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Utilization of cathodically-produced hydrogen from mild steel by *Desulfovibrio* species with different types of hydrogenases

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SUMMARY

Desulfovibrio (D.) vulgaris Hildenborough with a highly active Fe-containing periplasmic hydrogenase, *D. salexigens* British Guiana with a Fe-Ni-Se periplasmic hydrogenase, and *D. multispirans* with a Fe-Ni cytoplasmic hydrogenase utilized cathodically-produced hydrogen from mild steel as the only energy source for activity and growth. Changes on the mild steel surface occurred during growth of these bacteria. The concentration of iron sulfide, a corrosion product of mild steel, increased over time, and *Desulfovibrio* species had an active hydrogenase when they were grown in lactate/sulfate media. This hydrogenase may be any of the three types found in the genus, *Desulfovibrio*. The concentration of iron in the media affected the production and activity of the Fe-hydrogenase from *D. vulgaris* Hildenborough. With an iron-limited medium, the specific activity and the total amount of the periplasmic hydrogenase was less than found with a non-iron limited media.

INTRODUCTION

Many species of sulfate-reducing bacteria in the genus *Desulfovibrio* can use hydrogen as an energy source, with acetate and CO_2 as carbon sources and sulfate as terminal electron acceptor [28]. It is not surprising then that some species of *Desulfovibrio* can use cathodically-generated hydrogen from metal as the sole electron source for the reduction of sulfate [13], as an energy source for limited growth [20], or as a supplementary energy source for increased reduction of sulfate [8]. This removal of hydrogen by sulfate-reducers from the surface of a metal could be an essential step in the corrosion process, e.g. cathodic depolarization [27] or alternative cathodic depolarization [15].

Recent work on species of *Desulfovibrio* has demonstrated that members of this genus may contain three types of hydrogenases [13]. These are classified by their metal reaction centers: (1) iron, (2) iron-nickel, and (3) iron-nickel-selenium. These enzymes have been found in the cytoplasm, periplasmic space, and attached to the membrane [13].

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Desulfovibrio species are found at the surface of metals undergoing corrosion [7,12], but it has not been determined if the utilization of cathodically-produced hydrogen is related to the type of hydrogenase or to its location in the bacterial cells.

An important corrosion product is iron sulfide. The amount of this corrosion product can be determined by measuring the amount of sulfide in the iron sulfide precipitate. Iron sulfide measurement has been used to determine corrosion of mild steel [8]. The formation of iron sulfide can depend upon *Desulfovibrio* species having hydrogenase [26]. However, the effect of different types of hydrogenases and their locations in the cell on iron sulfide formation is unknown.

Sulfate-reducing bacteria of the genus *Desulfovibrio* have an exceptionally high requirement for iron [22]. However, anaerobic environments around corroded metals may be low in iron because sulfide forms a strong bond with iron. Iron may then be a limiting factor in growth of the bacteria and may affect production of hydrogenase enzyme.

This paper describes the utilization of cathodically-produced hydrogen from mild steel as the sole energy source of well characterized sulfate-reducing bacteria, which have any of the three types of hydrogenase [11]. In addition, it was found that more iron sulfide was formed in lactate media with bacterial species having active

hydrogenases than those not having active hydrogenases. It was also found that iron affects the production and activity of the periplasmic hydrogenase of *D. vulgaris* Hildenborough.

MATERIALS AND METHODS

Growth of Desulfovibrio species. *D. vulgaris* strain Hildenborough (NCIB 8303), *D. salexigens* strain British Guiana (NCIB 8403) and *D. multispirans* (NCIB 12078) were grown anaerobically at 32°C in acetate/sulfate medium with H_2 generated from mild steel coupons as the electron source. The medium contained in g/l: sodium acetate, 3; Na_2SO_4, 4; NH_4Cl, 2; KH_2PO_4, 0.5; $CaCl \cdot 2H_2O$, 0.035; $FeCl_2 \cdot 4H_2O$, 0.012; $MgSO_4 \cdot 7H_2O$, 2; EDTA, 0.002; and oligoelement solution, 3 ml [1]. *D. multispirans* was also grown with nitrate instead of sulfate as electron acceptor, using $NaNO_3$, 4 g/l and $Mg(NO_3)_2 \cdot 6H_2O$, 2 g/l. For *D. salexigens* 3% NaCl was added to the medium. Medium was added to serum bottles containing argon and a 5-g acid cleaned, mild steel (type 304) coupon. The pH of the medium was adjusted to and maintained at 7 with $NaHCO_3$. Sodium sulfide additions lowered the redox potential of the medium to about −200 mV. Cathodically-produced hydrogen was measured by gas chromatography, in the gas phase of the serum bottles. Total volume of the gas space was 45 to 50 ml. Hydrogen in the gas phase is reported as mmoles H_2/total volume gas phase.

