

Analysing the Metabolic Capabilities of *Desulfovibrio* Species through Genetic Manipulation

KELLY S. BENDER¹, HUEI-CHE YEN² AND JUDY D. WALL^{2*}

¹Department of Microbiology, Southern Illinois University, 1125 Lincoln Drive, Carbondale, IL 62901, USA and ²Biochemistry Department, University of Missouri-Columbia, Columbia, Missouri 65211, USA

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

*To whom correspondence may be addressed (wallj@missouri.edu)

Abbreviations: 5FU, 5-fluorouracil; Cm^R, chloramphenicol resistant; G418, geneticin derivative; *G.I.O.*, gene of interest; Gm^R, gentamycin resistant; Kan^R, kanamycin resistant; Kan^S, kanamycin sensitive; MV, methyl viologen; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); *sacB*, levansucrase; SRB, sulfate-reducing bacteria; Tet^R, tetracycline resistant; Tn, transposon; Xgal, 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside.

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: $\text{Fe}^0 + \text{H}_2\text{S} \rightarrow \text{FeS} + \text{H}_2$ (King and Müller, 1971). This dissolution of iron is compounded by cathodic depolarization, proposed as a model for biocorrosion by von Wolzogen Kühn and van der Vlugt in 1934. In this model, protons from water act as an oxidant to drive the formation of Fe^{2+} , and thereby form cathodic H_2 on the metal surface. The 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 Fe^0 as a source of reductant has yet to be determined and may point to an, as yet, unrecognized substrate, since Fe^0 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 H_2 via a cytoplasmic hydrogenase. The 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 H_2 transformation model (Lupton *et al.*, 1984). In this scenario, cells diluted into fresh medium convert excess electrons from carbon oxidation to 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), Tc(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), Tc(VII), 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 μ M (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 overlayer 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^r 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^r 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 10⁵ 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-I tetraheme cytochrome *c*₃ (Voordouw *et al.*, 1990b; Mus-Veteau *et al.*, 1992) and prismane (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; Seyedirashti *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.

TRANSPOSITION

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. Ringbauer 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 10^5 recipient cells (J. Ringbauer 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 *zruP* (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. Ringbauer, unpublished).

ELECTROPORATION

Electroporation has been explored as an alternative to conjugation for DNA introduction. In SRB, the technique was first successful in *Desulfovibrio fructosovorans* (Rousset *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. 2×10^9 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^{-8} , 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*₁ mutant was created via gene interruption by homologous recombination with a 292 bp internal portion of the *c*₃ 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*₁ 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^S, 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 *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 *sacB* gene and sucrose resistance. Voordouw and co-workers also found that movement of endogenous insertion elements into *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 *D. vulgaris*, a deletion strategy was developed that used a mutagenic cassette generated without restriction enzymes (Figure 9.1) (B. Emo 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 10⁹–10¹⁰ cells of *D. vulgaris* (Bender *et al.*, 2005). Deletion mutants were selected by resistance to 400 µg G418/ml. With this approach, deletion of the target gene apparently occurred by double recombination in a single selection, as was observed for *Desulfovibrio fructosovorans* (Rousset *et al.*, 1991). Unlike the conjugation approach where the mutagenic plasmid was first integrated in the region adjacent to the target gene and *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 *Desulfovibrio*, other technologies are being pursued. Currently, *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 *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*® (IBA) are cloned into an unstable vector. Following introduction into *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

