Roles of HynAB and Ech, the Only Two Hydrogenases Found in the Model Sulfate Reducer Desulfovibrio gigas

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Sulfate-reducing bacteria are characterized by a high number of hydrogenases, which have been proposed to contribute to the overall energy metabolism of the cell, but exactly in what role is not clear. Desulfovibrio spp. can produce or consume H2 when growing on organic or inorganic substrates in the presence or absence of sulfate. Because of the presence of only two hydrogenases encoded in its genome, the periplasmic HynAB and cytoplasmic Ech hydrogenases, Desulfovibrio gigas is an excellent model organism for investigation of the specific function of each of these enzymes during growth. In this study, we analyzed the physiological response to the deletion of the genes that encode the two hydrogenases in D. gigas, through the generation of ΔechBC and ΔhynAB single mutant strains. These strains were analyzed for the ability to grow on different substrates, such as lactate, pyruvate, and hydrogen, under respiratory and fermentative conditions. Furthermore, the expression of both hydrogenase genes in the three strains studied was assessed through quantitative reverse transcription-PCR. The results demonstrate that neither hydrogenase is essential for growth on lactate-sulfate, indicating that hydrogen cycling is not dispensable. In addition, the periplasmic HynAB enzyme has a bifunctional activity and is required for growth on H2, or by fermentation of pyruvate. Therefore, this enzyme seems to play a dominant role in D. gigas hydrogen metabolism.

Hydrogenases are key enzymes in the hydrogen metabolism of Desulfovibrio spp. that catalyze the reversible oxidation of molecular hydrogen into protons and electrons (1). However, their role during sulfate respiration has not been clearly established. Odom and Peck proposed a hydrogen cycling model to explain energy conservation during growth on lactate and sulfate by Desulfovibrio spp., which belong to the deltaproteobacteria subgroup of the sulfate-reducing bacteria (SRB) (2). The model predicts that protons and electrons produced in the oxidation of lactate are used for the production of molecular hydrogen by a cytoplasmic hydrogenase. This hydrogen then diffuses across the membrane to the periplasm, where it is reoxidized by a periplasmic hydrogenase. Electrons are transferred back to the cytoplasm for sulfate reduction, thus creating a proton gradient across the membrane that leads to ATP formation. In this model, the presence of at least two hydrogenases on opposite sides of the membrane is a requirement for growth. In contrast, other studies suggested that the physiological role of these enzymes was to regulate the redox potential of the cell, controlling the flow of protons and electrons and generating a proton motive force (3). More recent models, proposed for Desulfovibrio vulgaris, suggested dual pathways for electron transfer from lactate to sulfate, one involving the cycling of H2 and the other a route involving a membrane-associated electron transfer chain (4, 5). Several membrane complexes have been identified in SRB that could be involved in this process (reviewed in reference 6). It has been estimated that about 48% of the electrons transported from lactate to sulfate involve H2 production (4).

Several studies have tried to elucidate the function of hydrogenases in Desulfovibrio spp., but because most of these organisms present a multiplicity of hydrogenases, in the periplasm and/or in the cytoplasm, identification of the role of each enzyme is complex. In addition, the expression patterns of different hydrogenases were shown to be different and to depend on the substrate, fermentative or respiratory growth, or metal availability (5, 7–13). Furthermore, the function of each hydrogenase in terms of hydrogen production or oxidation may vary depending on the conditions presented to the cell. Numerous studies have reported hydrogenase mutant strains of Desulfovibrio fructosovorans and in D. vulgaris Hildenborough (9, 14–17). However, in most cases, because of the multiplicity of enzymes present, these studies were not conclusive. This indicates that each hydrogenase may contribute to the overall energy metabolism of the cell and that the loss of one enzyme might be compensated for by the presence of the remaining ones. In spite of the extensive work performed with hydrogenase deletion strains and also transcriptomic analyses, the results obtained so far have not permitted a complete elucidation of the function and importance of each hydrogenase under different growth conditions.

