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Anaerobic Biocorrosion of Mild Steel

by

K.F.A. Van Ommen Kloeke

A THESIS

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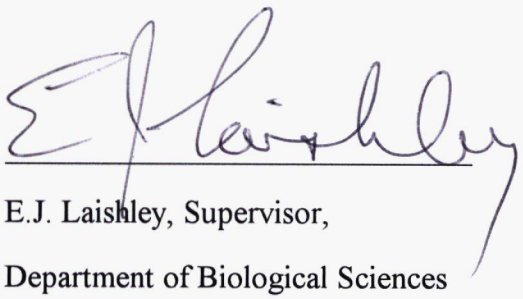

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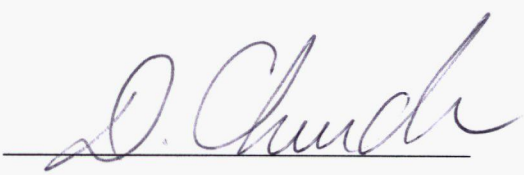

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ABSTRACT:

A protocol was developed whereby the outer membrane of the sulfate-reducing bacteria *Desulfovibrio vulgaris* Hildenborough could be isolated and partially characterized. The isolated outer membrane fractions from cultures grown under high (100 ppm) and low (5 ppm) Fe^{2+} conditions were compared by SDS-PAGE, and showed that several protein bands were derepressed under the low iron conditions, most notably at 50 kDa, and 77.5 kDa. Outer membrane isolated from low iron cultured cells was found to contain two proteins, 77.5 kDa and 62.5 kDa in size, that reacted with a heme specific stain and were referred to as high molecular weight cytochromes. Studies conducted to examine the interaction of low iron isolated outer membrane on a phosphate/mild steel hydrogen evolution system, showed that addition of the membrane fraction caused an immediate acceleration of H_2 evolution. Periplasmic fractions isolated from cultures grown under high and low iron conditions were compared via SDS-PAGE. Several of the protein bands, including those corresponding to the [Fe] hydrogenase subunits, were found to be derepressed under low iron conditions. A new model for the anaerobic biocorrosion of mild steel is proposed.

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I would also like to thank the members of my committee, Dr. M. Kapoor, and Dr. D. Church, for their time in reading this manuscript, and their input that followed.

Extra thanks go to my brother Gerry for lending me the use of his photographic expertise.

DEDICATION

I dedicate this work to my family, especially my parents and brother, whose love, support, sacrifice and guidance have enabled me to make a dream come true. I also dedicate this to my friends, who encouraged me along the way.

Finally I dedicate this thesis to the loving memory of my Grandmother Mrs. Maureen O'Toole (R.I.P. Dec 6th/1993).

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1.0 INTRODUCTION

Microbially induced corrosion accounts for a portion of all the corrosion of metal surfaces. While there is disagreement on the proportion of corrosion due to biological rather than chemical factors, several studies have indicated that the dollar cost is quite significant. In 1983 it was estimated that the United Kingdom spent between 300 to 500 million pounds per annum to repair corrosion damaged equipment and oil pipelines (Tiller, 1983), while Hamilton (1985) places the cost for the same problem at between 150 and 350 million pounds per annum. The Brazilian oil company PETROBRAS estimated that at the offshore platforms in the Campos Basin (Rio de Janeiro), 6.5 million U.S. dollars per year was being spent on infusion of biocides in an effort to control the effects of biocorrosion (De Araujo-Jorge *et al*, 1992). Due to the large dollar cost of biologically mediated corrosion, much research has been conducted in this area in an effort to understand, and ultimately control, the problem.

2.0 LITERATURE REVIEW

2.1 Corrosion

Corrosion of materials refers to the degradation of the material due to chemical or electrochemical action. Specifically, corrosion of metals may be described as an oxidation of a metal surface, with concomitant formation of metallic ions and loss of electrons to an oxidizing agent, resulting in a loss of metal mass. The loss of metal ions occurs as an anodic reaction, while the loss of electrons is a cathodic reaction; both reactions occurring at the same time gives rise to a corrosion cell. In an aqueous, aerated environment, water and oxygen act as cathodic agents, gaining electrons from the metal to form hydroxyl ions. These hydroxyl ions react with the metal ions released from the anode to produce $Fe(OH)_2$, more commonly recognized as rust. In anaerobic, aqueous environments the reaction is much different. Under these conditions, and without the presence of any other

corrosive factors, it has been suggested that water dissociates ($H_2O \leftrightarrow H^+ + OH^-$), and the protons react with electrons from the cathode to form atomic hydrogen. This hydrogen would then form a polarized protective envelope around the metal, preventing any further corrosion.

2.2 Depolarization Theory

In 1934, the Depolarization theory was put forward by von Wolzogen Kuhr and van der Vlugt to explain how sulfate-reducing bacteria (SRB) can disrupt the hydrogen envelope, and enhance corrosion by oxidizing the hydrogen via their hydrogenase enzyme system. The equations governing this reaction, as put forward in the 1934 theory, are listed in Table 1.

Thus it is an attempt to re-establish the anodic/cathodic equilibrium that causes more Fe^o to be oxidized, resulting in pit formation on the metal surface and metal weight loss. Under anaerobic conditions a number of bacterial groups can utilize the hydrogen as an electron donor, though it is now generally accepted that the SRB are the main corrosion culprits due to their ability to both oxidize H_2 and produce highly reactive sulfides as metabolic end products via H_2S production.

2.3 The Sulfate-Reducing Bacteria

In 1895 Beijerinck brought to the attention of the scientific community a group of bacteria which grew anaerobically and produced a large amount of sulfide when grown on sulfate. Upon subsequent investigation it was found that the bacteria used sulfate as their terminal electron acceptor, and hence they were named "sulfate-reducing bacteria" (Postgate, 1979) or SRB.

Table 1: Cathodic depolarization theory (von Wolzogen Kuhr and van der Vlugt, 1934).

Anodic reaction: $4Fe$	⟷	$4Fe^{2+} + 8e^-$
Water dissociation: $8H_2O$	⟷	$8H^+ + 8OH^-$
Cathodic reaction: $8H^+ + 8e^-$	⟷	$8H \longleftrightarrow 4H_2$
Hydrogen oxidation: $SO_4^{2-} + 4H_2$	⟷	$H_2S + 2H_2O + 2OH^-$
Sulfide Precipitation: $Fe^{2+} + H_2S$	⟷	$FeS + 2H^+$
Hydroxide formation: $3Fe^{2+} + 6OH^-$	⟷	$3Fe(OH)_2$
Overall reaction: $4Fe + SO_4^{2-} + 4H_20$	⟷	$FeS + 3Fe(OH)_2 + 2HO^-$

In the SRB, energy is conserved via two processes. The first is substrate level phosphorylation (SLP), as for example in the final step of acetate production from acetyl ~(P) in which lactate oxidation is coupled to ATP production; the second method associates electron transport phosphorylation (ETP) with the reduction of sulfate to sulfide (Pankhania, 1988). The SRB as a group possess multiple mechanisms for coupling the oxidation of various substrates with concomitant reduction of sulfate, and oxyanions of sulfur, to ETP. While the SRB are defined by their physiological ability to use sulfate as their terminal electron acceptor, over the past fifteen years it has been shown that they can use a wide range of electron donors. It has been suggested that while initial evolutionary pressures may have been toward utilization of geologically produced substrates, subsequent evolution involved the utilization of organic substrates generated by the degradation of photosynthetic products. Today this is seen in the ability of the SRB to further oxidize reduced organic end-products, produced by fermentative bacteria from more complex organic compounds (Postgate, 1979; Pankhania, 1988; Peck Jr., 1993). Due to their diverse substrate utilization the SRB can be broadly divided into two groups:

Group I organisms which can oxidize their substrates incompletely to acetate;

Group II organisms which can oxidize their metabolites, including acetate, directly to carbon dioxide.

2.4 Hydrogen Utilization

Many of the lactate utilizing SRB in groups I and II can also use H_2 as an electron donor, *Desulfovibrio sapovorans* and *Desulfovibrio multivorans* being the two exceptions to this generalization. Most of the H_2-utilizing Group II SRB have the ability to grow autotrophically with H_2, CO_2, and sulfate, while many of the *Desulfovibrio* species in Group I are able to gain their energy solely from H_2 and sulfate via ETP. The oxidation of the H_2 in the periplasm directly generates a proton gradient, and this gradient can be

used to form ATP in the ratio of one ATP molecule per three protons (Peck Jr., 1993). However these bacteria require a carbon source in addition to CO_2 since they are not able to grow autotrophically (Pankhania, 1988).

2.5 Hydrogenase

The ability of the SRB to utilize and produce hydrogen indicates that they posses the enzyme hydrogenase. Hydrogenase enzymes catalyze the reversible reaction:

$$2\,H^+ + 2e^- \leftrightarrow H_2$$

The hydrogenase class of enzymes is widespread throughout the plant and animal kingdoms. Hydrogenases have been found in many prokaryotes, as well as some eukaryotes such as algae, protozoa and higher plants (Fauque *et al*, 1988). The presence of a hydrogenase system in many anaerobic microorganisms allows them to oxidize and produce molecular hydrogen. The concept of interspecies hydrogen transfer by which different physiological classes of bacteria are united into consortia, is founded upon this single physiological characteristic. An example of this involves the genus *Desulfovibrio* and hydrogen utilizing methanogens. The interaction can be expressed via the following chemical reactions:

Desulfovibrio:

$$2\ \text{Lactate} + 4\ H_2O \rightarrow 2\ \text{Acetate} + 2\ CO_2 + 4\ H_2$$

Hydrogen-utilizing methanogens:

$$CO_2 + 4\ H_2 \rightarrow 4\ CH_4 + 2\ H_2O$$

Net reaction:

$$2\ \text{Lactate} + 4H2O \rightarrow 2\ \text{Acetate} + CO_2 + CH_4 + 2\ H_2O$$

(Singleton Jr., 1993)

In the past the hydrogenase system was perceived as being comprised of a single enzyme responsible for both H_2 consumption and production. This enzyme was envisioned as being connected to various metabolic activities via low molecular weight electron carriers. This view was upheld even when faced with evidence of multiple hydrogenase activities on polyacrylamide gels and chromatography columns. At the time the single enzyme view persisted due to three main reasons. Firstly, the techniques employed to demonstrate multiple hydrogenase enzyme forms were prone to artifacts. Secondly, there existed no physiological rationale for the existence of multiple hydrogenase activities. Finally, hydrogenases appear active in all the standard assays for hydrogenase activity, these being; 1) the proton-deuterium exchange assay, 2) H_2 evolution from reduced methyl viologen, and 3) H_2 utilization with artificial electron acceptors (Fauque *et al*, 1988). Due to renewed interest in the physiology of hydrogenases over the past twenty years, hydrogenases have been purified to homogeneity from crude extracts of a wide number of bacteria including the SRB. While hydrogenases show wide diversity with respect to their molecular weight, specific activity, subunit structure, sensitivity to various inhibitors including O_2, electron donors and acceptors etc., they have been broadly divided into six classes based on their sequence homologies, metal content, and physiological function. The first class is made up of nine H_2-uptake membrane bound [NiFe] hydrogenases from eight aerobic, facultative anaerobic, and anaerobic bacteria. The second class contains four periplasmic, and two membrane bound H_2-uptake [NiFe(Se)] hydrogenases from the SRB. The third class is comprised of four periplasmic [Fe] hydrogenases from strict anaerobic bacteria. The fourth is made up of eight soluble hydrogenases from methanobacteria and *Alcaligenes euthropus* H16, which are capable of reducing methyl viologen, factor F_{420}-(F_{420}), or NAD. The fifth is comprised of the H_2-producing labile hydrogenase isoenzyme 3 of *Escherichia coli*, while the sixth contains two soluble tritium-exchange hydrogenases of cyanobacteria. In the case of the *Desulfovibrio* genus, class two

and class three are represented. *D. vulgaris* Hildenborough has a class three [Fe] hydrogenase, as well as a [NiFe] hydrogenase and a [NiFeSe] hydrogenase of the class two variety (Wu and Mondrand, 1993).