Deleted genes*	Mutant phenotypes†	Reference
I. Deletion by marker exchange [2 steps: conjugation; Cm^R selection and <i>sacB</i> enrichment]		
<i>dsrA</i> (oxygen sensor protein)	Increased transcription of the <i>rbs-rnb</i> operon	Fu and Voordouw, 1997
<i>rpfA</i> and <i>rpf2</i> (regulatory proteins of <i>hmc</i> operon)	<i>hmc</i> operon is highly expressed on hydrogen sulfate medium	Keon <i>et al.</i> , 1997
<i>rbo</i> (rubredoxin oxidoreductase, desulfoferrodoxin)	More sensitive to internal superoxide than wild type	Voordouw and Voordouw, 1998
<i>hmc</i> (high molecular weight cytochrome complex)	Growth is impaired on hydrogen/sulfate medium	Della <i>et al.</i> , 2000
<i>hyd</i> (Fe-only hydrogenase)	Growth is impaired on hydrogen/sulfate medium	Pohorelec <i>et al.</i> , 2002
<i>rbr</i> (rubrerythrin)	No obvious oxidative stress phenotype	Fourmier <i>et al.</i> , 2003
<i>sod</i> (periplasmic superoxide dismutase)	More sensitive to air than wild type	Fourmier <i>et al.</i> , 2003
<i>adh</i> (alcohol dehydrogenase)	Cannot use alcohol as carbon and electron donor	Haveman <i>et al.</i> , 2003
<i>nrfA</i> (nitrite reductase)	Ten times more sensitive to nitrite than wild type	Haveman <i>et al.</i> , 2004
<i>hvaAB</i> (NiFe hydrogenase isoenzyme 1)	Lower final cell yield on lactate/sulfate medium	Goenka <i>et al.</i> , 2005
II. Deletion by marker exchange [1 step: electroporation; Kan^R selection]		
<i>fur</i> (ferric uptake regulator)	<i>fccAB</i> are derepressed; more resistant to MnCl ₂ ; more sensitive to salt stress and oxidative stress	K. Bender, unpublished
<i>perR</i> (peroxide responsive regulator)	Cells lyse at stationary phase; <i>ahpC</i> is not derepressed	K. Bender, unpublished
<i>zur</i> (zinc uptake regulator)	More resistant to salt stress and high pH; same sensitivity as wild type to added ZnCl ₂	K. Bender, unpublished
<i>img</i> (uracil DNA-glycosylase)	Unknown	J. Ringbauer, unpublished
<i>norM</i> (Na ⁺ -driven multidrug efflux pump)	Unknown	E. Drury, unpublished
<i>rnfC</i> (NADH:quinone oxidoreductase subunit)	Unknown	E. Drury, unpublished
DVU0164 (cation efflux family protein)	Unknown	E. Drury, unpublished
III. Deletion by marker exchange [1 step: electroporation; liquid selection]		
<i>hvaABC</i> (NiFe hydrogenase, also called <i>hvaN</i>)	Ten per cent of hydrogenase activity relative to wild type; no growth differences (Kan ^R)	Roussel <i>et al.</i> , 1991
<i>hmdD</i> (NADP-reducing hydrogenase)	No obvious phenotype (Cm ^R)	Malki <i>et al.</i> , 1997
<i>hvaABC</i> , <i>hmdD</i> (NiFe and NADP-reducing hydrogenases, respectively)	Growth with organic acids/sulfate inhibited 25–70% relative to wild type (Kan ^R Cm ^R)	Malki <i>et al.</i> , 1997
<i>hvaABC</i> , <i>hmdD</i> , <i>hvaAB</i> (NiFe, NADP-reducing, and Fe hydrogenases, respectively)	Hydrogenase activity absent as measured by MV reduction; mutant grew on hydrogen/sulfate (Kan ^R Cm ^R Gm ^R)	Casalot <i>et al.</i> , 2002

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

† Only the most outstanding phenotype is listed here.

Kan^R, kanamycin-resistant; Cm^R, chloramphenicol-resistant; Gm^R, gentamycin-resistant; MV, methyl viologen.