Desulfovibrio gigas is an excellent biological model for investigation of the function and importance of hydrogenases in energy metabolism, since its genome, recently sequenced in our laboratory, encodes only two hydrogenases, the HynAB and Ech enzymes. Furthermore, because each hydrogenase is located in a different cell compartment, D. gigas is also an excellent model for the study of the importance of hydrogen cycling in energy conservation. The D. gigas periplasmic HynAB enzyme is one of the most extensively studied enzymes of the [NiFe] type and was the first [NiFe] hydrogenase to have its crystal structure solved (18). In Desulfovibrio spp., as in other bacteria, the periplasmic hydrogenases are believed to be involved in the consumption of hydrogen, and their role during sulfate respiration has not been clearly established. Odom and Peck proposed a hydrogen cycling model to explain energy conservation during growth on lactate and sulfate by Desulfovibrio spp., which belong to the deltaproteobacteria subgroup of the sulfate-reducing bacteria (SRB) (2). The model predicts that protons and electrons produced in the oxidation of lactate are used for the production of molecular hydrogen by a cytoplasmic hydrogenase. This hydrogen then diffuses across the membrane to the periplasm, where it is reoxidized by a periplasmic hydrogenase. Electrons are transferred back to the cytoplasm for sulfate reduction, thus creating a proton gradient across the membrane that leads to ATP formation. In this model, the presence of at least two hydrogenases on opposite sides of the membrane is a requirement for growth. In contrast, other studies suggested that the physiological role of these enzymes was to regulate the redox potential of the cell, controlling the flow of protons and electrons and generating a proton motive force (3). More recent models, proposed for Desulfovibrio vulgaris, suggested dual pathways for electron transfer from lactate to sulfate, one involving the cycling of H2 and the other a route involving a membrane-associated electron transfer chain (4, 5). Several membrane complexes have been identified in SRB that could be involved in this process (reviewed in reference 6). It has been estimated that about 48% of the electrons transported from lactate to sulfate involve H2 production (4).

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coming either from the environment or from intracellular H₂ cy-
clling, generating protons and electrons. The electrons are then
shuttled to the cytoplasmin through the type I cytochrome c₅₅₃ and
the Qr and QmO complexes (19–21) to be used for sulfate reduc-

The D. gigas cytoplasmic EcH hydrogenase (22) belongs to the
subgroup of multisubunit membrane-bound energy-conserving
[NiFe] hydrogenases (23, 24), similar to the EcH hydrogenase
from Methanosarcina barkeri (25, 26). These enzymes have sub-
units that show high similarity to energy-conserving complex I
(NADH:quinone oxidoreductase). The EcH hydrogenase in methanogen-
archaea can catalyze H₂ formation from reduced ferredoxin, generat-
ing a proton motive force, or the reduction of ferredoxin by H₂, driven by reverse electron transport (26–29). However, the function of the EcH hydrogenase in Desulfovibrio spp. is not still clear.

To elucidate the function of each of these two hydrogenases in
D. gigas metabolism, we have generated single deletion (ΔechBC
and ΔhynAB) mutants. These strains were tested under respira-
tory versus fermentative conditions with different energy sources
(lactate, pyruvate, and H₂), and their hydrogenase expression pro-
files were studied at the mRNA level.

**MATERIALS AND METHODS**

**Generation of mutants.** To construct the mutant strains, recombinant
cassettes containing the DNA fragments of the regions flanking the genes
of interest were amplified from D. gigas wild-type (WT) ATCC 19364. A mutant D. gigas strain lacking a functional EcH hydrogenase (Δech) was produced by replacement of the echB (integral membrane subunit) and echC (electron transfer subunit) genes with a kanamycin resistance (kan) gene amplified from plasmid pRD215 (27) by homologous recombina-
tion (30). Oligonucleotides (ech, see Table S1 in the supplemental mate-
rual) were used to amplify ~1-kb adjacent DNA regions upstream of subunit echB (flank I) and downstream of subunit echC (flank II), respec-
tively, from a DNA fragment containing the ech operon. Pfx DNA poly-
merase (Invitrogen) was used for amplification. The PCR products were ligated with T4 DNA ligase (Fermentas) into plasmid pZERO-1 (Invitro-
gen), and this construct was transformed in Escherichia coli XL-1 Blue.
Plasmid DNA was extracted with the plasmid purification kit from
Eppendorf.