Cells were initially grown on acetate/sulfate or acetate/nitrate medium with hydrogen gas (80% H_2/20% CO_2) added as an electron donor. A 1% (v/v) bacterial inoculum was added to serum bottles containing the steel coupon. Activity and growth of the bacteria was determined by measuring the decrease in hydrogen, and by microscopic counts of cells in the media. Precipitate on the coupon was examined for viable cells by incubating the precipitate in lactate-sulfate medium [25]. The coupon surfaces were examined by optical microscopy by Dr. D. Feron, CEA, La Hague, SECA-LETC, Cherbourg, France.

To determine the effect of the different types of hydrogenase on metal corrosion, measured as iron sulfide production, *D. vulgaris*, *D. salexigens*, *D. multispirans* and *Desulfolobus (Db.) elongatus* were grown anaerobically for up to 168 h in lactate/sulfate medium [25] containing an acid-cleaned mild steel upon (3.3 g). The quantity of residual iron from the medium components was less than 0.1 mg/l (< 2 μmol/l) as measured by plasma emission spectroscopy. Therefore, most of the iron in iron sulfide was from the dissolution of the mild steel coupon. The amount of iron sulfide was determined at 46 and 168 h, and was correlated to the hydrogenase activity determined in the different species of bacteria. Growth of the bacteria was measured at 540 nm, after acidifying the bacterial-medium to remove the interference of iron sulfide on absorbancy. Para-aminobenzoic acid was added as a growth factor for *Db. elongatus* [22]. The bacteria were initially grown in lactate-sulfate medium without added iron.

Different concentrations of iron in lactate-sulfate medium [25] were examined for the effect on hydrogenase activity in *D. vulgaris* Hildenborough grown for 18 and 40 h. The concentrations of iron in the media were 0.5, 1, 1.5, and 2 mg Fe/l. In some experiments, 50 μmol EDTA was added to the media. The periplasmic hydrogenase was separated from the cells by the freeze-thaw procedure [3] and cells were then lysed. Total hydrogenase consisted of enzyme from both the lysed cells and the periplasmic fraction.

Hydrogenase from *D. vulgaris* was purified from the periplasm of cells grown for 38 h with low (0.5 mg Fe powder/l) and high (2 mg Fe/l) iron concentrations. The enzyme was purified in three steps using DEAE Biogel and hydroxylapatite column chromatography [14].

Measurement of iron sulfide. Iron sulfide in the reaction vials was determined after centrifuging the vials, pouring off the supernatant fluid, gassing the vials with argon, and then measuring the amount of iron sulfide in the precipitate. This was done by acidifying the precipitate, releasing sulfide [16] which was then measured by the methylene blue method of Siegel [24]. Iron sulfide is reported as μmol S^{2-}/l.

Hydrogenase activity and metal determinations. Hydrogenase activity was measured at 32°C either by the hydrogen evolution assay from dithionite-reduced methyl viologen as described by Peck and Gest [21] using an Aerograph A-90 P3 gas chromatograph as described by Chatelus et al. [6], or by hydrogen consumption with benzyl viologen as electron acceptor using Warburg respirometry [2]. One unit of hydrogenase activity is defined as the amount of enzyme which catalyzes the evolution or consumption of 1 μmol H_2/min and the specific activity is expressed in μmol/min/mg protein.

Protein was determined by a modification of the Lowry method as proposed by Markwell et al. [18]. Iron was determined by plasma emission spectroscopy using a Jarrel-Ash Model 75 Atomcomp.

Optical and EPR spectra. Ultraviolet and visible absorption spectra were recorded on a Beckman DU 7 spectrophotometer. EPR measurements were made with a Varian E-109 Spectrometer as previously described [14].

