UNIVERSITY OF CALGARY

Hydrogen Metabolism of the Sulfate-Reducing Bacterium *Desulfovibrio vulgaris*

by

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Abstract

Hydrogen metabolism in *Desulfovibrio vulgaris* was investigated. Two mutants were used: H801, which is missing the *hmc* operon, proposed to encode the transmembrane redox complex involved in electron transport from hydrogen oxidation to sulfate reduction, and Hyd100, which lacks the *hyd*A,B genes encoding the Fe-only hydrogenase.

The H801 mutant had a substantially lower growth rate when hydrogen was used as the electron donor, confirming the importance of the Hmc complex in efficient electron transport from hydrogen. On plates, delayed growth was observed, indicating a deficiency in anaerobic niche establishment. The Hmc complex does not play a role in hydrogen production from lactate.

The Hyd100 mutant had a similar cell yield with lactate and a lower cell yield with hydrogen as electron donor for sulfate reduction as compared to wild type. It produced less hydrogen from lactate under fermentative conditions, indicating a role for Fe-only hydrogenase in this process.

Acknowledgements

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Most of all I would like to thank my wife Joy who has supported me through everything, big and small.

For Joy who makes everything worthwhile

Table of Contents

List of Tables

List of Figures

Abbreviations and Symbols

A: alanine
ADP: adenosine diphosphate
AMP: adenosine monophosphate
Amp^r: ampicillin resistant
APS: aadenosine-5'-phosphosulfate
atm: atmoshere
ATP: adenosine triphosphate
ATPase: ATP synthesising enzyme
BSA: bovine serum albumen
bp: base pairs
C: cystein
Ci: Curies
Cm: chloramphenicol
Cm^r: chloramphenicol resistant
CoA: coenzyme A
D: aspartate
Δ: indicates gene deletion
*Dv*H: *Desulfovibrio vulgaris* Hildenborough wild type
dCTP: dimethyl triphosphate
DmsB: anaerobic dimethyl sulfoxide reductase from *E. coli*
DNA: deoxyribonucleic acid
dNTPs: deoxynucleoside triphosphates
E: glutamine
e^-: electrons
e^-_{in}: electrons within the cytoplasm
EDTA: ethylenediaminetetraacetic acid
F: phenylalanine
FdnH: nitrate-inducible formate dehydrogenase from *E. coli*
G: glycine
GC: gas chromatograph
H: histidine
H^+: a proton
H^+_{in}: cytoplasmic proton
H^+_{out}: periplasmic proton
HGT: high gelling temperature agarose
Hmc: high molecular weight cytochrome of *Desulfovibro vulgaris* Hildenborough
HmcA-HmcF: proteins encoded by the *hmc* operon of *Desulfovibrio vulgaris* Hildenborough
Hrs: hours
I: isoleucine
K: lysine
Kbp: kilobase pair (10^3 base pairs)
Km^r: kanamycin resistant

L: leucine
λ: lambda virus DNA
LGT: low gelling temperature agarose
M: methionine
mer: number of nucleotides within a short DNA molecule
mRNA: mesanger RNA
N: asparagine
OD_{600}:Optical Density at 600nm wavelength
P: proline
PAGE: polyacrylamide gel electrophoresis
PCR: polymerase chain reaction
% (v/v): percent volume by volume = ml/100 ml total volume
% (w/v): percent weight by volume = g/100 ml total volume
P_i: inorganic phosphate
ppm: concentration in part per million
Q: glutamine
R; arginine
RNA: ribonucleic acid
rrf1, rrf2: genes encoding regulatory proteins involved in transcriptional control of the *hmc* operon
S: serine
SDS: sodium dodecylsulfate
spp.: species (plural)
SRB: sulfate-reducing bacteria
SSC: standard sodium citrate
T: threonine
TCA: trichloroacetic acid
TE: tris-EDTA
Tris: Tris(hydroxymethyl)methylamine
TY: tryptone-yeast extract
UV: ultraviolet light
V: valine
W: tryotophan
Y: tyrosine
$Y_{sulfate}$: cell yield per mol of sulfate reduced
$Y_{hydrogen}$: cell yield per mol of hydrogen oxidized

1. Introduction

Desulfovibrio vulgaris Hildenborough (*Dv*H), an obligately anaerobic, gram-negative eubacterium, derives energy from the dissimilatory reduction of sulfate using hydrogen or organic acids as an electron donor (Postgate 1984). This organism belongs to a distinctive and ubiquitous group of anaerobic prokaryotes known as the sulfate-reducing bacteria (SRB), which are of great economic and academic interest. Sulfate-reducing bacteria play a role in the contamination of petroleum products, resulting in the souring of gas wells over time. They also play a role in the anaerobic corrosion of steel. They produce large amounts of noxious hydrogen sulfide production in such places as gas wells and sewage pipes. It was in Dutch canals that H_2S production caused the original interest in these organisms (Beyerinck 1895). Biochemical and genetic evidence shows that the evolutionary divergence of SRB began early in the history of life, thus they have had a long time in which to fill a large niche and play a unique ecological and evolutionary role (Odom & Singleton, 1993).

Many proteins involved in the metabolism of SRB have been studied. They show a wide array of unique electron carriers and redox enzymes (LeGall & Fauque 1988). The molecular study of these enzymes has lead to an understanding of their cellular localization, and function through sequence analysis and comparison to other known redox proteins. However, many aspects of the elementary question of how these organisms derive energy from their environment remain to be answered. Research described in this thesis was undertaken to further the understanding of hydrogen metabolism in *Dv*H. It also describes the role of a trans-membrane redox complex directly involved in the metabolism of hydrogen.

2. Literature Review

2.1 Metabolism

SRB have been studied for over 100 years; most information has been obtained for the eubacteria, specifically for those of the genus *Desulfovibrio*. SRB are defined by their use of sulfate as the principal electron acceptor for anaerobic respiration. As with most organisms electron transport, creating a proton gradient, is linked to ATP generation through a membrane bound ATPase. Many redox proteins have been discovered, and much is known about how they transfer electrons. However the complete understanding of how these organisms use sulfate as a terminal electron acceptor to create a proton gradient across the inner membrane is still incomplete.

2.1.1 Metabolism of organic acids

There are several models to explain the metabolism of organic acids by *Desulfovibrio* spp. with sulfate as the terminal electron acceptor. With the investment of two high-energy phosphate bonds, sulfate is reduced to sulfide within the cytoplasm (Fig. 2.1), as described in equation 1(Voordouw, 1995):

$$SO_4^{2-} + ATP + 8H^+_{in} + 8e^- \rightarrow HS^- + AMP + 2Pi \quad (1)$$

The protons are labeled "in" because they are produced within the cytoplasm. The investment of energy is recouped through proton-driven and/or substrate level phosphorylation (Voordouw, 1995). Exactly how this investment of energy is recovered is still a matter for debate.

When lactate is metabolized completely to acetate and CO_2, 4 hydrogen protons, 4 electrons, and 1 ATP are produced by substrate level phosphorylation (Fig. 2.2); as described in equation 2 (Voordouw, 1995):

$$\text{lactate} + \text{ADP} + \text{Pi} \rightarrow \text{acetate} + CO_2 + \text{ATP} + 4H^+_{in} + 4e^- \qquad (2)$$

From Figs. 2.1 and 2.2 it is evident that if two moles of lactate are consumed per mole of sulfate reduced there is no net gain of ATP. Moreover, there is no apparent proton transport to create a gradient, since production and consumption of protons and electrons takes place in the cytoplasm. In other words, there is no compartmental separation of the reactions (Voordouw, 1995; LeGall & Fauque 1988).

While the reactions involved in the use of lactate for sulfate reduction have been identified, the process by which a proton gradient is established (a requirement to obtain net ATP synthesis) is not at all clear. There are three theories worth mentioning. The first theory proposes that redox proton pumps, associated with sulfate reduction, create a proton gradient (Kobayashi *et al* 1982). This has yet to be substantiated.

Odom and Peck (1981) suggested a second theory called the hydrogen cycling mechanism, in which cytoplasmic hydrogenases produce hydrogen from the protons and electrons resulting from lactate metabolism (Fig. 2.3). This hydrogen diffuses to the periplasm, where periplasmic hydrogenases oxidize the hydrogen releasing protons and transfer electrons to a trans-membrane electron carrier. The electrons passed back through the membrane are used for cytoplasmic sulfate reduction. This proposal is questionable because a cytoplasmic hydrogenase has yet to be discovered. Moreover, it has been shown that hydrogenases are mostly (>95%) located within the periplasm (Badziong & Thauer 1980). The hydrogen cycling mechanism argument is also problematic when considering

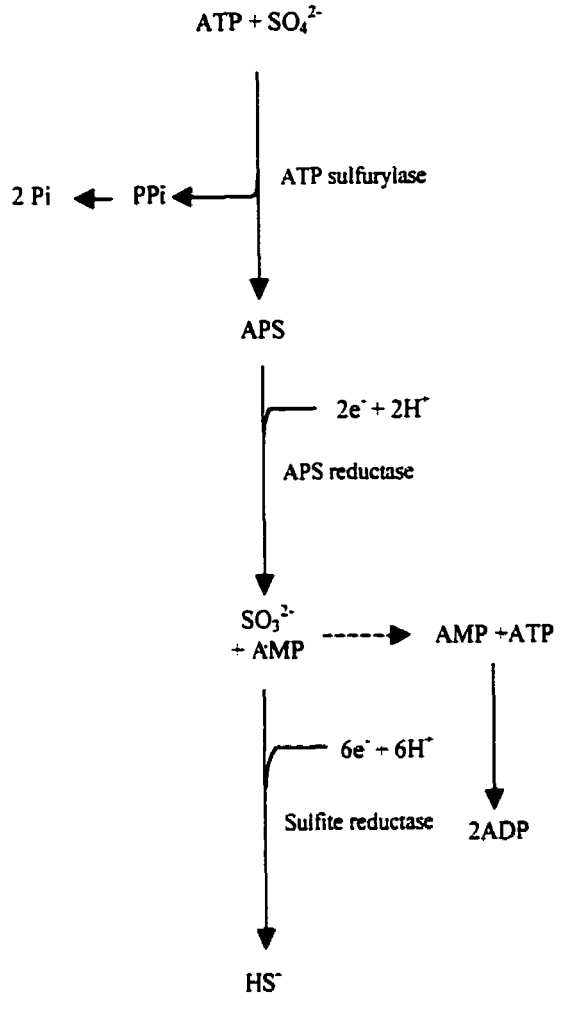

Figure 2.1. Dissimilatory sulfate reduction pathway that takes place within the cytoplasm of *Desulfovibrio* spp.

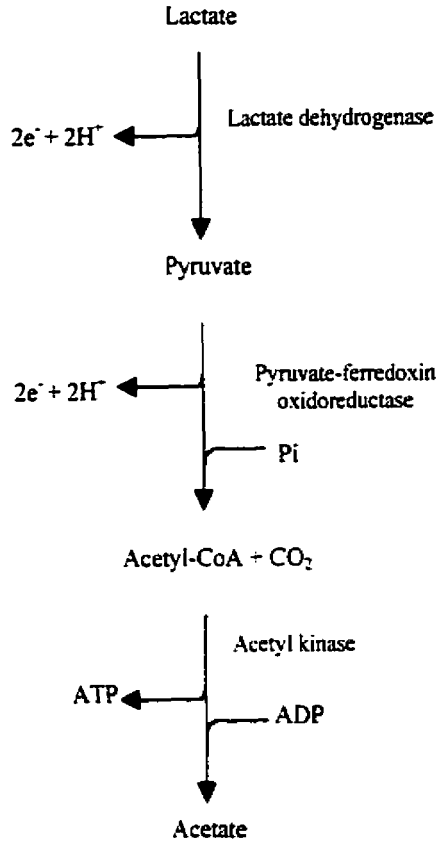

Figure 2.2. Oxidation of lactate pathway which takes place in the cytoplasm for *Desulfovibrio* spp.

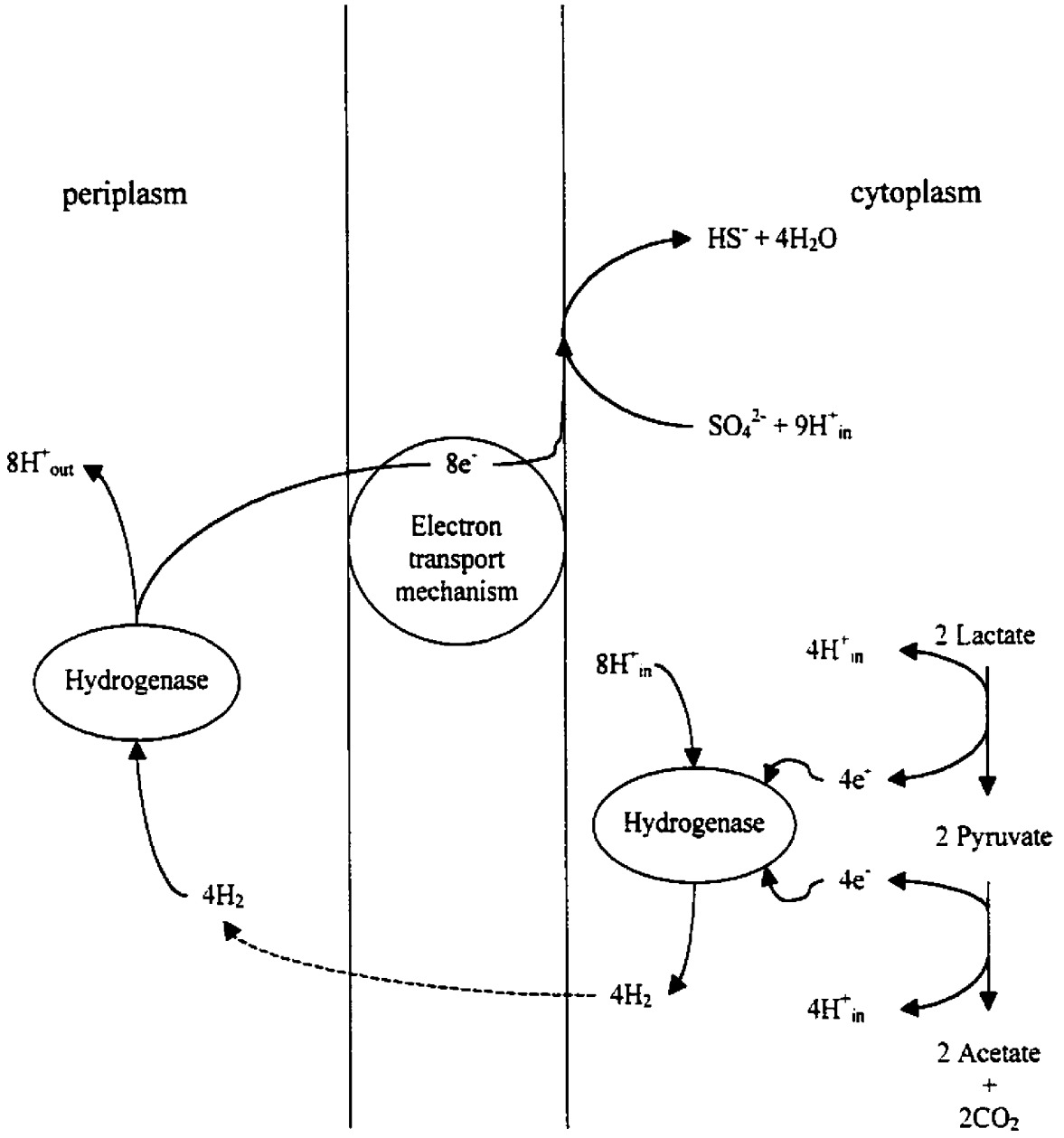

Figure 2.3. Hydrogen cycling mechanism proposed by Odom and Peck (1981)

the redox potentials of the half reactions, which produce hydrogen from lactate within the cytoplasm. The overall reaction is endergonic, and due to the equilibrium of this reaction the oxidation of lactate would only work with low concentrations of hydrogen. This is inconsistent with the ability of *Desulfovibrio* to grow on lactate with high concentrations of hydrogen (Pankhania *et al* 1986). Although the hydrogen cycling mechanism argument has faced substantial criticism, there is evidence that hydrogenases play a role in lactate metabolism. When the expression of soluble periplasmic [Fe] hydrogenase (one of 3 hydrogenases in *Desulfovibrio* spp.) was reduced (due to antisense RNA production from an engineered plasmid), a 50-65% decrease in the growth rate on lactate was observed (Van der Berg *et al*, 1991). While not supporting the H_2 cycling theory, this evidence does lend credence to the suggestion that hydrogenases play a role in growth on lactate.

There is also a third possibility of an as yet undiscovered means of proton export involving the membrane-bound lactate dehydrogenase. This enzyme could possibly couple export of protons with oxidation of lactate to pyruvate (Voordouw, 1995). The rapidly emerging genomic sequence of *D. vulgaris* Hildenborough (http://www.tigr.org) will help in illuminating this problem.

2.1.2 Metabolism of hydrogen

Growth on organic acids still has fundamental questions to be answered on how a proton gradient is formed, however chemolithotrophic growth with hydrogen as the sole electron donor provides an easyer explanation of proton gradient establishment. Since hydrogenases are located in the periplasm, electrons from hydrogen oxidation can be passed through the membrane to the cytoplasm for sulfate reduction (Fig 2.4). This

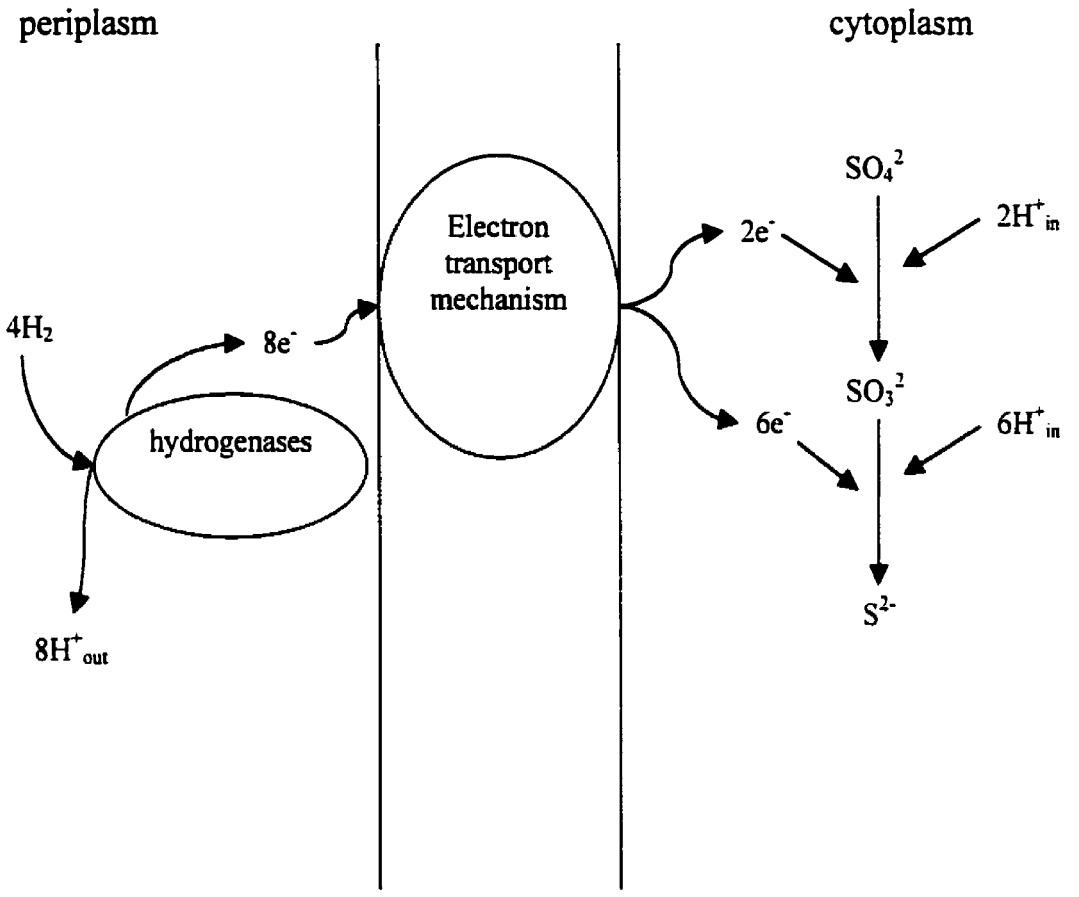

Figure 2.4. Hydrogen metabolism demonstrating the transfer of electrons through a membrane bound electron transport mechanism.

compartmentalization allows a proton gradient to be formed, hence ATP generation to occur through a membrane-bound, proton-driven ATP synthase; as described in equation 3 (Voordouw 1995):

$$4H_2 + ATP + SO_4^{2-} + 8H^+_{in} \rightarrow HS^- + AMP + 2P_i + 8H^+_{out} \quad (3)$$

The exact mechanism of electron transport from the periplasm to the cytoplasm will be discussed later in this review (section 2.2) and in the results and Discussion (section 5.1 and 6.1).

When growing on hydrogen, *Desulfovibrio* spp. derive approximately 70% of the cell carbon from acetate and 30% from CO_2 (Badziong & Thauer 1978). No energy is derived from the anabolic metabolism of acetate. When both lactate and hydrogen are available, biphasic growth is observed. Lactate is consumed preferentially, with the production of acetate, and then hydrogen is consumed and acetate used as a carbon source (Pankhania *et al* 1986).

2.1.3 Role of hydrogen metabolism in iron corrosion

Hydrogen metabolism is central to explaining how SRB are capable of corroding iron:

$$Fe^\circ \rightarrow Fe^{2+} + 2e^- \quad (4)$$

In fact, SRB were identified as the main cause of anaerobic corrosion (Von Wolzogen Kühr *et al* 1934). Controversy about this subject has centered on the question of whether the corrosion is due to active bacterial metabolism, or to its subsequent metabolites and products (Odom 1993). The end-product of sulfate-reduction, H_2S, is corrosive in itself. However, it is possible that *Desulfovibrio* spp. are capable of cathodic depolarisation using

hydrogenases (Postgate 1984). The classical mechanism for this type of corrosion can be described as follows: in aqueous environments iron dissolves to Fe^{2+} leaving behind electrons, which polarize the metal surface. Protons from the solution combine with electrons to form atomic hydrogen, which coats the metal surface, and an equilibrium is established. Corrosion takes place with cathodic depolarisation, which is the removal of the polarizing hydrogen layer. This causes further Fe^{2+} dissolution from the iron, allowing continued corrosion to take place (Fig. 2.5) (Odom 1993). The removal of the protecting hydrogen layer is thought to be the result of oxidising hydrogenases which pass the electrons from the oxidation of hydrogen to cytoplasmic sulfate reduction. Dissolved Fe^{2+} then reacts with sulfide to produce the characteristic iron-sulfide deposits of SRB.

