeled with T4 Polynucleotide Kinase (Promega) and [γ -³²P] ATP (Perkin Elmer, Waltham MA) according to the manufacturer's protocol. The unlabeled nucleotides were removed by QIAquick Nucleotide Removal Kit (Qiagen, Valencia CA).

Figure 7-4: Protein purification of 6XHis-Rex (Rex_{DVH}). A) Elution of protein samples during protein purification in 1-ml fractions. Protein concentration (µg/ml) is plotted versus fraction. Fractions 1-3 were pooled together and passed over a desalting column. B) SDS-PAGE, stained with Coomassie Brilliant Blue G-250, of the purification process for 6XHis-Rex. Non-Induced (NI). Induced (I). Lanes as follows: (1) Empty, (2) Ladder, (3) Whole cell extract (NI), (4) Whole cell extract (I), (5) Lysed cell extract (NI), (6) Lysed cell extract (I), (7) After column purification (NI), (8) After column purification (I), (9) Ladder, (10) Ladder. Protein ladder is BLUEstain™ 3 (Gold Biotechnologies®). Expected size of Rex monomer is ~25 kDa.

Figure 7-5: Depletion of NADH in purified Rex_{DVH} sample. NADH is detected at A_{340} . Purified His-Rex_{DVH} protein (blue) is absent of A_{340} peak and therefore, does not contain NADH.

Section 7. 5. Rex operon determination by RT-PCR

Samples to be used for Reverse-Transcriptase PCR (RT-PCR) were initially collected from DNase-treated RNA samples that were stored at -80°C. A 5 µl aliquot (20 ng/µl) of DNase-treated RNA was reverse transcribed to single stranded cDNA via iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA). The amplification protocol is as follows: in a 20 µl reaction, 13.5 µl water, 1 µl template (water, gDNA, mRNA, or cDNA), 2 µl 10X *Taq* Polymerase Buffer, 1 µl dNTP mix (each dNTP at 1.5 mM), 1 µl each of specified Forward and Reverse primers (Table 7-4) and 0.5 µl *Taq* DNA Polymerase (NEB). In a thermocycler, 50 cycles of amplification were carried out (20 sec at 94°C, 20 sec at 57°C, and 90 sec at 72°C) followed by a final extension for 5 min at 72°C. A 10-µl aliquot of each product was electrophoresed (50 min at 110 V) on a 0.8% wt/vol agarose gel and analyzed for a single product.

Table 7-4: Primer list for Real-Time PCR (RT-PCR)

Name	Sequence (5'-3')	length	Source	Descriptor
DVU0847-3F	TGCTCGGTTTCGCA CTCCG	18	(Zane et al. 2010)	For amplification of <i>apsA</i> with primer DVU0847-4R for DNA contamination screen of RNA sample and RT-PCR. Forward primer.
DVU0847-4R	GGTGTAGTAGGTA CCCACGCCA	22	(Zane et al. 2010)	For amplification of <i>apsA</i> with primer DVU0847-3F for DNA contamination screen and RT-PCR. Reverse primer.
DVU0917-int-F	CTCTGGGTCTCACC TGCCTC	20	This study	For amplification of DVU0917 (<i>atpE</i>) with primer DVU0917-int-R for analysis by RT-PCR. Template is from DvH gDNA, mRNA, and cDNA. Forward primer
DVU0917-int-R	GGTTGGCGAACAG GAGGATC	20	This study	For amplification of DVU0917 (<i>atpE</i>) with primer DVU0917-int-F for analysis by RT-PCR. Template is from DvH gDNA, mRNA, and cDNA. Reverse primer.
DVU0916-int-F	CTACTACGTCAAGA GTCTCATCGAATCC	29	This study	For amplification of DVU0916 (<i>rex</i>) with primer DVU0916-int-R for analysis by RT-PCR. Template is from DvH gDNA, mRNA, and cDNA. Forward primer.
DVU0916-int-R	CGTAGAGATGGTG GAAGAAGTCC	23	This study	For amplification of DVU0916 (<i>rex</i>) with primer DVU0916-int-F for analysis by RT-PCR. Template is from DvH gDNA, mRNA, and cDNA. Reverse primer.
DVU0915-int-F	GCTTCGTGCTTCTG ACCGATTTC	23	This study	For amplification of DVU0915 with primer DVU0915-int-R for analysis by RT-PCR. Template is from DvH gDNA, mRNA, and cDNA. Forward primer.
DVU0915-int-R	GACATGAAGGATG CGGGCTAC	21	This study	For amplification of DVU0915 with primer DVU0915-int-F for analysis by RT-PCR. Template is from DvH gDNA, mRNA, and cDNA. Reverse primer.

Section 7. 6. mRNA expression quantification by qRT-PCR

Initial transcript expression of *apsBA* was determined by northern blot as described by Zane et al. (2010). Refer to Chapter 5 for discussion. Subsequent analyses for all samples and conditions were performed by qRT-PCR as discussed in the following section.

