

Energy Metabolism and Uranium (VI) Reduction by *Desulfovibrio*

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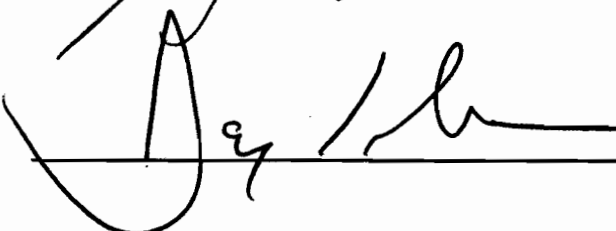
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*DESULFOVIBRIO***

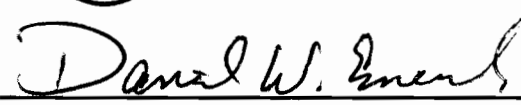
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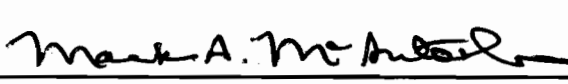
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Energy Metabolism and Uranium (VI) Reduction by *Desulfovibrio*

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Dr. Judy D. Wall, Dissertation Supervisor

Abstract

Sulfate reducing bacteria (SRB) of the genus *Desulfovibrio* can reduce uranium (VI) to uranium (IV) enzymatically. The reduction of U(VI) to U(IV) alters the solubility state of the uranium ion from a soluble to an insoluble, and therefore less biologically available, species. Because SRB are commonly found in uranium contaminated groundwater and soil, it is theoretically possible that we could use them to bioremediate uranium contaminated environments. However, before we attempt to manipulate the system, we must first understand the SRB genes and enzymes involved in uranium reduction and energy metabolism. Previous *in vitro* work by Lovley and coworkers suggested that the biochemical pathway for U(VI) reduction by *Desulfovibrio* was hydrogenase-to cytochrome c_3 -to U(VI). In this pathway, cytochrome c_3 was suggested to be the sole U(VI) reductase. First, we tested this model *in vivo* with strains carrying mutations in the dominant Fe-hydrogenase or cytochrome c_3 . We determined that the Lovley model is the primary pathway for U(VI) reduction *in vivo* when hydrogen gas is the electron donor; however, alternate pathways utilizing lactate or pyruvate for U(VI) reduction exist. In addition, at least one other cellular protein must be capable of acting as a U(VI) reductase in cytochrome c_3 -lacking cells. Second, we grew *Desulfovibrio desulfuricans* G20 in the presence of a non-lethal concentration of uranium in order to

understand some of the effects of uranium on its physiology. By doing so, we showed that G20 cells grown in the presence of uranium are impaired for U(VI) reduction. While exploring this observation, we found that the electron carrier protein cytochrome c_3 tightly adsorbs to insoluble uranium (IV) oxide, as well as copper oxide and iron oxide. Finally, we observed that sodium ions play an important role in energy metabolism and antibiotic resistance of *Desulfovibrio* when grown on lactate sulfate medium. We speculate that *Desulfovibrio* is capable of coupling lactate, but not pyruvate, oxidation and subsequent electron transport to the generation of a transmembrane sodium gradient. We propose that *Desulfovibrio* uses this “sodium circuit” to complement the proton motive force, for growth and for the efflux of some toxic compounds.

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1. Introduction

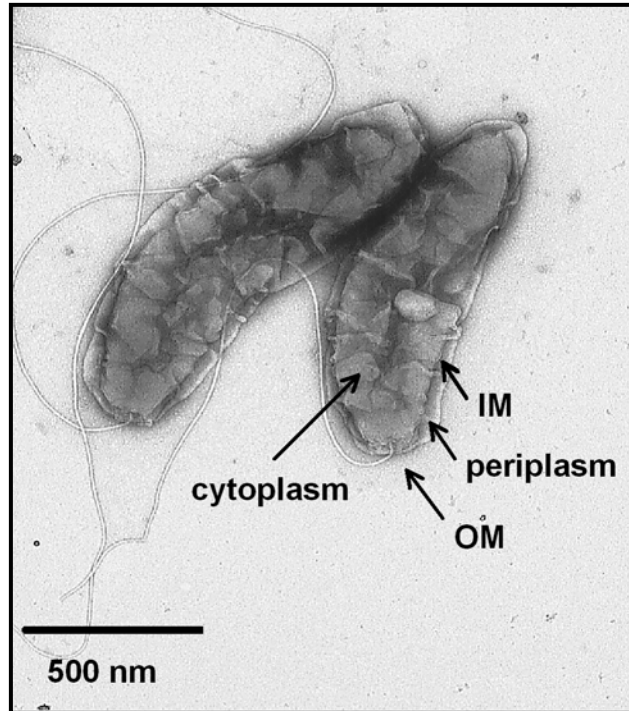
1.1. The Sulfate-Reducing Bacteria

The sulfate reducing bacteria (SRB) are a diverse group of microorganisms that are characterized by the ability to reduce sulfate enzymatically as the sole electron acceptor for growth (Postgate, 1979). The process of reducing sulfate as the terminal electron acceptor for anaerobic growth with the production of sulfide is termed dissimilatory sulfate reduction. This contrasts with assimilatory sulfate reduction, whereby sulfate is reduced enzymatically first to sulfide, incorporated into cysteine, and then finally used to make proteins (Kredich, 1996). Illustrative of their diversity, SRB include both Gram-negative and Gram-positive Bacteria and Archaea (Stetter, 1988). Most, but not all, strains of SRB can utilize hydrogen gas as the sole electron donor for growth by sulfate reduction (Postgate, 1979). Although typically thought of as strict anaerobes, meaning exposure to oxygen is lethal, SRB show various levels of tolerance to oxygen. In fact, members of the genus *Desulfovibrio* have even been shown to reduce molecular oxygen, perhaps as a defense mechanism (Fournier et al., 2003; van Niel and Gottschal, 1998). There is evidence that some strains of SRB can even couple the reduction of molecular oxygen to proton translocation and ATP synthesis (Cypionka, 2000; Johnson et al., 1997; Lemos et al., 2001).

Strains of the genus *Desulfovibrio* are among the better-characterized SRB due in part to their relative ease of cultivation in the laboratory. *Desulfovibrio* are Gram-negative, slightly curved rods, typically about 1 μm in length (Fig. 1.1). Representative strains used in this study were *Desulfovibrio desulfuricans* strain G20 and *Desulfovibrio vulgaris* strain Hildenborough. *D. desulfuricans* G20 was isolated from an oil well

Figure 1.1. Electron micrograph of *Desulfovibrio desulfuricans* G20.

Transmission electron micrograph of *D. desulfuricans* G20. Cells were grown to early stationary phase on medium containing 60 mM lactate as carbon and reductant source and 50 mM sulfate as terminal electron acceptor. The location of the cytoplasm, periplasm, inner membrane (IM), and outer membrane (OM) are indicated with arrows.



corrosion site (Weimer et al., 1988) and *D. vulgaris* Hildenborough was isolated from mud in Hildenborough, England (Postgate, 1979). In addition to using hydrogen gas as the electron donor (albeit poorly in the case of *D. desulfuricans* G20), both *D. desulfuricans* G20 and *D. vulgaris* can utilize the short chain organic acids lactate or pyruvate as the electron donor for growth via sulfate reduction (Widdel and Hansen, 1992). Standard molecular genetic techniques such as conjugation (Voordouw et al., 1990), transduction (Rapp and Wall, 1987), and electrotransformation (Rousset et al., 1998) have been demonstrated in *Desulfovibrio* and have been used to construct mutants by plasmid insertion (Rapp-Giles et al., 2000), marker exchange (Fu and Voordouw, 1997), or transposon insertion (Wall et al., 1996). The genomes of both *D. desulfuricans* G20 (DOE Joint Genome Institute and genome annotation can be found at The Virtual Institute for Microbial Stress and Survival, VIMSS, <http://www.escalante.lbl.gov>) and *D. vulgaris* Hildenborough (The Institute for Genomic Research, TIGR, <http://www.tigr.org>; (Heidelberg et al., 2004) were recently sequenced and were made available during the course of this dissertation. The resulting genomic data have greatly enhanced our understanding of these organisms.

1.2. The Lovley Model for U(VI) reduction

Microbial growth by mineral respiration was demonstrated in 1988 by two independent labs. Lovley and Phillips isolated a novel strictly anaerobic bacterium from freshwater sediments obtained from the Potomac River, Maryland that could reduce iron (III) to iron (II) enzymatically and couple that reduction to growth (Lovley and Phillips, 1988). This Gram-negative bacterium was *Geobacter metallireducens* (formerly strain GS-15; (Lovley et al., 1993). At the same time, Nealson and Myers isolated a

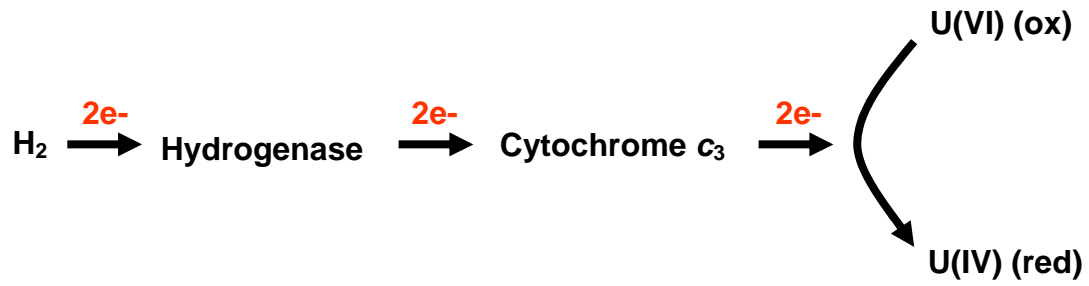
facultatively anaerobic bacterium from freshwater sediments obtained from Lake Oneida, New York, that could utilize Fe(III) or manganese (IV), as well as oxygen, nitrate, or chromate (VI) as the terminal electron acceptor (Myers and Nealson, 1988). This Gram-negative bacterium was *Shewanella oneidensis* (formerly *Shewanella putrefaciens* and *Alteromonas putrefaciens* (Venkateswaran et al., 1999), The reduction of Fe(III) to Fe(II) and Mn(IV) to Mn(II) by *Geobacter* or *Shewanella* strains was therefore truly a form of anaerobic respiration using a mineral as the terminal electron acceptor. *Geobacter* and *Shewanella* strains are ubiquitous and have been isolated from both freshwater and marine sediments, and terrestrial subsurface environments. They, along with the SRB, represent a significant proportion of the biological activity in those environments (Caccavo et al., 1992; Coates et al., 1998; Coleman et al., 1993; Nealson and Saffarini, 1994).

Geologic uranium (IV) deposits are often found in Fe(III)- or sulfide-rich rocks (Lovley et al., 1991). Historically, it was supposed that U(VI) was reduced and deposited as U(IV) in uranium rocks by geochemical means, not by the direct action of microbes. Lovley and coworkers reasoned that since Fe(III) and U(IV) are often found together in uranium deposits, dissimilatory Fe(III) reducing microorganisms might also reduce U(VI) enzymatically and couple this reduction to growth. This was the result observed with whole cells of *G. metallireducens* and *S. oneidensis* in an *in vitro* system (Lovley et al., 1991). Using the same reasoning, Lovley speculated that SRB might also enzymatically reduce U(VI) and tested SRB of the genus *Desulfovibrio* for their ability to reduce U(VI). They found that *Desulfovibrio desulfuricans* strain Essex (ATCC 29577) could reduce this radionuclide; however, the reduction did not support growth under the conditions

used (Lovley and Phillips, 1992). After additional biochemical experiments, Lovley et al. proposed a metabolic pathway for U(VI) reduction by *Desulfovibrio* (Fig. 1.2) (Lovley et al., 1993). Using partially purified cell extracts containing hydrogenase and cytochrome c_3 of *D. vulgaris*, they coupled hydrogen oxidation to the reduction of U(VI) *in vitro*. When they removed either hydrogenase or cytochrome c_3 from the *in vitro* system, reduction did not occur. When hydrogenase or the cytochrome c_3 was added back to the system, reduction was restored (Lovley et al., 1993). Based upon these observations, they proposed that cytochrome c_3 acts as the U(VI) reductase (Fig. 1.2).

Figure 1.2. Lovley Model of uranium (VI) reduction for *Desulfovibrio vulgaris*.

Each arrow indicates electron flow and implies a single step process.



1.3. Energy Metabolism in *Desulfovibrio*

It has long been known that most strains of *Desulfovibrio* can grow with hydrogen gas as the sole electron donor (Postgate, 1979) that is suggested to be oxidized in the periplasm primarily by the periplasmic Fe-hydrogenase (Pohorelic et al., 2002). Biochemical experiments demonstrated that the Fe- or NiFe-hydrogenase can directly interact with and reduce the high molecular mass cytochrome *c* complex (HMC), a transmembrane complex; however, the addition of catalytic amounts of cytochrome *c*₃ increased the rate of reduction of HMC by two orders of magnitude (Pereira et al., 1998). Thus it has been proposed that electrons liberated from hydrogen oxidation are first transferred to the dominant periplasmic cytochrome *c*₃, then passed through the cytoplasmic membrane via HMC where they subsequently are used to reduce sulfate to sulfide. Analysis of *D. vulgaris* and *D. desulfuricans* mutants of these proteins suggests that this pathway of electron flow from hydrogen to sulfate is likely true; however, other proteins can compensate for each of the single mutants (Dolla et al., 2000; Pohorelic et al., 2002 ; Rapp-Giles et al., 2000). Protons generated from the periplasmic oxidation of hydrogen are then available for the synthesis of ATP via a classical membrane bound F₁F₀ ATPase.

The mechanism for generation of a transmembrane proton gradient in *Desulfovibrio* when organic compounds like lactate or pyruvate are the electron donors and sulfate is the terminal electron acceptor has been intensely debated. The classical “Mitchell Hypothesis” for chemiosmotic ATP synthesis by *Desulfovibrio* envisions electrons generated from lactate or pyruvate oxidation being carried through membrane bound electron carrier components and eventually used to reduce sulfate to sulfide.

Electron flux through the electron transport chain is coupled to the translocation of cytoplasmic protons into the periplasm (Mitchell and Moyle, 1965). The consequent separation of ions (more protons in the periplasm) and charge (more positive in the periplasm) across the cytoplasmic membrane creates an energy gradient that is utilized to synthesize ATP via a membrane bound ATPase (Fig. 1.3). The energy potential of the transmembrane proton gradient can also be used for flagella rotation, substrate transport, and other processes in addition to ATP synthesis (for a recent review of the chemiosmotic hypothesis, see Harold, 2001). Researchers have reported classical proton translocation coupled to electron transport after the addition of hydrogen as the electron donor to resting cell suspensions of *Desulfovibrio* (Barton et al., 1983; Fitz and Cypionka, 1989). The evidence for classical proton translocation by *Desulfovibrio* strains is as follows. When 1 mole of hydrogen was added to resting cells supplied with an excess of electron acceptor, up to 6 moles of protons were released into the periplasm. Since the oxidation of 1 mole of hydrogen yields 2 moles of protons, the additional protons (up to 4 moles) released must come from cytoplasmic protons translocated into the periplasm via electron transport.

Many researchers have observed that *Desulfovibrio* strains generate, then consume, hydrogen gas when grown with an organic compound such as lactate as the sole electron donor (Tsuji and Yagi, 1980). Various models have been put forward to explain this hydrogen transient. One model proposes that the hydrogen burst is used to control the redox state of various intracellular electron carriers (Lupton et al., 1984). That is, the hydrogen burst is used to release excess electrons generated by the initial oxidation of organic compounds. In 1981, Odom and Peck proposed another

explanation: that *Desulfovibrio* strains produce and consume hydrogen as the primary means to generate a transmembrane proton gradient rather than the classical Mitchell model of electron transport driven proton translocation (Odom and Peck, 1981). The model proposed by Odom and Peck is commonly referred to as the “Hydrogen Cycling” model, and it has been quite controversial (Fig. 1.4). The periplasmic Fe-hydrogenase and cytochrome c_3 in the hydrogen cycling model have been proposed to re-oxidize hydrogen that is generated in the cytoplasm and then diffuses across the cytoplasmic membrane. Evidence has accumulated both for and against the hydrogen cycling model as a general means for generation of a transmembrane proton gradient. As might be expected if hydrogen gas is an intermediary in metabolism, a high concentration of external hydrogen gas inhibited organic substrate consumption when *D. vulgaris* strain Madison was cultured on pyruvate alone (Lupton et al., 1984). However, high external hydrogen concentrations did not inhibit growth of the same organism when cultured on lactate sulfate or pyruvate sulfate (Lupton et al., 1984). Curiously, a mutant of *D. desulfuricans* strain 27774 was isolated that was incapable of growth on lactate with sulfate under a hydrogen atmosphere or on hydrogen with sulfate; however, under an argon atmosphere, the mutant was still capable of growth on lactate with sulfate (Odom and Wall, 1987). Analysis of this mutant suggested that hydrogen was not an obligate intermediate during the oxidation of lactate in the wild-type strain 27774.

Figure 1.3. Chemiosmotic Hypothesis for energy metabolism.

Electrons liberated by the oxidation of reduced carbon compounds travel through a membrane bound electron transport chain driven by the different redox potential of the components and eventually are used to reduce a terminal electron acceptor (TEA), for example sulfate in the case of *Desulfovibrio*. The movement of electrons drives protons across the membrane into the periplasm. The separation of ions (more protons in the periplasm) and charge (a more positive charge in the periplasm) creates an energy gradient that can be used to synthesize ATP via the membrane bound ATPase, as well as rotate flagella, transport substrates, etc.

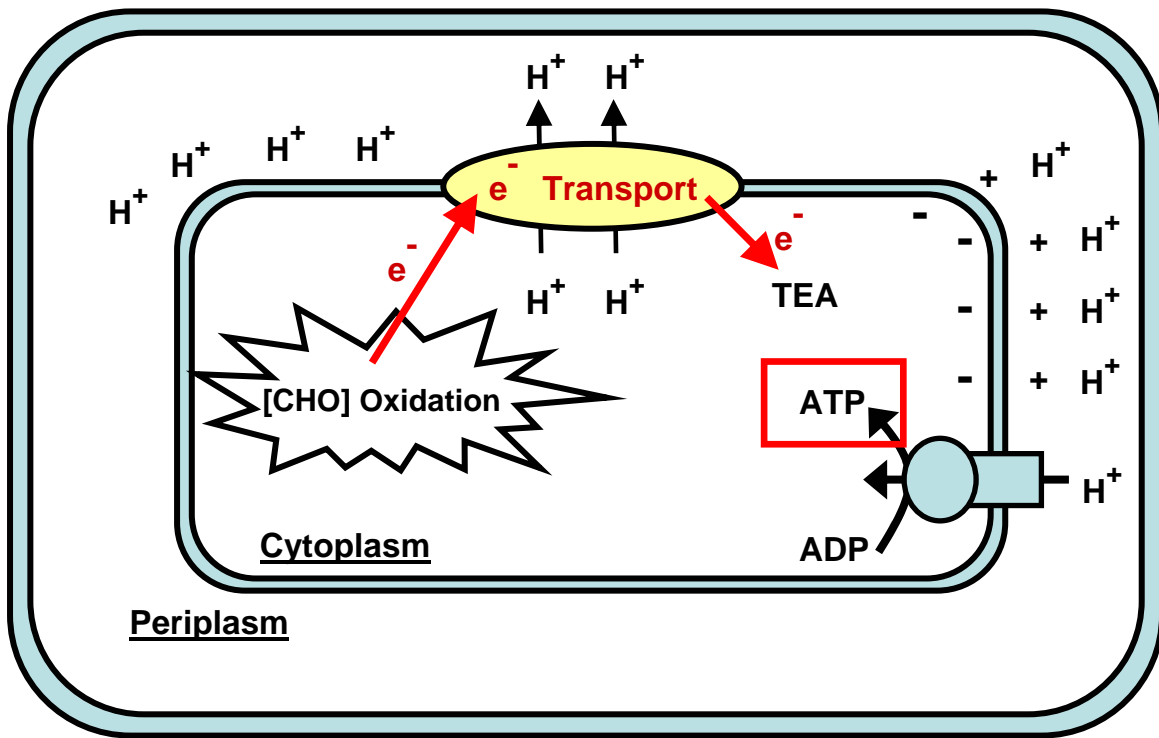
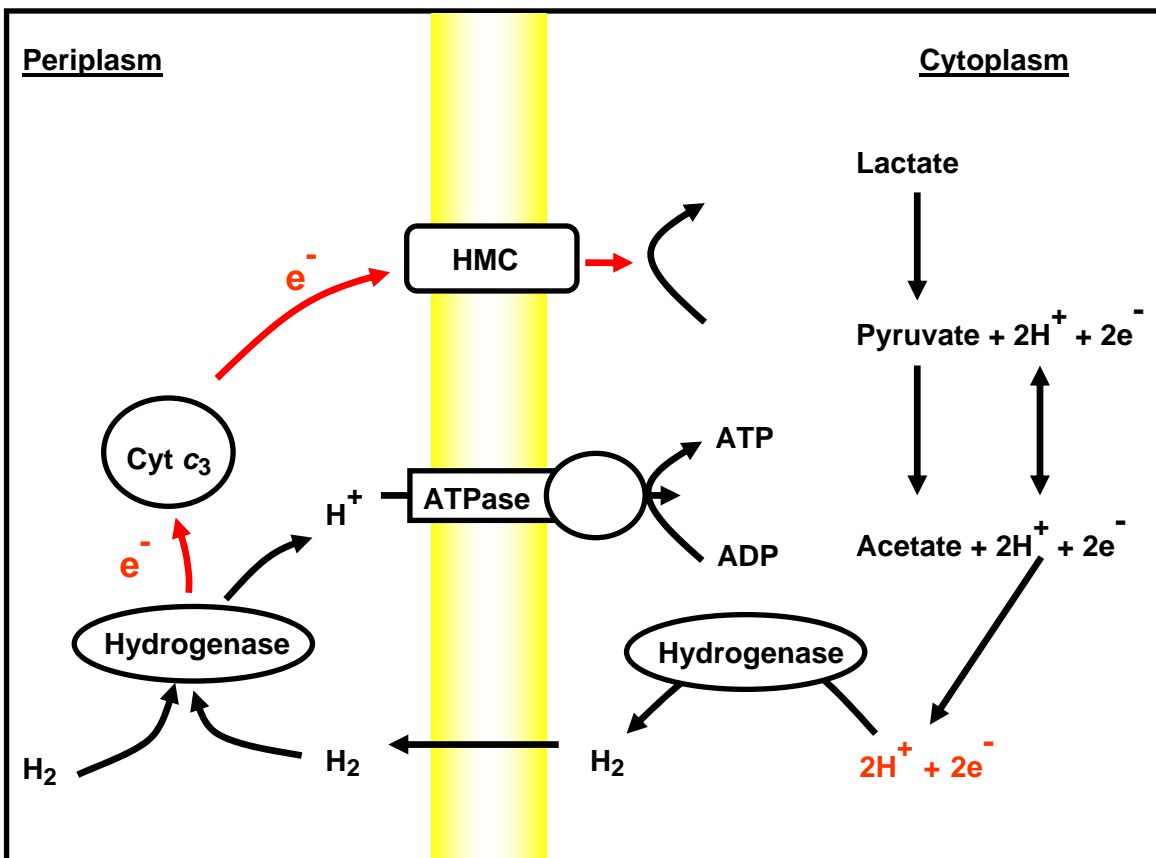


Figure 1.4. Hydrogen Cycling model for energy metabolism in *Desulfovibrio* (Odom and Peck, 1981). Electrons and protons liberated by the oxidation of reduced carbon compounds are the substrate of a cytoplasmic hydrogenase which generates hydrogen gas. Hydrogen gas diffuses across the cytoplasmic membrane and is oxidized by a periplasmic hydrogenase. Electrons are passed to cytochrome c_3 and then through a transmembrane complex like the high molecular mass cytochrome c complex (HMC). Once across the membrane, they are used to reduce sulfate to sulfide. The separation of protons and charge across the membrane is used to synthesize ATP through a membrane bound ATPase. *Arrows do not necessarily imply a single step process.*



The question remains: does hydrogen cycling as envisioned by Odom and Peck actually function in any strain of *Desulfovibrio*? Hydrogen cycling as a means to generate a proton gradient is not mutually exclusive with electron transport chain coupled proton translocation. Bacteria are frequently over designed and *Desulfovibrio* are clever organisms. Recent studies by Noguera and coworkers suggested that *D. vulgaris* can use both electron transport and hydrogen cycling to establish a periplasmic proton gradient from the oxidation of lactate or pyruvate (Noguera et al., 1998). This could benefit the bacterium in several ways. Rapid hydrogen oxidation would allow the bacterium to reuse hydrogen that may be unavoidably released during the oxidation of organic compounds. Alternately it might allow the SRB to out compete other bacteria for trace amounts of H₂ in the environment. This second advantage is supported by the observation that when sulfate is present in the environment, hydrogen-oxidizing SRB typically out compete hydrogen-oxidizing methanogenic bacteria (Kristjansson et al., 1982; Lovley and Goodwin, 1988; Robinson and Tiedje, 1984).

1.4. Aim of this dissertation

Our nation has used nuclear energy for over 50 years, and as a result our country is faced with cleaning or decontaminating a great deal of hazardous and radioactive waste. The U.S. Department of Energy is the steward of more than 120 sites, which contain approximately 475 billion gallons of contaminated water and 75 million cubic meters of contaminated soil (U.S. Department of Energy, <http://www.lbl.gov/NABIR/>).

