Analysing the Metabolic Capabilities of Desulfovibrio Species through Genetic Manipulation

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Introduction

Sulfate-reducing bacteria (SRB) are an environmentally significant group belonging to the anaerobic delta-Proteobacteria that respire sulfate for growth. From an industrial standpoint, SRB pose a threat through corrosion of ferrous metals and production of toxic sulfides. The more positive aspects of the metabolism of the SRB include a robust but poorly understood hydrogen metabolism that is of interest to alternative energy studies. SRB also immobilize a number of heavy metals through sulfide precipitation or through changing the redox state of the metal, and thus its solubility. When metals are made less soluble, as is the case with chromium (Cr(VI) to Cr(III)) or uranium (U(VI) to U(IV)), toxicity is reduced by limiting biological availability. Despite the economic and environmental impacts associated with SRB activities, our current knowledge of their metabolism is inadequate. Among the SRB, members of the Desulfovibrio genus have received most attention because these strains are grown most readily in pure culture. Therefore, Desulfovibrio strains have been the focus of biochemical and biophysical analyses; however, genetic studies have been more difficult. Over the past 15 years, progress has been made in developing techniques for DNA transformation, gene mutagenesis and over-expression, and protein tagging. Ten years have passed since the last genetics of SRB review by van Dongen (1995), and the complete genome sequences of a few strains are now available (Heidelberg et al., 2004). This review highlights the current advances in the genetic manipulation of Desulfovibrio species.

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Abbreviations: SFU, 5-fluorouracil; CmR, chloramphenicol resistant; G418, geneticin derivative; GLO, gene of interest; GmR, gentamicin resistant; KanR, kanamycin resistant; KmR, kanamycin sensitive; MV, methyl viologen; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); sucB, levansucrase; SRB, sulfate-reducing bacteria; TETR, tetracycline resistant; Tr, transposon, Xgal, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside.

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and the potential use of these tools in understanding the metabolism of sulfate reducers for biotechnological purposes.

The SRB are best known for their negative economic impact in the industrial world, most conspicuously affecting the petroleum industry. First, the product of microbial sulfate reduction, hydrogen sulfide, is directly responsible for oil souring and the formation of precipitates that plug pipelines (Singleton, 1993). Second, the bacterial capacity for anaerobic corrosion of iron has altered storage and engineering practices. Mechanisms of microbially influenced corrosion include sulfide production, cathodic depolarization, and direct metal oxidation. Biogenic sulfide generates a chemical attack that results from pyrite formation by the following reaction: \( \text{Fe}^{2+} + \text{H}_2\text{S} \rightarrow \text{FeS} + \text{H}_2 \) (King and Miller, 1971). This dissolution of iron is compounded by cathodic depolarization, proposed as a model for biocorrosion by von Woberey Kühr and van der Vlugt in 1934. In this model, protons from water act as an oxidant to drive the formation of \( \text{Fe}^{2+} \), and thereby form cathodic \( \text{H}_2 \), on the metal surface. The \( \text{H}_2 \) is then scavenged by the SRB, resulting in cathodic depolarization and stimulation of further corrosion (Hamilton, 2003). Finally, the effect on the metal surface of concentrated organic acid end products released by the metabolism of SRB in biofilms (Boopathy and Daniels, 1991) also may be a factor in anaerobic metal corrosion.

All the preceding mechanisms result in corrosion from indirect influences of the metabolism of SRB. Recently, a Desulfobacterium-like strain was isolated that is capable of corroding ferrous metals directly by coupling the reduction of sulfate to metallic iron oxidation, bypassing the need for a hydrogen intermediate (Dinh et al., 2004). The ubiquity of the use of \( \text{Fe}^{2+} \) as a source of reductant has yet to be determined and may point to an as yet unrecognized substrate, since \( \text{Fe}^{2+} \) is essentially anthropogenic. These negative biocorrosive impacts associated with SRB have prompted physiological studies aimed at controlling SRB metabolism.