For the hynAB gene, a D. gigas mutant strain lacking the entire dicis-
tronic operon was produced by replacement with kan in as in the Δech mu-
tant. Oligonucleotides (hynAB; see Table S1 in the supplemental material)
were used to amplify ~1-kb adjacent DNA regions upstream and down-
stream of the operon directly from WT D. gigas genomic DNA (gDNA).
Phusion high-fidelity DNA polymerase (Thermo Scientific) was used for
amplifications. The PCR products were ligated and transformed into the
detectant YipLac211 according to the In-Fusion HD cloning kit (Clontech)
protocol.

Kanamycin-resistant colonies of both constructs were selected, and
their plasmids were analyzed by restriction enzyme pattern. These plas-
mids were used for D. gigas transformation.

D. gigas cells to be transformed were prepared as previously described
(30) from 500 ml of an early stationary-phase culture. Immediately before
transformation, 6 μg of the plasmid construct was mixed with the cells.
Transformation was done aerobically in 0.1-cm cuvettes by electropora-
tion in a Bio-Rad Gene Pulser Apparatus, setting the resistance to ∞ and
using a 0.7-kV voltage and a 3-μF capacitance.

Immediately after electroporation, cells were inoculated into lactate-
sulfate medium at 37°C. After a 5-h recovery period, kanamycin was
added to the medium (50 μg/ml) and the cultures were allowed to grow
overnight. Cells were then subcultured in lactate-sulfate medium with
kanamycin (50 μg/ml) three consecutive times.

Colonies were grown for approximately 15 days to 1 month in me-
dium supplemented with agar (15 g/liter) and kanamycin (50 μg/ml) in
Hungate culture tubes by the roll tube technique and/or in plates inside an
AnaeroPack Rectangular Jar 7L (Mitsubishi Gas Chemical Company, Inc.)
with AnaeroPack System sachets (bioMérieux).

The deletions of the echBC and hynAB genes from the respective mu-
tant strains were confirmed by PCR and Southern blot analyses (see
the supplemental material).

**Culture media and growth conditions.** D. gigas cells were grown an-
erobically at 37°C in 100-ml flasks with 50 ml of medium. All media
were inoculated with 10% (vol/vol) fresh preculture cells grown in lactate-
sulfate medium.

For most phenotypic analyses, WT and mutant strains were grown in
basal medium modified from reference 14 and containing (per liter) 1 g
NH₄Cl, 0.15 g CaCl₂·2H₂O, 1 g NaCl, 0.5 g KCl, 0.4 g MgCl₂·7H₂O, 4.9
KH₂PO₄, 0.1 g yeast extract, and 1.5 ml of trace elements (31). This
medium was then supplemented with either lactate or pyruvate as an
electron donor at a concentration of 40 mM. Sulfate was added as an
electron acceptor at either 40 or 5 mM or not added. The pH of all solu-
tions was brought to 7.0 with NaOH. The growth conditions tested were
lactate and sulfate at 40 and 40 mM, respectively; pyruvate and sulfate at
40 and 40 mM; pyruvate and limiting sulfate at 40 and 5 mM; and pyru-
vate at 40 mM.

For growth with H₂ as the sole energy source, cells were grown on
modified Postgate medium C containing (per liter) 0.82 g sodium acetate,
0.5 g KH₂PO₄, 1 g NH₄Cl, 1.14 g NaNO₃, 0.05 g CaCl₂·7H₂O, 0.5 g
MgSO₄·7H₂O, 0.2 g yeast extract, 0.0071 g FeSO₄·7H₂O, 0.3 g sodium
citrate, 0.1 g ascorbic acid, 0.1 g sodium thioglycolate, 1 μM Ni, 1 μM Se,
and 0.1 μM Mo. The cultures were inoculated in 100-ml flasks containing
50 ml of medium and then gassed with H₂:CO₂ (80:20, vol/vol) at a
pressure of 1 atm. The cultures were grown with the flasks at 37°C in a
horizontal position to enhance the gas-liquid surface area.

