

Effects of Deletion of Genes Encoding Fe-Only Hydrogenase of *Desulfovibrio vulgaris* Hildenborough on Hydrogen and Lactate Metabolism

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The physiological properties of a *hyd* mutant of *Desulfovibrio vulgaris* Hildenborough, lacking periplasmic Fe-only hydrogenase, have been compared with those of the wild-type strain. Fe-only hydrogenase is the main hydrogenase of *D. vulgaris* Hildenborough, which also has periplasmic NiFe- and NiFeSe-hydrogenases. The *hyd* mutant grew less well than the wild-type strain in media with sulfate as the electron acceptor and H₂ as the sole electron donor, especially at a high sulfate concentration. Although the *hyd* mutation had little effect on growth with lactate as the electron donor for sulfate reduction when H₂ was also present, growth in lactate- and sulfate-containing media lacking H₂ was less efficient. The *hyd* mutant produced, transiently, significant amounts of H₂ under these conditions, which were eventually all used for sulfate reduction. The results do not confirm the essential role proposed elsewhere for Fe-only hydrogenase as a hydrogen-producing enzyme in lactate metabolism (W. A. M. van den Berg, W. M. A. M. van Dongen, and C. Veeger, *J. Bacteriol.* 173:3688–3694, 1991). This role is more likely played by a membrane-bound, cytoplasmic Ech-hydrogenase homolog, which is indicated by the *D. vulgaris* genome sequence. The physiological role of periplasmic Fe-only hydrogenase is hydrogen uptake, both when hydrogen is and when lactate is the electron donor for sulfate reduction.

Sulfate-reducing bacteria of the genus *Desulfovibrio* contain the genes for several hydrogenases, including the *hynBA* and *hysBA* genes for the NiFe- and NiFeSe-hydrogenases, the *hydAB* genes for Fe-only hydrogenase, and the *hndABCD* genes for NADP-reducing hydrogenase. The first three enzymes are translocated by the twin-arginine translocation (*tat*) system and are thus either periplasmic or membrane bound with the active site facing the periplasm. Only the NADP-reducing hydrogenase has been shown elsewhere to be cytoplasmic (11). *Desulfovibrio vulgaris* strain Hildenborough has the Fe-only hydrogenase as well as the NiFe- and NiFeSe-hydrogenases. The former is a soluble periplasmic enzyme, whereas the latter two are membrane bound. Searching of the database for the *D. vulgaris* genome at <http://www.tigr.org> indicated that *D. vulgaris* has an HndD homolog but lacks the *hndA*, *hndB*, and *hndC* genes. *D. vulgaris* is thus unlikely to have a cytoplasmic, NADP⁺-reducing hydrogenase.

The *hydAB* genes encode the 46-kDa α and the 10-kDa β subunit of Fe-only hydrogenase from *D. vulgaris* (25). The structures of Fe-only hydrogenase have been determined recently both for CpI, the cytoplasmic enzyme from *Clostridium pasteurianum* (16), and for the periplasmic enzyme from *Desulfovibrio desulfuricans* (14). The sequences of the α and β subunits of the periplasmic enzyme in *D. desulfuricans* and *D. vulgaris* form a contiguous, single polypeptide of 60 kDa in CpI. The splitting of the sequence into two polypeptides in *Desulfovibrio* spp. is for export: the β subunit has a long twin-

arginine-type signal sequence for this purpose (24). The function of CpI in the fermentative metabolism of *C. pasteurianum* is to reoxidize reduced ferredoxin, using protons as the electron acceptor to produce H₂ (2). A similar function has been proposed elsewhere for Fe-only hydrogenase in lactate metabolism by *D. vulgaris* (23). Reduction of the Fe-only hydrogenase content by expression of *hydAB* antisense RNA reduced the growth rate and growth yield of *D. vulgaris* in lactate- and sulfate-containing medium. Observation of a reduced H₂ burst in the initial stages of growth on this medium also pointed to a decreased H₂ production activity.

Desulfovibrio spp. are the only microorganisms with a periplasmic Fe-only hydrogenase. If the physiological function of this enzyme is indeed in H₂ production, then the question arises why it is not simply cytoplasmic as in *Clostridium* spp. A cytoplasmic, H₂-producing hydrogenase contributes to the transmembrane pH gradient, whereas a periplasmic, H₂-producing hydrogenase consumes this gradient. Because Fe-only hydrogenase is the most abundant periplasmic hydrogenase in *D. vulgaris* (10), one expects it to also catalyze hydrogen uptake at least under certain metabolic conditions. We have now constructed a *hydAB* mutant with a modification of the *sacB* replacement mutagenesis method first used to mutate the *dcrA* gene of *D. vulgaris* (7). The physiological properties of the resulting *hyd* mutant are compared with those of the wild-type strain.

MATERIALS AND METHODS

Growth of bacteria. All bacterial strains used are listed in Table 1. Tryptone-yeast extract medium was used for growth of *Escherichia coli* strains at 37°C (27). *D. vulgaris* strains were stored in Postgate's medium B and grown in medium C and on solid medium E (17) at 32°C in a mixed-gas atmosphere (5% [vol/vol] H₂,

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TABLE 1. Bacterial strains, primers, vectors and plasmids used

Strain, primer, vector, or plasmid	Genotype, comment(s), and/or reference
<i>Desulfovibrio vulgaris</i> subsp. <i>vulgaris</i> Hildenborough	NCIMB 8303; isolated from clay soil near Hildenborough, United Kingdom (17)
<i>D. vulgaris</i> Hyd 3-2	Plasmid pΔHydAB-CTB integrated into the chromosome through the downstream homologous regions; Suc ^s Cm ^r (this study)
<i>D. vulgaris</i> Hyd 3-7	Plasmid pΔHydAB-CTB integrated into the chromosome through the upstream homologous regions; Suc ^s Cm ^r (this study)
<i>D. vulgaris</i> Hyd100	Δ <i>hydAB</i> Suc ^r Cm ^r (this study)
<i>E. coli</i> TG2	<i>supE hsdΔ5 thi Δ(lac-proAB) Δ(srl-recA)306::Tn10(Tet^r) F' [traD36 proAB⁺ lacI^q lacZΔM15]</i> (18)
<i>E. coli</i> S17-1	<i>thi pro hsdR hsdM⁺ recA</i> RP4-2 (Tc::Mu Km::Tn7) (20)
pUC19Cm	pUC19 containing the <i>cat</i> gene (7)
pNOT19	Cloning vector pUC19; <i>Nde</i> I site replaced by a <i>Not</i> I site (19)
pMOB2	Contains <i>oriT</i> of plasmid RP4 and <i>Bacillus subtilis sacBR</i> genes on a 4.5-kb <i>Not</i> I fragment; Km ^r Cm ^r (19)
pHV15	Contains the <i>hydAB</i> genes on a 4.7-kb <i>Sal</i> I- <i>Eco</i> RI insert in pUC9 (26)
pHV15Not, pΔHydAB, pΔHydABcat, pΔHydAB-CTB	This study; see text
P173-r	5'-ATGCATCTGCGTTGCCGGAG; 3827-3808 ^a
P174-f	5'-ATCACACGCGGGCACATGCT; 1751-1770 ^a