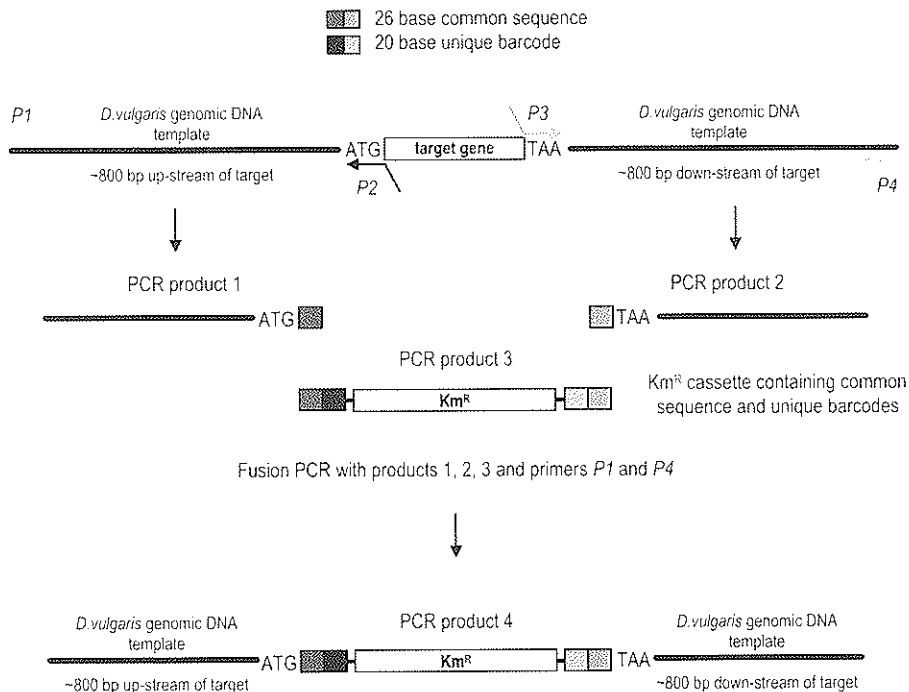


Figure 9.1. Cloning without restriction enzymes, scheme for generating deletion cassettes. PCR product 1 corresponded to the up-stream region and start codon of the target gene and was generated using primers *P1* and *P2*. A 26 bp common sequence was added to the 5' end of *P2* for future priming. PCR product 2 corresponded to the stop codon and down-stream region of the target gene, and was generated using primers *P3* and *P4*. A 26 bp common sequence was also added to the 5' end of *P3* for future priming. PCR product 3 corresponded to the Km^R determinant with flanking common sequences and unique bar codes (generated by primers containing both the common and unique sequences). The deletion cassette, PCR product 4, was generated by combining PCR products 1, 2, and 3 in a final amplification using primers *P1* and *P4*. The common sequences facilitated fusion of the three products.

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; Heim *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

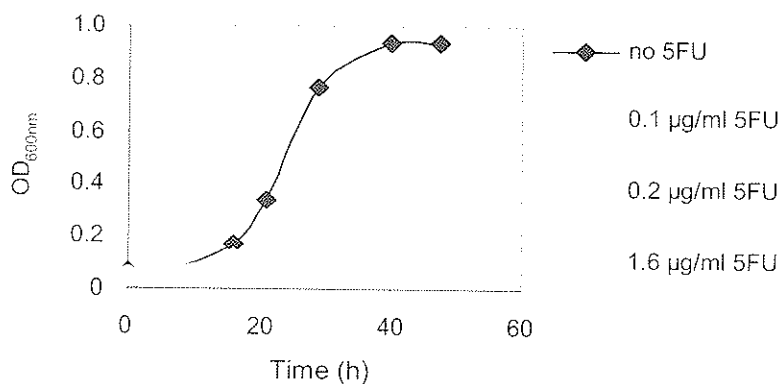


Figure 9.2. *Desulfovibrio vulgaris* Hildenborough grown anaerobically at 37°C with increasing concentrations of 5-fluorouracil. Growth was determined by optical density of the culture.

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^R determinant in *D. vulgaris* also expands the selectable markers for making a second deletion in a Kan^R background. However, markerless exchange may be a more desirable strategy for engineering

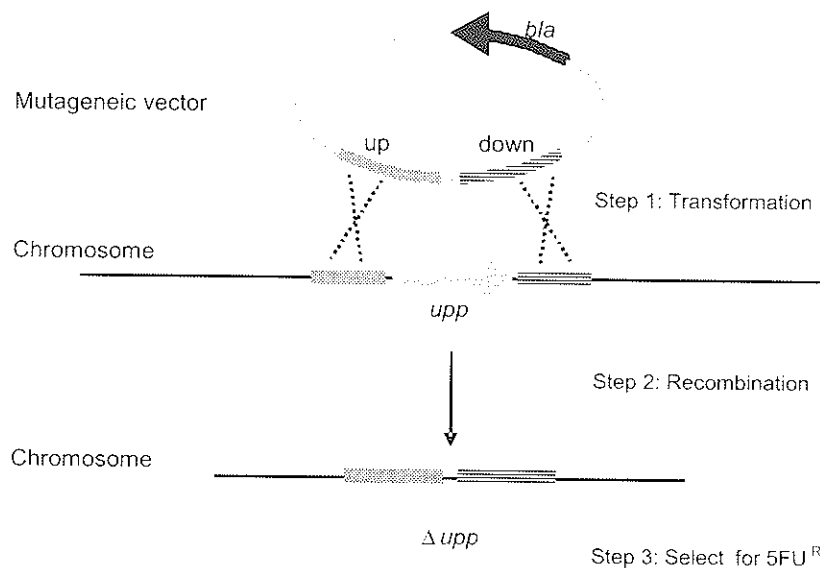


Figure 9.3. Deletion of *upp* gene using markerless exchange. The mutagenic vector (unstable in *Desulfovibrio*) contained DNA homologous to the up- and down-stream regions (shaded boxes) of the *D. vulgaris upp* gene (grey arrow), as well as a beta-lactamase gene (black arrow) for selection in *E. coli*. 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).