A potentially valuable biological trait of SRB is the ability to produce hydrogen, a biofuel. To realize the potential benefits from this capacity, the pathway for hydrogen generation and the role of hydrogen in the metabolism of the SRB has been, and continues to be, explored. Several models have been proposed. In 1981, the ‘hydrogen cycling’ model was outlined by Odom and Peck (1981). Energy sparing and additional ATP generation were proposed to result from moving protons from the cytoplasm to the periplasm by an energy neutral process. First, electrons from lactate or pyruvate oxidation are converted to \( \text{H}_2 \) via a cytoplasmic hydrogenase. The \( \text{H}_2 \) then diffuses across the membrane, where it is re-oxidized by a periplasmic hydrogenase generating protons for ATP generation. However, in 1984, Lupton and co-workers challenged the hydrogen cycling model with a trace \( \text{H}_2 \) transformation model (Lupton et al., 1984). In this scenario, cells diluted into fresh medium convert excess electrons from carbon oxidation to \( \text{H}_2 \), for redox balance. The hydrogen is subsequently reused when electron donors are more limiting. Both of these models are dependent on the presence of a cytoplasmic hydrogenase in SRB, which has not yet been experimentally identified (Voordouw et al., 1990a; Pohorelic et al., 2002). A completely different method for hydrogen generation by SRB may involve the activity of a CO dehydrogenase (\( \text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + \text{H}_2 \)) recently found in Desulfovibrio (Voordouw, 2002). Studies are ongoing to evaluate the proposed hydrogen generation models, as well as to identify the factors that allow increased hydrogen production by SRB.
A role for the SRB in toxic metal remediation has been suggested based on their apparent resistance to a number of heavy metals, their robust metal metabolism, and their production of sulfide. Sulfide, the product of sulfate reduction, can be used to immobilize metals through sulfide-precipitate formation. Remarkably, the SRB have been shown to be capable of direct enzymatic reduction of heavy metals, such as U(VI), Cr(VI), Te(VI), and As(V), generating insoluble forms (Aubert et al., 1998; Macy et al., 2000; Smith and Gadd, 2000; de Luca et al., 2001). Specifically, *Desulfovibrio* species have been shown to reduce toxic U(VI), Te(VI), and Cr(VI) to less soluble forms via a c-type cytochrome (Payne et al., 2002; Chardin et al., 2003). Both sulfide precipitation and enzymatic reduction immobilize the toxic metals and allow for subsequent environmental abatement. For efficient bioremediation of heavy metals, SRB strains need to be engineered for optimal metal reduction as well as oxygen and metal tolerance. While the SRB exhibit resistance to several metals, they are rather sensitive to inhibition by Cd(II), Zn(II), and Ni(II) at levels between 20 and 200 μM (White and Gadd, 1998). Also, as anaerobes, these bacteria are limited to environmental sites with reduced oxygen concentrations. Engineering strains to expand their niches and to gain the ability to produce biosurfactants might also increase access to metals for immobilization (Valls and de Lorenzo, 2002).

To impact the SRB processes of microbially influenced corrosion, hydrogen production, or immobilization of toxic metals, an understanding of the intrinsic metabolism must be accomplished. Also, it will be necessary to determine the responses of the bacteria to environmental stresses and possible adaptation responses. Future bioengineering of SRB for industrial or environmental purposes will rest on this knowledge. An important tool for the elucidation of microbial metabolism is the development of a genetic system. Here, we discuss the limitations and advances in genetic manipulation of *Desulfovibrio* and the application of the technologies.

**Genome highlights**

In 2004, the genome of *Desulfovibrio vulgaris* strain Hildenborough was released (Heidelberg et al., 2004). This bacterium possesses a 3.57 Mb genome and a 202.3 kb megaplasmid that contains the genes for nitrogen fixation. The genome has about 3395 putative coding sequences with over 850 genes corresponding to unconserved hypothetical proteins. *Desulfovibrio desulfuricans* G20 (to be renamed *Desulfovibrio alaskensis* G20; Wall et al., in preparation) has also been sequenced and exhibits 3862 total genes, apparently all on one replicon of 3.73 Mb. Access to the sequence is available from the Joint Genome Institute (Markowitz et al., 2006). While these attributes are not unusual, the occurrence of previously unknown hydrogenases and cytochromes is significant because of their potential roles in promoting corrosion and metal reduction (Heidelberg et al., 2004). However, a comparison among the genome sequences of *D. vulgaris*, *D. desulfuricans* G20, and *Desulfotalea psychrophila* revealed a lack of conserved cytoplasmic hydrogenases, weakening the two proposed hydrogen production models (see above). Numerous genes encoding formate dehydrogenases were also annotated for the *Desulfovibrio* as well as the *Desulfotalea* strains, indicating a putative second method for building a proton gradient through transfer of uncharged formate to the periplasm and
its subsequent oxidation (Heidelberg et al., 2004). These genes are excellent targets for mutagenesis to establish their roles in the metabolism of SRB. While a targeted mutagenesis system is now available, several developments are still lacking.

**Genetic system**

The first molecular analysis of SRB occurred in 1985 when Voordouw and Brenner cloned hydrogenase genes into *Escherichia coli* (Voordouw and Brenner, 1985). However, the heterologous expression of genes encoding SRB electron transfer proteins in *E. coli* was problematic due to the formation of apoproteins lacking metal clusters (Voordouw et al., 1987; van Dongen et al., 1988). Thus, the need for a facile method for expression of SRB genes in a homologous background was recognized. One of the major hindrances to molecular work in SRB has been the low and variable plating efficiencies. Whereas 30–80% efficiencies have been achieved (Singleton et al., 1988; van den Berg et al., 1989), often they are far less. This low efficiency may be caused by contaminating oxygen, since single cells are more sensitive to oxygen than biofilms (Cypionka, 2000). Generally, the simple use of an overlay of solidified medium improves colony formation. An additional difficulty with growth of lawns on the surface of solidified medium results from pH control. The generation of sulfide and the release of hydrogen sulfide into the atmosphere drive the pH up, often to a range that is inhibitory to growth. Regardless of plating limitations, the first stable vectors were introduced to *Desulfovibrio* via conjugation in 1989 (Powell et al., 1989; van den Berg et al., 1989).

