

Involvement of a single periplasmic hydrogenase for both hydrogen uptake and production in some *Desulfovibrio* species

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SUMMARY

Various sulphate-reducing bacteria differing in the number of genes encoding hydrogenase were shown to ferment lactate in coculture with *Methanospirillum hungatei*, in the absence of sulphate. The efficiency of interspecies H₂ transfer carried out by these species of sulphate-reducing bacteria does not appear to correlate with the distribution of genes coding for hydrogenase.

Desulfovibrio vulgaris Groningen, which possesses only the gene for [NiFe] hydrogenase, oxidizes hydrogen in the presence of sulphate and produces some hydrogen during fermentation of pyruvate without electron acceptor. The hydrogenase of *D. vulgaris* was purified and characterized. It exhibits a molecular mass of 87 kDa and is composed of two different subunits (60 and 28 kDa). *D. vulgaris* hydrogenase contains 10.6 iron atoms, 0.9 nickel atom and 12 acid-labile sulphur atoms/molecule, and the absorption spectrum of the enzyme is characteristic of an iron-sulphur protein. Maximal H₂ uptake and H₂ evolution activities were 332 and 230 units/mg protein, respectively. *D. vulgaris* cells contain exclusively the [NiFe] hydrogenase, whatever the growth conditions, as shown by biochemical and immunological studies. Immunocytochemical localization in ultrathin frozen sections of cells grown on lactate and sulphate, on H₂ and sulphate and on pyruvate showed that the [NiFe] hydrogenase was located in the periplasmic space. Labelling was enhanced in cells grown on H₂ and sulphate and on pyruvate.

The results enable us to conclude that *D. vulgaris* Groningen contains a single hydrogenase of the [NiFe] type, located in the periplasmic space like that described for *D. gigas*. This enzyme appears to be involved in both H₂ uptake and H₂ production, depending on the growth conditions.

Key-words: Hydrogenase, *Desulfovibrio*, *Desulfomicrobium*, Hydrogen; Metabolism, Interspecies H₂ transfer, [NiFe] hydrogenase, Immunogold labelling, Bioenergetics.

INTRODUCTION

Hydrogen metabolism plays a central role in the energy metabolism of strictly anaerobic sulphate-reducing bacteria (Odom and Peck,

1984; Fauque *et al.*, 1988; Hatchikian *et al.*, 1990a). *Desulfovibrio* species can either consume or produce hydrogen, depending on the growth conditions. In the presence of sulphate, hydrogen oxidation by these microorganisms is

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coupled to ATP synthesis through vectorial electron transfer (Badziong and Thauer, 1980). On the other hand, they can produce hydrogen when growing fermentatively on pyruvate in the absence of any electron acceptor (Postgate, 1952; Vosjan, 1975; Traore, 1981), or by lactate fermentation in the absence of sulphate in mixed cultures with hydrogen-consuming bacteria such as methanogens (Bryant *et al.*, 1977). Furthermore, it has been shown that *Desulfovibrio* species can produce or consume hydrogen when grown on organic substrates in the presence of sulphate (Hatchikian *et al.*, 1976; Tsuji and Yagi, 1980; Traore *et al.*, 1981; Lupton *et al.*, 1984a). The physiological significance of H₂ metabolism in these growth conditions is still a matter of debate (Lupton *et al.*, 1984a; Peck *et al.*, 1987; Pankhania *et al.*, 1986; Rohde *et al.*, 1990; Van den Berg *et al.*, 1991). Two possible mechanisms involving H₂ metabolism have been proposed: (i) obligate H₂-cycling linked to the generation of a proton gradient by the periplasmic hydrogenase itself (Odom and Peck, 1981a, 1984), and (ii) proton translocation through a typical Mitchell loop in which hydrogen production linked with lactate oxidation is required to regulate the redox states of internal electron carriers (Lupton *et al.*, 1984a). Both of the bioenergetic mechanisms proposed are dependent upon the existence of at least two distinct hydrogenases: one located in the cytoplasm and involved in H₂ evolution and another, periplasmic one, involved in H₂ consumption.

Different types of hydrogenases have been isolated from various *Desulfovibrio* species (Fauque *et al.*, 1988). These hydrogenases, termed [Fe], [NiFe] and [NiFeSe] hydrogenase, differ in their metal centre composition, catalytic properties, sensitivity to inhibitors, amino acid sequences and immunological properties. Most of the hydrogenases isolated from these bacteria have been found to be confined to the periplasmic space (Bell *et al.*, 1974; Hatchikian *et al.*, 1978; Van der Westen *et al.*, 1978; Glick *et al.*, 1980; Hatchikian *et al.*, 1990b) or associated

with the membrane (Lalla-Maharajh *et al.*, 1983; Lissolo *et al.*, 1986). The existence of different types of hydrogenase as well as their location within the cells of a single species of *Desulfovibrio* (Van der Westen *et al.*, 1978; Lalla-Maharajh *et al.*, 1983; Rieder *et al.*, 1984; Lissolo *et al.*, 1986; Rohde *et al.*, 1990) is of particular interest in relation to the bioenergetics of these microorganisms (Odom and Peck, 1984; Lupton *et al.*, 1984a). Four classes of *Desulfovibrio* species have been distinguished on the basis of the distribution of genes for [Fe], [NiFe] and [NiFeSe] hydrogenases (Voordouw *et al.*, 1990; Voordouw, 1992). All species contain the genes for the [NiFe] hydrogenase and can express this enzyme, which is characterized by its high affinity for H₂ (K_m = 1 μM). Furthermore, a few species of *Desulfovibrio* contain hydrogenase genes only for the [NiFe] enzyme (Voordouw *et al.*, 1990). In agreement with the distribution of genes for hydrogenases, it was shown recently by biochemical and immunocytochemical studies that *Desulfovibrio gigas* indeed contains a single hydrogenase of the [NiFe] type located in the periplasmic space (Nivière *et al.*, 1991).