*Dv*H has been shown to grow with acetate and CO_2 as the carbon source, in the presence of a mild steel electrode kept at an appropriately negative potential. Since any hydrogen is solely produced from polarizing the mild steel electrode, this indicates that the hydrogen produced from the steel is used for bacterial growth (Pankhania *et al* 1986). A refinement of the proposed mechanism for metal corrosion by SRB is possible. A cytochrome found in the outer membrane, following separation of membrane fractions, might siphon electrons directly from the metal, thereby bypassing the production of hydrogen from protons polarizing the metal surface by hydrogenases. These electrons are then donated to hydrogenases that eventually pass them through an electron transport system to cytoplasmic sulfate reduction (Kloeke *et al* 1995).

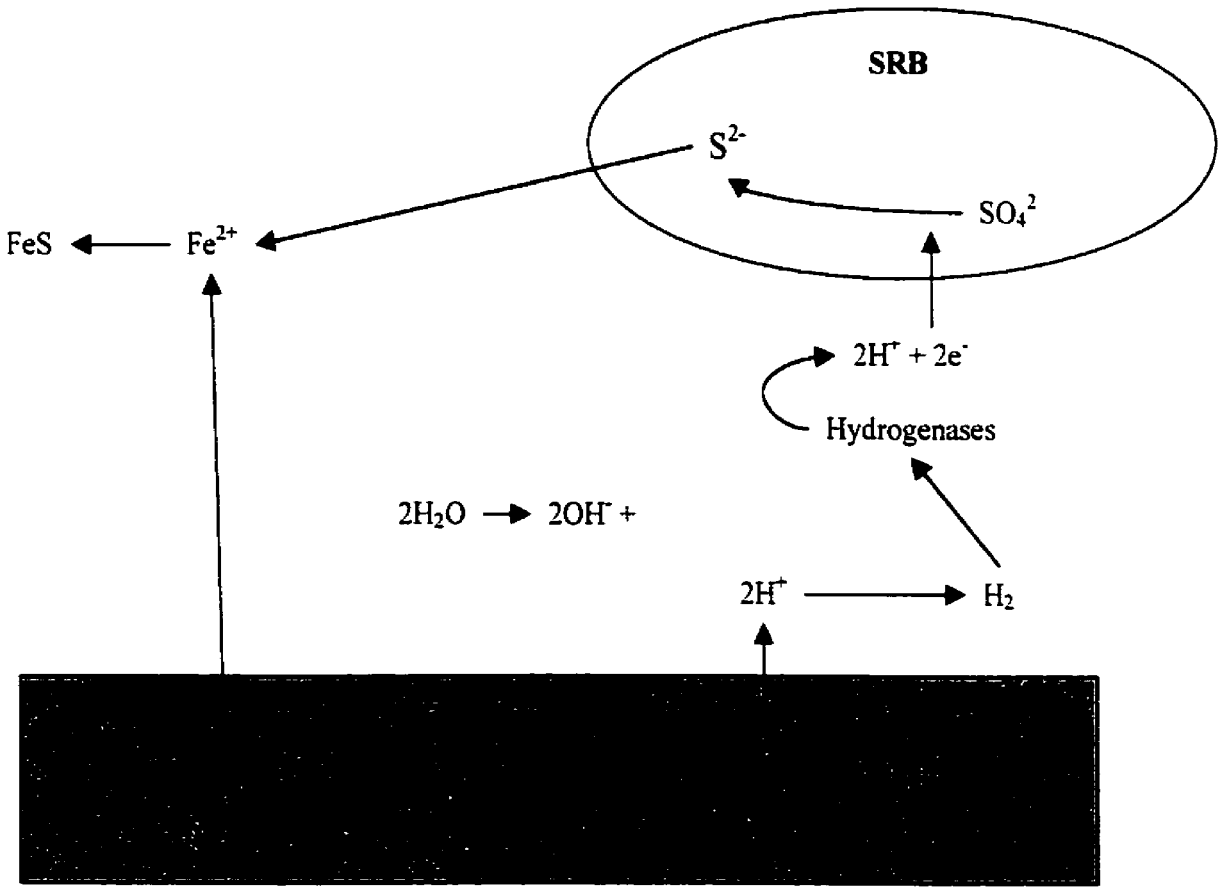

Figure 2.5. Proposed mechanism for active bacterial metabolism causing the anaerobic corrosion of iron. A black precipitate (FeS) is eventually formed.

2.1.4 Hydrogen production Through Lactate Metabolism and its Significance

Lactate metabolism is more complex than the simple use of lactate as an electron donor for sulfate reduction, *Desulfovibrio* spp. can grow on lactate in the absence of sulfate, in syntrophic association with methanogenic bacteria (Postgate 1994), summarized in the equation 5 (Pankhania *et al* 1988):

$$Lactate^- + H_2O \rightarrow Acetate^- + CO_2 + 2H_2 \quad (5)$$

Because the oxidation of lactate to the intermediate pyruvate (not shown in equation (5)) with protons acting as the terminal electron acceptor is endergonic, methanogenic bacteria are required to keep the H_2 partial pressure low.

Hydrogen production from lactate metabolism in the presence of sulfate has been observed. This phenomenon occurs in lag phase, just before exponential growth (Tsuji and Yagi 1980, Van den Berg *et al* 1991). A burst of hydrogen production takes place just as the cells begin to rapidly grow and divide. Once in exponential growth, hydrogen production stops and hydrogen is consumed for growth (Tsuji and Yagi 1980, Van den Berg *et al* 1991), although hydrogen production throughout the growth curve has been reported by other authors (Traore *et al* 1981).

The reason for hydrogen production prior to the start of exponential growth has yet to be discovered. There are two theories to explain this phenomenon. After inoculation into fresh medium, stationary phase cells have a low ATP content. This does not allow activation of sulfate and subsequently sulfate reduction. Tsuji and Yagi (1980) gave another explanation for this phenomenon. They proposed that rapid synthesis of cellular molecules, at the start of exponential growth, decreases the cellular content of ATP, preventing the activation of sulfate and hence sulfate reduction. Since sulfate activation is prevented from

low levels of ATP, electrons from lactate metabolism must be redirected to protons for hydrogen production.

Another possible reason for the hydrogen burst is that the redox potential of the new medium is not sufficiently low for sulfate reduction. A boiled medium that has been flushed under a nitrogen atmosphere has a redox potential of ~ +100 mV. *D. vulgaris* requires a redox potential of ~ -200 mV for growth (Postgate 1984). This requirement for a low redox potential might result in the use of protons as the electron acceptor from the lactate metabolism during cellular maintenance.

The mechanism driving the hydrogen burst needs to be elucidated, specifically in determining where hydrogen production takes place. Since all the hydrogenases are in the periplasm (Badziong & Thauer 1980), it is assumed that the electrons from lactate metabolism would be passed through some membrane bound transport system to the periplasm for hydrogen production. This membrane bound transport system might be the same as used for transporting electrons into the cytoplasm during hydrogen consumption.

2.2 The *hmc* Operon

Hydrogen metabolism in *Desulfovibrio* spp. illustrates how a proton gradient can be established as a result of compartmental separation of hydrogen oxidation and sulfate reduction. However, a redox link between these two processes must exist within the cytoplasmic membrane. Electrons from periplasmic hydrogenases must be passed to an electron transport chain, which would deliver the electrons into the cytoplasm.

2.2.1 The role of the Hmc complex in hydrogen metabolism

Since cytochromes are the principal electron carriers in many electron transport chains they are logical candidates for possible transmembrane redox proteins. *Desulfovibrio vulgaris* Hildenborough contains at least three different *c*-type cytochromes.

The high molecular weight cytochrome (HmcA) has been sequenced. It contains 16 heme-binding sequences of the form C-X-X-C-H in 4 domains (Pollock *et al* 1991). Three of the domains are complete c_3-like domains, and the fourth is an incomplete c_3-like domain (Bruschi *et al* 1992). Cytochrome c_3 is a tetraheme protein, which readily accepts electrons from periplasmic hydrogenases with each heme exhibiting an individual redox potential of –200 to 400 mV (Bruschi *et al* 1992). The presence of c_3 domains within HmcA indicates that it can possibly accept electrons directly from periplasmic hydrogenases.

The *hmc*A gene is the first in a large operon encoding 6 membrane bound redox proteins and 2 regulatory proteins (Fig 2.7) (Rossi *et al* 1993). HmcA is a periplasmic protein associated with the outer surface of the cytoplasmic membrane (Pollock *et al* 1991). Rossi *et al* (1993) used nucleic acid sequence comparisons between HmcB and other proteins and showed high homology to the iron-sulfur sub-units of anaerobic dimethyl sulfoxide reductase (DmsB) and to nitrate-inducible formate dehydrogenase (FdnH) from *E. coli*. These are both membrane-associated electron-transferring enzymes. Sequence analysis showed that HmcC, HmcD, and HmcE are highly hydrophobic, integral membrane proteins (Rossi *et al* 1993). HmcF was predicted to be a membrane-bound protein due to sequence analysis. As a result of hydrophobicity analysis, HmcF was proposed to be located on the cytoplasmic side of the inner membrane (Keon & Voordouw 1996). The N-

terminus of HmcB was shown to serve as an export signal, thus giving the protein a topology in which its FeS clusters were on the outside surface of the cytoplasmic membrane (Keon & Voordouw 1996). These observations indicate that this operon encodes a multi-subunit, membrane associated, electron-transferring complex as shown in Fig. 2.6.

Regulation of the *hmc* operon appears to be through proteins encoded by the *rrf1* and *rrf2* genes located just downstream of the other *hmc* genes (Fig. 2.7). *rrf2* shows sequence similarity to helix-turn-helix-containing DNA-binding proteins. Deletion of the two genes gives rise to an increase in *hmc* operon expression (Keon *et al* 1997). In wild type *Desulfovibrio vulgaris*, the *hmc* operon is expressed constitutively at low levels, although subject to some upregulation. It shows maximal expression when hydrogen is used as a sole electron donor, intermediate expression when organic acids are used as electron donors in the presence of hydrogen, and the least expression when organic acids are used as sole electron donors in the absence of hydrogen (Keon 1995). A deletion mutant, which does not express the *rrf1* or *rrf2* genes, grew faster with hydrogen as the sole electron donor when compared to the wild type (Keon *et al* 1997). This suggests that expression of the *hmc* operon is downregulated except in the presence of hydrogen.

There is also biochemical evidence showing the link between periplasmic hydrogenases and HmcA. Reduction of HmcA with [Fe] hydrogenase takes place in the presence of cytochrome c_3. This indicates the transfer of electrons from oxidation of hydrogen by hydrogenases to HmcA through cytochrome c_3 (Pereira *et al* 1998) (Fig. 2.6).

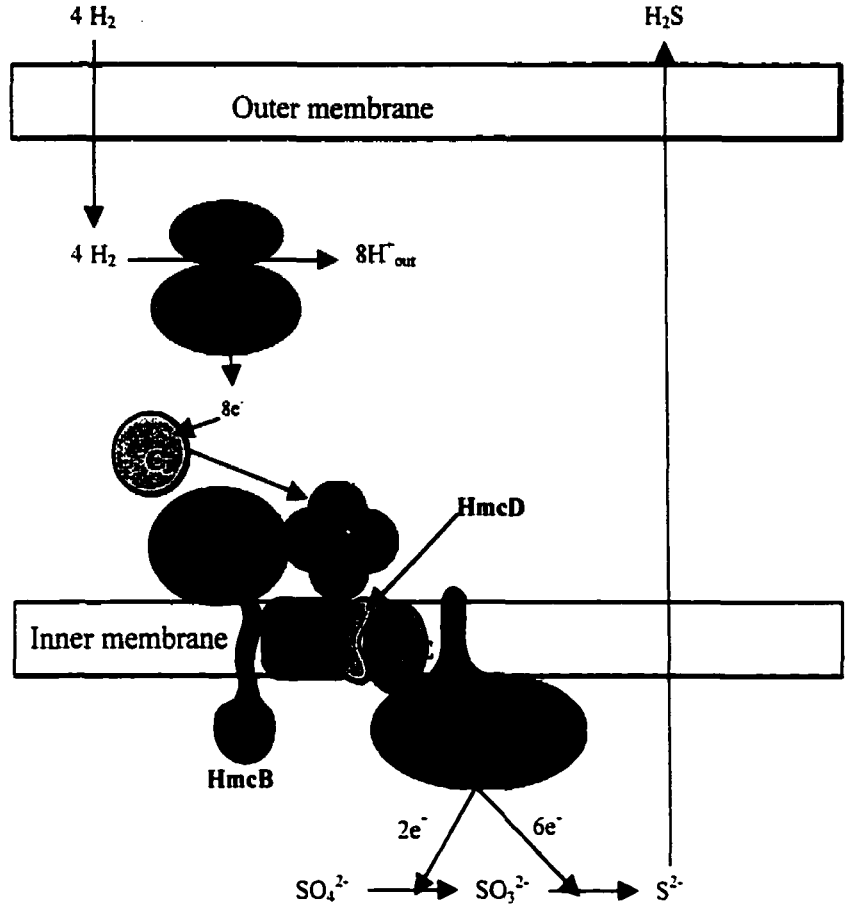

Figure 2.6. Model for the structure and function of the membrane-bound Hmc complex.

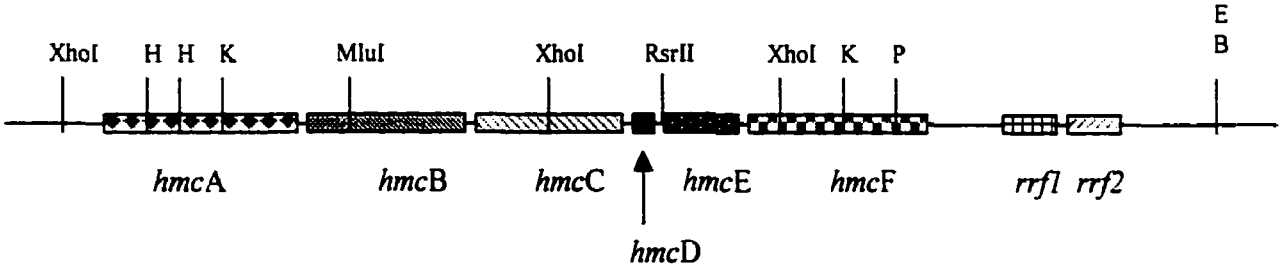

Figure 2.7. The *hmc* operon: genes *hmc*A through *hmc*F, as well as *rrf1* and *rrf2* are indicated. Restriction sites for *Xho*I, *Hin*dIII (H), *Kpn*I (K), *Mlu*I, *Pst*I (P), *Eco*RI (E), *Rsr*II, and *Bam*HI (B) are also indicated.

2.2.2 Construction of an *hmc* operon deletion mutant

Deletion of the Hmc operon (resulting in the mutant H801) has been accomplished with the removal of the 3' end of *hmc*A, all of *hmc*B, C, D, E, and most of *hmc*F (Dolla *et al* 2000). This was done using a methodology described previously (Keon *et al* 1997, Fu & Voordouw 1997). A plasmid containing the chloramphenicol resistance (*cat*) gene, flanked by small segments of the *hmc* operon, was constructed. The plasmid also contained *mob* genes, to allow conjugational transfer of the plasmid and the *sac*B gene, which confers sucrose sensitivity. The plasmid was grown in *E. coli*, which was conjugated to wild type *Dv*H. After conjugation, bacteria were grown on medium containing chloramphenicol to ensure integration of the plasmid into the chromosome since the plasmid is unable to replicate in *Dv*H. The resulting bacteria were then plated on sucrose to force the deletion of the *hmc* operon (Fig. 2.8) (Dolla *et al* 2000). Southern blot analysis confirmed the replacement of the majority of the *hmc* operon with the *cat* gene and the removal of the *sac*B gene (Dolla *et al* 2000). SDS-PAGE immunoblotting showed an absence of HmcA however a smaller form did react indicating the presence of a truncated form of the protein (Dolla *et al* 2000). The availability of an *hmc*-deletion mutant offers unique opportunities to study the role of the Hmc complex in the growth physiology of *Desulfovibrio vulgaris* Hildenborough.

2.3 Iron Hydrogenase

To understand hydrogen metabolism one must first look at the key enzyme involved in the reaction:

$$H_2 \leftrightarrow 2H^+ + 2e^- \qquad (6)$$

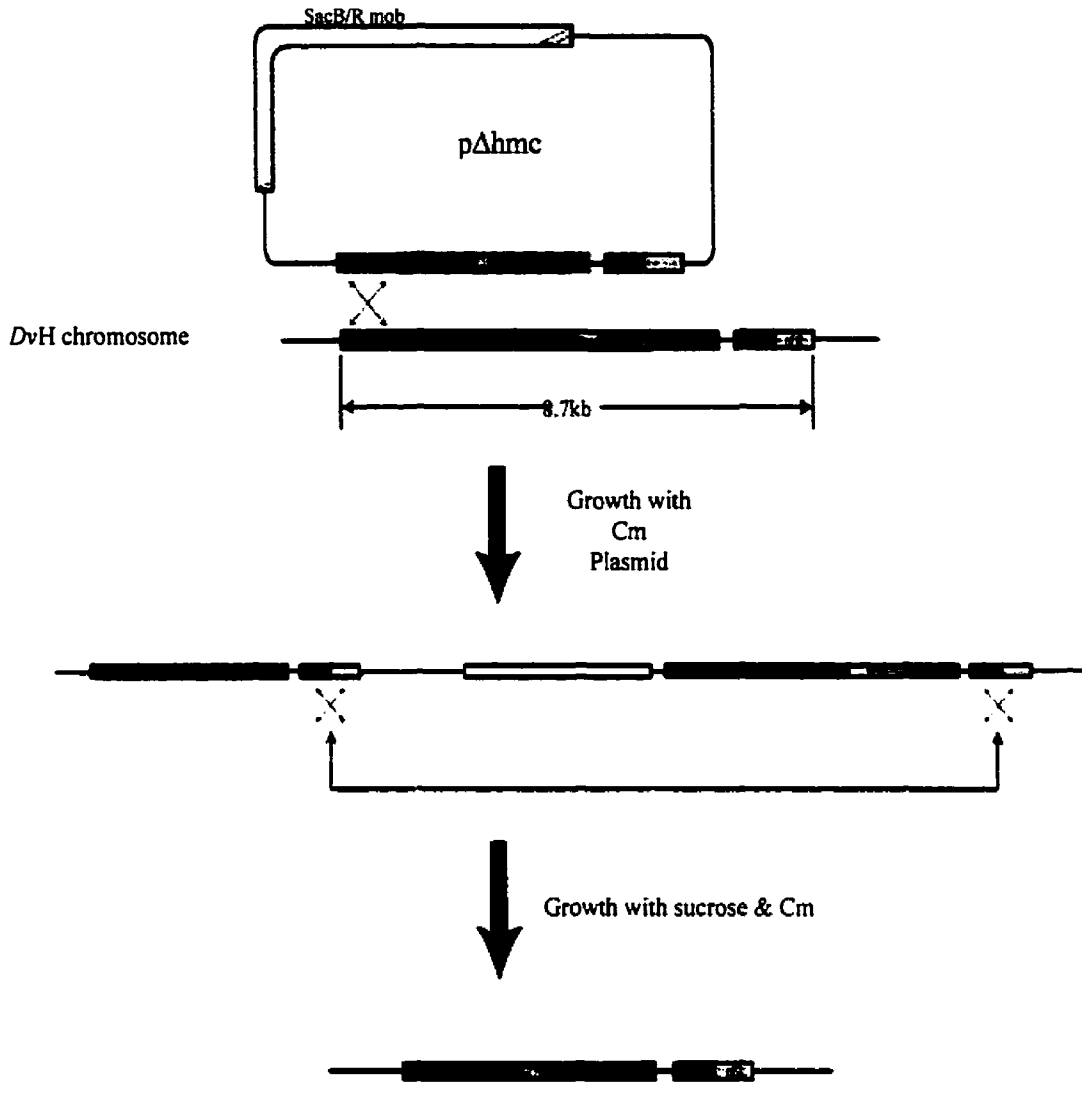

Figure 2.8. Mutagenesis strategy for removing the *hmc* operon from the chromosome of *D. vulgaris* (Dolla *et al* 2000).

The enzyme that catalyzes this reaction is hydrogenase. *Desulfovibrio* ssp. can have three different hydrogenases; [Fe], [NiFe] and [NiFeSe] hydrogenases. The only one that is universal to all *Desulfovibrio* spp. is the [NiFe] hydrogenase (Voordouw 1990). The non-universal distribution of hydrogenase enzymes in the different *Desulfovibrio* spp. makes understanding the specific roles of each difficult. It has been proposed that because the [NiFe] hydrogenase is universal to all *Desulfovibrio* spp., it is the general hydrogenase responsible for the utilization of hydrogen as an electron donor (Voordouw 1995).

2.3.1 Role of Iron Hydrogenase

*Dv*H [Fe] hydrogenase is localized in the periplasm. The enzyme exhibits high catalytic activity. It has higher catalytic H_2 uptake activity than catalytic H_2 evolution activity (Hatchikian *et al* 1992). This enzyme has been proposed to be involved in lactate metabolism, since a decrease in [Fe] hydrogenase due to expression of hydrogenase gene antisense RNA led to a decrease in growth rates and growth yields on lactate containing medium (Van den Berg *et al*, 1991). Elucidation of the role of this hydrogenase remains to be completed. A deletion mutant is required to further determine its function.