Electrophoresis and molecular weight determination. Purity of the hydrogenase was established by polyacrylamide disc gel electrophoresis [5]. Subunit structure and molecular weight were determined by SDS–polyacrylamide gel electrophoresis [10].

RESULTS AND DISCUSSION

Growth on hydrogen generated from metal surfaces

Desulfovibrio vulgaris with a highly active Fe-containing periplasmic hydrogenase, *D. salexigens* with a Fe-Ni-Se periplasmic hydrogenase, and *D. multispirans* with a Fe-Ni cytoplasmic hydrogenase utilized cathodically-generated hydrogen from mild steel coupons. Hydrogen was utilized within one to 4 days (Table 1). Media without the bacteria but with the coupon showed a steady increase in hydrogen (Table 1). Comparisons of hydrogen utilization among the different species of bacteria were not made because of differences in initial hydrogen concentrations. These differences may reflect the length of time the metal coupons were in the media prior to inoculation with bacteria. Growth was determined by counting the numbers of bacteria. All the species of *Desulfovibrio* grew with greatest growth found with those species having the highest initial concentration of hydrogen in the vial. After utilization of hydrogen, the number and activity of the cells in the media decreased. *D. vulgaris* and *D. multispirans* (sulfate- or nitrate-grown) decreased rapidly until none were found. However, some *D. salexigens* cells were active (motile) in the medium even when there was no detectable level of hydrogen in the gas phase. Motility of these bacteria was found in the media 30 days after the experiment began. Since the only source of energy for the cells was hydrogen generated from the coupon, *D. salexigens* appears to be more efficient in hydrogen uptake than the other *Desulfovibrio* species.

The periplasmic hydrogenase in anaerobic bacteria has been proposed to be a specific adaptation for utilization of low levels of hydrogen for activity and growth [3,17,19]. The metal composition of this enzyme appears not to be important as *Desulfovibrio* bacteria with Fe or Fe-Ni-Se periplasmic hydrogenases were able to utilize hydrogen for growth. Hydrogen utilization by *D. multispirans* was unexpected as this bacterium has so far been found to have only a cytoplasmic hydrogenase [9]. Cytoplasmic hydrogenases are thought to be involved in the oxidation of reduced electron transfer proteins leading to the production of hydrogen [19]. Utilization of hydrogen would indicate that the hydrogenase is bidirectional. This would necessitate a strict specialization of electron transfer proteins and carriers for the directional regulation of the enzyme as proposed for *D. gigas* [11].

Scraping off the black precipitate which had formed on the metal coupons during the growth of bacteria on sulfate, and incubating it in lactate/sulfate media, resulted in the immediate growth of the *Desulfovibrio*. *D. multispirans* with nitrate as electron acceptor formed a reddish-brown precipitate. Viable cells could be isolated from this precipitate.

Micrographic examination of the coupons revealed deterioration on the surface, seen as a discontinuous multiple layer of corrosion products, 5–10 μm in depth. Removal of the corrosion layer revealed a rough metal surface. Coupons in media without bacteria exhibited some roughness on the surface, but not as great as coupons in media with bacteria. Gaylarde and Johnson [12] showed that biocorrosion occurs only when bacteria are in contact with metal surfaces. The fact that the bacteria were viable in the precipitate and that changes occurred on the surface indicated that the surface changes were due to bacterial metabolic processes, such as hydrogen uptake by bacterial hydrogenase and sulfide formation by sulfate reduction.

Table 1

Utilization of cathodically-produced H_2 by *Desulfovibrio* species

Time (days)	*D. vulgaris* H_2 (mmol/TV)[a]		*D. multispirans* H_2 (mmol/TV)[a]		*D. salexigens* H_2 (mmol/TV)[a]		Control H_2 (mmol/TV)[a] (no bacteria)
	a	b	SO_4^{2-}	NO_3^-	a	b	
0	150	250	17.2	49	3.6	6.9	5.2
1	nd	nd	0.1	37	0	0	5.7
2	55	82	0	15	0	0	5.7
3	nd	nd	0	5	nd	nd	nd
4	2	2	nd	0	nd	nd	10.4
7	0	0			nd	nd	11.3
12	0	0			0	0	15.7

Cells were grown in acetate/CO_2 medium with SO_4^{2-} and NO_3^- as electron acceptors and cathodically generated hydrogen from mild steel as energy source.