The aim of this work was to compare the ability of various *Desulfovibrio* species containing different numbers of hydrogenase genes (Voordouw *et al.*, 1990) to grow on lactate by interspecies hydrogen transfer. Furthermore, it was of interest to focus on species containing one hydrogenase gene not only to show their ability to grow on lactate by syntrophic association but also to confirm previous genetic approaches by biochemical and immunological studies. For this purpose, *D. vulgaris* Groningen was chosen since it exhibited high efficiency in interspecies H₂ transfer during lactate oxidation and grew well on hydrogen and sulphate. This strain contains a periplasmic reversible hydrogenase of the [NiFe] type as the sole hydrogenase. The enzyme was characterized, and its level was investigated under different growth conditions.

HPLC = high-performance liquid chromatography.
PAGE = polyacrylamide gel electrophoresis.

SDS = sodium dodecyl sulphate.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The sulphate-reducing bacteria used in this study as well as the distribution of their genes coding for hydrogenase are indicated in table I. Further details are provided by Voordouw *et al.* (1987).

Axenic and cocultures were cultivated on medium containing (per liter) 1.0 g NH₄Cl, 0.3 g KH₂PO₄, 0.2 g MgCl₂ · 6H₂O, 0.6 g NaCl, 0.1 g CaCl₂ · 2H₂O, 1 g CH₃COONa · 3H₂O, 0.001 g resazurin and 1.5 ml trace element solution (Imhoff-Stuckle and Pfenig, 1983). The pH was adjusted to 7.0 with KOH (10 M). The medium was prepared as previously described (Fardeau *et al.*, 1993), and 5 ml or 20 ml were distributed into Hungate tubes or serum bottles, respectively. After autoclaving (110°C, 30 min), 0.05 ml of 2% Na₂S · 9H₂O, 0.2 ml of 10% NaHCO₃ (from sterile anoxic solutions), 0.05 ml of filter-sterilized vitamin solution (Widdel, 1980) and 0.05 ml of 0.1% sodium dithionite solution were added to 5 ml of medium. Stock solutions of sodium lactate (1 M) and Na₂SO₄ (2 M) were prepared separately and were added to the medium if required.

D. vulgaris Groningen used for biochemical studies and immunocytolocalization experiments was grown anaerobically on lactate-sulphate (LeGall *et al.*, 1965), hydrogen-sulphate (Brandis and Thauer, 1981) and pyruvate media. In the latter medium, which was devoid of sulphate, pyruvate (40 mM) was substituted for lactate. For immunocytochemical assays, cells were grown in rubber-sealed tubes that contained 30 ml of growth medium and were subsequently harvested either in the mid- or late-exponential phase. For measurement of hydrogenase activities in cell extracts, *D. vulgaris* was cultivated in 2-l flasks and

harvested at the end of the exponential phase (Nivière *et al.*, 1991). Mass cultures of *D. vulgaris* on lactate-sulphate were carried out in a 300-l fermentor.

Preparation of cell fractions

Crude extract was prepared by suspending the cell paste at 1:2 (wt/wt) in 10mM Tris-HCl buffer (pH 7.6). The suspension was then passed through a French pressure cell at 110 MPa. Centrifugation of the broken cell suspension at 30,000g for 20 min yielded the crude extract.

Enzyme assays

Hydrogenase activity was routinely measured spectrophotometrically at 30°C by the hydrogen uptake assay (Fernandez *et al.*, 1985). The hydrogen evolution activity of the purified enzyme was determined manometrically at pH 8 and 30°C (Hatchikian *et al.*, 1978); 1 unit of hydrogenase activity is the amount of enzyme which catalyses the consumption or the production of 1 μmol H₂/min under the assay conditions. The inhibitory effect of carbon monoxide on hydrogenase activity of *D. vulgaris* Groningen extract was investigated. Carbon monoxide was anoxically added as small aliquots (2 μl) of aqueous saturated solutions (1 mM) during the H₂ uptake assay with extract of *D. vulgaris*.

Purification of hydrogenase from *D. vulgaris* Groningen

All operations were performed under air. Freshly thawed cells of *D. vulgaris* (130 g wet cells) previ-

Table I. Distribution of genes coding for hydrogenase in sulphate-reducing bacteria used in this study (from Voordouw *et al.*, 1990).

Species	Strain	Source (*)	Hybridization with		
			hyn BA [NiFe]	hys BA [NiFeSe]	hyd AB [Fe]
<i>Desulfovibrio desulfuricans</i>	Berre Sol	NCIMB 8388	+	+	+
<i>Desulfovibrio africanus</i>	Walvis Bay	NCIMB 8397	+	+	+
<i>Desulfovibrio multispirans</i>		NCIMB 12078	+	-	+
<i>Desulfomicrobium baculatum</i>	Norway 4 (**)	NCIMB 8310	+	+	-
<i>Desulfovibrio gigas</i>		NCIMB 9332	+	-	-
<i>Desulfovibrio vulgaris</i>	Groningen	NCIMB 11779	+	-	-
<i>Desulfovibrio desulfuricans</i>	Teddington R	NCIMB 8312	+	-	-

(*) NCIMB = the British National Collection of Industrial and Marine Bacteria.