^a Position of primer in 4,678-bp *Hind*III-*Eco*RI insert of pHV15; the *hydAB* operon sequence (accession no. X02416 [25]) is located from nt 1904 to 3970 in this sequence. The deleted region extends from nt 1789 to 3796.

10% CO₂, and 85% N₂) as described previously (7, 8). Antibiotics kanamycin (50 μg/ml) and chloramphenicol (CHL) (10 μg/ml) were added, when appropriate. Media B, C, and E contain 1 g of yeast extract/liter. *D. vulgaris* strains were also grown on defined BT media without yeast extract (4-6). Growth in these media was under conditions of exchange with the mixed-gas atmosphere of the anaerobic hood. BT media were made by combining, per 100 ml, 90 ml of minimal salt solution, 1.25 ml of trace element solution, 1 ml of CaCl₂ solution, 1 ml of sodium lactate solution (for BT lactate-sulfate medium) or 1 ml of sodium acetate solution (for BT H₂-sulfate medium; acetate and CO₂ serve as the carbon source, and H₂ serves as sole electron donor in this medium), 0.5 to 5 ml of 1 M Na₂SO₄, and 3 to 6 ml of water. All these were formulated as described before (6), except for the minimal salt solution. Because sulfate was a variable in the growth studies, it was removed from the minimal salt solution, which contained, per liter, 1.65 g of NH₄Cl, 0.9 g of KH₂PO₄, and 0.36 g of MgCl₂ · 6H₂O at pH 7. Cultures of 100 ml were grown in 300-ml nephelometer (Erlenmeyer) flasks (Bellco Glass Inc., Vineland, N.J.) equipped with a 1.2- by 13-cm sidearm and closed with a sponge cap allowing gas exchange. Cell densities were measured with a Klett-Summerson colorimeter installed in the anaerobic chamber (Forma Scientific, Marietta, Ohio) or with a Shimadzu spectrophotometer at 600 nm. A cell density of 147 Klett units corresponded to an optical density at 600 nm (OD₆₀₀) of 1, which in turn corresponded to a cell dry weight of 0.162 g/liter (6). BT medium plates were prepared by adding 15 g of agar per liter of medium.

Growth in 500-ml closed serum bottles, containing 250 ml of headspace consisting of 90% (vol/vol) N₂ and 10% (vol/vol) CO₂, was in defined Widdel-Pfennig (WP) medium. Use of BT medium in this closed culture system led to formation of significant amounts of precipitated sulfides due to buildup of H₂S, which interfered with the OD₆₀₀ determination. WP media were formulated according to the work of Widdel and Bak (28) using 38 mM lactate and 28 mM sulfate.

Plasmid construction and replacement mutagenesis. All plasmids and vectors used are listed in Table 1. The 4,678-bp *Hind*III-*Eco*RI insert from plasmid pHV15 was ligated to pNOT19, digested with *Hind*III and *Eco*RI, to give plasmid pHV15Not. pHV15Not was digested with *Sac*II (nucleotide [nt] 1789) and *Mst*II (nt 3796) to delete a 2-kb fragment containing the *hydAB* genes. The digested plasmid was end repaired with Klenow polymerase and deoxyribose diphosphates and ligated in the presence of *Bam*HI linkers (5'-CCGGATCC GG) to give plasmid pΔHydAB. A 1.4-kb *Bam*HI fragment from plasmid pUC19Cm, containing the *cat* gene, was ligated into the *Bam*HI site of plasmid pΔHydAB to give plasmid pΔHydABcat. Plasmid pΔHydABcat was next digested with restriction endonuclease *Not*I and ligated with the 4.5-kb *Not*I fragment from pMOB2, containing the *oriT* locus and the *sacBR* genes, to give pΔHydAB-CTB. A map of this 11.2-kb plasmid is shown in Fig. 1.

E. coli S17-1(pΔHydAB-CTB) was conjugated with *D. vulgaris*, and single-crossover integrants were selected on medium E plates with CHL and kanamycin (7). Southern blot analysis was used to verify the integration of pΔHydAB-CTB into the *D. vulgaris* chromosome. A 2-kb upstream region probe (Fig. 1, up region) was prepared by digesting pHV15 with *Hind*III and *Sac*II and agarose gel

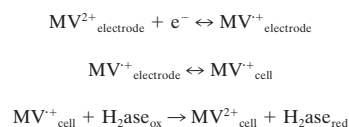
electrophoresis to isolate the 2-kb fragment and radioactive labeling with the random hexamer procedure using [α -³²P]dCTP. The probe was hybridized with the blots under highly stringent conditions (18, 27).

Gene replacement was achieved by growing a mapped, single-crossover integrant, either *D. vulgaris* Hyd 3-2 or *D. vulgaris* Hyd 3-7, in 5 ml of medium C with CHL, followed by growth in 5 ml of BT H₂-sulfate medium with CHL. Aliquots (50 to 100 μl) of the latter culture were then plated on BT H₂-sulfate medium plates containing CHL and 5% (wt/vol) sucrose. Colonies, appearing after 3 to 4 weeks, were grown in 5 ml of medium C with CHL. DNA was isolated from these cultures and used to test their genotype by Southern blotting, yielding *D. vulgaris* Hyd100 for further study.