2.3.2 Construction of a Deletion Mutant

Deletion of the [Fe] hydrogenase gene (resulting in the mutant Hyd100) has been accomplished as described previously (Keon *et al* 1997, Fu & Voordouw 1997). A plasmid was constructed that contained flanking regions of the hydrogenase *hydA,B* genes on either side of a *cat* gene conferring chloramphenicol resistance. The plasmid also contained *mob* genes for conjugational transfer and the *sacB* gene for sucrose sensitivity. After

conjugation, the bacteria were grown on medium containing chloramphenicol to ensure integration of the plasmid into the chromosome. The resulting bacteria were then plated on sucrose and chloramphenicol to cause the deletion of the desired genes resulting in the mutant strain *D. vulgaris* Hyd100 (Voordouw 2000). Deletion of the *hydA,B* genes was confirmed through Southern blotting and immunoblotting using anti-HydA antibodies. This showed the absence of the *hydA,B* genes and the absence of the Fe-only hydrogenase within the mutant Hyd100. Having a mutant that does not produce the [Fe] hydrogenase will help elucidate the role it plays in the metabolism of *Desulfovibrio vulgaris* Hildenborough.

3. Objectives

Although evidence for a role of the Hmc complex in transporting electrons from periplasmic hydrogenases to cytoplasmic sulfate reduction enzymes has already been obtained, proof and a more complete understanding of this role requires study of a deletion mutant. The role of Fe-only hydrogenase, the most active periplasmic hydrogenase in *Dv*H may also be defined by study of a deletion mutant.

This research aims to describe the roles of the Hmc complex as well as of Fe-hydrogenase in hydrogen metabolism in greater detail. Specific, objectives were as follows:

(1) A detailed physiological characterisation of the deletion mutants H801 and Hyd100 in comparison to the wild type strain. Determination of the efficiency by which organic acids (lactate, pyruvate) or hydrogen are used as electron donors for sulfate reduction.

(2) Development of a method to complement the mutant H801 with the wild type *hmc* operon, with the eventual goal to complement portions of the operon in order to study individual subunit function.

(3) An improved description of the phenomenon of hydrogen production from lactate in the presence of sulfate, i.e. the hydrogen burst at the start of the growth curve or in the absence of sulfate, i.e. the fermentation of lactate to acetate, CO_2 and $2H_2$.

(4) Determination of the effects of mutation of the hydrogen metabolism in H801 on iron corrosion

3.1 Characterization of growth on various electron donors

The Hmc complex has been implicated in hydrogen metabolism. Hence, characterisation of growth of the mutant H801 on various electron donors will help to pinpoint the function of the complex. Growth on various organic acids will be tested. If growth of the deletion mutant H801 is affected when using organic acids as electron donors for sulfate reduction, then the Hmc complex is not only involved in the transfer of electrons for hydrogen metabolism.

A complete characterization of mutant growth on hydrogen as an electron donor will also be undertaken. There are two different ways in which the Hmc complex can contribute to hydrogen metabolism. The complex might be involved proton pumping, leading to the export of more than 8 protons per mol of sulfate reduced. If this were the case the deletion mutant would have a reduced cell yield ($Y_{sulfate}$) per mole of sulfate consumed. Determination of cell yield per mole of hydrogen consumed ($Y_{hydrogen}$) gives similar information. A second way the Hmc complex can contribute to hydrogen metabolism may only influence the rate of transfer of electrons across the periplasmic membrane. This would be reflected in a lower growth rate for the mutant H801 relative to the growth rate of the wild type strain. However, $Y_{sulfate}$ and $Y_{hydrogen}$ remain unchanged.

The deletion mutant Hyd100, which is missing the Fe-hydrogenase, could have deficiencies in growth relative to the wild type strain when using hydrogen as the electron donor. *Dv*H has three hydrogenases and a deletion mutant missing one of them may help to explain the individual roles of this particular redox enzyme. The theory that Fe-only hydrogenase is important for hydrogen production from lactate will be tested by

measuring hydrogen concentration in cultures with lactate both in the absence and in the presence of sulfate.

3.2 Development of a method to complement the H801 mutant phenotype

The *hmc* operon encodes 6 protein subunits of the Hmc complex and 2 regulatory proteins. The role of the regulatory proteins has been investigated using a deletion mutant (Keon *et al* 1997). However, understanding the roles of any of the 6 subunits has been limited to sequence analysis and comparison to other protein sequences. Further analysis of essential function is possible by developing a method to reinsert individual genes and evaluate if this complements the mutant H801 phenotype. A suicide vector containing the entire operon will first be created in order to determine if complementation of the mutant H801 phenotype is possible. This suicide vector will also contain an *oriT* site for its transfer into *D. vulgaris* H801. Complementation of mutant phenotype following plasmid integration will be investigated by monitoring improved kinetics of hydrogen metabolism.

3.3 Characterization of the production of hydrogen from lactate

*Dv*H produces hydrogen from lactate in the presence of sulfate during the growth lag phase (the "hydrogen burst"). The reason for this phenomenon has yet to be fully explained. The size of the hydrogen burst will be compared for mutant and wild type strains by measuring hydrogen concentration during growth on defined lactate-sulfate medium throughout the growth curve. Efficiency of hydrogen production from lactate

will also be evaluated by measuring hydrogen concentrations during fermentative growth on lactate, i.e. in the absence of sulfate.

3.4 Iron corrosion studies

Anaerobic iron corrosion is a significant issue for industry and hydrogen metabolism has been theorized to play a role in this corrosion. If the Hmc complex is involved in hydrogen metabolism and further, if hydrogen metabolism does indeed play a role in iron corrosion, then the deletion mutant H801 (which is deficient in that metabolism) should also demonstrate a deficiency in iron corrosion. This will be determined by comparing the weight loss of iron corrosion coupons in cultures with either wild type or the mutant H801 strains.

4. Materials and Methods

4.1 Materials

4.1.1 Biochemical reagents

DNA manipulation enzymes were obtained from Pharmacia, Roche Diagnostics, Promega and New England Biolabs. Klenow DNA polymerase I, *Taq*I polymerase, λ DNA (0.5 mg/ml) and lysozyme were purchased from Pharmacia. Hybond-N hybridization transfer membrane was purchased from Amersham. Proteinase K was purchased from Roche Diagnostics. The antibiotics chloramphenicol and ampicillin (sodium salt) were obtained from Sigma Chemical Company and kanamycin sulfate was from Roche Diagnostics. Low-(LGT) and high-(HGT) gelling temperature agarose were bought from Gibco BRL and Roche Diagnostics, respectively. Ethidium bromide, used to stain DNA for UV illumination in gel electrophoresis, was from Sigma Chemical Company. [α-^{32}P]-dCTP (10 mCi/ml; 3000 Ci/mmol) was from ICN Biomedicals Inc. RNAseA from bovine pancreas was purchased from Sigma Chemical Company. Deoxyoligonucleotides, used as primers for PCR for sequencing reactions, as well as all linkers were synthesized by the Regional DNA Synthesis Laboratory of the University of Calgary. All compressed gases were purchased from Praxair. All other chemical and biochemical reagents were either obtained from Sigma Chemical Company, Roche Diagnostics, Difco Laboratories, Fisher Scientific, BDH or Aldrich Chemical Corporation Inc.

4.1.2 Bacterial strains

All bacterial strains used are described in Table 4.1. The methodology for the construction of mutant *D. vulgaris* H801 has been described previously (Fu and Voordouw 1997) and is described in detail in Dolla *et al* (2000). The construction of *D. vulgaris* Hyd100 been described in Voordouw (2000).

4.1.3 Plasmids

All plasmids used are described in Table 4.1. W. Pollock generated plasmid pP6A (Pollock *et al* 1991). M. Rossi generated pMR+ (Rossi *et al* 1993). Plasmid pMOB1 was obtained from H. Schweizer (Schweizer 1992).

4.1.4 PCR primers

All PCR primers used are listed in Table 5.1.

4.1.5 Growth media and conditions

E. coli cultures were grown in TY (tryptone-yeast extract) broth or on TY plates which are described in Sambrook *et al* (1989). Plasmid-containing strains of either *E. coli* TG2 or *E. coli* S17-1 were grown in TY with 20 µl of ampicillin (10 mg/ml) in 5 ml broth cultures or 100 µl ampicillin (10 mg/ml) spread on agar plates(~ 25 ml).

Stock cultures of *D. vulgaris* Hildenborough strains were grown in Postgate medium C without the poising agents ascorbic acid and thioglycollic acid (Postgate 1984). Long-term storage of *D. vulgaris* strains was done in 20 ml Postgate medium B (Postgate 1984) at 4°C in sealed Hungate tubes, flushed with mixed gas (10% CO_2, 5%

Table 4.1 Bacterial strains, linkers plasmids used

Strain, linker or plasmid	Genotype, Comments and/ or references
D. vulgaris Hildenborough	NCIMB 8303; isolated from clay soil near Hildenborough, Kent, United Kingdom (Postgate 1984).
D. vulgaris Hildenborough strain H801	Δhmc Cm^r (Dolla et al 2000)
E. coli TG2	Δ(*lac-pro*) *sup*E *thi hsd*M *hsd*R *rec*A F' [*tra*D36 *pro*AB^+ *lacZ*Δ$M1^q$]
E. coli S17-1	*thi pro hsd*R *hsd*M+ *rec*A RP4-2 (Tc::Mu, Km::Tn7) (Simon *et al* 1983)
pMOB1	Km^r; pHSS21 with 1.5kb EcoRI-SalI *ori*T fragment in *Eco*RI-*Xho*I (Schweizer 1992)
pP6A	Contains the *hmc* operon of *D. vulgaris* Hildenborough on a 3.7 kbp *Xho*I insert in pUC8 (Pollock *et al* 1991)
pMR+	Contains the *hmc* operon on an 8.7 kbp *Eco*RI-*Bam*HI fragment in a pUC vector (Rossi *et al* 1993)
pMR-MOB	This study, see Section 5.2.2.2
pRK-MOB	This study, see Section 5.2.2.2
pRsR-Blunt	This study, see Section 5.2.2.2
p179	5'-CAAGGACAGCTACGGGGTGC 2235 to 2254[a] Forward primer to PCR amplify DNA for sequencing and used for the sequencing reaction for pMX-MOB
p180	5'-GGCCTTGACGAAACACGCCG 2486 to 2505[a] Reverse primer to PCR amplify DNA for sequencing and used for the sequencing reaction for pMX-MOB
p194-f	5'-TCGTCGAATGGTGCGCTGCC 3668 to 3687[a] Forward primer to PCR amplify DNA for sequencing and used for the sequencing reaction for pRK-MOB and pRsr-Blunt
p170-r	5'-CAGGCTGAAGCCGAAGCGTT 4908 to 4889[a] Reverse primer to PCR amplify DNA for sequencing reaction for pRK-MOB and pRsr-Blunt
*Bam*HI Linker	5'-CCGGATCCGG (UCDNA)
Linker p-177	5'-CGCGACTCGAGT (UCDNA) Contains *Xho*I site
Linker p-178	5'-GACGGTACC (UCDNA) Contains *Kpn*I site

[a] Position of primer in *hmc* operon sequence (accession number L16784; Rossi *et al* 1993)

H_2 balance N_2), after cells had grown to saturation. Plate counts were done on Postgate medium E with 15 g/l agar (Postgate 1984).

Defined media without yeast extract (Brandis and Thauer 1981) were used for growth studies. Defined media contained 97 ml minimal salt solution, 1 ml $CaCl_2$ solution, 1.25 ml of trace element solution and either 1 ml of sodium lactate, 1.1 ml or sodium pyruvate or 1 ml of sodium acetate solution (pH 7.0-7.2) per 100 ml. Minimal salt solutions contained per litre: 6.6 g $(NH_4)_2SO_4$, 1.8 g NaCl, 0.9 g KH_2PO_4, 0.36 g $MgCl_2 \cdot 6H_2O$; pH 7.0. $CaCl_2$ solution contained per litre: 26 g $CaCl_2 \cdot 2H_2O$. Trace element solution contained per litre: 1.8 g nitrilotriacetic acid, 1 g $FeCl_2 \cdot 4H_2O$, 0.5 g $MnCl_2 \cdot 4H_2O$, 0.3 g $CoCl_2 \cdot 6H_2O$, 0.2 g $ZnCl_2$, 50 mg $Na_2MoO_4 \cdot 2H_2O$, 20 mg H_3BO_3; pH 6.5 with NaOH. Sodium lactate solution contained 3.75 M sodium lactate. Sodium acetate solution contained 3 M sodium acetate. Sodium pyruvate solution contained 3.1 M sodium pyruvate. All solutions were heat sterilized separately except sodium pyruvate, which was filter sterilized. All solutions were mixed aseptically to obtaiun media that will be referred to as lactate-sulfate, pyruvate-sulfate and hydrogen-sulfate medium, respectively, to indicate main electron donor for metabolism. Acetate and CO_2 serve as carbon sources in defined hydrogen-sulfate medium (Badziong *et al* 1979). Defined medium plates had 15 g/l agar added to minimal salt solution. After sterilization and while agar was still fluid, other solutions were added in volumes listed above.

All growth of *D. vulgaris* cultures took place in liquid medium or on 25 ml plates. The appropriate antibiotics were often but not always added (10 µg/ml chloramphenicol or 10 µg/ml chloramphenicol and 50 µg/ml kanamycin for mutant strains or 50 µg/ml kanamycin for the wild type strain). Cultures were incubated at 32°C in a Forma

Scientific Anaerobic Chamber filled with an atmosphere of 5% (v/v) H_2, 10% (v/v) CO_2 with balance N_2. For liquid cultures a 10% (v/v) inoculum was used, unless otherwise stated.

4.2 Methods

4.2.1 Standard protocols

4.2.1.1 Chromosomal DNA isolation

Chromosomal DNA of *D. vulgaris* strains was isolated using a miniprep protocol adapted from Marmur (1961). 5 ml cultures were harvested through centrifugation, washed with 0.5 ml of NaCl-EDTA (0.15 M NaCl, 0.1 M EDTA, pH 8.0) solution, and resuspended in 300 µl of the same NaCl-EDTA solution in an 1.5 ml microcentrifuge tube. 20 µl of lysozyme (5 mg/ml) was added and the tubes were incubated for 10 min in a 37°C water bath. To each cell suspension 32 µl of 25% SDS solution was added and the tubes were then incubated in a 60°C water bath to cause cell lysis. 60 µl of 5 M $NaClO_4$ and 400 µl of chloroform/isoamylalcohol (24:1) were added to the suspensions, tubes were capped tightly and mixed gently on a rotary wheel for 1 hour. After mixing, suspensions were centrifuged at 4°C for 2 min. The supernatant was transferred to new microcentrifuge tubes. 2.5x the volume of 95% ethanol at -20°C was added and tubes were mixed by inversion. DNA was collected by centrifugation by spinning at 4°C for 15 min. DNA was washed with 500 µl of cold (-20°C) 70% ethanol and dissolved in 300 µl of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA; pH 8.0). 20 µl of RNAseA (10 mg/ml) was added and the tubes were left for >15 min at room temperature. 10 µl of proteinase K (10mg/ml) was added and the tubes were left at room temperature for >1 hour. DNA

was precipitated with 600 µl of DNA precipitation mixture (90% (w/w) ethanol, 10% (w/w) 2.5 M NaOAc; pH 5.5) and centrifuged for 15 min. at 4°C. The DNA pellet was washed with 70% ethanol. After drying at room temperature DNA was dissolved in 150 µl of TE buffer and stored at 4°C.

4.2.1.2 Plasmid DNA isolation

Plasmids were isolated from *E. coli* and *D. vulgaris* using a modified 10 min mini-prep described by Zhou *et al* (1990) or using the Triton X-100 lysis method described by Voordouw *et al* (1985).

4.2.1.3 DNA electrophoresis

Electrophoresis was done using a BRL Model H5 gel apparatus. Restriction endonuclease digested chromosomal or plasmid DNA was electrophoresed in 0.7% (w/v) HGT agarose for routine use and 0.7% (w/v) LGT agarose for DNA fragment isolation. Agarose was dissolved in TAE (40 mM Tris-acetate, 2.0 mM EDTA, pH 8.0) buffer. Electrophoresis was in ~800 ml of TAE buffer.

4.2.1.4 DNA enzymatic manipulations

Restriction endonuclease digestions, ligations using T_4 DNA ligase, 5'-end dephosphorylation with calf intestine alkaline phosphatase, blunt end-repair reactions using DNA polymerase I Klenow fragment and incorporation of [α-^{32}P]-dCTP using random hexamer primers and Klenow polymerase were performed as described by Sambrook *et al* (1989).

4.2.1.5 PCR

Typical PCR reaction conditions included 5 µl template DNA (~200 ng), 4 µl dNTPs 10 mM each, 2.5 µl of each primer (10 pmol/µl), 0.5 µl of *Taq*I polymerase (5 U/µl), 2 µl DMSO, 5 µl of 10x *Taq* buffer (500 mM KCl, 10 mM $MgCl_2$, 100 mM Tris-HCl; pH 9.0) and 28.5 µl ddH_2O. A Perkin-Elmer Gene Amp 2400 PCR system was programmed for 25 cycles of 30 seconds at 90°C, 30 seconds at 55-65°C depending on melting temperature of primer DNA (annealing temperature for 20 mers = [4(G+C content) + 2(A+T)] -5°C) and 30 seconds to 5 minutes depending on size of amplified fragment (1 min per Kbp) (PCR Techniques, Life Technologies Training Handbook)

4.2.1.6 Southern blotting

Restriction endonuclease digested DNA was electrophoresed through 0.7% (w/v) HGT agarose gels as described in Section 5.2.1.3. Following electrophoresis, the DNA contained within the agarose was denatured using 0.5 M NaOH, 1.5 M NaCl and then neutralized with 1 M Tris-Cl, 1.5 M NaCl; (pH 8.0) and transferred to Hybond-N membrane overnight using Southern blot methodology described by Sambrook *et al* (1989). The Hybond-N blots were washed in 1 x SSC (15 mM sodium citrate, 150 mM NaCl; pH 7.2), air-dried and UV-irradiated for 3 minutes on a Fisher Biotech 321 nm transilluminator (8000 mW/cm^2) to cross-link the DNA to the Hybond-N. The membrane was pre-hybridized by incubation in a solution of 6 x SSC, 0.5% (w/v) SDS, 5 x Denhardt solution (1 x Denhardt solution is 1% (w/v) Ficoll 400, 1% (w/v) polyvinyl pyrrolidone, 1% (w/v) BSA) and 3 mg salmon sperm DNA at 68°C for 3 hours. For probe preparation, a 3.7 kbp *Xho*I fragment from plasmid pP6A was isolated by agarose

gel electrophoresis and labled by random hexamer priming and incorporation of deoxynucleotide triphosphates (dNTPs) and [α-^{32}P]-dCTP. After the 3 hour incubation the labeled probe was boiled and added to the pre-hybridization solution. This was further incubated overnight at 68°C. Following hybridization, the membrane was washed at room temperature for 15 minutes in 1 x SSC. A second wash was done for 1 hour at 68°C in 1 x SSC, 0.5% (w/v) SDS. The membrane was dried at room temperature and exposed to BAS-III Imaging Plates for 2 to 4 hours. These were then scanned with a Fuji BAS 1000 Bio-Imaging analyzer. MacBAS 2.2 software was used to visualize the Southern blot.

4.2.1.7 Transformation and conjugation

Transformation of *E. coli* strains was done using the $CaCl_2$ method to introduce plasmid DNA as described by Sambrook *et al* (1989). Plasmid DNA was conjugated into *D. vulgaris* using *E. coli* strain S17-1 containing the desired plasmids and was described previously (Rossi *et al* 1993). The mating mixtures were spotted on nitrocellulose disks on Postgate medium E plates supplemented with 1.5 g/l of KNO_3 to allow growth of *E. coli* in the anaerobic chamber (Powell *et al* 1989) overnight. Mixtures were then suspended by placing the disks in 1 ml defined hydrogen-sulfate medium and vortexing. Aliquots (10-100 µl) of this suspension were plated on Postgate medium E containing kanamycin (125 µl of 10 µg/ml) and ampicillin (100 µl of 10 µg/ml). Aliquots (500 µl) of this suspension were also placed into defined hydrogen-sulfate medium to cause enrichment of integrants. After multiple generations, these enrichments were plated on hydrogen-sulfate agar plates.

4.2.1.8 Sulfide Determination

Sulfide concentrations were done according to Cord-Ruwisch (1985) by the addition of 950 µl $CuSO_4$ solution (5 mM $CuSO_4$, 50 mM HCl) to 50 µl of sample to a total volume of 1 ml. This was analyzed using a Shimadzu UV–265 recording spectrophotometer at 480 nm. Concentrations were determined relative to standard solutions diluted from 1 M Na_2S. Prior to experiments 100 µl of this standard solution was diluted with 9.9 ml of de-ionized water to a concentration of 10 mM which was used for a standard curve.

4.2.1.9 Sulfate Determination

Sulfate concentrations were determined through precipitation with barium chloride (APAH 1992) and measurement with a Shimadzu UV–265 recording spectrophotometer at 420 nm.

4.2.1.10 Protein determination

For protein analysis, 5 M NaOH was added to samples to a final concentration of 0.5 M. These samples were then heated in a 60°C water bath for 30 minutes to lyse cells. Protein content was then measured using a DC protein assay kit (Bio-Rad 500-0116) by adding 250 µl reagent A, vortexing, adding 2 ml reagent B, vortexing and determining the absorbance at 750 nm using a Shimadzu UV–265 recording spectrophotometer.

4.2.1.11 Lactate Determination

D/L-lactic acid determination was done using a D-lactic acid/L-lactic acid Enzymatic BioAnalysis Food Analyser Kit from Boehringer Mannheim (Roche Diagnostics) according to the instructions of the manufacturer. After 30 minutes incubation at room temperature to allow enzymatic reaction to take place the absorbance at 340 nm were read in a Shimadzu UV–265 recording spectrophotometer.

4.2.2 Other experimental procedures

4.2.2.1 Determination of growth rates and growth yields

Growth rates and final cell yields of *D. vulgaris* Hildenborough wild type strain and mutant strains H801 and Hyd100 were determined generally in liquid defined medium with hydrogen or lactate (and hydrogen), or pyruvate (and hydrogen) as electron donors for sulfate reduction. Cell densities were determined using a Klett-Summerson Photoelectric Colorimeter. Vessels used for growth were either 5 ml in 1.2 x 10-cm tubes fitted with screw caps that were kept loose to allow gas exchange or 50 or 100 ml volumes grown in 300 ml nephelometer culture flasks (Bellco Glass, Vineland, NJ). The latter were equipped with a 1.2 x 13 cm sidearm and closed with a sponge-cap to allow gas exchange. Care was taken to ensure equal cell densities for the inocula for growth studies. The tubes and side arm fitted in the sample holder of the Klett meter.