(**) Formerly called *Desulfovibrio desulfuricans* Norway 4.

ously stored at -80°C were suspended in 180 ml of 10 mM Tris-HCl containing $1\ \mu\text{M}$ deoxyribonuclease I, and the crude extract was prepared as previously described. The extract was then centrifuged at $180,000\ g$ for 90 min, and the soluble fraction was obtained from the supernatant. Total purification of hydrogenase could be achieved in five chromatographic steps using a procedure similar to that previously reported (Hatchikian *et al.*, 1990b). The following steps were carried out: ion exchange chromatography on DEAE-cellulose (DEAE 52, Whatman) and "Q-sepharose FF" (Pharmacia), gel filtration on "Ultrogel AcA-34" (IBF), adsorption chromatography on hydroxylapatite (Bio-Gel HTP, Bio-Rad) and a final ion exchange chromatography on a preparative HPLC column (Ultropac TSK DEAE-5 PW from LKB, $2.15 \times 15\ \text{cm}$). This last chromatography step was performed as described previously (Hatchikian *et al.*, 1992) using a linear gradient from 50 mM Tris-HCl (pH 7.6) to 100 mM NaCl in 50 mM Tris buffer. The enzyme was eluted from the column at approximately 70 mM NaCl and concentrated by ultrafiltration. The purification procedure yielded 13 mg of pure hydrogenase.

Analytical procedures

Optical density of the cultures was determined at 580 nm with a "Shimadzu model UV-160" spectrophotometer (Shimadzu Corporation). Methane and hydrogen were quantified using a "Girdel series 30 gas chromatograph" equipped with a thermal conductivity detector. The column was filled with "Carbosphere SS 60/80 mesh". Lactate and acetate were assayed in diluted samples of the cocultures by high performance liquid chromatography using an "Analprep" pump (Touzart and Matignon) and an "ORH 801" type column (Interaction Chemicals Inc.); the flow rate was 0.6 ml/min; the volume of the injection loop was 20 μl ; the column temperature was 35°C , and the detector was a differential refractometer (Knauer). D-lactate was also determined enzymatically (Boehringer, Mannheim).

Analytical gel electrophoresis was performed according to the method of Davis (1964). SDS/PAGE was performed following the method of Laemmli (1970). The H_2 uptake activity was located in native gels by the method of Ackrell *et al.* (1966). The molecular mass of hydrogenase from *D. vulgaris* Groningen was determined by analytical ultracentrifugation with a "Beckman model E" analytical ultracentrifuge. It was estimated by equilibrium sedimentation (Yphantis, 1964). The molecular mass of the protein was also estimated from the masses of its subunits using SDS/PAGE as reported previously (Hatchikian *et al.*, 1990b). Protein concentrations were measured by the method of Lowry *et al.* (1951). Inorganic sulphide was estimated by the procedure of

Lovenberg *et al.* (1963). Iron and nickel were determined by plasma emission spectroscopy using a "Jobin Yvon model JY" spectrometer.

Antibody preparation

Antisera were obtained after immunization of rabbits against purified [NiFe] hydrogenase from *D. vulgaris* Groningen as reported previously (Nivière *et al.*, 1991). Polyclonal antibodies were also raised against the [NiFeSe] hydrogenase from *Desulfomicrobium baculatum* Norway 4 (Rieder *et al.*, 1984) and [Fe] hydrogenase from *Desulfovibrio desulfuricans* ATCC 7757 (Hatchikian *et al.*, 1992). Immunoblotting of the proteins from the soluble extract and of the pure hydrogenase was carried out as reported by Towbin *et al.* (1979), and the peroxidase-conjugated anti-rabbit IgG method was used to detect the hydrogenase specificity of the polyclonal antibodies (Hawkes *et al.*, 1982). Antiserum was used at a dilution of 1:2,000.

Immunogold labelling

The detailed procedures for fixation, cryosectioning and labelling have been previously described (Anba *et al.*, 1984). Antiserum dilution was 1:100. Control was performed with rabbit serum obtained before immunization.

RESULTS

Growth of different *Desulfovibrio* species cultured on lactate with sulphate or *Methanospirillum hungatei* acting as electron acceptors

All the sulphate reducers tested oxidized lactate when sulphate was supplied in the medium. In the absence of sulphate, *M. hungatei* served as H_2 scavenger for all species (table II). The optical density of cocultures differed from each other, depending on the type of sulphate reducer. In the case of *D. gigas* and *D. baculatum*, possessing one and two hydrogenase(s), respectively, growth with a methanogen was rather slow (fig. 1A and 1B). In contrast, *D. vulgaris* Groningen, which possesses one hydrogenase, grew faster with *M. hungatei* (fig. 1C). Thus, there was no correlation between the number of genes coding for hydrogenase and growth of the different cocultures.

Table II. Growth of different species of sulphate reducers cultivated on lactate with sulphate as electron acceptor or *M. hungatei* as H₂ scavenger.

