Evidence for a Fourth Hydrogenase in *Desulfovibrio fructosovorans*

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A strain devoid of the three hydrogenases characterized for *Desulfovibrio fructosovorans* was constructed using marker exchange mutagenesis. As expected, the H₂-dependent methyl viologen reduction activity of the strain was null, but physiological studies showed no striking differences between the mutated and wild-type strains. The H⁺-D₂ exchange activity measured in the mutated strain indicates the presence of a fourth hydrogenase in *D. fructosovorans*.

Molecular hydrogen plays an important role in the energy-generating metabolism of sulfate reducers belonging to the genus *Desulfovibrio*. *Desulfovibrio* species can alternatively utilize hydrogen as the sole source of electron and energy (2, 3) or can produce hydrogen when growing fermentatively on a suitable carbon source in the absence of sulfate as an electron acceptor (17). Furthermore, hydrogen is successively produced and consumed during the degradation of organic compounds in the presence of sulfate (7, 22, 23). It has not yet been established whether hydrogen plays only a role in the regulation of the redox status of the electron transfer chains (11) or a central role as a key intermediate in the electron transfer across the membrane (14, 15). Hydrogenases are the key enzymes of energy-generating metabolism because of their ability to catalyze the splitting or the synthesis of molecular hydrogen. The number (generally more than one), the type ([Fe], [NiFe], or [NiFeSe] on the basis of their metal contents), and the cellular location of hydrogenases vary considerably from one *Desulfovibrio* species to another (6, 25). This diversity makes the role of these various hydrogenases difficult to determine.

With the aim to study the role of the hydrogenases in *Desulfo-
vibrio*, we chose *D. fructosovorans* DSM 3604 (16) as a model. In this species, three hydrogenases have already characterized: a periplasmic [NiFe] hydrogenase which represents about 1% of the total proteins (8, 20), a cytoplasmic NADP-reducing hydrogenase (13), and a periplasmic [Fe] hydrogenase (4). In order to elucidate the relative importance of these various hydrogenases in the energy-generating metabolism of *D. fructosovorans*, deletions were first made by marker exchange mutagenesis of the genes encoding the [NiFe] hydrogenase (19) and the NADP-reducing hydrogenase (12). All mutants (single or double) showed significant growth on organic substrates as well as on medium containing H₂ as the sole energy source.

Construction and molecular characterization of a triple mutant depleted of all three hydrogenases. In order to perform the marker exchange experiment, a 5-kb fragment containing the two structural genes (*hydAB*) coding for the [Fe] hydrogenase of *D. fructosovorans* (obtained by PCR amplification performed on genomic DNA by using oligonucleotides 1 [5′-AAACGCGCACCCGTGGTCGGCAAGGTCAA-3′] and 2 [5′-CGATGTCGGTCCGGATATT-3′]) was cloned in pMosblue-T-vector (Amersham) to give the recombinant plasmid pMBE9 (Fig. 1). A 1.3-kb fragment containing the gentamicin resistance gene (*accI*) was obtained by PCR amplification performed on pML122 (10), using two oligonucleotides, one introducing a BspEI restriction site (in boldface) (Gm 1, 5′-TTAAAATCCGGATGAAGGCACCGAACCAGTT-3′) and one located downstream from the BstEII restriction site (Gm 2, 5′-GACGCTTATGCCACCTCTCTATGTT-3′). The amplification product was digested with BstEII and BspEI and cloned into pMBE9 with a deletion of the BstEII-BspEI fragment containing *hydAB*, to give pE9Gm (Fig. 1). This recombinant suicide plasmid was introduced into the DM4 strain (ΔhydABC Kan²; ΔhndD Cm²) (12) depleted of the [NiFe] and NADP-reducing hydrogenases, using an electrotransformation procedure as previously described (19). The electrotransformed cells were first grown for 6 h without any antibiotics and then subcultured in the presence of antibiotics (50 μg of kanamycin ml⁻¹, 34 μg of thiamphenicol ml⁻¹, and 20 μg of gentamicin ml⁻¹) in liquid medium. The start of growth was observed within 2 weeks. Afterwards, recombinant cultures were isolated on plates under the anaerobic atmosphere of a glove box (N₂-H₂, 95:5 [vol/vol]) and incubated in hyperbaric (2 × 10⁵ Pa) (N₂-CO₂, 80:20 [vol/vol]) anaerobic jars. The genotype of the isolated gentamicin-resistant strain, called TM4, was then analyzed by Southern blot hybridization.