2.5.1 The [NiFeSe] Hydrogenase

The [NiFeSe] hydrogenases most extensively studied have been isolated from the species *Desulfovibrio desulfuricans* (Reider *et al*, 1984) and *Desulfovibrio baculatus* (Teixeira *et al*, 1985). In *Desulfovibrio vulgaris* Hildenborough, this enzyme has been found to be localized in the cytoplasm and is believed to be associated with the cytoplasmic membrane (Rohde *et al*, 1990). This enzyme has, unlike the [NiFe] and [Fe] hydrogenases, a higher activity in H_2 production than H_2 uptake. The [NiFeSe] hydrogenase is made up of two subunits approximately 57 and 31 kDa in size (Menon *et al*, 1987). While the physiological role of this enzyme is still uncertain, it has been suggested that the [NiFeSe] hydrogenase is responsible for cytoplasmic hydrogen production from organic substrates (Fauque *et al*, 1988; Rohde *et al*, 1990).

2.5.2 The [NiFe] Hydrogenase

Data from antibody cross reaction studies suggest that the [NiFe] hydrogenases represent a ubiquitous class of immunologically related enzymes (Fauque *et al*, 1988). [NiFe] hydrogenases of the type found in *Dv. vulgaris* and *Dv. gigas* are probably the most widespread among prokaryotes. In *Desulfovibrio vulgaris* Miyazaki the [NiFe] hydrogenase has been found to be comprised of two subunits of approximately 29 and 62 kDa, and has 80% homology to the [NiFe] hydrogenase of *Desulfovibrio gigas* (Deckers *et al*, 1990). Localization studies in *Dv. vulgaris* place this enzyme on the periplasmic aspect of the cytoplasmic membrane (Rohde *et al*, 1990). It is proposed by Fauque *et al*

(1988) that the [NiFe] hydrogenase activates hydrogen for the reduction of sulfite and thiosulfate.

2.5.3 The [Fe] Hydrogenase

The [Fe] Hydrogenase of *Dv. vulgaris* Hildenborough is a dimeric enzyme made up of 46 and 10 kDa subunits (Voordouw *et al*, 1985). This iron-sulfur hydrogenase contains two ferredoxin-type (4Fe-4S) clusters plus an atypical iron-sulfur centre believed to be involved in the activation of H_2. The small subunit actually codes for a peptide 13,500 daltons long (Prickril *et al*, 1986). The extra 3,500 daltons comprises a signal peptide that is thought to be cleaved during translocation across the cytoplasmic membrane to yield the mature small subunit.

The [Fe] hydrogenase is periplasmically located, and has no apparent membrane associations. This allows the [Fe] hydrogenase to be removed from the cells by washing in a Tris-HCl, Sodium Carbonate, EDTA pH 9.0 buffer solution (Van der Westen *et al*, 1978). In *Dv. vulgaris* Hildenborough much attention has been focused on this hydrogenase due to the fact that while it comprises ~0.2% of the total cell protein (Van der Westen *et al*, 1978), it is responsible for ~95% of the cells total hydrogenase activity (Badziong and Thauer, 1980). The [Fe] hydrogenase is among the most active enzymes in this group (Hoogvliet *et al*, 1988), far outstripping the activities of the [NiFe] or [NiFeSe] hydrogenases. It has recently been found that the [Fe] hydrogenase of *Dv. vulgaris* Hildenborough is regulated by the levels of free Fe^{2+} in the system. Under conditions of low Fe^{2+} (<1 ppm) availability the hydrogenase is derepressed by some, as yet unknown, mechanism (Bryant *et al*, 1993). Although this phenomenon has not been observed before in SRB, the regulation of proteins by iron is well established in other genera (Bagg and Neilands, 1987; Dai *et al*, 1992; O'Halloran, 1993).

2.6 Hydrogenase Inhibition

The hydrogenase enzymes exhibit a range of diversity with respect to inhibitors such as O_2, CO, NO, NO_2^- and C_2H_2 (Serebryakova and Gogotov, 1991). As a group the Nickel-containing hydrogenases are generally oxygen stable, that is to say they can be reversibly inactivated. The iron hydrogenase of *Dv. vulgaris* Hildenborough is not rapidly inactivated by oxygen. This fact was not recognized until 1978 when work done by van der Westen *et al* showed the [Fe] hydrogenase to be highly stable in its oxidized form. If purified under anaerobic conditions, in its reduced form, the enzyme is very unstable. The [Fe] and [NiFeSe] hydrogenases from *Dv. vulgaris* Hildenborough are also very sensitive to low levels of CO. In contrast the [NiFe] hydrogenase of *Dv. vulgaris* Hildenborough is relatively insensitive requiring 20 to 30 times the amount of CO for the same percentage of inhibition to occur (Berlier *et al*, 1987). All three enzymes are very sensitive to low levels of nitric oxide, the [Fe] and [NiFeSe] being the most sensitive of the three. Nitrite produces a very selective inhibition, affecting the [Fe] and [NiFeSe] hydrogenases. The [NiFe] hydrogenase is insensitive to the NO_2^- effects (Berlier *et al*, 1987). Acetylene produces an opposite effect on the hydrogenases. The [Fe] hydrogenase is unaffected by C_2H_2 while the nickel-containing hydrogenases are inhibited by the acetylene, [NiFe] hydrogenase being 10-50 fold more sensitive than the [NiFeSe] hydrogenase.

2.7 The Role of Hydrogenases In Corrosion

Since the 1934 work of von Wolzogen Kuhr and van der Vlugt, it has been shown that the SRB can mediate corrosion of iron either indirectly through metabolic end products such as H_2S, or via direct depolarization of cathodic hydrogen. A main player in both of these scenarios is the hydrogenase enzyme. In work done by Bryant and Laishley (1990) using mild steel rods and cell free hydrogenase extract, several interesting effects were noticed. Iron rods, in the presence of dd H_2O and under anaerobic conditions, did not produce

hydrogen gas spontaneously. An additional requirement in the form of phosphate, was required for H_2 gas to be produced (Bryant and Laishley, 1990; Bryant and Laishley, 1993). Under anaerobic conditions phosphate buffer (pH 7.0), reacted with mild steel to produce an iron/phosphate complex, subsequently identified as vivianite, and H_2 gas in an approximate ratio of 1:1 of Fe:H_2. The following chemical reaction has been proposed to account for the observations:

$$3\ Fe^o + 4\ H_2PO_4^{1-} \rightarrow Fe_3(PO_4)_2 + 3\ H_2 + 2\ HPO_4^{2-}$$

This hydrogen generating system was used to explore the biocorrosion mechanism for mild steel. When H_2 gas was being evolved from iron rods it was found that addition of either artificial or natural electron acceptors of hydrogenase, could temporarily halt H_2 gas evolution. The electron acceptors preferentially siphoned electrons from the cathodic pole of the corrosion cell until they were fully reduced, at which point H_2 gas evolution resumed. It was found that this hydrogen evolution lag due to the electron acceptors was concentration dependent, and the addition of cell free hydrogenase extract could lower the time required for electron acceptor reduction quite significantly. Finally it was observed that once the electron acceptor was fully reduced by cathodic depolarization, addition of hydrogenase to this reaction caused the enzyme to switch into its reverse catalytic mode, oxidizing the reduced electron acceptor, and producing hydrogen gas at an accelerated rate (Bryant and Laishley, 1990). As long as phosphate was present in sufficient concentration to elicit H_2 gas evolution through its reaction on mild steel, the hydrogenase enzyme did not even have to be in contact with the iron rods in order to accelerate corrosion. This effect was demonstrated through the dual flask experiments of Bryant and Laishley (1993). In these experiments dual flasks were used in which H_2 evolving metal in one flask was physically separated from a second flask containing hydrogenase. The gas phases of these two flasks were connected via glass tubing. The solubilization rate of the

metal rods, in the presence of phosphoric acid (pH 2.2), was 19% higher when active hydrogenase was present in the second flask. A similar flask setup, but minus the acid, showed no metal solubilization even when active hydrogenase was present in the second flask. This increased corrosion via hydrogenase agreed with similar type whole cell studies by Ragagopal and Le Gall (1989), and Belay and Daniels (1990).

The relationship between hydrogenase activity level in mixed populations of SRB and corrosion was studied by Bryant *et al* in 1991. In this study two mixed populations of SRB were isolated from the field. One population was taken from a corroding oil pipeline (hydrogenase positive activity), whilst the second was isolated from a non-corroding oil pipeline (hydrogenase negative activity). These two populations were used to inoculate two separate Robbin's devices. The Robbin's device, a circular tube (15 mm diameter by 1 meter) made of Admiralty brass, had cleaned, pre-weighed cylindrical steel studs inserted into sample ports, which were separated from the brass casings by rubber O rings. Medium was circulated through the devices at a flow rate of 4 liters/minute. Studs were periodically removed from the devices, and the biofilm analyzed for a most probable number (MPN) count, as well as for hydrogenase level via the semi-quantitative Caproco test. Biofilms developed in both devices, with MPN values of the corrosive population ranging from 1.14×10^4 to 1.4×10^7 organisms/0.5 cm^2 stud, and numbers of the non-corrosive population reaching 1.5×10^4 organisms/0.5cm^2. The hydrogenase activity level in the corrosive population, as measured by the Caproco© test, was high (+++) and was associated with a significantly higher corrosion rate (7.79 mm/year) than the non-corrosive biofilm (0.48 mm/year) with no measurable hydrogenase activity (Bryant *et al*, 1991).

While the link between SRB accelerated corrosion and the derepressible periplasmic [Fe] hydrogenase has been implied (Bryant and Laishley, 1990; Bryant *et al*, 1991; Bryant and

Laishley, 1993), one very important aspect of the corrosion model remains unclear. While the hydrogen film on the metal surface lies exterior to the bacterium and the [Fe] hydrogenase is located within the periplasmic space, the question remains as to how the organism strips the hydrogen from the metal surface.

2.8 The Outer Membrane

Initially the outer membrane (OM) of the gram negative bacteria was viewed as a simple lipid bilayer for protection and sieving of compounds up to ~500 daltons. Now it is considered a dynamic layer interfacing with the surrounding environment. The modern view of the outer membrane is that of a fully asymmetric bilayer, the inner layer being comprised of lipid or phospholipid, while the outer layer contains the lipopolysaccharide (LPS). Many of the outer membrane's unique barrier properties stem from the self association of the anionic LPS through divalent cation cross bridging plus the strong association of LPS with proteins (Hancock, 1991).