Section 7. 6. 1. Primer design, protocol and analysis

Primers to be used for qRT-PCR were manually designed to be 18-30 bp in length, have a GC-content ~50%, $T_m = 58-60^{\circ}\text{C}$, amplify a ~100-150 bp region at the 3'-end of the transcript for each gene (Table 7-5). Additional discussion is covered in Chapter 5. qPCRs were set up according to the manufacturer's protocol with SsoFastTM EvaGreen® Supermix (Bio-Rad) on a CFX96TM and analyzed with CFX ManagerTM (version 3.1, Bio-Rad), with the curve fit to regression (Pfaffl 2001; Vandesompele et al. 2002; Hellemans et al. 2007). The amplification protocol was initiated with a 3 min pre-incubation at 95°C and processed through 40 cycles of denaturation (30 sec at 95°C) and annealing/extension (30 sec at 65°C). The fluorescent signal was acquired at the end of each annealing/extension step. The melt curve protocol included annealing at the annealing/extension temperature (65°C) and melting at a ramp rate of 0.5°C/5 sec up to 95°C, with the fluorescent signal acquired continuously during the melt curve. Additional discussion is provided in the appendix.

Table 7-5: Primers list for quantitative Real-Time PCR (qRT-PCR)

Name	Sequence (5'-3')	bp	Source	Gene
DVU0173-qPCR-F1	CGACCAGGACCTCATGCC	18	This study	<i>phsA</i>
DVU0173-qPCR-R	CTACCTGACCCCTGCTTTGC	20	This study	
DVU0402-qPCR-F1	CAGAAGCTGCTCGAAGTGACCGAAAT	26	(Christensen et al. 2014)	<i>dsrA</i>
DVU0402-qPCR-R1	ATCTCAGGTGTCTCTTGCGGTATTCC	26	(Christensen et al. 2014)	
DVU0414-qPCR-F	GCGTGTTCATCGAGTGGGTC	19	This study	<i>tme</i>
DVU0414-qPCR-R1	CGTAGCTGTCTGATGAAGGCC	20	This study	
DVU0835-qPCR-F	TGTCTTCCCTGCACTCG	19	(Christensen et al. 2014)	<i>rpIS</i>
DVU0835-qPCR-R1	CTTGATGCGGGCAGCCTTAC	20	(Christensen et al. 2014)	
DVU0847-qPCR-F3	ACTCCAAGTGGAAGTGCTTCGTGAA	25	(Christensen et al. 2014)	<i>apsA</i>
DVU0847-qPCR-R3	CTATTCGGGGATGATCTGGTAGTAGG	26	(Christensen et al. 2014)	
DVU0848-qPCR-F2	CGTTCGATGTACCTGTGATGAAGAC	26	This study	<i>qmoA</i>
DVU0848-qPCR-R1	GCAGAGCGCATGACATCAAGG	21	This study	
DVU0851-qPCR-F2	GAAATCGAAAGCAGCGTGAAGGACAT	26	This study	<i>qmoD</i>
DVU0851-qPCR-R2	ACGCCACCGTCGGAAGA	17	This study	
DVU0915-qPCR-F2	GCTTTTATGCGGAAGTCAGGGATACTG	27	This study	DVU0 915
DVU0915-qPCR-R1	CGCTATCCCCGTGTGC	17	This study	
DVU0916-qPCR-F1	GGCATCAAGGGCATTCTCAACTAC	24	(Christensen et al. 2014)	<i>rex</i>
DVU0916-qPCR-R2	CTATTTGTGCGCGAGAACGTGAT	24	(Christensen et al. 2014)	
DVU1290-qPCR-F2	CGCAACCTTCCGAACAACCTCC	21	This study	<i>dsrM</i>
DVU1290-qPCR-R	TTACTCGGCAGCAGCTTCGT	20	This study	
DVU1295-qPCR-F2	GCAAGGCCCTTTCCGAAGG	19	(Christensen et al. 2014)	<i>sat</i>
DVU1295-qPCR-R	TTACATCACAGAGCCGGAAGCG	22	(Christensen et al. 2014)	
DVU1311-qPCR-F	AACTGTTCAATCTGCGCTTCCGT	23	(Christensen et al. 2014)	<i>rpmC</i>
DVU1311-qPCR-R	CTATTCCTTTTCTTCAGAATGGTCTGAATC	32	(Christensen et al. 2014)	
DVU1636-qPCR-F1	GTTCTGTCTGCTTACCGACATC	22	This study	<i>ppaC</i>
DVU1636-qPCR-R1	GCTTCTTGCGGCTCATCACG	20	This study	
DVU1932-qPCR-F	CCACGACATCTACTACAACACCG	23	This study	<i>adk</i>
DVU1932-qPCR-R	CTAGGAAAGCTGTGCGAGGAG	21	This study	
DVU2776-qPCR-F	AACGGTATCGCTCCGATGGTTC	22	This study	<i>dsrC</i>
DVU2776-qPCR-R2	AGGCCGGCCATCTTGCA	17	This study	
DVU2791-qPCR-F2	CAGTGCAAGCTGCCAGACC	19	This study	<i>dhcA</i>
DVU2791-qPCR-R	GGCCCTTGCCCTCTTCT	18	This study	
DVU2792-qPCR-F1	GCGGACTCTGCGGGTAC	17	This study	<i>rnjC</i>
DVU2792-qPCR-R	CGAGCTTCTGCTTCGAGAGACG	22	This study	
DVU3262-qPCR-F	TCGCCACATGGCAGGAG	17	This study	<i>fdxA</i>
DVU3262-qPCR-R2	AGACGGCCTCCTCGGAAG	18	This study	
DVU3264-qPCR-F	CCATCAACGCTCTCGGCATC	20	This study	DVU3 264
DVU3264-qPCR-R	TCAGAGCGTCACCTCCCC	18	This study	
DVU3360-qPCR-F	GTCAGCGGGACAGAACAGAAG	21	This study	<i>parB</i>
DVU3360-qPCR-R	TTAGTGGTTGGAAAGCCCAAGGC	23	This study	
DORF6830-qPCR-F1	GAAGATGTGGCAGTGCGAGC	20	This study	<i>grvA</i>
DORF6830-qPCR-R	TTACTTGTGGCACACGAAGCAG	22	This study	