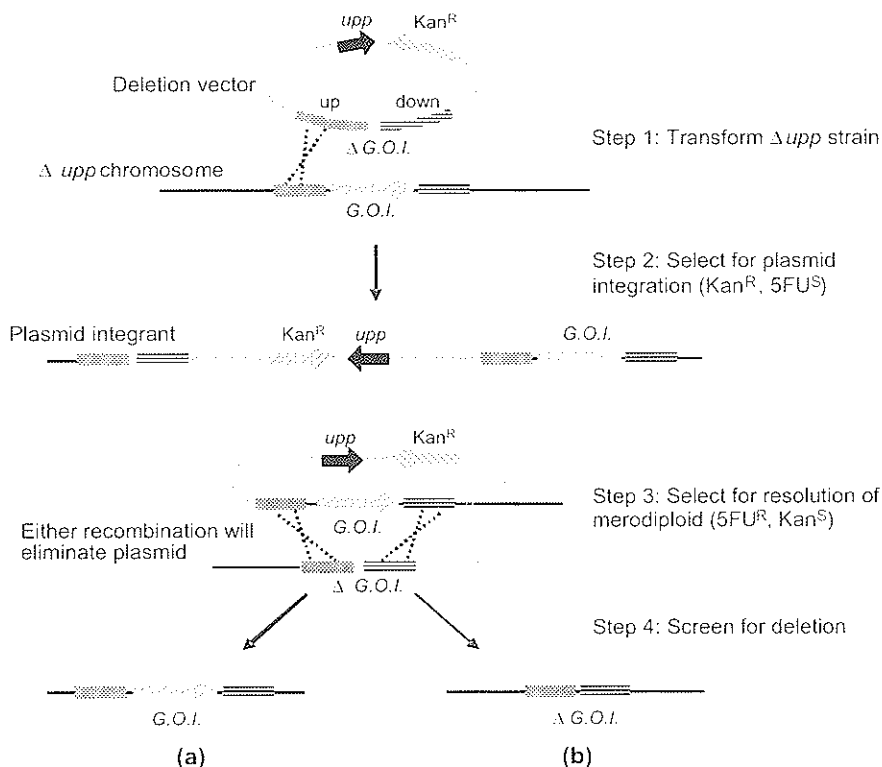


Figure 9.4. Markerless exchange using *upp* as a counterselectable marker. The gene of interest (*G.O.I.*) is deleted from the Δupp strain using a vector containing DNA homologous to the up- and down-stream regions (shaded boxes) of the *G.O.I.* (grey arrow), a Kan^R determinant (striped arrow), and the *upp* gene (black arrow). Following transformation of the Δupp strain, integration of the plasmid is selected by Kan^R and screened by $5FU^S$ (Step 2). Because the deletion vector is unstable in *Desulfovibrio*, Kan^R $5FU^S$ transformants can only arise via a single recombination event (dotted lines) (Step 2). (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 (5FU). *D. vulgaris* was subsequently shown to be quite sensitive to 5FU (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,

Δupp 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). Reintroduction 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 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_7 gene in *D. desulfuricans* (Aubert *et al.*, 1998). Physiological studies of the mutant indicated that cytochrome c_7 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 I cytochrome c_3 (Rapp-Giles *et al.*, 2000). Elimination of that cytochrome caused impaired reduction of U(VI) to 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 (*rnf1* and *rnf2*) (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 *dcrA*, 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 rubrerythrin exhibits NADH-dependent H_2O_2 reductase activity, no oxidative stress phenotype was observed when a deletion of the encoding gene (*rbr*) was constructed (Lumppio *et al.*, 1997; Fournier *et al.*, 2003). Studies are ongoing to further understand the oxygen defence 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.

Acknowledgements

We would like to thank A. Mukhopadhyay, D. Stahl, J. Ringbauer, E. Drury, and R. Payne for communication of results before publication. This work was supported by a subcontract (#6700892) from the DOE Genomics:GTL programme (DE-AC03-76SF00098), the Virtual Institute for Microbial Stress and Survival.