The outer membrane may have a very diverse amount of proteins present. Generally there are only three to eight major protein species in the OM, but these species can have 50,000 to 200,000 copies per cell, making them the most predominant proteins in the bacterium. Apart from the major protein species there may be 50 to 100 minor species also present. Many of these proteins are associated with lipid moieties and, in addition to their various functions, also play a part in anchoring the outer membrane bilayer to the underlying peptidoglycan layer (Hancock, 1991) e.g. Braun's lipoprotein.

2.9 Functions Of The Outer Membrane

2.9.1 Cell Uptake

One of the primary functions of the outer membrane is to allow the passing into the cell of a restricted variety of molecules, while at the same time excluding others. With few exceptions all hydrophilic molecules above a certain limit are excluded from gram negative bacteria. This size limit cut off varies from species to species. Many of the gram negative bacteria also exclude some hydrophobic and amphiphilic molecules.

There are two classes of uptake pathways. These pathways are of either the porin, or non-porin types and determine the selective permeation of molecules across the outer membrane. The porin class of proteins are found throughout the different gram negative bacteria, and can aggregate to form water-filled channels through which small hydrophilic molecules can pass. While most form sodium dodecylsulfate (SDS) resistant trimers, their individual molecular weights can range between 30,000 and 48,000 daltons. Porins can be either specific or non-specific. The non-specific type forms a water-filled channel through which hydrophilic molecules smaller than a given size limit can pass, thus determining the exclusion limit of the outer membrane, and the cell. They are typically only weakly ion selective, and are chemically non-selective. The specific class of porins is made up of only six examples, and of these six only the OmpP of *P. aeruginosa* and LamB of *E. coli* have been well studied. It has been suggested that the iron-regulated outer membrane proteins, which are assumed to be important in bacterial pathogenesis, may act as a specific receptor proteins for iron-siderophores or iron-transferin complexes (Hancock, 1991).

2.9.2 Cell Export

Gram negative bacteria must also be able to selectively export or excrete end products, secondary metabolites, siderophores etc., into their surrounding environment. The export

of these products must not come at the expense of letting in unwanted substances, nor must periplasmically located proteins be lost to the environment. At this point there is no well understood method by which such specific export could occur (Hancock, 1991).

2.9.3 Receptors

Since the outer membrane is the first line of interaction that the gram negative bacterium has with its surrounding environment, it is not surprising that it contains receptors for binding specific nutrients and proteins. These receptors have been placed into three classes, the first of which are the nutrient receptors. These receptors act to concentrate scarce nutrients on the surface of the cell in preparation for uptake. A second class of receptors is involved in the cross bacterial or cross species transfer of DNA, via the binding of pili involved in conjugation. While the specific receptors (Postgate, 1979) involved are unknown, LPS has been implicated in binding other types of pili. The final class of receptors are those which allow the binding of bacteriophage or bacteriocins. While having these receptors puts the cells at risk, they also play other roles in the outer membrane and are required for cell functioning.

2.9.4 Interaction with the Environment

The outer membrane by its very nature, is in close contact with the environment around the organism, even if the organism is surrounded by a capsule. It is therefore no surprise that outer membranes can interact directly with environmental surfaces. Recently the first evidence showing components of an electron transport system to be present in the OM was put forward by Myers and Myers (1992). They discovered that cytochromes were preferentially located in the outer membrane of anaerobically grown *Shewanella putrefaciens*, and that these cytochromes could shuttle electrons to externally located,

insoluble Mn (IV) and Fe (III) oxides. These oxides were being used by the bacterium as external terminal electron acceptors.

2.10 Cytochromes

Since the discovery of cytochrome C_3 in the SRB, a variety of cytochromes have been discovered. A tentative classification of these cytochromes has been suggested by Moura *et al* (1991). This classification of the sulfate and sulfur reducing bacteria is based on the number of hemes per monomer, heme axial ligation, heme spin state, and primary structures. A brief overview of the cytochrome classification system follows:

2.10.1 Monoheme Cytochromes

Cytochrome C-553 is a 9 kDa protein containing a single covalently attached heme-C group with methionine and histidine as axial ligands. C-553 has a mid point redox potential that is around 0 mV. This value is quite negative when compared to other methionine histinide-ligated monochrome cytochromes. It has been proposed that C-553 acts as a natural electron acceptor for the formate dehydrogenase system in *Dv. vulgaris* Miyazaki.

2.10.2 Diheme Cytochromes

When grown under sulfate or nitrate respiring conditions *Dv. desulfuricans* produces a 52 kDa dimeric cytochrome protein. Two C-hemes are present per 26 kDa subunit. This cytochrome has bis-histidinyl axial coordination with distinct midpoint potentials of -168 and -330 mV. The N-terminus of this protein shows no homology to any other known cytochrome

2.10.3 Triheme Cytochromes

Cytochrome C-551.5 is a triheme cytochrome isolated from the sulfur-reducing bacteria *Desulfuromonas acetoxidus.* This cytochrome, named C-7, has a molecular mass of 9 kDa. Two of the hemes have midpoint potentials of -177 mV while the third has a midpoint potential of -107 mV. This heme shows a high degree of homology with the tetraheme proteins. While the bacterium does not contain any hydrogenase, the cytochrome is still reduced by *Desulfovibrio* hydrogenase. This casts doubt on the "specificity" of cytochrome C_3 as a cofactor for hydrogenases (Moura *et al,* 1991).

2.10.4 Tetraheme Cytochromes

The 13 kDa tetraheme protein is characteristic of the family *Desulfovibrionacae*. In this protein four hemes are covalently bound to the polypeptide chain via the thioether linkages provided by cysteine residues. The axial ligands in this protein are histidines. The four hemes are localized in protein environments that are quite distinct from each other. The tetraheme cytochromes have been suggested to act as cofactors of hydrogenase and as such are required for the electron transfer to redox partners like ferredoxin, flavodoxin and rubredoxin.

2.10.5 Hexadecaheme Cytochrome

A high molecular weight cytochrome (HMC) with sixteen heme groups has been isolated from *Dv. vulgaris* Hildenborough by Yagi *et al* (Higuci *et al,* 1987). The gene encoding this large cytochrome has been cloned and sequenced. This work has revealed that the HMCholoprotein is a single polypeptide of molecular weight 65.5 kDa. Of the 16 heme groups, 15 have bis-hisdinyl coordination. When the arrangement of heme binding sites and coordinated histidines are compared between cytochrome C_3 and HMC, it appears that the HMC has three cytochrome C_3-like domains. The gene for HMC encodes for a 31

amino acid signal peptide with characteristics similar to those of *Dv. vulgaris* Hildenborough cytochromes C_3 and C553, though of greater overall length. The HMC signal sequence also differs in that its three NH_2-terminal Arginine residues are spaced out rather than adjacent, and the signal sequence peptide cleavage site is not of the A↓A form as it is in the other two procytochromes (Pollock *et al*, 1991).

Recent sequence findings show that HMC is the first protein of an operon containing a total of eight open reading frames, encoding the proteins Orf 1 to 6, Rrf 1 and Rrf 2. Orf 1 is the high molecular weight cytochrome, Orf 2 has been described as a transmembrane redox protein with four iron-sulfur clusters as determined by its similarity to DmsB from *E. coli*. Orf 3, 4, and 5 are highly hydrophobic proteins with similarities to subunits of NADH dehydrogenase, or cytochrome c reductase. Due to its similarity to the ferredoxin domain of [Fe] hydrogenase from *Desulfovibrio,* the Orf 6 protein was determined to be a cytoplasmic redox protein containing two iron-sulfur clusters. The Rrf 1 protein was identified as belonging to the family of response regulator proteins, while the function of Rrf 2 is as yet unknown (Rossi *et al*, 1993). Rossie *et al* believe that the proteins from this operon form a large redox protein complex that spans the cytoplasmic membrane (CM), and is involved in the transport of electrons across the membrane. The electrons being provided by periplasmically located hydrogenases (Rossi *et al*, 1993).

2.11 Purpose of Research

It is evident that SRB action is an important part of the anaerobic biocorrosion process, and that the hydrogenase enzyme system plays a key role (Bryant and Laishley, 1990; Bryant *et al*, 1991; Bryant and Laishley, 1993; Bryant *et al*, 1993). Our recent finding involving iron regulation of the [Fe] hydrogenase, and preferential electron siphoning from mild steel via electron carriers needed for [Fe] hydrogenase reduction activity, differs from

the current cathodic depolarization theory. The latter assumes atomic H forms a protective film on the metal surface, essentially neutralizing any further electrochemical reaction, however the SRB disrupt this delicate electrochemical balance by utilizing this hydrogen as an energy source, causing further iron oxidation in trying to maintain the initial electrochemical state.

We believe the electrons at the cathodic site on mild steel are removed via an electron carrier system located in the OM of *Dv. vulgaris*, which then interacts with the organism's periplasmic derepressed [Fe] hydrogenase to produce H_2 in the periplasmic space. This gas is then oxidized by the cytoplasmic membrane associated [NiFe] hydrogenase for energy and metabolic reducing equivalents. Therefore the purpose of this study was to isolate the outer membrane of the SRB *Dv. vulgaris* Hildenborough, under iron stressed conditions, and determine if there exists an electron carrier system capable of coupling the metals electrons, to the bacterium's derepressible periplasmic [Fe] hydrogenase, activating its reductive activity for evolving H_2.

3.0 METHODS AND MATERIALS

3.1 Culture Conditions

Cultures of *Desulfovibrio vulgaris* Hildenborough DSM 8303 were maintained in culture tubes containing lactate mineral salts medium (Pankhania et al, 1986), supplemented with 0.1 g of sterile iron powder. These tubes were incubated in a stainless steel chamber stored at 37°C. This chamber was rendered anaerobic through the use of a BBL H_2/CO_2 gas pack backed with palladium catalyst; H_2S was removed through the use of traps containing 10% wt./vol. cadmium acetate to precipitate H_2S as CdS (Laishley and Krouse, 1978). To gather enough material for experimentation, 2 L cultures of *D. vulgaris* were grown up in the following manner. All glassware used in the experiments was washed in 18N HCl and rinsed in doubly distilled (dd) H_2O, to remove possible Fe contamination from the glass surface. A 200 mL culture of lactate mineral salts medium, supplemented with 100 ppm of Fe^{2+}, in the form of $FeCl_2 \cdot 4H_2O$, was inoculated with a four day old test tube culture (10 mL). This 200 mL culture was incubated at 37°C under an atmosphere of N_2. After 48 hours this 200 mL culture was in turn used to inoculate 2 L of lactate mineral salts medium supplemented with either 5 ppm or 100 ppm of Fe^{2+}, in the form of $FeCl_2 \cdot 4H_2O$. Cultures were grown at 37°C under an atmosphere of N_2. All culture flasks were connected to bottle traps containing 10% wt./vol. cadmium acetate (Laishley and Krouse, 1978). Cultures were harvested at mid to late log phase as determined by pH, and acetate production.

3.2 Growth Analysis

3.2.1 pH Analysis

Culture medium pH was determined using a Fisher Accumet Selective Ion Analyzer model 750 pH meter.