Variation of growth at varying inoculum size were done in 5 ml Postgate medium C in 1.2 x 10-cm tubes fitted with screw caps that were kept loose to allow gas exchange. No antibiotics were used in quantitative growth studies to prevent reduction of the growth rate and cell yield by the presence of the antibiotic.

Plate counts were done using either Postgate medium E or defined hydrogen-sulfate medium with the addition of 15 g/l agar. Cell cultures were diluted 10^4 to 10^6 fold

with defined hydrogen-sulfate liquid medium before 100 µl of each dilution was spread on the agar plates.

4.2.2.2 Plasmid construction

4.2.2.2.1 Construction of pMR-MOB

To create a conjugational plasmid that contains the *hmc* operon, *ori*T and *mob* genes, the plasmids pMR+ and pMOB1 were used. pMR+ contains the *hmc* operon with its putative promoter and regulatory genes *rrf*1 and *rrf*2 (Rossi *et al* 1993). pMOB1 contains the *ori*T and *mob* genes (Schweizer 1992).

The *ori*T and *mob*BR genes within pMOB1 are flanked by *Eco*RI and *Bam*HI sites. The *Eco*RI site of pMOB1 was altered into a *Bam*HI site using *Bam*HI linkers. The plasmid was cut with *Eco*RI, blunt-end repaired using Klenow DNA polymerase and dNTPs. It was then mixed with *Bam*HI linker (UCDNA) and religated using T_4 DNA ligase using the methodology described by Sambrook *et al* (1989). It was confirmed that the *Eco*RI had been changed to a *Bam*HI site in the new plasmid pMOB1-B using restriction digestion and gel electrophoresis. Plasmid pMR+ was then digested with *Bam*HI. The phosphates from the 5' ends were removed by CAP treatment (Sambrook *et al* 1989). The *Bam*HI digested pMOB1-B was added to this along with ligase to ligate the *Bam*HI flanked fragment containing the *ori*T and *mob* genes into plasmid pMR+ to create pMR-MOB.

4.2.2.2.2 Construction of pMX-MOB

Plasmid pMX-MOB was created to allow identification of possible double crossover integrants from that of the wild type chromosome. pMR-MOB was digested with with *Mlu*I (a single site within the *hmc*B gene) and ligated with linker p-177, which contains an *Xho*I site. The presence of the linker in pMX-MOB was verified with restriction digestion and with dideoxysequencing using primers p179 and p180.

4.2.2.2.3 Construction of pRK-MOB

Plasmid pRK-MOB was also created in order to be able to discriminate possible double crossover integrants from the wild type chromosome. This was done by digesting pMR-MOB with *Rsr*II (a single site within the non-coding region between *hmc*D and *hmc*E genes) and ligation of linker number p-178, which contains a *Kpn*I site. The presence of this linker in pRK-MOB was verified with restriction digestion and with dideoxysequencing using primers p194-f and p170-r.

4.2.2.2.4 Construction of pRsr-Blunt

Plasmid pRsr-Blunt was also created to discriminate possible double crossover integrants from the wild type chromosome. pMR-MOB was digested with *Rsr*II (a single site within the non-coding region between *hmc*D and *hmc*E genes). This was problematic because *Rsr*II is a poor cutting restriction endonuclease and required at least 16 hours to digest plasmid DNA to completion. Restriction digestion was aided by addition of fresh *Rsr*II enzyme to the reaction mixture after 16 hours for a further 2-hour digestion. After complete digestion was determined through gel electrophoresis, blunt-end repair was performed using Klenow polymerase and religation using T_4 ligase (Sambrook *et al*

1989). The removal of the *Rsr*II site was verified with restriction digestion and with dideoxysequencing using primers p194-f and p170-r.

4.2.2.3 Warburg assay of hydrogen uptake

Hydrogen uptake by whole cells was measured manometrically using a Warburg apparatus. The wild type and H801 mutant strains were grown in 50 ml defined hydrogen-sulfate medium to mid-exponential phase. Samples of 20 ml were taken, centrifuged and re-suspended to approximately the same cell density in hydrogen-sulfate medium without acetate or other carbon sources. 2 ml of these samples were placed in Warburg flasks and flushed with 100% (v/v) hydrogen for 15 minutes before the hydrogen uptake was measured as a function of time. Only experiments in which a constant uptake rate was observed for 20 minutes were used to calculate hydrogen uptake rates.

4.2.2.4 Iron corrosion assay

Cultures (75 ml) in defined lactate-sulfate or hydrogen-sulfate medium were grown in 138 ml serum bottles (Kimble VWR) sealed and flushed with mixed gas (5% (v/v) H_2, 10% (v/v) CO_2, with balance N_2). Within each culture vessel two small (1x2 cm, less than 2 g) soft rolled steel coupons held in plastic brackets to prevent contact between the coupons were placed. Before placement in the serum bottles each coupon was cleaned by abrasion using soft 400 grit sand paper and washed with 1 M HCl and neutralized in a saturated sodium bicarbonate solution. Finally, coupons were washed with acetone and dried before weight measurement. After the coupons were added culture

vessels were sealed, flushed and then sterilized. Cultures were inoculated with the wild type strain and the mutant strain H801 and incubated for 1 month at 30°C. Coupons were again washed as described above and weighed. Controls were done under the same conditions as above, but without bacterial inoculation.

4.2.2.5 Gas chromatographic determination of hydrogen concentration

Hydrogen concentration determinations were done for both hydrogen consumption and hydrogen production experiments. Hydrogen concentration analysis was performed using a Hewlett-Packard HP5190 gas chromatograph (GC) equipped with a thermal conductivity detector. The column used was a stainless steel packed column (6m x 0.5 mm ID) packed with Poropac R 80/100 mesh (Supelco). For hydrogen consumption analysis the flow rate of the carrier gas (helium) was 15.6 ml/min. For hydrogen production analysis the flow rate of the carrier gas (nitrogen) was 15.6 ml/min. For both types of analysis the injector temperature was 37°C, detector temperature was 80°C and oven temperature was 35°C isothermal. Peaks were identified by injecting pure hydrogen gas, mixed gas (5% (v/v) H_2, 10% (v/v) CO_2, with balance N_2) and pure nitrogen.

Hydrogen consumption assays were done using 50 ml cultures of defined hydrogen-sulfate medium in 138 ml serum bottles flushed with pure nitrogen. 10 ml of headspace gas was removed and replaced with 10 ml of pure hydrogen gas. Samples of wild type and mutant H801 strains were then used to inoculate the serum bottles and

hydrogen consumption was followed over time by withdrawing 100 µl headspace samples and injecting into the GC.

Hydrogen production assays were done with 130 ml cultures in 158 ml serum bottles. Serum bottles were flushed with 10% CO_2 (v/v) with balance N_2. A 2% (v/v) inoculum of the wild type strain or the mutant strains H801 or Hyd100 were then injected. Hydrogen production was followed over time by withdrawing 200 µl headspace samples and injecting into the GC.

5 Results

5.1 Physiological characterization of *D. vulgaris* H801

Confirmation of the role of the Hmc complex as a transmembrane redox electron carrier will necessarily begin by describing the growth of the mutant *D. vulgaris* H801 with various electron donors. Growth is the simplest measure of metabolism. According to previous research, growth on hydrogen as an electron donor should be affected most by deletion of the entire operon (Rossi *et al* 1993 and Keon & Voordouw 1997). If the *hmc* operon encodes the sole complex involved in transporting electrons across the membrane, growth on hydrogen should be completely impaired. Comparison of the growth of wild type and the mutant in a defined medium in which hydrogen is the sole electron donor for sulfate reduction requires some preparation. *Desulfovibrio* spp. form an endogenous polysaccharide carbon reserve that can be used as the energy source if an external one is not provided (Fareleira *et al* 1997). This requires that all growth data obtained using hydrogen as an electron donor be done with cultures transferred multiple times into defined medium to ensure that only hydrogen metabolism is being compared. Growth on lactate or pyruvate should be unaffected, if metabolism of these organic acids is independent of electron transport through the Hmc complex.

5.1.1 Growth kinetics on various electron donors

Because growth is the simplest measure of metabolism, the study of growth kinetics is the most straightforward means to determine the physiological characteristics of the mutant H801. Growth on various electron donors was quantified using a Klett meter as a function of time. Equal inocula of the deletion mutant H801 and the wild type

strain were injected in defined medium. A Klett meter inside the anaerobic chamber was used to monitor growth, minimizing disruption to the cultures. Comparison of multiple Klett readings to optical density at 600 nm allowed the conversion of Klett readings into the more standard OD_{600} with the conversion further being 155±7 Klett units/ OD_{600} with a linear relationship between 10 to 80 Klett units. Because all the cultures were done in an atmosphere containing 5% (v/v) H_2, hydrogen was always available as an electron donor.

The growth rate with either lactate or pyruvate as the electron donor was similar for the mutant H801 and the wild type strain (Fig. 5.1). Final cell densities were particularly identical when pyruvate was used (Table 5.1). Although there was a lower final cell density for the H801 mutant when lactate was used as the electron donor the difference was unremarkable since the variance between the values for the mutant and wild type were within the standard deviation of the measurement (Table 5.1). The concentrations of pyruvate and lactate were 37.4 mM and 37.5 mM in these experiments, respectively.

There was a marked difference in growth on hydrogen as an electron donor between the mutant H801 and wild type strains. The final cell densities were between 20-60% lower for the mutant (Table 5.1). Large differences were observed with hydrogen as electron donor in 5 ml tubes (Table 5.1) Some of these cultures had differences in duration of the lag phase, the time before growth started. This may have been caused by the presence of residual oxygen, which could be removed only by passive diffusion from the tubes. More consistent results were obtained for growth in flasks. Due to higher surface to volume ratio these cultures achieved a more consistent anaerobicity. Details of

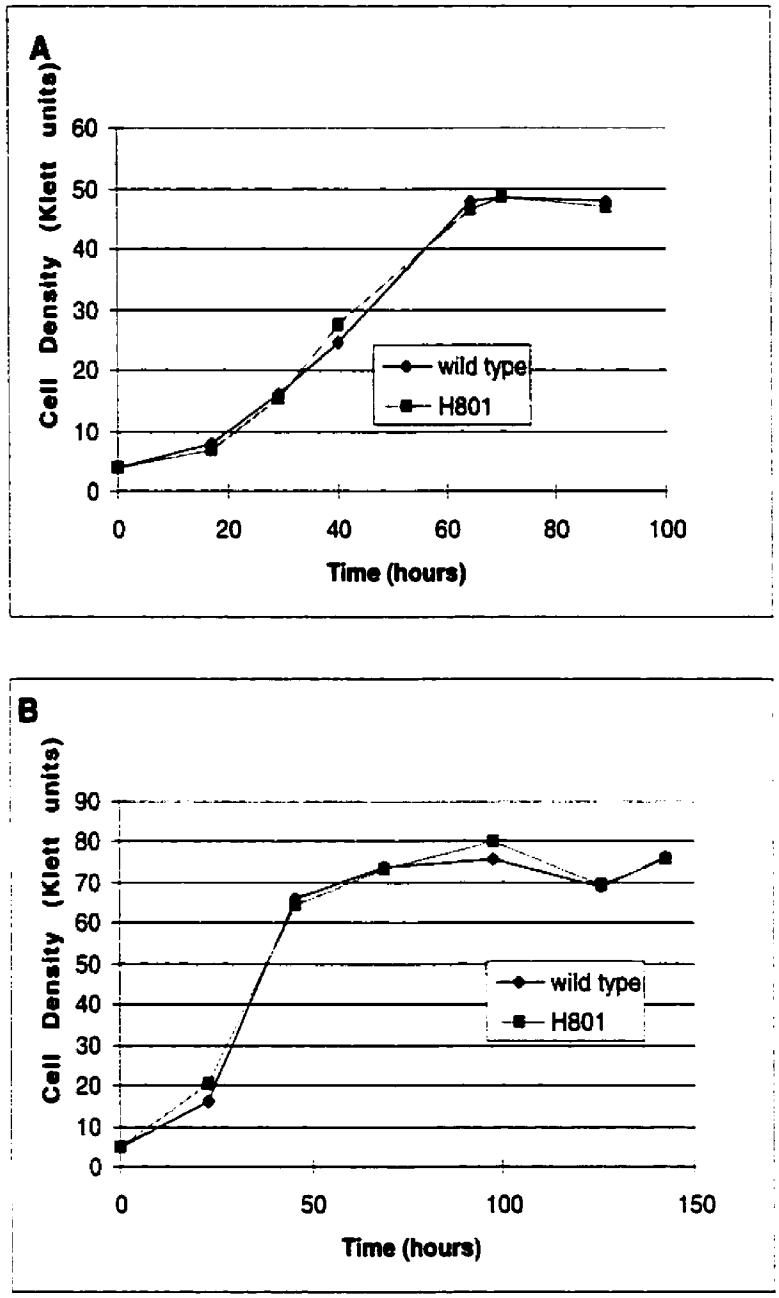

Figure 5.1: Growth on defined medium with pyruvate (A) or lactate (B) as the electron donor for sulfate reduction. Hydrogen was also available.

Table 5.1 Cell densities (OD_{600}) of liquid cultures of *D. vulgaris* H801 and wild type in defined medium after 100-200 hrs of growth.

Culture Vessel[a]	Volume (ml)[b]	Electron donor[c]	n[d]	Strain	OD_{600}[e]	Time (Hrs)
Tube	5	Lactate	5	Wild type	0.57±0.03	100
Tube	5	Lactate	5	H801	0.53±0.01	
Tube	5	Pyruvate	3	Wild type	0.35±0.03	100
Tube	5	Pyruvate	3	H801	0.34±0.02	
Tube	5	Hydrogen	3	Wild type	0.25±0.01	200
Tube	5	Hydrogen	3	H801	0.12±0.01	
Flask	50	Hydrogen	3	Wild type	0.48±0.03[f]	120
Flask	50	Hydrogen	3	H801	0.39±0.02[f]	
Flask	100	Hydrogen	4	Wild type	0.37±0.03	150
Flask	100	Hydrogen	4	H801	0.16±0.01	

[a]Tube: 1.2×10cm, 10ml, kept upright with loose screw cap; flask: 300ml nephelometer (Erlenmeyer) flask
[b]Volume of defined medium in a tube or flask
[c]Hydrogen was also a possible electron donor when lactate or pyruvate were provided; acetate and CO_2 served as carbon sources when hydrogen was the exclusive electron donor for sulfate reduction
[d]Number of cultures incubated
[e]Average value for all cultures
[f]Growth over time shown in Figure 6.2

the experiment are shown in Fig. 5.2. Initial inoculation was from cultures growing in Postgate C medium (PC). Subsequent inoculations were made with samples from the previous growth curve taken at the indicated time (↓). Four experiments were done. The data from experiment 1 (DvH-1, H801-1) were not used to determine growth rates or final cell densities because growth was thought to be influenced by polysaccharide storage molecules. From the data for experiments 2 to 4 specific growth rates were determined for the mutant ($\mu = 0.38 \pm 0.02\ h^{-1}$) and the wild type strain ($\mu = 0.50 \pm 0.05\ h^{-1}$). From all liquid culture experiments it is clear that the mutant H801 grows more poorly than the wild type. However, H801 did grow using hydrogen as the sole electron donor.

The growth of H801 and the wild type strain was also markedly different when monitored on agar plates. On defined hydrogen-sulfate plates, colonies of the wild type strain formed in 10-15 days, while the mutant did not form colonies in this period (Fig. 5.3 C & D). The mutant required 4 weeks to form (not shown). Colony morphology was the same, the only difference was the delay of growth.

The mutant and wild type strains had the same colony morphology when grown on rich medium lactate-sulfate plates (Postgate E plates; Fig. 5.3 A & B). The formation of colonies by the mutant H801 occurred one day after the wild type strain. This delay of growth with lactate as the electron donor demonstrates a phenotypic difference between the mutant and wild type strains. The main difference between growth in liquid cultures and on plates is in the size of the inoculum. In liquid cultures a standard 10% (v/v) inoculum was used, whereas on plates colonies grew from single cells. The delayed

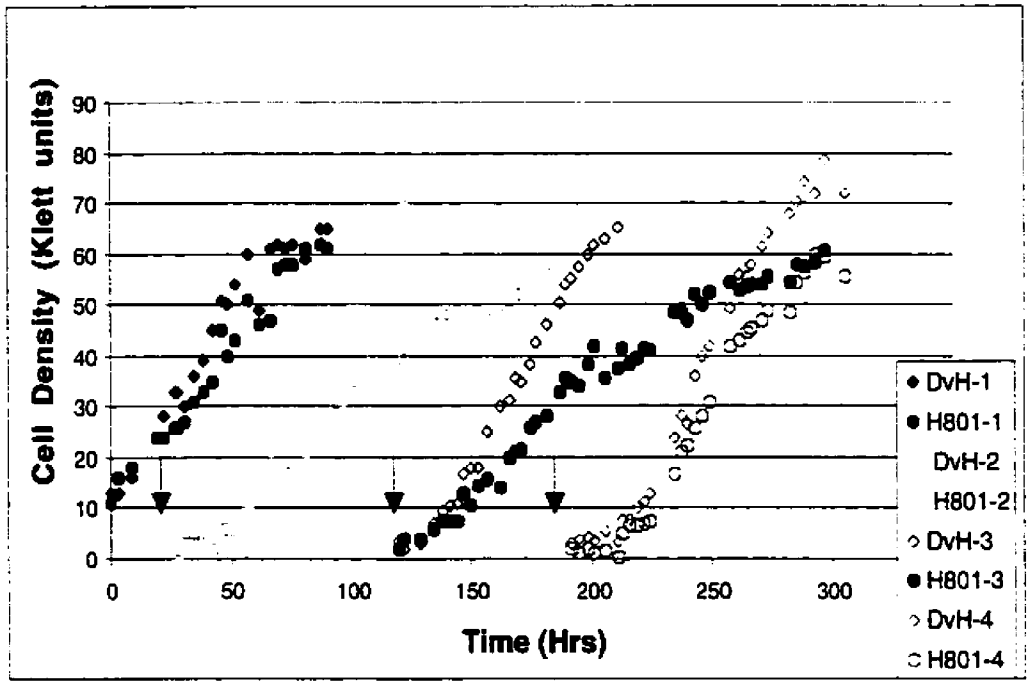

Figure 5.2 Growth in defined hydrogen-sulfate medium with hydrogen as the electron donor and acetate and CO_2 as carbon sources.

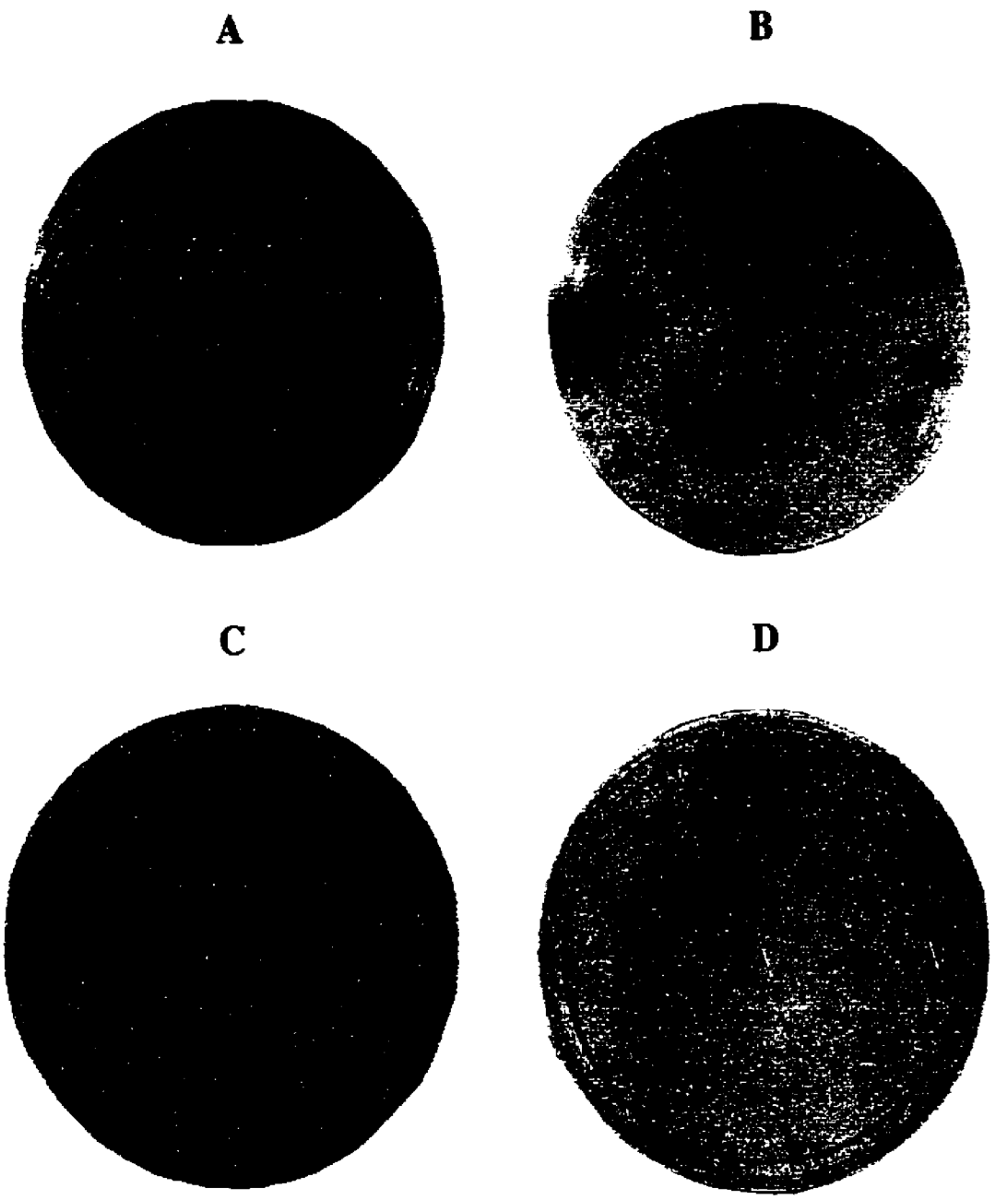

Figure 5.3 Growth of *D.vulgaris* H801 colonies (A, C) and wild type colonies (B, D) on solid medium with lactate (Postgate E) (A, B) or hydrogen (defined medium) (C, D) as the electron donor for sulfate reduction. Plates were incubated at 32°C in a mixed gas atmosphere (5% H_2, 10% CO_2 balance nitrogen). Plates A and B were incubated for 4 days. Plates C and D were incubated for 10 days. Inoculation was from exponentially growing cells at a 10^{-6} dilution.

growth phenotype was, therefore, investigated by comparing growth in liquid lactate-sulfate medium using various sizes of inoculum (Fig 5.4). As inoculation size decreased an increase in the lag phase was observed for both the mutant H801 and the wild type strain. However, for the mutant H801 strain the duration of the lag phase increased more than for the wild type strain.