References

- ARGYLE, J.L., RAPP-GILES, B.J. AND WALL, J.D. (1992). Plasmid transfer by conjugation in *Desulfovibrio desulfuricans*. *FEMS Microbiology Letters* **94**, 255–262.
- AUBERT, C., LOJOU, E., BIANCO, P. ET AL. (1998). The *Desulfuromonas acetoxidans* tri-heme cytochrome *c₂* produced in *Desulfovibrio desulfuricans* retains its metal reductase activity. *Applied and Environmental Microbiology* **64**, 1308–1312.
- BENDER, K.S., YEN, H.C. AND WALL, J.D. (2005). Analysis of a ferric uptake regulator (Fur) mutant of *Desulfovibrio vulgaris* Hildenborough. abtr. H-109. In: *Abstracts of the General Meeting of the American Society of Microbiology 2005*. Atlanta, GA, USA: American Society for Microbiology.
- BOOPATHY, R. AND DANIELS, L. (1991). Effect of pH on anaerobic mild steel corrosion by methanogenic bacteria. *Applied and Environmental Microbiology* **57**, 2104–2108.
- BURLAGE, R.S. AND KUO, C.T. (1994). Living biosensors for the management and manipulation of microbial consortia. *Annual Review of Microbiology* **48**, 291–309.
- CASALOT, L., DE LUCA, G., DERMOUN, Z., ROUSSET, M. AND DE PHILIP, P. (2002). Evidence for a fourth hydrogenase in *Desulfovibrio fructosovorans*. *Journal of Bacteriology* **184**, 853–856.
- CHARDIN, B., GIUDICI-ORTICONI, M.-T., DE LUCA, G., GUIGLIARELLI, B. AND BRUSCHI, M. (2003). Hydrogenases in sulfate-reducing bacteria function as chromium reductase. *Applied Microbiology and Biotechnology* **63**, 315–321.
- COPPI, M.V., LEANG, C., SANDLER, S.J. AND LOVLEY, D.R. (2001). Development of a genetic system for *Geobacter sulfurreducens*. *Applied and Environmental Microbiology* **67**, 3180–3187.
- CYPIONKA, H. (2000). Oxygen respiration by *Desulfovibrio* species. *Annual Review of Microbiology* **54**, 827–848.
- DE LUCA, G., DE PHILIP, P., DERMOUN, Z., ROUSSET, M. AND VERMEGLIO, A. (2001). Reduction of technetium (VII) by *Desulfovibrio fructosovorans* is mediated by the nickel-iron hydrogenase. *Applied and Environmental Microbiology* **67**, 4538–4587.
- DINH, H.T., KUEVER, J., MUßMANN, M., HASSEL, A.W., STRATMANN, M. AND WIDDEL, F. (2004). Iron corrosion by novel anaerobic microorganisms. *Nature* **427**, 829–832.
- DOLLA, A., POHORELIC, B.K.J., VOORDOUW, J.K. AND VOORDOUW, G. (2000). Deletion of the *hmc* operon of *Desulfovibrio vulgaris* subsp. *vulgaris* Hildenborough hampers hydrogen metabolism and low-redox potential niche establishment. *Archives of Microbiology* **174**, 143–151.
- FABRET, C., EHRLICH, S.D. AND NOIROT, P. (2002). A new mutation delivery system for genome-scale approaches in *Bacillus subtilis*. *Molecular Microbiology* **46**, 25–36.
- FOURNIER, M., ZHANG, Y., WILDSCHUT, J.D., DOLLA, A., VOORDOUW, J.K. AND VOORDOUW, G. (2003). Function of oxygen resistance proteins in the anaerobic sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. *Journal of Bacteriology* **185**, 71–79.
- FU, R. AND VOORDOUW, G. (1997). Targeted gene-replacement mutagenesis of *dcrA*, encoding an oxygen sensor of the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. *Microbiology* **143**, 1815–1826.