Strain	Sulphate reducer + <i>M. hungatei</i>	Optical density (580 nm)	
		Sulphate reducer (*) - SO ₄	Sulphate reducer (*) + SO ₄
<i>D. multispirans</i>	0.184 (*)	0.024	0.308
<i>D. baculatum</i> Norway 4	0.088 (**)	0.027	0.180
<i>D. gigas</i>	0.082 (***)	0.018	0.350
<i>D. africanus</i> Walvis bay	0.125 (***)	0.009	0.243
<i>D. desulfuricans</i> Berre sol	0.119 (***)	0.037	0.115
<i>D. vulgaris</i> Groningen	0.209 (*)	0.030	0.275
<i>D. desulfuricans</i> Teddington	0.215 (*)	0.025	0.320

Initial sulphate and lactate concentrations were 20 mM; *M. hungatei* was cultivated on H₂/CO₂ before inoculation. The inoculum was 5 % and experiments were performed after two successive inoculations into the same medium.

(*) Measured after 8 days of incubation at 37°C; (**) measured after 27 days of incubation at 37°C; (***) measured after 22 days of incubation at 37°C.

Furthermore, *D. vulgaris* Groningen actively ferments pyruvate in the absence of sulphate. Under these growth conditions, a small amount of hydrogen is produced, which accounts for approximately five per cent of the electrons derived from pyruvate oxidation. The major end product of pyruvate fermentation has been found to be D-lactate (N. Forget, M.-L. Fardeau and E.C. Hatchikian, unpublished results). Since *D. vulgaris* Groningen exhibits a very active hydrogen metabolism, the part of this work which follows is devoted to the hydrogenase of this microorganism.

Properties of the hydrogenase of *D. vulgaris* Groningen

Hydrogenase from *D. vulgaris* was purified 55-fold, and the overall recovery was 15%. The protein was judged to be homogeneous using the following criteria: (a) PAGE yielded a single band of protein which catalysed the H₂-dependent reduction of methyl viologen (fig. 2); (b) the absorbance ratio ($A_{400\text{nm}}/A_{280\text{nm}}$) remained constant after further chromatographic steps.

The molecular mass of hydrogenase determined by analytical ultracentrifugation was estimated to be 87 ± 3 kDa using a partial specific volume (0.73 ml/g) determined from

amino acid composition (Cohn and Edsall, 1943). The subunit masses of hydrogenase were estimated to be 60 kDa and 28 kDa by SDS/PAGE. These results indicate that the enzyme molecule contains one subunit of each type.

The UV-visible absorption spectra of oxidized and hydrogen-reduced hydrogenase are shown in figure 3. The oxidized enzyme exhibits a spectrum typical of an iron-sulphur protein with a broad absorption peak around 400 nm and a shoulder in the 310 nm region. The absorbance ratio ($A_{400\text{nm}}/A_{280\text{nm}}$) of the pure enzyme is 0.29 and the absorption coefficients at 400 and 280 nm were $48 \text{ mM}^{-1} \text{ cm}^{-1}$ and $165 \text{ mM}^{-1} \text{ cm}^{-1}$, respectively. Reduction of the enzyme with hydrogen results in a 35 % decrease of absorbance in the 400 nm region.

Analysis of the pure hydrogenase yielded values of 10.6 ± 1 iron atoms, 12 ± 1 acid-labile sulphur atoms and 0.9 ± 0.15 nickel atom/molecule. No selenium was detected in the protein sample. The most reasonable interpretation of these results is that the enzyme contains 11 Fe, 12 Se and 1 Ni atoms/molecule.

The hydrogenase from *D. vulgaris* isolated under air exhibited a specific activity of 40 units/mg of protein in the H₂ uptake assay and 96 units/mg of protein in the H₂ evolution

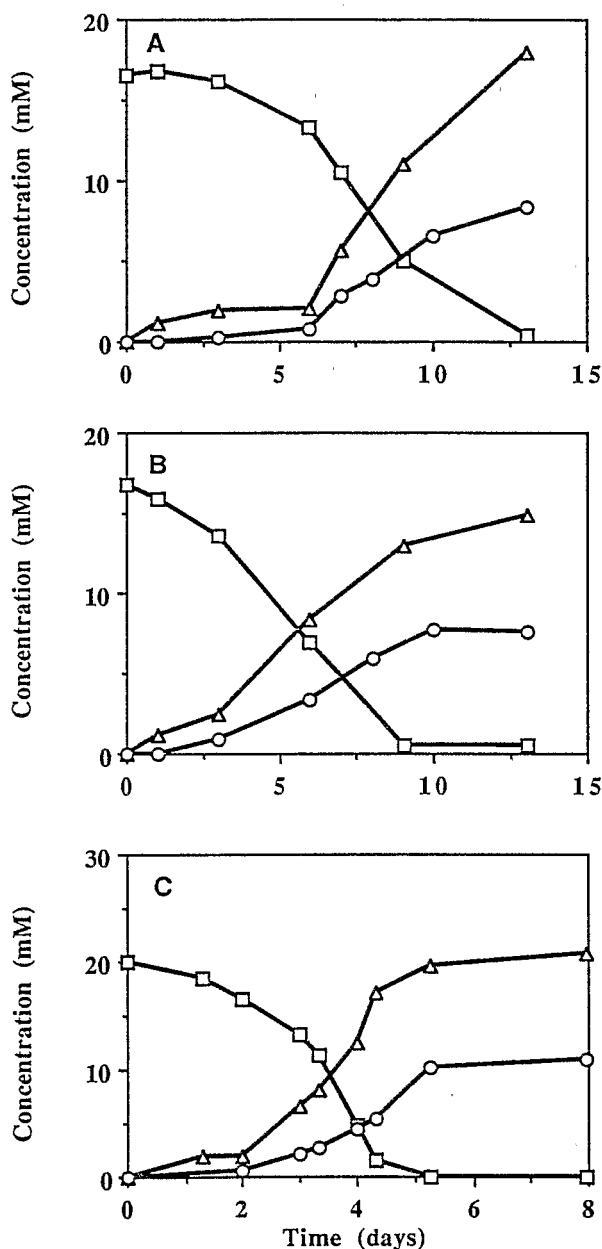


Fig. 1. Lactate metabolism through interspecies H_2 transfer of *D. baculatum* Norway 4 (A), *D. gigas* (B) and *D. vulgaris* Groningen (C).