Section 7. 6. 2. Reference gene determination

Genes to be used as internal (reference) controls were *rplS* (DVU0835) and *rpmC* (DVU1311) (Zhou et al. 2010) because they are expressed at similar levels to the genes to be assessed in this study and their expression levels were not found to change during exposure to environmental stresses (<http://www.microbesonline.org/>). To validate these reference genes, the strategy by Hellemans and coworkers was implemented (Hellemans et al. 2007) and the genes were shown to have minimal transcriptional differences among the strains, mutants and conditions tested (Mean Coefficient of Variance (CV<0.25) and Mean M<0.5) (data not shown).

Section 7. 6. 3. Sample preparation for transcript analysis

Samples to be used for quantitative Reverse Transcriptase PCR (qRT-PCR) were initially collected from DNase-treated RNA samples that were stored at -80°C. Purity of RNA from DNA is confirmed by a PCR screen with primers DVU0847-3F and DVU0847-4R (Figure 7-1). A 5 µl aliquot (20 ng/µl) of DNase-treated RNA was reverse transcribed to single stranded cDNA via iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA). For each gene to be analyzed, a standard curve (6 logs, serial dilution from 100 ng/µl stock cDNA) was performed to calculate efficiency. For each transcript the relative abundance was normalized to the reference gene transcripts (*rplS* and *rpmC*) in the specified sample.

Section 7. 7. *in vitro* protein-DNA interaction studies

Rex_{DvH} protein was isolated as described previously in Section 7. 4.

Section 7. 7. 1. Electrophoretic Mobility Shift Assay (EMSA)

EMSAs were performed according to Ravcheev et al. (Ravcheev et al. 2012). Primers used for generating dsDNA fragment are listed in Table 7-6. In brief, dsDNA fragments (0.1 nM) were incubated with Rex_{DvH} at specified concentrations (0-2000 nM) in a final volume of 30 μ l. The binding buffer contained 20 mM Tris-HCl pH 8.0, 10% vol/vol glycerol, 1 mM MgCl₂ and 40 mM KCl. Samples were incubated at 37°C for 25 min, placed on ice for 2 min and then separated (90 V, 70 min, 4°C) on a 5% (wt/vol) native Tris-Borate-EDTA (TBE) polyacrylamide gel that was preincubated (200 V, 30 min at 4°C) in 0.5X TBE buffer (Bio-Rad). In the cases when pyridine nucleotides were examined, desired concentrations were added after the initial incubation and were incubated for an additional 10 min at 37°C. The gel was removed from the apparatus, wrapped in plastic wrap and exposed to a Kodak Imaging Screen K (Bio-Rad) typically 15-60 min in a sealed cassette, followed by imaging with Personal Molecular ImagerTM (PMI) (Bio-Rad).

Table 7-6: Primers list for Electrophoretic Mobility Shift Assay (EMSA)