**Selectable markers**

Another obstacle associated with genetic manipulation of SRB is the small number of facile selectable markers. SRB are naturally resistant to many antimicrobials (Postgate, 1984). Conversely, *Geobacter sulfurreducens*, another metal-reducing delta-Proteobacteria, has shown sensitivity to chloramphenicol, nalidixic acid, tetracycline, kanamycin, spectinomycin, streptomycin, and ampicillin at varying concentrations on plates (Coppi et al., 2001). In fact, the annotation of the *Desulfovibrio* genome has several multi-drug exporters. Recent studies indicate that these exporters may be sodium dependent, since the salt concentration of the medium was shown to influence the level of antimicrobial resistance (Payne et al., 2004).

Kanamycin resistance has been shown to be a facile marker in *D. desulfuricans* G20 (Argyle et al., 1992). However, *D. vulgaris* Hildenborough requires concentrations of 1 mg kanamycin/ml or more to be effective for colony selection. Recently, it was observed that G418, an aminoglycoside that selects for genes encoding enzymes for kanamycin resistance, could be used effectively at a concentration of 200 to 400 µg/ml to obtain Kan<sup>+</sup> colonies of *D. vulgaris* (Ringbauer et al., 2004). This observation has made a number of plasmid constructs and transposons bearing this marker available for manipulation of *D. vulgaris*.

Tetracycline sensitivity of *D. vulgaris* in liquid cultures also has been shown at levels as low as 20 µg/ml, and the Tet<sup>+</sup> determinant from pBBRMCS-3 (Kovach et
al., 1995) confers resistance (H.C. Yen and J.D. Wall, unpublished). Use of tetracycline to select rare resistant colonies from a culture of sensitive cells is currently being explored. The limited range of Desulfovibrio antibiotic resistance markers for colony selection may be related in part to the number of cells plated. Fu and Voordouw showed that chloramphenicol selection was feasible at a level of 10 µg/ml. However, clean selection occurred only when plating no more than 10⁶ cells/cm² per plate or 10⁷ cells/ml in liquid (Fu and Voordouw, 1997). Additional selectable traits such as auxotrophic markers have not yet been applied to genetic manipulation of the SRB.

CONJUGATION

Transfer of mobilizable plasmids from E. coli donors was the first successful genetic transfer method in Desulfovibrio (Powell et al., 1989; van den Berg et al., 1989). These broad host range vectors belonged to the IncQ incompatibility group. Similarly, IncQ plasmids have been shown to be stable in Geobacter (Coppi et al., 2001). A second replicon for Desulfovibrio was discovered in 1993. Designated pBG1, it is a small cryptic plasmid from Desulfovibrio desulfuricans G100A (Weimer et al., 1988). Parent strain of G20, pBG1 replicates in Desulfovibrio strains, but not in E. coli, and is compatible with IncQ plasmids (Wall et al., 1993). This native plasmid has allowed shuttle vectors to be generated by fusion with E. coli plasmids that offer the convenience of blue/white screening in E. coli (Rousset et al., 1998). Although plasmids based on the pBG1 replicon are apparently stably replicated in D. desulfuricans G20, loss of these vectors from D. vulgaris can be detected in about 5% of cells grown to stationary phase in the absence of antibiotic selection (H.C. Yen, unpublished observation). However, pBG1 replicons have not shown stability in Geobacter (Coppi et al., 2001). The ability to introduce foreign DNA into the SRB via conjugation allowed for the first over-expression of Desulfovibrio genes to produce fully functional proteins. Both type-1 tetraheme cytochrome c₅ (Voordouw et al., 1990b; Mus-Veau et al., 1992) and prismatic (Stokkermans et al., 1992) genes were successfully over expressed in alternate Desulfovibrio hosts. Although conjugation works well, counter selection of the E. coli donor remains a concern due to the resistance of E. coli to aminoglycosides under anaerobic conditions.

TRANSDUCTION

Only one case of transduction in SRB has been reported (Rapp and Wall, 1987). A defective bacteriophage was found in Desulfovibrio desulfuricans 27774 capable of intraspecies transfer of antibiotic resistance and auxotrophic markers. The transfer of genetic markers occurred via bacteriophage-like particles that had packaged random pieces of bacterial DNA (generalized transduction). Because transfer of antibiotic resistances to other Desulfovibrio species was not observed, it was concluded that the vector had a restricted host range (Rapp and Wall, 1987). With this system, no plaque-forming ability was detected, impeding the development of more useful constructs. That limitation, along with the inability to observe conjugation with the host strain, D. desulfuricans 27774, discouraged its further development.