5.1.2 Comparison of lactate/sulfate ratios

When lactate is used to reduce sulfate, 0.5 mol of sulfate is reduced per mol of lactate oxidized (Voordouw 1995). However, some lactic acid is also used for cell synthesis. This would increase the lactic acid to sulfate ratio. Also experiments were carried out in the presence of hydrogen. If the cells used hydrogen as an alternative electron donor for sulfate reduction this would decrease the lactate to sulfate ratio. The growth studies (Table 5.1, Fig 5.1) have already indicated that the Hmc complex is not critically important for growth with lactate as the electron donor. Based on these results we would expect a similar ratio of lactate oxidized to sulfate reduced.

Lactate and sulfate consumption was measured. The lactate/sulfate ratios were 1.353 mol of lactate consumed per mol sulfate reduced for the wild type strain and 1.421 mol of lactate consumed per mol sulfate reduced for the H801 strain. Ignoring use of lactate for cell biosynthesis (usually less than 10% in aerobic growth) we conclude that for the wild type, 67% of sulfate reduction is though use of lactate as electron donor (and 33% through hydrogen) whereas for the H801 mutant, 71% of sulfate reduction is through the use of lactate and 29% through the use of hydrogen. These differences are not

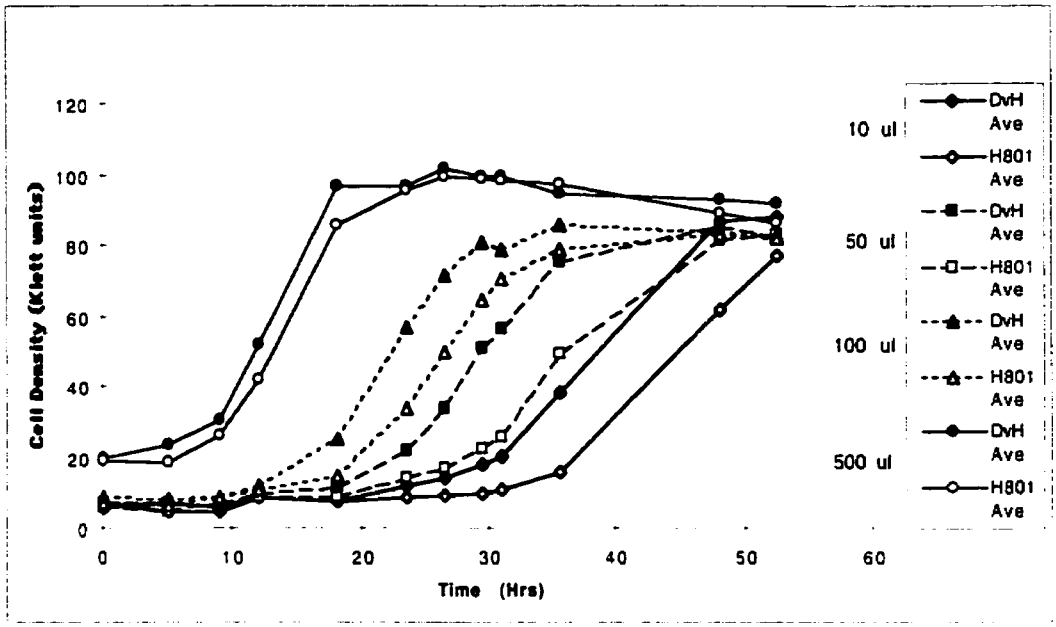

Figure 5.4 Comparison of the effect of different inoculum sizes on the lag time of growth of *D. vulgaris* H801 and the wild type strain in liquid cultures. Cultures were grown in 5 ml rich lactate-sulfate medium (Postgate C) in 10 ml Klett tubes with the screw cap left loose for gas exchange. Cultures were incubated at 32°C in a mixed gas atmosphere (5% H_2 10% CO_2, balance N_2).

large. It is also possible that the measured lactate consumption was low due to the slowness and inefficiency of the enzymatic reaction used to determine lactate content. However, results were consistent between trials and strains. HPLC measurements could be used to produce more accurate lactate measurements. Thus the lactate/sulfate ratios for the wild type strain and H801 were not very different, confirming that the Hmc complex plays only a minor role in lactate metabolism under conditions of excess sulfate.

5.1.3 Metabolism of hydrogen - energy conservation

Although metabolism of hydrogen has been studied in the past, and much is known about how *Desulfovibrio* ssp. utilize hydrogen, fundamental questions about this process still need to be answered. Since the Hmc complex has been proposed as the transmembrane redox complex involved in electron transfer between periplasmic hydrogenases and cytoplasmic sulfate reduction enzymes, it is important to study the deletion mutant H801 in order to further understand this metabolism.

5.1.3.1 Cell yield per mol of sulfate ($Y_{sulfate}$) when hydrogen is the electron donor

$Y_{sulfate}$ provides an indication of the efficiency with which the free energy of hydrogen oxidization for sulfate reduction is used to build cell mass. If the Hmc complex is missing, and there is a lower $Y_{sulfate}$, then it suggests that this efficiency has been lowered, and that the Hmc complex is possibly involved in proton pumping leading to an altered cell mass production per mol of sulfate reduced.

During growth in liquid cultures of the mutant H801 and the wild type strain on hydrogen as the electron donor for sulfate reduction (Fig. 5.2), the wild type strain gained more cell mass than the strain H801 per unit time. Analysis of sulfate concentrations conducted after these growth experiments indicated that sulfate was not limiting. One cannot conclude therefore, that the wild type strain was capable of greater cell mass production than the mutant H801 per mol of sulfate reduced.

The relationship between cell mass formation and sulfate utilization was investigated in several experiments in which the cell density (OD_{600} or Klett) or protein concentration was monitored as a function of sulfate concentration. The results of all experiments are given in Table 5.2. An experiment in which the protein concentration was determined as a measure of sulfate reduction is shown in Fig. 5.5. The amount of protein produced per mol of sulfate consumed was the same for both the wild type strain and the mutant H801 (Fig. 5.5). The wild type cultures produced 0.0091±0.0021 mg/ml protein per mM of sulfate reduced, and the mutant produced 0.0099±0.0001 mg/ml protein per mM sulfate reduced.

5.1.3.2 Hydrogen uptake rates

Since $Y_{sulfate}$ was similar for both the mutant H801 and the wild type strain, further investigation was required to understand why growth kinetics for the two strains was different (fig. 5.2). Hydrogen uptake rates were determined to elucidate the efficiency of the Hmc complex in transferring electrons from periplasmic hydrogenases to cytoplasmic sulfate reduction. Direct manometric hydrogen uptake assays, using a Warburg apparatus, were performed on cells that had been grown to mid-exponential

Table 5.2 Relationship between cell density (protein content and cell density, OD_{600} and Klett units) as a function of sulfate concentration. 10 data points during exponential growth were related for each experiment.

	Experiment number	Strain	$Y_{sulfate}$ value	r^2 value	Averages
$Y_{sulfate}$ (Δmg/ml protein per mM sulfate reduced)	1	Wild type	0.0076	0.916	Wild type average 0.0091±0.0021
		H801	0.0098	0.871	
	2	Wild type	0.0106	0.871	H801 average 0.0099±0.0001
		H801	0.0100	0.753	
$Y_{sulfate}$ (Δcell density in Klett units per mM sulfate reduced)	1	Wild type	3.309	0.926	Wild type average 3.692±0.542
		H801	3.085	0.791	
	2	Wild type	4.076	0.966	H801 average 2.991±0.133
		H801	2.897	0.820	
$Y_{sulfate}$ (Δcell density in OD_{600} per mM sulfate reduced)	1	Wild type	0.0282	0.965	Wild type average 0.0308±0.0037
		H801	0.0359	0.905	
	2	Wild type	0.0334	0.984	H801 average 0.0346±0.0018
		H801	0.0333	0.937	

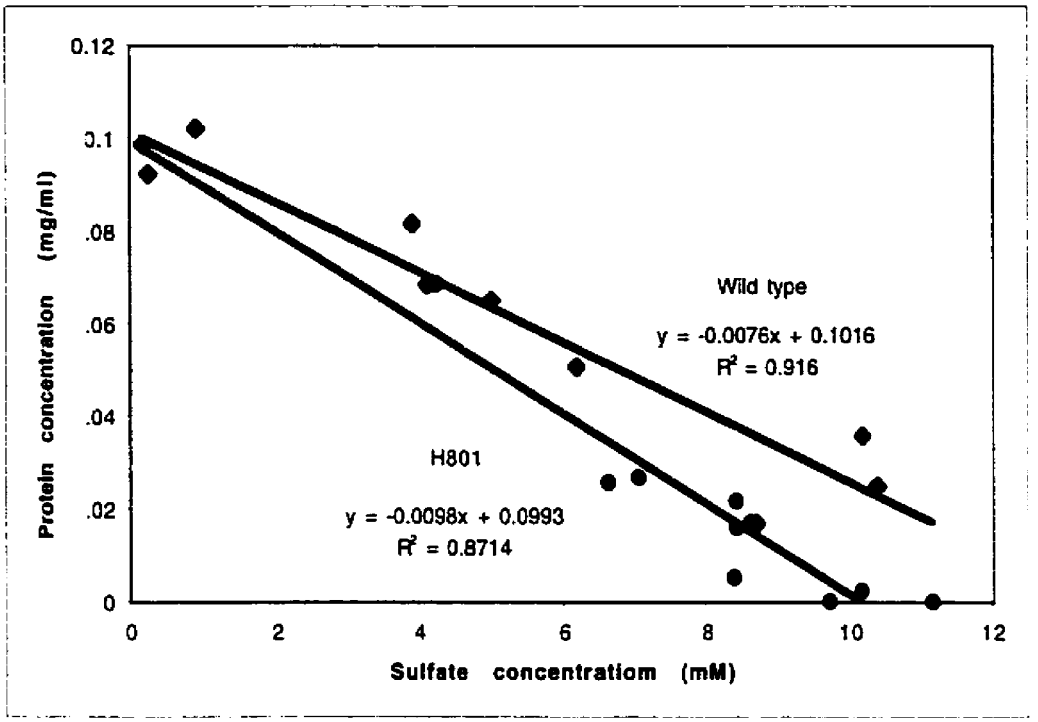

Figure 5.5 Comparison of cellular protein production from the reduction of sulfate by the *D. vulgaris* wild type strain and H801 mutant strain.

growth phase, centrifuged and resuspended in defined medium without a carbon source. These cultures were flushed with pure hydrogen gas for 15 minutes before measurement of the hydrogen uptake rate began. The cells took up hydrogen at a steady rate for approximately 20 minutes before a decline in the rate of uptake was observed. During this steady uptake period, the mutant H801 demonstrated a 3-fold lower hydrogen uptake rate than the wild type strain. The average values for two assays were 0.33 µmol of hydrogen min^{-1} (mg $protein^{-1}$) for the mutant H801 and 1.00 µmol of hydrogen min^{-1} (mg $protein^{-1}$) for wild type *Dv*H.

5.1.3.3 Determination of $Y_{hydrogen}$

In order to complete the study of hydrogen metabolism, $Y_{hydrogen}$ was also determined. Hydrogen consumption was measured in batch cultures of 50 mL defined hydrogen-sulfate medium in sealed serum bottles flushed with pure nitrogen and injected with 10 mL of pure hydrogen. Hydrogen consumption was measured over time using a GC. The amount of cell mass formed was determined in protein assays. The wild type strain consumed hydrogen more readily during growth than did the mutant strain H801 (Fig. 5.6). An average of 3 assays showed that wild type *Dv*H had a $Y_{hydrogen}$ of 16.51±1.42 g protein produced per mol of hydrogen consumed. The mutant H801 had a $Y_{hydrogen}$ of 12.56±0.92 g protein produced per mol of hydrogen consumed. The data are summarized in Table 5.3.

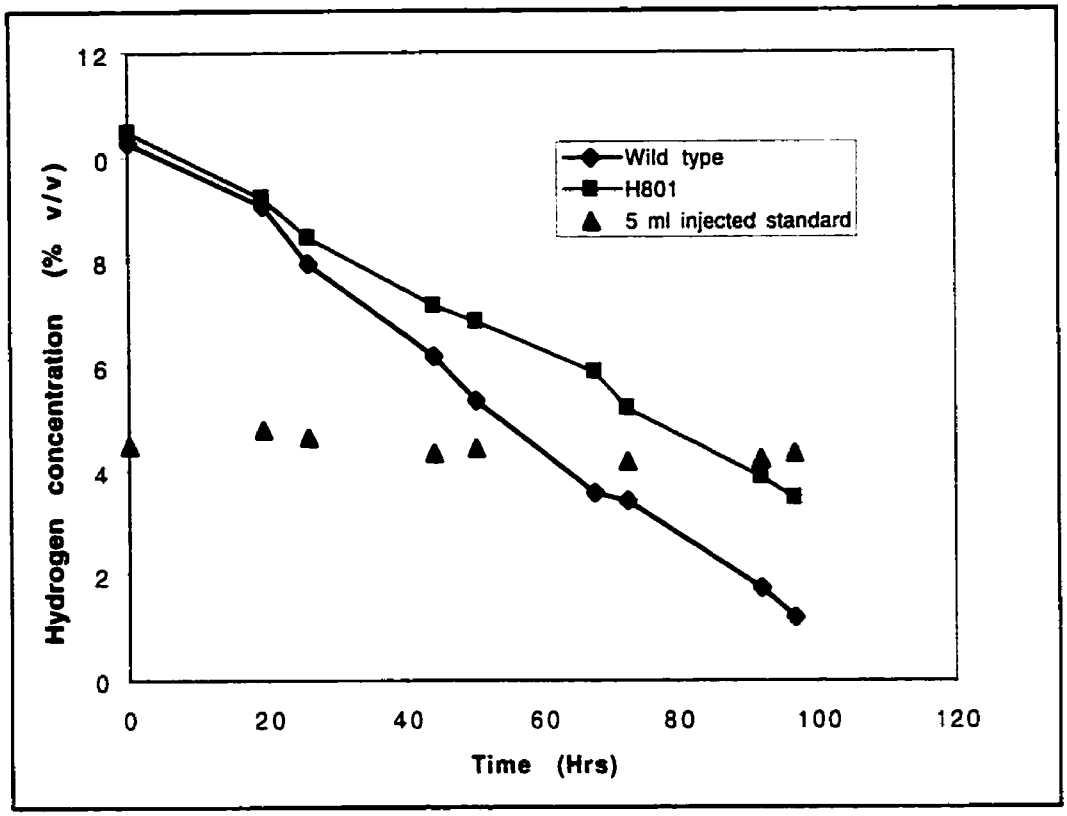

Figure 5.6 The utilization of hydrogen by exponentially growing *D. vulgaris* wild type strain and H801 mutant strain over time. Values are an average of three trials.

Table 5.3 Relationship between cell density as a function of hydrogen consumption

	Experiment number	Strain	$Y_{hydrogen}$	Averages[a]
$Y_{hydrogen}$ (g protein per mol hydrogen consumed) Data Set #1	1	Wild type	16.60	Wild type average 16.51±1.42
		H801	12.40	
	2	Wild type	15.05	
		H801	11.74	H801 average 12.56±0.92
	3	Wild type	17.89	
		H801	13.55	
$Y_{hydrogen}$ (g protein per mol hydrogen consumed) Data Set #2	1	Wild type	8.40	Wild type average 14.68±11.55
		H801	31.64	
	2	Wild type	7.63	
		H801	43.51	H801 average 34.87±7.56
	3	Wild type	28.01	
		H801	29.46	

[a] Data Set #1 was used for $Y_{hydrogen}$ determination due to the consistency of results between experiments.

5.1.4 Iron corrosion study

Hydrogen metabolism has been directly implicated in the ability of *Desulfovibrio* ssp. to anaerobically corrode iron (Odom 1993 and Postgate 1984). Since it has been shown that the Hmc complex is involved in hydrogen metabolism (Dolla *et al* 2000 and Rossi *et al* 1993), the deletion mutant H801, which is deficient in hydrogen metabolism, may also be less able to corrode iron if corrosion is directly due to active hydrogen metabolism.

Iron corrosion of small soft rolled steel coupons was measured in batch cultures of wild type *Dv*H and the mutant strain H801. More corrosion occurred in the presence of bacteria than in sterile medium alone. However, there was no appreciable difference between corrosion by the wild type strain and the mutant strain H801 (Table 5.4). Both gave approximately 0.4% weight loss after 1 month of incubation.

5.2 Physiological characterization of *D. vulgaris* Hyd100

Physiological characterization of the mutant Hyd100, in which the *hyd*A,B genes, encoding the Fe-only hydrogenase, were deleted and replaced with the *cat* gene conferring chloramphenicol resistance, gives an understanding of the role of Fe-only hydrogenase in *Dv*H metabolism. It is notable that the creation of this deletion mutant required the use of defined hydrogen-sulfate medium. Since the mutant can grow on this medium, the Fe-only hydrogenase can not be critically important in hydrogen uptake for its use as an electron donor for metabolism. The Hyd100 mutant can grow

Table 5.4 Comparison of iron corrosion, measured by weight loss.

Conditions[a]	Strain	Percent Weight loss[b] (%)
35.5 mM lactate under 5% hydrogen	Wild type	0.420±0.062
	H801	0.397±0.021
	control	0.251±0.007
18.74 mM lactate under 5% hydrogen	Wild type	0.303±0.009
	H801	0.397±0.014
30.0 mM Acetate[c] under 15% hydrogen[d]	Wild type	0.415±0.024
	H801	0.405±0.021

[a]Corrosion experiment was performed in sealed serum bottles under a mixed gas atmosphere (5% H_2, 10%CO_2, balance N_2) Defined medium was used with concentrations of lactate or acetate as indicated. All incubations were for 1 month at 30°C.
[b]Average weight loss of 5 or 6 coupons. Control coupons had an average weight loss of 0.251%.
[c]Hydrogen-sulfate medium was used acetate and CO_2 were the carbon source
[d]Headspace was mixed gas (5% H_2, 10% CO_2, balance N_2) plus 10 ml pure H_2.

well on lactate medium indicating that it can also use lactate as an electron donor for sulfate reduction.

The growth rates and cellular yields were compared for the Hyd100 mutant and the wild type strain in defined hydrogen-sulfate or lactate-sulfate media. Hydrogen was available in unlimited amounts in both cases because the growth flasks were stoppered with foam plugs and incubation was in a 5% hydrogen atmosphere.

Growth rates of the wild type strain and the Hyd100 mutant, with hydrogen as the electron donor, were very similar. There were however, differences in cell yields between the two cultures (Table 5.5). At low sulfate concentrations (5 to 20 mM), where it was the limiting substrate, the wild type strain grew to approximately 10% higher cell yield than the Hyd100 mutant. At higher concentrations of sulfate, the wild type strain had approximately 30% greater cell yields than the mutant (Table 5.5). These results indicate a role for Fe-only hydrogenase in hydrogen uptake. This role is partially (but not completely) taken over by the other nickel-containing hydrogenases of the organism in the mutant Hyd100.

With lactate as the electron donor, cell yields were the same for the two strains at limiting sulfate concentrations (5 to 20 mM). When sulfate was not the limiting substrate (25 to 50 mM), the wild type strain showed an approximately 13% higher cell yield (Table 5.5). Under these conditions a contribution of hydrogen, as electron donor to the growth of the organism, may be expected (see section 5.1.3.1). The mutant is less efficient in creating cell mass under these conditions although the difference is small.

Table 5.5 Final cell densities (OD_{600}) of *D. vulgaris* wild type strain and Hyd100 strains in defined medium[a] with hydrogen only, or lactate and hydrogen[b] as the electron donors for sulfate reduction at various sulfate concentrations (C).

	H_2 wild type		H_2 Hyd100			Lactate & H_2 wild type		Lactate & H_2 Hyd100		
C (mM)	n[d]	OD_{600}	n[d]	OD_{600}	R[c]	n[d]	OD_{600}	n[d]	OD_{600}	R[c]
5	1	0.139	1	0.150	0.93	1	0.272	2	0.278±0.041	0.98
10	1	0.231	1	0.184	1.26	1	0.340	2	0.327±0.020	1.04
15	1	0.340	1	0.272	1.25	1	0.510	2	0.612±0.129	0.83
20	1	0.374	1	0.388	0.96	2	0.660±0.068	2	0.612±0.034	1.08
				Average 1.10±0.16						Average 0.98±0.10
25	1	0.429	1	0.374	1.15	2	0.735±0	2	0.592±0.007	1.24
30	2	0.558±0.014	2	0.422±0.041	1.32	3	0.667±0.068	3	0.571±0.088	1.17
35	1	0.558	1	0.531	1.05	1	0.680	2	0.707±0.027	1.96
40	2	0.605±0.051	2	0.408±0.034	1.48	2	0.639±0.020	3	0.551±0.095	1.16
45	1	0.680	1	0.422	1.54	2	0.660±0.020	3	0.626±0.122	1.05
50	1	0.612	1	0.497	1.23	1	0.591	1	0.497	1.19
				Average 1.30±1.15						Average 1.13±0.08

[a]100 ml of medium in 300 ml nephelometer (Erlenmeyer) flask incubated at 32° C.
[b]Lactate concentration was 38 mM; available H_2 was unlimited because cultures were grown in a 5% (v/v) H_2 atmosphere and allowed constant gas exchange.
[c]Ratio of final cell densities between the wild type and Hyd100 strain cultures, grown under identical conditions
[d]Number of independent observations; the average deviation of the average cell density is given for n > 1

5.3 Creation of a suicide vector for complementation of H801

The Hmc transmembrane complex has 6 proteins, HmcA to HmcF involved in the transfer of electrons (Rossi *et al* 1993). HmcA is the high molecular weight cytochrome, whereas HmcB and HmcF contain iron sulfur subunits involved in electron transfer. In order to understand the specific role of each of these individual proteins, a method to reinsert portions of the complex back into the deletion mutant H801must be established.