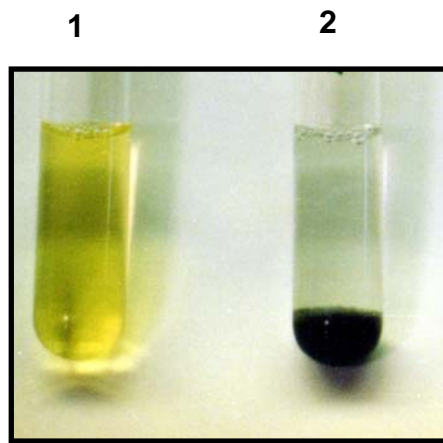
SRB have been shown to reduce radionuclides like uranium (VI) and technetium (VII) *in situ* in contaminated waters and soils and this reduction can be stimulated by the addition of nutrients such as lactate to the contaminated environment (Abdelouas et al.,

2000; Abdelouas et al., 2002). Promisingly, the reduced species U(IV) and Tc(IV) are less soluble in water and precipitate out of aqueous environments as minerals (Fig. 1.5). Although the reduction of these radioactive metals does not alter their radioactivity, the reduced species are less environmentally mobile and therefore less biologically available.

Before we can use SRB to decontaminate uranium-polluted environments we must first understand the SRB genes and enzymes involved in energy metabolism in general, and U(VI) reduction in particular. The idea of creating a “super bug” that is very efficient at reducing U(VI) enzymatically is somewhat appealing; however, the release of genetically modified organisms into the environment is problematic due to both rightful public concern, and to the observation that genetically modified laboratory organisms are typically at a disadvantage in the environment compared to their wild-type counterparts. Even though it is not technologically feasible to genetically engineer such a “super bug” at this time, understanding the pathway and regulation of electron flow from electron donor to U(VI) will allow us to make predictions on which sites are amenable to bioremediation by SRB, and how to increase the efficiency of the natural bioremediation, for example by nutrient addition.

Figure 1.5. U(VI) reduction by *Desulfovibrio desulfuricans* G20.

Pictured are test tubes containing 5 ml of 30 mM sodium bicarbonate buffer, pH 7.0 supplemented with 10 mM lactate as the electron donor (as described in section 2.3). *Test tube 1* contains cells (*ca.* 1 mg cell protein) heat-killed by boiling for 15 min; *Test tube 2* contains living cells (*ca.* 1 mg cell protein). Test tubes were incubated anaerobically at 31°C for 16 hours. No U(VI) reduction was seen in the absence of electron donor (data not shown). U(IV) precipitates from solution as the black mineral uraninite (UO₂) in the presence of living cells and an electron donor.



This dissertation began as a study on the pathway of electron flow to U(VI) in *Desulfovibrio*. By studying mutants previously constructed in cytochrome c_3 and in Fe-hydrogenase, I found that the Lovley model for electron flow from hydrogen gas to U(VI) was consistent with all experimental observations; that is, the enzymatic pathway *in vivo* with hydrogen as the electron donor was hydrogenase-to-cytochrome c_3 -to-U(VI) (Fig. 1.2; see section 3, below). However, when lactate or pyruvate was the electron donor, an alternate pathway that did not involve cytochrome c_3 was utilized for U(VI) reduction. In addition, at least one other cellular protein other than cytochrome c_3 must act as a U(VI) reductase (see section 3, below). Interestingly, studies of the Fe-hydrogenase mutant indicated that Fe-hydrogenase was in the pathway of electron flow from lactate to U(VI) (and sulfate) but not from hydrogen or pyruvate to U(VI) (see section 4, below). These observations were consistent with either the hydrogen cycling model or classical chemiosmotic mechanism for generation of energy via a transmembrane proton gradient (Figs. 1.3 and 1.4). Further studies explored the effect of a non-inhibitory concentration of uranium on the metabolism of *D. desulfuricans* G20. I found that prior exposure of cells to uranium impaired those cells in the rate, but not extent, of U(VI) reduction. In addition, I found that cytochrome c_3 protein forms a tight association with insoluble metal oxides which would limit any role of this cytochrome as an extracellular electron shuttle (see section 5, below). Studies using metabolic inhibitors indicated a further difference in the pathway of energy generation from lactate compared to pyruvate. Apparently *D. desulfuricans* G20 can couple electron transport from lactate, but not pyruvate, to the generation a transmembrane sodium gradient that complements the well-known proton motive force. Data suggest that the transmembrane sodium

gradient is used for both growth during lactate supported sulfate respiration and for drug resistance by the export of some toxic compounds (see section 6, below). Not surprisingly, *Desulfovibrio* has multiple pathways for energy generation. In *Desulfovibrio*, it appears that both a hydrogen cycling mechanism and a classical electron transport chain coupled mechanism are used to generate a transmembrane proton or sodium ion gradient.

2. Experimental Procedures

2.1. Growth of *Desulfovibrio*

2.1.1. Growth of *Desulfovibrio desulfuricans* G20

D. desulfuricans strain G20 is a spontaneously nalidixic acid-resistant derivative of the parent strain G100A that is also cured of the endogenous cryptic plasmid pBG1 (Wall et al., 1993; Table 2.1). The cytochrome c_3 mutant, I2, was created by insertion of a plasmid containing the kanamycin resistance cassette into the single chromosomal copy of the monocistronic *cycA* operon of the parent strain G20 (Wall et al., 1993; Table 2.1).

Cells were grown anaerobically in medium (LS) containing lactate (60 mM) as the primary electron donor and carbon source with sodium sulfate (50 mM) as the terminal electron acceptor (Rapp and Wall, 1987). Kanamycin (175 $\mu\text{g/ml}$) was added to all media used to grow I2 to select for maintenance of the inserted plasmid.

Growth curves were started by the subculture of early stationary phase cells (about 16 hour old cultures with an $\text{OD}_{600\text{nm}}$ of about 0.9) 1:20 into fresh growth medium. Growth was at 37 °C in 13 \times 100 mm test tubes containing 3 ml of medium and a headspace of 4 ml that were sealed with black rubber stoppers. The headspace was the atmosphere from the anaerobic chamber (Coy Laboratory Products, www.coylab.com) that was predominantly 90:10 $\text{N}_2:\text{H}_2$.

I2 cultures were always started from freezer stocks and were subcultured no more than three times. Since suppressors that restore cytochrome c_3 have been shown to accumulate in I2 cultures after an extended time in stationary phase, I2 cultures were monitored by Western analysis (see below) to ensure that the majority of the cells were not expressing cytochrome c_3 (data not shown) (Rapp-Giles et al., 2000).

2.1.2. Growth of *Desulfovibrio desulfuricans* G20 in iron-limiting medium

In experiments testing the requirement of iron for the synthesis of cytochrome c_3 , iron salts were omitted from the trace mineral solution used to make LS medium. Yeast extract was also omitted. Growth in this modified medium has been shown to be limited by available iron (Postgate, 1956).

2.1.3. Growth of *Desulfovibrio vulgaris*

D. vulgaris strain Hildenborough wild-type and the Fe-hydrogenase mutant were a generous gift of the Voordouw laboratory (University of Calgary, Alberta, Canada).

The Fe-hydrogenase mutant was created by gene replacement of the operon coding for the Fe-hydrogenase, *hydAB*, with the chloramphenicol resistance cassette (Pohorelic et al., 2002; Table 2.1). Wild-type and mutant strains were grown in LS medium.

Chloramphenicol (10 $\mu\text{g/ml}$) was added to media used to grow the Fe-hydrogenase mutant.

2.1.4. Growth of *D. desulfuricans* G20 in the presence of 1 mM uranium

One mM uranyl acetate was added from a sterile stock solution to autoclaved LS medium to make LSU medium. Growth of G20 in LSU medium was identical to that in LS medium as measured by $\text{OD}_{600\text{nm}}$ or by protein yield (Payne et al., 2002).

Table 2.1. Plasmids and bacterial strains used in this study

Strains or plasmids	Relevant features ^a	Source or reference
Plasmids		
pVIK111	R6K origin of replication, promoterless <i>lacZ</i> for translational fusions, Km ^R	(Kalogeraki and Winans, 1997)
pLC111	<i>cycA</i> 2kb promoter region- <i>lacZ</i> translational fusion in pVIK111, Km ^R	Casalot and Wall, published in Payne et al., 2004)
pGEMT-Easy	PCR Cloning vector, ColE1 origin, Ap ^R	Promega
pMON5003	IncQ, <i>bla</i> promoter- <i>lacZ</i> fusion, Km ^R	(Barry, 1988)
pBG1	Cryptic plasmid of <i>D. desulfuricans</i> G100A	(Wall et al., 1993)
pRK2073	ColE1 mobilization helper (Tra ⁺ Mob ⁺), Sp ^R , Sm ^R	(Kim et al., 1986)
Bacterial Strains		
<i>Desulfovibrio desulfuricans</i> G20	Nx ^R	(Wall et al., 1993)
<i>Desulfovibrio desulfuricans</i> G25	<i>D. desulfuricans</i> G20 with pLC111 integrated in chromosome, Km ^R , Nx ^R	Casalot and Wall, published in Payne et al., 2004)
<i>Desulfovibrio desulfuricans</i> I2	<i>D. desulfuricans</i> G20, <i>cycA</i> , Km ^R	(Rapp-Giles et al., 2000)
<i>Desulfovibrio vulgaris</i> Hildenborough	Wild-type	(Postgate, 1979)
<i>Desulfovibrio vulgaris</i> Hyd100	In <i>D. vulgaris</i> Hildenborough background, <i>hydAB</i> , Cm ^R	(Pohorelic et al., 2002)
<i>Escherichia coli</i> CC118	Host for <i>pir</i> dependent plasmids; [Δ (<i>ara, leu</i>)7697 Δ <i>lacX74</i> Δ <i>phoA20 galE galK thi rpsE rpoB argE(am) recA1</i>]; λ <i>pir</i> lysogen	(Manoil, 1991)
<i>Escherichia coli</i> DH5 α	Cloning strain; [F ⁻ Φ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (r _k ⁻ , m _k ⁺) <i>phoA supE44 thi-1 gyrA96 relA1</i> λ]	Invitrogen
<i>Escherichia coli</i> HB101	Donor for conjugations; [<i>supE44 hsdS20</i> (r _B ⁻ m _B ⁻) <i>recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 leuB6 thi-1</i>]	(Boyer and Roulland-Dussoix, 1969)

2.1.5. Growth of *D. desulfuricans* G20 under low sodium- or low proton-motive force conditions

Standard LS medium contains approximately 160 mM sodium, primarily from the addition of sodium lactate and sodium sulfate (Rapp and Wall, 1987). To remove sodium from the LS medium, the following modifications were made. Lactate was added from concentrated lactic acid (Fisher Scientific, www.fishersci.com) and sulfate was added from a 0.5 M potassium sulfate stock solution. Yeast extract was omitted, as was sodium nitrilotriacetic acid used as a chelator in the trace element solution. The sodium salts of molybdate, selenate, and tungstate were also omitted from the trace mineral solution. The 0.5 M HEPES stock solution used in LS was adjusted with dry KOH to pH 7.0. The pH of the medium was adjusted with dry KOH to 7.2 before the medium was sterilized by autoclaving. Sterile cysteine•HCl was added (1.5 mM final concentration) to reduce the medium after autoclaving. Sodium carbonate was omitted. When necessary, the pH of the medium was readjusted after being reduced with sterile 1 N KOH or 1 N HCl to obtain the desired pH (7.2 or 9.0). In spite of efforts to remove sodium ions, inductively coupled plasma-mass spectrometry (ICP) analysis of the medium (Agricultural Experiment Station Laboratories of the University of Missouri-Columbia) revealed that it contained about 0.7 mM sodium still, likely due to sodium leaching from glassware or as a contaminant introduced with other minerals. Medium prepared in this fashion was termed LS0 medium (for LS + 0 mM sodium medium). To prepare media with different NaCl concentrations, NaCl was added before autoclaving.

Pyruvate sulfate (PS) medium was prepared as for LS medium, except lactate was omitted and pyruvate was added from a sterile 0.5 M sodium pyruvate stock solution to

autoclaved medium to reach a final concentration of 50 mM. The final concentration of sodium in PS medium was approximately 160 mM. The addition of inhibitors (see below) sometimes caused LS and PS media to have a yellowish color that prevented an accurate OD_{600nm} reading. Therefore OD_{620nm} was used where indicated to follow cell growth. High concentrations of inhibitors (monensin, ethidium bromide, or methylene blue, in particular) sometimes formed a precipitate in uninoculated media that prevented an accurate OD_{620nm} reading. In this case, both protein measurements (Bradford, 1976) and cell counts were used to follow cell growth.

2.1.6. Antibiotic resistance of *D. desulfuricans* G20 under low sodium- or low proton-motive force conditions

Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and sodium monensin (Fisher Scientific) were made fresh weekly in isopropanol as 20 mM stock solutions. As a control, isopropanol alone was added to medium to reach the same final concentration as would be present with CCCP or monensin (typically 0.5 to 1.0% vol/vol). The addition of isopropanol at these concentrations had no noticeable effect on the growth of *D. desulfuricans* G20 (data not shown). Kanamycin, ethidium bromide, methylene blue, and nalidixic acid were made as stock solutions in water (25 mg/ml). The pH of nalidixic acid stock solutions was adjusted with KOH to about pH 7.0. All stock solutions were filter sterilized and kept at -20C.

Cells to be tested for growth in medium containing low sodium were harvested from complete LS medium by centrifugation at $7,000 \times g$ for 5 min and then subcultured into LS0 medium three times sequentially before growth data were collected.

2.2. Western analysis for cytochrome c_3

2.2.1. Protein extraction from *Desulfovibrio* grown on LS medium

Proteins were extracted from cells by a pH shift (van der Westen et al., 1978). Briefly, *D. desulfuricans* cells were grown on LS medium to early stationary phase (OD_{600nm} of about 0.9) and 50-ml samples were harvested by centrifugation at $7000 \times g$ for 5 min. The cell pellet was resuspended in 0.5 ml of buffer containing 50 mM EDTA and 50 mM Tris-HCl (pH 9.0, TE) and the suspension stirred on ice for 30 min to release cell proteins. Whole cells, insoluble precipitates, and debris were removed from the soluble extract by a second centrifugation at $7000 \times g$ for 5 min at room temperature and supernatants were retained for Western analysis where indicated.

While original studies suggested this procedure allowed fractionation of periplasmic proteins of *E. coli* (Nossal and Heppel, 1966), recent analyses of released proteins showed that both periplasmic and cytoplasmic proteins were extracted (Vazquez-Laslop et al., 2001). The majority of both periplasmic and cytoplasmic proteins recovered were that subset that passed through a 100-kDa-cutoff membrane filter (Vazquez-Laslop et al., 2001). Vazquez-Laslop and coworkers suggested that during the osmotic shock procedure, cellular proteins (periplasmic and cytoplasmic) are strained through the extracellular peptidoglycan mesh. The peptidoglycan layer therefore acts to retain proteins or complexes of proteins that are greater than approximately 100 kDa. The high pH TE wash procedure has been used to characterize the proteome of *D. vulgaris* (Fournier et al., 2003; Haveman et al., 2003). We have observed that the high pH TE wash procedure releases about 10% of the total whole cell protein of *D. desulfuricans* G20; about 90% of the whole cell protein remains in the insoluble pellet as

measured by Bradford analysis using bovine serum albumin (Fisher Scientific) as a standard (Bradford, 1976; Payne and Wall, unpublished).

2.2.2. Western analysis for cytochrome c_3 .

The separated protein fractions were mixed with an approximately equal volume of loading buffer for SDS PAGE (8 M urea, 4% wt/vol SDS, 4% vol/vol β -mercaptoethanol, 2% wt/vol bromophenol blue), heated for 30 min in a 65°C water bath, and then loaded onto a denaturing polyacrylamide gel (12.5% acrylamide in the separation phase and 3% acrylamide in the stacking phase). Polyacrylamide was 29.2% (w/v) acrylamide and 0.8% (w/v) bis-acrylamide in dH₂O. Running buffer was Tris (3.3 g/l), glycine (14.4 g/l), SDS (1 g/l). Gels were transferred to 0.45 micron nitrocellulose membrane (Micron Separations Inc., Westborough, MA) and analyzed for cytochrome c_3 by Western analysis using a polyclonal antibody generated against purified *D. desulfuricans* G20 cytochrome c_3 as the primary antibody and anti-rabbit IgG (alkaline phosphatase-conjugated) as the secondary antibody (Sigma, <http://www.sigmaladrich.com>) (Rapp-Giles et al., 2000). Bands were quantitated with Kodak 1D version 3.6 (<http://www.kodak.com>).

2.2.3. Adsorption of cytochrome c_3 to uraninite

D. desulfuricans G20 cytochrome c_3 was purified by the procedure of Pattarkine and Wall (unpublished). Briefly, the soluble fraction from the high pH TE wash was loaded onto a hydroxyapatite column (Biorad, www.bio-rad.com) equilibrated with 10 mM Tris-HCl buffer, pH 7.6. The column was washed with 10 mM Tris-HCl, pH 7.6, and then the pink cytochrome c_3 band was eluted from the column by a 0.2-0.5 M gradient of phosphate buffer, pH 7.6. Purified *D. desulfuricans* G20 cytochrome c_3 was

tested for its ability to adsorb to the insoluble precipitates of the metals uranium, copper, and iron. Samples of the insoluble oxides, 13.5 mg UO₂ (uranium (IV) oxide, Strem Chemicals, Newburyport, MA), 4 mg CuO (cupric oxide, Fisher Scientific), and 8 mg Fe₂O₃ (ferric oxide, Fisher Scientific), were suspended in 50 ml of deionized water. The final concentration of each metal oxide suspension was 1 mM, if the metal oxide would have been soluble. The metal oxides were pelleted by centrifugation at 7000 × *g* for 5 min at room temperature and the supernatant was decanted. To duplicate the high pH TE wash extraction procedure, the metal oxide pellet (about 0.5 ml) was resuspended in 0.5 ml of TE buffer and purified cytochrome *c*₃ was then added to reach a final concentration of 5 ng/μl. The metal oxide-TE suspension containing cytochrome *c*₃ was then stirred on ice for 30 min, then centrifuged a second time at 7000 × *g* for 5 min to separate the insoluble metal oxide from the solution. The supernatant was diluted 1:1 in loading buffer, heated for 30 min at 65°C and subjected to Western analysis for cytochrome *c*₃. A portion of the metal oxide precipitate was estimated by volume in the microfuge tube and an equal volume of loading buffer was added and processed as for the supernatant. Another portion of metal oxide precipitate was washed by resuspending in an equal volume of 2 M NaCl followed by centrifugation at 7000 × *g* for 5 min at room temperature. The supernatant from the NaCl wash was analyzed as above for release of cytochrome *c*₃ by Western analysis.

2.2.4. Separation of cells and uraninite by ultracentrifugation

To examine the effect of uraninite on cytochrome *c*₃ recovery during the high pH TE wash procedure, cells grown on LSU medium were separated from the mineral uraninite by sedimentation through a step gradient of sucrose before cytochrome *c*₃ was

released by TE extraction. For the gradient separation, a 50-ml early stationary phase ($OD_{600\text{nm}}$ of about 0.9) culture of *D. desulfuricans* G20 grown on LSU medium was harvested by centrifugation at $7000 \times g$ for 5 min at room temperature. The pellet, containing both unextracted cells and uraninite, was then layered onto a 36 ml sucrose gradient of three steps, 65, 85, and 100% (wt/vol) sucrose in H_2O . The gradient was subjected to centrifugation at $72,000 \times g$ for 30 min at room temperature. Cells banded at the 85-100% interface and uraninite pelleted to the bottom of the tube. The separation of uraninite and cells was visually confirmed by light microscopy (data not shown). The separated uraninite and cells were resuspended in equal volumes of TE buffer (about 0.5 ml each). Cells and uraninite precipitates were both subjected to extraction as described above in section 2.2. The final volumes of the uraninite fraction and the soluble protein fraction were equalized by addition of TE buffer, if necessary, so that both were concentrated ca. 50-fold. The presence of cytochrome c_3 was assayed in both the soluble protein fraction and the uraninite fraction by Western analysis as above.

2.3. Uranium (VI) reduction assay

Early exponential and late exponential phase ($OD_{600\text{nm}}$ of about 0.3 and 0.9, respectively, when grown on complete LS medium) cultures of the wild-type and the cytochrome c_3 mutant of *D. desulfuricans* G20 were used for the U(VI) reduction assay. Mid-exponential phase cells of the wild-type and the Fe-hydrogenase mutant of *D. vulgaris* ($OD_{600\text{nm}}$ of about 0.5 and about 0.3, respectively, when grown on complete LS medium) were used for the U(VI) reduction assay. Cells were harvested by centrifugation at $6000 \times g$ for 10 min and washed once in an equal volume anaerobic sodium bicarbonate buffer. This buffer, 2.5 g $NaHCO_3$ per liter, was always made fresh

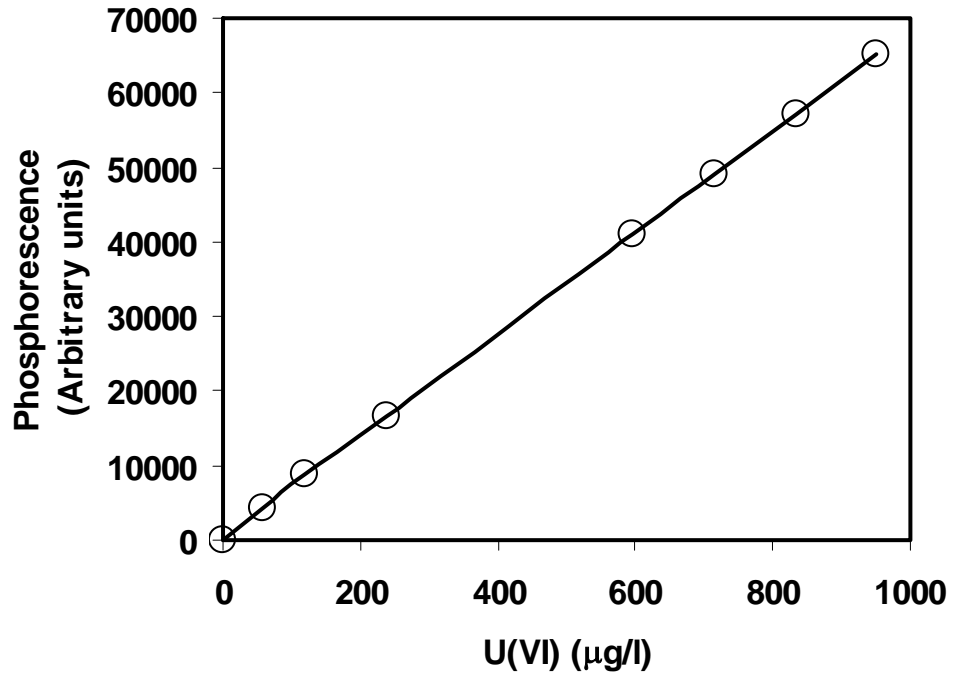
the day before use, boiled under CO₂ for 20 min to degas, and taken into the anaerobic chamber (atmosphere of N₂:H₂, 95:5) while still warm. The day of the assay, the pH of the buffer was adjusted to 7.0-7.2 with 5 M HCl. The washed cell pellet was resuspended in 1 ml of this buffer inside of the anaerobic chamber. To initiate the assay, a sample of the culture equivalent to 0.5-1.0 mg total cell protein was transferred to a tube containing 5 ml of an assay solution (1 mM uranyl acetate in anaerobic sodium bicarbonate buffer plus 10 mM Na pyruvate or 10 mM Na lactate as the electron donor). For experiments investigating H₂ as the electron donor, alternate electron donors were omitted from the medium, the headspace (~12 ml) of a Hungate tube (Bellco, Vineland, NJ) was replaced with 100% H₂, and the tubes were incubated horizontally to maximize the surface area for gas exchange. All assays were incubated and sampled in the anaerobic chambers that were maintained at 31°C. During the 24 h assays, the pH of the assay buffer increased less than 0.4 pH units.

U(VI) reduction was followed by the disappearance of U(VI) from the assay solution with a Kinetic Phosphorescence Analyzer (KPA; KPA-10, Chemchek Instruments, Richland, Washington). Samples of 100 µl were removed at the indicated times, appropriately diluted with anaerobic H₂O, and then transferred from the anaerobic chambers in chilled microfuge tubes. The samples were mixed with Uraplex™ complexant and the U(VI) concentration determined according to the manufacturer's directions (Chemchek Instruments), essentially by phosphorescence following excitation by a pulsed nitrogen dye laser with comparison of the response to a standard curve (Fig. 2.1). U(VI) at 1mM is 238 mg/l, therefore, excellent sensitivity in the µM range is achieved with the KPA. Since spontaneous reoxidation of U(IV) to U(VI) occurs in

aerobic conditions, tests were made to determine whether reoxidation was occurring during the dilution and reading of samples. None was detected in diluted samples left for over two hours, although reoxidation did occur after standing overnight.

Figure 2.1. Standard curve of U(VI).

A representative standard curve of uranium (VI) acetate as measured by the Kinetic Phosphorescence Analyzer (Chemchek Instruments). Data points represent one measurement.



Hydrogenase inhibitor studies. For studies measuring U(VI) reduction in the presence of copper or nitrite added as hydrogenase inhibitors, cells were pre-incubated in assay buffer lacking uranyl acetate but containing 1 mM NaNO₂ (Berlier et al., 1987) or 0.5 mM CuCl₂ (Fitz and Cypionka, 1989) for 10 min. The U(VI) reduction assay (using cells exposed to inhibitor) was started by the addition of sterile U(VI) acetate to a final concentration of 1 mM.