Genome sequences of Desulfovibrio strains have revealed the presence of a
number of gene clusters that might encode endogenous bacteriophage (BacMap at http://wishart.biology.ualberta.ca/BacMap/). Earlier reports from Akagi and co-workers showed mitomycin C induction of phage production from *D. vulgaris* Hildenborough (Handley et al., 1973, 1991; Seyedarashi et al., 1992). Subsequent restriction analysis of the DNA obtained from the particles was interpreted as evidence that at least two phages were being produced. No plaque-forming ability was demonstrated, likely because a susceptible host was not available. Recently, Stahl isolated *D. vulgaris* strain DP4 from the contaminated Lake DePue that, by DNA hybridization to Hildenborough microarrays, appeared to lack the bacteriophage genes (D. Stahl, personal communication). Testing of that strain as a host has revealed plaques from supernatants of mitomycin C-induced and -uninduced *D. vulgaris* Hildenborough cultures (H.C. Yen, unpublished data). Further work is needed to determine the number and transduction capacity, if any, of the phages being produced.

**TRANSPPOSITION**

Transposons have been shown to be effective tools for random mutagenesis in *Desulfovibrio*. Early studies indicated successful, but rather low, efficiencies of transposition of Tn5 derivatives and Tn7, but no transfer of Tn9 or Tn10 was observed in *D. desulfuricans* G20 (Wall et al., 1996). More recently, a mini-Tn5 derivative developed for *Xanthobacter*, Tn5-RL27 (Larsen et al., 2002), has been used to generate transposon mutant libraries of *D. vulgaris* and *D. desulfuricans* G20 (J. Ringsbauer and R. Payne, unpublished). The transposase in this element was mutated to increase the efficiency of transposition (Larsen et al., 2002). Importantly, the gene encoding the mutant transposase was placed outside of the transposed DNA; thus, successive transposition in the cell is prevented. Although not optimized for SRB as yet, the transposition efficiency in preliminary experiments was observed to be in the range of 1 in 10⁶ recipient cells (J. Ringsbauer and R. Payne, unpublished). Random sequencing of about 50 transposition sites showed that transposition was essentially random. Mutations were generated in such putative genes as *livG* (high-affinity branched-chain amino acid ABC transporter, ATP binding protein), *flgG* (flagellar basal-body rod protein), and *zraP* (zinc resistance-associated protein), as well as several apparent histidine kinases. Introduction of the transposition vector by electroporation eliminated the need for counter selection of a conjugal donor, although fewer mutants were obtained (J. Ringsbauer, unpublished).

**ELECTROPORATION**

Electroporation has been explored as an alternative to conjugation for DNA introduction. In SRB, the technique was first successful in *Desulfovibrio fructosovorans* (Roussel et al., 1991). However, success with the technique has been slower in *D. vulgaris* and *D. desulfuricans* G20. In an early attempt at electroporation of *D. vulgaris* by a procedure similar to that used for *D. fructosovorans*, Fu and Voordouw were unable to obtain transformants (Fu and Voordouw, 1997). In this attempt, chloramphenicol was used as the selective agent. Electroporation trials with the pBG1-based vector, pSC27, encoding a kanamycin resistance determinant that was
selected with G418, were successful in *D. vulgaris* (Ringbauer et al., 2004). The procedure used ca. 2 × 10^6 late exponential *D. vulgaris* cells re-suspended in either 30 mM PIPES buffer or 10% glycerol in 1 mM MgCl₂. To overcome restriction barriers in the SRB, 0.5–5.0 μg of plasmid DNA purified from *E. coli* was introduced. This protocol generally gave a minimum efficiency of transfer of 10⁻⁶, or about 20–100 CFU (G. Zane, unpublished observations). However, with differential cell lysis from the electroporation and cell death from oxygen contamination occurring with manipulation, the actual efficiency is certainly higher. Efforts at continued optimization will prove invaluable in increasing the efficiency of genetic manipulation of *Desulfovibrio* species.

**TARGETED MUTAGENESIS**

While random mutagenesis is a useful tool in analysing the biology of SRB, a method for studying specific genes, such as hydrogenases and cytochromes, is integral to the elucidation of metabolism. In 1991, van den Berg and co-workers were the first to utilize antisense RNA in *D. vulgaris* to decrease expression of the Fe-hydrogenase (van den Berg et al., 1991). While this technique gave some insight to the function of the hydrogenase, the interpretation of the results was clouded by the residual expression of the gene. It is now well known that suppression of translation of mRNA by antisense RNA is incomplete.

Mutagenesis by plasmid integration or interruption is an alternative method that abolishes activity of the target gene. In *D. desulfuricans* G20, a tetraheme cytochrome *c*₅₅ mutant was created via gene interruption by homologous recombination with a 292 bp internal portion of the *c*₅₅ gene (Rapp-Giles et al., 2000). The fragment was cloned into a plasmid constructed from pBluescript® (Stratagene) that does not replicate in the SRB. Integration of the entire plasmid into the gene was selected by requiring inheritance of the plasmid antibiotic resistance (Rapp-Giles et al., 2000). In *D. vulgaris*, the same strategy is being used for ongoing studies on histidine kinases (A. Mukhopadhyay, personal communication). While plasmid insertion results in mutated genes, the complete sequences of the target genes remain in the genome, raising questions regarding the stability of these constructs. Genome rearrangements restoring a wild-type copy of cytochrome *c*₅₅ gene were documented in the strain with the plasmid-interrupted mutation, while the selection determinant was retained (Rapp-Giles et al., 2000). Thus, deletion of target genes in *Desulfovibrio* yields mutants that are more stable, and thus easier to analyse.