Analysis of the *D. vulgaris* Hyd100 phenotype. For immunoblotting, the Hyd100 and wild-type strains were grown in 5 ml of medium C to stationary phase. Cells were pelleted and suspended in 250 μl of water. An equal volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) incubation buffer (8) was then added, and the samples were boiled immediately. Aliquots (30 μl; ca. 100 μg of protein) were loaded onto 12.5% (wt/vol) polyacrylamide gels together with molecular mass markers and a known amount of purified Fe-only hydrogenase. SDS-PAGE was performed according to the method of Laemmli (9). The separated proteins were electroblotted onto nitrocellulose (22). The blots were blocked with gelatin, incubated with a 2,000-fold dilution of anti-HydA antibodies for 4 h and then with a 2,000-fold dilution of the anti-rabbit, alkaline phosphatase-conjugated secondary antibody for 2 h, and then stained by incubation with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-β-D-phosphopyranoside (BCIP).

Hydrogenase activity in the wild-type and Hyd100 strains was qualitatively evaluated by PAGE under nondenaturing conditions using the Phast System (Amersham Pharmacia Biotech). Following culturing of *D. vulgaris* Hyd100 and wild type in 100 ml of medium C to stationary phase, cells were pelleted and broken with a French press (590 MPa). After centrifugation for 20 min at 4,000 × g, a volume of supernatant containing 1 μg of total protein was loaded onto a PhastGel gradient gel of 8 to 25% polyacrylamide (pH 8.3). A 1-μl sample (2.5 μM) of pure Fe-only hydrogenase from *D. vulgaris* Hildenborough was also loaded. The gel was stained for hydrogenase activity according to the method of Ackrell et al. (1).

Measurement of hydrogenase activity by CV. Enzyme activities were derived from enzyme-catalyzed electrochemical reactions (21) as measured by cyclic voltammetry (CV). An electrode modified with whole cells or cell fractions of *D. vulgaris* wild-type or Hyd100 strains produced a methyl viologen (MV)-mediated catalytic current (*I*_k) for evolution or consumption of H₂. The catalytic scheme for H₂ evolution can be summarized as follows:



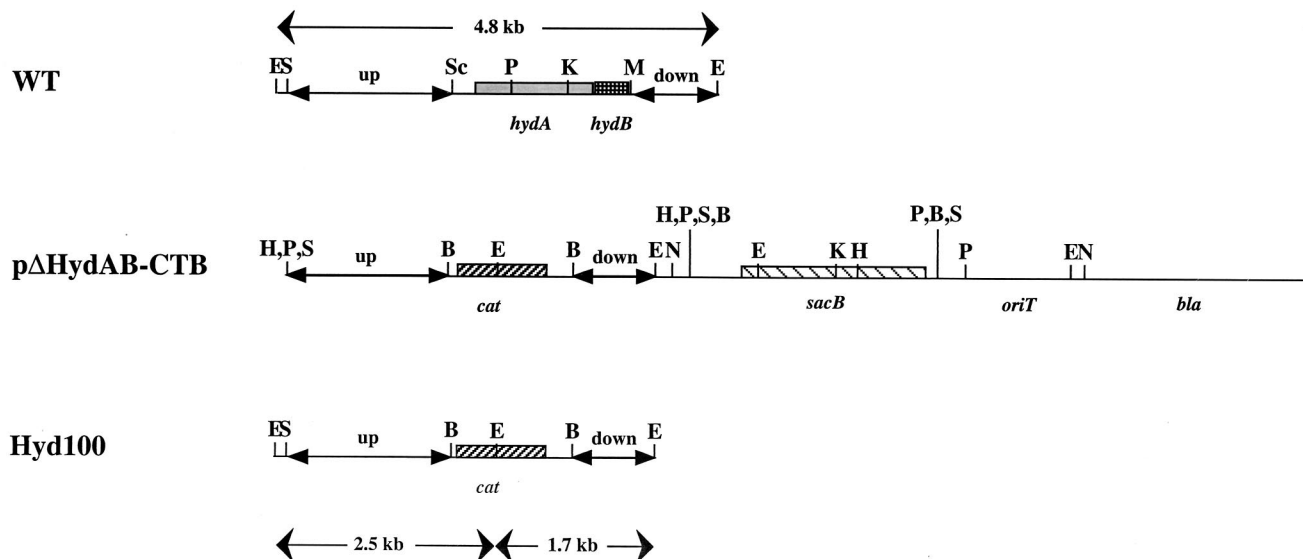
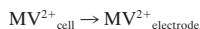
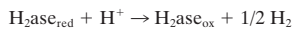


FIG. 1. Maps of the integration plasmid p Δ HydAB-CTB and the DNA region containing the wild-type and mutated *hydAB* locus (WT and Hyd100, respectively). Positions of restriction sites for *Bam*HI (B), *Eco*RI (E), *Hind*III (H), *Kpn*I (K), *Mst*II (M), *Nor*I (N), *Pst*I (P), *Sal*I (S), and *Sac*II (Sc) are shown. The 2-kb *Sac*II-*Mst*II fragment, containing the *hydAB* genes, in the wild-type strain was replaced by a 1.4-kb *Bam*HI fragment containing the *cat* gene in *D. vulgaris* Hyd100.



CV measurements were carried out using an EGG 263A potentiostat modulated by EGG PAR M270 software. Cyclic voltammograms were recorded at a sweep rate of 20 mV s⁻¹ at room temperature. A three-electrode system consisting of a Metrohm Ag-AgCl-NaCl-saturated reference electrode, a platinum wire auxiliary electrode, and the glassy carbon membrane electrode (GCME) as the working electrode was used throughout. All potentials were relative to the Ag-AgCl-NaCl-saturated reference electrode. Potential versus the standard H₂ electrode can be obtained by adding 0.210 V. For each H₂ evolution measurement, the solution (100 mM acetate [pH 5.6], 0.1 mM MV²⁺) was flushed with high-purity nitrogen, whereas for each H₂ uptake measurement the solution (100 mM Tris-HCl [pH 8.5], 0.1 mM MV²⁺) was flushed with H₂ gas. Prior to use, the GCME (*A* = 0.07 cm² from Tokay) was first polished with 0.05- μ m alumina slurry. A 2- μ l aliquot of either whole cells or cellular fraction suspensions of known protein concentration, prepared as described below, was then dropped onto the surface of the glassy carbon electrode. After the solvent had evaporated under N₂ flow, the electrode was pressed against a square piece (1 by 1 cm) of dialysis membrane (Visking; molecular weight cutoff, 12,000 to 16,000; 30 μ m thick). A rubber ring was fitted around the electrode body so that a uniform thin layer existed between the electrode surface and the membrane. The membrane electrode was then placed in the electrochemical cell, The dialysis membrane prevented loss of cells or protein molecules from the electrode surface, whereas small substrate and product molecules freely diffused through the membrane. The limiting current *I*_k was independent of the sweep rate, as expected for a catalytic process.