Name	Sequence (5'-3')	bp	Source	Descriptor
DVU1295-UP-F-EMSA	AGCGAAATGTGCCCCGCCA	18	(Christensen et al. 2014)	For amplification of promoter sequence of <i>sat</i> with primer DVU1295-200-R-EMSA (fragment ALL, or promoter mutation) or DVU1295-UP-R-EMSA (fragment UP). Forward primer.
DVU1295-200-R-EMSA	ACCTTACATCCTCCAGATGCGTGA	24	(Christensen et al. 2014)	For amplification of promoter sequence of <i>sat</i> with primer DVU1295-UP-F-EMSA (fragment ALL or promoter mutation) or DVU1295-DWN-F-EMSA (fragment DOWN). Reverse primer
DVU1295-UP-R-EMSA	ATGTCAAGCGGCAAAGCGCG	20	(Christensen et al. 2014)	For amplification of promoter sequence of <i>sat</i> with primer DVU1295-UP-F-EMSA (fragment UP). Reverse primer.
DVU1295-DWN-F-EMSA	ACGGAATCAACGCGACGCCA	20	(Christensen et al. 2014)	For amplification of promoter sequence of <i>sat</i> with primer DVU1295-200-R-EMSA (fragment DOWN). Forward primer.
DVU1295-RegPLUS-F-EMSA	CGCTTGACATTTTGTAAATTTTTTC ACAAGACGGAATCAA	40	(Christensen et al. 2014)	Rex binding site upstream of <i>sat</i> . Annealed with DVU1295-RegPLUS-R-EMSA (fragment RegPLUS). Forward primer
DVU1295-RegPLUS-R-EMSA	TTGATTCCGTC TTGTGAAAAAAATT TACAAAAATGTCAAGCG	40	(Christensen et al. 2014)	Rex binding site upstream of <i>sat</i> . Annealed with DVU1295-RegPLUS-F-EMSA (fragment RegPLUS). Reverse primer
DVU1295-RegPLUS-GtoA-F	CGCTTGACATTTTATAAATTTTTTC ACAAGACGGAATCAA	40	(Christensen et al. 2014)	For mutant GtoA Rex binding site mutation upstream of <i>sat</i> . Annealed with DVU1295-RegPLUS-GtoA-R. Forward primer.
DVU1295-RegPLUS-GtoA-R	TTGATTCCGTC TTGTGAAAAAAATT TATAAAATGTCAAGCG	40	(Christensen et al. 2014)	For mutant GtoA Rex binding site mutation upstream of <i>sat</i> . Annealed with DVU1295-RegPLUS-GtoA-F. Reverse primer.
DVU1295-RegPLUS-IR1-F	CGCTTGACATTTTACGAATTTTTTC ACAAGACGGAATCAA	40	(Christensen et al. 2014)	For mutant IR1 Rex binding site mutation upstream of <i>sat</i> . Annealed with DVU1295-RegPLUS-IR1-R. Forward primer.
DVU1295-RegPLUS-IR1-R	TTGATTCCGTC TTGTGAAAAAAATT CGTAAAAATGTCAAGCG	40	(Christensen et al. 2014)	For mutant IR1 Rex binding site mutation upstream of <i>sat</i> . Annealed with DVU1295-RegPLUS-IR1-F. Reverse primer.
DVU1295-RegPLUS-IR2-F	CGCTTGACATTTTGTAAATTTTTTT GTAAGACGGAATCAA	40	(Christensen et al. 2014)	For mutant IR2 Rex binding site mutation upstream of <i>sat</i> . Annealed with DVU1295-RegPLUS-IR2-R. Forward primer.
DVU1295-RegPLUS-IR2-R	TTGATTCCGTC TTACAAAAAAATT TACAAAAATGTCAAGCG	40	(Christensen et al. 2014)	For mutant IR2 Rex binding site mutation upstream of <i>sat</i> . Annealed with DVU1295-RegPLUS-IR2-F. Reverse primer.
DVU1295-RegPLUS-IR1and2-F	CGCTTGACATTTTACGAATTTTTTT GTAAGACGGAATCAA	40	(Christensen et al. 2014)	For mutant IR1and2 Rex binding site mutation upstream of <i>sat</i> . Annealed with DVU1295-RegPLUS-IR1and2-R. Forward primer.
DVU1295-RegPLUS-IR1and2-R	TTGATTCCGTC TTACAAAAAAATT CGTAAAAATGTCAAGCG	40	(Christensen et al. 2014)	For mutant IR1and2 Rex binding site mutation upstream of <i>sat</i> . Annealed with DVU1295-RegPLUS-IR1and2-F. Reverse primer.

Section 7. 7. 2. Fluorescent Polarization Assay (FPA)

Fluorescent polarization was performed as described (Novichkov et al. 2014) by Dr. Dmitry Rodionov. Fluorescently labeled DNA was designed (Table 7-7) and supplied along with REX_{DvH} to his laboratory for his contribution to this work. The binding assay was performed in a 96-well black plate (VWR, Radnor PA) with 1 nM fluorescently labeled (6-FAMTM) oligonucleotides. Different concentrations of protein (10, 25, 50, 100, 250, 500 and 1000 nM) were incubated with 1 nM labeled oligonucleotides in 100-μl reaction mixture in the binding buffer: 20 mM Tris-HCl, pH 7.5; 100 mM NaCl; 0.3 mg/ml of BSA; and 1 μg of herring sperm DNA. The fluorescence reading was taken on a Beckman multimode plate reader (DTX 880) with excitation and emission filters at 495 and 520 nm. The background fluorescence from buffer was subtracted and the Fluorescence Polarization values were defined as follows.

$$P_{(mp)} = \frac{I_{(parallel)} - ((G\text{-factor}) * I_{(perpendicular)})}{I_{(parallel)} + ((G\text{-factor}) * I_{(perpendicular)})} * 1000$$

I (parallel) and I (perpendicular) are the fluorescence intensity in the parallel and perpendicular orientation respective to the orientation of the excitation polarizer. The G-factor is an experimental correction for the polarization bias of the detection system (Titolo et al. 2003).

Table 7-7: Primer List for Fluorescent Polarization Assay (FPA)