[a] H_2 (mmol/TV): H_2 in the gas space above the medium; nd, not determinated; a and b are results of duplicate experiments.

Table 2

Corrosion measured as 'FeS' formation and the hydrogenase activity of 2 genera of sulfate-reducing bacterial species

Bacteria	Growth Abs[a] (540 nm)		'FeS' μmol S^{-2}/l		Hydrogenase μmol/min/Abs[a] cells			
					evolution		consumption	
	46 h	168 h	46 h	168 h	46 h	168 h	46 h	168 h
D. vulgaris	0.7	0.62	60	102	13	10	9	6
	0.7 (63 h)	0.64 (144 h)	101	188	7	4	5	2
D. salexigens	0.8	0.34	65	96	16	26	13	6
D. multispirans	0.66	0.42	19	172	14	7	4	5
Db. elongatus	0.6	0.43	79	75	not found		not found	

The bacteria were grown on lactate/sulfate media at 32 °C. Iron sulfide was acidified which released sulfide that was measured by the methylene blue method [34]. Hydrogenase activity (evolution and consumption of H_2) was determined with lysed cells as found in Materials and Methods.
Abs[a], absorbance.

Hydrogenase activity and iron sulfide production

Iron sulfide was present in all the cultures after 46 h (Table 2). However, at 168 h, the concentration of iron sulfide had increased only in those bacterial cultures having active hydrogenases (Table 2). These bacteria exhibited hydrogenase activity in both evolution and consumption. The active hydrogenases may be located in the cytoplasm as observed with *D. multispirans*, or in the periplasm as observed with *D. vulgaris* and *D. salexigens*. In addition the chemical composition of the hydrogenases appears not to be important, since increase in iron sulfide formation was observed with *Desulfovibrio* species having highly active Fe, Fe-Ni or Fe-Ni-Se hydrogenases.

The amount of iron sulfide formed in lactate/sulfate media with a metal coupon but without bacteria was 1 μmol/l after 46 h, and 4 and 19 μmol/l after 168 h.

After 168 h there was no increase, and even a decrease, in turbidity in cultures compared to 46 h (Table 2). This decrease in growth may be due to depletion of nutrients or toxic products of metabolism. However, iron sulfide levels increased (Table 2). Chatelus et al. [6] observed that even when *D. vulgaris* Hildenborough was not viable, the hydrogenase may still be active under reduced conditions. Therefore, depolarization of a metal surface by hydrogenase could occur. The same phenomenon could play a part in the increase in iron sulfide observed in this experiment.

There was no direct correlation between the amount of hydrogenase activity, as either hydrogen production or consumption, and iron sulfide formation.

Iron and hydrogenase activity

When the amount of iron in the medium was decreased from 2.0 to 0.5 mg/l, there was a corresponding decrease in the level of hydrogenase activity of *D. vulgaris* Hildenborough as measured by hydrogen production (Table 3). Postgate [22] found that the content of hydrogenase in *D. desulfuricans* was also lower in iron-starved cells. However, decreasing iron in the medium from 2 to

Table 3

Effect of iron on the hydrogenase from *D. vulgaris* Hildenborough

Iron (mg/l)	Hours of culture growth	Periplasmic hydrogenase activity[a]	Total hydrogenase activity[a]
0.5	18	30	95
0.5	40	36	73
1	40	1123	1406
1	40[b]	1645	1730
1.5	40	1121	1226
2	18	1005	1404
2	39	2618	2728
2	40[b]	4210	4333

Cells were grown on lactate-sulfate medium with the appropriate amount of added iron. Initial iron concentration in the medium was less than 0.1 mg/l, as measured by plasma emission spectroscopy. At least 2 g (wet weight) of cells were used for each experiment.
[a] Activity expressed in units/g.
[b] 50 μmol EDTA added.

0.5 mg/l did not affect the total amount of cell mass produced, as 600 g of cells were formed with either iron concentration. The decrease in hydrogenase activity in *D. vulgaris* was almost entirely in the periplasmic fraction (Table 3). Hydrogenase activity in the cytoplasmic and membrane fractions (total hydrogenase activity minus periplasmic hydrogenase activity) was affected only slightly by the amount of iron in the medium. The decrease in periplasmic hydrogenase was a result of a lesser amount of enzyme in the cell (50 mg enzyme/600 g wet wt. at 0.5 mg Fe/l versus 142 mg enzyme/600 g wet wt. at 2 mg Fe/l in media) with less specific activity (275.3 at 0.5 mg Fe powder/l versus 4800 at 2 mg Fe powder/l). This indicates that a very active periplasmic hydrogenase may not play an important part in the metabolism of these organisms when growing on lactate/sulfate medium.