- FU, R. AND VOORDOUW, G. (1998). *ISDI*, an insertion element from the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough: structure, transposition, and distribution. *Applied and Environmental Microbiology* **64**, 53–61.
- GIAEVER, G., CHU, A.M., NI, L. ET AL. (2002). Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* **418**, 387–391.
- GOENKA, A., VOORDOUW, J.K., LUBITZ, W., GARTNER, W. AND VOORDOUW, G. (2005). Construction of a [NiFe]-hydrogenase deletion mutant of *Desulfovibrio vulgaris* Hildenborough. *Biochemical Society Transactions* **33**, 59–60.
- HAMILTON, W.A. (2003). Microbially influenced corrosion as a model system for the study of metal-microbe interactions: a unifying electron transfer hypothesis. *Biofouling* **19**, 65–76.
- HANDLEY, J., ADAMS, V. AND AKAGI, J.M. (1973). Morphology of bacteriophage-like particles from *Desulfovibrio vulgaris*. *Journal of Bacteriology* **115**, 1205–1207.
- HANDLEY, J., ADAMS, V. AND AKAGI, J.M. (1991). Induction and partial purification of bacteriophages from *Desulfovibrio vulgaris* (Hildenborough) and *Desulfovibrio desulfuricans* ATCC 13541. *Journal of General Microbiology* **137**, 1545–1549.
- HAVEMAN, S., BRUNELLE, V., VOORDOUW, J.K., VOORDOUW, G., HEIDELBERG, J.F. AND RABUS, R. (2003). Gene expression analysis of energy metabolism mutants of *Desulfovibrio vulgaris* Hildenborough indicates an important role for alcohol dehydrogenase. *Journal of Bacteriology* **185**, 4345–4353.
- HAVEMAN, S.A., GREENE, E.A., STILWELL, C.P., VOORDOUW, J.K. AND VOORDOUW, G. (2004). Physiological and gene expression analysis of inhibition of *Desulfovibrio vulgaris* Hildenborough by nitrite. *Journal of Bacteriology* **186**, 7944–7950.
- HEIDELBERG, J.F., SESHADRI, R., HAVEMAN, S.A. ET AL. (2004). The genome sequence of the anaerobic, sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. *Nature Biotechnology* **22**, 554–559.
- HEIM, R., PRASHER, D.C. AND TSIEN, R.Y. (1994). Wavelength mutations and posttranslational autooxidation of green fluorescent protein. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 12501–12504.
- KEON, R.G., FU, R. AND VOORDOUW, G. (1997). Deletion of two downstream genes alters expression of the *hmc* operon of *Desulfovibrio vulgaris* subsp. *vulgaris* Hildenborough. *Archives of Microbiology* **167**, 376–383.
- KING, R.A. AND MILLER, J.D.A. (1971). Corrosion by sulphate-reducing bacteria. *Nature* **233**, 491–492.
- KOVACH, M.E., ELZER, P.H., HILL, D.S. ET AL. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175–176.
- LARSEN, R.A., WILSON, M.M., GUSS, A.M. AND METCALF, W.W. (2002). Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria. *Archives of Microbiology* **178**, 193–201.
- LUMPIO, H.L., SHENVI, N.V., GARG, R.P., SUMMERS, A.O. AND KURTZ, D.M.J. (1997). A rubrerythrin operon and nigerythrin gene in *Desulfovibrio vulgaris* (Hildenborough). *Journal of Bacteriology* **179**, 4607–4615.
- LUPTON, F.S., CONRAD, R. AND ZIEKUS, J.G. (1984). Physiological function of hydrogen metabolism during growth of sulfidogenic bacteria on organic substrates. *Journal of Bacteriology* **159**, 843–849.
- MACY, J.M., SANTINI, J.M., PAULING, B.V., O'NEILL, A.H. AND SLY, L.I. (2000). Two new arsenate/sulfate-reducing bacteria: mechanisms of arsenate reduction. *Archives of Microbiology* **173**, 49–57.
- MALKI, S., DE LUCA, G., FARDEAU, M.L., ROUSSET, M., BÉLAÏCH, J.P. AND DERMOUN, Z. (1997). Physiological characteristics and growth behaviour of single and double hydrogenase mutants of *Desulfovibrio fructosovorans*. *Archives of Microbiology* **167**, 38–45.
- MARKOWITZ, V., KORZENIEWSKI, F., PALANIAPPAN, K. ET AL. (2006). The Integrated Microbial Genomes (IMG) System. *Nucleic Acids Research* (special database issue) **1** (34), D344–348.
- MUS-VETEAU, I., DOLLA, A., GUERLESQUIN, F. ET AL. (1992). Site-directed mutagenesis of