Name	Sequence (5'-3')	bp	Source	Descriptor
DVU0694-3FAM-F	gggggTTCGTGAAATATTTTCAC CTTgggggt	30	(Christensen et al. 2014)	Rex binding site upstream of DVU0694 (<i>qrB</i>). Annealed with DVU0694-R. Contains 3-FAM. Forward Primer.
DVU0694-R	accccAAGGTGAAATATTTTCAC GAAccccc	30	(Christensen et al. 2014)	Rex binding site upstream of DVU0694 (<i>qrB</i>). Annealed with DVU0694-3FAM-F. Reverse Primer.
DVU0780-3FAM-F	gggggTTTGAGCTTTAATTCAC AACgggggt	30	(Christensen et al. 2014)	Rex binding site upstream of DVU0780 (<i>atpF1</i>). Annealed with DVU0780-R. Contains 3-FAM. Forward Primer.
DVU0780-R	accccGTTGTGAATTAAAGCTC AAAccccc	30	(Christensen et al. 2014)	Rex binding site upstream of DVU00780 (<i>atpF1</i>). Annealed with DVU0780-3FAM-F. Reverse Primer.
DVU0402-3FAM-F	gggggTTTGTCCAAAAAATTCAC GAGgggggt	30	(Christensen et al. 2014)	Rex binding site upstream of DVU0402 (<i>dsrA</i>). Annealed with DVU0402-R. Contains 3-FAM. Forward Primer.
DVU0402-R	accccCTCGTGATTTTTTGGAC AAAccccc	30	(Christensen et al. 2014)	Rex binding site upstream of DVU0402 (<i>dsrA</i>). Annealed with DVU0402-3FAM-F. Reverse Primer.
DVU1636-3FAM-F	gggggATTGTGCTATTTGGCAC AAAgggggt	30	(Christensen et al. 2014)	Rex binding site upstream of DVU1636 (<i>ppaC</i>). Annealed with DVU1636-R. Contains 3-FAM. Forward Primer.
DVU1636-R	accccTTTGTGCCAAATAGCAC AATccccc	30	(Christensen et al. 2014)	Rex binding site upstream of DVU1636 (<i>ppaC</i>). Annealed with DVU1636-3FAM-F. Reverse Primer.
DVU1917-3FAM-F	gggggCGAGCTATATATTCAC AAAggggt	30	(Christensen et al. 2014)	Rex binding site upstream of DVU1917 (<i>hysB</i>). Annealed with DVU1917-R. Contains 3-FAM. Forward Primer.
DVU1917-R	accccTTTGTGAAATATATAGC TCGccccc	30	(Christensen et al. 2014)	Rex binding site upstream of DVU1917 (<i>hysB</i>). Annealed with DVU1917-3FAM-F. Reverse Primer.
DVU1295-3FAM-F	gggggTTTGTAAATTTTTCAC AAGgggggt	30	(Christensen et al. 2014)	Rex binding site upstream of DVU1295 (<i>sat</i>). Annealed with DVU1295-R. Contains 3-FAM. Forward Primer.
DVU1295-R	accccCTTGTGAAAAAATTTAC AAAccccc	30	(Christensen et al. 2014)	Rex binding site upstream of DVU1295 (<i>sat</i>). Annealed with DVU1295-3FAM-F. Reverse Primer.
DVU0920-3FAM-F	gggggCITGTGAACGATTGCAC GAAGgggt	30	(Christensen et al. 2014)	Rex binding site upstream of DVU0920 (<i>atpI</i>). Annealed with DVU0920-R. Contains 3-FAM. Forward Primer.
DVU0920-R	accccTTCGTGCAATCGTTCAC AAGccccc	30	(Christensen et al. 2014)	Rex binding site upstream of DVU0920 (<i>atpI</i>). Annealed with DVU0920-3FAM-F. Reverse Primer.
DVU0846-3FAM-F	gggggATTGTTAATTCCATCAC AAGgggggt	30	(Christensen et al. 2014)	Rex binding site upstream of DVU0846 (<i>apsB</i>). Annealed with DVU0846-R. Contains 3-FAM. Forward Primer.

Name	Sequence (5'-3')	bp	Source	Descriptor
DVU0846-R	accccCTTGTGATGGAATTAAC AATcccc	30	(Christensen et al. 2014)	Rex binding site upstream of DVU0846 (<i>apsB</i>). Annealed with DVU0846-3FAM-F. Reverse Primer.
DVU2791-3FAM-F	gggggCTTGTGAAATAATGTTC TTTgggg ^g _g ^g _g ^g _g	30	(Christensen et al. 2014)	Rex binding site upstream of DVU2791 (<i>dhcA</i>). Annealed with DVU2791-R. Contains 3-FAM. Forward Primer.
DVU2791-R	accccAAAGAACATTATTTCAC AAGcccc	30	(Christensen et al. 2014)	Rex binding site upstream of DVU2791 (<i>dhcA</i>). Annealed with DVU2791-3FAM-F. Reverse Primer.
DVU1932-3FAM-F	gggggCTCGTGAAATTAATGAC AAGgggg ^g _g ^g _g ^g _g	30	(Christensen et al. 2014)	Rex binding site upstream of DVU1932 (<i>adk</i>). Annealed with DVU1932-R. Contains 3-FAM. Forward Primer.
DVU1932-R	accccCTTGTTCATTAATTTTCAC GAGcccc	30	(Christensen et al. 2014)	Rex binding site upstream of DVU1932 (<i>adk</i>). Annealed with DVU1932-3FAM-F. Reverse Primer.
DVU2286-3FAM-F	gggggATTGGGAATCGATTTCAC AAAgggg ^g _g ^g _g ^g _g	30	(Christensen et al. 2014)	Rex binding site upstream of DVU2286 (<i>cooM</i>). Annealed with DVU2286-R. Contains 3-FAM. Forward Primer.
DVU2286-R	accccTTTGTGAATCGATTCCC AATcccc	30	(Christensen et al. 2014)	Rex binding site upstream of DVU2286 (<i>cooM</i>). Annealed with DVU2286-3FAM-F. Reverse Primer.
DVU1292-3FAM-F	gggggTATGTGAAAAAATCAT TTTgggg ^g _g ^g _g ^g _g	30	(Christensen et al. 2014)	Rex binding site upstream of DVU1292 (hypothetical). Annealed with DVU1292-R. Contains 3-FAM. Forward.
DVU1292-R	accccAAAATGATTTTTTTTCAC ATAcccc	30	(Christensen et al. 2014)	Rex binding site upstream of DVU1292 (hypothetical). Annealed with DVU1292-3FAM-F. Reverse Primer.
FDde2476_L	ccccctcagtTTATCAACTTAGTTT GATATagtgaccccc ^t	41	(Novichkov et al. 2014)	Negative control. Random AT-rich sequence. Annealed with GC_FDde2476_L. Contains 3-FAM. Forward Primer.
GC_FDde2476_L	agggggtcactATATCAAATAAGT TGATAAactgaggggg ^g _g ^g _g ^g _g	41	(Novichkov et al. 2014)	Negative control. Random AT-rich sequence. Annealed with FDde2476_L. Reverse Primer.
Base “t” at 3'- end for addition of 3-FAM.				