The first deletion mutant generated by marker exchange in *D. vulgaris* was obtained using a two-step recombination method with *sacB* as the counter selection determinant (Fu and Voordouw, 1997). Briefly, an unstable Kan^R^ vector containing a Cm^R^ marker flanked by DNA sequences up- and down-stream of the target gene was introduced via conjugation. Integration of the plasmid in the first recombination event was selected for by Cm^R^ and Kan^R^. The vector also contained a *sacB* gene for enrichment of the second recombination event removing plasmid sequences. Because the *sacB* gene encodes levansucrase, which confers sensitivity to sucrose, the merodiploid generated by plasmid insertion was sensitive to sucrose. Removal of the kanamycin pressure, in the presence of sucrose, enriched for cells in which the unstable merodiploid had resolved via a second recombination event yielding cells
that were Cm\(^r\), Kan\(^r\), and sucrose resistant. Mutants deleted for the target gene were obtained. When attempts were made to introduce this mutagenic construct by electroporation, no mutants were identified (Fu and Voordouw, 1997).

Marker exchange with this two-step procedure of homologous recombination has provided a number of important deletion mutants in \textit{D. vulgaris} (Table 9.1). However, a recombinational event that removes plasmid sequences is not the only event that can result in loss of the \textit{sacB} gene and sucrose resistance. Voordouw and co-workers also found that movement of endogenous insertion elements into \textit{sacB} accounted for almost 50\% of the sucrose resistant colonies (Fu and Voordouw, 1998). These events had to be distinguished from among the desired deletion mutants (Fu and Voordouw, 1997). Thus, this procedure for construction of mutants remains an investment of time and talent.

To expedite targeted mutagenesis in \textit{D. vulgaris}, a deletion strategy was developed that used a mutagenic cassette generated without restriction enzymes (Figure 9.1) (B. Eno and J.D. Wall, unpublished). The cassette, made by sequential PCR reactions, contained a gene encoding a Kan\(^r\) determinant flanked by \(-\)800 bp of the up- and down-stream regions of the target gene, as well as unique nucleotide sequences for mutant tracking (see below). No counter selection determinant was present or needed. The cassette was ligated into an unstable vector and directly electroporated into \(10^8\)–\(10^9\) cells of \textit{D. vulgaris} (Bender et al., 2005). Deletion mutants were selected by resistance to 400 \(\mu\)g G418/ml. With this approach, deletion of the target gene apparently occurred by double recombination in a single selection, as was observed for \textit{Desulfovibrio fructosovorans} (Rouset et al., 1991). Unlike the conjugation approach where the mutagenic plasmid was first integrated in the region adjacent to the target gene and \textit{sacB} employed for enrichment of plasmid loss, plasmid integration (single cross-over event) occurred only in a minority of cases following electroporation. The double recombination event is believed to be necessitated by the linearization of the incoming plasmid by endogenous nucleases. A single recombination event with linear DNA would result in a lethal double-strand break. Thus, stable acquisition of the marker replacing the target gene would require two recombination events.

**Current studies**

While progress has been made in developing an efficient genetic system in \textit{Desulfovibrio}, other technologies are being pursued. Currently, \textit{D. vulgaris} deletion mutants in the Wall laboratory (K. Bender and J.D. Wall, unpublished) are being marked with a bar coding strategy first proposed in \textit{Saccharomyces cerevisiae} (Shoemaker et al., 1996; Giaever, 2002). This tracking method allows identification and quantitation of deletion strains subjected to growth competition.

Another technique being explored is the addition of peptide tags to proteins for the isolation of functional protein complexes and the identification of the interactome (G. Zane and J.D. Wall, unpublished). The modified target genes containing the Strep-tag\(^\text{®}\) (IBA) are cloned into an unstable vector. Following introduction into \textit{D. vulgaris} by electroporation, integration into the genome occurs by homologous recombination with the target gene inserting the plasmid into the chromosome. Analysis of these constructs is now under way. The tagged proteins will be isolated.
### Table 9.1. Deletion mutants constructed in *Desulfovibrio* strains