The blot of total DNA digested with EcoRI and BgII was first hybridized with a DNA fragment encompassing the majority of the *hydA* gene (probe A) (Fig. 2A). After removal of the probe, the blot was subsequently hybridized with the *accI* gene (probe B) (Fig. 2B). The pMBE9 and pE9Gm plasmids digested with *BspEI* (unique restriction site) were used as controls with the two probes. Restriction analysis of the region containing the wild-type *hydAB* genes revealed the existence of one *EcoRI* site and two *BgII* sites, producing three fragments (0.65, 0.8, and 1 kb) which specifically hybridized with probe A (Fig. 1). The hybridization pattern of the genomic DNA from
wild-type *D. fructosovorans* showed the 0.8- and 0.65-kb bands (Fig. 2A). The absence of the 1-kb band can be explained by the very short sequence (52 bp) of this fragment hybridizing with probe A. In strain TM4, the replacement of *hydAB* by *acc1* leads to the loss of one *EcoRI* site and one *BglII* site (Fig. 1). Thus, a 1.3-kb fragment should hybridize with probe A. The hybridization pattern of genomic DNA from the TM4 strain (Fig. 2A) showed that a double crossing over, rather than a single crossing over event, occurred. With probe B (Fig. 2B), two bands (1.3 and 2.2 kb) were revealed in the genomic DNA of the mutated strain TM4. Indeed, one *BglII* site is located in the middle of the gentamicin resistance reporter gene (Fig 1). As expected, pE9Gm hybridized specifically with probe B, and neither the genomic DNA from wild-type *D. fructosovorans* nor the pMBE9 plasmid was detected. The results of the Southern blot experiments demonstrated that the *hydAB* genes were deleted and replaced by the *acc1* gene. The TM4 mutant obtained is Δ*hydAB* Gm’ in addition to the Δ*shyABC* Km’ and Δ*hndD* Tm’ of the primary DM4 strain.

**Methyl viologen reduction activity of the triple mutant.** Hydrogenase activity in *D. fructosovorans* is usually determined by measurement of hydrogen-dependent methyl viologen reduction activity in soluble cellular extract obtained from cultures grown on a 30 mM fructose–50 mM sulfate medium (4, 5, 12, 19). The presence of the hydrogenase activities in the *D. fructosovorans* strains was tested in native polyacrylamide gel electrophoresis (Fig. 3). None of the three hydrogenase activities which are observed in the wild-type strain were visualized in the TM4 soluble cellular extract. In addition, total methyl viologen reduction activities measured in soluble cellular extracts of different strains are given in Table 1. As expected, no hydrogenase activity was detected in the TM4 strain by using methyl viologen as a redox mediator.

Complementation experiments were performed by cloning *hydAB* in shuttle vectors harboring chloramphenicol or streptomycin resistance (18). A synthetic linker (5′-AGCTTGCCC CCGCTGCA-3′/5′-GGGCCGGCCA-3′) was designed to introduce an *FseI* (boldface) site between the polylinker *PstI* (italics) and *HindIII* (underlined) sites, was cloned into plasmid pBMC6 digested with *PstI* and *BglII* and *HindIII* (18) to give the recombinant plasmid pC6Fse. A 2.6-kb *BbrPI/FseI* fragment from pMBE9 containing *hydAB* was cloned in pC6Fse digested with *SmaI* and *FseI*. This recombinant plasmid, pC6HF, was used to construct pS7HF. A 2.7-kb *HindIII/Asp718* fragment of pC6HF containing *hydAB* was blunted using Klenow enzyme (Roche Molecular Biochemicals) and cloned into the *SmaI*-digested pBMS7 plasmid harboring the streptomycin resistance gene (18). Electrottransformation of the TM4 strain was performed with the replicative pS7HF plasmid, and transformant TM4(pS7HF) colonies were isolated in medium containing 50 μg of kanamycin ml⁻¹, 34 μg of thiamphenicol ml⁻¹, 20 μg of gentamicin ml⁻¹, and 300 μg of streptomycin ml⁻¹. On a native gel with soluble cellular extracts prepared from the TM4(pS7HF) strain, a unique hydrogenase activity corresponding to the [Fe] hydrogenase was revealed (Fig. 3).

**Physiological studies.** Growth parameters of the wild-type and mutated strains were determined on fructose-sulfate medium as previously described (Table 2) (12). High-pressure liquid chromatography (Bio-Rad Aminex Fast Acid Analysis
HPAH column) analysis of the metabolism products in the culture medium did not give any evidence of intermediate accumulation or that an alteration in the metabolism had occurred during the growth of the mutant strain compared to the wild-type strain (data not shown). Fructose was completely oxidized into acetate and CO₂. The growth rate and the molar growth yield of strain TM4 were lower than those of the wild-type strain (Table 2), but surprisingly, no striking differences were observed compared to the results obtained in previous studies with the double mutant strain DM4 (12). Thus, the depletion of three hydrogenases did not have more of an effect on energy-generating metabolism than the depletion of two hydrogenases. It is possible to assume that the [Fe] hydrogenase is not necessary for growth on fructose-sulfate medium.