3.2.2 Acetate Analysis

Acetate was measured by first filtering samples through a 0.45 μm-pore-size syringe filter. The filtrate (1 μL) was injected into a Perkin-Elmer 3B gas chromatograph equipped with a flame ionization detector and a 183 cm glass column containing 10% Flourad FC-431 plus 1% H_3PO_4 on a Chromosorb W-HP 80/100 mesh. The temperature of the column was maintained at 130^{o}C, and the nitrogen carrier gas flowed at a rate of 30 mL/min.

3.3 Extraction of Periplasmic Fraction

The culture was centrifuged at 15,000 x g in a Sorval RC5B centrifuge, for 20 minutes at 4^{o}C. The supernatant was discarded, the cell pellet resuspended in a small amount of fresh salts medium and centrifuged at 15,000 x g for 20 minutes. After the wash, the cell pellet was resuspended in 10 mL of 10 mM Tris (pH 8.5), 1 M NaCl to help break up extracellular polymeric substances (EPS), and the centrifugation cycle repeated as per above. The resulting pellet was resuspend in extraction buffer pH 9.0 (1 mL per 100 mL of culture spun down). The extraction buffer was comprised of 50 mM EDTA, 50 mM Tris base, and 170 mM Na_2CO_3 (Van der Westen *et al*, 1978).

The cell/buffer mixture was incubated at 37^{o}C for 30 minutes, stirring occasionally. The extraction solution was centrifuged at 20,000 rpm for 20 minutes at 4^{o}C. The supernatant containing the periplasmic fraction was decanted, and the pellet containing the cells was saved for membrane isolations.

3.4 Membrane Isolation

The isolation of the outer membrane (OM) and cytoplasmic membrane (CM) was carried out using a modification of the protocols of Booth and Curtis (1977), and Myers and Myers (1992). The pellet obtained from the hydrogenase extraction procedure was

resuspend in 10 mM Tris (pH 8.5), 1 M NaCl. To this cell suspension was added a 10% volume of disodium EDTA (20 mg/mL), a 10% volume of Lysozyme (6.4 mg/mL), and a few crystals of DNase. The mixture was shaken gently at room temperature for 30 minutes. Brij 58 (polyoxyethylene cetyl ether) was then added to a final concentration of 3%, to lyse the cells (Myers and Collins, 1987), and the mixture was incubated for 30 minutes at room temperature with gentle stirring. $MgCl_2$ was added to a final concentration of 10 mM. The resulting suspension was layered onto 5 mL of 70% sucrose, and centrifuged at 45,000 rpm in a Beckman Ultracentrifuge using a Ti 70.1 rotor, at $4^{o}C$ for 60 minutes. Due to its lower density the CM will remain suspended at the top of the 70% sucrose while the higher density OM will be pelleted out (Booth and Curtis, 1977). After centrifugation the top layer (CM) was removed and dialyzed against 10 mM Tris pH 7.5, 20 mM NaCl for 15 hours at $4^{o}C$. This fraction was subsequently centrifuged at 45,000 rpm in a Ti 70.1 rotor for 1 hour to pellet the cytoplasmic membrane.

To obtain the OM, pellets obtained from the Brij 58 treatment were re-suspended in 10 mM Tris pH 7.5, 20mM NaCl, and Triton X-100 added to a final concentration of 3%. The mixture was incubated at room temperature for 30 min while stirring gently. During this time period the Triton X-100 solubilized any remaining contaminating CM, while leaving the OM untouched (Schnaitman, 1971a; Schnaitman, 1971b). The resulting suspension was layered onto 5 mL of 70% sucrose, and centrifuged at 45,000 rpm in a Beckman Ti 70.1 rotor, at $4^{o}C$ for 60 minutes. The outer membrane pellets were resuspend in ddH_20, divided into 0.5 mL aliquots, and frozen at $-20^{o}C$.

3.5 Protein Estimation

Protein was estimated by two methods. Periplasmic extract protein concentration was measures via the Bio-Rad protein dye binding assay as outlined in Bio-Rad Technical Bulletin No: 1051.

The membrane fractions could not be measured by the Bio-Rad assay due to the interfering effects of detergents and sucrose. In these cases a modified Lowry protein assay was carried out as described by Markwell *et al* in 1978. 100 parts of Reagent A (2.0% Na_2CO_3, 0.4% NaOH, 0.16% Sodium Tartrate, and 1% SDS) were mixed with one part of Reagent B (4% $CuSO_4 \cdot 5$ H2O) to make Reagent C. Samples containing 10 to 100 µg of protein were made up in 1 mL volumes of dd H_2O. To these samples 3 mL of Reagent C were added. The samples were incubated at room temperature for 15 minutes. 0.3 mL of Folin-Ciocalteau phenol reagent (diluted 1:1 with dd H_2O just prior to use) was added to each of the tubes and mixed in vigorously. The tubes were incubated for 45 minutes at room temperature, then the optical densities measured at 660 nm.

In both cases the optical densities were measured on a Perkin-Elmer Lambda 3 spectrophotometer. Bovine serum albumin was used in construction of the standard curves.

3.6 Warburg Respirometry

3.6.1 Hydrogenase Activity Assay

Assays for hydrogenase activity of periplasmic fractions by hydrogen uptake, were carried out using a Warburg respirometer apparatus. The main reaction compartment contained 0.1 M Tris buffer (pH 8.5), 0.05 M NO_2^-, and 10 mM $MgCl_2$, while 15 mM methyl viologen (M.V.) was placed in a side arm. A volume of the periplasmic fraction to be

tested, containing 10 µg of protein was added to the main reaction chamber. These flasks were attached to manometers and incubated at 37°C. Prior to the commencement of any reactions, the vessels were gassed with high purity H_2 for 15 minutes. After the flask was sealed, the reaction was started by tipping the M.V. from the side arm into the main vessel. One unit of enzyme activity was defined as one µmole of H_2 uptake per minute per mg of protein. All reactions were monitored by standard manometric techniques (Umbriet *et al.* 1959). To test the inhibitory effect of NO_2^- on the periplasmic [Fe] hydrogenase, NO_2^- was added to the main reaction chamber to a final concentration of 50 mM, and reacted in the manner described above. Since NO_2^- selectively inhibits the [Fe] and not the [NiFe] hydrogenase, any hydrogenase activity in the presence of NO_2^- was attributed to the [NiFe] hydrogenase (Berlier *et al*, 1987).

3.6.2 Detection of Enzymatic Marker for CM and OM

Warburg vessels were set up to test for the [NiFe] hydrogenase, a known cytoplasmic membrane marker (Rohde *et al*, 1990). The main reaction compartment contained 0.1 M Tris buffer (pH 8.5), 0.05 M NO_2^-, and 10 mM $MgCl_2$, while 15 mM methyl viologen was placed in a side arm. A volume of the CM or OM fraction to be tested, containing 75 µg was added to the main compartment, and the vessel gassed under H_2 for 15 minutes at 37°C. After the flask was sealed, the reaction was started by tipping the M.V. from the side arm into the main vessel. One unit of enzyme activity was defined as one µmole of H_2 uptake per minute per mg of protein. Warburg vessels to test for total hydrogenase activity were set up in a similar manner, but with the [Fe] hydrogenase inhibitor NO_2^- replaced by dd H_2O.

3.6.3 Investigation of the Effect of the Isolated Outer Membrane Fraction on Mild Steel

The phosphate buffer/mild steel H_2 generation system developed by Bryant and Laishley (1990) was used to observe the effects of the isolated OM on the production of H_2. The Warburg vessels contained 2.2 mL of 0.1 M phosphate buffer and 3 mild-steel rods in the center well, while the OM fraction was placed in the side arm. The vessel was incubated under an atmosphere of N_2 at 37°C for 15 minutes before the vessel was sealed, and readings commenced. Outer membrane was tipped from the side arm of the Warburg vessel into the reaction mixture after 50 minutes. This time period allowed the metal to equilibrate to a constant hydrogen production rate, before addition of the outer membrane. Gas production during this time period was compared to a control containing mild steel rods and phosphate buffer, but no OM. As an added control a Warburg flask containing mild-steel rods, 2.2 mL of de-ionized H_2O, and OM in the side arm, was also run to check if OM could elicit hydrogen production from metal without the action of phosphate.

3.7 SDS-PAGE Analysis of Isolated Membrane Fractions

Samples of isolated CM and OM preparations Isolated from 5 ppm and 100 ppm iron cultures were mixed with Laemmli sample buffer and boiled at 100°C for 5 minutes. Samples were analyzed using the Phastsystem™ (Pharmacia LKB Biotechnology Inc.). The gels had an acrylamide gradient of 8 to 25%, and were stained for total protein via Coomassie Brilliant Blue R250, or a combination of Coomassie followed by silver staining, for more dilute samples. Protein standards run on the gel were Gibco BRL Protein Molecular Weight Standards; Myosin (H-chain) (200 kDa), Phosphorylase B (97.4 kDa), Bovine serum albumin (68 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (29 kDa), β-Lactoglobulin (18.4 kDa), and Lysozyme (14.3 kDa).

3.8 Detection of Cytochromes in OM Fractions Via Heme Staining

Heme containing proteins were detected on 10% SDS-PAGE through the use of the hemes peroxidase activity. A 6.3 mM solution of 3,3',5,5'-tetramethylbenzidine (TMBZ) was prepared in methanol. Immediately prior to use 3 parts of the TMBZ solution was added to 7 parts of 0.25 M sodium acetate (pH 5.0). The gel was immersed in this mixture at room temperature, in the dark, for several hours. H_2O_2 was added to a final concentration of 30 mM (Thomas *et al.*, 1976). A positive band test was indicated by a blue colour reaction. To stop the reaction, and for short term storage, the gel was moved to a container containing dd H_2O.

3.9 Photography of Protein Gels

All photography was carried out using Ilford XP2™ 400 ASA black and white film. The camera was a Canon™ Eos™ A2E, and the lens was an Ultrasonic™ 28-105, 1:3.5/4.5. Gels to be photographed were placed on a bottom illuminated light table, and photos were taken with 2 to 3 stops compensation, to adjust for the light table intensity.

4.0 RESULTS

4.1 Isolation of *D. vulgaris* Cytoplasmic and Outer Membranes

D. vulgaris cultures were grown in 2 L of Lactate mineral salts medium supplemented with 5 and 100 ppm ferrous iron (Bryant *et al*, 1993), and harvested at late log phase as indicated by their acetate production and medium pH profiles (Figure 1). The isolation of the two membranes and periplasmic fraction from these cultures was outlined in Figure 2. Furthermore the [NiFe] hydrogenase known to be located in the cytoplasmic membrane, was chosen as the marker for this membrane fraction. The isolated fractions were measured for hydrogenase activity as shown in Table 2. Nearly all the hydrogenase activity (>95%) was located in the periplasm (Supernatant I), which agrees with the findings of Badziong and Thauer (1980), while minor amounts of the hydrogenase activity occurred in the membrane fractions.