For sample preparation, all buffers were deaerated by flushing with argon. Cultures of *D. vulgaris* wild-type and Hyd100 strains, grown in 10 ml of medium C for 13 h, were centrifuged (5,700 \times g, 15 min), and the cell pellets were resuspended in 1 ml of 0.1 M Tris-HCl-0.15 M NaCl (pH 7.6) and recentrifuged. The cell pellets were then resuspended in 1 ml of 0.01 M Tris-HCl, pH 7.6. For preparation of subcellular fractions, 50-ml cultures in medium C were centrifuged and the pellets were resuspended in 0.5 ml of 0.1 M Tris-HCl-0.1 M EDTA (pH 9.0) and incubated at 37°C for 30 min. After centrifugation (5,700 \times g, 15 min, 4°C), periplasmic fractions were collected as the supernatants. These were concentrated and desalted by ultrafiltration on Centricon YM10 (Millipore) filters using 0.01 M Tris-HCl, pH 7.6. The pellets were resuspended in 4 ml of 0.01 M Tris-HCl, pH 7.6, and then passed twice through a French press unit at 590 MPa. After centrifugation (5,700 \times g, 60 min, 4°C), the supernatant was centrifuged at 125,000 \times g (12 h, 4°C). The cytoplasmic fractions were collected as the supernatants, and the membrane fractions were collected as the pellets

from the latter centrifugation. The pellets were washed once by resuspension in 4 ml of 0.01 M Tris-HCl, pH 7.6, and centrifugation (125,000 \times g, 12 h, 4°C). The membrane fractions were then resuspended in 500 μ l of 0.01 M Tris-HCl buffer, pH 7.6. Purity of periplasmic and cytoplasmic fractions was verified by spectroscopic determination of the presence of cytochrome *c*₃ and of desulfoviridin, respectively.

Analytical procedures. H₂ concentrations in the headspace of serum bottles containing cultures in WP media were analyzed by gas chromatography using a Shimadzu GC-8A gas chromatograph with a Molecular Sieve 5A column (80 to 100; 0.125 in. by 2 m; 32 ml of N₂/min at 40°C) and an RGD2 reduction gas detector (Trace Analytical). Headspace samples of 1 ml were withdrawn and appropriately diluted in closed serum bottles filled with air. A 1-ml diluted sample was then withdrawn with a gastight syringe and injected into the gas chromatograph. H₂ concentrations were calculated by comparing the peak area for the diluted sample with that of an H₂ standard (20 ppm in helium).

Molecular biology reagents and (bio)chemicals. DNA manipulation enzymes were obtained from Pharmacia or Roche Molecular Biochemicals. [α -³²P]dCTP (3,000 Ci/mmol; 10 mCi/ml) was from ICN Biomedicals. Deoxyoligonucleotide primers P173-r and P174-f (Table 1) were obtained from University Core DNA Services of the University of Calgary. Rabbit antiserum generated against HydA purified from *D. vulgaris* was as described before (26). The chromogenic substrates nitroblue tetrazolium and BCIP, as well as anti-rabbit and anti-mouse immunoglobulin G-alkaline phosphatase conjugates, were from Promega. Nitrogen and mixed gas were from Praxair Canada. MV dichloride was purchased from Aldrich. All other chemicals were of reagent grade and used as received.

RESULTS

Construction of an *hydAB* deletion mutant. Conjugation of *E. coli* S17-1(p Δ HydAB-CTB) and *D. vulgaris* gave rise to single-crossover integrants in which the suicide plasmid recombined with the chromosome through either the upstream (Fig. 1, up region) or the downstream (Fig. 1, down region) homologous regions. Digestion of chromosomal DNA of these and of the wild type with *Eco*RI and hybridization of a Southern blot with the upstream region probe resulted in hybridizing fragments of 7.2 and/or 4.8 and/or 2.5 kb, as expected based on the maps shown in Fig. 1. It appeared impossible to achieve the second gene-replacing crossover by growing single-crossover integrants in medium C or medium E with CHL and sucrose.

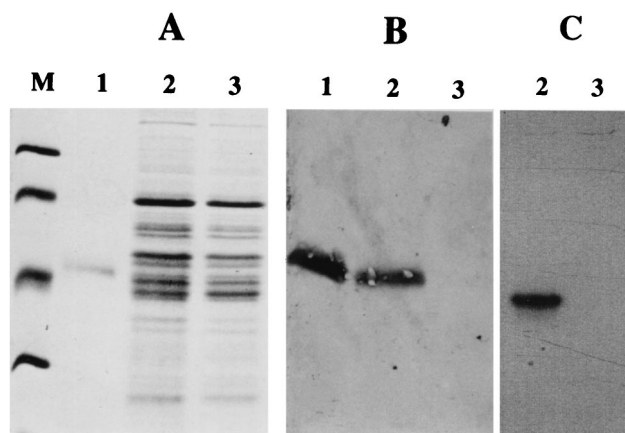


FIG. 2. Electrophoretic comparison of *D. vulgaris* wild-type and Hyd100 strains. (A) Gel stained with Coomassie blue following SDS-PAGE. Lane M, molecular mass markers; from top to bottom, 94, 67, 43, and 30 kDa; lane 1, 1 μg of purified Fe-only hydrogenase; lane 2, *D. vulgaris* wild type, 50 μg of protein; lane 3, *D. vulgaris* Hyd100, 50 μg of protein. (B) Immunoblot of gel as in panel A incubated with a polyclonal antiserum specific for the 46-kDa α subunit of Fe-only hydrogenase. (C) Native gel stained for hydrogenase activity. Lane 2, cell extract from *D. vulgaris* wild type, 4 μg of protein; lane 3, cell extract from *D. vulgaris* Hyd100, 4 μg of protein.