2.4. Northern analysis and RT-PCR

Cultures of 30 ml of *D. desulfuricans* G20 were grown to early exponential phase (an OD_{620nm} of about 0.3 when grown on LS medium), then harvested by centrifugation for 5 min at 4,000 × g and the spent medium decanted. Cell pellets were immediately resuspended in 1 ml of RNAWiz (Ambion, www.Ambion.com), and total RNA was extracted according to the manufacturer's directions.

For Northern analysis, approximately 5 µg of the extracted RNA was mixed with one-half volume of formamide and ethidium bromide-containing RNA loading buffer (Sigma, <http://www.sigma-aldrich.com>), then loaded onto a denaturing gel [1.2% (wt/vol) agarose/1% (vol/vol) formaldehyde]. Molecular weight standards were RNA Markers (R-4268, Sigma) and were used according to the manufacturer's directions. The gel was subjected to a field strength of 75 volts over 10 cm for *ca.* 1 hour. The gel was documented with a UV transilluminator and then blotted to a 0.45-micron pore sized Magnacharge nylon membrane (Micron Separations Inc.). Probes for Northern analysis were PCR products labeled with [α -³²P]dCTP (10 mCi/ml, 3000Ci/mmol; Perkin Elmer, <http://las.perkinelmer.com>) using the Prime It II kit (Stratagene, www.stratagene.com) according to the manufacturer's directions. The probe for the nitrite reductase was the

360-bp internal fragment of the nitrite reductase small subunit generated using primers nir_left and nir_right (Table 2.2). The probe for the NQR complex operon was the 560-bp internal fragment of the decaheme cytochrome *c* subunit generated using primers 10c_left and 10c_right. The probe for *cycA* was the 377-bp internal fragment of the monocistronic *cycA* gene using primers c3_left and c3_right (Rapp-Giles et al., 2000). Membranes were pre-hybridized and hybridized using Ultrahyb (Ambion), and washed as described by Ambion. Approximately 0.02 μCi of ^{32}P -labelled probe was used to probe each Northern blot.

Table 2.2. Primers used in this study.

Gene amplified encodes	Primer Name	Sequence 5' to 3'	Reference
Nitrite Reductase small subunit (<i>nfrH</i>)	nir_left	GCGGACTGTATGGACTGTCA	This work
	nir_right	TACCGGTATTGAGGCCTGTC	This work
Decaheme cyt <i>c</i> of Na ⁺ -translocating NADH-Quinone Oxidoreductase (NQR)	10c_left	AGCCCATGCGTATTTTGTTTC	This work
	10c_right	CAGGGTTCCGTTTTTCAGAG	This work
1st subunit of NQR	nqr_left	TACTCCGGAATGTGTGGTCA	This work
	nqr_right	GCGGTGTCATCTTTGCCTAT	This work
Tetraheme cyt <i>c</i> (<i>cycA</i>)	c3_left	GAAGGAGGTATCACAGTTATGAGGA	Rapp-Giles et al., 2000
	c3_right	AGTTCCTTTTTTCAGGTCCTTGTC	Rapp-Giles et al., 2000

2.5. Nitrite reduction assay

Cells were grown and harvested as described for the U(VI) reduction assay in section 2.3. The buffer was anaerobic (2.5 g/l) NaHCO₃ buffer, pH 7.0-7.2, and electron donors tested were hydrogen, lactate, and pyruvate as described in section 2.3. In experiments measuring nitrite reduction alone, uranyl acetate was omitted from the assay buffer and NaNO₂ was added at a final concentration of 1 mM from a sterile 100 mM stock solution. For experiments measuring simultaneous reduction of nitrite and U(VI), U(VI) (as uranyl acetate) was also included in the assay buffer at a final concentration of 1 mM.

Nitrite was measured spectrophotometrically (Hamilton, 1976). Briefly, 100- μ l subsamples were obtained from the nitrite reduction assay and diluted with 900 μ l of anoxic H₂O so that the nitrite concentration was within the range of the standard curve. Then, 100-300 μ l of the diluted subsample was added to a 13 \times 100 mm screw-capped test tube containing a volume of assay mix (0.017 mg/ml *N*-(1-naphthyl)ethylenediamine dihydrochloride, 0.17 mg/ml sulfanilic acid, and 0.00083% (wt/vol) phosphoric acid) to make the final volume 3.0 ml. Under acidic conditions, nitrite quantitatively converts the sulfanilic acid to a salt, which then couples to *N*-(1-naphthyl)ethylenediamine dihydrochloride to form an azo dye (Hamilton, 1976). The azo dye is quantified spectrophotometrically based on its absorbance at 548 nm following comparison to a standard curve of known nitrite concentrations.

2.6. Hydrogenase assay

Cells were grown and harvested as described for the U(VI) reduction assay except that anaerobic Tris buffer (50 mM, pH 8.5) was used in place of sodium bicarbonate

buffer. Tris buffer was made anaerobic by boiling under nitrogen for 20 min and then taken immediately into the anaerobic chamber. Sodium dithionite (Fisher) was added as dry powder to the Tris buffer to reach a final concentration of 3 μ M.

Hydrogenase activity by whole cells was quantitated as follows. A 5-ml volume of Tris buffer amended with sodium dithionite and 1 mM methyl viologen were added to Hungate tubes (Bellco, www.bellcoglass.com) that were then sealed with stoppers. The tubes were removed from the anaerobic chamber and the headspace (approximately 12 ml) was exchanged with hydrogen gas. The assay was started by the addition of 10 μ g of whole cell protein, as determined by Bradford (Bradford, 1976), to each Hungate tube. Hydrogenase activity was followed by the reduction of methyl viologen (as increasing absorbance at 602 nm) and comparison to a standard curve of known reduced methyl viologen concentrations. Negative controls with no added hydrogen showed no methyl viologen reduction during the time frame of this assay.

2.7. β -galactosidase assay

Gene-fusion plasmids were derived from pVIK111 (Table 2.1), a plasmid allowing translational fusions with *lacZ* as the reporter gene (Kalogeraki and Winans, 1997). An approximately 2-kb fragment was amplified by PCR that contained the deduced start codon of the monocistronic cytochrome c_3 gene (*cycA*) gene at one end, a *Sall* site for cloning in-frame, and the upstream region of the gene. After cloning the uncut PCR product into pGEMT-Easy (Promega, Madison, WI) and confirming that the correct sequence had been obtained, an approximately 2-kb *KpnI/SaII* fragment upstream of the *cycA* coding sequence containing the putative *cycA* promoter was cloned in frame into pVIK111 creating pLC111 (Casalot and Wall, Table 2.1). β -galactosidase activity

assays with *o*-nitrophenol- β -D-galactoside as substrate were performed as described by Miller (1972). Light scattering cell debris was removed by centrifugation. The specific activity (SA) was calculated using the following formula with an optical density at 420 nm of 0.0045 for 1 nmole/ml of *o*-nitrophenol in a 10 mm light path:

$$\text{SA(U/mg protein)} = \frac{\text{OD}_{420\text{nm}}}{0.0045 \bullet \text{Time (min)} \bullet \text{Protein (mg/ml)} \bullet \text{Volume (ml)}}$$

3. Uranium reduction by a cytochrome c_3 mutant of *Desulfovibrio*

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**Text, Table 3.1, and Figure 3.1 were modified and used with permission from
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3.1. Introduction

The Lovley model posited that the pathway of electron flow from hydrogen to U(VI) in *Desulfovibrio* was hydrogen-to-hydrogenase-to-cytochrome c_3 -to-U(VI) (Fig. 1.2). To determine if cytochrome c_3 was essential for U(VI) reduction, a cytochrome c_3 mutant of *D. desulfuricans* strain G20, named I2, was assayed for its ability to reduce U(VI) enzymatically. In addition, wild-type cells, severely reduced in cytochrome c_3 content due to growth in iron-limiting medium, were tested for their ability to reduce U(VI). Cells lacking cytochrome c_3 , either due to plasmid interruption of the *cycA* gene or due to iron starvation, were severely impaired in U(VI) reduction when hydrogen was the electron donor. When the organic acid lactate or pyruvate was the electron donor, the impairment was less severe. These data suggest that cytochrome c_3 is the primary U(VI) reductase when hydrogen is the electron donor, and that other pathways for U(VI) reduction independent of cytochrome c_3 function when lactate or pyruvate are the electron donors.

Our experiments with cells lacking cytochrome c_3 indicated that at least one protein other than cytochrome c_3 could function as a U(VI) reductase. Two possible alternate U(VI) reductases were suggested by work from other labs: (1) the *c*-type cytochrome nitrite reductase; and (2) hydrogenase. Previous mutagenesis work by Wade and DiChristina suggested that the *c*-type cytochrome nitrite reductase of *Shewanella oneidensis* could act as a U(VI) reductase (Wade and DiChristina, 2000). The first candidate for an alternate uranium reductase in the I2 mutant that we tested was the *c*-type cytochrome nitrite reductase. The completed genome sequence of *D. desulfuricans* G20 revealed a possible candidate for a *c*-type cytochrome nitrite reductase, and G20

cells were shown to reduce nitrite enzymatically. To determine if the nitrite reductase of *D. desulfuricans* G20 could act as a U(VI) reductase, simultaneous reduction kinetics of U(VI) and nitrite were measured in resting cells. At the concentrations tested, U(VI) reduction and nitrite reduction proceeded independently of each other. We interpreted this result to mean that the nitrite reductase of *D. desulfuricans* G20 was unlikely to function as a significant U(VI) reductase.

The second candidate for an alternate uranium reductase in I2 mutant that we tested was hydrogenase. The Fe-hydrogenase of *D. vulgaris* was reported to act as a chromium reductase *in vitro*, reducing Cr(VI) to Cr(III) (Chardin et al., 2003), and the NiFe-hydrogenase of *Desulfovibrio fructosovorans* was implicated as a Tc(VII) reductase by assaying a mutant in the dominant NiFe-hydrogenase for its ability to reduce Tc(VII) (De Luca et al., 2001). Although neither lab tested U(VI) reduction in their systems, these data suggest that hydrogenase, in addition to cytochrome *c*₃, may act as a reductase for metal oxoanions in *Desulfovibrio*. Since the uranyl ion forms a negatively charged complex with carbonate (Ganesh et al., 1997), it is feasible that the hydrogenases might be functional in reducing it. If the hydrogenase activity of I2 were repressed because of the lack of cytochrome *c*₃, then the inability of I2 cells to reduce U(VI) with hydrogen as the electron donor could be due to an impairment of hydrogenase activity and not due to the lack of cytochrome *c*₃ *per se*.

To test the possibility that hydrogenase were impaired in the I2 mutant, we assayed whole cell hydrogenase activity. We found that the I2 mutant had more hydrogenase activity than wild-type G20, and we interpret this result to mean that the hydrogenase of *D. desulfuricans* is unlikely to act as a U(VI) reductase. Further, the role

of hydrogenase in U(VI) reduction by a Fe-hydrogenase mutant of *D. vulgaris* was also explored and the results are shown in section 4.

3.2. Results

3.2.1. Uranium(VI) reduction by cytochrome c_3 mutant

U(VI) reduction assays of wild-type *D. desulfuricans* G20 showed no difference in the specific rate of reduction when cultures were harvested at early exponential phase as compared to early stationary phase (Fig. 3.1A), suggesting there was no major difference in U(VI) reduction capacity with growth phase. Therefore early stationary phase cells were selected for assays to provide cell material. The mutant I2 lacking cytochrome c_3 reduced U(VI) with hydrogen as the electron donor poorly. However, I2 was still capable of reducing U(VI) at about 10% of the rate of the wild-type with hydrogen as the electron donor (Fig. 3.1 B,C). I2 was still capable of reducing U(VI) at a rate about 50% that of wild-type using lactate as the electron donor, and at a rate of about 33% of wild-type using pyruvate as the electron donor (Fig. 3.1 B,C and Table 3.1).

Figure 3.1. U(VI) reduction by a cytochrome c_3 mutant of *D. desulfuricans* G20.

U(VI) reduction by *D. desulfuricans* strain G20 (A,B) or by the cytochrome c_3 mutant I2 (C). All samples have 1 mM uranyl acetate and 200 μ g whole cell protein/ml. (A) 10 mM sodium lactate is included as the electron donor. (\circ) cells harvested at early exponential phase, (\bullet) cells harvested at late exponential phase. Data points are the average of three measurements and the figure is representative of four independent trials. (B,C) cells are harvested at late exponential phase. 10 mM sodium lactate (\circ), 10 mM sodium pyruvate (Δ), 1 atm hydrogen gas (\square), or no electron donor (\diamond) was added. Each point is the average of three or more U(VI) measurements from two different experiments. The figure is representative of four trials of no reductant and hydrogen as the electron donor, and a minimum of five trials each of lactate or pyruvate as the electron donor.

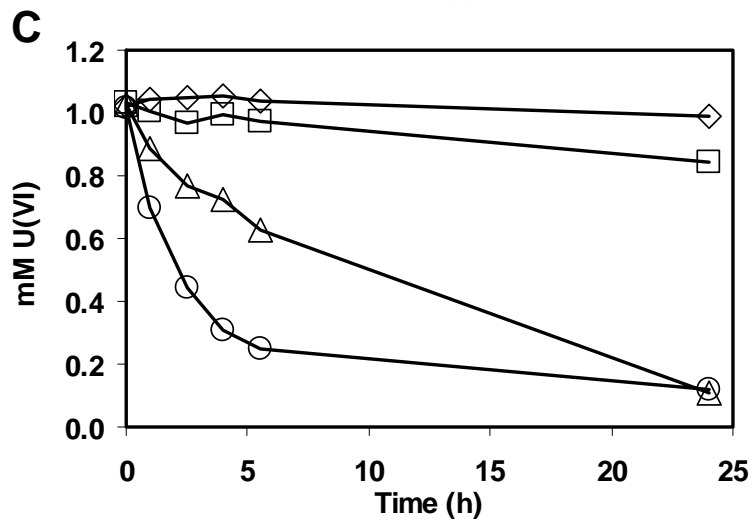
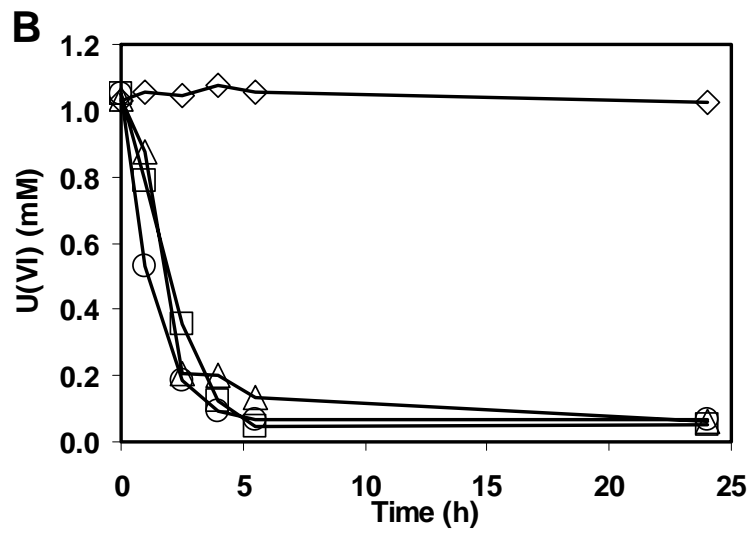
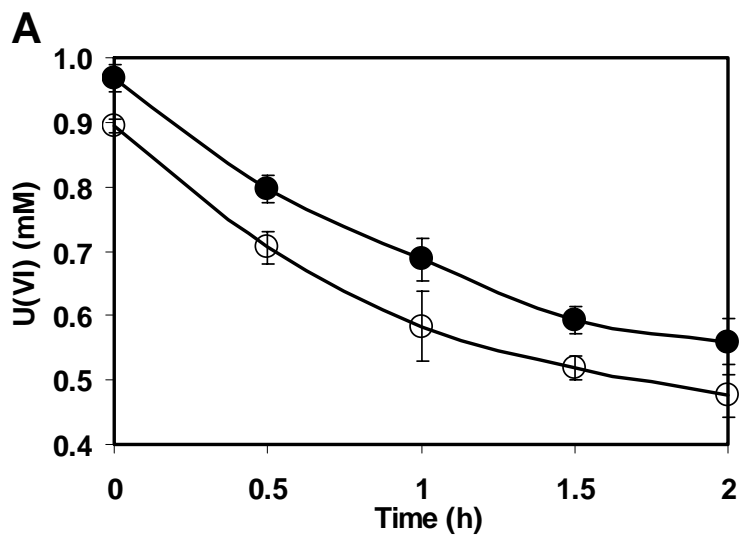


Table 3.1. U(VI) reduction by a cytochrome *c*₃ mutant of *D. desulfuricans* G20^a

Strain (relevant genotype)	Electron Donor		
	Lactate ^b	Pyruvate ^c	Hydrogen ^d
G20 (wild type)	2.2 ± 0.3	2.2 ± 0.3	1.5 ± 0.2
I2 (<i>cycA</i> mutant)	1.0 ± 0.2	0.6 ± 0.2	0.2 ± 0.1

^aRates are from assays with whole cells, 200 µg cell protein/ml, determined from the first 2.5 h of the assay; µmol U(VI) reduced x mg cell protein⁻¹ x h⁻¹. No U(VI) reduction rate was measured in the absence of electron donor.

^bLactate, 10 mM; averages of seven or more determinations.

^cPyruvate, 10 mM; averages of five or more determinations.

^dHydrogen, 1 atm; averages of four determinations.

3.2.2. Fe-requirement for the synthesis of cytochrome c_3

Western analysis of periplasmic extracts showed that wild-type *D. desulfuricans* G20 cells grown in complete LS medium expressed abundant cytochrome c_3 . As expected, the I2 mutant did not contain measurable cytochrome c_3 . Wild-type cells grown in LS medium lacking iron have no more than 10% of the normal levels of cytochrome c_3 as wild-type cells grown in medium with excess iron (Fig. 3.2).

3.2.3. Uranium (VI) reduction by iron-limited cells

Wild-type cells grown in complete LS medium, and therefore expressing abundant cytochrome c_3 , reduce U(VI) with lactate, pyruvate, or hydrogen gas as the electron donor at about the same rate and extent for all three electron donors (about 2 $\mu\text{mol U(VI)}/\text{mg cell protein}/\text{hour}$; Table 3.1). Cells lacking wild-type levels of cytochrome c_3 due to growth in iron-limiting medium reduced U(VI) poorly with all electron donors tested (Fig. 3.3). Typically, no increase in the rate or the amount of U(VI) reduction was detected in cells grown in iron-limiting medium when electron donor was included in the assay as compared to the rate seen in the absence of exogenous electron donor (Fig. 3.3). We interpret the endogenous rate of U(VI) reduction seen with no added external electron donor as being due to the oxidation of an internal storage reserve of reductant, like polyglucose. *Desulfovibrio gigas* has been shown to store reductant as polyglucose (Santos et al., 1993)

Figure 3.2. Cytochrome c_3 expression in iron-limited cells of *D. desulfuricans* G20.

Western analysis showing *cycA* mutant cells and wild-type cells limited for iron do not make cytochrome c_3 . Lane M: Molecular mass markers, 14 kDa is indicated; lane 1: 10 μ g high pH TE wash protein fraction from wild-type cells grown in complete medium; lane 2: 10 μ g high pH TE wash protein fraction from wild-type cells grown in iron limiting medium; lane 3: 10 μ g high pH TE wash protein fraction from *cycA* cells grown in complete medium; lane 4: 25 ng of pure cytochrome c_3 .

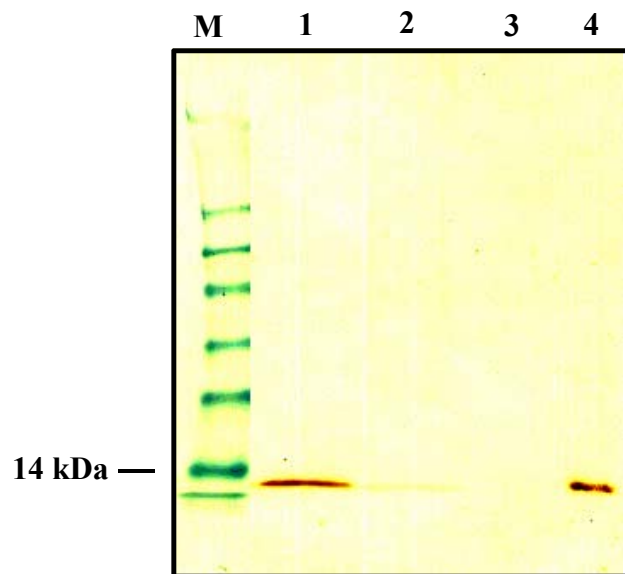
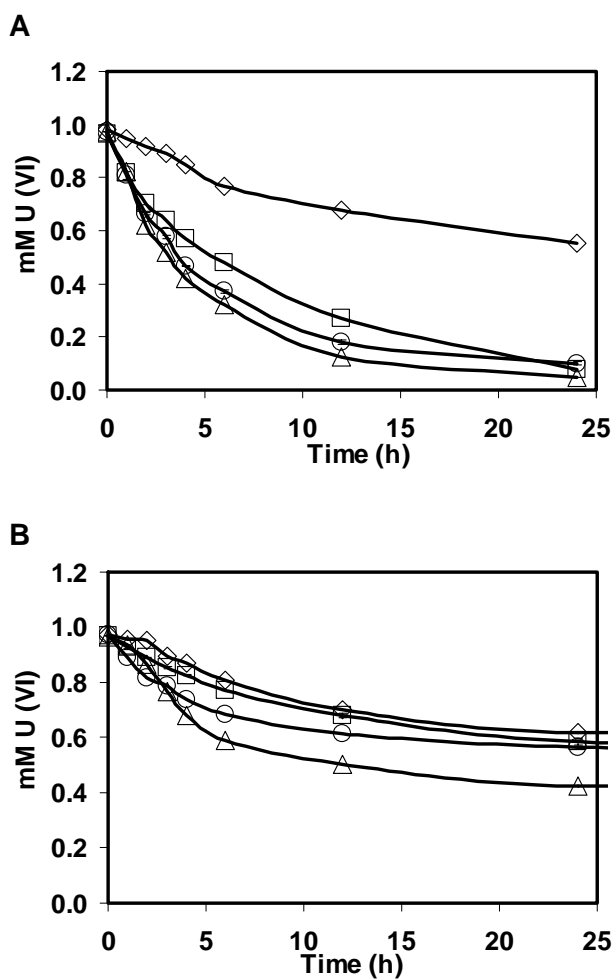


Figure 3.3. U(VI) reduction by iron-limited cells of *D. desulfuricans* G20.

U(VI) reduction by *D. desulfuricans* strain G20 grown in complete medium with iron (A) or grown in iron-limiting medium (B). All samples have 1 mM uranyl acetate and 200 μ g whole cell protein/ml. 10 mM sodium lactate (\circ), 10 mM sodium pyruvate (Δ), 1 atm hydrogen gas (\square), or no electron donor (\diamond) was added. Each point is the average of three U(VI) determinations, in most cases the error bars are within the symbols. The figure is representative of three trials.



3.2.4. Nitrite reduction by a cytochrome *c*₃ mutant

DiChristina and coworkers screened a library of chemically mutagenized *Shewanella oneidensis* and found a mutant incapable of growth using U(VI) or nitrite as the sole electron acceptor, but this mutant was still capable of aerobic growth or anaerobic growth using Fe(III) or Mn(IV) (Wade and DiChristina, 2000). They interpreted this observation to mean that in *Shewanella*, U(VI) reduction and nitrite respiration shared a common component that the other forms of respiration did not. Furthermore, they hypothesized that the component shared between U(VI) and nitrite respiration was the *c*-type cytochrome, nitrite reductase. Some strains of *Desulfovibrio*, such as *D. desulfuricans* strain 27774, can utilize nitrate or nitrite as the terminal electron acceptor for respiration (Almeida et al., 2003). In these strains, the periplasmic *c*-type cytochrome, nitrite reductase, is the terminal enzyme in the respiratory pathway of nitrite respiration (Liu and Peck, 1981). Nitrite reductase has been purified from *D. desulfuricans* strain 27774 and found to be a heterodimer that is membrane-bound with the active site periplasmic-facing (Almeida et al., 2003). Each subunit is a multiheme *c*-type cytochrome and the two subunits are encoded in an operon *nfrHA*. The first gene, *nfrH*, encodes a tetraheme *c*-type cytochrome and is an integral membrane protein with a predicted transmembrane helix motif composed of the first 33 amino acids, using the transmembrane helix prediction programs TMHMM (Almeida et al., 2003). NfrH is a member of the NapC/NirT family of oxidoreductases, whose members in other proteobacteria accept electrons from the quinone pool of the electron transport chain and transfer those electrons to the catalytic subunit encoded by the gene *nfrA* (Almeida et al., 2003; Simon et al., 2000). The second subunit, NfrA, is a pentaheme *c*-type cytochrome.

Interestingly, one of the heme-binding sites is comprised of the amino acids CXXCK rather than the typical CXXCH motif (Cunha et al., 2003). The second subunit contains no transmembrane domains and is more loosely associated with the membrane. NfrA is the catalytic subunit, reducing nitrite to ammonia, a six electron transfer event (Almeida et al., 2003; Simon et al., 2000).