Section 7. 8. **Select protein abundance determination**

Samples for protein analysis by targeted proteomics (Redding-Johanson et al. 2011) were grown and collected as described for the qRT-PCR analysis. Instead of 5-ml cultures, these samples were 100-ml cultures grown in 250-ml bottles. Preliminary transcript analysis between cultures grown in the two separate containers (tube vs bottle) had similar expression (data not shown). For each sample, 40 ml of the respiring cultures (OD₆₀₀ = 0.250) or 80 ml of the pyruvate-fermenting cultures (OD₆₀₀ = 0.125) were pelleted by centrifugation (~3700 x g 4°C 12 min), and the supernatant was decanted. Cells were flash frozen in liquid nitrogen and stored at -80°C until use. Protein extracts were then digested with trypsin (1 µg/µl) at 37°C overnight. Each sample was analyzed on an Agilent Technologies (Santa Clara, CA) 6460QQQ mass spectrometer operating in multiple-reaction monitoring (MRM) mode coupled to an Agilent 1100 system. Five micrograms of peptides were injected onto a Sigma Ascentis Peptide Express C-18 column (2.1 mm x 50 mm) via an Agilent G1377A autosampler. A 23-min method with a 10-min gradient was used and consisted of a 400 µl/min flow rate, starting with 95 % (all % in this section are vol/vol) Buffer A (98 % water, 2 % acetonitrile, 0.1 % formic acid) and 5 % Buffer B (98 % acetonitrile, 2 % water, 0.1 % formic acid) for one min, followed by an increase to 40 % Buffer B over 10 min, followed by a rapid increase to 90 % B in 5 min, where it was held for 4 min. The solvent composition was quickly ramped to 5 % B, where it was subsequently held for 2 min to allow the column to equilibrate for the next run. The peptides eluting from the column were ionized by an Agilent Jet Stream source (Sheath Gas flow: 11 l/min nitrogen, Sheath Gas Temperature: 350 C, Nozzle Voltage: 1000 V, Nebulizing Pressure: 30 psi, Chamber Voltage: 4500 V) operating in positive-ion mode. All selected-reaction monitoring (SRM) methods were designed, analyzed, and processed with Skyline software

version 1.4 (MacLean et al. 2010). Two or three peptides for each protein were selected and validated from a reference DvH protein digest for quantification by SRM mass spectrometry. The top 2-4 ionizing tryptic peptides (2+ or 3+ charge states) of 8-20 amino acids in length were selected with the default Agilent 6460 collision energy equations for each target protein. The most intense two or three y-series fragment ions for each peptide were monitored and the sum of their integrated peak areas was given for peptide quantification. Peptide areas for multiple peptides of the same protein were summed to assign relative area abundance to that protein. This information along with the experimentally determined retention times were used to build a scheduled MRM method with a retention time window of 3 min to optimize speed and sensitivity. The error bars represent the standard deviation of three biological replicates. The data were plotted with Spotfire 3.2.1 (TIBCO Software, <http://spotfire.tibco.com>), a data analysis and visualization package.

Section 7. 9. Transcription start site (TSS) of *sat* determined by 5'-RACE

Samples were initially collected from DNase-treated RNA samples. The procedure used was adapted from Scotto-Lavino et al. (Scotto-Lavino et al. 2006). A schematic is shown in Figure 7-6 and primers in Table 7-8. In brief, 100 ng of DNase treated RNA was reverse transcribed to single-stranded cDNA (iScript™ Select cDNA Synthesis Kit, Bio-Rad) with primer DVU1295-sat-GSP1, degraded with RNase A/T1 Mix and RNaseH (Thermo Fisher Scientific) and the cDNA was purified by column purification. With terminal deoxynucleotidyl transferase (Thermo Fisher Scientific), an adenosine tail was added to the cDNA, followed by generation of the second DNA strand with iScript™ and primer RACE-

2nd Strand. The RACE-2nd Strand primer was adapted from primer AUAP, Invitrogen 5' RACE System for Rapid Amplification of cDNA Ends Version 2.0, to include additional T residues and a "V" at the 3' position ("V" as A/G/C, but not T) to allow for better anchoring to the modified cDNA. dsDNA was purified, diluted 1:1000 and amplified by PCR to generate DNA fragment PCR1, with primers: DVU1295-GSP2 and AUAP. Thirty cycles of amplification were carried out (30 sec at 94°C, 30 sec at 58°C, 1 min at 72°C) followed by a final extension for 5 min at 72°C with *Taq* DNA Polymerase (NEB). An additional PCR (PCR2) followed, which was the same protocol as PCR1, but with 1:1000 diluted PCR1 as template and nested primers (AUAP and DVU1295-GSP3). Fragments were purified, sequenced, and mapped to the genome. Additional discussion of the complexities of the 5'-RACE experiment is described in Chapter 5.