In view of this lack of success in rich media (both medium C and medium E contain 1 g of yeast extract per liter), we decided to attempt BT H_2 -sulfate medium. Growth in this medium is slow ($\mu = 0.050 \text{ h}^{-1}$ [6]), and colonies emerged on solid BT H_2 -sulfate medium containing CHL and sucrose only after 3 to 4 weeks of incubation at 32°C. However, when these were subsequently cultured in liquid BT H_2 -sulfate medium with CHL or medium C with CHL a large fraction (50 to 100%) was found to have the required genotype both by Southern blot analysis and by PCR using primers P173-r and P174-f. One of these, *D. vulgaris* Hyd100, was selected for further study.

Immunoblotting and native PAGE. Comparison of whole-cell extracts following SDS-PAGE and immunoblotting indicated the presence of the 46-kDa α subunit of Fe-only hydrogenase in *D. vulgaris* wild type (Fig. 2B, lane 2) and its absence in the Hyd100 strain (Fig. 2B, lane 3). Native gel electrophoresis also showed definitively that active Fe-only hydrogenase was present in the wild type but not in the Hyd100 strain. The membrane-bound NiFe- and NiFeSe-hydrogenases did not give an activity band under the conditions used. Some hydrogenase activity remained in the wells (Fig. 2C). In order to accurately evaluate the remaining hydrogenase activity in Hyd100 compared to that in wild-type cells, H_2 uptake and evolution were quantitated in both whole cells and subcellular fractions using an electrochemistry approach.

H_2 uptake and production activity by bioelectroanalysis. CV curves for measurement of H_2 production and consumption activities are shown in Fig. 3A and B. Figure 3C indicates a reversible electrochemical wave with a midpoint potential of -630 mV , representing the MV^{2+} - MV^+ electrochemical couple. This signal turned into an S-shaped pH-dependent wave when using the *D. vulgaris* cell-modified GCME (Fig. 3A and B). The value of the limiting current I_k is directly proportional to the enzyme activity in the linear part of the curve of I_k versus

protein concentration. This is illustrated in Fig. 3D for whole cells of the *D. vulgaris* wild-type and Hyd100 strains. It appeared that I_k first linearly increased with protein concentration and then leveled off as protein concentration was increased further. Estimates of the specific hydrogenase activity (relative units) were obtained by dividing the limiting current I_k by the current obtained at the GCME with MV^{2+} alone and by the protein concentration. The results are summarized in Table 2, which shows that the specific hydrogenase activity of the Hyd100 strain is about 50% less than that of the wild-type strain in both H_2 uptake and H_2 evolution reactions. A large difference was observed in the periplasmic fraction, which is in agreement with the deletion of the gene encoding periplasmic Fe-only hydrogenase in the Hyd100 strain. On the other hand, no significant changes were observed in either the cytoplasmic or the membrane fractions (Table 2).

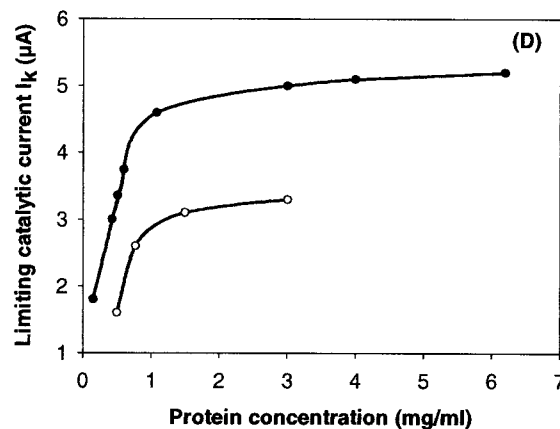
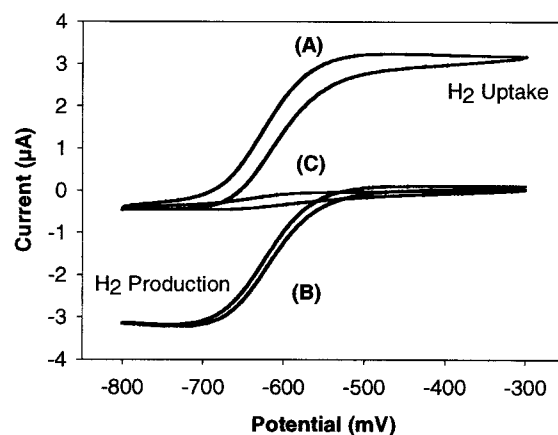


FIG. 3. Determination of hydrogenase activities with CV. (A to C) Cyclic voltammograms obtained with a GCME. (A) *D. vulgaris* cell-modified GCME (1 μg of protein) in N_2 -saturated 0.1 M Na-acetate buffer, pH 5.6, with 100 μM MV^{2+} ; (B) *D. vulgaris* cell-modified GCME (1 μg of protein) in H_2 -saturated 0.1 M Tris-HCl buffer [pH 8.5] with 100 μM MV^{2+} ; (C) bare GCME under same conditions as in panel A. (D) Limiting catalytic current I_k plotted against protein concentration (milligrams per milliliter) for experiments with cell-modified GCME under the same conditions as for panel A. Data are for whole cells of *D. vulgaris* wild-type (closed circles) and Hyd100 (open circles) strains.

TABLE 2. Specific hydrogenase activities derived from CV experiments on whole cells or subcellular fractions prepared from *D. vulgaris* wild-type and Hyd100 strains

Cell or fraction	Hydrogenase activity (relative units)/protein concn (mg/mL) for strain			
	Uptake		Production	
	Wild type	Hyd100	Wild type	Hyd100
Whole cells	47	24	33	16
Periplasm	36	ND ^a	13	3
Membrane	1	0.5	8	11
Cytoplasm	ND	ND	6	6

^a ND, not detected.