Although *D. desulfuricans* G20 and *D. vulgaris* cannot grow by nitrate or nitrite respiration (data not shown; Postgate, 1979), sequence analysis of the completed genomes of *D. desulfuricans* G20 and *D. vulgaris* revealed an operon similar to the biochemically characterized nitrite reductase of *D. desulfuricans* 27774 (Fig. 3.4). The intergenic region between the *nfrH* and *nfrA* genes of G20 is 100 bp and operon structure is a possibility (Oak Ridge National Labs, <http://genome.ornl.gov/microbial/ddes/.html>). The *nfrHA* orthologs found in *D. vulgaris* Hildenborough overlap by 76 bp supporting operon structure (VIMSS, <http://www.escalante.lbl.gov>). The first subunit encoded in the predicted *D. desulfuricans* G20 and *D. vulgaris* nitrite reductase operon (*nfrH*) has the characteristic tetraheme *c*-type cytochrome heme-binding motif. In addition, both the *D. desulfuricans* G20 and *D. vulgaris* NfrH proteins have the predicted transmembrane motif characteristic of an integral membrane protein of the NapC/NirT family (VIMSS, <http://www.escalante.lbl.gov>; TMHMM, www.cbs.dtu.dk/services/TMHMM-2.0.html). Interestingly, the second subunit of the predicted *D. desulfuricans* G20 nitrite reductase operon, NfrA, has an octaheme *c*-type cytochrome binding motif. All eight of the heme-binding motifs of the *D. desulfuricans* G20 NfrA are of the CXXCH type. Preliminary computer annotation by VIMSS identifies the *D. vulgaris* operon as a nitrite reductase, while preliminary computer annotation by VIMSS identifies the *D. desulfuricans* G20

operon as a member of the NapC/NirT family of reductases, possibly a trimethylamine or hydroxylamine reductase (Simon et al., 2000). The true nitrite reductase from *D. desulfuricans* 27774 was also shown to have hydroxylamine reductase activity in addition to nitrite reductase activity (Liu and Peck, 1981). This circumstantial evidence suggested that these two genes of *D. desulfuricans* G20 encode nitrite reductase; however, the assignment is still tentative.

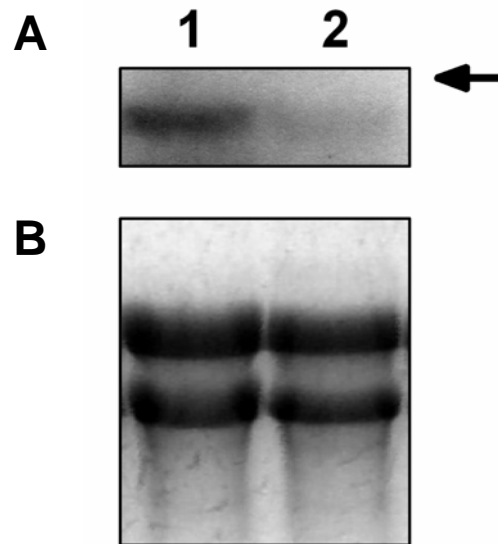
Northern analysis indicated that the putative *D. desulfuricans* G20 nitrite reductase operon was expressed in wild-type G20 and in the cytochrome *c*₃ mutant I2. Interestingly, the expression of the nitrite reductase was at least 8-fold higher in the wild-type than in the cytochrome *c*₃ mutant (Fig. 3.5). In addition, the *ca.* 2.5-kb transcript observed when using a probe from the internal region of the *nfrH* gene suggested that the putative *nfrH* and *nfrA* genes were cotranscribed on a single transcript.

Figure 3.4. The nitrite reductase operon of *Desulfovibrio* strains.

Diagrammatic representation of the gene order of the putative nitrite reductase operons of the SRB. VIMSS (<http://www.eslcante.lbl.gov>) orf numbers of each putative gene are shown for *D. vulgaris* and *D. desulfuricans* G20. The predicted number of heme-binding motifs deduced from the translated protein is shown above each gene. The percent identity/percent similarity of each putative protein as compared to the nitrite reductase translated from the corresponding gene of *D. desulfuricans* 27774 is indicated beneath each. The genomic regions surrounding the putative nitrite reductase operons from *D. desulfuricans* G20 and *D. vulgaris* share no synteny.

Figure 3.5. Nitrite reductase expression in a cytochrome *c*₃ mutant.

Lane 1: G20 RNA. Lane 2: I2 RNA. *Panel A*: Northern blot probed with the internal fragment of the small subunit of the G20 nitrite reductase. The *Arrow* indicates the position of the 3-kb marker on the gel. *Panel B*: Gel showing the 23S and 16S rRNA. 5 μ g total RNA added per lane in gels shown in both panels. These results are representative of RNA from two independent experiments.



In experiments measuring nitrite reduction, both wild-type G20 and cytochrome c_3 mutant I2 reduced nitrite. Interestingly, when pyruvate was the electron donor, I2 reduced nitrite about twice as fast as G20 (Table 3.2, Fig. 3.6 A). Hydrogen served as a poor electron donor for nitrite reduction in G20. The rate of hydrogen-supported nitrite reduction by G20 was higher than the rate of nitrite reduction when no electron donor was added ($P=0.039$, Table 3.2). Apparently hydrogen gas did not support nitrite reduction by I2, as the rate when hydrogen was the electron donor was not faster than the rate when no electron donor was added ($P=0.24$, Table 3.2).

Viamajala and coworkers tested the nitrite reductase of *Shewanella* for its ability to reduce Cr(VI) (Viamajala et al., 2002). They observed that Cr(VI) inhibited nitrite reduction, and nitrite inhibited Cr(VI) reduction in *Shewanella* when the two compounds were added in equal molar amounts (0.1-0.5 mM) (Viamajala et al., 2002). Since electron donor was in excess in their system, they interpreted this result to mean that the inhibition was due to competition at the site of the common reductase. In our experiments measuring the simultaneous reduction of 1 mM nitrite and 1 mM U(VI), uranyl acetate failed to inhibit nitrite reduction when 10 mM lactate or 10 mM pyruvate was the electron donor in either G20 or I2 (Fig. 3.6 A, data not shown). Similarly, nitrite failed to inhibit U(VI) reduction by either G20 or I2 when lactate or pyruvate were the electron donor (Fig. 3.6 B,C). Nitrite also failed to inhibit U(VI) reduction in G20 when hydrogen was the electron donor (Fig. 3.6 D). Curiously, 1 mM nitrite did inhibit the residual rate of U(VI) reduction measured in I2 (Fig 3.1, Table 3.1) when hydrogen was the electron donor. (Fig. 3.6 D).

Table 3.2. Nitrite reduction by *D. desulfuricans* G20 wild-type and the cytochrome *c*₃ mutant I2^a

Strain (relevant genotype)	Nitrite reduction rate ^a (avg ± SD) with electron donor ^b			
	Lactate	Pyruvate	Hydrogen	None
G20 (wild-type)	0.52 ± 0.13	1.0 ± 0.36	0.15 ± 0.06	0.07
I2 (<i>cycA</i> mutant)	0.64 ± 0.04	2.0 ± 0.6	0.06 ± 0.02	0.07

^aRate units are μmol NO₂ reduced/mg cell protein/h. Rates are from assays with whole cells, 100-200 μg cell protein/ml, determined from the first 2.5 h of the assay when lactate or pyruvate were the electron donors, or from the first 20 h of the assay when hydrogen gas or no electron donor was added. All rates are the average of four independent determinations except control lacking added electron donor which was the average of two independent determinations. The endogenous rate of nitrite reduction is not subtracted.

^bLactate, 10 mM; pyruvate, 10 mM; hydrogen, 1 atm.

Figure 3.6. Nitrite reduction by a cytochrome c_3 mutant.

Panel A: Nitrite reduction by G20 (*squares*) and I2 (*circles*) in the presence or absence of 1 mM uranyl (VI) acetate. G20 without (\square) and with (\blacksquare) 1 mM U(VI), and I2 without (\circ) and with (\bullet) 1 mM U(VI). 10 mM sodium pyruvate added as the electron donor.

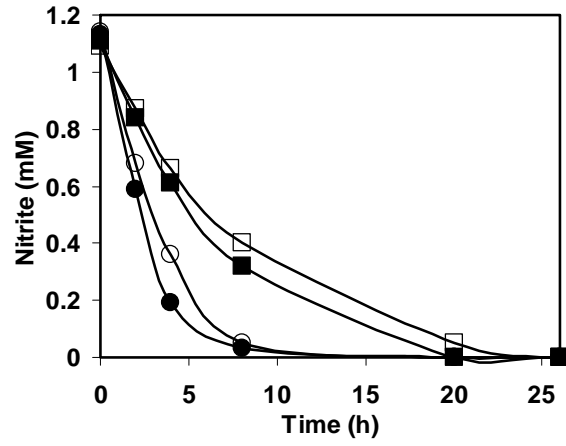
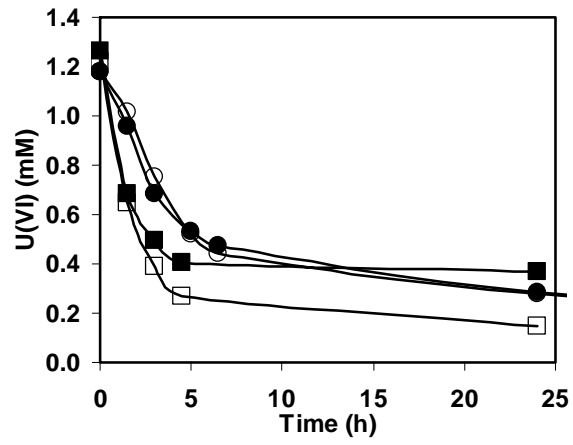
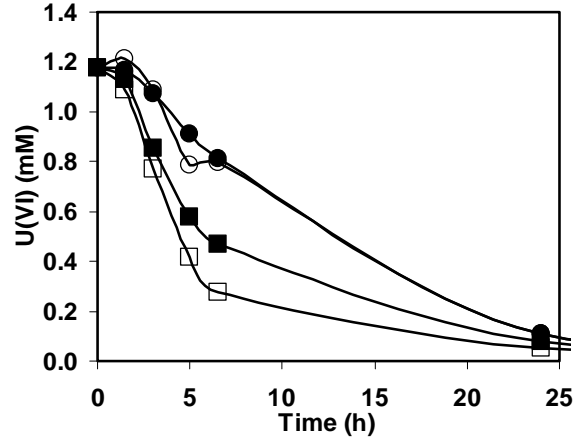
Panel B-D: U(VI) reduction by G20 (*squares*) and I2 (*circles*) in the presence or absence of 1 mM sodium nitrite. G20 without (\square) and with (\blacksquare) 1 mM NO_2^- , and I2 without (\circ) and with (\bullet) 1 mM NO_2^- .

Panel B: 10 mM sodium lactate added as the electron donor;

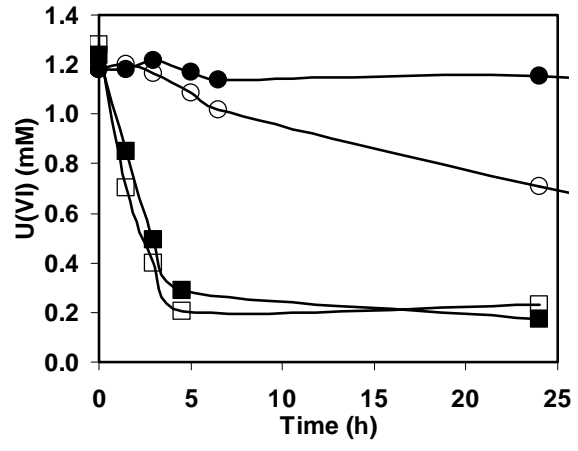
Panel C: 10 mM sodium pyruvate added as the electron donor;

Panel D: 10 mM sodium pyruvate added as the electron donor.

Data points are the average of at least two independent measurements, and graphs are representative of at least two independent experiments.

A**B****C**

D

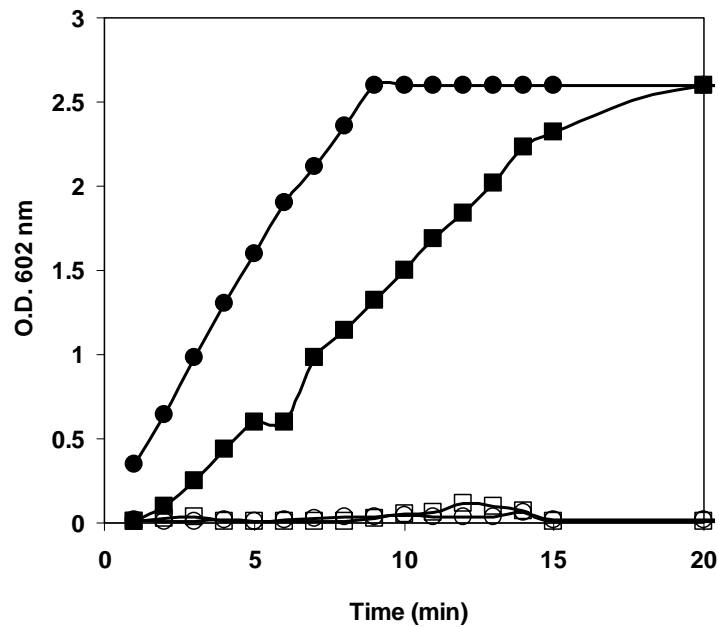


3.2.5. Hydrogenase activity in the cytochrome c_3 mutant of *D. desulfuricans*.

Hydrogenase has been reported to reduce Cr(VI) *in vitro* (Chardin et al., 2003) and to reduce Tc(VII) *in vivo* (De Luca et al., 2001), although the ability of hydrogenase to reduce U(VI) to U(IV) was not tested in these two systems. The cytochrome c_3 mutant of *D. desulfuricans* G20 was severely impaired for sulfate respiration (Rapp-Giles et al., 2000), and for U(VI) reduction (Fig. 3.1) using hydrogen as the electron donor. We tested the possibility that these two observations were due to repression of hydrogenase activity in the cytochrome c_3 mutant rather than the lack of the protein cytochrome c_3 *per se*. Hydrogenase activity was measured in resting cells of both wild-type G20 and the cytochrome c_3 mutant, I2 grown on LS medium. When activity was measured in the direction of hydrogen oxidation with methyl viologen as the electron acceptor, I2 cells reduced methyl viologen about twice as fast as G20 (Fig 3.7). Negative controls with no added hydrogen showed no reduction of methyl viologen during the time frame of this assay (30 min), although negative controls with no electron donor showed a similar extent of methyl viologen reduction after 16 hours as when hydrogen was included (data not shown). Therefore, the *cycA* mutant did not downregulate hydrogenase.

Figure 3.7. Hydrogenase assay of a cytochrome c_3 mutant of *D. desulfuricans*.

Hydrogenase activity as measured by methyl viologen reduction by whole cells of wild-type G20 (*squares*) and the cytochrome c_3 mutant I2 (*circles*). G20 cells with (■) and without (□) hydrogen as the electron donor; I2 cells with (●) and without (○) hydrogen as the electron donor. Each tube contains 2.5 $\mu\text{g/ml}$ whole cell protein. Each point is one measurement. This figure is representative of 3 independent trials.



3.3. Discussion

Both the mutant lacking cytochrome c_3 and wild-type cells lacking cytochrome c_3 due to growth in iron-limiting medium reduced U(VI) poorly with hydrogen as the electron donor (Table 3.1, Fig. 3.1). However, some reduction of U(VI) with hydrogen was consistently seen during assays of I2. This likely resulted from a bypass of cytochrome c_3 (for example through reduction of U(VI) directly by the Fe-hydrogenase, see below) rather than from the accumulation of small numbers of suppressors that restore cytochrome c_3 since Western analysis of a portion of the I2 cells used in the U(VI) reduction assay did not detect cytochrome c_3 (data not shown). This observation supports the earlier report that cytochrome c_3 was necessary for U(VI) reduction by extracts of *D. vulgaris* when hydrogen was the source of electrons (Lovley et al., 1993). Surprisingly, I2 was still capable of reducing U(VI) with lactate as the electron donor at a rate about one-half that of the wild-type and of reducing U(VI) with pyruvate as the electron donor at a rate of about one-third that of the wild-type (Table 3.1, Fig. 3.1). However, wild-type cells, containing low amounts of cytochrome c_3 when grown in iron-limiting medium reduced U(VI) poorly, if at all, with lactate or pyruvate as the electron donor (Fig. 3.1). Since iron is required for the synthesis of many cofactors needed by electron transport proteins such as Fe-S clusters and hemes, growth in iron-limiting medium may cause a general repression of other electron transport proteins in addition to cytochrome c_3 . Previous experiments using sulfate as the electron acceptor showed that I2 grew at the same rate and to 85% of the extent of the wild-type with lactate as the electron donor but was unable to grow with pyruvate as the sole electron donor (Rapp-Giles et al., 2000). We inferred that cytochrome c_3 is apparently involved in the transfer of electrons from

pyruvate to sulfate to support growth but is not the sole carrier for electrons from lactate to sulfate. We also expected that the cytochrome c_3 mutant might be unable to reduce U(VI) with pyruvate. However, the linkage between growth and U(VI) reduction with pyruvate as the electron donor was not absolute suggesting multiple pathways for electrons to reach toxic metals.

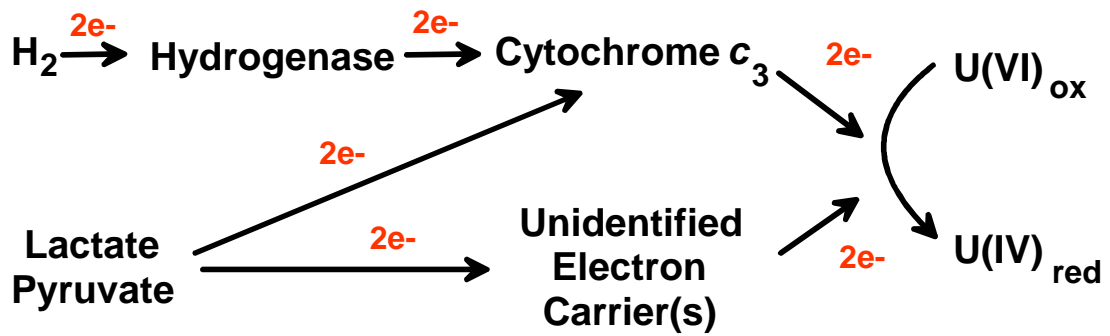
Clearly, electron flow independent of cytochrome c_3 provides reductant for U(VI) in *D. desulfuricans* strain G20 when organic acids are oxidized. We suggest that essentially all of the electrons from hydrogen must go through the cytochrome c_3 pathway for U(VI) reduction, but only some of the electrons from lactate or pyruvate go through the cytochrome c_3 pathway for U(VI) reduction (Fig. 3.8). This is consistent with the hydrogen cycling model of electron flow for some of the electrons from organic acids coupled with alternative pathways for others.

The periplasmic nitrite reductase of *Shewanella* (a facultative anaerobe capable of growing by nitrate, Fe(III), Cr(VI), or U(VI) respiration (Myers and Nealson, 1988; Lovley et al., 1991) has been suggested to be a carrier in the pathway for U(VI) reduction in that organism (Wade and DiChristina, 2000). In addition, the nitrite reductase of *Shewanella* has been proposed to also act as a Cr(VI) reductase (Viamajala et al., 2002). Some strains of SRB (like *D. desulfuricans* strain 27774) can grow by nitrate respiration (Mitchell et al., 1986) and, therefore, must have a bona fide nitrite reductase. Although *D. desulfuricans* G20 and *D. vulgaris* are incapable of this respiration (data not shown), the presence of an operon encoding a possible periplasmic nitrite reductase is present in the *D. desulfuricans* G20 and *D. vulgaris* genome sequences (VIMSS, <http://www.escalante.lbl.gov>; Fig 3.4). The function of nitrite reductase in SRB

Figure 3.8. Modified Lovley Model for U(VI) reduction in a cytochrome c_3 -lacking cells.

Arrows indicate electron flow and do not necessarily imply a single step in vivo.

Although essentially all of the electrons from hydrogen are transferred to U(VI) via cytochrome c_3 , some of the electrons from lactate or pyruvate can bypass cytochrome c_3 .



which are unable to grow by nitrate respiration has been suggested to be for the detoxification of nitrite produced by neighboring sulfate-oxidizing nitrate-reducing bacteria such as *Thiomicrospira* strains (Greene et al., 2003). We hypothesized that the putative *D. desulfuricans* G20 nitrite reductase (a *c*-type cytochrome) might act as an alternate U(VI) reductase in wild-type G20 or in the cytochrome *c*₃ mutant cells. The putative nitrite reductase of G20 was shown to be expressed by Northern analysis when cells are grown in LS medium in the absence of nitrite, and whole cells were shown to have nitrite reducing activity (Fig. 3.5 and Fig. 3.6).

Viamajala and coworkers observed that Cr(VI) inhibited nitrite reduction, and nitrite inhibited Cr(VI) reduction in *Shewanella* when the two compounds were added in equal molar amounts (0.1-0.5 mM) (Viamajala et al., 2002). Because of this result, it was suggested that the nitrite reductase of *Shewanella* was capable of acting as a Cr(VI) reductase (Viamajala et al., 2002). We reasoned that if the nitrite reductase of the wild-type or cytochrome *c*₃ mutant of *D. desulfuricans* G20 acted as a U(VI) reductase, then the addition of nitrite should inhibit U(VI) reduction, and the addition of uranium should inhibit nitrite reduction. The addition of a sub-inhibitory concentration of nitrite (1mM) did inhibit U(VI) reduction by the I2 mutant, but not wild-type G20 cells when hydrogen was the electron donor (Fig. 3.6). The interpretation of this observation is somewhat complicated by the fact that nitrite has been shown to inhibit the Fe-hydrogenase of *D. vulgaris* (Berlier et al., 1987). However, nitrite and U(VI) reduction proceeded independently of each other in both G20 and the I2 mutant under the conditions of these assays when lactate or pyruvate were the electron donors and the electron acceptors were in equal molar concentrations (1 mM; Fig. 3.6). In particular, since nitrite and U(VI)

reduction proceeded independently of each other in cells lacking cytochrome c_3 , we interpret this result to mean that the nitrite reductase is unlikely to act as a U(VI) reductase.

Interestingly, the I2 mutant lacking cytochrome c_3 reduced nitrite about twice as fast as the wild-type G20 when pyruvate was the electron donor (Fig. 3.6). This suggested to us that either nitrite reductase is up-regulated in I2, or that the blockage of normal electron flux in the cytochrome c_3 mutant caused more electron flow from pyruvate through the nitrite reductase. Northern analysis showed that the transcription of the putative nitrite reductase of I2 is not up-regulated, but is about 8-fold down-regulated in the cytochrome c_3 mutant (Fig. 3.5). However, it must be pointed out that cultures used for Northern analysis and cultures used for nitrite reduction assays were not identical. Northern analysis was performed on RNA isolated from early- to mid-exponential phase cultures, as older cultures yielded degraded RNA (data not shown), while the nitrite reduction assays were performed on early stationary phase cultures.

It is possible that the absence of cytochrome c_3 in I2 causes an alteration in the electron flow in this organism so that more electrons are available to the nitrite reductase (Fig. 3.9) and that the additional electron flow accounts for the mutant's faster rate of nitrite reduction. We speculate that the build-up of electrons in the electron transport chain of I2 might cause the mutant to downregulate the components of its electron transport chain that are partnered with cytochrome c_3 in the wild-type. The redox state of the quinone pool has been shown to regulate the expression of electron carrier proteins in the facultative anaerobe *Paracoccus denitrificans* (Otten et al., 2001) and the phototroph *Rhodobacter sphaeroides* (Oh and Kaplan, 2000). Northern analyses (see sections 6 and

7, below) indicate that other membrane-bound *c*-type cytochromes are down-regulated in the cytochrome *c*₃ mutant. A similar phenomenon has been observed for the Fe-hydrogenase mutant of *D. vulgaris* (Haveman et al., 2003; Pohorelic et al., 2002). The gene encoding a cytoplasmic alcohol dehydrogenase is highly expressed in wild-type *D. vulgaris* when cells are grown on media in which ethanol, lactate, pyruvate, formate, or hydrogen provide energy for sulfate reduction (Haveman et al., 2003). The authors interpreted this to suggest that electron flow in the wild-type from lactate or pyruvate, as well as formate or hydrogen using acetate as the carbon source, requires alcohol dehydrogenase (Haveman et al., 2003). In the Fe-hydrogenase mutant of *D. vulgaris*, the alcohol dehydrogenase was at least 10-fold down-regulated (Haveman et al., 2003).