□ = lactate; △ = acetate; ○ = CH_4 .

assay. The kinetics of the H_2 uptake reaction showed a lag period, followed by an induction phase in which the activity gradually increased. Both methyl viologen-dependent activities, *i.e.*

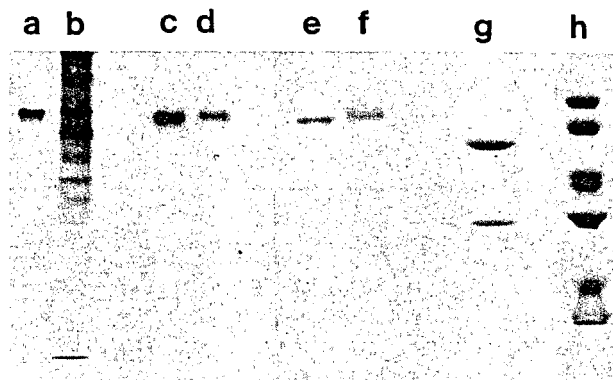


Fig. 2. Homogeneity of purified [NiFe] hydrogenase from *D. vulgaris* Groningen, and characterization of the antibody preparation with immunoblotting.

Crude soluble extract and purified hydrogenase were subjected to non-denaturing PAGE: lanes *a* and *b* = staining of pure hydrogenase (3 μ g) and crude soluble extract (40 μ g), respectively, by Coomassie brilliant blue; lanes *c* and *d* = activity staining of pure hydrogenase (0.10 μ g) and crude soluble extract (40 μ g), respectively, with methyl viologen under an atmosphere of H_2 ; lanes *e* and *f* = immunoblotting of pure hydrogenase (0.10 μ g) and crude soluble extract (40 μ g), respectively. Purified hydrogenase was also subjected to SDS/PAGE: lane *g* = SDS/PAGE of *D. vulgaris* Groningen hydrogenase; lane *h* = SDS/PAGE of the following molecular markers: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α -lactalbumin (14 kDa).

H_2 uptake and H_2 evolution, increased after prolonged preincubation of the enzyme with hydrogen plus 1 mM methyl viologen as reported previously with other [NiFe] hydrogenases (Lissolo *et al.*, 1984; Fernandez *et al.*, 1985). Under our activation conditions, the enzyme exhibited its maximal activity after 10 h incubation at 30°C, when the kinetics of H_2 uptake reaction showed neither a lag nor an induction phase. Maximal H_2 uptake and H_2 evolution activities were 332 and 230 units/mg of protein, respectively.

These data clearly indicate that *D. vulgaris* Groningen contains a [NiFe] type of the hydrogenase. The possibility of a second hydrogenase, distinct from the [NiFe] enzyme, was investigated in the bacteria grown on lactate-sulphate, H_2 -sulphate and pyruvate media. The

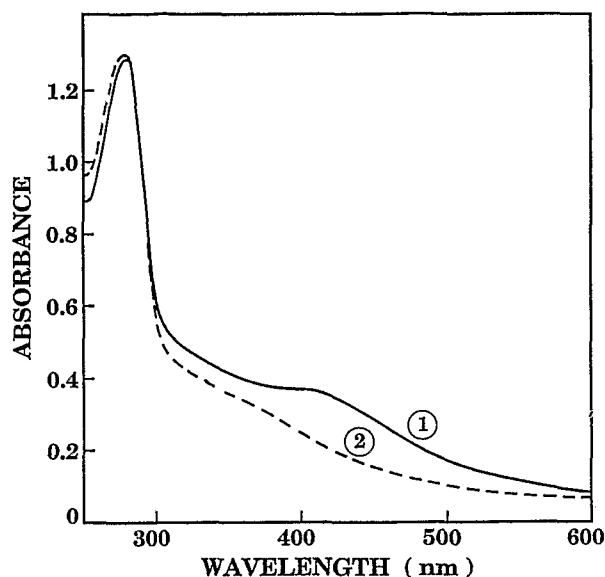


Fig. 3. Optical absorption spectra of *D. vulgaris* Groningen hydrogenase.

1) Native enzyme (7.7 μ M) in 50 mM Tris buffer pH 8, under argon; 2) H₂-reduced enzyme after incubation under H₂ for 3 h.

investigation of H₂ uptake activity directly in PAGE, using both soluble and membrane fractions from the three different extracts in all cases indicated the presence of a single activity band, located at the same R_f as the pure [NiFe] hydrogenase (fig. 2). Immunoblotting of the proteins from these extracts showed cross-reactivity with antibodies against the [NiFe] hydrogenase from *D. vulgaris* (fig. 2) but failed to cross-react with anti-[Fe]- or anti-[NiFeSe]-hydrogenase (data not shown). The pattern of cross-reactivity in figure 2 proves the monospecificity of the antibodies against [NiFe] hydrogenase from *D. vulgaris* used in this work.