Table 7-8: Primer List for 5'-Rapid Amplification of cDNA Ends (5'-RACE)

Name	Sequence (5'-3')	bp	Source	Descriptor
DVU1295-sat-GSP1	GACGAGAGCGATTTC	16	(Christensen et al. 2014)	Gene specific primer (GSP1) for cDNA generation of <i>sat</i> transcript. Reverse Primer.
RACE-2nd Strand	<u>GGCCACGCGTCGAC</u> <u>TAGTACTTTTTTTT</u> TTTTTTTTTV	39	(Christensen et al. 2014)	For generation of complementary strand of <i>sat</i> cDNA. Underlined portion contains overhang region for amplification by PCR. "V" denotes base A/G/C but not T.
AUAP	GGCCACGCGTCGAC TAGTAC	20	Invitrogen 5'-RACE system	Abridged Universal Amplification Primer. For amplification of <i>sat</i> cDNA for PCR1 (with primer DVU1295-sat-GSP2) or PCR2 (with primer DVU1295-sat-GSP3).
DVU1295-sat-GSP2	CGTCGTCCTTGGAC ACGTCA	20	(Christensen et al. 2014)	(GSP2) For amplification of <i>sat</i> cDNA with primer AUAP (PCR1).
DVU1295-sat-GSP3	ATTTCGATCTGCTT GAGGCCGG	22	(Christensen et al. 2014)	(GSP3) For nested amplification of PCR1 fragment with primer AUAP (PCR2). For sequencing of PCR2 fragment.

Figure 7-6: Method to determine TSS. Simplified schematic of 5'-Rapid Amplification of cDNA Ends (5'-RACE) for *sat*.

Section 7. 10. Whole-genome fitness study by TnLE-seq

This Rex mutant gene-fitness study presented here for the first time was part of a larger study (Fels et al. 2013; Korte et al. 2014), where five TnLE-seq pools were multiplexed for deep sequencing by the IlluminaTM procedure. Complete data are available at <http://desulfovibriomaps.biochem.missouri.edu/fitness/>. Gene fitness is shown by log₂R format, where R is the ratio of the number of insertions in a gene in the Rex mutant compared to the insertions in that gene in the parental strain. A positive R correlates to a fitness advantage and a negative R to a fitness defect. A gene whose fitness was >-1.7 was considered non-essential. For those genes with a fitness score between -1.7 and -2.2, they were classified as “synthetic sick” (Fels et al. 2013). As previously published (Fels et al. 2013), the fitness was only determined for genes with insertions within the 5-85% region of the coding sequence, as these mutations are most likely to affect the protein function of the gene product. Interestingly, there are sequences for transposon insertions within the *rex* gene in the strain deleted for *rex*. This phenomenon was attributed to “barcode bleed,” an artifact of assaying multiple pools on the same IlluminaTM HiSeq lane (Kircher et al. 2010).

Section 7. 11. NAD⁺ and NADH determination

The NADH/NAD⁺ quantification kit (Sigma) was used to measure the concentration of pyridine nucleotides. The experiment was followed according to manufacturer’s protocol. As a proof of principal experiment, α -select silver efficiency *E. coli* cells were first tested. In brief, 5 ml LC was inoculated with 50 μ l cells and incubated 12 hrs while shaking at 37°C. Cells were washed twice with PBS by centrifugation at ~2,000 x g for 5 min. Cells were extracted in 400 μ l NADH/NAD Extraction buffer by freeze/thaw methods (20 min dry ice/ 10 min at room temperature, twice). Samples were then vortexed and centrifuged

(13,000 x g 10 min) and the supernatant was passed over a PD-10 desalting column (GE Healthcare) and stored on ice. The assay to determine the pyridine concentration is a cycling reaction that converts NADH to NAD⁺. Therefore, the supernatant is split into two samples, one to determine NAD_{total} and one to determine NAD⁺. NADH is determined by subtracting NAD⁺ from NAD_{total}. Following the manufacturer's protocol the samples are read (A450) in a 96-well format with a Synergy Mx monochromator-based multi-mode microplate reader (BioTek, Winooski, VT) and the concentration of the pyridine nucleotides are determined by comparing the unknowns to a standard curve. A discussion of the practicality of this experiment is described in Chapter 5.

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Appendix

Table A-1: Additional transcript data for Rex MED

Strain	Name	Media	Mean Cq ^a			
			<i>sat</i>	<i>rex</i>	reference genes	
					<i>rplS</i>	<i>rpmC</i>
JW710	Parental	MOLS4	21.72 ± 0.33	22.60 ± 0.02	21.17 ± 0.01	23.14 ± 0.01
JW3311	Δrex	MOLS4	19.50 ± 0.31	>35	22.64 ± 0.01	24.62 ± 0.00
JW3311 (pMO3313)	Complement of <i>rex</i>	MOLS4	19.42 ± 0.21	19.32 ± 0.01	21.76 ± 0.01	23.42 ± 0.02
JW710	Parental	MOYPyr	20.88 ± 0.26	24.05 ± 0.01	23.56 ± 0.05	25.10 ± 0.03
JW3311	Δrex	MOYPyr	20.27 ± 0.42	>35	23.78 ± 0.01	25.56 ± 0.01
JW3311 (pMO3313)	Complement of <i>rex</i>	MOYPyr	21.71 ± 0.13	20.47 ± 0.01	24.32 ± 0.00	25.57 ± 0.02

OD600 was monitored through growth for kinetic studies and samples were collected for analysis at early-exponential and generation time was determined. Approximately 100 ng of TURBO™ DNase-treated RNA was converted to cDNA and 1 μ L of cDNA (5 ng of RNA) was used per qRT-PCR. Critical threshold (Cq) is presented for all genes tested, including two reference genes, *rplS* and *rpmC*. ^a Critical threshold (Cq) for specified gene, with technical error determined as standard error of the mean (n=3).