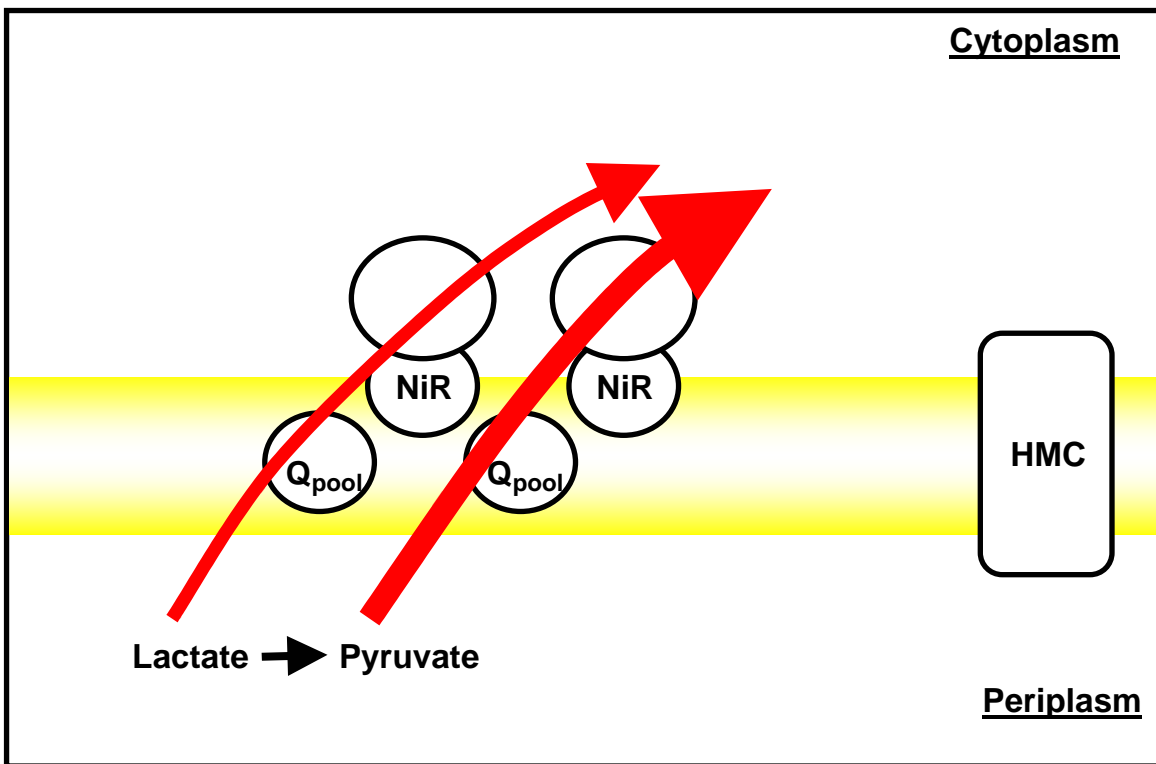
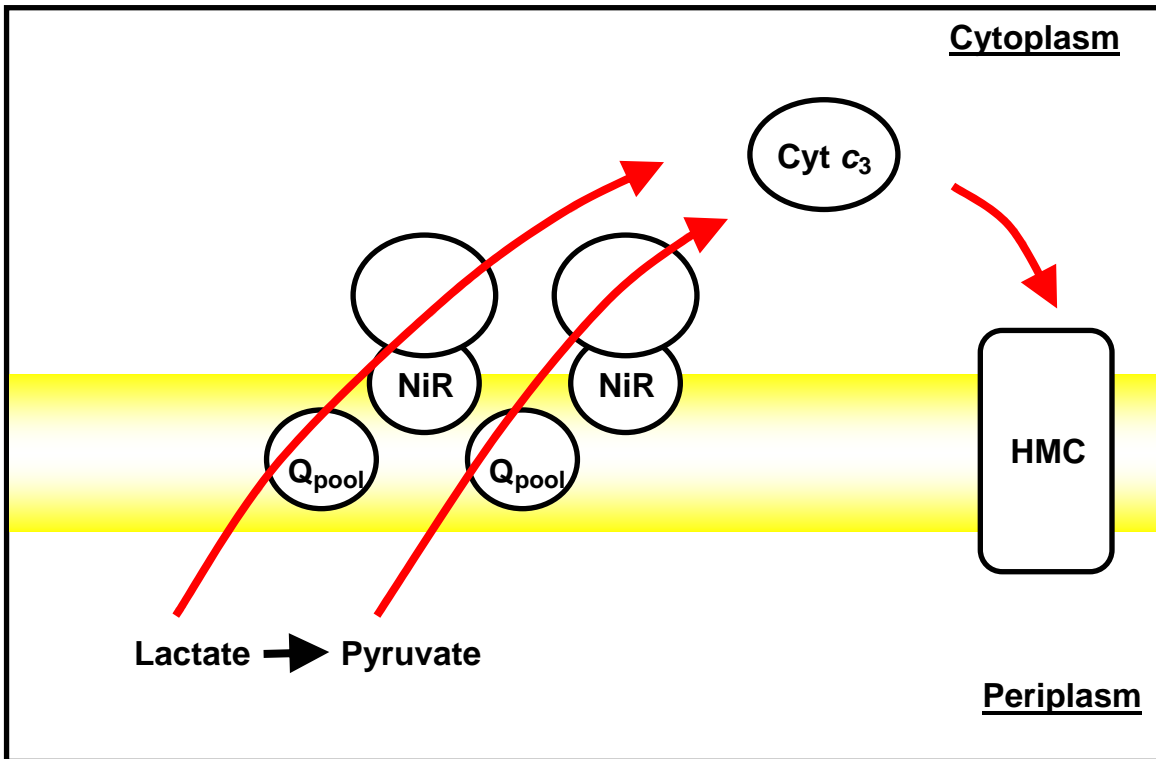
Alternatively, it is also possible that the proteins encoded by the putative G20 nitrite reductase operon may not be responsible for the measured nitrite reductase activity. This would also account for the paradoxical observation between the nitrite reductase activity and nitrite reductase expression in the cytochrome *c*₃ mutant. A mutant constructed in the putative G20 nitrite reductase, or biochemical analysis on the purified NfrHA complex from G20 would help to answer these questions.

The final U(VI) reductase candidate explored was hydrogenase. The NiFe-hydrogenase of *Desulfovibrio fructosovorans* has been reported to act as a technetium (VII) reductase by DeLuca and coworkers (De Luca et al., 2001), and the Fe-hydrogenase of *D. vulgaris* has been shown to act as a chromium (VI) reductase (Chardin et al., 2003). These data suggest that low redox-potential Fe-S clusters (non-heme Fe cofactors) may be sufficient for metal reduction *in vivo* (Chardin et al., 2003). We think it is unlikely that the hydrogenase of wild-type G20 or the mutant I2 acts as a U(VI)

reductase, since the I2 mutant has at least as much, if not more, hydrogenase activity than the wild-type, yet reduces U(VI) poorly with hydrogen as the electron donor. However, it is possible that the Fe-hydrogenase shown to be responsible for greater than 90% of the periplasmic hydrogen oxidation activity of *D. vulgaris* (Pohorelic et al., 2002) acts as a U(VI) reductase, but with a much slower rate than cytochrome *c*₃ (about 10% the rate of cytochrome *c*₃). This would account for the small rate of U(VI) reduction seen in I2 when hydrogen is the electron donor. The role of hydrogenase will be further tested in the next chapter by assaying a Fe-hydrogenase mutant of the related species *D. vulgaris* for U(VI) reduction.

Figure 3.9. Altered electron flux in a cytochrome c_3 mutant.

Electron flow from lactate and pyruvate oxidation in the wild-type (*Top panel*) and in the *cycA* mutant (*Bottom panel*). *Cyt c_3* represents the dominant periplasmic *c*-type cytochrome encoded by *cycA*. *Qpool* represents the quinone pool. *NiR* is the putative nitrite reductase. *Hmc* represents the transmembrane high molecular mass cytochrome *c* complex which is proposed to transfer electrons to the cytoplasmic enzymes responsible for sulfate reduction (Dolla et al., 2000). In the *cycA* mutant, the absence of *cyt c_3* causes a blockage of electron flow from the quinone pool. Those electron transport proteins linked to the quinone pool are “over-reduced” with electrons. *Red arrows* indicate electron flow and do not necessarily imply a single step *in vivo*.



4. Uranium(VI) reduction by a hydrogenase mutant of *Desulfovibrio vulgaris*

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Part of the work described here was carried out by Kelly R. Titkemeier as part of a semester project for a non-thesis Master's degree in Geological Sciences completed in May 2004.

4.1. Introduction

Three classes of hydrogenase have so far been identified in the periplasm of *D. desulfuricans* G20 and *D. vulgaris* Hildenborough; each is named for the metal ion(s) at the active site of the enzyme. The three classes are Fe-, NiFe-, and NiFeSe-hydrogenase (Fauque et al., 1988; Lissolo et al., 1986; Pohorelic et al., 2002; Prickril et al., 1987). The genome sequence of *D. vulgaris* suggests the existence of an additional NiFe-hydrogenase isozyme and at least two cytoplasmic hydrogenases (Heidelberg et al., 2004). The genome sequence of *D. desulfuricans* G20 suggests the existence of an additional NiFe-hydrogenase isozyme, an additional Fe-hydrogenase isozyme, and one cytoplasmic hydrogenase (VIMSS, <http://www.escalante.lbl.gov>). The existence of a cytoplasmic hydrogenase to create hydrogen gas from protons and electrons generated by the oxidation of organic acid, and the existence of a periplasmic hydrogenase to re-capture cytoplasmically generated hydrogen, are essential for the generation of a proton gradient across the cytoplasmic membrane by the mechanism illustrated in the hydrogen cycling model (Odom and Peck, 1981; Fig. 1.4).

Since the 16S ribosomal RNA genes of *D. desulfuricans* G20 and *D. vulgaris* Hildenborough are about 91% identical, and the cytochrome and hydrogenase content are quite similar between the two organisms (VIMSS, <http://www.escalante.lbl.gov>), it seems likely that many electron transport components used to reduce U(VI) will be shared. A mutant in the Fe-hydrogenase encoding gene of *D. vulgaris* was constructed by Voordouw and coworkers by marker exchange of the gene encoding the periplasmic Fe-hydrogenase (*hydAB*) with a gene encoding chloramphenicol resistance (Pohorelic et al., 2002). Whole, intact cells of the Fe-hydrogenase mutant of *D. vulgaris* have about 50%

of the hydrogen uptake activity of the wild-type *D. vulgaris* and have no detectable periplasmic hydrogen uptake activity compared to the wild-type *D. vulgaris* when measured by cyclic voltammetry (Pohorelic et al., 2002). These data suggest that the Fe-hydrogenase is responsible for essentially all of the periplasmic hydrogen uptake activity in the wild-type *D. vulgaris*, and that the contribution of the NiFe- and NiFeSe-hydrogenases to periplasmic hydrogen uptake is negligible.

In previous experiments, a mutant in the related strain *D. desulfuricans* G20 lacking cytochrome c_3 was shown to be impaired about 50% compared to the wild-type in U(VI) reduction when lactate or pyruvate were the electron donors. The cytochrome c_3 mutant was more severely impaired, about 90%, in U(VI) reduction when hydrogen gas was the electron donor (Payne et al., 2002). In many *Desulfovibrio* strains, this cytochrome has been proposed to act in series with the hydrogenases. Thus we would predict that a mutation decreased in hydrogenase activity might also be impaired in U(VI) reduction with hydrogen as the source of reductant. To determine the role played by the Fe-hydrogenase in U(VI) reduction, we assayed the Fe-hydrogenase mutant of *D. vulgaris* for its ability to reduce U(VI). We anticipated that the Fe-hydrogenase mutant of *D. vulgaris* would have a similar phenotype as the cytochrome c_3 mutant of *D. desulfuricans* G20. In other words, the Fe-hydrogenase mutant of *D. vulgaris* was anticipated to be about 90% impaired when hydrogen gas was the electron donor, and about 50% when lactate or pyruvate were the electron donors. This was not the result observed. We found that the Fe-hydrogenase mutant reduced U(VI) at the same rate as wild-type cells when hydrogen gas or pyruvate were the electron donors. However, when lactate was the electron donor the Fe-hydrogenase mutant was about 50% impaired in

U(VI) reduction. This suggests that the periplasmic Fe-hydrogenase is involved in the flow of electrons to U(VI) when lactate, but not hydrogen or pyruvate, is the electron donor.

4.2. Results

4.2.1. Growth of *Desulfovibrio vulgaris*

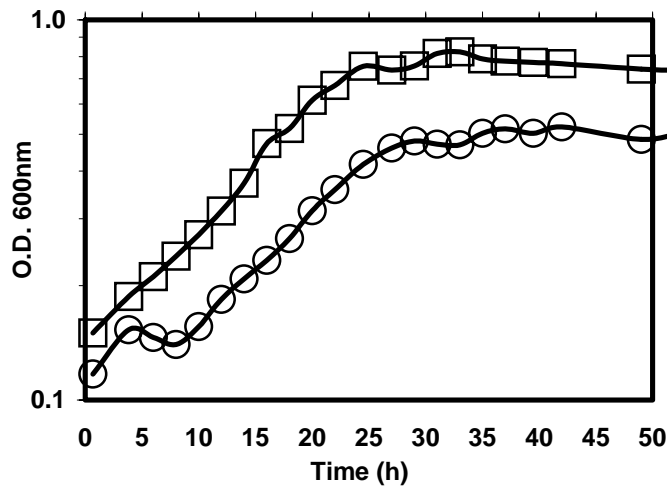
A representative growth curve of wild-type *D. vulgaris* and the Fe-hydrogenase mutant of *D. vulgaris* on LS medium is shown (Fig. 4.1). The Fe-hydrogenase mutant consistently grew at about a 30-50% slower rate, and reached ca. a 50% lower final protein yield (data not shown) than the wild-type when lactate was the electron donor. No attempts to grow *D. vulgaris* on PS or hydrogen plus sulfate were made during the course of this study.

4.2.2. U(VI) reduction by a Fe-hydrogenase mutant of *Desulfovibrio vulgaris*

Comparative U(VI) reduction rates are shown in Table 4.1 for wild-type and the Fe-hydrogenase mutant. These rates were corrected for those observed when no external source of reductant was added to cultures. In these cultures that endogenous rate was about one-half that of pyruvate supported U(VI) reduction activity. As expected, wild-type cells expressing the Fe-hydrogenase reduced U(VI) with lactate, pyruvate, or hydrogen gas as the electron donor. Unlike the result for *D. desulfuricans* wild-type (Table 3.1), the rates of reduction were about twice as fast for lactate or hydrogen as for pyruvate (Table 4.1). The Fe-hydrogenase mutant reduced U(VI) at the same rate as wild-type when hydrogen gas or pyruvate was the electron donor, but was impaired about 50% in U(VI) reduction when lactate supplied the electrons (Table 4.1).

Figure 4.1. Growth of a Fe-hydrogenase mutant of *Desulfovibrio vulgaris*.

Growth of wild-type (\square) and Fe-hydrogenase mutant (\circ) of *D. vulgaris* on LS medium as measured by optical density at 600_{nm} (Titkemeier and Wall, 2004). Data points represent one measurement and this figure is representative of five independent trials.



4.2.3. U(VI) reduction by *Desulfovibrio vulgaris* in the presence of inhibitors

To further explore the contribution of the various periplasmic hydrogenases (Fe-, NiFe-, and NiFeSe-) to U(VI) reduction, we exposed resting cells of *D. vulgaris* to various hydrogenase inhibitors, then tested those cells for U(VI) reduction. 0.5 mM Cu²⁺ has been shown to inhibit greater than 90% of the periplasmic hydrogen uptake activity of whole cells of *D. vulgaris* (Cypionka and Dilling, 1986; Fitz and Cypionka, 1991), and *Desulfovibrio fructosovorans* (De Luca et al., 2001). When wild-type *D. vulgaris* was preincubated with 0.5 mM CuCl₂, there was about a 90% inhibition of the U(VI) reduction rate with all electron donors tested (Table 4.1). This suggests that periplasmic hydrogenase activity or its associated activities that may be copper inhibited are essential for U(VI) reduction when hydrogen or the organic acids lactate or pyruvate supply the electrons.

Nitrite at 100 μM was reported to inhibit the Fe- and NiFeSe-hydrogenase, but not the NiFe-hydrogenase in cell-free extracts of *D. vulgaris* (Berlier et al., 1987). The concentration of an inhibitor needed to cause inhibition is likely to be greater in whole cells than in cell-free extracts. Therefore a concentration of 1 mM NaNO₂ was chosen to test for hydrogenase inhibition in whole cells of *D. vulgaris*. One mM NaNO₂ does not inhibit the growth of *D. desulfuricans* G20 on LS medium (data not shown). When wild-type *D. vulgaris* was preincubated with 1 mM NaNO₂, there was about a 50% inhibition of the U(VI) reduction rate using lactate or hydrogen as the electron donors tested (Table 4.1). However, no inhibition of U(VI) reduction by nitrite was observed when pyruvate was the source of electrons (Table 4.1).

Table 4.1. U(VI) Reduction by a Fe-hydrogenase mutant of *D. vulgaris* Hildenborough^a

<i>D. vulgaris</i> strain (relevant genotype)	Inhibitor	Electron Donor		
		Lactate	Pyruvate	Hydrogen
Wild-type ^b	None	4.2 ± 0.4	1.5 ± 0.6	4.4 ± 1.0
<i>hydAB</i> mutant ^b	None	1.4 ± 0.5	1.5 ± 0.3	4.5 ± 0.8
Wild-type ^c	0.5 mM CuCl ₂	0.15	0.1	0.1
Wild-type ^d	1 mM NaNO ₂	2.0 ± 0.2	1.6 ± 0.2	1.6 ± 0.5

^aRates are from assays with whole cells, 100µg cell protein/ml, determined from the first 2 h of the assay; µmol U(VI) reduced x mg cell protein⁻¹ x h⁻¹.

^bRates are four independent determinations.

^cRates are two independent determinations.

^dRates are three independent determinations.

4.3. Discussion

Lovley and coworkers reconstituted a pathway for U(VI) reduction *in vitro* using hydrogen gas as the electron donor, purified hydrogenase, and purified cytochrome c_3 (Lovley et al., 1993). We have a cytochrome c_3 mutant in *D. desulfuricans* G20 and a Fe-hydrogenase mutant in *D. vulgaris* Hildenborough. Because these strains share about 91% identity in 16S ribosomal RNA and have many physiological and biochemical phenotypes (such as substrates utilized, incomplete oxidation of organic acids, antibiotic resistances, etc.) in common, we assumed that the electron transport chain components in the two strains would be comparable. This assumption is supported by both biochemical (Aubert et al., 1998) and genome sequence analysis (VIMSS, <http://www.escalante.lbl.gov>) of the cytochrome and hydrogenase content of the two strains. A mutant in the dominant periplasmic cytochrome c_3 , constructed in the parent strain *D. desulfuricans* G20, was impaired about 90% with hydrogen gas as the electron donor for U(VI) reduction (Payne et al., 2002). This observation supported the Lovley model derived from *D. vulgaris* Hildenborough for U(VI) reduction when hydrogen is the electron donor. The Fe-hydrogenase constitutes the majority of the periplasmic hydrogenase activity of *Desulfovibrio* strains, and a mutant of *D. vulgaris* Hildenborough lacking that enzyme had no detectable periplasmic hydrogen uptake activity (Pohorelic et al., 2002). When I tested that mutant for U(VI) reduction, the rate and extent were not impaired with hydrogen gas as the electron donor. This result was consistent with those from Elias and coworkers who also found that the Fe-hydrogenase mutant of *D. vulgaris* was not impaired in hydrogen supported U(VI) reduction (Elias et al., 2004). Two possible explanations for this observation are: (1) the electrons generated from hydrogen

oxidation by the Fe-hydrogenase are not in the pathway of U(VI) reduction in wild-type *D. vulgaris*; therefore, the mutant is not impaired in this process; or (2) though the electrons generated from hydrogen oxidation by the Fe-hydrogenase are in the pathway of U(VI) reduction in wild-type *D. vulgaris*, in the mutant the additional hydrogenases compensate for the absence of the Fe-isozyme. Although Pohorelic and coworkers showed that the NiFe- or NiFeSe-hydrogenases apparently were responsible for only trace amounts of periplasmic hydrogen oxidation activity with methyl viologen as the electron acceptor (Pohorelic et al., 2002), these isozymes might be altered in activity or abundance when the Fe-hydrogenase is removed by mutation. The expression of the alternate hydrogenase isozymes was not followed (by Northern analysis or RT-PCR) during the course of this dissertation, but explanation (2) is appealing as the NiFe- or NiFeSe-hydrogenase have been shown to have a higher affinity for hydrogen than the Fe-hydrogenase (Fauque et al., 1988). This possibility is supported by the observation that Cu^{2+} , an inhibitor of all periplasmic hydrogenases (Cypionka and Dilling, 1986; De Luca et al., 2001), inhibits greater than 90% of U(VI) reduction by both the Fe-hydrogenase mutant and the wild-type when hydrogen is the electron donor (Table 4.1).

My experiments showed that the cytochrome c_3 mutant of *D. desulfuricans* G20 was impaired by about 66% in U(VI) reduction compared to the wild-type *D. desulfuricans* G20 when pyruvate was the electron donor. Surprisingly, the rate of U(VI) reduction by the Fe-hydrogenase mutant of *D. vulgaris* Hildenborough with pyruvate as the electron donor was not impaired compared to the wild-type. At least one alternate electron transfer pathway apparently exists that does not require the Fe-hydrogenase. It is possible that the NiFe- or NiFeSe-hydrogenase are involved in this alternate pathway.

This model is supported by the observation that Cu^{2+} inhibits greater than 90% of U(VI) reduction by both the Fe-hydrogenase mutant and the wild-type when pyruvate is the electron donor (Table 4.1)

With lactate as the electron donor, however, I found there was a statistically significant difference in the rates of U(VI) reduction by the wild-type and the Fe-hydrogenase mutant of *D. vulgaris* Hildenborough. The Fe-hydrogenase mutant had about 30% of the rate of the wild-type *D. vulgaris* (Table 4.1). Interestingly, the mutant was also impaired for growth on sulfate when lactate was the electron donor (Figure 4.1). However, Voordouw and coworkers did not report an impairment in the growth of the Fe-hydrogenase mutant on lactate sulfate medium (Pohorelic et al., 2002). However, van den Berg and coworkers constructed via antisense RNA a *D. vulgaris* Hildenborough mutant that expressed about 30% of the Fe-hydrogenase activity (van den Berg et al., 1991). They observed that their Fe-hydrogenase mutant was impaired by two- to three-fold in growth rate, and about 20% in final yield when lactate was the electron donor (van den Berg et al., 1991). Although we cannot directly resolve these conflicting data, we interpret our observations to mean that the Fe-hydrogenase is involved in the flow of electrons from lactate to both U(VI) and to sulfate. However, an alternate pathway for electron transport from lactate to U(VI) or sulfate (that may involve the NiFe- or the NiFeSe-hydrogenases) must be present in the wild-type or accessible in the mutant. This model is supported by the observation that 1 mM nitrite, an inhibitor of the Fe- and NiFe-hydrogenase (De Luca et al., 2001), inhibits lactate supported U(VI) reduction by in wild-type cells by about 50% (Table 4.1), and by the observation that Cu^{2+} inhibits about 90%

of U(VI) reduction by both the Fe-hydrogenase mutant and the wild-type when lactate is the electron donor.

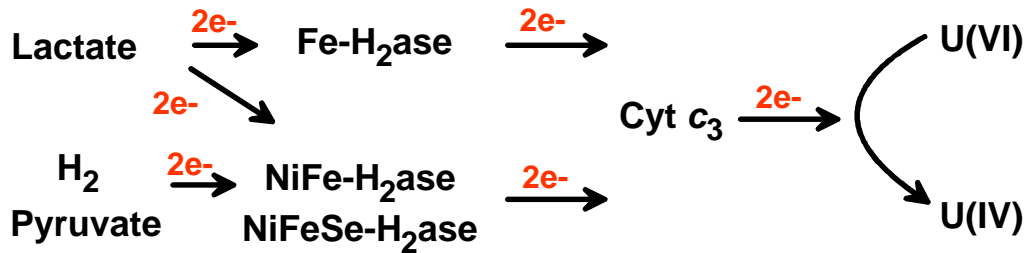
Hydrogen cycling as a means used by *Desulfovibrio* to generate a transmembrane proton gradient has been intensely debated. We believe the experiments here support the hydrogen cycling model of electron flow from the cytoplasmic oxidation of organic molecules, at least with respect to the electrons transferred to U(VI) (Fig. 1.4). This does not necessarily imply that all of the electrons generated from lactate or pyruvate oxidation are used to make cytoplasmic hydrogen. Some electrons generated from the oxidation of lactate or pyruvate may still be transferred through a classical membrane bound electron transport chain (Fig. 1.3; Fig 1.4; Fig. 3.8). The reason for the existence of multiple periplasmic hydrogenases in *Desulfovibrio* is still obscure. We interpret the data here to suggest that the different individual hydrogenases are involved in the flow of electrons from different organic substrates, or, in other words, the electrons from hydrogen, lactate, and pyruvate are not equivalent. This is somewhat surprising in the case of electrons generated from the oxidation of lactate and pyruvate as these two organic compounds are linked by one redox reaction step. The evidence presented here suggests that at least some of the electrons generated by the cytoplasmic oxidation of lactate or pyruvate go through a periplasmic hydrogen intermediate, and that these electrons can be used for U(VI) or sulfate reduction.

In conclusion, the Fe-hydrogenase is apparently involved in electron flow from lactate, but not from pyruvate or hydrogen, to U(VI) in *D. vulgaris*. The NiFe- and the NiFeSe-hydrogenase may be involved in electron flow from all three substrates to U(VI)

(Fig. 4.2). In addition, we observed that the Fe-hydrogenase appears to function in the pathway from lactate to sulfate.

Figure 4.2. Modified Lovley Model for U(VI) reduction in a Fe-hydrogenase mutant of *D. vulgaris*.

Arrows indicate electron flow and do not necessarily imply a single step *in vivo*. Some of the electrons from the oxidation of lactate are eventually transferred by the Fe-hydrogenase to U(VI), but some of the electrons bypass the Fe-hydrogenase pathway. Apparently none of the electrons from the oxidation of hydrogen or pyruvate are transported through the Fe-hydrogenase pathway. The NiFe- and NiFeSe-hydrogenases also are likely involved in electron flow from all three substrates to U(VI).