The presence of different types of hydrogenase in *D. vulgaris* cells was also investigated using the inhibitory effect of carbon monoxide. The three types of *Desulfovibrio* hydrogenase can be distinguished by their sensitivity to this inhibitor (Berlier *et al.*, 1987; Fauque *et al.*, 1988). No inhibition of hydrogenase activity of

Table III. Hydrogenase activity in extracts of *D. vulgaris* Groningen grown under various conditions.

Growth conditions	Hydrogenase activity (U/mg)	
	Extract prepared under air	Extract after reactivation(*)
Lactate/sulphate	0.24	1.73
H ₂ /sulphate	3.20	7.64
Pyruvate	1.29	3.80

(*) After reactivation of hydrogenase present in the extract by incubation of the crude extract for 10 h under H₂ in the presence of 1 mM methyl viologen (cf. "Materials and Methods").

D. vulgaris extracts was observed with 2 μ M carbon monoxide, a concentration known to inhibit approximately 100% and 60% of [Fe]- and [NiFeSe]-type hydrogenase, respectively (Berlier *et al.*, 1987). Only the [NiFe]-type hydrogenase is insensitive to this concentration of carbon monoxide. All together, these data indicate that *D. vulgaris* cells grown in lactate-sulphate, hydrogen-sulphate or pyruvate media contain exclusively a hydrogenase of the [NiFe] type.

The specific activity of hydrogenase in the extracts of *D. vulgaris* grown on the three different media was measured using the H₂ uptake assay with extracts prepared under air, and was compared with that of extracts activated several hours under reducing conditions (table III). The content of hydrogenase in the cells can be estimated accurately from the reactivated extracts, which express maximal activity. Under these conditions, the content of [NiFe] hydrogenase was 4.4- and 2.2-fold higher in cells grown with H₂-sulphate and pyruvate than in the bacteria grown on lactate-sulphate, respectively (table III). In addition, the data indicate that the extent of activation of hydrogenase was clearly greater with the extract of bacteria grown in lactate-sulphate medium (7-fold) than that obtained with the extracts from bacteria grown on H₂-sulphate or pyruvate (2.4- and 3-fold, respectively).

In conclusion, the results show that hydrogenase activity in *D. vulgaris* Groningen is due to