**Growth of the cultures was monitored by determining the optical
density at 600 nm (OD₆₀₀).** Biomass was determined by measuring the dry
weight (dcw) and correlating it with the OD₆₀₀. One unit of OD₆₀₀
corresponded to 0.365 g (dcw)/liter.

**Analytical procedures.** H₂ quantification in the headspace of culture-
containing serum bottles was performed by gas chromatography with a
Thermo Electron Corporation TRACE GC Ultra gas chromatograph fit-
ted with a Alltech Molecular Sieve 5A 80/100 column. The carrier gas was
N₂, and measurements were done at 130°C. Headspace volumes of 30 μl
were withdrawn with a gas-tight syringe and injected into the gas chro-
matograph. The detection limit was 5 mmol of molecular hydrogen. Cul-
tures of WT D. gigas and both mutant strains were compared.

Identification and quantification of the organic substrates and prod-
ucts generated during growth were performed by high-performance liq-
uid chromatography (HPLC) analyses with a Waters chromatograph
(Waters Chromatography, Milford, MA) consisting of a Waters 510
pump, a Waters 715 Autosampler, and a Waters temperature control
module connected to an LKB 2142 Differential Refractometer detector
(LKB, Bromma, Sweden). Chromatographic separation was undertaken
with an Aminex HPX-87H column (300 by 7.8 mm) with a 9-μm particle size
(Bio-Rad, Hercules, CA) at 45°C. Elution was carried out isocratically at
a flow rate of 0.6 ml·min⁻¹ with 0.005 M H₂SO₄, and the injection
volume was 20 μl. The retention times of the compounds were compared
with standards for identification, and the peak area was used for quanti-
fication.

Sulfate concentration was measured by HPLC analysis (32) and/or by
the SulfaVer4 method (Hach-Lange). HPLC analyses were performed with
a Hitachi LaChrom Elite HPLC apparatus with a photodiode array
detector. Injections of 20 μl were made into a 10-μl loop operated in
full-loop mode, and separation was achieved on a PRP-X100 (4.1 by 150
mm) with a 10-μM-particle-size column (Hamilton Company, Reno,
NV) and a thermostat set at 25°C. Isocratic conditions of the mobile phase
consisted of 3% (vol/vol) methanol and 97% (vol/vol) 4 mM 4-hydroxy-
benzoic acid (pH adjusted to 10). The flow rate of the eluent was 2 ml
Function of Hydrogenases in *Desulfovibrio gigas*

**RESULTS**

A search of the *D. gigas* genome sequenced in our laboratory revealed the presence of genes encoding only the two known hydrogenases, the periplasmic HynAB and cytoplasmic Ech hydrogenases. Two mutant strains with these enzymes deleted, the ΔechBC and ΔhynAB mutant strains, were generated and then tested under different growth conditions. The deletions of *echBC* and *hynAB* were confirmed by both Southern blot and PCR analyses (see Table S2 in the supplemental material). The 16S rRNA gene was used as an internal reference gene for each sample analyzed. qRT-PCRs were performed in a LightCycler 480 real-time PCR system (Roche) with LightCycler Fast Start DNA Master SYBR green I (Roche). Relative standard curves and gene expression were calculated as described in reference 31. For the final results, three biological replicates and two technical replicates were used for each condition.

**Nucleotide sequence accession numbers.** The NCBI accession number of the *D. gigas* genome sequence described in this work is CP006585. In addition, the accession numbers of the hydrogenases and specific membrane complexes are as follows: aprAB qmoABCD, KF113859; dsrMKJOP, KF113860; echABCDEF, AY282786; hdrABC floxABC, KF113861; hynAB, M18083; qrcABC, KF113862.