However, periplasmic hydrogenase is important in utilizing low levels of hydrogen from the environment [3]. If iron is limiting, utilization of hydrogen from the metal surface and, therefore, depolarization of the surface may decrease. Decrease in polarization has been seen in long term corrosion experiments. The reason given for this decrease was sulfide covering the metal surface, thus preventing the electrochemical process [4]. The addition of iron is believed to stimulate corrosion rates by removing the sulfide film from the metal surface [4,26]. However, our results indicate that iron is important for full production and activity of the Fe-containing periplasmic hydrogenase, permitting utilization of hydrogen and causing depolarization of the metal surface.

Periplasmic hydrogenase activity was also dependent upon the length of cell incubation time. With 2 mg Fe/l, and a chelating agent in the medium to keep the iron in solution, hydrogenase activity reached maximum values in about 39–40 h.

Purification and characterization of low activity periplasmic hydrogenase in D. vulgaris

The periplasmic hydrogenase of *D. vulgaris* Hildenborough grown with 0.5 mg Fe/l was purified and partially characterized. Total activity of hydrogenase in the periplasmic fraction prepared by the freeze-thaw method was 13437 μmol of H_2 produced/min/600 g wet wt. of cells. The enzyme (13437 units) was purified in three steps using DEAE Biogel and hydroxylapatite column chromatography (Table 4). The purity index (ratio of $A_{400\ nm}/A_{280\ nm}$) was 0.36 and the final specific activity was 275.3 μmol H_2 evolved/ min/mg protein. This corresponds to about 50 mg of hydrogenase per 600 g wet wt. of cells. Hydrogenase did not exhibit a lag phase before maximum activity occurred. The enzyme was brown in color with a typical iron-sulfur-containing protein absorption spectrum. Non-denaturing gel electrophoresis revealed only one band and SDS electrophoresis showed two bands, one with a MW of 50000 Da and the other with a MW of 10500 Da. Plasma emission spectroscopy indicated the presence of only iron in the purified fraction, about 13 ± 2 irons/molecule of enzyme. EPR spectroscopy of the "as isolated" enzyme gave an isotropic $g = 2.02$ signal; no EPR signal for nickel was found (J.J.G.Moura, CQE-IST, New University of Lisbon, Lisbon, Portugal, personal communication). A high activity periplasmic hydrogenase was isolated from bacteria grown with 2 mg Fe/l and the amount of hydrogenase was 142 mg enzyme/600 g wet wt., with a specific activity of 4800 units/mg protein.

The low activity periplasmic hydrogenase appears to be similar in molecular weight, subunit structure and iron content to the high activity periplasmic hydrogenase [14]. The reason for the low specific activity of the periplasmic hydrogenase is unknown.

These results show that Fe, Fe-Ni, or Fe-Ni-Se hydrogenases in sulfate-reducing bacteria of the genus *Desulfovibrio* utilize cathodically-produced hydrogen from metal, causing changes in the metal surface. These changes may involve iron dissolution from the mild steel, the iron combining with sulfide from sulfate reduction to form iron sulfide. Iron sulfide increased only with those sulfate-reducers having an active hydrogenase of any of the types found in the genus *Desulfovibrio*. One such hydrogenase,

Table 4

Purification of a low activity periplasmic Fe-hydrogenase from *D. vulgaris* Hildenborough

Fraction	Protein (mg)	Total activity (μmol H_2/min)	Specific activity[a] (μmol H_2/min/mg)	$A_{400\ nm}/A_{280\ nm}$
Wash from cells	4827	13437	2.78	nd
DEAE Biogel A column I	1523	6242	4.1	nd
Hydroxylapatite column	47.2	2204	46.7	0.26
DEAE Biogel A column II	4.9	1349	275.3	0.36

[a] In H_2 evolution. nd, not determined.

Fe-hydrogenase, is affected by iron concentration. Low amounts of iron in the medium resulted in a decrease in the production and the activity of this hydrogenase in the cell.

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