- tetraheme cytochrome c_3 . Modification of oxidoreduction potentials after heme axial ligand replacement. *Journal of Biological Chemistry* **267**, 16851–16858.
- ODOM, J.M. AND PECK, H.D., JR. (1981). Hydrogen cycling as a general mechanism for energy coupling in the sulfate-reducing bacteria *Desulfovibrio* sp. *FEMS Microbiology Letters* **12**, 47–50.
- 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. *Applied and Environmental Microbiology* **68**, 3129–3132.
- PAYNE, R.B., HEMME, C.L. AND WALL, J.D. (2004). A new frontier in genomic research. *World Pipelines* **4**, 53–55.
- POHORELIC, B.K., VOORDOUW, J.K., LOJOU, E., DOLLA, A., HARDER, J. AND VOORDOUW, G. (2002). Effects of deletion of genes encoding Fe-only hydrogenase of *Desulfovibrio vulgaris* Hildenborough on hydrogen and lactate metabolism. *Journal of Bacteriology* **184**, 679–686.
- POSTGATE, J.R. (1984). *The sulphate-reducing bacteria*. London, UK: Cambridge University Press.
- POWELL, B., MERGEAY, M. AND CHRISTOFLI, N. (1989). Transfer of broad host-range plasmids to sulphate-reducing bacteria. *FEMS Microbiology Letters* **59**, 269–274.
- PRIEFER, U.B., SIMON, R. AND PÜHLER, A. (1985). Extension of the host range of *Escherichia coli* vectors by incorporation of RSF1010 replication and mobilization functions. *Journal of Bacteriology* **163**, 324–330.
- PRITCHETT, M.A., ZHUANG, J.K. AND METCALF, W.W. (2004). Development of a markerless genetic exchange method for *Methanosarcina acetivorans* C2A and its use in construction of new genetic tools for methanogenic archaea. *Applied and Environmental Microbiology* **70**, 1425–1433.
- RAPP, B.J. AND WALL, J.D. (1987). Genetic transfer in *Desulfovibrio desulfuricans*. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 9128–9130.
- RAPP-GILES, B.J., CASALOT, L., ENGLISH, R.S., RINGBAUER, J.A., JR., DOLLA, A. AND WALL, J.D. (2000). Cytochrome c_3 mutants of *Desulfovibrio desulfuricans*. *Applied and Environmental Microbiology* **66**, 671–677.
- RINGBAUER, J.A., JR., ZANE, G.M., EMO, B.M. AND WALL, J.D. (2004). Efficiencies of various transformation methods for the mutagenesis of *Desulfovibrio vulgaris* Hildenborough. abtr. H-202. In: *Abstracts of the General Meeting of the American Society for Microbiology 2004*. New Orleans, LA, USA: American Society for Microbiology.
- ROUSSET, M., DERMOUN, Z., CHIPPAUX, M. AND BÉLAÏCH, J.P. (1991). Marker exchange mutagenesis of the *hydN* genes in *Desulfovibrio fructosovorans*. *Molecular Microbiology* **5**, 1735–1740.
- ROUSSET, M., CASALOT, L., RAPP-GILES, B.J. ET AL. (1998). New shuttle vectors for the introduction of cloned DNA in *Desulfovibrio*. *Plasmid* **39**, 114–122.
- SEYEDIRASHTI, S., WOOD, C. AND AKAGI, J.M. (1992). Molecular characterization of two bacteriophages isolated from *Desulfovibrio vulgaris* NCIMB 8303 (Hildenborough). *Journal of General Microbiology* **138**, 1393–1397.
- SHOEMAKER, D., LASHKARI, D.A., MORRIS, D., MITTMANN, M. AND DAVIS, R.W. (1996). Quantitative phenotypic analysis of yeast deletion mutants using a highly parallel molecular bar-coding strategy. *Nature Genetics* **14**, 450–456.
- SINGLETON, R., JR. (1993). *The sulfate-reducing bacteria: contemporary perspectives*, pp 189–210. New York, USA: Springer-Verlag.
- SINGLETON, R., JR., KETCHUM, R.B. AND CAMPBELL, L.L. (1988). Effect of calcium cation on plating efficiency of the sulfate-reducing bacterium *Desulfovibrio vulgaris*. *Applied and Environmental Microbiology* **54**, 2318–2319.
- SMITH, W.L. AND GADD, G.M. (2000). Reduction and precipitation of chromate by mixed culture sulphate-reducing bacterial biofilms. *Journal of Applied Microbiology* **88**, 983–991.
- STOKKERMANS, J.P., HOUBA, P.H., PIERIK, A.J., HAGEN, W.R., VAN DONGEN, W.M. AND VEEGER, C. (1992). Overproduction of prismane protein in *Desulfovibrio vulgaris* (Hildenborough): evidence for a second $S = 1/2$ -spin system in the one-electron reduced state. *European Journal of Biochemistry* **210**, 983–988.