**TABLE 1** Doubling times and cell yield coefficients of WT *D. gigas* and ΔechBC and ΔhynAB hydrogenase mutant strains during growth under different conditions.

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Doubling time (h)</th>
<th>Yc (g [dry wt] cells/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>ΔechBC mutant</td>
</tr>
<tr>
<td>Lactate-sulfate</td>
<td>8.7</td>
<td>11.1</td>
</tr>
<tr>
<td>Pyruvate-sulfate1</td>
<td>6.7</td>
<td>6.7</td>
</tr>
<tr>
<td>Pyruvate-sulfate2</td>
<td>6.9</td>
<td>7.1</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>14.2</td>
<td>16.7</td>
</tr>
<tr>
<td>H2-sulfate</td>
<td>33.3</td>
<td>24.1</td>
</tr>
</tbody>
</table>

1 Pyruvate-sulfate (40 mM–40 mM).
2 Pyruvate-limiting sulfate (40 mM–5 mM).
first case, although sulfate was limiting, the growth of the \(D. \text{gigas}\) WT and mutant strains reached cell densities similar to those seen when sulfate was present in excess (Fig. 1), revealing that enough sulfate was present to allow respiratory growth. In agreement with this, the doubling time was similar to pyruvate respiratory conditions and once again the deletion of the \(\text{hynAB}\) genes slowed growth (Table 1). When yield coefficients were compared, a decrease in cell yield was observed for all of the strains under this condition, especially in the case of the \(\Delta \text{hynAB}\) mutant, which possibly correlates with the lower sulfate concentration available. Once again, it is noticeable that the absence of the \(\text{echBC}\) genes appears to increase the cell yield of the mutant strain over that of the WT.

Interestingly, after sulfate was completely reduced at 20 to 24 h (Fig. 3A), the cells stopped growing and started to accumulate \(\text{H}_2\) (Fig. 3E), and the \(\Delta \text{echBC}\) mutant strain, containing only the periplasmic hydrogenase, accumulated much more \(\text{H}_2\) than did the WT strain. On the other hand, the \(\Delta \text{hynAB}\) mutant strain,
having the cytoplasmic hydrogenase, did not accumulate any H\textsubscript{2}. No difference in pyruvate consumption or acetate formation was observed, as shown in Fig. 3C, indicating that the difference in H\textsubscript{2} accumulation was due to HynAB activity.

Under fermentative conditions when no sulfate was added, a small amount of sulfate (\(\sim 1 \text{ to } 3 \text{ mM}\)) was nevertheless present in the beginning of growth, coming from the inoculum. After this sulfate was reduced (\(\sim 16 \text{ h}\)), we observed a phenotype different from that seen during respiratory growth on pyruvate. The \(\Delta\text{hynAB}\) mutant strain did not show fermentative growth once the small amount of residual sulfate was consumed, whereas the \(\Delta\text{echBC}\) mutant and WT strains were able to grow, albeit to lower cell densities (Table 1) than in pyruvate-sulfate. Furthermore, the doubling times of these strains in pyruvate fermentation were at least double those seen when sulfate was present. From the metabolite analysis, it is clear that the absence of the HynAB hydrogenase prevented the cells from fermenting pyruvate, resulting in the absence of fermentative growth of this strain (Fig. 3B and D). Furthermore, the expression of the ech genes is also lower under this condition in the \(\Delta\text{hynAB}\) mutant strain than in the WT strain (see below), which may also have contributed to the lack of growth.

Similarly to what happened under sulfate-limiting conditions, H\textsubscript{2} accumulation in the \(\Delta\text{echBC}\) strain was much higher than that in the WT (Fig. 3F). Interestingly, this accumulation started only after all of the pyruvate was consumed, as previously reported for \textit{D. vulgaris} (39). A slight accumulation of H\textsubscript{2} could be observed in the \(\Delta\text{hynAB}\) mutant strain as well, showing that the Ech hydrogenase can contribute to some H\textsubscript{2} production under these conditions.