<table>
<thead>
<tr>
<th>Deleted genes</th>
<th>Mutant phenotypes</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td><strong>I. Deletion by marker exchange [2 steps: conjugation; Cm&lt;sup&gt;+&lt;/sup&gt; selection and sacB enrichment]</strong></td>
<td></td>
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</tr>
<tr>
<td><em>dcrA</em> (oxygen sensor protein)</td>
<td>Increased transcription of the <em>rbo-rnh</em> operon</td>
<td>Fu and Voordouw, 1997</td>
</tr>
<tr>
<td><em>rfl</em> and <em>rfl2</em> (regulatory proteins of <em>rnh</em> operon)</td>
<td><em>rnh</em> operon is highly expressed on hydrogen sulfide medium</td>
<td>Keen et al., 1997</td>
</tr>
<tr>
<td><em>rbo</em> (rubredoxin oxidoreductase, desulfuradoxin)</td>
<td>More sensitive to internal superoxide than wild type</td>
<td>Voordouw and Voordouw, 1998</td>
</tr>
<tr>
<td><em>hox</em> (high molecular weight cytochrome complex)</td>
<td>Growth is impaired on hydrogen/sulfate medium</td>
<td>Dolta et al., 2000</td>
</tr>
<tr>
<td><em>hox</em> (Fe-only hydrogenase)</td>
<td>Growth is impaired on hydrogen/sulfate medium</td>
<td>Polthoric et al., 2002</td>
</tr>
<tr>
<td><em>rubr</em> (ruberythrin)</td>
<td>No obvious oxidative stress phenotype</td>
<td>Fournier et al., 2003</td>
</tr>
<tr>
<td><em>sod</em> (periplasmic superoxide dismutase)</td>
<td>More sensitive to air than wild type</td>
<td>Fournier et al., 2003</td>
</tr>
<tr>
<td><em>adh</em> (alcohol dehydrogenase)</td>
<td>Cannot use alcohol as carbon and electron donor</td>
<td>Haveman et al., 2003</td>
</tr>
<tr>
<td><em>nrfA</em> (nitrile reductase)</td>
<td>Ten times more sensitive to nitrite than wild type</td>
<td>Haveman et al., 2004</td>
</tr>
<tr>
<td><em>hynAB</em> (NiFe hydrogenase isoenzyme 1)</td>
<td>Lower final cell yield on lactate/sulfate medium</td>
<td>Goenka et al., 2005</td>
</tr>
<tr>
<td><strong>II. Deletion by marker exchange [1 step: electroporation; Kan&lt;sup&gt;+&lt;/sup&gt; selection]</strong></td>
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<tr>
<td><em>perR</em> (peroxide responsive regulator)</td>
<td><em>feoAB</em> are derepressed, more resistant to MnCl₂; more sensitive to salt stress and oxidative stress</td>
<td>K. Bender, unpublished</td>
</tr>
<tr>
<td><em>zir</em> (zinc uptake regulator)</td>
<td>Cells lyse at stationary phase; <em>adpC</em> is not derepressed</td>
<td>K. Bender, unpublished</td>
</tr>
<tr>
<td><em>ung</em> (tunicin DNA-glycosylase)</td>
<td>More resistant to salt stress and high pH; same sensitivity as wild type to added ZnCl₂</td>
<td>K. Bender, unpublished</td>
</tr>
<tr>
<td><em>norM</em> (Na⁺-driven multidrug efflux pump)</td>
<td>Unknown</td>
<td>J. Ringbauer, unpublished</td>
</tr>
<tr>
<td><em>rufC</em> (NADH:quinone oxidoreductase subunit)</td>
<td>Unknown</td>
<td>E. Drury, unpublished</td>
</tr>
<tr>
<td>DVU0164 (cation efflux family protein)</td>
<td>Unknown</td>
<td>E. Drury, unpublished</td>
</tr>
<tr>
<td><strong>III. Deletion by marker exchange [1 step: electroporation; liquid selection]</strong></td>
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<tr>
<td><em>hynABC</em> (NiFe hydrogenase; also called <em>hylAB</em>)</td>
<td>Ten per cent of hydrogenase activity relative to wild type; no growth differences (Kan&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Rouset et al., 1994</td>
</tr>
<tr>
<td><em>hynBC, hynD</em> (NiFe and NADP-reducing hydrogenases, respectively)</td>
<td>No obvious phenotype (Cm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Matki et al., 1997</td>
</tr>
<tr>
<td><em>hynABC, hynD, hylAB</em> (NiFe, NADP-reducing, and Fe hydrogenases, respectively)</td>
<td>Growth with organic acids/sulfate inhibited 25–70% relative to wild type (Kan&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Matki et al., 1997</td>
</tr>
<tr>
<td>Hydrogenase activity absent as measured by MV reduction; mutant grew on hydrogen/sulfate (Kan&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt; Gm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Hydrogenase activity absent as measured by MV reduction; mutant grew on hydrogen/sulfate (Kan&lt;sup&gt;+&lt;/sup&gt; Cm&lt;sup&gt;+&lt;/sup&gt; Gm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>Casalot et al., 2002</td>
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</table>

* Genes indicated were deleted from the genome of the strain.

* Only the most outstanding phenotype is listed here.

Kan<sup>+</sup>: kanamycin-resistant; Cm<sup>+</sup>: chloramphenicol-resistant; Gm<sup>+</sup>: gentamycin-resistant; MV: methyl viologen.
along with other associated proteins, giving a glimpse into the intricate protein interactions that occur during different growth stages and environmental stresses.