The creation of a conjugational suicide plasmid to reinsert selected genes into the deletion mutant was the first step in understanding the individual roles of the proteins in the Hmc complex. A suicide vector is a plasmid that can be conjugated from one bacterial species to another and contains genes of interest that can integrate into the chromosome of the target strain. It does not contain an origin for replication in the recipient strain, therefore any expression of those genes requires their integration into the chromosome. Selection for conjugational integration of reintroduced genes is problematic in *Dv*H because there is only one suitable antibiotic marker gene to ensure that integration has occurred. This marker (chloramphenicol) was used to create the H801 mutant, therefore it cannot be used to score for reinsertion of the *hmc* genes. However, since the H801 mutant is inefficient in its ability to metabolize hydrogen, conjugational integrants can be screened for on defined hydrogen-sulfate medium, especially on plating media where colony formation of the H801 mutant is delayed by 2 weeks (Fig 5.3).

5.3.1 Complementation using pMR-MOB

The conjugational plasmid pMR-MOB, containing the *hmc* operon and mobilization genes for conjugational transfer between *E. coli* and *D. vulgaris* H801, was

created. Chromosomal DNA was purified from subsequent integrant colonies that grew on defined hydrogen-sulfate agar plates at the same rate as the wild type strain. This DNA was digested with the restriction enzymes *Xho*I and *Sal*I, then analyzed using Southern blot homology to a *Xho*I *hmc* fragment from plasmid pP6A (Rossi *et al* 1993), containing *hmc*A, *hmc*B and the 5' end of *hmc*C genes. The resulting blot (Fig 5.7) showed that the two possible integrants had the same digestion pattern as the wild type strain. The probe used was a *Xho*I fragment of the *hmc* operon that contains *hmc*A, *hmc*B and a portion of *hmc*C. If the integrant contained the wild type operon from the plasmid pMR-MOB and the deleted operon present in H801 the probe should have hybridized to 2 *Xho*I fragments within the integrant. It should have hybridized to a 3.7 kbp fragment from the wild type operon and another much larger fragment from the deleted region of the genome, which contains no *Xho*I sites within it. The *Xho*I digestion pattern seen in figure 5.7 shows only one 3.7 kbp band, which is the same as that for the wild type strain. The digestion pattern should not be the same for recipients as for the wild type strain if other genes inserted to make the H801 mutant were still present. There are two possibilities to explain this result. The first is that cultures of the H801 mutant were contaminated with the wild type strain during the conjugation and subsequent plating experiment. The other possibility is that, after conjugation and insertion of the *hmc* operon into the genome, a second crossover event that excised the plasmid DNA took place, resulting in a genome that is indistinguishable from wild type. To test this, identifiable modifications to the *hmc* operon, within the conjugational plasmid (pMX-MOB, pRK-MOB and pRsr-Blunt), were made.

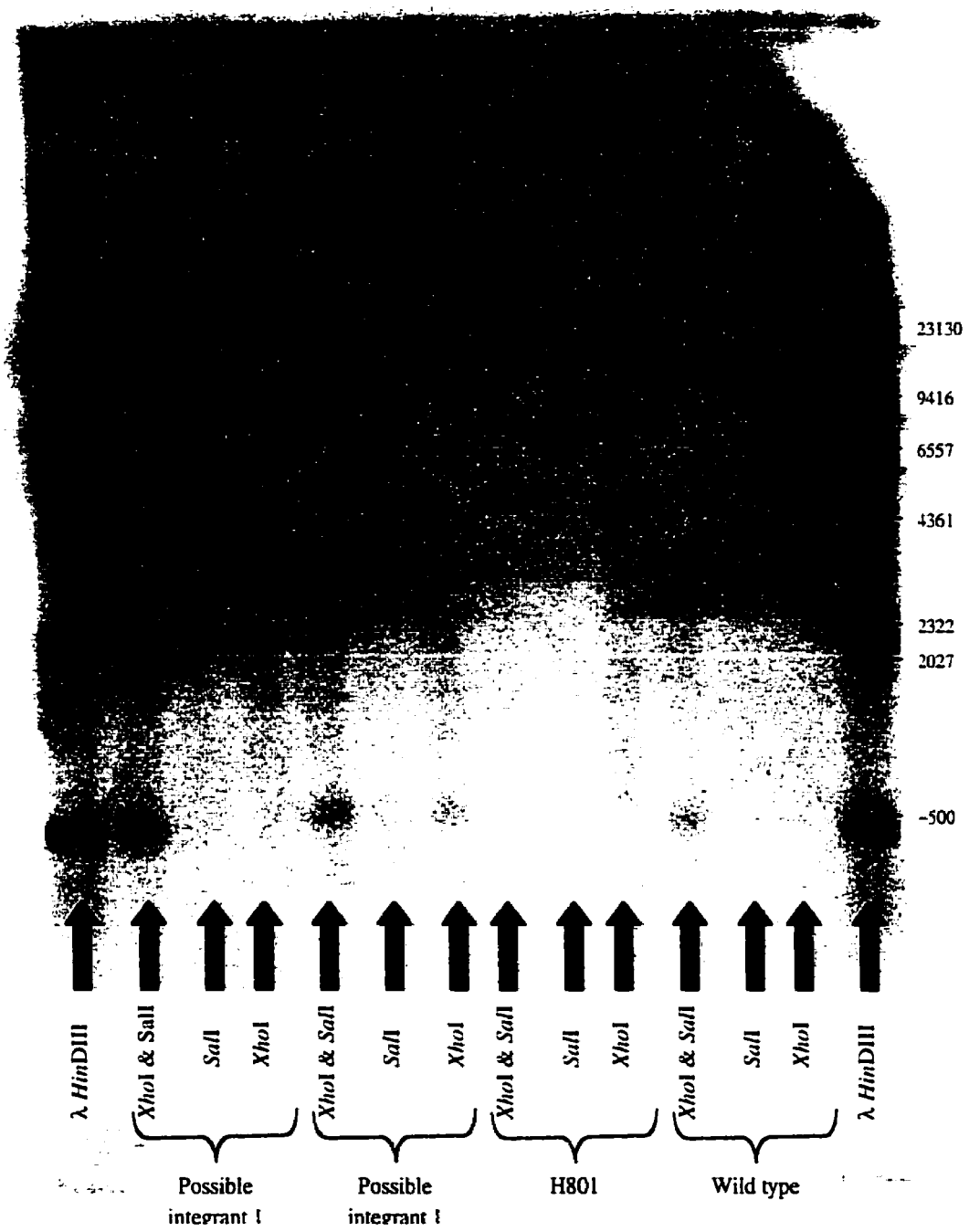

Figure 5.7 Southern blot analysis of *Xho*I, *Sal*I and *Xho*I & *Sal*I digested chromosomal DNA from the H801 and wild type strains and 2 possible integrants. The Southern blot was probed using a radiolabelled XhoI *hmc* fragment from plasmid pP6A. Lambda DNA digested with *Hin*DIII was used as a molecular marker. Sizes of fragments are given in bp.

5.3.2 Complementation using pMX-MOB

Plasmid pMR-MOB was modified to remove the *Mlu*I restriction endonuclease site in the *hmc*B gene and replace it with a *Xho*I site. This modification involved the insertion of 12 nucleotides into the DNA, hence 4 amino acids were added into the encoded protein (Fig. 5.8). This insertion was not in any of the catalytic regions involved in binding to iron sulfur clusters. However it cannot be predicted that it would not inhibit the redox protein function. Multiple conjugational experiments never resulted in wild type growth on hydrogen sulfate medium. The screening process to obtain an integrant using pMX-MOB was unsuccessful, therefore it was assumed that insertion of the 4 amino acids into the protein sequence disabled the protein in such a way as to inhibit its activity in electron transfer across the periplasmic membrane.

5.3.3 Complementation using pRK-MOB

Plasmid pRK-MOB was modified to remove the *Rsr*II restriction endonuclease site in the noncoding region between the *hmc*D and *hmc*E genes and replace it with a *Kpn*I site. This modification involved the insertion of 9 nucleotides into the DNA in a noncoding region. No modifications to any protein structure were produced. However, no successful integration of pRK-MOB was obtained. The modified region of the DNA was sequenced to ensure that it was correct, and it was found that multiple copies of the inserted linker were present (Fig. 5.9). These multiple linkers can form a large stem-loop structure in any resulting mRNA. Since a large stem-loop structure would inhibit ribosomes from translating downstream sequences, this modification could decrease or prevent the production of *hmc*E and *hmc*F. The resulting absence of these two subunits

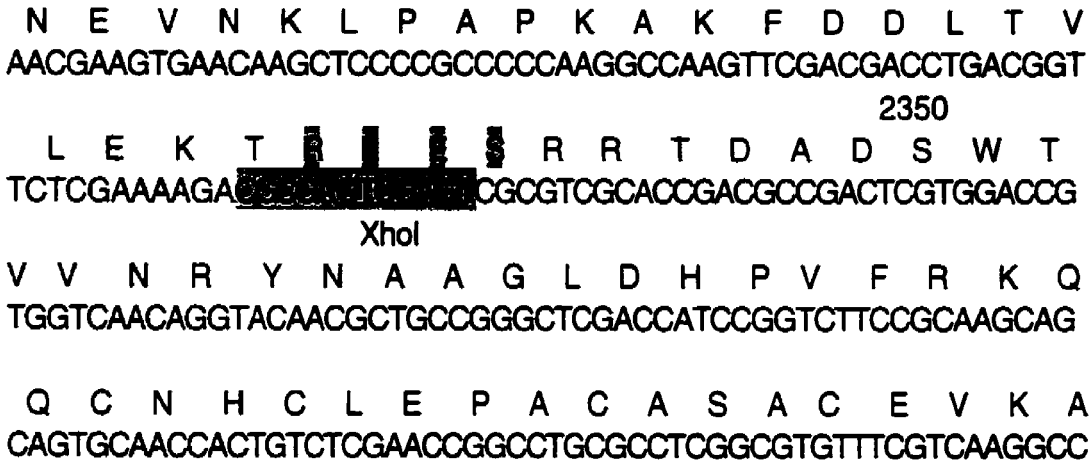

Figure 5.8 Nucleotide sequence within pMX-MOB of the segment of *hmc*B that was altered by the addition of 12 nucleotides in the shaded region. The protein sequence is shown above the nucleotide sequence, with the added amino acids shaded.

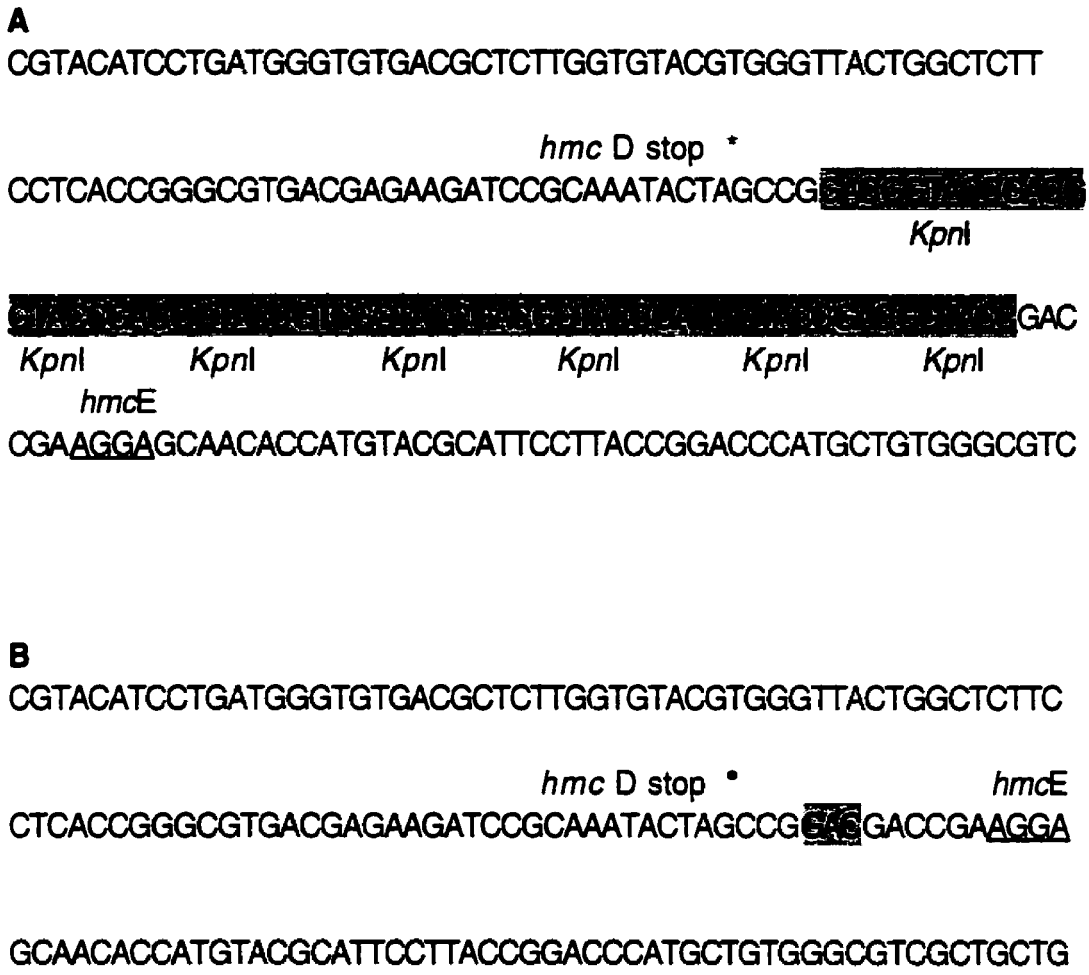

Figure 5.9 Sequence of pRK-MOB (A). Multiple linker insertion is displayed in the shaded region. The *Rsr*II site (CGG(TA)C↓CG) is altered by this insertion. Sequence of pRsr-Blunt (B) in the noncoding region between *hmc*D and *hmc*E. Digestion with *Rsr*II and blunt-end repair with DNA polymerase resulted in the addition of three nucleotides, shaded above.

of the Hmc complex may not have conferred any advantage in hydrogen metabolism over the H801 deletion mutant, therefore the screening process did not identify any integrants.

5.3.4 Complementation using pRsr-Blunt

Plasmid pRsr-Blunt was modified to remove the *Rsr*II restriction endonuclease site in the non-coding region between *hmc*D and *hmc*E by cutting the *Rsr*II, site, blunt-end repairing, and religating. In effect, this changed the *Rsr*II site by the addition of 3 nucleotides, making the site ineffective for *Rsr*II digestion (Fig. 5.9). Integration of this plasmid has been performed and wild type-like colonies were obtained. At the time of writing, Southern blot probes are being conducted on the integrant DNA.

5.4 Hydrogen production from lactate metabolism

The role of hydrogen in the overall metabolism of *Dv*H is not simply limited to its use as an electron donor. Hydrogen production from lactate in the presence of sulfate has been observed. Hydrogen production occurs in a burst during lag phase, just before exponential growth commences (Tsuji and Yagi 1980 and Van Den Berg *et al* 1991). This phenomenon has been investigated to determine if the *hmc* operon and the Fe-only hydrogenase play a role. Hydrogen production from lactate also occurs in the absence of sulfate when *Dv*H grows fermentatively (lactate → acetate + CO_2 + H_2). This yields one ATP per lactate by substrate level phosphorylation.

5.4.1 Comparison of sulfate-limited hydrogen production by wild type *D. vulgaris* to *D. vulgaris* H801 and *D. vulgaris* Hyd100

Hydrogen production by cultures of wild type *Dv*H and the H801 and Hyd100 strains in sealed serum bottles was monitored by GC. The bottles contained 28 ml headspace and 130 ml of defined lactate sulfate medium. This medium contained 37.5 mM lactate and 10 mM sulfate. The culture headspace was 90% N_2 and 10% CO_2. To eliminate disparities in lag time between the three strains large inocula were used. Cultures grown in 50 mL of Postgate medium C to stationary phase, were centrifuged, resuspended in approximately 3 ml distilled water to equal cell densities for the three strains and then injected into the serum bottles. The results of 2 separate experiments A and B (1 and 2) are summarized for the wild type strain, the H801 mutant and Hyd100 mutant in Figs 5.10, 5.11 and 5.12, respectively. In experiments A sulfate, sulfide and hydrogen concentration (but not cell density) were monitored. In experiment B cell density, hydrogen, sulfate and sulfide were measured. All three strains produced hydrogen just before exponential growth, but to a much smaller extent than was expected from the literature (Tsuji and Yagi 1980 and Van Den Berg *et al* 1991) (Fig 5.10, 5.11, and 5.12). These cultures were grown in medium that was sulfate-limited and production of hydrogen occurred again in stationary phase after all the sulfate was consumed. This production much larger than associated with the "hydrogen burst". Burst concentrations (at 0 to 24 Hrs) were several 100 ppm, where fermentative hydrogen production was 5000 – 6000 ppm for the wild type, 6000 – 8000 ppm for the H801 strain and 2000 – 2500 ppm for the Hyd100 strain. Fermentative hydrogen production was not associated with strong growth. It started when sulfate was depleted. The significantly lower

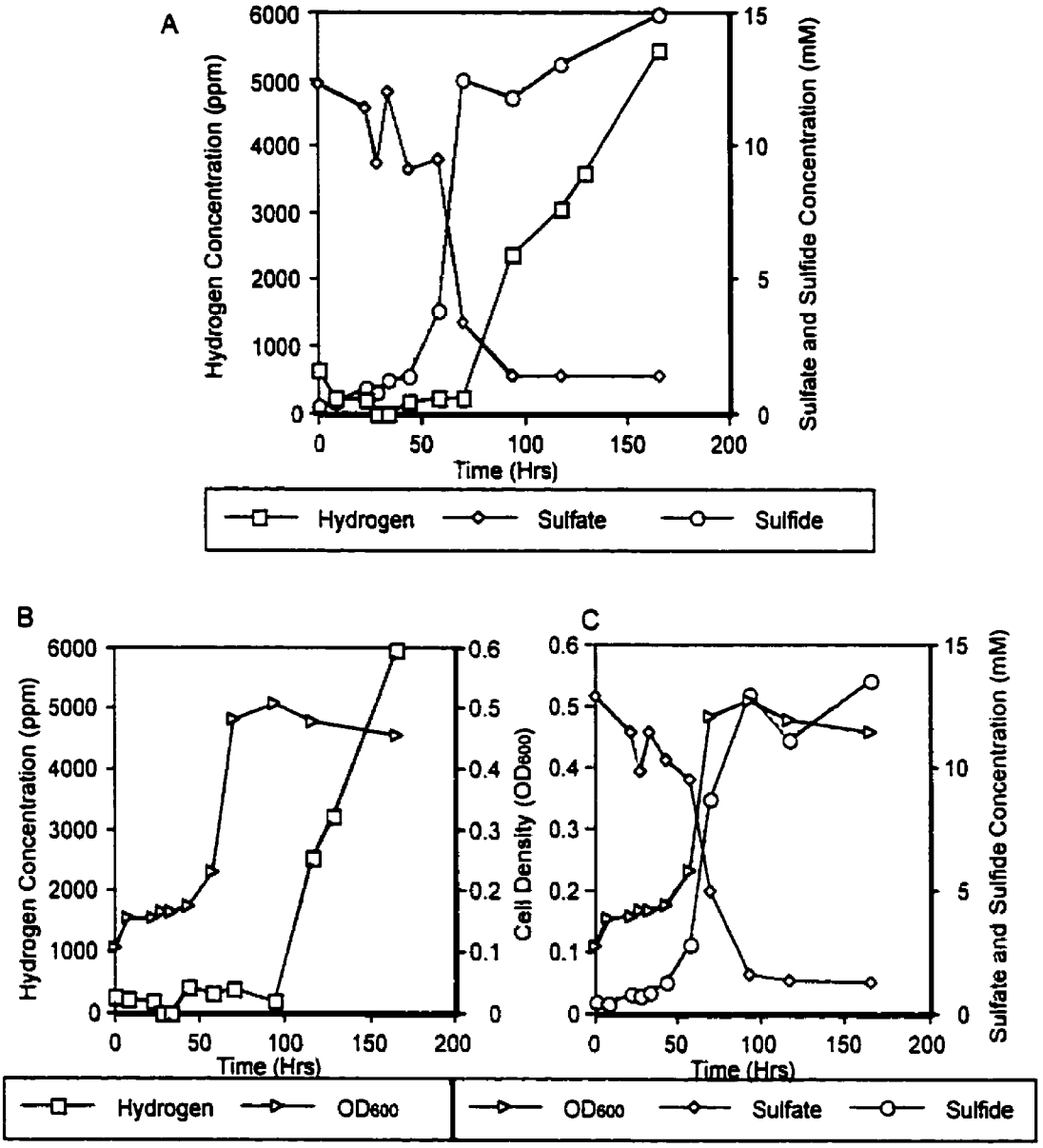

Figure 5.10 Growth of wild type *Dv*H, and hydrogen production in defined lactate-sulfate media (A). Hydrogen, sulfate, and sulfide concentrations (cell density was not measured in this experiment because more sampling would have interfered with accurate hydrogen measurement). Second experiment with hydrogen concentration and cell density (B) and sulfate, sulfide concentration and cell density (C).