The wild-type and TM4 strains were grown mixotrophically on medium containing H₂ as the sole energy source, 50 mM fructose and 20 mM fumarate, rather than sulfate, was used to prevent formation of sulfate precipitates. Argon was bubbled in the reaction vessel filled with 1 ml of 50 mM Tris·HCl (pH 8) until saturation to eliminate the majority of O₂, and then 0.5 ml of the culture was added to the reaction vessel and D₂ was bubbled until saturation. Changes in concentrations of gases (D₂, HD, H₂, and O₂) were automatically scanned and recorded immediately after the vessel was closed, as described previously (24). Production of H₂ and HD was used to calculate the exchange activity. The results presented in Table 1 show that an exchange reaction occurred in the triple mutant strain (TM4), which was about 10-fold lower than that in the wild-type strain but equivalent to the activity measured in the double mutant strain (DM4).

As methyl viologen reduction activity was null for soluble cellular extracts of the TM4 strain grown on fructose-sulfate

**TABLE 1.** Methyl viologen reduction and H⁻→D₂ exchange activities in wild-type and mutated (DM4 and TM4) strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Methyl viologen reduction activity (U mg⁻¹) on:</th>
<th>H⁻→D₂ exchange activity (μmol of HD and H₂ produced min⁻¹ mg⁻¹) on fructose-fumarate medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fructose-sulfate medium</td>
<td>Fructose-fumarate medium</td>
</tr>
<tr>
<td>Wild type</td>
<td>8</td>
<td>8.8</td>
</tr>
<tr>
<td>DM4</td>
<td>0.15</td>
<td>ND³</td>
</tr>
<tr>
<td>TM4</td>
<td>0.005</td>
<td>0.375</td>
</tr>
</tbody>
</table>

* Methyl viologen reduction activities were measured in soluble cellular extracts from cultures grown on fructose-sulfate or fructose-fumarate medium, and H⁻→D₂ exchange activities were measured in whole-cell suspensions obtained from cultures grown on fructose-fumarate medium.
³ ND, not determined.

**TABLE 2.** Growth parameters of *D. fructosovorans* wild-type and mutated (DM4 and TM4) strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth rate (h⁻¹)</th>
<th>Yield relative to fructose (g mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.05 ± 0.001</td>
<td>36 ± 2.8</td>
</tr>
<tr>
<td>DM4³</td>
<td>0.041 ± 0.005</td>
<td>25.6 ± 2.8</td>
</tr>
<tr>
<td>TM4</td>
<td>0.037 ± 0.006</td>
<td>29.1 ± 1.5</td>
</tr>
</tbody>
</table>

³ Values were measured with 10, 20, and 30 mM fructose and 50 mM sulfate and are reported as means ± standard deviations.

FIG. 3. Detection, by methyl viologen reduction in a native gel, of the three hydrogenase activities in the wild-type strain (WT), the triple mutant strain (TM4), and the complemented strain [TM4(pS7HF)] grown on fructose-sulfate medium. Electrophoresis was performed in a glove box under an atmosphere of N₂:H₂ (95:5, vol/vol). The hydrogenase activities were detected by bubbling the gel with H₂ in the presence of methyl viologen and stained by adding 2,2,5-triphenyltetrazolium chloride.

FIG. 4. Kinetics of mixotrophic growth on medium containing H₂ as the sole energy source (H₂-acetate-CO₂-sulfate medium) of the wild-type (WT), double mutant (DM4), and triple mutant (TM4) strains. O.D., optical density.
medium and H$^+\cdot$D$_2$ exchange activity could be measured in whole cells of the same strain grown on fructose-fumarate medium (Table 1), we measured methyl viologen reduction in cultures grown on fructose-fumarate medium. Results obtained with whole-cell suspensions and soluble cellular extracts were quite similar (data not shown). Interestingly, a significant methyl viologen reduction activity was measured in the triple mutant TM4, representing 4% of the wild-type activity (Table 1). Thus, H$^+\cdot$D$_2$ exchange activity and methyl viologen reduction activity in cells grown on fructose-fumarate indicated the presence of a fourth hydrogenase in *D. fructosovorans*. This activity was not seen in strain TM4 grown on fructose-sulfate medium. Thus, it can be assumed that the induction or the derepression of a gene encoding a fourth hydrogenase occurred when TM4 was cultivated in fructose-fumarate medium.

In this medium, the metabolism of the bacteria is mostly fermentative, as the amount of exogenous fumarate (electron acceptor) is limiting. Thus, the energy-generating metabolism may be quite different than that in fructose-sulfate medium. It is possible to assume that the fermentative growth has induced the expression of one enzyme presenting an hydrogenase activity in order to dispose of an excess of reducing power. On the other hand, this hydrogenase is likely to be responsible for the growth on H$_2$ as a sole energy source.

The presence of multiple hydrogenases in bacteria is relatively widespread. *Escherichia coli* possesses four hydrogenases (1). In *D. vulgaris*, three hydrogenases of different types have been characterized ([Fe], [NiFe], and [NiFeSe]) (6), and a gene encoding a potential fourth one has been isolated (21). In this study, we investigated the characteristics and growth behavior of a fourth hydrogenase activity in *D. fructosovorans* sp. nov. Arch. Microbiol. 154:41–49.