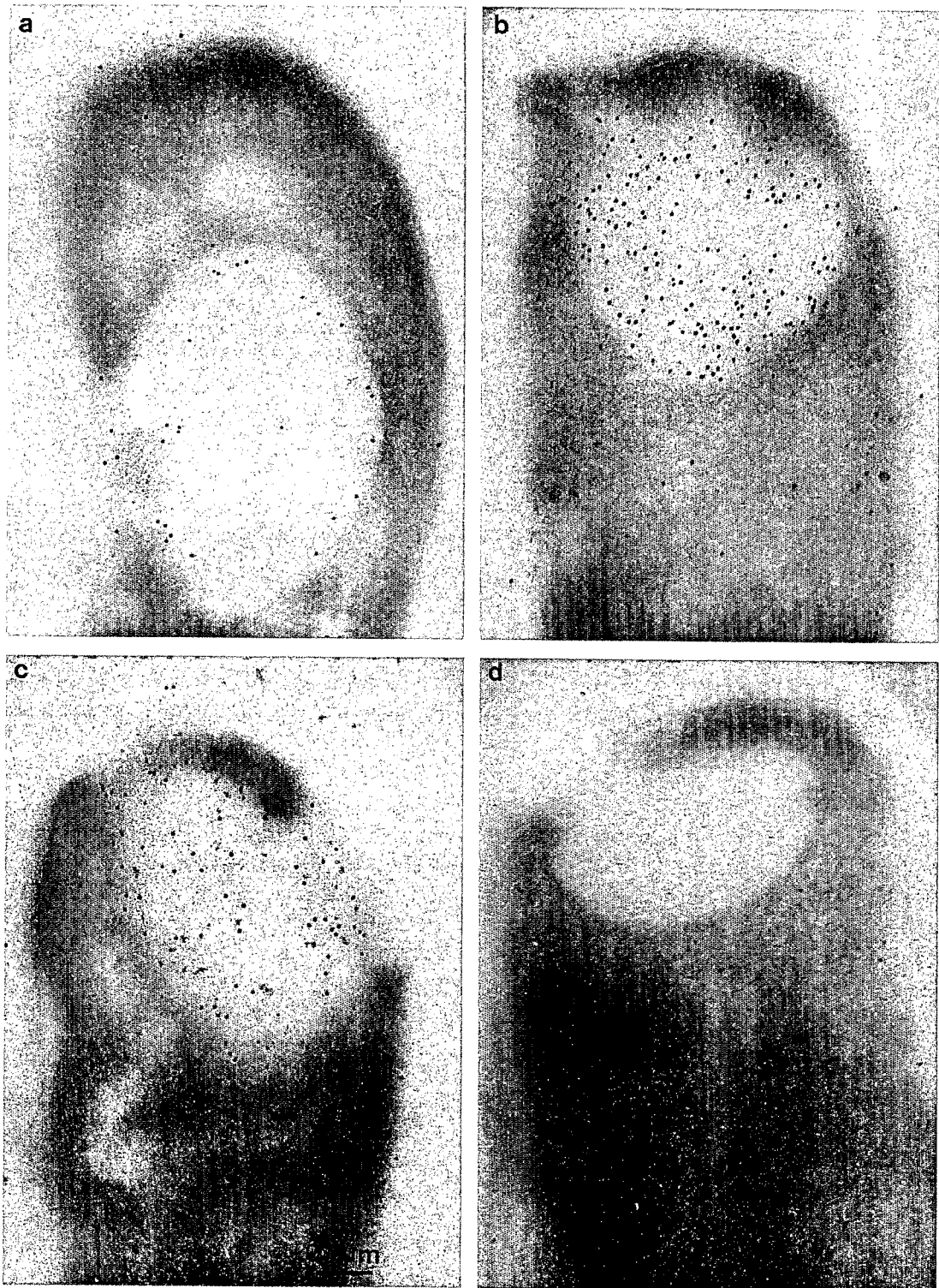


Fig. 4. Localization of hydrogenase in *D. vulgaris* Groningen cells grown on lactate-sulphate (a), H_2 -sulphate (b) and pyruvate (c and d).

Ultra-thin frozen sections were incubated with anti-(NiFe)-hydrogenase serum (a, b and c) or normal serum (d) for 60 min (1 : 100 dilution). The sections were subsequently treated with protein A-gold (7 nm).

the [NiFe] hydrogenase, whatever the energy source is. In order to establish the cellular localization of the enzyme, we used immunogold labelling.

Immunogold labelling

Hydrogenase of *D. vulgaris* was visualized by immunogold labelling of cryosections of cells grown in different media. These cells have a partly dilated periplasmic space, with a well-separated inner and outer membrane. On cells grown in lactate-sulphate medium, gold particles were clearly visible in a large, dilated part of the periplasmic space close to the inner membrane (fig. 4a). In these cells, labelling was weak compared with that of cells in H₂-sulphate medium (fig. 4b), where the periplasmic bays were filled with gold particles, showing that more hydrogenase had been produced. In pyruvate medium (fig. 4c), periplasmic gold particles were present in slightly lower numbers than those shown in figure 4b. In all cases, the outer membrane and the cytoplasm, where black precipitates of sulphide can be seen, were devoid of labelling. As a control, immunogold labelling of cryosections was performed with rabbit serum obtained before immunization. No labelling was observed with cells grown in media containing lactate-sulphate, H₂-sulphate (not shown) or pyruvate (fig. 4d). The sera against [Fe] and [NiFeSe] hydrogenases were tested but failed to show any labelling (date not shown).

DISCUSSION

Bryant *et al.* (1977) and McInerney and Bryant (1981) demonstrated the anaerobic degradation of lactate by syntrophic associations of sulphate reducers with hydrogenotrophic methanogens such as *Methanobacterium* strain MOH, *Methanosarcina barkeri* or *Methanobrevibacter smithii*. These hydrogenotrophs increased the free energy of lactate oxidation by lowering the partial pressure of H₂. Our experiments showed that similar syntrophic

associations can be obtained between *Desulfovibrio* species having various numbers of hydrogenases and *M. hungatei*, which proved to be an efficient H₂ scavenger. The different species of sulphate reducers used in this study were chosen according to the distribution of genes for hydrogenase (table I) (Voordouw *et al.*, 1990). Interspecies H₂ transfer was obtained with all species of sulphate-reducing bacteria, and its efficiency does not appear to be related to the number of hydrogenases present in the cells.

To our knowledge, we first evidenced growth of *D. gigas* by interspecies H₂ transfer from lactate to a methanogen. In a former study, *D. gigas* was unable to oxidize lactate in the absence of sulphate when associated with *M. barkeri* (A.S., Traore, personal communication), probably because the latter hydrogenotrophic methanogen had an insufficient affinity for hydrogen as compared with that of *M. hungatei*. Furthermore, Kremer *et al.* (1988) concluded that ethanol dissimilation can take place in *D. gigas* if associated with a hydrogenotrophic methanogen. Our results show effective growth of the sulphate reducers, including *D. gigas* in coculture with *M. hungatei*. This implies that lactate oxidation by all the sulphate reducers tested occurred with ATP synthesis *via* substrate-level phosphorylation whatever the number of hydrogenases they possess. All cultures converted lactate to acetate and methane with the same stoichiometry (1 lactate yielding 1 acetate and 0.5 CH₄).

D. vulgaris Groningen was shown to contain only an [NiFe] hydrogenase, on the basis of biochemical and immunological studies. This hydrogenase is similar in most respects to the other [NiFe] hydrogenases previously isolated from *Desulfovibrio* species (Fauque *et al.*, 1988; Hatchikian *et al.*, 1990a). The results are in agreement with the data of Voordouw *et al.* (1990) indicating the lack of hybridization of *D. vulgaris* DNA with gene probes for [Fe] and [NiFeSe] hydrogenases.

The differences in the specific activity of hydrogenase in extracts of *D. vulgaris* grown on lactate-sulphate, hydrogen-sulphate and

pyruvate were related to the changes in content of [NiFe] hydrogenase. These data indicate a regulation by H₂ and pyruvate of the genes encoding hydrogenase. The differences observed in the extent of activation of the [NiFe] hydrogenase in the extracts (table III) could be explained if the proportion of the enzyme in the ready state is higher in the extracts of the cells grown on H₂-sulphate and pyruvate than in the extracts of the cells grown on lactate-sulphate (Fernandez *et al.*, 1985; Hatchikian *et al.*, 1990a).

