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The relationship between hydrogen metabolism, sulfate reduction and nitrogen fixation in sulfate reducers

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SUMMARY

Hydrogenase and nitrogenase activities of sulfate-reducing bacteria allow their adaptation to different nutritional habits even under adverse conditions. These exceptional capabilities of adaptation are important factors in the understanding of their predominant role in problems related to anaerobic metal corrosion. Although the D_2-H^+ exchange reaction indicated that *Desulfovibrio desulfuricans* strain Berre-Sol and *Desulfovibrio gigas* hydrogenases were reversible, the predominant activity in vivo was hydrogen uptake. Hydrogen production was restricted to some particular conditions such as sulfate or nitrogen starvation. Under diazotrophic conditions, a transient hydrogen evolution was followed by uptake when dinitrogen was effectively fixed. In contrast, hydrogen evolution proceeded when acetylene was substituted as the nitrogenase substrate. Hydrogen can thus serve as an electron donor in sulfate reduction and nitrogen metabolism.

INTRODUCTION

Sulfate-reducing bacteria are important in marine environments where they play a major role in the decomposition of organic compounds and the anaerobic corrosion of iron and steel [8]. The physiology of these bacteria is complex and involves several biochemical pathways, some of which are not fully elucidated [12,18]. Three major characteristics are exhibited by most sulfate-reducing bacteria: sulfate reduction including several steps leading to sulfide formation and a number of unique redox proteins [11,12]; hydrogen metabolism catalyzed by one or more hydrogenases [15a]; and most generally a nitrogenase activity responsible for dinitrogen fixation and also for an irreversible hydrogen production [13].

The relationship between hydrogenases and nitrogenase is well documented. In bacteria having an aerobic metabolism, such as *Azotobacter* [21] or *Azospirillum* [14], hydrogen evolved via nitrogenase activity or of exogenous origin can be recycled and

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serve as an electron-donor in nitrogen fixation [4,22]. In bacteria following a fermentative metabolism, such as *Clostridium* [16] or *Klebsiella* grown under strictly anaerobic conditions [9], hydrogenase activity results in hydrogen production which, in the absence of electron acceptors other than protons, releases excess reducing power from the cells. With anaerobic bacteria endowed with an oxidative metabolic pathway with an electron acceptor such as nitrate [10] or sulfate, the situation is more complex, since they are able to either evolve or consume hydrogen according to their nutritional status and to the electron source available [1].

The present work shows that hydrogen metabolism turns to either production or uptake according to the sulfate level and to the nitrogen nutritional status. Hydrogen can act as an electron donor and as an energy source when carbon substrate is limiting.

MATERIALS AND METHODS

The strains used were *Desulfovibrio desulfuricans* strain Berre-Sol (NCIB 8388) and *D. gigas* (NCIB 9332). The bacteria were either subcultured daily in batch culture or grown continuously in a chemostat with complete Starkey's medium [19] with ammonia as nitrogen source plus 0.1% (w/v) of yeast extract under an atmosphere of argon or nitrogen. When necessary they were adapted to diazotrophic conditions by eliminating combined nitrogen from the renewal medium entering the chemostat (save for 0.01% (w/v) of yeast extract to provide vitamins) and keeping an atmosphere of pure N_2.

For experiments with resting cells, cultures kept under anaerobic conditions were centrifuged (10 min at 7000 $\times$ *g*) then resuspended in one-tenth of their initial volume of 0.35 M NaCl or of lactate-free, sulfate-free and eventually nitrogen-free medium. The suspension thus obtained was used to inoculate the appropriate medium for further experiments.

Kinetic experiments on hydrogen evolution or uptake, and on dinitrogen or acetylene reduction, were performed in penicillin vials or serum vials under the appropriate atmosphere. Hydrogen production or uptake and acetylene reduction to ethylene were followed by gas-chromatography [3]. Nitrogen fixation was measured with aliquots of cultures as the total amount of ^{15}N incorporated during the incubation period. The D_2-H^+ exchange activity was determined in 50 mM Tris-HCl buffer at pH 7.6 by a direct mass-spectrometric technique with a membrane inlet system [2]. All the results were expressed as specific activity (micromoles per milligram of cell protein).