- VALLS, M. AND DE LORENZO, V. (2002). Exploiting the genetic and biochemical capacities of bacteria for remediation of heavy metal pollution. *FEMS Microbiology Reviews* **26**, 327–338.
- VAN DEN BERG, W.A.M., STOKKERMANS, J.P.W.G. AND VAN DONGEN, W.M.A.M. (1989). Development of a plasmid transfer system for the anaerobic sulphate-reducer, *Desulfovibrio vulgaris*. *Journal of Biotechnology* **12**, 173–184.
- VAN DEN BERG, W.A., VAN DONGEN, W.M. AND VEEGER, C. (1991). Reduction of the amount of periplasmic hydrogenase in *Desulfovibrio vulgaris* (Hildenborough) with antisense RNA: direct evidence for an important role of this hydrogenase in lactate metabolism. *Journal of Bacteriology* **173**, 3688–3694.
- VAN DONGEN, W.M.A.M. (1995). Molecular biology of redox-active metal proteins from *Desulfovibrio*. In: *Sulfate-reducing bacteria*. Ed. L. Barton, pp 185–208. New York, USA: Plenum Press.
- VAN DONGEN, W., HAGEN, W., VAN DEN BERG, W. AND VEEGER, C. (1988). Evidence for an unusual mechanism of membrane translocation of the periplasmic hydrogenase of *Desulfovibrio vulgaris* (Hildenborough), as derived from expression in *Escherichia coli*. *FEMS Microbiology Letters* **50**, 5–9.
- VON WOLZOGEN KÜHR, C.A.H. AND VAN DER VLUGT, L.S. (1934). De grafiteering gietijzer als electrobiochemische process in anaerobe gronden. *Water (Netherlands)* **18**, 147–165.
- VOORDOUW, G. (2002). Carbon monoxide cycling by *Desulfovibrio vulgaris* Hildenborough. *Journal of Bacteriology* **184**, 5903–5911.
- VOORDOUW, G. AND BRENNER, S. (1985). Nucleotide sequence of the gene encoding the hydrogenase from *Desulfovibrio vulgaris* (Hildenborough). *European Journal of Biochemistry* **148**, 515–520.
- VOORDOUW, J.K. AND VOORDOUW, G. (1998). Deletion of the *rbo* gene increases the oxygen sensitivity of the sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. *Applied and Environmental Microbiology* **64**, 2882–2887.
- VOORDOUW, G., HAGEN, W.R., KRÜSE-WOLTERS, K.M., VAN BERKEL-ARTS, A. AND VEEGER, C. (1987). Purification and characterization of *Desulfovibrio vulgaris* (Hildenborough) hydrogenase expressed in *Escherichia coli*. *European Journal of Biochemistry* **162**, 31–36.
- VOORDOUW, G., NIVIERE, V., FERRIS, F.G., FEDORAK, P.M. AND WESTLAKE, D.W.S. (1990a). Distribution of hydrogenase genes in *Desulfovibrio* spp. and their use in identification of species from the oil field environment. *Applied and Environmental Microbiology* **56**, 3748–3754.
- VOORDOUW, G., POLLOCK, W.B.R., BRUSCHI, M., GUERLESQUIN, B., RAPP-GILES, B.J. AND WALL, J.D. (1990b). Functional expression of *Desulfovibrio vulgaris* Hildenborough cytochrome c_3 in *Desulfovibrio desulfuricans* G200 after conjugational gene transfer from *Escherichia coli*. *Journal of Bacteriology* **172**, 6122–6126.
- WALL, J.D., RAPP-GILES, B.J. AND ROUSSET, M. (1993). Characterization of a small plasmid from *Desulfovibrio desulfuricans* and its use for shuttle vector construction. *Journal of Bacteriology* **175**, 4121–4128.
- WALL, J.D., MURNAN, T., ARGYLE, J., ENGLISH, R.S. AND RAPP-GILES, B.J. (1996). Transposon mutagenesis in *Desulfovibrio desulfuricans*: development of a random mutagenesis tool from Tn7. *Applied and Environmental Microbiology* **62**, 3762–3767.
- WEIMER, P.J., VAN KAVELAAR, M.J., MICHEL, C.B. AND NG, T.K. (1988). Effect of phosphate on the corrosion of carbon steel and on the composition of corrosion products in two-stage continuous cultures of *Desulfovibrio desulfuricans*. *Applied and Environmental Microbiology* **54**, 386–396.
- WHITE, C. AND GADD, G.M. (1998). Accumulation and effects of cadmium on sulphate-reducing bacterial biofilms. *Microbiology* **144**, 1407–1415.