5. Interaction of Cytochrome c_3 and Uranium

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Text, Tables 2.1 and 5.1, and Figures 5.1, 5.2, 5.3, and 5.4 were modified and used with permission from Archives of Microbiology, 2004, 181(6):398-406.

5.1. Introduction

Since SRB are found in significant numbers at uranium-contaminated sites, their response to low levels of toxic metals might indicate an interaction that could affect U(VI) reduction. The hypothesis was tested that exposure to a subinhibitory concentration of uranium might induce the cellular machinery of *D. desulfuricans* G20 responsible for the reduction of this metal. Prior exposure of *D. desulfuricans* G20 cells to uranium actually decreased the ability of those cells to reduce U(VI). Interestingly, growth of *D. desulfuricans* G20 in the presence of uranium did not affect the expression of the periplasmic cytochrome c_3 ; however, cytochrome c_3 was no longer among the soluble proteins from a high pH wash of those cells. Instead, periplasmic cytochrome c_3 was found associated with precipitated uraninite. The adsorption of cytochrome c_3 to uraninite appeared to be non-specific since purified cytochrome c_3 was shown to bind to CuO (cupric oxide), Fe₂O₃ (ferric oxide), or commercially available U(IV) oxide in a buffer system.

5.2. Results

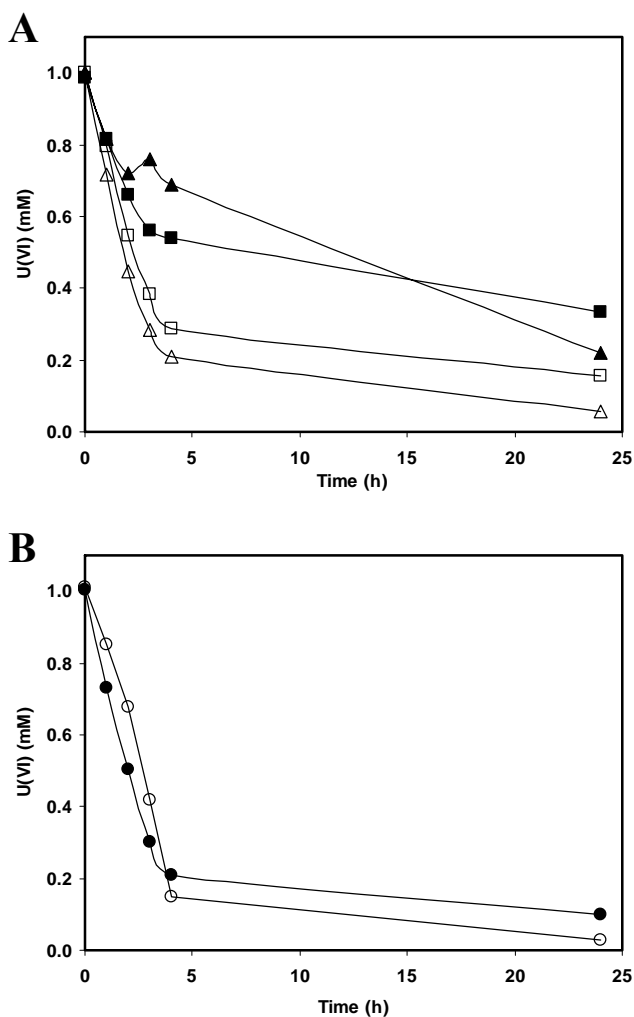
5.2.1. Uranium(VI) reduction by cells grown in the presence of uranium

The possibility was tested that prior growth in the presence of a non-lethal concentration of U(VI) would cause an increase of cellular machinery responsible for U(VI) reduction, therefore such cells would reduce U(VI) at a faster rate than cells that had not been previously exposed to U(VI). Cells were grown on LS medium amended with a subinhibitory concentration of uranium (1 mM) (Payne et al., 2002) and tested for their ability to reduce U(VI). These cells exhibited a slower rate of U(VI) reduction than cells grown in the absence of uranium when the organic acids lactate or pyruvate were

the electron donors in the assay (Fig. 5.1). With hydrogen as the electron donor, the rate of U(VI) reduction was not impaired by growth in the presence of uranium (Fig. 5.1).

Figure 5.1. U(VI) reduction by *D. desulfuricans* G20 previously exposed to uranium.

U(VI) reduction kinetics of *D. desulfuricans* G20 grown in the absence (open symbols) or in the presence (closed symbols) of 1 mM uranyl acetate. All samples have 1 mM uranyl acetate and 100 μ g whole cell protein/ml. Symbols are the average of two data points, and the graphs are representative of 4 independent trials with each electron donor. (A) 10 mM sodium lactate (\square , \blacksquare), or 10 mM sodium pyruvate (Δ , \blacktriangle) was the electron donor for U(VI) reduction. (B) 1 atm hydrogen gas (\circ , \bullet) was added as the electron donor for U(VI) reduction.



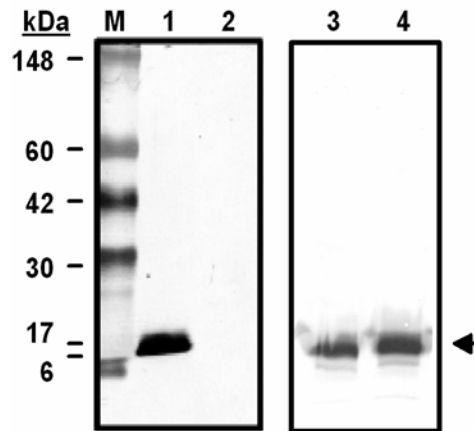
5.2.2. Cytochrome c_3 recovery from cells grown with uranium

The cytochrome c_3 of *D. vulgaris* Hildenborough participates in the electron transfer to uranium in a partially purified cell extract system (Lovley et al., 1993), and a cytochrome c_3 mutant of *D. desulfuricans* G20 is impaired in uranium reduction (Payne et al., 2002). Since these data support the involvement of cytochrome c_3 in U(VI) reduction, it was speculated that the impairment observed in U(VI) reduction after exposure of cells to the subinhibitory concentration of uranium might have resulted from an alteration in cytochrome c_3 content. Immunoblot analysis with antibodies to purified cytochrome c_3 was performed on protein extracts from cells grown in the presence of 1 mM uranium to test this possibility. Cytochrome c_3 was not detected in the soluble extracts of cells grown on LSU medium, in contrast to control cultures grown without exposure to uranium (Fig. 5.2). Interestingly, cytochrome c_3 was detected in the insoluble fraction (containing both whole cells and uraninite) remaining after the soluble protein was harvested from *D. desulfuricans* G20 cells (Fig. 5.2). The cytochrome c_3 recovered from the insoluble fraction of LSU grown cells was roughly equivalent to the amount of cytochrome c_3 recovered from the insoluble fraction from LS grown cells. The tetraheme cytochrome c_3 of *D. vulgaris* Hildenborough has been found both in the periplasm and associated with the cytoplasmic membrane (Valente et al., 2001). Thus significant cytochrome c_3 should be recovered in the insoluble cell material of *D. desulfuricans* G20 after the periplasmic contents of cells were harvested by a pH shift. This was the result observed. The inability to detect cytochrome c_3 in the soluble extracts obtained from LSU grown cells was due to the quantitative adsorption of the protein to the mineral uraninite, as shown in following sections. If in fact cytochrome c_3 adsorbs to uraninite,

then it might be expected that the insoluble fraction of LSU grown cells would have more cytochrome c_3 than the insoluble fraction of LS grown cells. However, the difficulty in loading the insoluble fraction of LSU cells (containing an approximately equal volume of the mineral uraninite) onto acrylamide gels makes a comparison of the cytochrome c_3 by Western analysis somewhat qualitative. A quantitative measurement of cytochrome c_3 expression by a translational fusion of *cycA* to *lacZ* will be presented in section 5.2.3; Western analysis of cytochrome c_3 obtained from cells grown on LSU medium, but separated from the insoluble uraninite before the TE extraction, will be presented in section 5.2.5.

Figure 5.2. Cytochrome c_3 is not recovered in the periplasm of cells grown in the presence of uranium.

Immunoblot comparison of the high pH TE wash extracted protein fractions from *D. desulfuricans* G20 cultures grown in the absence or in the presence of 1 mM uranyl acetate. Lane M, molecular mass markers; lane 1, 20 μ g pH extracted protein from cells grown on LS medium; lane 2, 20 μ g pH extracted protein from cells grown on LSU medium; lane 3, 30 μ g insoluble fraction protein from cells grown on LS medium; lane 4, 30 μ g insoluble fraction protein from cells grown on LSU medium. The arrow shows the migration of purified cytochrome c_3 on the gel.



5.2.3. Effect of uranium on transcription/translation of cytochrome c_3 .

To test the possibility that uranium might have also negatively influenced the expression of the gene encoding cytochrome c_3 , the transcription of *cycA* was monitored by reverse transcription-polymerase chain reaction (RT-PCR) and Northern analysis. Qualitatively both techniques revealed that transcripts for *cycA* were still present in *D. desulfuricans* G20 cells cultured in the presence of uranium (data not shown). Further proof that uranyl acetate did not quantitatively alter the level of transcription came from studies with a protein fusion of the promoter region and the start codon of *cycA* with the *lacZ* gene creating the reporter vector pLC111 (Casalot and Wall, published in Payne et al., 2004). The upstream region of *cycA* was confirmed to act as a strong promoter in *E. coli* harboring pLC111 (Table 5.1). A single copy of this fusion construct was then integrated into the chromosome of *D. desulfuricans* G20 at the site of the wild-type *cycA* gene creating *D. desulfuricans* G25. The *cycA* gene had previously been shown to be in a monocistronic operon (Rapp-Giles et al., 2000), therefore polar effects of reporter plasmid integration were not an issue. As can be seen in Table 5.1, there was no detectable difference in β -galactosidase activity when *D. desulfuricans* G25 cells (harboring the integrated *cycA-lacZ* translation fusion) were grown in LS or LSU medium. As a control, for β -galactosidase production in the sulfate-reducing bacterium, *D. desulfuricans* G20 cells harboring the plasmid pMON5003, with a constitutive *lacZ* under the control of the *bla* promoter, were assayed. There was also no detectable difference in β -galactosidase activity when *D. desulfuricans* G20 (pMON5003) cells were grown in LS or LSU medium (Table 5.1). The observation that the expression of cytochrome c_3 , which is clearly involved in U(VI) reduction, is not detectably regulated

by a subinhibitory concentration of uranium suggests that strategies designed to reveal genes involved in toxic metal reduction by altered gene transcription will clearly not reveal all genes important to the process.

Table 5.1. β -Galactosidase activities of the different strains.

Host bacterium	Strain	Specific activity - uranium ^a	Specific activity + uranium
<i>E. coli</i>	CC118 (pMON5003)	22,200	N.D. ^b
	CC118 (pVIK111)	100	N.D.
	CC118 (pLC111)	60,000 ^c	N.D.
<i>D. desulfuricans</i>	G20 (pMON5003)	2,700	3,000
	G20	<10	<10
	G25 ^d	3,300	3,600

^a Specific activity ($\mu\text{mol}/\text{min}\cdot\text{mg}$ protein) determined in cells grown to early stationary phase ($\text{OD}_{600\text{nm}} = 2$ for *E. coli* cultures and $\text{OD}_{600\text{nm}} = 0.8$ for *D. desulfuricans* cultures) in the absence or presence of 1 mM uranyl acetate. Numbers were the average of 3 duplicates and were representative of 7 independent determinations.

^b Not determined.

^c Corrected for plasmid stability.

^d G20 with pLC111 integrated in the chromosome in single copy.

5.2.4. Adsorption of cytochrome c_3 to uraninite

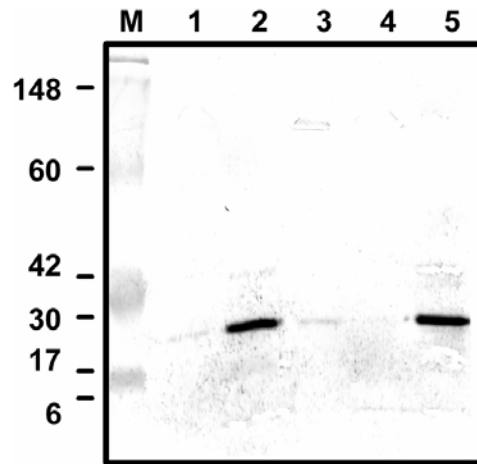
Other researchers reported the adsorption of purified *D. vulgaris* cytochrome c_3 , *Desulfuromonas acetoxidans* cytochrome c_7 , and *Desulfomicrobium norvegicum* (formerly *Desulfovibrio desulfuricans* Norway 4) (Genthner et al., 1997) cytochrome c_3 (M_r 26,000) onto the soil minerals kaolinite, montmorillonite, talc, goethite, and ochre (Sallez et al., 2000). The adsorption of the 3 cytochromes, each with different pIs (10.2, 7.8, and 4.8 respectively) onto the surface of the minerals was suggested to be due to both electrostatic and hydrophobic interactions. The association between negatively charged montmorillonite and the cytochromes c from all three SRB was essentially irreversible, while the association was much weaker between the other minerals and the cytochromes. In particular, the association of the *D. vulgaris* cytochrome c_3 to montmorillonite resulted in a loss of catalytic activity of the cytochrome c_3 (Sallez et al., 2000).

We hypothesized that the failure to detect cytochrome c_3 in soluble protein preparations from cells grown on LSU medium was due to the adsorption of the periplasmic cytochrome c_3 to the insoluble mineral uraninite generated from the subinhibitory concentration of uranyl acetate. Purified cytochrome c_3 was further tested for its ability to adsorb to commercially available, insoluble oxides of the metals uranium, copper, and iron in TE buffer. When soluble purified cytochrome c_3 was added to suspensions of U(IV) oxide or CuO, cytochrome c_3 was no longer detected in solution, but was detected in the insoluble pellet fraction (Fig. 5.3). By immunoblot analysis, the bound cytochrome c_3 was not dissociated from the U(IV) oxide pellet by a 2 M NaCl wash (data not shown), but components of the SDS-PAGE loading buffer were effective in removing some of the cytochrome c_3 from the insoluble metal precipitate (Fig. 5.3).

On occasion the purified cytochrome c_3 showed multiple bands of higher than expected molecular weight, suggesting that the protein formed multimers when concentrated under these conditions. When soluble purified cytochrome c_3 was added to suspensions of Fe_2O_3 , cytochrome c_3 was not detected in solution nor was it detected in the insoluble pellet fraction (Fig. 5.3), possibly due to a very strong association of the cytochrome c_3 to the ferric oxide. Neither a 2 M NaCl wash (data not shown) nor the SDS-PAGE loading buffer was effective in removing cytochrome c_3 from the insoluble ferric oxide precipitate.

Figure 5.3. Adsorption of cytochrome c_3 to uraninite.

Immunoblot showing adsorption of *D. desulfuricans* G20 cytochrome c_3 to uraninite and Fe_2O_3 . Lane M, molecular mass markers; lane 1, supernatant from the cytochrome c_3 - UO_2 suspension; lane 2, pellet from the cytochrome c_3 - UO_2 suspension; lane 3, supernatant from the cytochrome c_3 - Fe_2O_3 suspension; lane 4, pellet from the cytochrome c_3 - Fe_2O_3 suspension; lane 5, 125 ng purified cytochrome c_3 diluted in the same manner as the cytochrome c_3 -metal oxide suspensions; Cytochrome c_3 adsorption to CuO not shown.

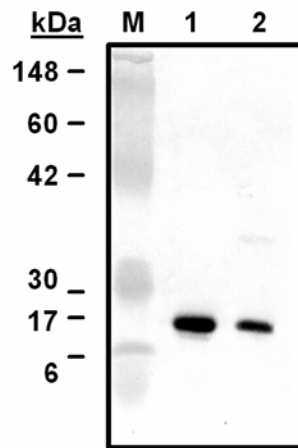


5.2.5. Cytochrome c_3 recovery from cells grown on LSU medium after separation of cells and uraninite by ultracentrifugation

Western analysis for cytochrome c_3 was performed on $15 \times$ concentrated spent LSU medium after cells had been removed by centrifugation. The tetraheme cytochrome c_3 , accounting for over 80% of the periplasmic c -type cytochromes (Aubert et al., 1998), was not detected in spent LS or LSU medium (data not shown). However, since cytochrome c_3 was shown to associate with UO_2 in a cell-free suspension (Fig. 5.3), any cytochrome c_3 released into the medium during growth would be predicted to adsorb to the insoluble uraninite and therefore not be detected in solution in the spent medium. After separation of LSU grown cells from uraninite by sedimentation in a sucrose gradient, the recovered cells were subjected to a high pH TE wash to recover periplasmic proteins. Greater than 50% of the cytochrome c_3 was recovered in the soluble extract from the LSU grown cells, supporting the interpretation that 1 mM uranium did not cause a drastic disruption of the outer membrane of *D. desulfuricans* G20 (Fig. 5.4). Lack of full recovery could be attributed to small losses of cytochrome c_3 during the ultracentrifugation and protein extraction steps. About 25% of the amount of cytochrome c_3 recovered by pH shift from LSU grown cells was found associated with the insoluble uraninite pellet separated by the sucrose gradient. This association could have occurred because of normal cell death and lysis or to osmotic changes occurring during the separation in sucrose (Fig. 5.4).

Figure 5.4. Cytochrome c_3 recovery from cells grown in the presence of uranium.

Cells were grown on LSU medium then separated from uraninite by sucrose gradient sedimentation before soluble protein was harvested by a high pH TE wash. Lane M, molecular mass markers; lane 1, 25 μ l of the 50-fold concentrated soluble protein obtained from LSU grown cells as described above; lane 2, 25 μ l of the 50-fold concentrated uraninite fraction obtained as described above.



5.3. Discussion

The initial observation that growth of *D. desulfuricans* G20 cells in the presence of a subinhibitory concentration of uranium impaired U(VI) reduction when lactate or pyruvate are the electron donors (Fig. 5.1) remains to be explained, especially since cells grown on LSU medium showed no detectable decrease in rate or extent of growth as determined by whole cell protein analysis (Payne et al., 2002). In addition, the impairment of U(VI) reduction was apparently not due to down-regulation of transcription/translation of cytochrome c_3 , or to destabilization of the outer membrane. Uranium might non-specifically inhibit a number of enzymes needed for energy and carbon metabolism resulting in a decrease in the reductive capacity for U(VI) when electron donor is supplied in low concentrations (10 mM in the U(VI) reduction assay compared to 60 mM in LS medium). The observation that cells previously exposed to uranium are not impaired in hydrogen-supported U(VI) reduction (Fig. 5.1) suggests to us that 1 mM uranium causes an impairment of carbon metabolism (or transport) rather than hydrogen oxidation alone. Microarray studies of cells exposed to non-inhibitory uranium concentrations may provide insight into this phenomenon.

The biological implications of the adsorption of cytochrome c_3 to uraninite and metal oxides. Dissimilatory metal-reducing bacteria use several strategies to access extracellular metal terminal electron acceptors (TEA) in the environment (Hernandez and Newman, 2001). Possible means include: 1) direct contact of an oxidized metal with an outer membrane electron transfer component (Beliaev and Saffarini, 1998; Lower et al. 2001; Magnuson et al., 2000; Nevin and Lovley, 2000); 2) the release of siderophores which complex and solubilize the metal TEA (Ledyard and Butler, 1997); or 3) the use of

extracellular electron shuttling molecules such as quinone-containing compounds or *c*-type cytochromes to transfer electrons from the cellular electron transport chain to the metal TEA (Nevin and Lovley, 2002; Newman and Kolter, 2000; Seeliger et al., 1998). The extracellular electron shuttles may either be already present in the environment due to decomposition of organic material or intentionally transported by cellular processes into the environment. The use of extracellular *c*-type cytochromes in Fe(III) reduction has been questioned (Lloyd et al., 1999). Our results here taken together with previous work suggest that the periplasmic cytochrome *c*₃ of *D. desulfuricans* G20 is unlikely to function as an significant extracellular electron carrier to U(VI), since the tight association of the protein with both clay minerals and metal oxides would preclude a catalytic role for the protein.

6. The role of the sodium motive force in *Desulfovibrio*

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6.1. Introduction

Most bacteria couple electron transport and respiration to the formation of a transmembrane proton motive force. The transmembrane proton motive force represents a potential source of energy which can be used to generate ATP (by passage of periplasmic protons down a concentration gradient through the membrane-bound F_1F_0 ATPase), to transport substrates, or to rotate flagella (Fig. 1.4; for a recent review see Harold, 2001). The electrochemical potential of protons ($\Delta\mu_{H^+}$) is composed of the sum of two separate components: (1) the pH gradient across the cytoplasmic membrane (ΔpH , typically the periplasm is more acidic than the cytoplasm); and (2) the membrane potential ($\Delta\Psi$, typically the periplasm is more positive than the cytoplasm). Under alkaline conditions, both neutrophilic and alkaliphilic bacteria are faced with the problem of generating a proton motive force with an inverse pH gradient, i.e. the cytoplasm must be maintained at a lower pH than the external environment and periplasm (Krulwich 1995; Padan et al., 1981). In fact, an external pH of greater than about 7.8 abolishes the ΔpH of *Escherichia coli* (Pan and Macnab, 1990). In the absence of a ΔpH , only the $\Delta\Psi$ contributes to the overall membrane activation. A sufficiently large $\Delta\Psi$, which can be created by transport of other positively charged ions into the periplasm, can allow the translocation of H^+ ions against an unfavorable ΔpH (Harold, 2001).

Slightly halophilic marine bacteria of the genus *Vibrio* can grow under low proton motive force conditions such as in high pH environments or in the presence of the protonophore CCCP (Tokuda and Unemoto, 1981; Tokuda and Unemoto, 1983). These bacteria are capable of using a transmembrane sodium motive force in addition to a transmembrane proton motive force for growth under such conditions (Hayashi et al.,

2001; Tokuda and Unemoto 1982). In the *Vibrio* strains, the sodium motive force can be generated directly (i.e. in a mechanism not dependent upon the proton motive force) by the translocation of cytoplasmic sodium ions into the periplasm coupled to the oxidation of energy substrates. The transmembrane complex responsible for sodium ion translocation coupled to electron transport is the NADH-dependent Na^+ -Translocating Quinone Oxidoreductase (NQR). This contribution to the membrane potential apparently supports growth in low proton motive force conditions (Hayashi et al., 2001; Tokuda and Unemoto 1982). Na^+ ions in the periplasm can then be exchanged for protons to reestablish a proton gradient; in addition, positively charged Na^+ ions contribute to the $\Delta\Psi$ component of the membrane potential, thus allowing protons to be used for ATP synthesis against an unfavorable ΔpH . Alternatively, *Vibrio alginolyticus* can use the sodium motive force directly for substrate transport, for flagella rotation (Chernyak et al., 1983; Unemoto et al., 1993), or for extrusion of toxic compounds via a Na^+ /multidrug exporter (Morita et al., 1998). The completed genome sequences of the sulfate-reducing bacteria *Desulfovibrio desulfuricans* G20, *Desulfovibrio vulgaris* Hildenborough and *Desulfotalea psychrophila*, revealed homologs of many of the sodium-related genes of *V. alginolyticus* and *Vibrio parahaemolyticus*. We found homologs of the NQR complex and homologs of the well-characterized Na^+ /multidrug exporters in all three sequenced genomes even though only *Dt. psychrophila* has been characterized as a marine bacterium (Rabus et al., 2004). This observation suggested to us the existence of a “sodium circuit” in the SRB that may play a major role their bioenergetics.

We sought to explore the role of the “sodium circuit” in the SRB with the model organism *D. desulfuricans* G20. Initial RT-PCR experiments showed that the *D.*

desulfuricans G20 *nqr* operon was expressed. We hypothesized that *D. desulfuricans* G20 used the sodium motive force in a complementary manner to the well known proton motive force. For example, we predicted that under some conditions *D. desulfuricans* G20 could grow in the absence of a proton gradient. We found that *D. desulfuricans* G20 could grow in the absence of a proton gradient when lactate, but not pyruvate, was the electron donor. We also reasoned that the resistance of *D. desulfuricans* G20 to some antimicrobials was dependent upon the sodium motive force and have established that this is true.

6.2. Results

6.2.1. The Na⁺-Translocating NADH Quinone Oxidoreductase (NQR Complex) of the Sulfate-Reducing Bacteria

Sequence analysis of the completed genomes of the three sulfate-reducing bacteria *D. desulfuricans* G20, *D. vulgaris* Hildenborough, and *Dt. psychrophila*, revealed an operon similar to the well-characterized *nqr* operon of *V. alginolyticus* (Fig. 6.1). While the amino acid identities of the SRB *nqr* encoded proteins to those of *V. alginolyticus* are relatively low, the conservation of similar amino acids is somewhat higher (Fig. 6.1). More importantly, the synteny of the operons is identical. To our knowledge, genes immediately downstream of *nqr6* in *V. alginolyticus* have not been characterized. A gene encoding a homolog of *apbE* of *Salmonella* is downstream of *nqr6* in the completed genome sequences of the *Vibrio cholerae* El Tor N16961, *Vibrio vulnificus* CMCP6, *Vibrio vulnificus* YJ016, and *Vibrio parahaemolyticus* RIMD 2210633 (TIGR, <http://www.tigr.org>). The presence of an *apbE* ortholog promoter distal is a conserved feature of the SRB NQR complex operons as well (TIGR,

<http://www.tigr.org>; VIMSS, <http://www.escalante.lbl.gov>). It is possible that *apbE* is the last gene in the *V. alginolyticus nqr* operon as well. In *Salmonella*, ApbE is a periplasmic lipoprotein that plays a role in thiamine biosynthesis or Fe-S cluster formation (Beck and Downs, 1998; Skovran and Downs, 2003). The *Salmonella apbE* gene appears to be in a monocistronic operon, and genes located nearby are not similar to those of the *nqr* operon. The role, if any, of AbpE in the function of the NQR complex is unknown; however, several of the NQR subunit proteins have Fe-S clusters.