Two limitations still plaguing the genetic analysis of *Desulfovibrio* are the lack of inducible promoters and easily visualized reporter genes. While the promoter of the tetracycline resistance gene in pSUP104 (Priefer *et al.*, 1985) has been shown to express high levels of cloned genes in *Desulfovibrio vulgaris* (van den Berg *et al.*, 1989; Stokkermans *et al.*, 1992), no controllable promoter has been found for molecular work in *Desulfovibrio*. Currently, facile reporter genes for anaerobic studies are limited. Common reporters such as LuxAB (luciferase) and GFP (green fluorescent protein) are not suitable because of the oxygen requirement for bioluminescence or fluorescence (Burlage and Kuo, 1994; Hein *et al.*, 1994). While β-galactosidase activity can be assayed in SRB, the dark sulfide precipitates in normal colonies makes colour differentiation problematic. In addition, β-galactosidase substrates such as X-gal also require oxidation for colour development.

Another desirable genetic tool is the ability to delete two or more genes in one
strain. Sequential deletion of three different hydrogenases was performed in *D. fructosovorans* (*Table 9.1; Casalot et al., 2002*). This was accomplished by marker replacement, such that the final triple mutant also carried three different antibiotic resistance determinants. These results showed that such an approach was possible. A recent study confirming expression of the Tet<sup>+</sup> determinant in *D. vulgaris* also expands the selectable markers for making a second deletion in a Kan<sup>R</sup> background. However, markerless exchange may be a more desirable strategy for engineering

Figure 9.3. Deletion of *upp* gene using markerless exchange. The mutagenic vector (unstable in *Desulfovibrio*) contained DNA homologous to the up- and down-stream regions (shaded boxes) of the *D. vulgaris* *upp* gene (grey arrow), as well as a beta-lactamase gene (black arrow) for selection in *E. coli*. No marker separated the up- and down-stream regions. The vector was transformed into *D. vulgaris* via electroporation (Step 1). Following recombination (Step 2), mutant cells were selected using 40 μg 5-fluorouracil/ml (Step 3).
Figure 9.4. Markerless exchange using upp as a counterselectable marker. The gene of interest (G.O.I.) is deleted from the Δupp strain using a vector containing DNA homologous to the up- and down-stream regions (shaded boxes) of the G.O.I. (grey arrow), a KanR determinant (striped arrow), and the upp gene (black arrow). Following transformation of the Δupp strain, integration of the plasmid is selected by KanR and screened by SFU (Step 2). Because the deletion vector is unstable in Desulfovibrio, KanR SFU transformants can only arise via a single recombination event (dotted lines) (Step 3). While insertion into the up-stream region is shown, integration is also possible via the down-stream homology. Integration of the plasmid creates an unstable merodiploid. Nonselective growth allows plasmid excision and resolution of the merodiploid allele via a second recombination event (dotted lines) (Step 3). Depending on which homologous region is used for the second event, the result is either allele retention (a) or deletion (b) (Step 4) (Pritchett et al., 2004).

multiple mutations and would allow complementation with genes introduced on antibiotic resistant vectors. One approach would be to use resistance or sensitivity to a nucleotide analogue such as 5-fluorouracil as either positive or negative selectable markers (Pritchett et al., 2004). Genome scanning indicated that D. vulgaris possesses a uracil phosphoribosyl transferase (upp) gene for salvage of pyrimidine bases and, therefore, was predicted to incorporate the toxic analogue 5-fluorouracil (SFU). D. vulgaris was subsequently shown to be quite sensitive to SFU (Figure 9.2). Based on these findings, a strategy similar to the one used by Bender and co-workers (Bender et al., 2005) for marker exchange mutagenesis was employed to delete the upp gene involved in the salvage of pyrimidines. This strategy was altered by fusing together the up- and down-stream regions of the upp gene (Figure 9.3). No selectable marker separated the two homologous DNA regions. Following electroporation,
\( \Delta upp \) transformants were selected by resistance to 5FU (K. Bender and J.D. Wall, unpublished). This strain was confirmed by Southern analysis and can now be used as the parent strain for the creation of multiple unmarked deletions (Figure 9.4). Reiduction of the upp gene restores the sensitivity to 5FU and provides the basis for selection of vector loss during the introduction of mutant alleles, tagged genes, or gene deletions by two recombination events (Figure 9.4). Already, this strategy has been employed successfully in *Bacillus* (Fabret et al., 2002) and *Methanosarcina* (Pritchett et al., 2004).

**Overview of current mutants**

A number of informative mutants of *Desulfovibrio* strains have been constructed through the years (Table 9.1). While this list is not inclusive, mutants in cytochrome, hydrogenase, oxygen resistance, and regulator genes have provided new insights into the metabolism of SRB. While studying the response of *D. vulgaris* to nitrite, Haveman and co-workers showed that deletion of the nitrite reductase cytochrome \( c \) (nrfA) increased the inhibitory effect of \( \text{NO}_2^- \) on sulfate reduction (Haveman et al., 2004), thus providing another potential counter selection method for generating markerless mutants in a \( \Delta nrfA \) background.