RESULTS

Desulfovibrio gigas and *Desulfovibrio desulfuricans* strain Berre-Sol were able to grow either with combined nitrogen in the medium or under diazotrophic conditions, but with different growth parameters (Table 1). For both species the generation time was approximately doubled and the growth yield was halved under diazotrophic conditions. This phenomenon has been previously described in a study of *D. desulfuricans* strain Berre-Sol grown in the same conditions [13].

The hydrogenase specific activities of whole resting cells prepared from each species were practically the same in the two types of nitrogen nutrition. In the absence of redox mediators the deuterium-proton exchange gave different values for the H_2/HD ratio for the two *Desulfovibrio* species (Table 2). With *D. desulfuricans* strain Berre-Sol growing cells the exchange rate was unaffected by acetylene but the initial velocity was decreased about 4-times by 9 mM carbon monoxide (1%) (Fig. 1).

Different experimental conditions were used to compare H_2 production and H_2 uptake activities of the two *Desulfovibrio* strains. Hydrogen uptake was increased in the presence of an electron acceptor such as sulfate for *D. gigas* (Fig. 2) or of combined nitrogen with *D. desulfuricans* strain Berre-Sol (Fig. 3).

In contrast, the presence of lactate as an electron-donor decreased but did not suppress H_2 uptake. The H_2 partial pressure corresponding to an equilibrium between H_2 production and uptake was

Table 1

Growth parameters of *D. desulfuricans* strain Berre-Sol and *D. gigas* grown on lactate-sulfate medium in the presence of combined nitrogen or under diazotrophic conditions

Strain	N source	Final O.D. at 450 nm	Generation time (h)	Growth yield (g/mol)
D. desulfuricans	ammonia	1.40[b]	4.3	6.3
	dinitrogen	0.4[a]	9.5	3.0
		0.8[b]		
D. gigas	ammonia	1.35[b]	5.8	4.2
	dinitrogen	0.35[a]	13	N.d.[c]
		0.75[b]		

[a] Continuous culture.
[b] Batch culture.
[c] N.d., not determined.

in fact very low. In the presence of lactate and in the absence of ammonium it established itself around 15 μM in the uptake of added H_2 (Fig. 3) as well as in the production from a zero level (Fig. 4). The latter case, i.e., the absence of nitrogen metabolism, was the only one in which H_2 production was observed.

An interesting pattern was observed under diazotrophic conditions (Fig. 4). As long as no nitrogenase activity was exhibited (lag-time in nitrogen fixation), hydrogen production occurred, but as soon as dinitrogen was being effectively fixed, the H_2 previously evolved was consumed. The concomitant kinetics of dinitrogen fixation appeared as biphasic, with an initial faster component leading to a linear part. In contrast, when the substrate for nitrogenase was acetylene instead of dinitrogen, H_2 evolution proceeded even after nitrogenase activity was established and the concomitant C_2H_2 reduction was linear. Acetylene also had an inhibitory effect upon hydrogen uptake by *D. gigas* resting cells [20] (Fig. 2).

Table 2

Comparison of the D_2-H^+ exchange activity of *D. desulfuricans* strain Berre-Sol and *D. gigas* cells after sparging by 20% deuterium.

The reported values represent initial velocities (expressed in μmol/min/mg protein).