Comparison of growth yields of the wild-type and Hyd100 strains in BT media. Growth of *D. vulgaris* Hyd100 colonies on solid BT H₂-sulfate medium already indicated that Fe-only hydrogenase is not critically important for H₂ uptake. Colonies of the wild-type and Hyd100 strains also grew similarly on medium E plates, in which lactate serves as the electron donor for sulfate reduction. The Hyd100 strain is thus capable of using both lactate and H₂ as the electron donor for sulfate reduction.

Growth rates and yields were compared quantitatively in BT media in which either H₂ or lactate and H₂ served as the electron donor for sulfate reduction. H₂ was in principle never limiting, because growth was in an atmosphere containing 5% (vol/vol) H₂ under conditions of gas exchange. These culture conditions allow a specific growth rate (μ) of 0.050 h⁻¹ and a molar growth yield, Y_{sulfate} , of 5.1 g of cells mol⁻¹ with H₂ (6), similar to values observed by Badziong and Thauer (3) for the same medium. Growth studies were carried out at different sulfate concentrations (5 to 50 mM). Growth rates of *D. vulgaris* wild-type and Hyd100 strains were similar. However, differences in final cell densities were found (Fig. 4). Final cell

densities were constant (Fig. 4A) or tended to display a maximum (Fig. 4B). The average of four determined values at or near the maximum (Fig. 4B; 70 to 100 h) was taken as the final cell density in these cultures. Comparison of final cell densities as a function of sulfate concentration indicated that the *hyd* mutant grew less well than did the wild-type strain when only H₂ was available, especially at a high sulfate concentration (Fig. 5A). Statistical analysis of the data indicated that the ratio (*R*) of final cell densities of the wild-type and Hyd100 strains was $R = (1.10 \pm 0.16)$ for sulfate concentrations from 5 to 20 mM and $R = (1.30 \pm 0.15)$ for sulfate concentrations from 25 to 50 mM. The limiting slope at low sulfate concentration was the same for both strains and indicated a Y_{sulfate} of 4.4 g of cells mol⁻¹ for H₂ as electron donor, in reasonable agreement with previously reported values. With lactate (38 mM) and hydrogen as the electron donor, no differences in final cell density between the two strains were found at sulfate concentrations of 0 to 15 mM [$R = (0.98 \pm 0.10)$]. The final cell density increased linearly with sulfate concentration in this range (Fig. 5B). Above 15 mM sulfate, this increase ceased abruptly, and final cell densities decreased with increasing sulfate concentration. Because the transition point is close to 19 mM sulfate, where the culture goes from sulfate to lactate limitation, one can conclude that lactate serves as the main electron donor in the cultures with 5 to 15 mM sulfate. The data in this range indicate a Y_{sulfate} of 6.2 g of cells mol⁻¹ for lactate as the electron donor. Above 19 mM sulfate, the wild-type strain grew to a higher final cell density than did the Hyd100 strain [Fig. 5B, $R = (1.13 \pm 0.08)$]. The reasons why final cell densities decrease in cultures with increasing sulfate concentration in the range 20 to 50 mM are not clear.

Comparison of growth and hydrogen production in a closed culture system. Cultures of the wild-type and Hyd100 strains in 500-ml serum bottles containing 250 ml of WP medium (38 mM lactate and 28 mM sulfate) and 250 ml of a 90% (vol/vol)

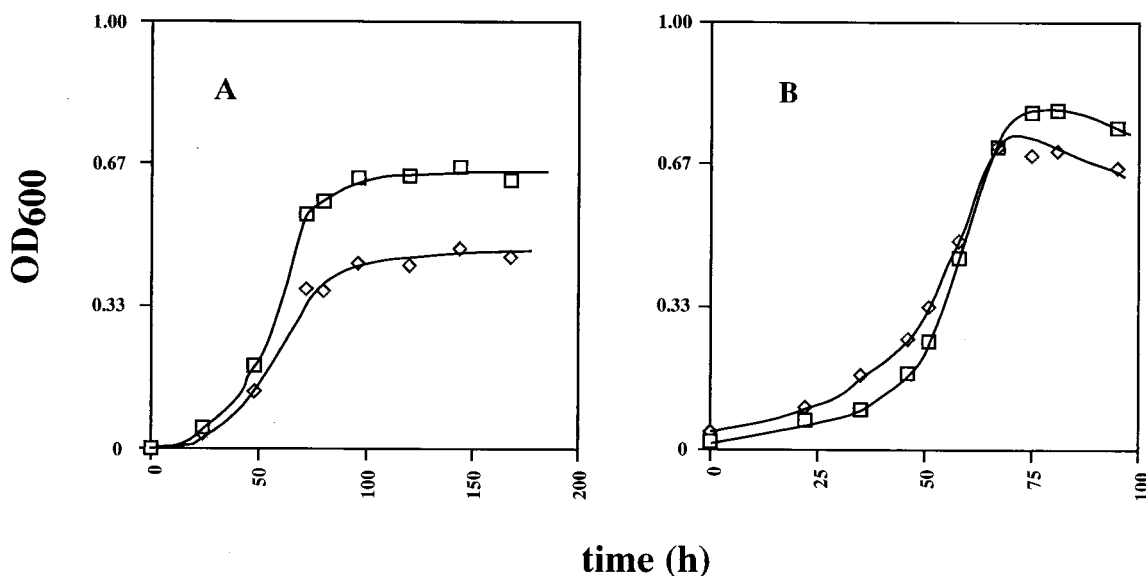


FIG. 4. Growth of *D. vulgaris* wild-type (\square) and Hyd100 (\diamond) strains in BT medium. (A) H₂ as sole electron donor for sulfate reduction; 40 mM sulfate. (B) Lactate (38 mM) and H₂ as electron donor for sulfate reduction; 30 mM sulfate.