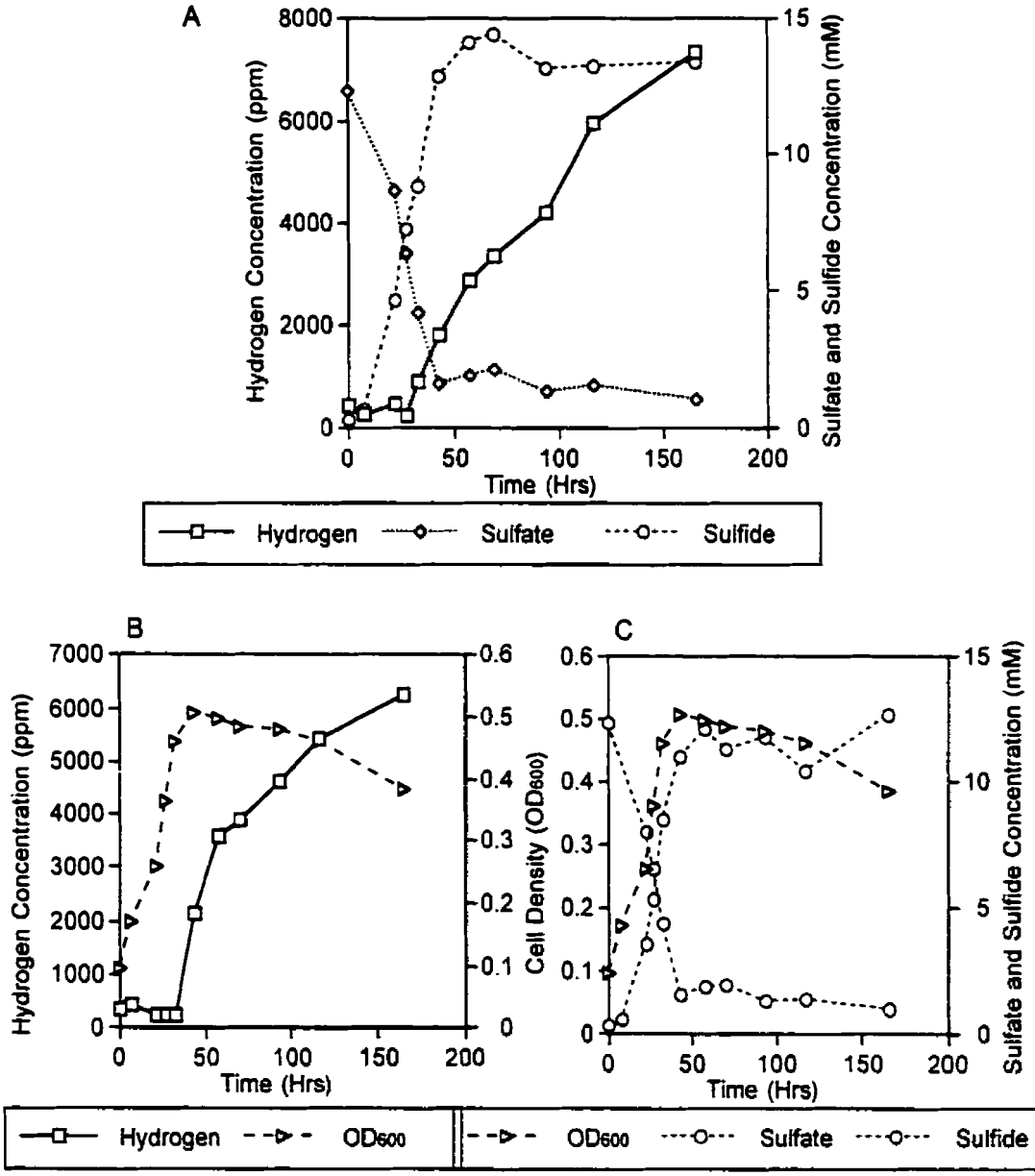

Figure 5.11 Mutant strain H801 growth and hydrogen production in defined lactate-sulfate media (A). Hydrogen, sulfate, and sulfide concentrations (cell density was not measured in this experiment because more sampling would have interfered with accurate hydrogen measurement). Second experiment with hydrogen concentration and cell density (B) and sulfate, sulfide concentration and cell density (C).

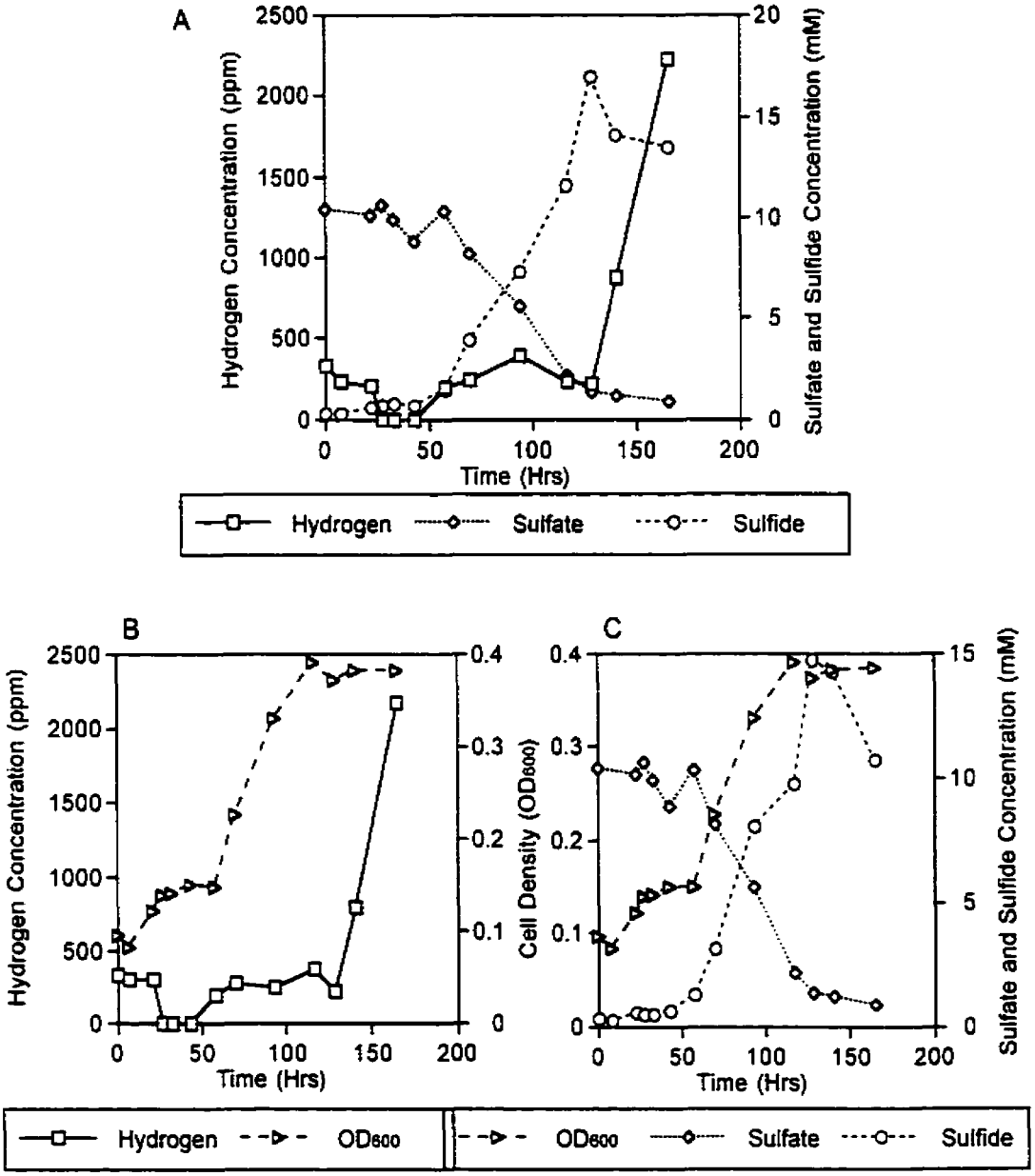

Figure 5.12 Mutant strain Hyd100 growth and hydrogen production in defined lactate-sulfate media (A). Hydrogen, sulfate, and sulfide concentrations (cell density was not measured in this experiment because more sampling would have interfered with accurate hydrogen measurement). Second experiment with hydrogen concentration and cell density (B) and sulfate, sulfide concentration and cell density (C).

production of hydrogen in the Hyd100 mutant indicates a role for Fe-only hydrogenase in the fermentative production from lactate. This has been suggested before (van den Burg *et al* 1991) for observations on cultures in which sulfate was not limiting, i.e. lactate was not in excess.

5.4.2 Hydrogen production from small inocula

Since large inocula displayed a far lower than expected hydrogen burst in the lag phase, smaller inocula (2%) of the wild type strain and the mutant H801 were tested. Both wild type and H801 displayed similar hydrogen burst characteristics, with a large hydrogen burst just before and during the commencement of exponential growth (Fig 5.13 and 5.14). It is of note that hydrogen production did continue to increase slightly into exponential growth. The reason why this hydrogen production continues into the beginning stages of exponential growth has yet to be explained. Following depletion of sulfate a large fermentative accumulation of hydrogen (5000 – 6000 ppm for the H801 strain; 8000 – 9000 ppm for the wild type strain) was again observed.

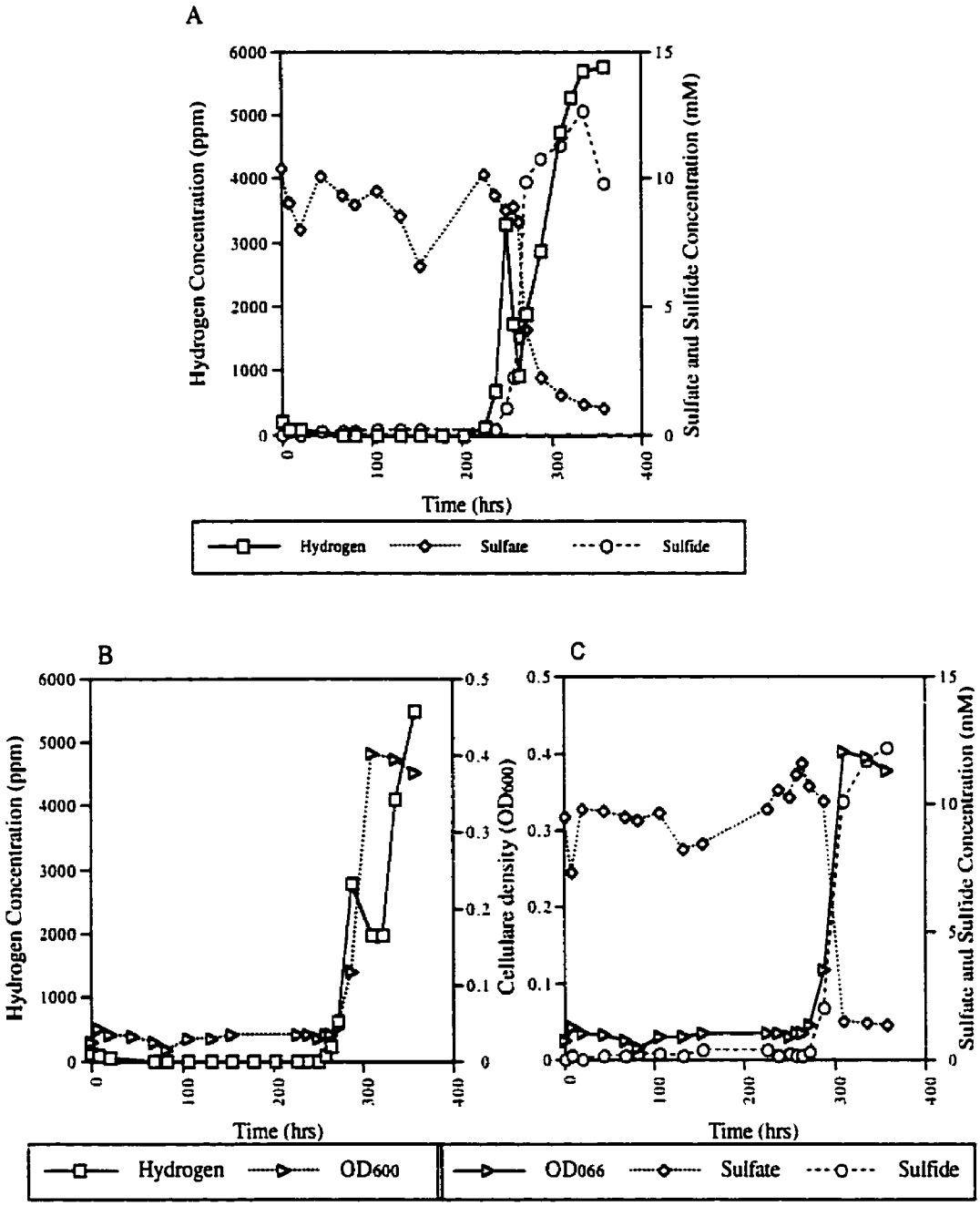

Figure 5.13 Mutant strain H801 growth and hydrogen production with a 2% inoculum in defined lactate-sulfate media (A). Hydrogen, sulfate, and sulfide concentrations (cell density was not measured in this experiment because more sampling would have interfered with accurate hydrogen measurement). Second experiment with hydrogen concentration and cell density (B) and sulfate, sulfide and cell density (C).

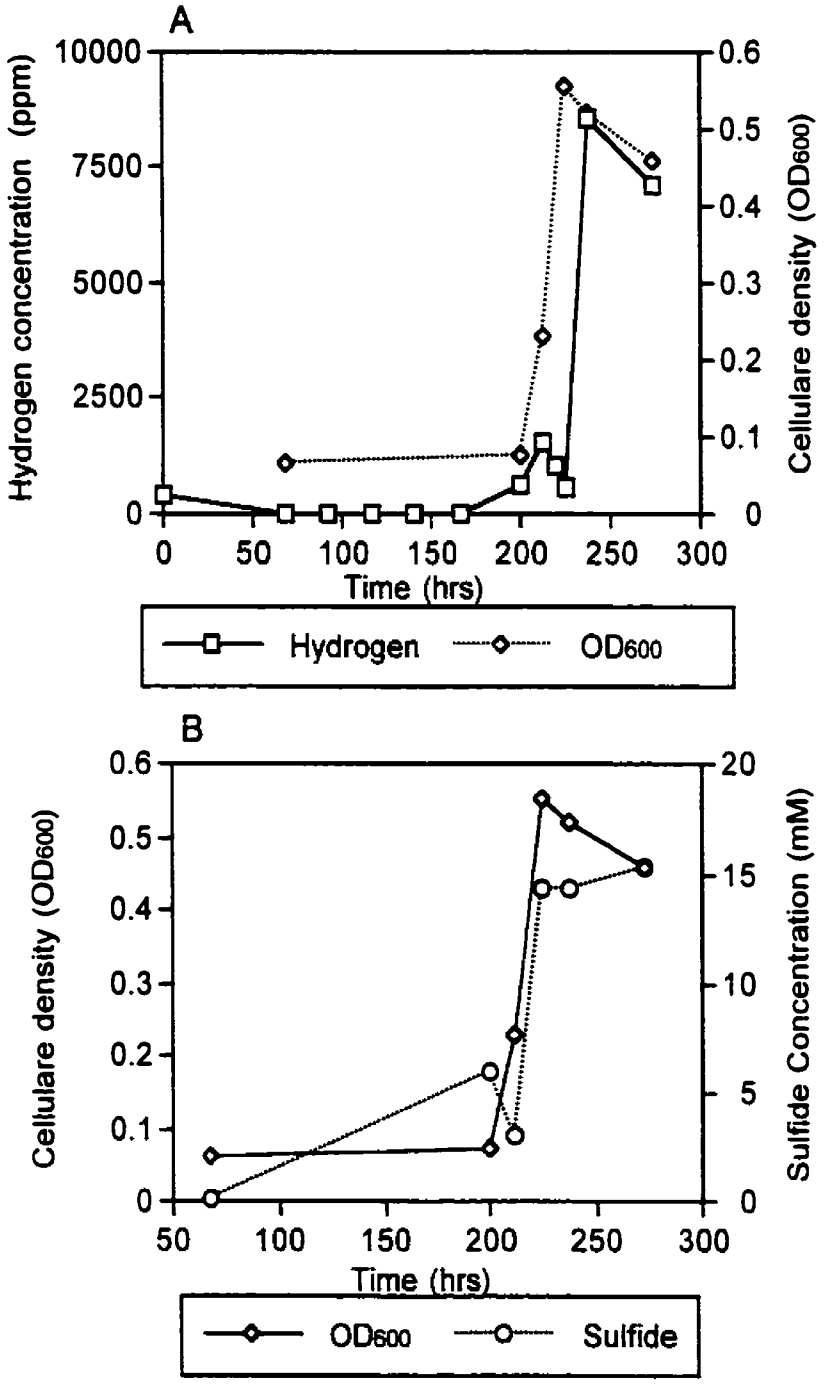

Figure 5.14 Wild type growth and hydrogen production using a 2% inoculum. (A): Hydrogen concentration and cell density. (B): Cell density and sulfide concentration.

6 Discussion

Hydrogen metabolism in *Dv*H has been extensively studied previously (Keon 1995, Keon and Voordouw 1996, 1997, Rossi *et al* 1993 and Van den Burg *et al* 1991). Specifically, the Hmc complex has been implicated as the transmembrane redox complex involved in transferring electrons from the periplasm, where oxidation of hydrogen by hydrogenases occurs, to the cytoplasm, where reduction of sulfate takes place.

In this research a detailed study of growth of *Dv*H on hydrogen and organic acids (lactate and pyruvate) was done using the recently created deletion mutant H801 that is missing the *hmc* operon (Dolla *et al* 2000). In Section 6.1, the detailed physiological characterization of this mutant is discussed through a close examination of hydrogen metabolism and the kinetics of growth using hydrogen as the sole electron donor. A novel role for the Hmc complex in reverse electron transport, which establishes a low redox potential niche for *Dv*H growth is proposed through the research in this thesis.

In addition to the Hmc complex, other redox enzymes are also involved in hydrogen metabolism; namely, the hydrogenases that oxidize hydrogen to electrons and protons. This research examined the Fe-only hydrogenase and its role in hydrogen metabolism, by studying the properties of Hyd100 a deletion mutant of the *hyd*A and B genes that encode the Fe-only hydrogenase.

In order to confirm the deduced roles of specific proteins in the *hmc* operon, a method for the reinsertion of the genes deleted from the mutant H801 was developed (Section 6.3). By using differing conjugational plasmids, it was demonstrated that the *hmc*B protein within the complex is very sensitive to amino acid sertion within the region 2369 to 2381 (Rossi *et al* 1993). A functional HmcB protein is required for the wild type

function of the Hmc complex. Proteins encoded by *hmc*E and *hmc*F were also required for Hmc complex function.

Further exploration of the role of hydrogen in lactate metabolism, specifically the production of hydrogen during lactate metabolism in the presence of sulfate and in sulfate-limited conditions, was done. Lactate metabolism is well understood; no production of hydrogen should occur in the presence if sulfate (Voordouw 1995). However, hydrogen production was observed during *Dv*H growth on lactate. A possible explanation for this phenomenon is presented in Section 6.4.

6.1 Physiological characterization of *D. vulgaris* H801

Any detailed study of a deletion mutant that is missing genes involved in metabolism necessitates that an understanding of the basic consequences (i.e. effect on growth) of the mutation be reached. This section discusses the physiological characterization of the mutant H801, which is missing the *hmc* operon.

6.1 1 Growth kinetics

Figure 5.1 and Table 5.1 show that growth of H801 on either pyruvate or lactate was unaffected by the deletion of the *hmc* operon. The cell yield for wild type *Dv*H grown on lactate was only slightly higher than that for H801. Although this difference is not large, it can be explained by the fact that cultures were incubated in a mixed gas atmosphere that contained 5% hydrogen and 10% CO_2. The end products of lactate metabolism are acetate and CO_2, which can subsequently be used as a carbon source for metabolism using hydrogen as an electron donor. Since hydrogen and CO_2 were available, it is possible that wild type culture underwent diauxic growth utilizing

hydrogen after all the lactate was consumed. Further, since the *hmc* operon is proposed to be involved in hydrogen metabolism, H801 was likely deficient in the hydrogen-utilizing growth phase, which could explain why wild type cells grew to a slightly higher cell density.

When H801 and the wild type strain were provided with hydrogen as a sole electron donor, a marked difference in growth became evident. Figure 5.2 shows the obvious difference between both average growth rates and cell yields for the two strains. Growth rates for H801 were 24% lower (note: growth rates were only analyzed for 2 growth curves) than that of wild type and final cell densities for H801 ranged from 20% to 60% lower (Table 5.1). The large range in final cell densities was dependent upon varying culture volumes and vessel shape, which changed surface areas and hence affected mass transfer of hydrogen. The greater the ratio of surface area to volume, of the cultures, the lower the difference in final cell densities between the two strains.

The Growth rates and final cell densities determined from figure 5.2 were done using the second and third growth curves. The first curve was not used because endogenous storage molecules affected the growth. The last growth curve was not used because the differences between the mutant H801 and wild type seemed less defined. This might have been due to the possibility that the mutant was compensating for the deletion by up-regulating another system for the transfer of electrons. However no cultures of the mutant H801 ever grew to the same cell density or with as a high a growth rate as the wild type strain no matter how many serial transfers were done.

It is notable, however, that H801 was able to grow using hydrogen as an electron donor. Given that all the known hydrogenases are in the periplasm (Badziong & Thauer

1980), there must be an another set of redox proteins other than the Hmc complex for transporting electrons across the plasma membrane. Since the growth rates and final cell densities were lower for the mutant H801 than the wild type, this other system for electron transport appears kinetically less efficient at electron transport, than the Hmc complex. Whether its use results in generation of a smaller proton gradient and thus in generation of less ATP and a smaller amount of cell mass per mol of sulfate reduced, can only be evaluated by measuring growth rates and rates of hydrogen or sulfate consumption.

The mutant H801 demonstrated an interesting phenotype with respect to colony development on solid medium. On lactate-sulfate plates, the mutant H801 showed the same colony morphology as wild type, except that a 1-day delay in growth occurred (Figure 5.3 A and B). Growth of H801 in lactate-sulfate liquid medium showed that the Hmc complex is not involved in lactate metabolism, implying that the Hmc complex has another function besides transport of hydrogen electrons from the periplasm to the cytoplasm. Understanding this other function first requires an understanding of the differences in growth between liquid cultures and colonies grown on solid medium. Typically, a large inoculum (10%) is used for liquid cultures of *Dv*H. This is because a lactate-sulfate medium stored for at least 4 days under a mixed gas atmosphere has a redox potential of +100 mV, while *Dv*H requires a redox potential of –200 mV in order to grow (Postgate 1984). When using a large inoculum the redox potential disparity is generally inconsequential because hydrogen sulfide produced by resting cells will reduce the medium sufficiently for growth.