	D. desulfuricans	*D. gigas*
D_2 uptake	7.00	11.30
HD evolution	2.95	9.70
H_2 evolution	2.60	4.70
H_2/HD	0.88	0.48

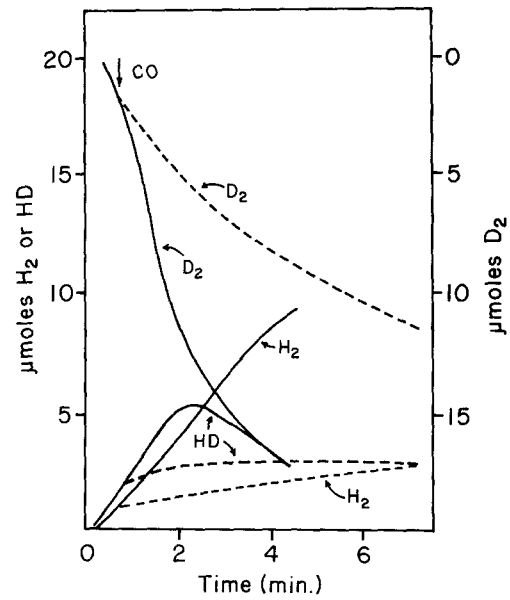

Fig. 1. D_2-H^+ exchange activity of *D. desulfuricans* strain Berre-Sol growing cells sparged with 20% D_2. Solid line: without addition of CO. Dashed line: with 9 mM CO added at 30 s.

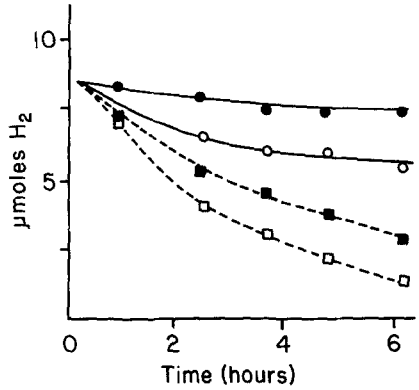

Fig. 2. Effect of sulfate and acetylene on hydrogen uptake by resting cells of *D. gigas*. Solid line: without added sulfate. Dashed line: with 30 mM sulfate. Open symbols: without addition of acetylene. Closed symbols: with 15% acetylene added in the atmosphere.

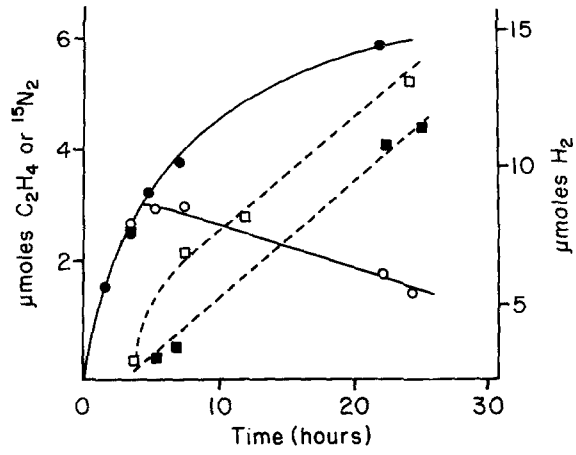

Fig. 4. Hydrogenase activity and nitrogen or acetylene reduction in cells of *D. desulfuricans* strain Berre-Sol. Solid line: hydrogen evolution or uptake. Dashed line: acetylene or $^{15}N_2$ reduction, both expressed as reducing-equivalents used. Open symbols: in the presence of $^{15}N_2$. Closed symbols; in the presence of C_2H_2.

DISCUSSION

Although hydrogenase activity was potentially reversible for the two *Desulfovibrio* species studied here, as can be seen from the D_2-H^+ exchange reaction, it was largely directed toward hydrogen uptake under most physiological conditions examined. This is consistent with a rather low K_m of 5–7 µM reported for H_2 in the uptake activity of the purified hydrogenase [15].

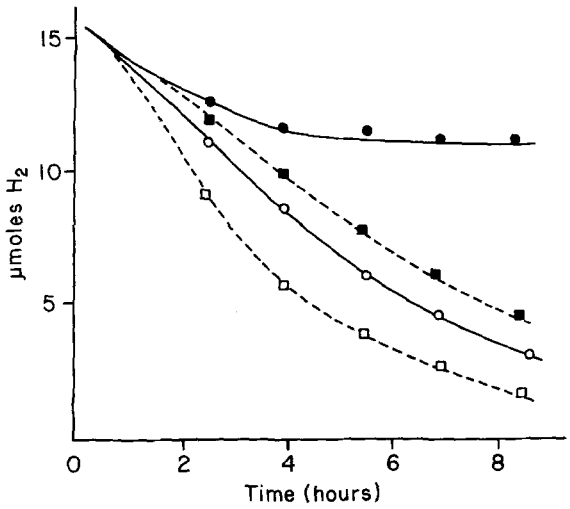

Fig. 3. Effect of lactate and ammonia on hydrogen uptake by resting cells of *D. desulfuricans* strain Berre-Sol. Solid line: without addition of ammonia. Dashed line: with 36 mM ammonia. Open symbols: without addition of lactate. Closed symbols: with 32 mM lactate.