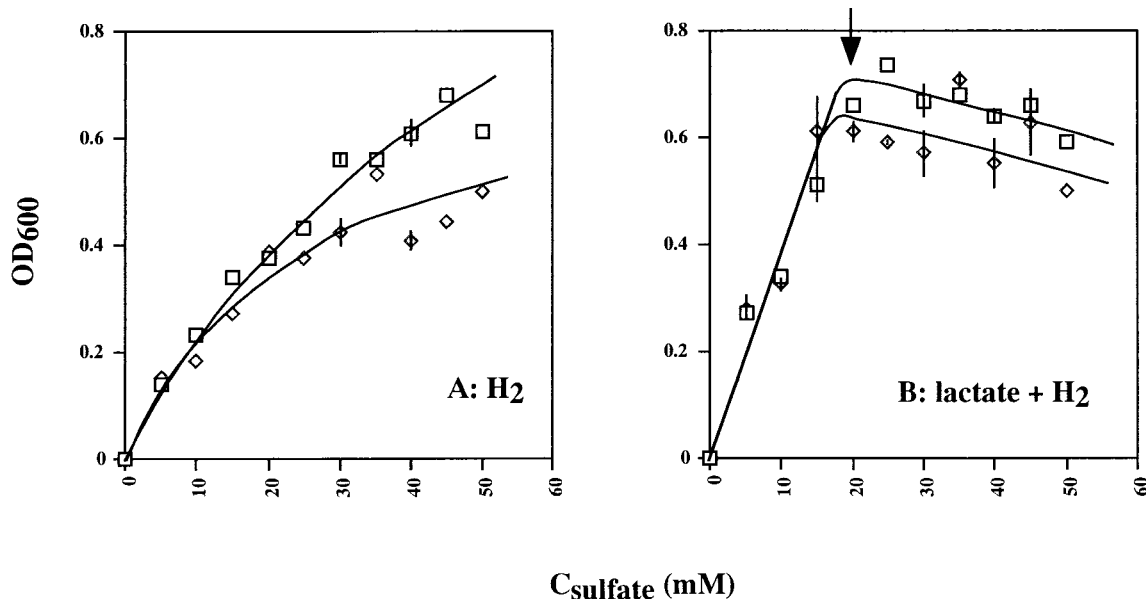


FIG. 5. Final cell density (OD_{600}) for cultures of *D. vulgaris* wild-type (\square) and Hyd100 (\diamond) strains in BT medium as a function of the sulfate concentration. All data points are for single cultures, except where the vertical bars indicate the spread of data obtained for duplicate cultures. Cultures were with H_2 only (A) or with lactate (38 mM) and H_2 as the electron donor for sulfate reduction (B). Lactate becomes limiting at sulfate concentrations above 19 mM (\downarrow). Final cell densities for wild type were $(13 \pm 8)\%$ larger than for Hyd100 cells at 25 to 50 mM sulfate. The downward-sloping lines drawn are best fits to the data in this range of sulfate concentrations and reflect this difference in cell density.

N_2 and 10% (vol/vol) CO_2 headspace were compared. The Hyd100 strain grew to a significantly lower cell density than did the wild type (Fig. 6A) while transiently producing significantly larger amounts of hydrogen (Fig. 6B). The levels of hydrogen indicated in Fig. 6B were reproducible in two other experiments (data not shown). The fact that the absence of Fe-only hydrogenase leads to increased headspace hydrogen concentrations points to a role of this enzyme in hydrogen consumption, not hydrogen production during lactate metabolism.

DISCUSSION

The genes for Fe-only hydrogenase were the first to be cloned and sequenced for *D. vulgaris* (25, 26). However, the isolation of a deletion mutant proved to be problematic. van den Berg et al. (23) were unsuccessful in their attempts to construct a mutant and resorted to antisense RNA expression technology to reduce *hydAB* gene expression. Following transfer of pWB1151, a broad-host-range plasmid that constitutively expresses *hydAB* antisense mRNA, a two- to threefold-reduced content of Fe-only hydrogenase of *D. vulgaris* was found. This decreased both growth rates and growth yields when lactate (70 mM) was the electron donor for sulfate (53 mM) reduction. The strain with reduced Fe-only hydrogenase content showed a reduced H_2 concentration in the headspace compared to that for the wild type. This H_2 was transient and was eventually all used for sulfate reduction. The effects of reducing Fe-only hydrogenase content on growth with H_2 as electron donor were not reported by these authors.

We have reduced expression of Fe-only hydrogenase to zero (Fig. 2), but we cannot confirm the phenotype suggested by van den Berg et al. (23). With lactate as the electron donor and H_2 in the headspace, there was no difference in growth yield be-

tween the Hyd100 and wild-type strains when sulfate was limiting (Fig. 5B; 5 to 20 mM). Because lactate (38 mM) was in excess (two lactate molecules reduce one sulfate molecule), it served as the main electron donor for sulfate reduction under these conditions. At sulfate concentrations in excess of available lactate, the wild-type strain reached a higher final cell density than did the Hyd100 strain (Fig. 5B, 20 to 50 mM), possibly indicating more efficient H_2 metabolism. Indeed, when H_2 was the sole electron donor for sulfate reduction, larger differences in final cell density were seen, especially at high sulfate concentrations (Fig. 5A). When strains were cultured on lactate- and sulfate-containing media, in the absence of H_2 (Fig. 6) the *hyd* mutant was found to generate more, not less, H_2 than that generated by the wild-type strain. Therefore, we cannot support the conclusion (23) that Fe-only hydrogenase has an important role in H_2 production from lactate when sulfate is in excess. Instead, the data in Fig. 5 and 6 suggest a role of Fe-only hydrogenase in H_2 uptake under these conditions. It should be pointed out that the culture conditions are not completely comparable, as van den Berg et al. (23) used rich medium, containing 1 g of yeast extract per liter, whereas we used defined medium in which lactate was the only organic molecule present. The strains used by van den Berg et al. and in this study are identical.