The *nqr* operons of the three SRB all have an interesting difference from the *Vibrio* spp. *nqr* operon. In the *Desulfovibrio* strains, the promoter proximal gene of the operon encodes a potential decaheme *c*-type cytochrome and in the same position in the *Dt. psychrophila* operon is a gene encoding a FAD flavoprotein (Fig. 6.1). It is possible that the decaheme *c*-type cytochrome (of the *Desulfovibrio* spp.) or the flavoprotein (of *Desulfotalea*) is intimately involved in electron flow from the NQR complex to other members of the electron transport chain. RT-PCR analysis shows that the putative *nqr* operon is expressed when *D. desulfuricans* G20 cells are grown on LS medium (Fig. 6.2). The expected products were obtained but quantitation was not possible from this experiment.

Figure 6.1. The Na⁺-Translocating NADH Quinone Oxidoreductase (NQR) operon of SRB.

Diagrammatic representation of the gene order of the putative NQR complex operons of the SRB compared with that from *Vibrio alginolyticus*. Arrows represent putative orfs and their relative sizes. Arrow direction indicates direction of transcription. Numbers below arrows report the level of identity/homology of the derived amino acid sequence from each SRB gene to the corresponding translation products of the *V. alginolyticus* genes. Note conserved order of *nqr* genes and the presence of an additional, promoter proximal gene in the SRB operons that encodes a potential electron carrier protein.

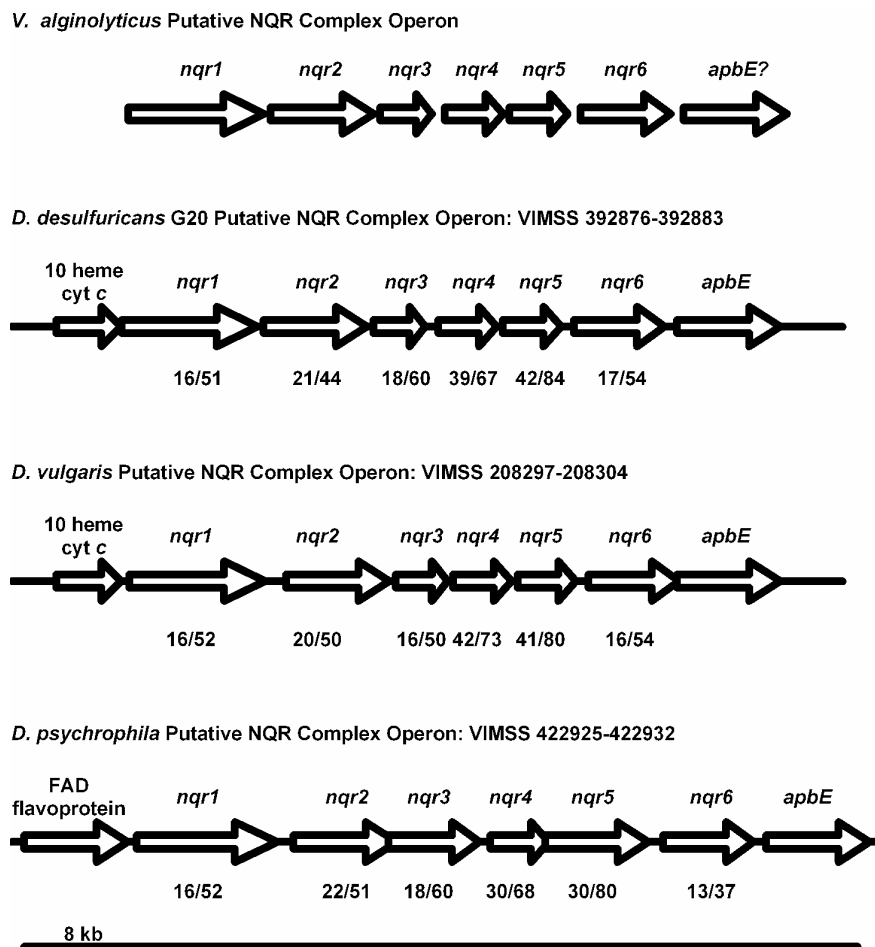


Figure 6.2. Expression of the NQR complex operon measured by RT-PCR.

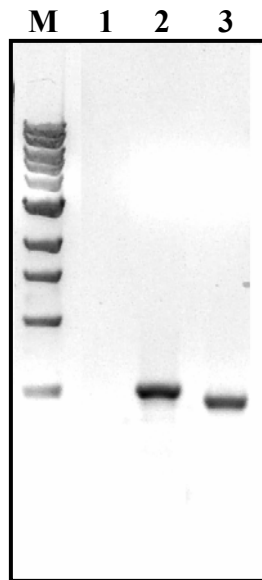
RT-PCR analysis of *nqr* operon expression by *D. desulfuricans* G20 cells grown on LS medium. Lanes 1, 2, and 3 have 50 ng of mRNA.

Lane: M, MW markers;

Lane 1, test for DNA contamination with *cycA* primers, primers c3_left and c3_right, no Reverse Transcriptase added;

Lane 2, test for decaheme cytochrome *c* gene expression, primers 10c_left and 10c_right with Reverse Transcriptase;

Lane 3, test for expression of gene encoding the transmembrane NADH oxidizing subunit of NQR, primers *nqr1_left* and *nqr1_right* with Reverse Transcriptase.



Growth on LS but not PS requires either the proton- or sodium- motive force. We tested *D. desulfuricans* G20 to determine its ability to grow in the presence of the protonophore CCCP. Cells were grown on LS medium or PS medium with or without CCCP added at 100 μM final concentration. No significant differences in growth rates were observed with or without CCCP when lactate was the electron donor and sodium was present (about 160 mM Na^+ in standardly prepared LS or PS; Fig. 6.3A). In addition, the same final optical density and protein concentration as cells grown in the absence of CCCP were documented (Fig. 6.3A, Table 6.1). The addition of 100 μM CCCP to PS medium completely abolished growth, even after cultures were incubated for 2 weeks (Fig. 6.3A, Table 6.1). The addition of 100 μM monensin, an electroneutral antiporter of sodium, to LS medium did not noticeably impair the growth rate or final yield of *D. desulfuricans* G20 when measured by OD or protein (Fig. 6.3B, Table 6.1). The addition of both 100 μM CCCP and 100 μM monensin together resulted in the decrease of both the growth rate and the final yield in LS medium (Fig. 6.3A,B, Table 6.1).

Figure 6.3. Growth of *D. desulfuricans* G20 under low proton- and sodium-motive force conditions.

The effect of a protonophore and a sodium electroneutral antiporter on the growth of *D. desulfuricans* G20. All media contained approximately 160 mM sodium.

Panel A. The effect of CCCP on the growth of G20 on LS or PS.

(□): LS growth medium.

(○): PS growth medium. Filled symbols had 100 μM CCCP added.

Panel B. The effect of CCCP and monensin on the growth of G20 on LS.

(□): LS growth medium.

(■): 100 μM CCCP added.

(◆): 100 μM monensin added.

(▲): 100 μM CCCP and 100 μM monensin added together.

All data points are the average of two independent cultures. The figure is representative of four independent experiments.

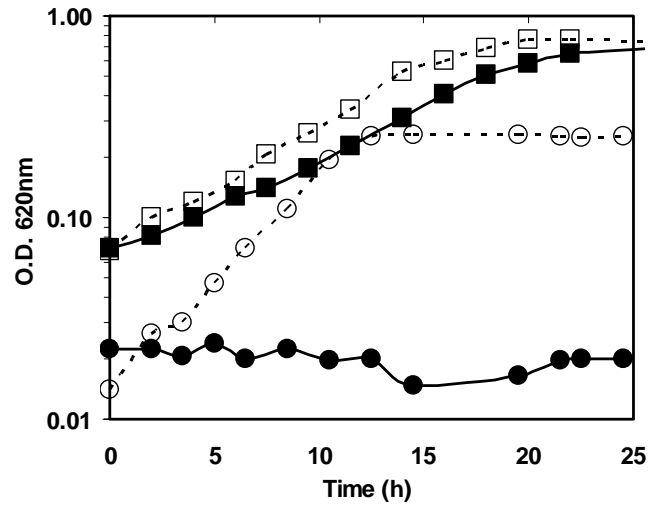
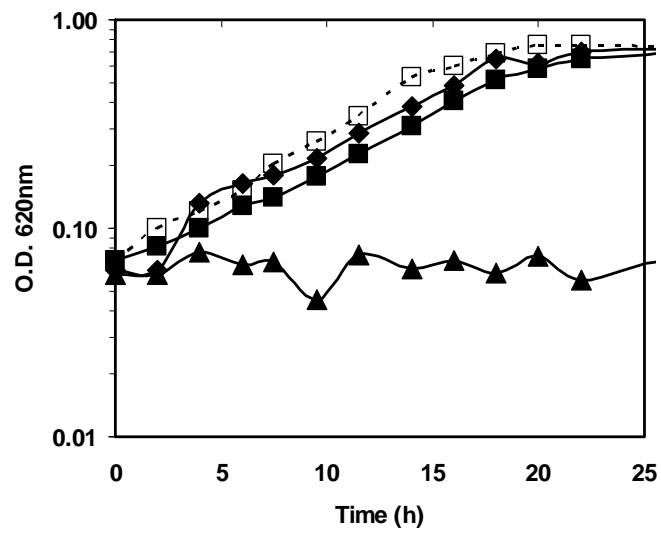
A**B**

Table 6.1. Protein yield of *D. desulfuricans* G20 cells grown in the presence of CCCP and monensin.

Growth Medium ^a	Inhibitor ^b	Final Protein Yield (µg/ml) ^c
Lactate Sulfate	None	206 ± 25 ^d
LS	CCCP	206 ± 7 ^d
LS	monensin	178 ± 4 ^d
LS	CCCP + monensin	7 ± 5 ^d
Pyruvate Sulfate (PS)	None	79 ^e
PS	CCCP	4 ^e

^aLS, 60 mM (D,L)-lactate and 50 mM Na₂SO₄; PS, 60 mM Na pyruvate and 50 mM Na₂SO₄.

^bCCCP, carbonyl cyanide m-chlorophenyl hydrazone, is a protonophore; monensin, is a sodium ionophore. Each inhibitor was used at a final concentration of 100 µM.

^cProtein measurements after 44 hours of growth at 37^oC of whole cell protein by Bradford procedure.

^dThe average and standard deviation obtained from three independent cultures from two different experiments.

^eThe average of two different cultures from one experiment.

6.2.2. Expression of the NQR complex operon

We used Northern analysis to see if the *D. desulfuricans* G20 NQR complex operon was differentially expressed under high pH or sodium concentrations. On complete LS medium at pH 7.2, we observed that the addition of monensin or CCCP alone did not affect the expression of the NQR. However, Northern analysis of RNA obtained from cells grown on complete LS medium at pH 9.0 showed about a 10 fold decrease of the expression of the NQR transcript (Fig. 6.4). Northern analysis was not performed on cells grown in the absence of sodium as we were unable to obtain high quality RNA from cells grown on LS0 medium during the course of this study. Interestingly, Northern analysis of the cytochrome *c*₃ mutant of *D. desulfuricans* showed that this mutant had about a 7 fold decrease in the expression of the NQR transcript compared to the wild-type when cultures were grown on complete LS medium (Fig. 6.4).

6.2.3. The Na⁺/multidrug exporters of the Sulfate-Reducing Bacteria

Sequence analysis of the completed genomes of the three SRB revealed homologs to the Na⁺/multidrug exporter NorM of *V. parahaemolyticus* (VIMSS, <http://www.escalante.lbl.gov>; Fig. 6.5). The identity at the amino acid level is somewhat low. However, all of the putative SRB sodium dependent drug efflux pumps were annotated as such by the VIMSS automatic annotation (<http://www.escalante.lbl.gov>), primarily based upon the characteristic 12 alpha helical transmembrane domains present in this family of proteins (TIGR, <http://www.tigr.org>). The NorM isozyme 3 from *D. desulfuricans* G20 and the NorM isozyme 2 from *D. vulgaris* have in common an unknown hypothetical protein at the promoter distal end of the operon (Fig. 6.5).

Figure 6.4. Expression of the NQR complex operon at pH 9, and by a cytochrome c_3 mutant of *D. desulfuricans* G20.

Panels A: Northern Blots probed with the internal fragment of the *D. desulfuricans* G20 decaheme cytochrome *c* of the *nqr* operon. The band corresponds to approximately 9 kb on the gel. *Panels B:* Gel showing the 23S and 16S rRNA stained with ethidium bromide. *Lanes 1-4:* RNA from wild-type cells grown on LS medium showing that the putative *nqr* operon is down-regulated at high pH. This Northern is representative of RNA from two independent experiments. The *Arrow* corresponds to the position of the 9 kb marker on the gel.

Lane 1: pH 7.2 medium.

Lane 2: pH 9.0 medium.

Lane 3: pH 7.2 medium + CCCP.

Lane 4: pH 7.2 medium + monensin.

Lanes 5-6: The putative *nqr* operon is down-regulated in the cytochrome c_3 mutant of *D. desulfuricans* G20.

Lane 5: RNA from wild-type *D. desulfuricans* G20.

Lane 6: RNA from the cytochrome c_3 mutant of *D. desulfuricans* G20.

5 μ g total RNA added per lane.

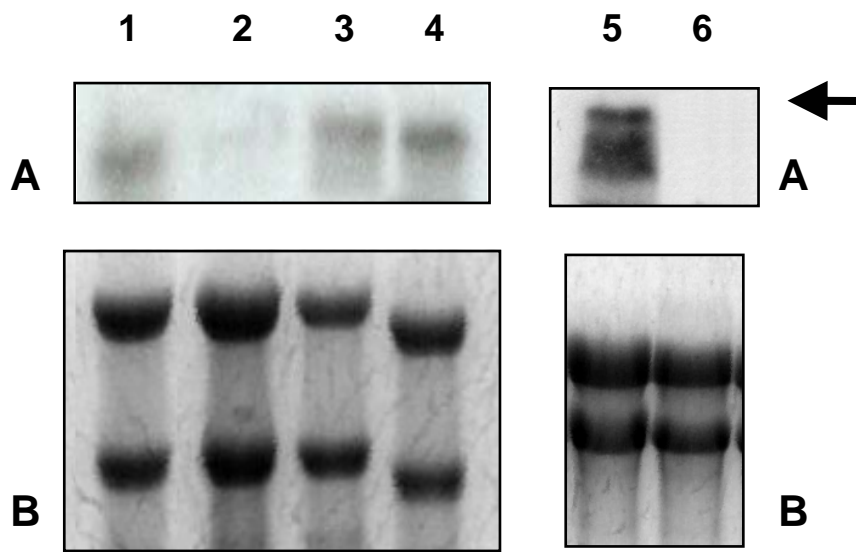


Figure 6.5. The Na⁺-dependent multidrug exporters (NorM) of the SRB.

Black arrows represent the NorM homologs of the sequenced sulfate-reducing bacteria.

Only the genetically and biochemically characterized NorM isozyme of *V.*

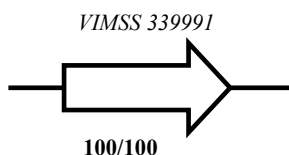
parahaemolyticus is shown, 9 other homologs of NorM in *V. parahaemolyticus* are

predicted based upon the sequence. Percent identity and percent similarity scores at the amino acid level are shown beneath each gene. VIMSS orf numbers

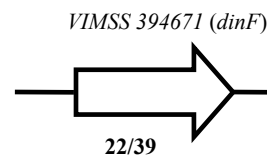
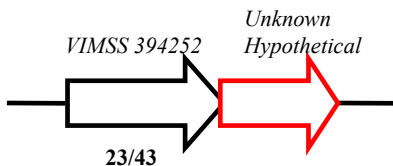
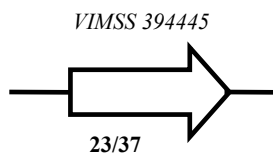
(<http://www.escalante.lbl.gov>) are shown above each gene. The *red arrows* represent

orfs encoding unknown hypothetical proteins promoter distal to the NorM homologs.

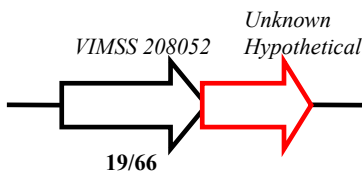
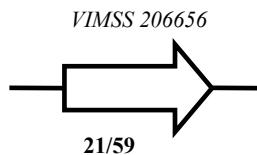
V. parahaemolyticus NorM 1 isozyme



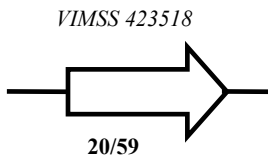
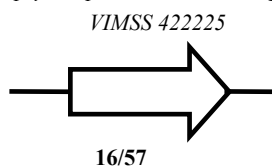
D. desulfuricans G20 NorM orthologs



D. vulgaris NorM orthologs



D. psychrophila NorM orthologs



6.2.4. The role of the sodium motive force during drug resistance

The NorM of *V. parahaemolyticus* functions to pump some antimicrobial compounds of the same general structure (like kanamycin) out of the cell. Typically the compounds are relatively small (MW ca. 300-500), ring-shaped, and cationic or hydrophilic. The activity of the *V. parahaemolyticus* NorM is dependent upon the sodium motive force. *D. desulfuricans* G20 is somewhat resistant to many inhibitors, for example kanamycin. We tested whether sodium was required for kanamycin resistance in *D. desulfuricans* G20. When sodium was omitted from the LS medium, or when the sodium ionophore monensin was added to LS medium containing sodium, *D. desulfuricans* G20 was much more sensitive to kanamycin (Fig. 6.6, Table 6.2). The resistance of *D. desulfuricans* G20 to methylene blue and ethidium bromide, as well as kanamycin, was dependent on the sodium concentration in the growth medium. Over a 60 fold increase in sensitivity to kanamycin with low sodium concentrations was observed (Table 6.2). Apparently nalidixic acid is not a substrate for any sodium driven efflux pump because G20 cells were not more sensitive to nalidixic acid when sodium was omitted from the LS medium or when LS medium contained both sodium and the sodium ionophore monensin (Table 6.2).

Figure 6.6. *D. desulfuricans* G20 is more sensitive to kanamycin in the absence of the sodium-motive force.

D. desulfuricans G20 is more sensitive to kanamycin in the absence of the sodium-motive force. All cells are grown on LS0 medium, pH 7.2, modified as follows:

Open squares: 160 mM NaCl present

Closed squares: 160 mM NaCl + 120 μ g kanamycin per ml.

Open circles: <1 mM NaCl.

Closed circles: <1 mM NaCl + 120 μ g kanamycin per ml.

Closed triangles: 160 mM NaCl + 100 μ M monensin + 120 μ g kanamycin per ml.

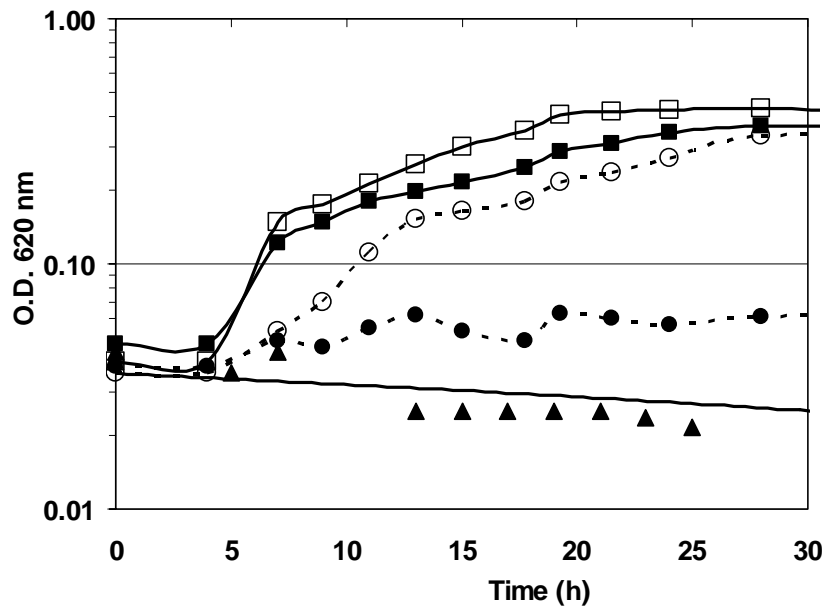


Table 6.2. MIC^a of inhibitors for *D. desulfuricans* G20 when grown with different NaCl.

Inhibitors	Medium Composition ^b		
	150 mM NaCl	150 mM NaCl + 100 μ M Monensin ^c	0 mM NaCl
Kanamycin	480 ^d	240 ^d	7.5 ^d
Ethidium Bromide	60 ^e	15 ^e	15 ^e
Methylene Blue	>800 ^e	>800 ^e	100 ^e
Nalidixic Acid	800 ^e	800 ^e	800 ^e

^aMinimum Inhibitory Concentration given as μ g/ml of the compound that completely inhibited growth in liquid LS0 medium supplemented as indicated.

^bAll experiments were performed in LS0 medium supplemented as indicated. LS0 medium actually contained about 0.7 mM sodium as determined experimentally by ICP analysis by the Agricultural Experiment Station Laboratories, University of Missouri-Columbia.

^cMonensin is an electroneutral H⁺/Na⁺ exchange compound.

^dMIC of at least 3 independent experiments done in duplicate.

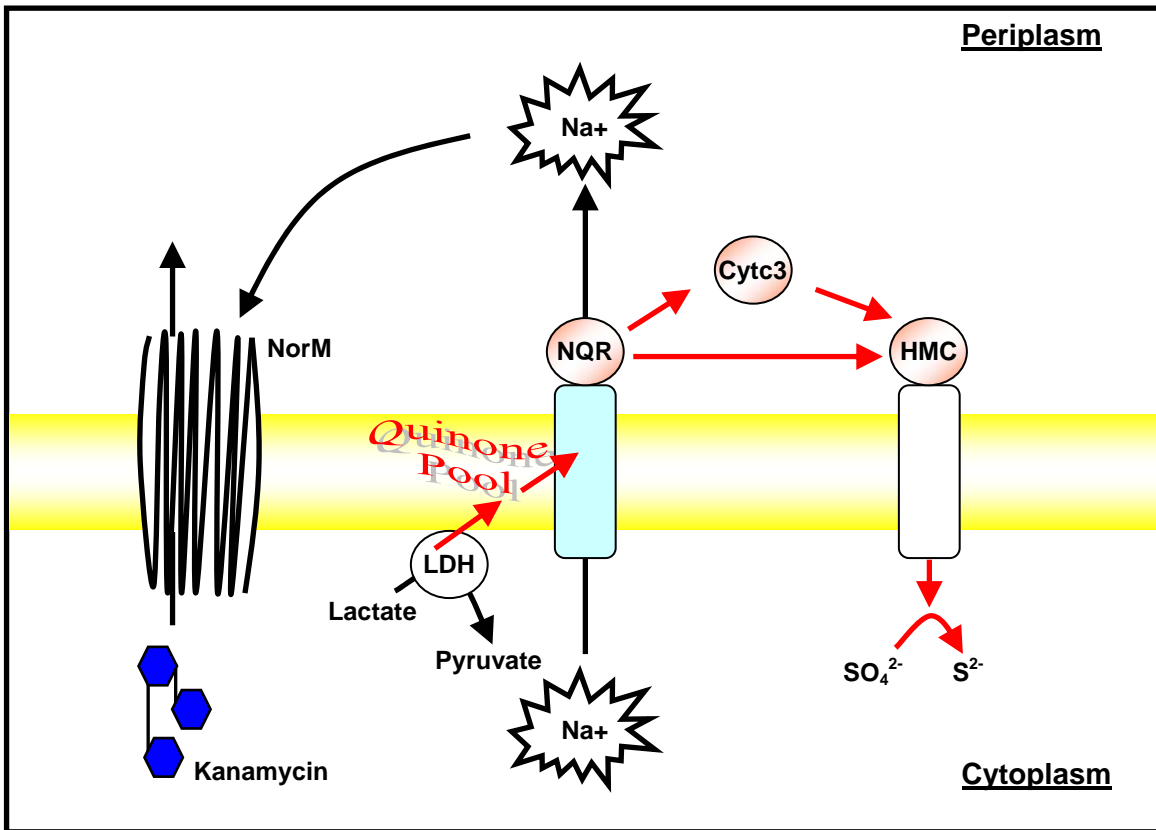
^eMIC of one experiment done in duplicate.

6.3. Discussion

The role of the proton- and sodium- motive force in *Desulfovibrio*. Sulfate-reducing bacteria raise the pH of their environment as they grow due to volatilization of sulfide, the end product of sulfate respiration. So-called neutrophilic SRB have been shown to raise the pH of their local environment to pH 9.0 (Fitz and Cypionka, 1989; Postgate, 1979). Since sulfate reduction can lead to alkanization of the media (and periplasm) and a corresponding loss of the ΔpH , we reasoned that SRB might use the alternate sodium motive force as a strategy to cope with low proton motive force conditions generated by their metabolism. It is important to note that the existence of classical electron transport driven proton translocation or “hydrogen cycling” as a mechanism to generate a periplasmic proton motive force does not necessarily rule out the use of other ion gradients by the sulfate-reducing bacteria. Cypionka and coworkers have previously explored the role of the proton motive force (Fitz and Cypionka, 1991) and the related sodium motive force (Kreke and Cypionka, 1994) in the SRB. Interestingly, they suggested that the alkaliphilic sulfate-reducing bacterium *Desulfonatronovibrio hydrogenovorans* coupled its electron transport chain to the generation of a sodium gradient (Sydow et al., 2002). The genome sequence of all three sequenced SRB indicated a homolog of the NQR complex, which in *Vibrio alginolyticus* is responsible for the generation of a periplasmic sodium gradient at the expense of electron transport chain. However, searches of the sequenced SRB genomes did not reveal a Na^+ -ATPase in addition to the classical H^+ -ATPase.