Significant hydrogen production occurs only under specific and/or transient circumstances. This was the case when there was a limitation of nitrogen metabolism resulting from omission of combined nitrogen from the medium or from a delay in the establishment of diazotrophic conditions. Nitrogen starvation and hydrogen evolution were both prolonged when acetylene was substituted for dinitrogen as nitrogenase substrate.

In contrast, the reverse of these conditions favored the consumption of added hydrogen, which could serve as an electron donor for sulfate or sulfite reduction. With some *Desulfovibrio* strains, sulfate reduction is dependent on electrons transferred from H_2 [1]. In the same manner, H_2 was used more extensively when nitrogen metabolism was taking place, probably due to an increased need for reducing equivalents when the carbon supply was used in anaplerotic reactions [5]. Under diazotrophic conditions, the appearance of nitrogen-fixing activity was immediately followed by a transition from hydrogen evolution to hydrogen uptake, indicating that the H_2 substituted for lactate as an electron donor.

It is possible that the net uptake corresponds to a single process, or it may result from a combination of production and uptake catalyzed by two or more enzymes associated in a recycling phenomenon. Such a cycling process is known to occur in diazotrophic species between the H_2-evolving activity of nitrogenase and a 'unidirectional' hydrogenase [20]. As a certain amount of nitrogenase-catalyzed H_2 evolution is unavoidable under diazotrophic conditions, such a recycling can be expected when dinitrogen is being fixed, but not under acetylene, since the latter gas suppresses H_2 production by nitrogenase [20]. Another recycling process between two differently located hydrogenases has been postulated in the genus *Desulfovibrio* [17]. In this scheme, hydrogen produced internally at the expense of lactate or pyruvate would diffuse across the cytoplasmic membrane and be oxidized at the external face by a periplasmic hydrogenase. The electrons thus produced would be transferred back into the cytoplasm where they would be used principally in sulfate reduction; the protons left outside would create a gradient able to drive the synthesis of ATP by a reversible ATPase [7]. Such a proton gradient could also be of importance in corrosion caused by *Desulfovibrio* [8]. The latter cycling process is difficult to demonstrate even by the use of deuterium, since it is masked by the exchange reaction which is catalyzed by a single hydrogenase, but it provides a logical explanation for the role of the several hydrogenases isolated in some *Desulfovibrio* species [6,15a].

The hydrogenase activities of sulfate-reducing bacteria help them to adapt to changes in sulfate and nitrogen sources. It has been demonstrated recently (W.A. Hamilton, personal communication) that *Desulfovibrio* species are able to grow on hydrogen as sole electron and energy source produced electrochemically at the surface of a steel cathode. Therefore, in marine environments where electron donors are sometimes scarce, metallic structures may act as cathode and favor a proliferation of sulfate-reducing bacteria leading to anaerobic corrosion.

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REFERENCES

1 Badziong, W., R.K. Thauer and J.G. Zeikus. 1978. Isolation and characterization of *Desulfovibrio* growing on hydrogen plus sulfate as the sole energy source. Arch. Microbiol. 116: 41–49.

2 Berlier, Y.M., B. Dimon, G. Fauque and P.A. Lespinat. 1985. Direct mass-spectrometric monitoring of the metabolism and isotope exchange in enzymic and microbiological investigations. In: Gas enzymology (Degn, H., et al., eds), pp. 17–35, Reidel Publishing Company, The Hague.

3 Berlier, Y.M. and P.A. Lespinat. 1978. Relationship between nitrogenase and hydrogenase activity in a rhizosphese diazotroph. FEMS Microbiol. Lett. 3: 187–190.

4 Dixon, R.O.D. 1972. Hydrogenase in legume root nodule bacteroids: Occurrence and properties. Arkh. Mikrobiol. 85: 193–201.