Reduced efficiency of growth on H_2 has also been found by Malki et al. (12), who described the effects of single and double mutations in hydrogenase genes of *Desulfovibrio fructosovorans*. This organism has *hyn*, *hyd*, and *hnd* genes, encoding a periplasmic NiFe-, a periplasmic Fe-only, and a cytoplasmic $NADP^+$ -reducing hydrogenase, respectively. The latter is absent from *D. vulgaris*. Strains with deletions in either the *hnd* or the *hyn* gene, as well as a strain with deletions in both the *hyn* and *hnd* genes, were still able to grow on H_2 as the sole

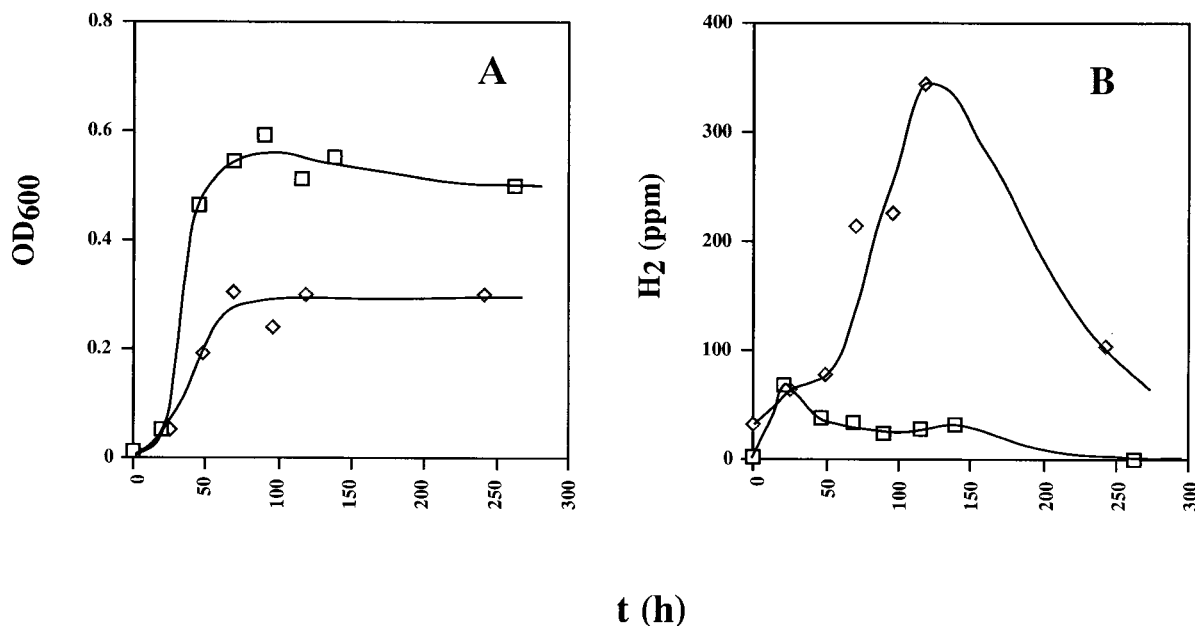


FIG. 6. Growth of wild-type and *hyd* mutant strains in WP medium containing lactate (38 mM), sulfate (28 mM), and a headspace of 10% (vol/vol) CO₂ and 90% N₂. Plotted as a function of time (hours) are cell density (OD₆₀₀) (A) and H₂ concentration in the headspace (parts per million) (B).

electron donor for sulfate reduction, which was credited to the presence of the remaining Fe-only hydrogenase. The double mutant also had reduced growth efficiency when lactate or pyruvate was used as the electron donor for sulfate reduction.

The data in Fig. 6B indicate that hydrogen production and consumption are an important feature of the use of lactate as the electron donor for sulfate reduction. However, the hydrogen-producing hydrogenase is not the periplasmic Fe-only hydrogenase, as we have shown. A good candidate is a homolog of the Ech-hydrogenase from *Methanosarcina barkeri*, which appears to be present in the *D. vulgaris* genome. This enzyme consists of six subunits (EchA to EchF), which together form a membrane-bound hydrogenase of which the active site faces the cytoplasm. The enzyme from *M. barkeri* has been purified previously as a six-subunit complex following its solubilization from membranes (13). The function ascribed to Ech-hydrogenase, based on biochemical studies of the purified enzyme, is to reoxidize reduced ferredoxin (Fd_{red}) formed during oxidative conversion of acetyl coenzyme A to methyl-tetrahydrosarcinapterin (CH₃-H₄SPT) and CO₂. Ech-hydrogenase reoxidizes Fd_{red} and uses the electrons together with cytoplasmic protons for hydrogen production. The produced hydrogen may then be captured by an externally located NiFe-hydrogenase of *M. barkeri*, with the electrons being used for cytoplasmic reduction reactions. This hydrogen cycling mechanism contributes to the proton gradient formed across the membrane by *M. barkeri*, when it metabolizes acetate to methane.

An H₂ cycling mechanism was first proposed for *Desulfovibrio* spp. by Odom and Peck (15). However, a cytoplasmic hydrogenase has so far not been identified. The Ech-hydrogenase homolog of *D. vulgaris* is encoded by an operon with the same gene order as that in the *ech* operon from *M. barkeri*. All subunits have the corresponding sequences from *M. barkeri* as

their closest homologs. Thus, *D. vulgaris* is likely to have a very similar cytoplasmic, energy-conserving Ech-hydrogenase. Fd_{red} is formed during lactate metabolism by *D. vulgaris* in the oxidative conversion of pyruvate to acetyl coenzyme A and CO₂, catalyzed by pyruvate-ferredoxin oxidoreductase. Fd_{red} may be reoxidized by the Ech-hydrogenase homolog, with the H₂ formed by this enzyme diffusing across the membrane to be captured by the periplasmic Fe-only and nickel-containing hydrogenases. The absence of the main periplasmic hydrogenase results in less efficient hydrogen capture and thus in higher hydrogen headspace concentrations. These are transient due to the presence of excess sulfate under the conditions of the experiment shown in Fig. 6.

Why is H₂ production and subsequent capture by the Hyd100 mutant bioenergetically less efficient than the proposed H₂ cycling catalyzed by the wild-type strain? One factor to consider is that thermodynamically the energy content of H₂ per mole is proportional to the logarithm of its concentration. During lactate metabolism, a high H₂ concentration may be maintained in the periplasm. Yet, little escapes due to the high periplasmic hydrogenase activity of wild-type *D. vulgaris* (Fig. 6B). In the case of the *hyd* mutant, H₂ escaping into the headspace is considerably diluted. Although this headspace H₂ is used for sulfate reduction, its availability at a reduced concentration leads to less energy conservation. This could be one of the factors contributing to the large difference in cell densities seen in Fig. 6A and could also explain why the presence of 5% (vol/vol) H₂ in the headspace reduces this difference (Fig. 5B). Another factor that may contribute to the larger difference in final cell densities between the cultures in Fig. 6A than between those in Fig. 5B is the buildup of H₂S, but this remains to be investigated.

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