Further characterization of the hydrogenase activities in these fractions was performed in order to determine the percentage of [Fe] to [NiFe] hydrogenase activity. To differentiate between these two hydrogenases, nitrite inhibition studies were conducted. It has been reported that NO_2^- is a potent inhibitor of the [Fe] hydrogenase while having no apparent effect on the [NiFe] hydrogenase (Berlier *et al*, 1987). After treatment with the NO_2^-, any remaining hydrogenase activity was assumed to be the [NiFe] hydrogenase. The data presented in Table 3 shows that the activities in both the periplasm and the OM (Pellet III) was mainly due to the [Fe] hydrogenase (~99% and 94% of activity respectively). Only the cytoplasmic membrane (Supernatant II) showed a significant proportion of activity due to the [NiFe] hydrogenase. In this case approximately 55% of the measured activity was attributed to the [NiFe] hydrogenase. This asymmetric distribution of the [NiFe] enzyme supports previous findings that associate the [NiFe] hydrogenase with the cytoplasmic

Figure 1: Acetate and pH profiles of *Desulfovibrio vulgaris* Hildenborough cultures growing on lactate mineral salts medium supplemented with either 5 or 100 ppm Fe^{2+}.

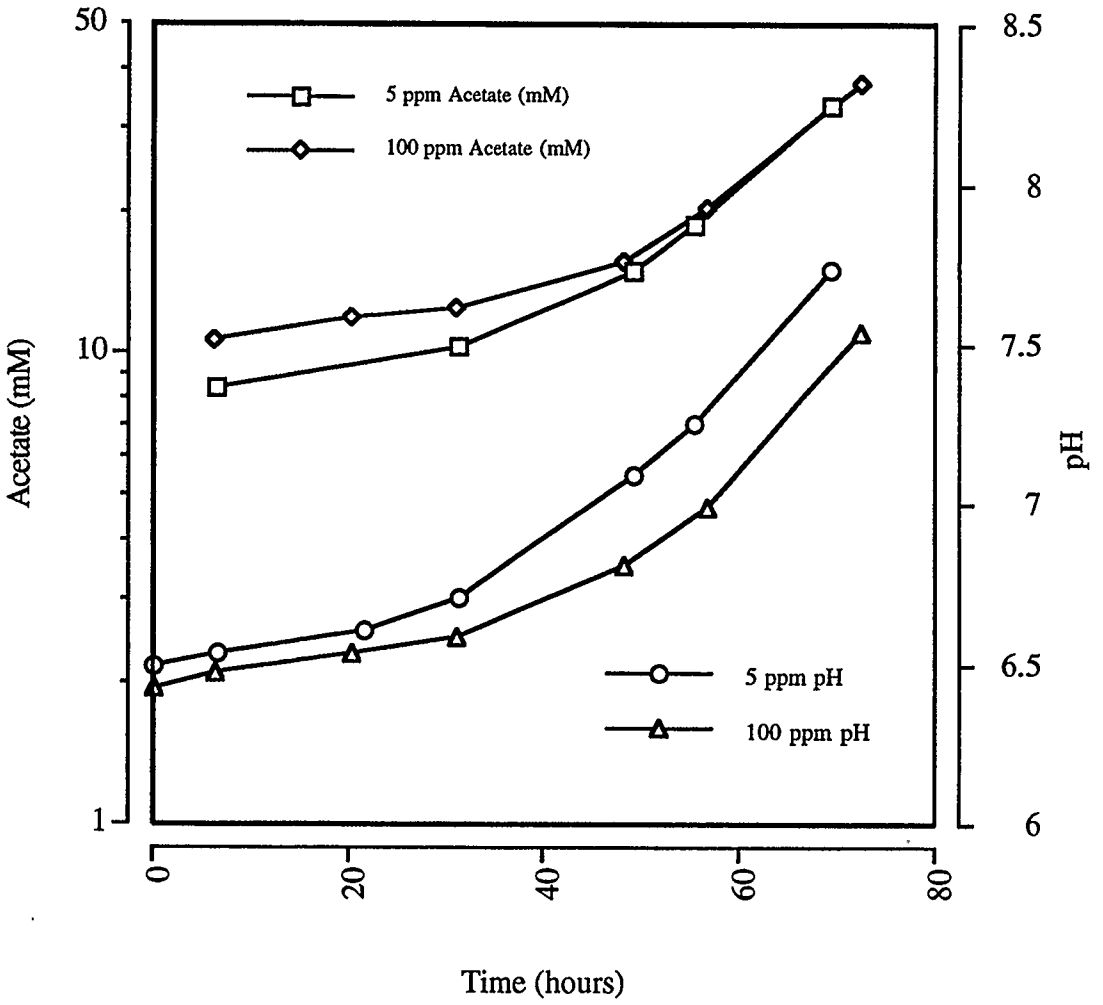

50
10
1
Acetate (mM)
8.5
8
7.5
7
6.5
6
pH
5 ppm Acetate (mM)
100 ppm Acetate (mM)
5 ppm pH
100 ppm pH
0
20
40
60
80
Time (hours)

Figure 2: Flow chart schematic for isolation of outer membrane, cytoplasmic membrane, and periplasmic fractions from *Desulfovibrio vulgaris* Hildenborough.

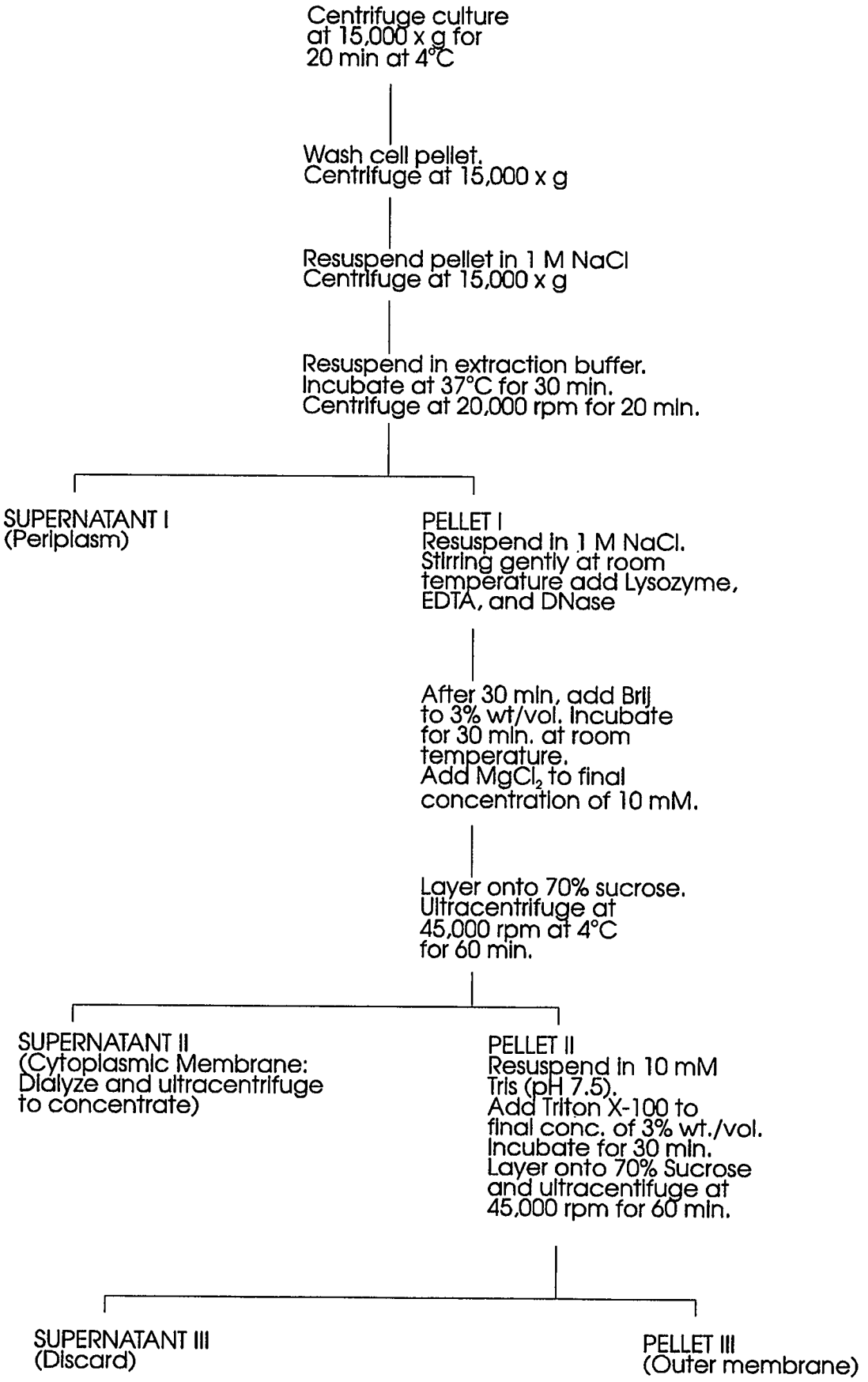

Centrifuge culture at 15,000 x g for 20 min at 4°C
Wash cell pellet. Centrifuge at 15,000 x g
Resuspend pellet in 1 M NaCl Centrifuge at 15,000 x g
Resuspend in extraction buffer. Incubate at 37°C for 30 min. Centrifuge at 20,000 rpm for 20 min.
SUPERNATANT I (Periplasm)
PELLET I Resuspend in 1 M NaCl. Stirring gently at room temperature add Lysozyme, EDTA, and DNase
After 30 min, add Brij to 3% wt/vol. Incubate for 30 min. at room temperature. Add $MgCl_2$ to final concentration of 10 mM.
Layer onto 70% sucrose. Ultracentrifuge at 45,000 rpm at 4°C for 60 min.
SUPERNATANT II (Cytoplasmic Membrane: Dialyze and ultracentrifuge to concentrate)
PELLET II Resuspend in 10 mM Tris (pH 7.5). Add Triton X-100 to final conc. of 3% wt./vol. Incubate for 30 min. Layer onto 70% Sucrose and ultracentifuge at 45,000 rpm for 60 min.
SUPERNATANT III (Discard)
PELLET III (Outer membrane)

Table 2: Distribution of hydrogenase activity in the isolated cytoplsmic membrane, periplasm, and outer membrane fractions of *D. vulgaris* Hildenborough.

Fraction*	Volume (mL)	Protein (mg/mL)	Total Protein (mg)	Hydrogenase Specific Activity†	Total Activity‡	% Total Hydrogenase Activity
Cytoplasmic Membrane (Sup II)	1.0	5.8	5.8	2.12	12.30	0.4
Periplasm (Sup I)	20.0	0.85	17	181	3077	99.1
Outer Membrane (Pellet III)	2.0	10.2	20.4	0.80	16.32	0.5

* Fractions were isolated from cells grown in 2 L of Lactate mineral medium. Sup: Supernatant (Figure 2).

† Expressed as μmoles of Hydrogen uptake min^{-1} mg^{-1}

‡ Expressed as μmoles of Hydrogen uptake min^{-1}

Table 3: The apparent % of [Fe] and [NiFe] hydrogenase activity in the isolated cytoplasmic membrane, periplasm, and outer membrane fractions of *D. vulgaris* Hildenborough as determined by NO_2^- inhibition.