Immunogold labelling of ultrathin frozen sections of *D. vulgaris* cells showed that [NiFe] hydrogenase was located in the periplasmic space, whatever the growth conditions. The differences in amounts of labelling observed with cells grown under various conditions was related to the activity of hydrogenase measured with the various extracts (table III and fig. 4).

From the results obtained, we conclude that the single hydrogenase of the [NiFe] type located in the periplasmic space of *Desulfovibrio* species such as *D. vulgaris* Groningen or *D. gigas* is involved in both H₂ uptake and H₂ production, depending on the growth conditions. The lack of an internal hydrogenase in these sulphate reducers, which are able to grow by interspecies hydrogen transfer, strongly suggests that the periplasmic hydrogenase accepts electrons *via* transmembrane electron transfer, since lactate dehydrogenase and pyruvate oxidoreductase are cytoplasmic enzymes (Odom and Peck, 1981b). The cytoplasmic oxidation of lactate to acetate and CO₂ results in the liberation of 4e⁻ and 4H⁺ which must be transferred to the periplasmic hydrogenase. The mechanisms of proton translocation are unknown (Odom and Peck, 1984).

The requirement of at least two distinct hydrogenases, a cytoplasmic one producing H₂ and a periplasmic one consuming H₂, has been proposed in two bioenergetic models of lactate-sulphate metabolism, namely the obligate H₂ cycling model (Odom and Peck, 1981a, 1984) and the trace hydrogen transformation model (Lupton *et al.*, 1984). Former reports on the effect of hydrogen and carbon monoxide on the

growth of *D. vulgaris* strains on lactate-sulphate media (Lupton *et al.*, 1984a, b; Pankhania *et al.*, 1986), growth studies of a hydrogen-inhibited mutant of *D. desulfuricans* ATCC 27774 (Odom and Wall, 1987), and experiments with antisense RNA complementary to periplasmic [Fe] hydrogenase mRNA of *D. vulgaris* Hildenborough (Van den Berg *et al.*, 1991) have led to arguments against the H₂-cycling hypothesis in some *Desulfovibrio* species. The presence of a single reversible hydrogenase located in the periplasm of *D. vulgaris* Groningen reported in this work rules out the possibility of an H₂ cycling mechanism during lactate-sulphate metabolism in this microorganism. Furthermore, for the same reason, the data do not support the trace hydrogen transformation model (Lupton *et al.*, 1984a). It can be deduced from the lack of hydrogen production during growth on lactate with sulphate by *D. gigas* (Traore *et al.*, 1982) and *D. vulgaris* Groningen (this work) that in these microorganisms the periplasmic [NiFe] hydrogenase is not involved in the cytoplasmic oxidation of lactate. These observations are in favour of the existence in these *Desulfovibrio* species of a vectorial proton translocation coupled to lactate oxidation as observed with *D. vulgaris* Marburg by Fitz and Cypionka (1991).

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Intervention d'une seule hydrogénase périplasmique dans la consommation et la production d'hydrogène chez quelques espèces du genre *Desulfovibrio*

Au cours de cette étude, nous avons montré que plusieurs bactéries sulfato-réductrices possédant un nombre différent de gènes codant pour des hydrogénases, oxydent le lactate en absence de sul-

fate lorsqu'elles sont en coculture avec *Methanospirillum hungatei*. L'efficacité du transfert d'hydrogène avec la bactérie méthanogène n'est pas corrélée avec le nombre de gènes codant pour l'hydrogénase chez ces bactéries sulfato-réductrices.

Desulfovibrio vulgaris Groningen, qui possède uniquement le gène de l'hydrogénase à nicker-fer (hydrogénase [NiFe]), oxyde l'hydrogène en présence de sulfate et produit de l'hydrogène au cours de la fermentation du pyruvate. L'hydrogénase de *D. vulgaris* Groningen a été purifiée et caractérisée. Son poids moléculaire est de 87 kDa et elle est constituée de deux sous-unités différentes (60 et 28 kDa). L'hydrogénase de cette bactérie contient 10,6 atomes de fer, 0,9 atome de nickel et 12 atomes de soufre par molécule et son spectre d'absorption est caractéristique d'une protéine à centre fer-soufre. Les activités catalytiques de consommation et production d'hydrogène sont de 332 et 230 unités/mg de protéine, respectivement. Les cellules de *D. vulgaris* Groningen contiennent exclusivement l'hydrogénase [NiFe] quelles que soient les conditions de croissance, ainsi que l'ont montré des études biochimiques et immunologiques. L'immunocytolocalisation de cryosections ultrafines de cellules ayant poussé sur différents milieux indique que l'hydrogénase [NiFe] est localisée dans l'espace périplasmique, le marquage étant plus important sur les cellules cultivées sur H₂ et sulfate ou pyruvate seul que sur celles cultivées sur lactate et sulfate.

Les résultats nous permettent de conclure que *D. vulgaris* Groningen contient une seule hydrogénase de type [NiFe] située dans l'espace périplasmique tel que cela a été décrit chez *D. gigas*. Cette enzyme serait impliquée à la fois dans la production et la consommation d'H₂ en fonction des conditions de croissance.

Mots-clés : Hydrogénase, *Desulfovibrio*, *Desulfomicrobium*, Hydrogène ; Métabolisme de l'H₂, Transfert interspèces d'H₂, Hydrogénase de type [NiFe], Marquage immunologique à l'or, Bioénergétique.

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