In an effort to engineer a SRB strain with increased metal bioremediative potential, Aubert and co-workers over expressed the *Desulfuromonas acetoxidans* \( c \), gene in *D. desulfuricans* (Aubert et al., 1998). Physiological studies of the mutant indicated that cytochrome \( c \), retained its reductive ability in the *D. desulfuricans* host, increasing the metal reduction capability of the strain versus wild type. Additional efforts to explore the metal-reducing capacity of the *Desulfovibrio* strains resulted in a plasmid insertion mutation of the gene for type 1 cytochrome \( c \), (Rapp-Giles et al., 2000). Elimination of that cytochrome caused impaired reduction of \( \text{U(VI)} \) to \( \text{U(IV)} \) with lactate and pyruvate as the electron donor, whereas with hydrogen as the electron source, the reduction was nearly abolished (Payne et al., 2002). This mutant illustrates the complexity of the pathways utilized by SRB for respiration and metal metabolism.

Based on the controversial hydrogen production models proposed for SRB, a great deal of molecular work has focused on understanding hydrogen metabolism. The Hmc complex (high molecular mass cytochrome redox complex) has been proposed as a conduit for electrons from hydrogen oxidation in the periplasm to sulfate reduction in the cytoplasm. The first deletion studies to explore hydrogen production targeted the Hmc regulatory genes (nrfl and nrf2) (Keon et al., 1997). A deletion mutant exhibited an increase in Hmc operon expression, as well as increased growth on hydrogen when coupled to sulfate reduction. Conversely, the mutant showed impaired growth on lactate during sulfate reduction. Later studies on mutants with the entire Hmc complex deleted indicated impaired growth on hydrogen as the electron donor, but not on lactate or pyruvate as the electron donor when coupled to sulfate reduction (Dolla et al., 2000). These results are consistent with the proposed role for the Hmc complex in electron transport across the cytoplasmic membrane.

Providing a glimpse into the controlling factors associated with hydrogen production, deletion of the periplasmic Fe hydrogenase (hyd) gene in *D. vulgaris*
indicated that more hydrogen was produced from lactate and formate if excess sulfate was present when compared to wild type (Voordouw, 2002). This result indicated that the Fe hydrogenase was not the sole enzyme producing or consuming hydrogen, and underscored the capacity for compensation exhibited by *D. vulgaris* in hydrogen metabolism. More studies are needed before the mechanisms for hydrogen production in SRB are fully realized.

Since SRB experience periodic exposure to oxygen in the environment, they need mechanisms to deal with reactive oxygen species. In an effort to understand this tolerance mechanism, several oxygen resistance genes have been mutated in *D. vulgaris*. The first gene to be deleted in *D. vulgaris* was *derA*, an apparent oxygen sensor. This strain proved to be more resistant to oxygen than the wild type (Fu and Voordouw, 1997). Further analysis of the genes encoding superoxide reductase (*sor*) and superoxide dismutase (*sod*) indicated that the superoxide reductase was involved in superoxide resistance, while the superoxide dismutase was involved in protection against both oxygen and superoxide (Fournier et al., 2003). While ruberythrin exhibits NADH-dependent \( \text{H}_{2}\text{O}_2 \) reductase activity, no oxidative stress phenotype was observed when a deletion of the encoding gene (*rbr*) was constructed (Lunppio et al., 1997; Fournier et al., 2003). Studies are ongoing to further understand the oxygen defense mechanisms of SRB for future biotechnological applications in aerobic environments.

Recently constructed *D. vulgaris* deletions of the Ferric Uptake Regulator encoding paralogs, *fur*, *perR*, and *zur* have shown interesting phenotypes (K. Bender and J.D. Wall, unpublished). Compared to results for Fur in well-studied gamma-Proteobacteria, Fur appears to play a less significant role in Fe regulation in this anaerobe and a more dominant role in oxygen protection. As in *Bacillus*, the PerR regulator is involved in protection against oxidative stress. However, the Zur mutant may have a more global role than the predicted zinc uptake regulation. This deletion strain shows an increased resistance to high salt exposure and to high pH when compared to the wild type. No distinguishing phenotype was observed when the strain was exposed to various concentrations of zinc. As more regulators are targeted for mutagenesis, and transcriptional profiles are determined through microarray analyses, an increased understanding of the SRB stress response will result.

**Conclusion**

Over the past 15 years, numerous advances have been made in the genetics of *Desulfovibrio*. Currently available *Desulfovibrio* cytochrome, hydrogenase, oxygen response, and transcriptional regulator mutants have provided the first glimpse into the complicated SRB metabolism. Global analyses derived from genome sequences of a few SRB have allowed pathway predictions from gene annotations. However, the inherent limitations in annotation accuracy require that deletion studies be carried out to verify the predictions. The advent of transcriptional profiling and proteomics analyses for *D. vulgaris* have also provided data for pathway modelling but, again, targeted mutagenesis is needed to confirm these models. As further improvements are made in the molecular manipulation of SRB, a greater understanding of the intrinsic SRB metabolism is likely to follow. Since engineered systems can be successful only if entire pathways and overall
energy budgets are taken into account, these new techniques will prove integral to biotechnological strategies for optimizing bioremediation and reducing corrosion caused by SRB.

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