Fraction	Hydrogenase Specific Activity†		Apparent % Hydrogenase Activity	
	minus NO_2^-	plus NO_2^-‡	[Fe]	[NiFe]*
Cytoplasmic Membrane	2.12	1.17	44.8	55.2
Periplasm	181.00	2.15	98.8	1.2
Outer Membrane	0.8	0.048	94.0	6.0

† Expressed as μmoles of Hydrogen uptake min^{-1} mg^{-1}

‡ 50 mM NO_2^-

* Calculations for NO_2^- inhibition data, assuming NO_2^- inhibited [Fe] hydrogenase activity and had no effect on the [NiFe] hydrogenase.

membrane (Rohde *et al*, 1990). These results confirm our use of the [NiFe] hydrogenase as a marker for the CM. When the membrane fraction [NiFe] hydrogenase activities are compared (Table 3) it is seen that the [NiFe] hydrogenase activity is 24 fold higher in the CM compared to the OM. While the small amount of [NiFe] hydrogenase activity present in the OM reflects a slight cross contamination, the membrane isolation protocol that was developed did separate the majority of the CM from the OM. These results are also in agreement with other investigators who have shown that the majority of the CM of other genera can be isolated in Supernatant II as indicated in Figure 2 (Hancock and Nikaido, 1978; Schnaitman, 1981).

4.2 SDS-PAGE Analysis of the Isolated Cytoplasmic and Outer Membrane Fractions of *D. vulgaris*

In comparing the protein profiles of the OM (Lane A) and CM (Lane B) fractions isolated from cells grown on 5 ppm Fe^{2+} by SDS-PAGE, major and minor protein band differences can be observed in Figure 3. These data validates the isolation procedure developed for these two different membrane fractions. The OM of cultures grown under 5 and 100 ppm of Fe^{2+} was next examined to see if the iron concentration had any effect on the OM protein composition. Striking differences of the OM protein profiles are shown by SDS-PAGE (Figure 4), between the 100 ppm Fe^{2+} (Lane A) and 5 ppm Fe^{2+} (Lane B) growth conditions. The protein bands at ~77.5 (top arrow head) and 50 kDa (bottom arrow head) in Lane B, were very much reduced in stain intensity under 100 ppm iron conditions (Lane A), with the 77.5 kDa band being almost absent. Protein bands above 78 kDa were also absent under 100 ppm iron conditions.

The presence of heme-containing proteins was tested on the 5 ppm Fe^{2+} isolated OM fraction, by the method of Thomas *et al* (1976) employing a 10% isocratic SDS

Figure 3: SDS-polyacrylamide gel electrophoresis (8-25%) of OM (A) and CM (B) fractions isolated from 5 ppm Fe^{2+} cultured *Desulfovibrio vulgaris* Hildenborough. Membrane fractions containing 1.3 μg of protein was applied per lane. Staining was carried out by Coomassie blue followed by Silver staining. Numbers on the right indicate molecular weight markers in kDa.

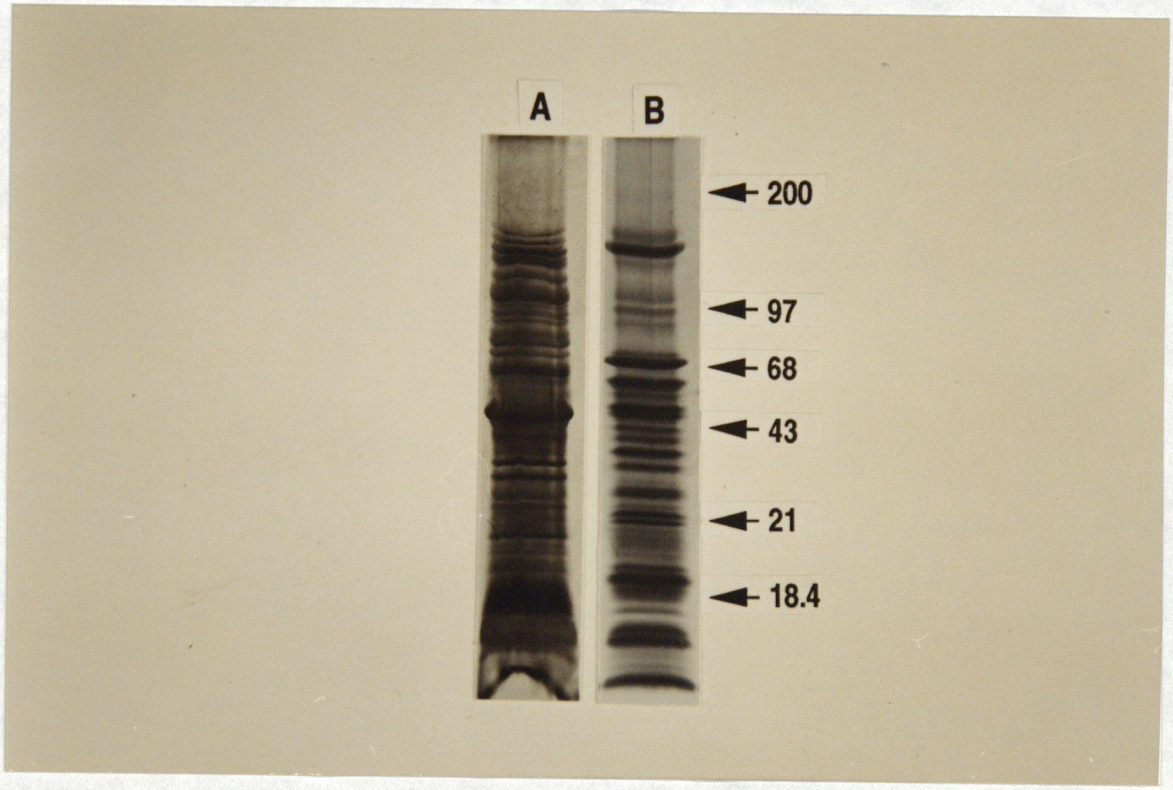
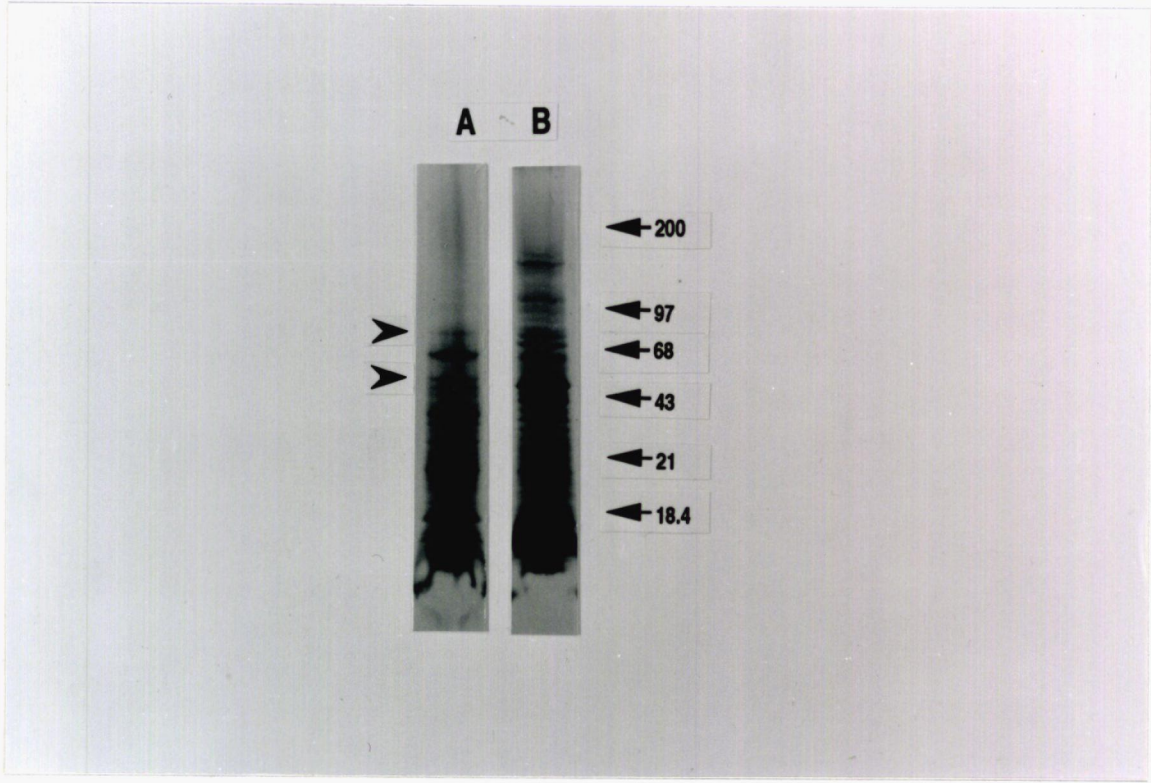

A
B
200
97
68
43
21
18.4

Figure 4: Comparison of SDS-polyacrylamide gel electrophoresis (8-25%) protein band profiles of OM fractions isolated from *Desulfovibrio vulgaris* Hildenborough cultures, supplemented with 5 or 100 ppm Fe^{2+}. OM fractions, lane A (5 ppm Fe^{2+}) and lane B (100 ppm Fe^{2+}), were applied at 0.4 μg of protein per lane. Staining was carried out by Coomassie blue followed by Silver staining. Numbers on the right indicate molecular weight markers in kDa.


A
B
200
97
68
43
21
18.4

acrylamide gel exposed to TMBZ and H_2O_2. Figure 5 shows that two protein bands in the OM fraction reacted positively to the heme specific stain (lane A), giving a blue colour reaction. A comparison was made to a second 10% isocratic SDS acrylamide gel which had been run under identical conditions, and subsequently stained for total protein (lane B). The first heme containing protein correlated with the 77.5 kDa band (top arrowhead), while the second matched the 62.5 kDa band (bottom arrowhead). This positive heme test indicates the presence of two different high molecular weight cytochromes in the OM. Difficulties were encountered when trying to carry out heme staining on 100 ppm Fe^{2+} isolated OM. Low protein concentration, combined with a large amount of FeS contamination, prevented the testing for heme staining bands in this fraction.

4.3 Effect of Outer Membrane on Hydrogen Evolution System

The role of the newly found HMC in the isolated OM fraction from the 5 ppm Fe^{2+} grown cells, was investigated by using the phosphate/mild steel hydrogen generation system of Bryant and Laishley (1990). Upon addition of the OM fraction to the phosphate/mild steel hydrogen evolution system, an immediate acceleration of hydrogen gas production was observed (Figure 6). In contrast the experiment where the phosphate buffer was replaced by dd H_2O, the OM addition did not elicit hydrogen evolution from the mild steel even though it contained the cytochromes and [Fe] hydrogenase. This study showed again that phosphate was necessary to chemically react with the mild steel for hydrogen evolution (Bryant and Laishley, 1990; Bryant and Laishley 1993).

4.4 Analysis of SDS-PAGE of 5 ppm and 100 ppm Periplasm

The results of SDS-PAGE of periplasm isolated from *D. vulgaris* under high and low iron conditions are shown in Figure 7. Under low iron (5 ppm) conditions, strong bands are seen at ~46 kDa and ~13.5 kDa (Lane A), which represent the two subunits of the

Figure 5: SDS-polyacrylamide gel electrophoresis (10%) of the isolated OM from *Desulfovibrio vulgaris* Hildenborough grown on 5 ppm Fe^{2+}. Heme staining was carried out using TMBZ and H_2O_2 (A), staining for total protein was carried out using Coomassie blue (B). Outer membrane fractions were applied at 100 μg of protein per lane. Numbers on the right indicate molecular weight markers in kDa.