Table A-2: Sample collection and transcript analysis for *sat* promoter studies

Strain	Name	Media	Growth kinetics		Mean Cq ^b			
			OD600 collected ^a	Generation time (h)	<i>sat</i>	<i>rex</i>	reference genes	
							<i>rplS</i>	<i>rpmC</i>
JW710	Parental	MOLS4	0.38 ± 0.2	8.4	19.68 ± 0.08	24.18 ± 0.12	20.81 ± 0.12	22.18 ± 0.14
JW9293	ΔP_{sat}	MOLS4	---	---	---	---	---	---
JW9312	Restored P _{sat}	MOLS4	0.36 ± 0.0	9.3	21.34 ± 0.84	25.89 ± 0.76	22.13 ± 0.73	23.91 ± 0.77
JW9314	G -147 A	MOLS4	0.34 ± 0.0	8.5	18.75 ± 0.10	24.50 ± 0.15	21.36 ± 0.11	22.80 ± 0.09
JW9316	IR1	MOLS4	0.40 ± 0.0	8.2	18.21 ± 0.05	23.77 ± 0.06	20.26 ± 0.12	21.58 ± 0.15
JW9318	IR2	MOLS4	0.40 ± 0.1	9.1	17.50 ± 0.08	24.08 ± 0.12	20.61 ± 0.08	22.24 ± 0.12
JW9320	IR1and2	MOLS4	0.39 ± 0.0	8.1	18.27 ± 0.17	24.63 ± 0.29	21.64 ± 0.21	23.24 ± 0.14
JW710	Parental	MOYPyr	0.16 ± 0.0	31	22.30 ± 0.09	24.43 ± 0.11	21.26 ± 0.14	22.46 ± 0.09
JW9293	ΔP_{sat}	MOYPyr	0.21 ± 0.0	25	>30	24.89 ± 0.12	21.19 ± 0.12	22.35 ± 0.15
JW9312	Restored P _{sat}	MOYPyr	0.16 ± 0.0	36	26.09 ± 0.45	26.97 ± 0.46	25.47 ± 0.21	25.91 ± 0.35
JW9314	G -147 A	MOYPyr	0.16 ± 0.0	35	22.69 ± 0.35	28.34 ± 0.24	24.57 ± 0.31	26.63 ± 0.33
JW9316	IR1	MOYPyr	0.16 ± 0.0	33	22.61 ± 0.26	26.30 ± 0.16	22.74 ± 0.16	25.36 ± 0.12
JW9318	IR2	MOYPyr	0.15 ± 0.0	34	23.39 ± 0.23	26.42 ± 0.13	24.17 ± 0.09	24.80 ± 0.11
JW9320	IR1and2	MOYPyr	0.17 ± 0.0	31	23.85 ± 0.04	28.29 ± 0.12	25.40 ± 0.12	27.37 ± 0.10

OD600 was monitored through growth for kinetic studies and samples were collected for analysis at early-exponential and generation time was determined.

Approximately 100 ng of TURBO™ DNase treated RNA was converted to cDNA and 1 µL of cDNA (5 ng of RNA) was used per qRT-PCR. Critical threshold (Cq) is presented for all genes tested, including two reference genes, *rplS* and *rpmC*. a error determined as standard deviation (n=3). b Critical threshold (Cq) for specified gene, with technical error determined as standard error of the mean (n=3).

VITA

Geoff A. Christensen, Ph.D.

Geoff Christensen received his undergraduate degree from University of Illinois Urbana-Champaign (2008) and will receive his doctorate from University of Missouri-Columbia (2014), both in Biochemistry. He currently works with Dr. Judy Wall, where he is characterizing the redox repressor Rex and its role in regulating the metabolism of the sulfate reducer *Desulfovibrio vulgaris* Hildenborough. In pursuit of his degree he developed skills in molecular modeling, in culturing aerobes and anaerobes, in mutagenesis and purification and in analysis of RNA, DNA and protein. Additionally, he adapted qRT-PCR, RACE, and EMSA protocols for use in the Wall laboratory. Geoff has also become a valuable resource for several PIs starting to introduce transcript analysis techniques into their laboratories at Missouri. As a graduate student at Missouri, Geoff has been recognized for his efforts with many awards, including the National Institute of General Medical Sciences (NIGMS) Training Grant, Dr. Charles W. Gehrke Jr. Memorial Scholarship Fund and the Federation of European Microbiological Societies (FEMS) Young Scientist Grant. He has been active in the Biochemistry Graduate Student Organization and was the lead in many successful fundraisers that provided support for recruitment activities and development of collaborative interactions.