5 Dixon, R.O.D., Y.M. Berlier and P.A. Lespinat. 1981. Respiration and nitrogen fixation in nodulated roots of Soya Bean and Pea. Plant Soil 61: 135–143.

6 Gow, L.A., I.P. Pankhania, S.P. Ballantine, D.H. Boxer and W.A. Hamilton. 1986. Identification of a membrane-bound hydrogenase of *Desulfovibrio vulgaris* (Hildenborough). Biochim. Biophys. Acta 851, 57–64.

7 Guarria, L.J. and H.D. Peck, Jr. 1971. Dinitrophenol-stimulated adenosine-triphosphate activity in extracts of *Desulfovibrio*. J. Bacteriol. 106;: 890–898.

8 Hamilton, W.A. 1985. Sulphate-reducing bacteria and anaerobic corrosion. Annu. Rev. Microbiol. 39: 195–217.

9 Hill, S., J.W. Drozd and J.R. Postgate. 1972. Environmental effects on the growth of nitrogen-fixing bacteria. J. Appl. Chem. Biotechnol. 22: 541–558.

10 Keith, S.M. and R.A. Herbert. 1983. Dissimilatory nitrate reduction by a strain of *Desulfovibrio desulfuricans*. FEMS Microbiol. Lett. 18: 55–59.

11 LeGall, J., D.V. DerVartanian and H.D. Peck, Jr. 1979. Flavoproteins, iron proteins and hemoproteins as electron

transfer components of the sulfate-reducing bacteria. In: Current Topics in Bioenergetics (Sandy, R., ed.), pp. 237–265, Academic Press, New York.

12 LeGall, J. and G. Fauque. 1987. Dissimilatory reduction of sulfur compounds. In: Biology of Anaerobic Microorganisms (Environmental Microbiolgy of Anaerobes) (Zehnder, A.J.B., ed.), John Wiley and Sons, New York, in the press.

13 Le Gall, J. and J.C. Senez. 1960. Influence de la fixation de l'azote sur la croissance de *Desulfovibrio desulfuricans*. C.R. Acad. Sci. Paris 250: 404–406.

14 Lespinat, P.A. and Y. Berlier. 1981. The dependence of hydrogen recycling upon nitrogenase activity in *Azospirillum brasilense* Sp7. FEMS Microbiol. Lett. 10: 127–132.

15 Lissolo, T., S. Pulvin and D. Thomas. 1984. Reactivation of the hydrogenase from *Desulfovibrio gigas*. J. Biol. Chem. 9: 11725–11729.

15a Lissolo, T., E.S. Choi, J. LeGall, and H.D. Peck, Jr. 1986. The presence of multiple intrinsic membrane nickel-containing hydrogenases in *Desulfovibrio vulgaris* (Hildenborough). Biochem. Biophys. Res. Commun. 139: 701–708.

16 Mortenson, L.E. 1978. Hydrogenase of *Clostridium pasteurianum*. In: Mechanisms of oxidizing enzymes (Singer, T., ed.), pp. 119–125, Elsevier, New York.

17 Odom, J.M. and H.D. Peck, Jr. 1981. Hydrogen cycling as a general mechanism for energy coupling in the sulfate-reducing bacteria. FEMS Microbiol. Lett. 12: 47–50.

18 Peck, H.D., Jr. and J. LeGall. 1982. Biochemistry of dissimilatory sulfate reduction. In: Sulfur Bacteria (Postgate, J.R. and D.P. Kelly, eds.), pp. 448–466, The Royal Society, London.

19 Postgate, J.R. 1984. The sulphate-reducing bacteria. Cambridge University Press.

20 Smith, L.A., S. Hill and M.G. Yates. 1976. Inhibition by acetylene of conventional hydrogenase in nitrogen-fixing bacteria. Nature (Lond.) 262: 209–210.

21 Van der Werf, A.N. and M.G. Yates. 1978. In: Hydrogenases: Their Catalytic Activity, Structure and Function (Schlegel, H.G. and K. Schneider, eds.), pp. 307–326, E. Goltz, Gottingen.

22 Walker, C.C. and M.G. Yates. 1978. The hydrogen cycle in nitrogen-fixing *Azotobacter chroococcum*. Biochimie 60: 224–231.