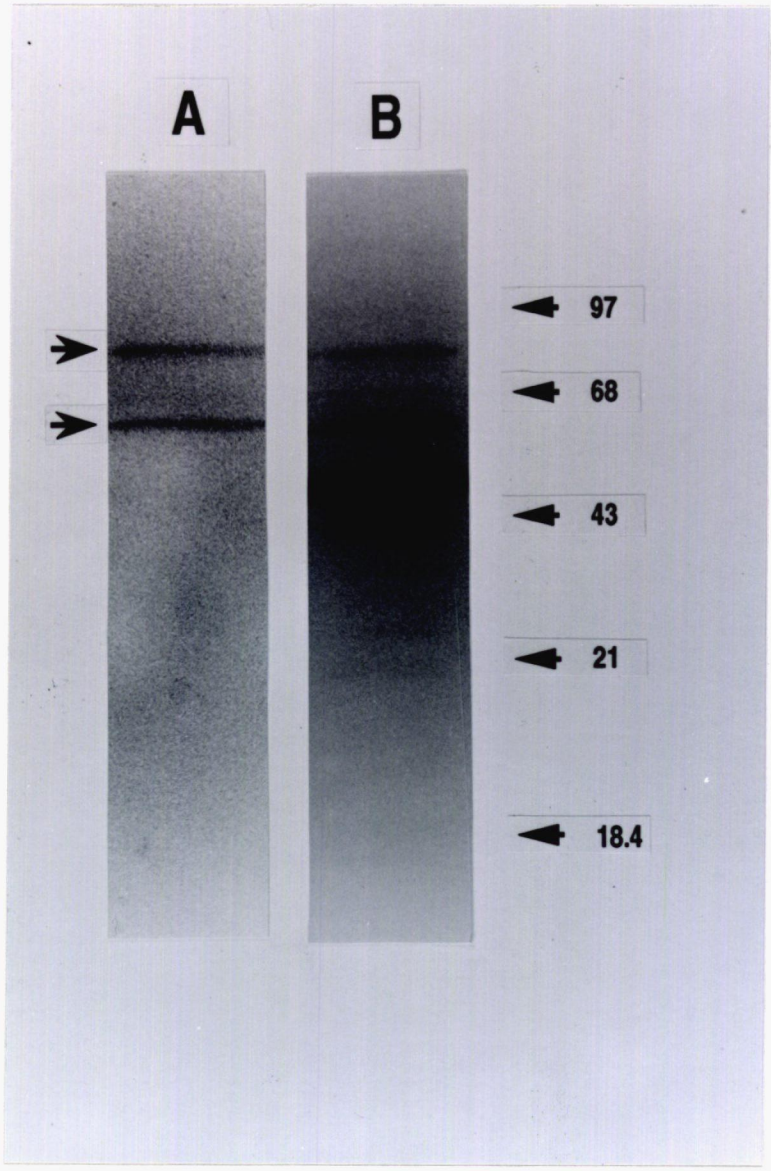

A
B
97
68
43
21
18.4

Figure 6: The effect on the phosphate buffer/mild steel hydrogen evolution system by the isolated OM fraction (100 µg of protein), from *Desulfovibrio vulgaris* Hildenborough grown on 5 ppm Fe^{2+}.

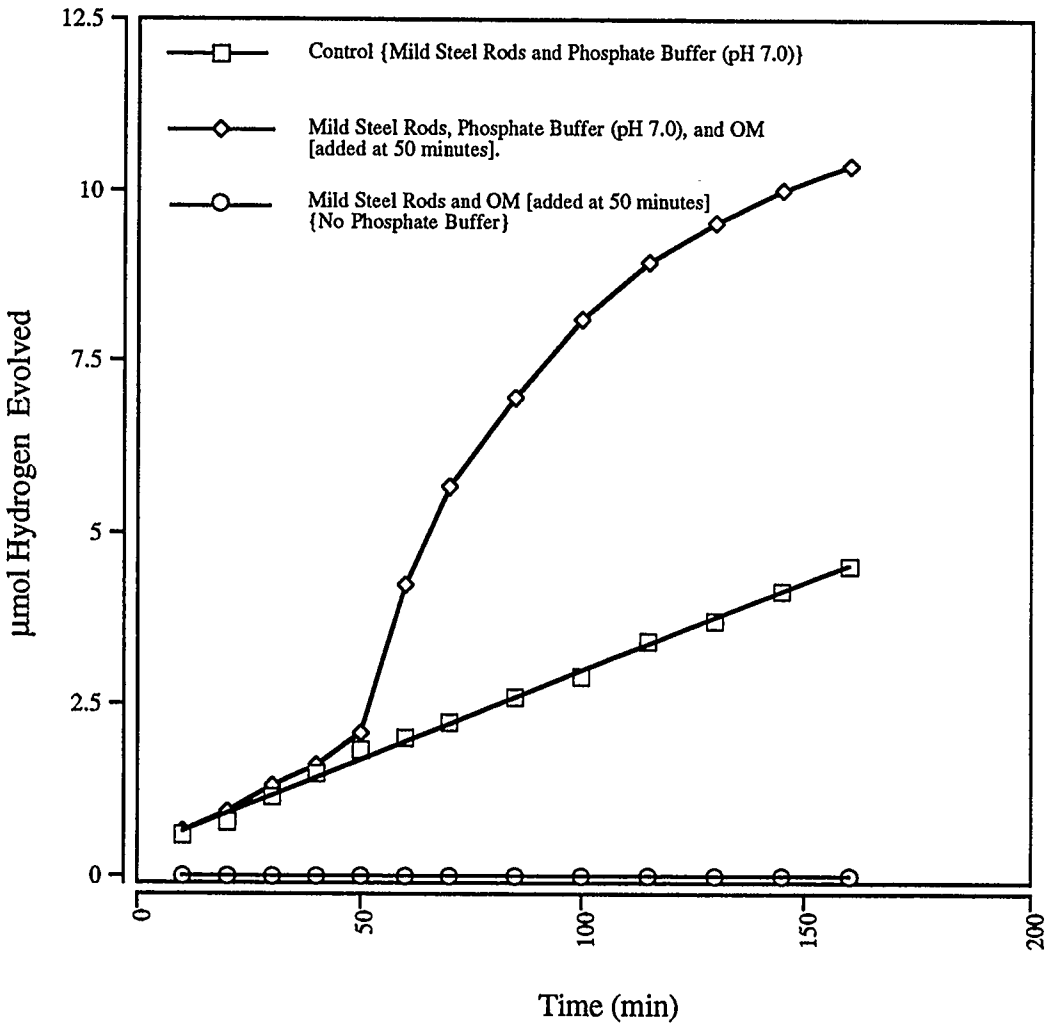
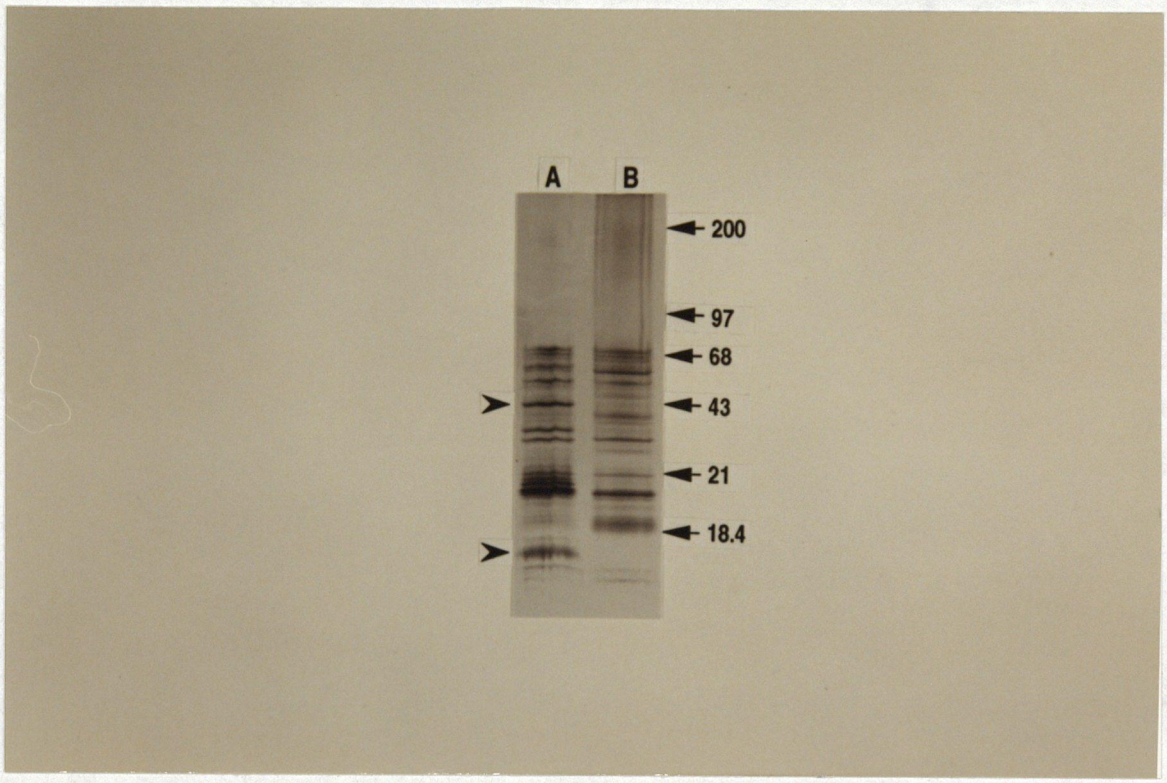

12.5
10
7.5
5
2.5
0
µmol Hydrogen Evolved
Control {Mild Steel Rods and Phosphate Buffer (pH 7.0)}
Mild Steel Rods, Phosphate Buffer (pH 7.0), and OM [added at 50 minutes].
Mild Steel Rods and OM [added at 50 minutes] {No Phosphate Buffer}
0
50
100
150
200
Time (min)

Figure 7: Comparison of SDS-polyacrylamide gel electrophoresis (8-25%) protein band profiles of extracted periplasmic fractions from *Desulfovibrio vulgaris* Hildenborough, grown on 5 ppm Fe^{2+} and 100 ppm Fe^{2+}. Periplasmic fractions A (5 ppm Fe^{2+}) and B (100 ppm Fe^{2+}) were applied at 31 ng of protein per lane. Staining was carried out by Coomassie blue followed by Silver staining. Numbers on the right indicate molecular weight markers in kDa.


A
B
200
97
68
43
21
18.4

derepressable [Fe] hydrogenase (actual molecular weights of 46 and 10 kDa respectively). These bands are not observed under the high iron (100 ppm) conditions (Lane B, Figure 7). Other differences are also noted between the high and low iron conditions. Protein bands with higher expression under low iron are also seen at ~38 kDa, 25.5 kDa, and 24.5 kDa, while under high iron a band with higher expression appears at ~18 kDa. These results demonstrate protein regulation by different Fe^{2+} concentration. The function of these iron regulated proteins is as yet unknown.