On solid medium, single CFUs are isolated and each must reduce its immediate environment individually. Therefore, the delayed growth phenotype displayed by H801 suggests that the Hmc complex is involved in establishing a low redox potential niche. This phenotype is even more pronounced when hydrogen is the sole electron donor (Figure 5.3 C and D). The phenotype of poor low redox potential niche establishment was further investigated by comparing the growth of the wild type strain and H801 using different inoculum sizes in liquid lactate-sulfate medium. The lag time increased as inoculum size decreased (Fig 5.4). The growth rates and final cell yields, once growth had commenced, were unaffected. This suggests that, as the co-operative effect of larger inoculum sizes was reduced, a greater amount of time was required for the establishment of a low redox potential by the mutant. Since the cytoplasm of *Dv*H has a redox potential of –300mV, this further implies that the Hmc complex might be involved in transporting electrons from the cytoplasm to the periplasm in order to reduce the immediate environment for the commencement of growth.

6.1.2 Comparison of lactate/sulfate ratios

Lactate and sulfate consumption were measured for the wild type and H801 strains: the wild type strain had a ratio of 1.353 mols lactate consumed per mol sulfate reduced; the ratio was 1.421 for the mutant H801. The low values can be explained by assuming that hydrogen also served as an electron donor. They could also be the result of underestimation of lactate concentrations. The similar ratios of lactate oxidation to sulfate reduced for the mutant strain H801 and the wild type *Dv*H support the concept that the Hmc complex is not involved in lactate metabolism. If it were involved in lactate

metabolism the lactate-sulfate ratios for the mutant H801 would be different than that of *Dv*H wild type, which is not the case. This difference would most likely increase the amount of lactate consumed per mol of sulfate reduced. If the lactate sulfate ratios were different in the mutant H801, indicating an involvement in the Hmc complex with lactate metabolism, this involvement would most likely result in a change in the proton gradient established from lactate metabolism. This change in proton gradient would implicate the Hmc complex in proton pumping for lactate metabolism and a deletion mutant would be impaired in this proton export, resulting a larger lactate consumption per mol sulfate reduced.

6.1.3 Metabolism of hydrogen - energy conservation

The results indicate that deletion of the *hmc* operon affected growth on hydrogen (Figure 5.2). When considering the 20% to 60% lower final cell yield (Table 5.1) and the 24% lower growth rate in the mutant H801, one might be tempted to think that less cell mass was produced per mol of sulfate consumed. However, sulfate was not the limiting factor; it was detected in the culture medium after growth had reached stationary phase. Since hydrogen was unlimited in these growth studies, and sulfate was not depleted, another factor, such as a change in pH must have triggered stationary phase.

To determine whether deletion of the *hmc* operon did affect the amount of cell mass produced per mol of sulfate consumed, $Y_{sulfate}$ during growth on hydrogen-sulfate medium was measured. $Y_{sulfate}$ for the mutant H801 was 0.0099±0.0021 mg/ml protein mM sulfate $^{-1}$ and for the wild type strain was 0.0091±0.0001 mg/ml protein mM sulfate^{-1}. There is no large difference between these two values, demonstrating that

deletion of the *hmc* operon does not affect the amount of cell mass produced per mol of sulfate consumed. One can therefore assume that the number of protons translocated per mol of sulfate reduced is the same, so equation 3 (Voordouw 1995):

$$4H_2 + ATP + SO_4^{2-} + 8H^+_{in} \rightarrow HS^- + AMP + 2P_i + 8H^+_{out} \quad (3)$$

is unaffected. In equation 3, one ATP is converted to AMP and two P_i for the activation of sulfate; eight H^+ are translocated from the inside (eight H^+_{in}) to the outside (eight H^+_{out}). The number eight implies no addition proton pumping. It is caused purely by the localization of the hydrogenases in the periplasm and the localization of sulfate reduction enzymes in the cytoplasm. The former produce eight H^+_{out} and the latter consume eight H^+_{in}. Use of the proton gradient for ATP synthesis would lead to synthesis of $2^2/_3$ ATP. Assuming the influx of 3 H^+ are required to form 1 ATP:

$$8H^+_{out} + 2.67ADP + 2.67P_i \rightarrow 8H^+_{in} + 2.67ATP \quad (7)$$

Conversion of ATP and AMP to 2 ADP is bioenergetically neutral:

$$ATP + AMP \rightarrow 2ADP \quad (8)$$

Adding equations (3), (7) and (8) gives (ignoring formation of $4H_20$):

$$4H_2 + SO_4^{2-} + 0.67ADP + 0.67P_i \rightarrow HS^- + 0.67ATP \quad (9)$$

Because $Y_{sulfate}$ appears similar for both wild type and H801 strains, it is likely that the same amount of ATP is formed per mol of sulfate reduced in both, e.g. 0.67ATP / mol of sulfate as indicated above assuming that the Hmc complex (and other complexes that transport of electrons from hydrogen to sulfate) do not pump additional protons.

Since $Y_{sulfate}$, is the same in the mutant H801 and the wild type strains, the contribution of the Hmc complex is mainly kinetic (Fig 5.2). Direct manometric measurement of hydrogen uptake rates showed that the mutant H801 had a 3-fold

reduced hydrogen uptake activity than the wild type strain. The decreased activity to directly take up hydrogen suggests that the Hmc complex is kinetically more efficient in the transfer of electrons from the periplasm to the cytoplasm. If the other systems involved in transferring electrons, which allow H801 to grow using hydrogen as the sole electron donor, are less active in that transport then hydrogenases in the periplasm would be less able to take electrons from hydrogen, reducing the hydrogen uptake activity of H801.

To fully understand different growth kinetics for the mutant H801, $Y_{hydrogen}$ must be considered. $Y_{hydrogen}$ specifically relates the production of cell mass to the metabolism of hydrogen. The mutant H801 produced a similar amount of cell protein per mol of hydrogen consumed as the wild type strain. Although the values are lower for the mutant H801 the values are of the same magnitude. The determination of hydrogen is problematic in that the concentration of hydrogen determined from a standard curve assumes atmospheric pressure and standard temperature. The samples were from sealed serum bottles incubated at 30°C. As the cultures grow, hydrogen sampling might be affected by changes in pressure from other gas production such as H_2S. These changes might not be the same for both strains and would result in variances between them. The possibility of errors in the determination of hydrogen concentration might also explain why the values of $Y_{hydrogen}$ are higher than expected when comparing them to the values of $Y_{sulfate}$. For the same amount of cell mass production 4 hydrogen are oxidized for every sulfate reduced (equation 3). The value of $Y_{hydrogen}$ should be 4 times less than that of $Y_{sulfate}$. The values obtained (tables 5.2 and 5.3) show that $Y_{sulfate}$ is ~10 g protein/mol

sulfate and $Y_{hydrogen}$ is ~16 g protein/mol sulfate. This variance might be the result of inaccuracies in the determination of hydrogen concentration.

Since the values of $Y_{hydrogen}$ and $Y_{sulfate}$ for the wild type and mutant H801 strains are similar it can be concluded that the amount of ATP formed per mol of hydrogen oxidized is the same for both strains. Since the mutant is less able to take up hydrogen this suggests that the Hmc complex is involved in electron transport and not involved in additional proton pumping.

6.1.4 Iron corrosion

The main controversy surrounding the ability of SRB to cause anaerobic corrosions centers on whether the corrosion is due to active bacterial metabolism or simply the result of corrosion by end metabolites of SRB; namely hydrogen sulfide. Active bacterial metabolism causing corrosion has been postulated to involve the use of hydrogen as an electron donor (Postgate 1984). The theory is that this corrosion takes place by cathodic depolarization through the removal of the polarizing hydrogen layer on metal in an aqueous environment (Fig 2.5) (Odom 1993). The removal of this hydrogen would be accomplished by hydrogenases, which would then pass electrons through an electron transfer mechanism to the cytoplasm for sulfate reduction. Since this work has shown that the Hmc complex is involved in electron transport for growth on hydrogen, the mutant H801 should be less able to corrode iron than wild type *Dv*H. However, the results obtained showed that there was no difference between corrosion by the mutant H801 and the wild type strain. This could indicate that hydrogen metabolism is not

involved in the corrosion of iron. Iron corrosion may thus be the result of reactions of iron and metabolites produced by *Dv*H such as H_2S.

6.2 Physiological characterization of the Hyd100 mutant

While investigating the role of hydrogenases in lactate metabolism, Van der Berg *et al* (1991) found a 20% reduction in growth rate and cell yields for a strain of *D. vulgaris* which contained a broad host range plasmid constitutively expressing antisense mRNA for *hyd*A,B. The constitutive expression of this antisense mRNA reduced the level of iron hydrogenase expressed and affected growth of this strain on lactate. A deletion mutant (Hyd100) which was completely missing the *hyd*A,B genes, resulting in no expression of the iron hydrogenase, was studied here. The growth phenotype described by Van der Berg *et al* (1991) was not observed when comparing growth of the Hyd100 and wild type strains. Specifically, no differences in growth yields were observed when lactate was in excess and sulfate was the limiting substrate (Table 5.5). A higher final cell density for wild type cultures than for the mutant Hyd100 was observed only when sulfate was in excess and lactate was limiting. When sulfate was not limiting, the result would be diauxic growth with a switch to hydrogen as the electron donor when lactate was depleted. Since Hyd100 is missing the iron hydrogenase, growth on hydrogen is adversely affected. A higher cell yield can be attributed to differences in hydrogen uptake – a factor that was not considered in the study conducted by Van den Berg *et al* (1991). The differences in hydrogen uptake, resulting in lower final cell densities for the mutant Hyd100, were most likely due to inefficiencies in hydrogen uptake. This is reflected in that the wild type strain had a 22% higher cell density, on

average, than the mutant Hyd100 when hydrogen was the sole electron donor. It should be pointed out that the growth studies on lactate conducted here are not completely comparable with those by Van den Berg *et al* (1991). Because these researchers used an atmosphere lacking hydrogen and used stoppered serum bottles in which hydrogen sulfide would continuously accumulate.

6.3 Complementation of the deletion mutant H801

Development of a method to reinsert the *hmc* operon into the deletion mutant H801 has been problematic, since only one antibiotic marker is currently available for *Dv*H, and it was used to create H801 therefore is not available for complementing the deletion. However, due to the slow growth phenotype of the mutant H801 on solid hydrogen-sulfide medium a marker to screen for complementation exists. Any restoration of the ability to efficiently transfer electrons from hydrogen to sulfate reduction, or transfer electrons from the cytoplasm to the periplasm for low redox potential niche establishment, would decrease the delay in colony development, which can be used as a screening tool.

6.3.1 Roles of *hmc*B, *hmc*E and *hmc*F

Complementation using the plasmid pMR-MOB resulted in integrants with no discernible difference between the genome or phenotype of the recipient cells and the wild type strain. Hence, it was likely that complementation was successful and DNA repair mechanisms had excised the unnecessary (inserted plasmid) DNA through a second cross-over event (Fig 6.1).

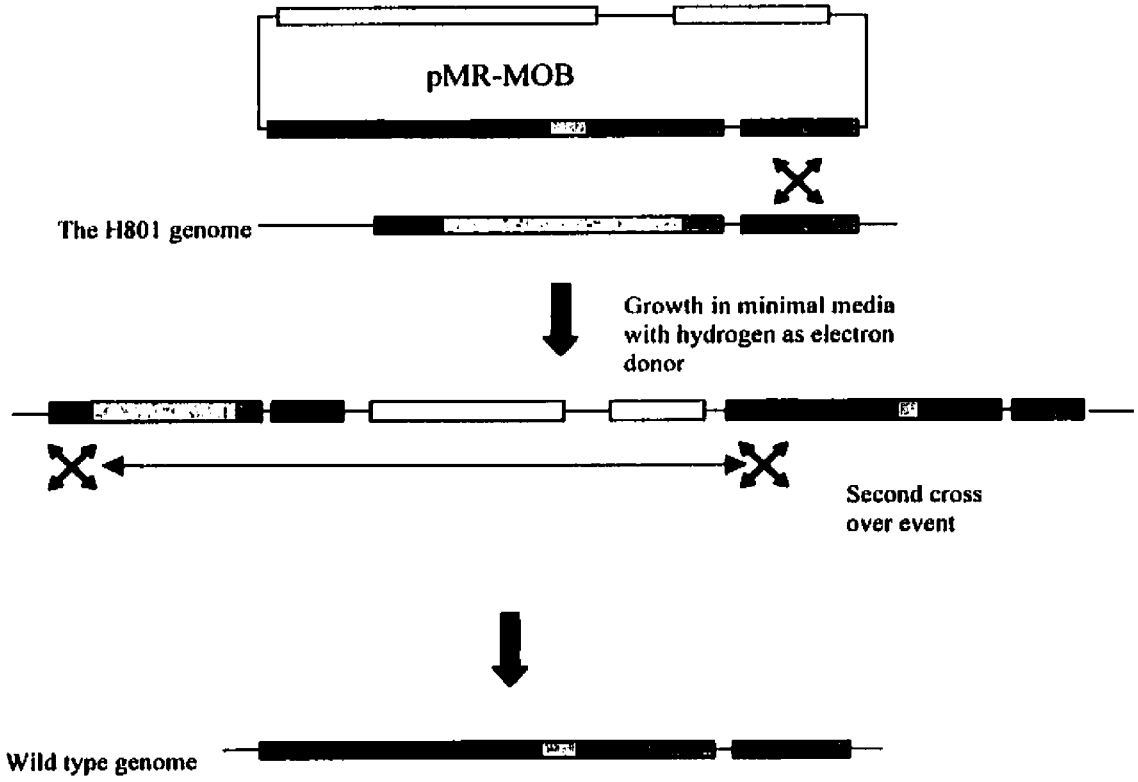

Figure 6.1 Proposed second crossover event that takes place after pMR-MOB integration into the genome of H801. Resulting in excision of plasmid DNA

Using plasmids modified to mark the insert DNA for identification resulted in the demonstration that some of the subunits within the Hmc complex are essential. The sequence of HmcB shares significant homology with DmsB and FdnH, the iron-sulfur subunits of anaerobic dimethyl sulfoxide reductase and nitrate-inducible formate dehydrogenase, respectively (Rossi *et al* 1993), suggesting that HmcB is an electron transporting protein. The HmcB protein contains iron-sulfur subunits, therefore any disruption to the tertiary structure of this protein could decrease its ability to transfer electrons. The introduction of 12 nucleotides (4 amino acids) into the *hmc*B sequence apparently affected its enzymatic ability greatly, because no integrants demonstrated the wild type phenotype when grown on hydrogen-sulfate solid medium. This indicates that *hmc*B is necessary for the function of the Hmc complex.

Integrants containing pRK-MOB failed to show wild type phenotype on hydrogen-sulfate solid medium. Analysis of the sequence showed a series of repeats which would allow the formation of a stem loop structure between *hmc*D and *hmc*E regions of the *hmc* mRNA (Fig 5.9). This stem loop structure could decrease the translation of *hmc*E and *hmc*F, rendering the Hmc complex incomplete. This may be why no colonies showing wild type growth on hydrogen-sulfate solid medium were obtained, and indicates that these two proteins are also necessary for function of the Hmc complex.

6.4 Hydrogen production from lactate metabolism

Lactate metabolism is more complex than the simple use of lactate as an electron donor for sulfate reduction; as demonstrated by growth of *Desulfovibrio* spp. in the

absence of sulfate, in syntrophic association with methanogenic bacteria (Postgate 1994). The oxidation of lactate to the intermediate pyruvate, with protons acting as the terminal electron acceptor, produces hydrogen. This hydrogen production was investigated in this thesis.

Hydrogen production from lactate in the presence of sulfate has been observed (Tsuji and Yagi 1980, Van den Berg *et al* 1991). This phenomenon occurs in lag phase, just before the commencement of exponential growth. Hydrogen is produced in a burst and, is consumed once exponential growth begins. It has been theorized that when cell components are rapidly synthesized to support exponential growth, all available ATP is consumed, leaving none for sulfate activation. Without activated sulfate, lactate metabolism requires that electrons be transferred to protons, hence hydrogen would be produced. This hydrogen production might also be due to the overly high redox potential of fresh medium resulting in the inability of ATP sulfurylase to activate sulfate with ATP. Therefore resting lactate metabolism would necessitate transfer of electrons to protons, producing hydrogen.

The hydrogen burst phenomenon was observed in *Dv*H (Fig 5.10, 5.11 and 5.12); however, the amount of hydrogen observed was much less (0 to 500 ppm) than that reported by Tsuji and Yagi (1980) (1000 to 4000 ppm) and Van den Berg *et al* (1991) 2000 to 4000 ppm). It is of note however, that the experiments reported in this thesis were done using defined lactate-sulfate medium whereas these others used rich medium that included yeast extract. This lower hydrogen production could be due to the use of large inoculum size. Smaller inocula produced larger hydrogen bursts (Figs 5.13 and 5.14). This suggests that the production of hydrogen is more a function of cellular

replication than of redox potential. If hydrogen was produced to counter a relatively high redox potential, then hydrogen production would be expected to occur throughout lag phase. However, because hydrogen production took place only at the begining of exponential growth, ATP consumption for the rapid synthesis of cell components is most likely the main cause of hydrogen production. These results show that hydrogen production continues briefly into the exponential growth phase, before a decline in the hydrogen content of culture headspace is observed. This suggests that the rapid synthesis of cell components throughout exponential growth might keep the cellular ATP concentrations sufficiently low to cause some inhibition of ATP sulfurylase, hence resulting in some hydrogen production. At some point in the exponential growth phase, the hydrogen concentration must become high enough to activate systems involved in hydrogen metabolism, then hydrogen is consumed faster than it is produced.

The fact that smaller inocula cause a larger hydrogen burst suggests that hydrogen production takes place throughout exponential growth. The assumption being that lactate metabolism is the primary metabolic process and that hydrogen metabolism is a secondary activity. Hydrogen consumption would therefore be minor in comparison to lactate metabolism. When large inocula are used, more cells can consume the relatively small amount of hydrogen produced; for small inocula secondary hydrogen metabolism is minor enough to allow the detection of hydrogen production.

This thesis also investigated the possibility that the Hmc complex might play a role in hydrogen production during lag phase. The theory that since the Hmc complex appears to be involved in reverse electron transport for low redox potential niche establishment, it might also be involved in reverse electron transport from cytoplasmic

lactate metabolism to periplasmic hydrogenases to produce hydrogen. However, this theory does not appear to be correct. The deletion mutant H801 showed no appreciable difference in hydrogen production from lactate metabolism compared to wild type (fig 5.13 and 5.14). This indicates that the reverse electron transport for niche establishment is not related to electron transport for the production of hydrogen from lactate metabolism.

7. Concluding remarks

The Hmc complex was thought to be the complex involved in transferring electrons from the oxidation of hydrogen by periplasmic hydrogenases to the reduction of sulfate within the cytoplasm. This research has shown that the Hmc complex is indeed important for hydrogen-dependent growth. However, the Hmc complex is not the only channel for flow of electrons from hydrogen to sulfate. The mutant strain H801 still grows when hydrogen is the sole electron donor. This implies that another mechanism is involved in transferring these electrons, although this electron transfer is not as efficient as with the Hmc complex. This other mechanism for electron transfer has yet to be discovered and therefore studied in any capacity. The entire genome of *Desulfovibrio vulgaris* Hildenborough is currently being sequenced (http://www.tigr.org), which will allow this other mechanism to be identified through sequence comparison to that of the *hmc* operon. Availability of the genomic sequence will lead to much more research into the metabolism of this organism.

These results obtained here have also shown an unusual phenotype for the mutant H801. The mutant is deficient in its ability to form a low redox potential niche. More work is required to further understand this phenomenon. In particular, the redox potential of developing cultures needs to be closely monitored in order to confirm the results obtained here. This can accomplished using accurate redox probes to follow the redox potential of a freshly inoculated culture of the mutant H801 in comparison to the wild type strain.

Due to the lack of antibiotic markers, characterizations of individual proteins of the Hmc complex are limited to creating deletion mutants of the sequences for those

proteins. The methodology available to create deletion mutants of *Dv*H is very labor intensive and difficult. The complementation method described in this research provides the possibility for testing the roles of individual proteins within the complex. *Dv*H has three cytochromes, one of which is encoded within the *hmc* operon. It is of interest whether the other cytochromes can act as a replacement for the HmcA protein in transferring electrons from hydrogenases to the rest of the Hmc complex. It has been shown that cytochrome c_3 is involved in the transfer of electrons from hydrogenases to the high molecular weight cytochrome (HmcA) and then through the membrane (Pereira *et al* 1998). It is of interest whether cytochrome c_3 can transfer electrons to the Hmc complex in the absence of HmcA. This can be accomplished using the complementation method of which development was attempted here.

Since *Dv*H contains three hydrogenases, and much previous work has gone into elucidating the specific role of each of these enzymes, deletion mutants of each hydrogenase should be constructed in order to understand fully their specific roles in hydrogen metabolism. This work has begun to characterize the role of the iron-only hydrogenase, but more work needs to be done as well the other enzymes.

Hydrogen production from lactate occurs when sulfate is not available to accept electrons. When lactate is oxidized, energy (in the form of ATP) is produced and electrons from this metabolism have to be transferred to an acceptor, usually sulfate. However, when sulfate is not available the electrons can be transferred to protons and hydrogen is produced. It is of note that *Dv*H uses the Hmc complex for reverse electron transport in anaerobic niche establishment, but does not appear to use this complex for reverse electron transport from cytoplasmic lactate to periplasmic hydrogen production

by hydrogenases. This is likely because the mutant H801, which lacks this electron-transferring complex, produces the same amount of hydrogen as the wild type strain when sulfate is not available. In contrast, the Hyd100 mutant produces less hydrogen from lactate. The electron-transferring mechanisms involved are still not fully clasified.

The exact reason why sulfate (a component of the medium) is not available as an electron acceptor during the hydrogen burst phenomenon observed in lag phase is still not completely understood. Further research should involve the use of redox probes to monitor potential change within the medium while measuring the hydrogen burst phenomenon.

Additional work is also required to determine which hydrogenases are involved in this hydrogen production. Deletion mutants of each of the three hydrogenases within *Dv*H need to be studied in reference to this hydrogen production.

Hydrogen metabolism within *Dv*H has been extensively characterized previously and this work adds to that characterization. But, in the nature of science, more questions are created from the answers that are discovered and there is always more research to be done.

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