Figure 6.7. The “Sodium Circuit” of the SRB.

The “Sodium Circuit” of *D. desulfuricans* G20. Red arrows represent proposed electron flow from lactate which generates a transmembrane sodium gradient through the NQR complex. *NorM*: Na⁺/multidrug exporter. *LDH*: lactate dehydrogenase. *NQR*: Na⁺-translocating NADH Quinone Oxidoreductase. *Cyt c3*: cytochrome *c*₃. *HMC*: high molecular weight cytochrome transmembrane complex.



The role of the NQR complex in energy metabolism of the sulfate-reducing bacteria. The role of the putative NQR complex of the SRB is as yet unknown. The true NQR of *Vibrio* couples electron transport to the translocation of cytoplasmic Na⁺ to the periplasm. The NQR complex also shares homology with the RNF transmembrane complex from *Rhodobacter capsulatus* (*Rhodobacter Nitrogen Fixation*) (Kumagai et al., 1997). The RNF complex is essential for growth on medium that requires *R. capsulatus* to fix nitrogen as the sole nitrogen source (Schmehl et al., 1993). Presumably, The RNF complex is involved in electron transport to nitrogenase (Schmehl et al., 1993). The NQR complex and the RNF complexes are members of a family of energy-coupling NADH oxidoreductases (Kumagai et al., 1997). It is unlikely that the NQR complex acts in nitrogen metabolism in *Desulfovibrio* and *Desulfotalea* since only *D. vulgaris* has genes for nitrogen fixation.

The simple interpretation of the effect of ionophores may not be accurate. Thus, it is also possible that CCCP and monensin are inhibiting some other cellular process rather than disrupting the proton- or sodium- motive force, since we have not directly measured H⁺ or Na⁺ ion translocation in this study. However, the inhibition of growth on lactate when both ionophores are added together and the disruption of antibiotic resistance by monensin, but not CCCP (Fig. 6.3, Table 6.1), suggest to us that the ionophores are in fact acting as proposed. Biochemical experiments measuring translocation of Na⁺ and H⁺ by whole cells or by purified NQR complex, as well as the construction and testing of *nqr* mutants, will help to elucidate the role of this protein complex in the SRB.

To the best of our knowledge, expression of the *nqr* operon in other organisms has received little attention. Initially, investigators thought that the expression of the NQR complex of *V. alginolyticus* was upregulated at alkaline pH. However, it was later shown that expression of the NQR complex was not in fact upregulated at alkaline pH, instead that the NQR complex had more activity at alkaline pH than at near-neutral pH (Tokuda and Unemoto, 1981). The regulation of other genes encoding sodium-related functions has been studied. For example, the *E. coli* H⁺/Na⁺ antiporter NhaA is regulated by pH, Na⁺ and Li⁺ concentrations, and growth phase (Dover and Padan, 2001; Karpel et al., 1991). We reasoned that if the putative NQR complex in *D. desulfuricans* G20 was involved in the “sodium circuit”, then it might be differentially expressed under various salt or pH conditions. While we were unable to obtain the RNA for analysis of low salt effects on expression, RNA from cells grown at pH 9.0 in LS medium showed down-regulation of the expression of the putative *nqr* operon. The addition of monensin or CCCP alone had no effect on the expression of the putative *nqr* operon. The responsiveness of the *nqr* operon to pH may support the suggestion that the NQR complex has a role in the “sodium circuit” of the SRB, but it is the opposite of what would be predicted if the expression of the NQR complex is needed at a higher level when cells are grown under low proton motive force conditions (like high pH).

Earlier in the course of the dissertation research, we observed that the cytochrome *c*₃ mutant of *D. desulfuricans* G20 had a higher nitrite reductase activity than the wild-type. Conversely, we observed that the expression of the putative nitrite reductase was down-regulated in the cytochrome *c*₃ mutant of *D. desulfuricans* G20 as compared to the wild-type. Interestingly, we found that the *nqr* operon is also down-regulated in the

cytochrome c_3 mutant of *D. desulfuricans* G20 as compared to the wild-type. Both the putative nitrite reductase and the putative NQR complex share common features: (1) they are transmembrane complexes; (2) they are predicted to be linked to electron transport at the level of the quinone pool; and (3) one of the subunits of the complex is a periplasmic facing c type cytochrome.

Growth of *D. desulfuricans* G20 on lactate sulfate at low sodium or at high pH. We sought to test if *D. desulfuricans* G20, like *V. alginolyticus*, could grow in the absence of a proton gradient. We found that pyruvate supported growth by *D. desulfuricans* G20 was dependent upon the proton motive force and was inhibited by CCCP. Surprisingly, growth on lactate by *D. desulfuricans* G20 was unaffected by CCCP. This observation led us to speculate that the *D. desulfuricans* G20 NQR complex might generate a transmembrane sodium motive force from the oxidation of lactate, but not pyruvate, and that the sodium motive force would therefore be sufficient to allow growth under low proton motive force conditions when lactate was the electron donor (Fig. 6.7). The addition of CCCP and monensin (a sodium ionophore) together inhibited growth when lactate was the electron donor suggesting that a proton- or sodium-motive force was necessary for the cells to grow. While we cannot demonstrate an absolute requirement for sodium ions *per se* during growth on LS medium, G20 cells in medium with very low sodium (about 0.7 mM), became more sensitive to the sodium ionophore monensin either because monensin is a substrate for the sodium-driven multidrug efflux pump or because the cell ultimately needs a sodium gradient for growth (data not shown). In our model, we speculate the uptake of lactate by cells is coupled to H^+ or Na^+ cotransport, while pyruvate may be taken up by H^+ cotransport alone. Lactate was

suggested to be taken up by cotransport with either H⁺ or Na⁺ in *Shewanella oneidensis* by inference from results with various inhibitors (Stenberg et al., 1984).

Role of the Na⁺/multidrug exporters in the sulfate-reducing bacteria. In 1998 a novel multidrug efflux pump, NorM, was characterized from *V. parahaemolyticus*. NorM is genetically and functionally related to its homolog YdhE of *E. coli* (Morita et al., 1998). These 2 proteins represented the first members of a new class of multidrug efflux pumps, termed the MATE class (*M*ultidrug *A*nd *T*oxic compound *E*xtrusion). This family of drug transporters is dependent upon the sodium motive force (Na⁺ ion antiport), not the proton motive force (H⁺ antiport) or ATP hydrolysis, for the translocation of toxic compounds (Morita et al., 2000). In *Vibrio parahaemolyticus* NorM functions to transport the drugs norfloxacin and ciprofloxacin and also the unrelated antimicrobial compounds ethidium, kanamycin, and streptomycin from the cell in a sodium motive force-dependent manner (Morita et al., 1998). The three sequenced SRB each have homologs to the characterized NorM Na⁺/multidrug efflux pumps of *V. parahaemolyticus*. *D. desulfuricans* G20 apparently has three Na⁺/multidrug efflux pump homologs. It has long been known that SRB are naturally resistant to many antimicrobial compounds, in part because of their anaerobic metabolism, but also because of their production of sulfide, which can inactivate many antimicrobials (Salleh et al., 1964). The extreme resistance of SRB makes it a challenge to prevent or treat SRB-induced corrosion.

We speculated that the resistance of the SRB to some antimicrobial compounds was due at least in part to the efflux of the antimicrobial by a NorM isozyme and therefore coupled to the sodium motive force (Fig. 6.7). Previous studies showed that the

NorM of *Vibrio* strains functioned in the efflux of the antimicrobial compounds kanamycin, ethidium bromide, and methylene blue, among others (Morita et al., 1998). Nalidixic acid was not a substrate of NorM from the tested *Vibrio* strains (Morita et al., 1998). As predicted, the growth of *D. desulfuricans* G20 in the presence kanamycin, ethidium bromide, and methylene blue was shown here to be dependent upon the sodium motive force. The sodium gradient was reduced by omitting sodium salts from laboratory growth media or by including a sodium ionophore like monensin in the growth medium. When this was done, the sensitivity of the SRB to some antimicrobial compounds was increased (Table 6.2). This observation has obvious implications for the control of SRB in an industrial setting. If sodium ions could be removed in an economic fashion from water before its use during industrial operations such as oil recovery or recirculating air-conditioning systems, then a lower concentration of an industrial biocide might inhibit SRB growth and corrosion. In addition, a biocide previously thought to be ineffective against SRB corrosion might be effective in the absence of sodium ions. In the future, development of new antimicrobials that target the sodium/drug exporter, or that disrupt the periplasmic sodium gradient (like monensin), would be an exciting avenue of research.

7. Overall conclusions of this dissertation

The classic compounds for growth of SRB in the laboratory are hydrogen, lactate, and pyruvate, although the metabolic versatility of some strains of SRB is much greater (Postgate, 1979). The mechanism by which sulfate-reducing bacteria generate a transmembrane proton motive force has been intensely debated. When using carbon compounds as electron donors, SRB have been shown to both produce and consume molecular hydrogen (Hatchikian et al., 1976). Odom and Peck first proposed a “hydrogen cycling” model for generation of a transmembrane proton gradient (Odom and Peck, 1981). In that model, the oxidation of organic electron donors in the cytoplasm was coupled to the generation of cytoplasmic hydrogen. Cytoplasmic hydrogen was then suggested to diffuse across the cytoplasmic membrane and be oxidized by a periplasmic hydrogenase, generating protons and allowing the recapture of electrons. Noguera and coworkers proposed that both hydrogen cycling and classical electron transport driven proton translocation functioned in *D. vulgaris* to generate a proton gradient (Noguera et al., 1998).

Experiments here support the suggestion that classical electron transport driven proton translocation functions in addition to hydrogen cycling to generate a transmembrane proton gradient in *Desulfovibrio* (Figs. 1.3 and 1.4). Our studies began by testing a single mutant in the predominant periplasmic cytochrome c_3 for its ability to reduce U(VI). The mutant reduced U(VI) at a rate of about 10% that of the wild-type with hydrogen as the electron donor; when lactate or pyruvate were electron donors the impairment was less severe. These observations were consistent with either hydrogen cycling or classical electron transport. Growth and U(VI) reduction experiments using

the Fe-hydrogenase mutant suggested that electrons from lactate oxidation, but not pyruvate oxidation, were transferred through the periplasmic Fe-hydrogenase. This observation suggests that hydrogen cycling from lactate does exist. Many experiments indicated that the electrons generated from oxidation of lactate are quite different from those electrons generated from the oxidation of pyruvate (for example, the experiments using the Fe-hydrogenase mutant). Measurements of nitrite reduction by the wild-type and the cytochrome *c*₃ mutant suggested that more electrons from pyruvate oxidation than from lactate oxidation enter the electron transport chain (ETC) at the level of the putative nitrite reductase (NiR). Inhibitor studies using the protonophore CCCP and the sodium ionophore monensin were interpreted to mean that electrons from lactate oxidation, but not from pyruvate oxidation, entered the ETC at the putative Na⁺-translocating NADH-dependent Quinone Oxidoreductase (NQR). Both of these protein complexes, NiR and NQR, are coupled to the quinone pool in other organisms (Hayashi et al., 2001; Simon et al., 2000)

Mutant studies of the HMC carried out by others were interpreted to support a role for HMC in electron transfer from the periplasm into the cytoplasm where sulfate reduction could take place (Dolla et al., 2000). In vitro studies suggested that although the HMC could accept electrons directly from the Fe-, NiFe-, or NiFeSe-hydrogenases, a catalytic amount of cytochrome *c*₃ increased the rate of electron transfer 100- to 1000-fold (Pereira et al., 1998). Northern analysis (Table 7.1) and protein analysis (Giles and Wall, unpublished) indicated that the high-molecular mass cytochrome *c* complex (HMC) was upregulated in the cytochrome *c*₃ mutant. Therefore it appears that the expression of

at least one electron carrier protein downstream of cytochrome c_3 (in the ETC) was upregulated in the cytochrome c_3 mutant.

Previous expression studies showed that some of the electron carrier proteins coupled to the Fe-hydrogenase were down-regulated in the Fe-hydrogenase mutant of *D. vulgaris* (Haveman et al., 2003). We found that the NiR and the NQR complexes were down-regulated (at least 8- and 7-fold, respectively) in the cytochrome c_3 mutant of *D. desulfuricans* G20 (Figs. 3.5 and 6.4, Table 7.1). In addition, we found that the cytochrome c_{553} (c_{553}), suggested by *in vitro* studies to act as an electron carrier between lactate dehydrogenase (LDH) or formate dehydrogenase and cytochrome c_3 , was at least 10-fold down-regulated in the cytochrome c_3 mutant (Table 7.1). Similarly, the operon encoding the predicted acidic tetraheme c type cytochrome (c_3 T-II) and its associated transmembrane complex was observed to be downregulated (Table 7.1). Other researchers have used gene expression profiling to model metabolic pathways. Often, but not always, functionally related genes are coordinately expressed (Hughes et al., 2000; Kabir and Shimizu, 2003; Thompson and Zhou, 2004). We have so far looked at the expression of only a small sample of the electron carrier proteins in the cytochrome c_3 mutant. The expression the HMC complex, which has been shown *in vitro* and *in vivo* to be downstream of cytochrome c_3 in the ETC (Dolla et al., 2000; Pereira et al., 1998), is upregulated in the cytochrome c_3 mutant compared to the wild type. In contrast, the expression of four other electron carrier transmembrane complexes (acidic tetraheme c type cytochrome, cytochrome c_{553} , NiR, and NQR) is downregulated in the cytochrome c_3 mutant. Previous *in vitro* work suggested that the acidic tetraheme c type cytochrome and the cytochrome c_{553} are electron donors to cytochrome c_3 (Pereira et al., 1998;

Valente et al., 2001). Our work suggests that cytochrome c_3 does not donate electrons to the putative NiR. Based upon these observations along with the differential transcription of these complexes, I hypothesize that the acidic tetraheme c type cytochrome, cytochrome c_{553} , NiR, and NQR transmembrane complexes are all upstream of cytochrome c_3 in the ETC of *Desulfovibrio* (Fig. 7.1).

Table 7.1. Expression of Genes Encoding Electron Carrier Proteins in *CycA*

Genes Downregulated in <i>CycA</i>		
Gene	Change in <i>CycA</i>^a	VIMSS^b
Acidic cytochrome <i>c3</i> operon	10× ^c	393426-393418
Cytochrome <i>c553</i>	8× ^d	394971
Nitrite Reductase operon	8× ^d	393132,393130
Na ⁺ -translocating NADH-Quinone Oxidoreductase (NQR) operon	7× ^e	392883-392876
Genes Upregulated in <i>CycA</i>		
Gene	Change in <i>CycA</i>^a	VIMSS
High molecular mass cytochrome <i>c</i> (HMC) operon	4.5× ^f	392827-392831

^aFold change (up- or down-regulated) as measured by Northern analysis in the *CycA* mutant as compared to the wild-type as quantitated by Kodak 1D version 3.6 (<http://www.kodak.com>).

^bOrf numbers of genes annotated by VIMSS (<http://www.escalante.lbl.gov>).

^cExpression from RNA obtained from 1 experiment.

^dAvg of expression from RNA obtained from 2 experiments.

^eAvg of expression from RNA obtained from 3 experiments.

^fExpression from RNA obtained from 1 experiment; 2-D gel analysis of proteins from late-log cells confirms Northern data (Giles and Wall, unpublished).

Figure 7.1. Overall Model for the Electron Transport Chain in *Desulfovibrio* from this dissertation.

Expansion of the “classical model” for electron transport via a membrane bound electron chain (ETC).

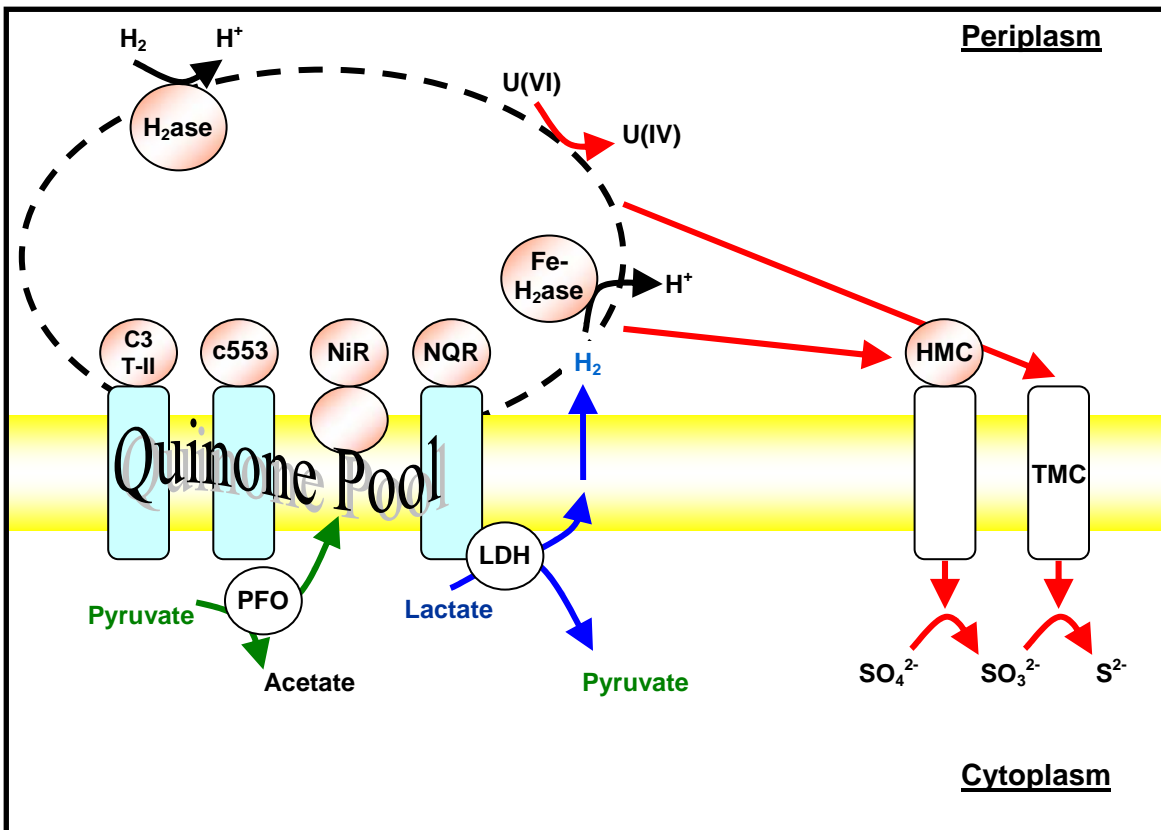
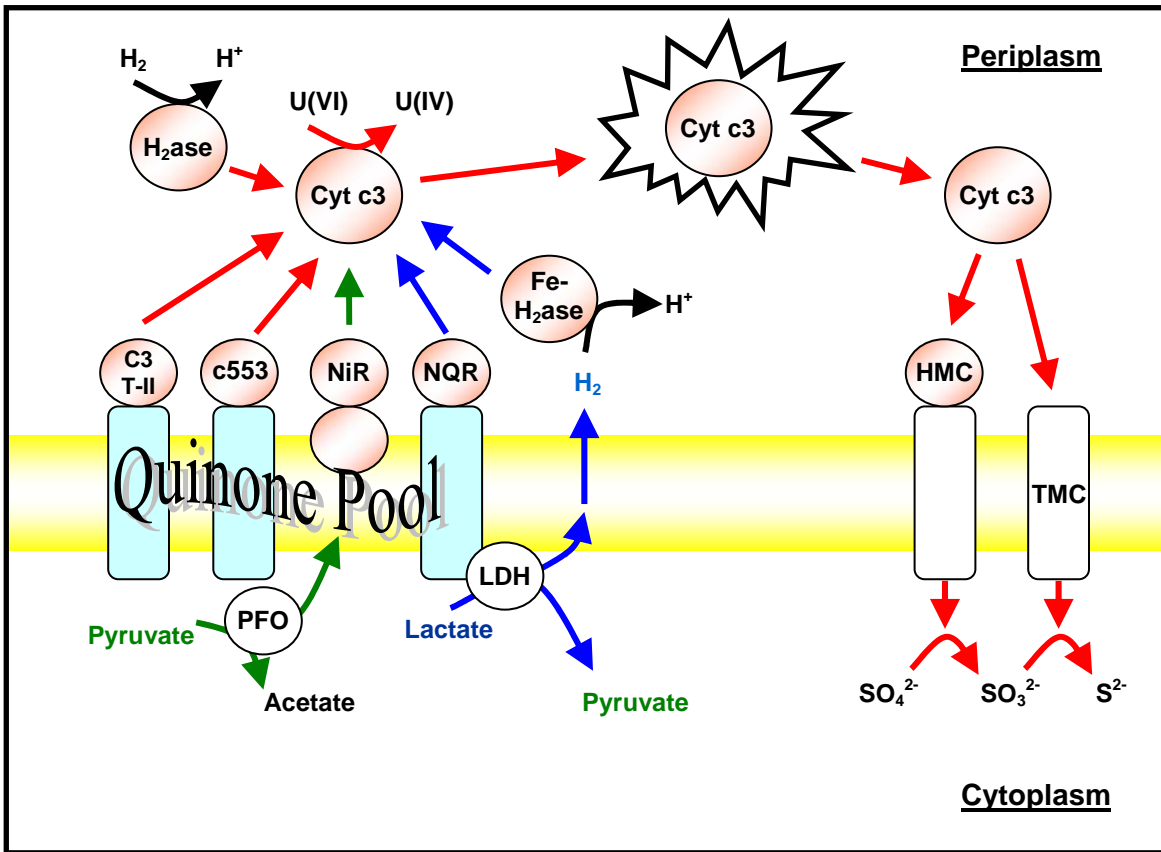
Top Panel: Electron flow in wild-type *Desulfovibrio*.

Cyt c3 represents cytochrome *c*₃, which is envisioned in this model as the central electron carrier protein in the ETC. Cytochrome *c*₃ has two roles: (1) to accept electrons from upstream proteins in the ETC (the left side of the figure); and (2) to donate electrons to downstream proteins in the ETC (the right side of the figure). Proteins upstream of cytochrome *c*₃ are the cytochrome *c*₅₅₃ (*c553*), the putative nitrite reductase (*NiR*), the putative Na⁺-translocating NADH-Quinone Oxidoreductase (*NQR*), the Fe-hydrogenase (*Fe-H₂ase*), and acidic tetraheme *c* type cytochrome (*C3 T-II*). Note that electrons generated from the oxidation of lactate and pyruvate are not equivalent, they enter the ETC at different points. Apparently electrons from the oxidation of lactate, but not pyruvate, are transferred through the NQR or the Fe-hydrogenase; however, more electrons from the oxidation of pyruvate are transferred through the NiR than the electrons from lactate. While not shown, pyruvate fermentation can yield ATP through substrate level phosphorylation (Postgate, 1979).

Bottom Panel: Electron flow in the cytochrome *c*₃ mutant.

Electron carrier proteins in the upstream part of the ETC (*C3 T-II*, *c553*, *NiR*, *NQR*, and *Fe-H₂ase*, in the *dotted circle*) donate electrons directly to downstream transmembrane complexes (like *HMC*) for the subsequent reduction of sulfate. Possibly some or all of

the electron carrier proteins in the *dotted circle* are responsible for U(VI) reduction in *cycA* mutant cells.



The knowledge gained of the electron transport pathways of the SRB can allow us to make predictions on bioremediation of uranium contaminated sites. For example, we know that SRB can utilize lactate, but not pyruvate, as a carbon and energy source when grown under low proton motive force conditions. Therefore we predict that we would need to add lactate as an energy source to stimulate SRB U(IV) reduction in high pH (low proton motive force) environments. The growth of SRB are not always desirable as they have been shown to sour oil wells and corrode ferrous metals in industrial settings (Hamilton, 1985). During oil recovery, water (often salt-containing sea water) is pumped into the reservoir to allow additional oil recovery. The process of flooding is associated with the growth of SRB blooms that hamper oil recovery. It is a great challenge for the oil industry to control the growth of SRB (Saleh et al., 1964). We have learned that the natural resistance of *D. desulfuricans* G20, which was isolated from an oil well corrosion site (Weimer et al., 1988), to some antimicrobial compounds is linked to the presence of the sodium motive force. The genome sequence of three sequenced Gram-negative SRB all have homologs to the biochemically and genetically characterized sodium-driven multidrug efflux pump (NorM) of *V. parahaemolyticus*. This observation may result in a practical improvement in management of SRB industrial contamination. If sodium ions could be removed from water in a cost effective fashion before oil well flooding, then it may be easier to control the resulting SRB blooms. Alternatively, the development and use of toxic compounds that inhibit the sodium motive force may allow for more control of SRB in the industrial setting.

8. References

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- Payne, R.B., Gentry, D.M., Rapp-Giles, B.J., Casalot, L., and Wall, J.D. (2002). Uranium reduction by *Desulfovibrio desulfuricans* strain G20 and a cytochrome *c*₃ mutant. *Appl Environ Microbiol* **68**: 3129-3132.
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