5.0 DISCUSSION

This is the first report on the isolation and partial characterization of a SRB's outer and cytoplasmic membranes. These membrane fractions were isolated from *Desulfovibrio vulgaris* Hildenborough employing a modified procedure of Booth and Curtis (1977), and Myers and Myers (1992). A major problem encountered in this membrane isolation was the removal of the FeS product associated with the cell surfaces. The FeS, which was produced during growth, initially interfered with the separation of the two membranes during the sucrose density gradient step. This problem was overcome by treating the cells with 1 M NaCl, a chaotropic agent, at the appropriate stages in the procedure (Figure 2). This treatment was very effective on cells grown with low iron, but less effective with the high iron grown cells.

Desulfovibrio vulgaris contains three hydrogenases, [Fe], [NiFe], and [NiFeSe], in various amounts and localized in special cell areas. The periplasmic [Fe] hydrogenase represents the major proportion of hydrogenase activity in the cell, approximately 95%, with the remaining activity mainly associated with the cytoplasmic membrane bound [NiFe] hydrogenase, and a minor presence due to the [NiFeSe] cytoplasmic hydrogenase (Badziong and Thauer, 1980). Thus the cytoplasmic membrane fraction could be detected from the others by the [NiFe] hydrogenase activity level, as was the case described in Table 3.

Membrane fractions from *Desulfovibrio vulgaris* Hildenborough, grown under high (100 ppm Fe^{2+}) and low (5 ppm Fe^{2+}) iron conditions, were isolated and partially characterized by hydrogenase content and SDS-PAGE analysis. It was found that the CM marker [NiFe] hydrogenase was 24 fold greater in the cytoplasmic membrane fraction than in the outer membrane fraction (Table 3). The particular membrane fractions identified in

this isolation procedure agreed with other investigators (Myers and Myers, 1992) using a similar type protocol (Figure 2). The modified membrane isolation technique was judged successful, however a small amount of cross contamination in the outer membrane fraction from the other fractions, was evident based on enzymatic data (Table 3). In addition the SDS-PAGE results comparing the cytoplasmic and outer membrane fractions supports the isolation of different membrane fractions, by showing major differences in their protein banding patterns (Figure 3). Of particular interest are the three protein bands in the outer membrane (low iron) located at 77.5 kDa, 62.5 kDa, and 50 kDa. The 77.5 kDa and 62.5 kDa protein bands were identified to be high molecular weight cytochromes by heme specific staining reactions (Figure 5). The 62.5 kDa protein band is in the same range as the known HMC (65.5 kDa) of *Desulfovibrio vulgaris* Hildenborough (Pollock *et al*, 1991), and may possibly be that cytochrome. Further analysis would be necessary to confirm it. When the high and low iron SDS-PAGE banding patterns for the outer membranes proteins were compared (Figure 4), the 77.5 kDa and 50 kDa had increased stain intensities inferring increased synthesis of these proteins under low iron growth conditions. It suggests that these two outer membrane proteins are under Fe^{2+} regulation control, possibly in a similar manner as the periplasmic [Fe] hydrogenase (Bryant *et al*, 1993).

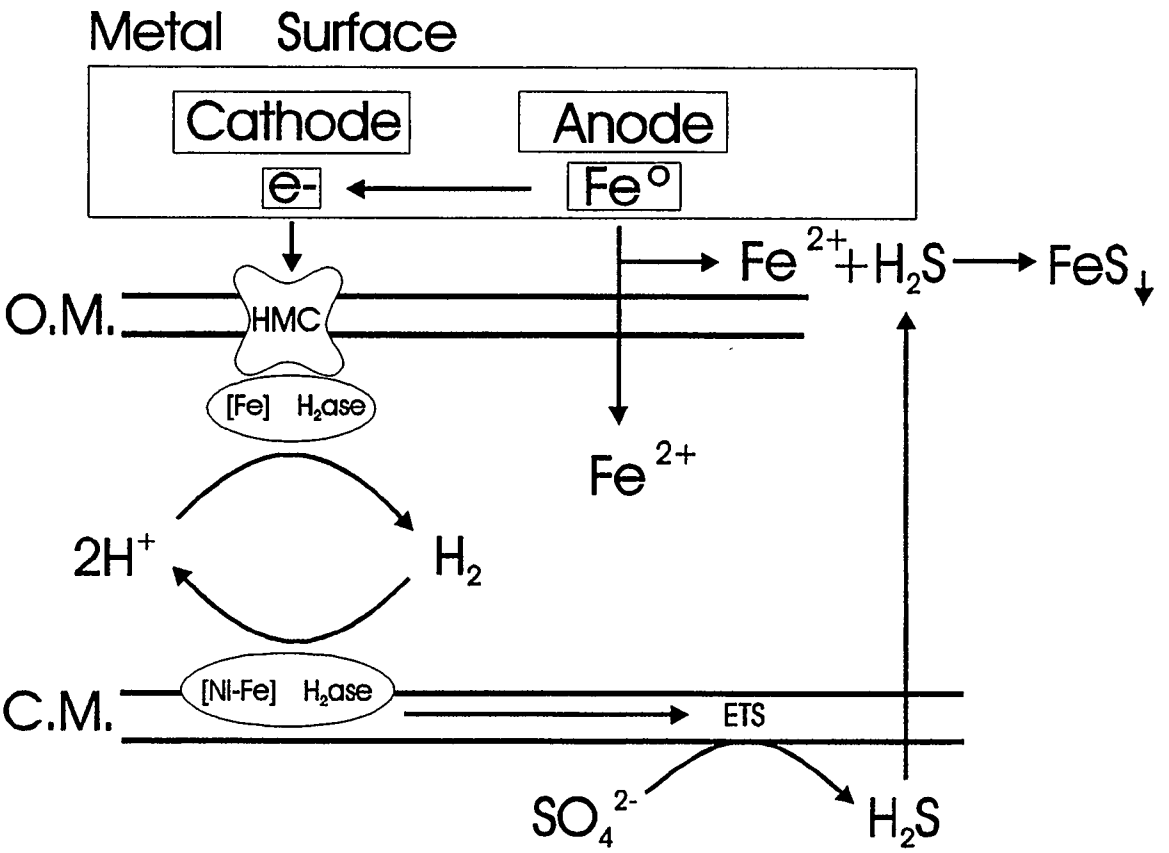
SDS-PAGE analysis of isolated periplasmic fractions from *Desulfovibrio vulgaris* Hildenborough grown under different iron conditions (Figure 7), showed that the [Fe] hydrogenase sub units (46 kDa and 10 kDa), were derepressed under low iron and nearly absent under high iron growth conditions. This observation is in agreement with the iron regulation of this enzyme reported by Bryant *et al* (1993). Several other proteins of low molecular weight (~38 kDa, 25.5 kDa, and 24.5 kDa), were also found to derepress under iron stress conditions, however the function of these iron regulated proteins is not known.

It is obvious that Fe^{2+} regulation plays a major role in controlling important metabolic and physiological activities of this organism.

The outer membrane fraction containing the newly found HMC's (Figure 5), when added to the phosphate/mild steel H_2 generating system, caused an immediate acceleration in H_2 evolution (Figure 6). Bryant and Laishley (1990) using the same H_2 generating system, reported that electron carriers required for hydrogenase activity could preferentially siphon electrons from the metal surface curtailing H_2 production, or in the presence of hydrogenase cause significant increase in H_2 evolution. Also, Rossi *et al* (1993) suggested that *Desulfovibrio vulgaris* HMC (65.5 kDa) should be able to couple with the [Fe] hydrogenase as three of its four heme domains are classified as being of the cytochrome C_3 type, a known physiological electron carrier for this enzyme. Therefore a plausible explanation for the outer membrane action on the metal is a preferential electron siphoning, via HMC's coupling with the contaminating [Fe] hydrogenase to cause this accelerated H_2 production. Whether one, or both of these HMC's play a major role in shuttling electrons across the outer membrane remains to be determined by using purified HMC proteins in the test system. In contrast, the outer membrane caused no H_2 evolution from the metal when dd H_2O replaced the phosphate buffer in the test system, even though the membrane fraction contained the biological components for H_2 generation. This indicated another factor was necessary to kick start the electrochemical reaction on the metal before significant cathodic depolarization was promoted by the outer membrane fraction. Once again, phosphate was shown to initiate this anodic/cathodic process on mild steel (Bryant and Laishley, 1990; Bryant and Laishley, 1993), which could be greatly enhanced by *Desulfovibrio vulgaris*'s outer membrane fraction.

These findings are translated into a new concept for biocorrosion as outlined in Figure 8, in which Fe^{2+} regulation plays a major role in controlling the synthesis of key proteinsassociated with *Desulfovibrio vulgaris*'s outer membrane and periplasm, for generating H_2 and its utilization by the cytoplasmic membrane bound [NiFe] hydrogenase, for reducing equivalents involved in metabolic reduction processes. The present depolarization theory suggests bacteria like the SRB disrupt a protective hydrogen layer on the metal surface via the hydrogenase enzyme. This implies that the organisms hydrogenase would have to be localized on the OM's outer surface, to utilize the surface hydrogen. However SRB hydrogenases have only been found in the periplasm, cytoplasmic membrane, and cytoplasm (Fauque *et al*, 1988), and our data also shows the overwhelming amount of hydrogenase was present in the periplasmic space (Table2, Figure 7). The proposed model suggests an alternative mechanism to this conundrum, and may offer an explanation for situations where large SRB populations with low hydrogenase levels, resulted in low metal corrosion rates, whereas SRB containing high hydrogenase levels were associated with severe metal corrosion (Bryant *et al*, 1991; Cord-Ruwisch and Widdel, 1986). More research is required to determine if this proposed biocorrosion model operates in other SRB.

Figure 8: Proposed biocorrosion model for cathodic electron depolarization of mild steel by *Desulfovibrio vulgaris* Hildenborough.


Metal Surface
Cathode
Anode
e-
Fe°
Fe 2+ + H2S → FeS↓
O.M.
HMC
[Fe] H2ase
Fe 2+
2H+
H2
C.M.
[Ni-Fe] H2ase
ETS
SO4 2-
H2S

6.0 SUMMERY

The sulfate-reducing bacteria, of which *Desulfovibrio vulgaris* Hildenborough is a member, have been associated with the corrosion of mild steel (Bryant *et al*, 1991; De Araujo-Jorge *et al*, 1992). A protocol was developed whereby the outer membrane could be isolated and partially characterized. The isolated outer membrane fractions from cultures grown under high (100 ppm) and low (5 ppm) Fe^{2+} conditions were compared by SDS-PAGE analysis and showed that several protein bands were derepressed under the low iron conditions, most notably protein bands at 50 kDa, and 77.5 kDa. Outer membrane isolated from 5 ppm Fe^{2+} cultured cells was found to contain two proteins, 77.5 kDa and 62.5 kDa, that reacted with a heme specific stain and were referred to as high molecular weight cytochromes. Studies were conducted to examine the interaction of isolated 5 ppm Fe^{2+} outer membrane fraction on the hydrogen evolution system of Bryant and Laishley (1990). Addition of the outer membrane fraction to this system was found to cause an immediate acceleration of H_2 evolution. Periplasmic fractions isolated from cultures grown under high (100 ppm) and low (5 ppm) Fe^{2+} conditions were compared via SDS-PAGE. Several of the protein bands, including those corresponding to the [Fe] hydrogenase subunits, were found to derepress under low iron conditions. Fe^{2+} regulation plays a major role in controlling important metabolic and physiological activities of this organism. A new model for the anaerobic biocorrosion of mild steel is proposed.

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