These results indicate that under fermentative conditions, HynAB is an essential enzyme for cell growth and that cells are unable to ferment pyruvate in its absence. Furthermore, periplasmic HynAB is the main hydrogenase responsible for the H\textsubscript{2} accumulation observed under fermentative conditions.

**Expression profiles of the ech and hyn genes.** The higher H\textsubscript{2} accumulation by the \(\Delta\text{echBC}\) mutant strain, relative to that by the WT, during pyruvate fermentation is very surprising, since it could be expected that the cytoplasmic Ech hydrogenase would be responsible for H\textsubscript{2} production from reduced ferredoxin produced by the pyruvate:ferredoxin oxidoreductase. To evaluate if this was due to an increase in HynAB transcripts in the deletion strain, we analyzed the expression levels of genes coding for both the HynAB and Ech hydrogenases in WT \textit{D. gigas} and the deletion strains.

Real-time qRT-PCR was performed to analyze the mRNA expression levels of the HynAB hydrogenase in both WT \textit{D. gigas} (Fig. 4A) and the \(\Delta\text{hynAB}\) mutant strain (Fig. 4B) in the mid-exponential (16 h) and stationary (32 h) phases in pyruvate-limiting sulfate and pyruvate fermentation, as well as under lactate-sulfate and H\textsubscript{2}-sulfate conditions, for which microarray expression data have been reported for other \textit{Desulfovibrio} spp. (5, 7). Expression of the Ech hydrogenase was similarly evaluated in WT \textit{D. gigas} (Fig. 5A)
and the ∆hynAB mutant strain (Fig. 5B). Quantification of all samples was performed relative to the 16S rRNA gene.

The expression of the hynB gene was highest in the WT strain during exponential growth in hydrogen respiration and in pyruvate fermentation (Fig. 4A), whereas it was lower in pyruvate-sulfate and even lower in lactate-sulfate. After cells entered stationary phase, hynB gene expression dropped to very low levels in hydrogen-sulfate, pyruvate-sulfate, and pyruvate fermentation, whereas it increased under lactate-sulfate conditions.

An increase in hynB gene expression, compared to that of the WT, was observed in the ∆echBC mutant strain under conditions in which the hynAB deletion prevented growth, i.e., hydrogen respiration and pyruvate fermentation (compare Fig. 4B and A). This increase was more prominent during the exponential phase but was also observed during the stationary phase, where we saw a more accentuated drop in the expression of hynB in the WT. Under respiratory conditions with organic substrates (lactate-sulfate and pyruvate-limiting sulfate), hynB expression in the exponential phase was similar to that of the WT, and once again an increase was observed in the stationary phase relative to the WT level. These increased levels of hynB in the stationary phase observed in the ∆echBC mutant may account for the greater accumulation of H₂ observed in pyruvate limitation fermentation during physiological analyses. Therefore, in the ∆echBC mutant strain, the absence of the Ech hydrogenase appears to be compensated for by an increased level of HynAB (except during exponential-phase growth in lactate-sulfate).

We also measured the levels of echE expression in the WT and ∆hynAB mutant strains. The echE mRNA levels were highest in the D. gigas WT strain during exponential growth on an organic substrate (lactate or pyruvate) in the presence of sulfate (Fig. 4A, 16 h). After cells entered the stationary phase (32 h), the levels of echE gene expression decreased significantly, especially during growth on lactate. During respiratory growth with H₂ as the energy source, the opposite behavior was observed, where the echE mRNA level was almost undetectable during the exponential phase (16 h) but increased after cells entered the stationary phase. During fermentative growth on pyruvate, the echE transcript levels were low in the exponential phase and decreased further to undetectable levels after cells entered the stationary phase. This suggests that Ech activity is related mainly to the initial phase of respiratory growth (i.e., when sulfate is still present) with organic substrates, whereas Ech appears to play a much less important role during fermentative growth.

When echE gene expression in the ∆hynAB mutant strain was analyzed (Fig. 5B), a major overall decrease in expression was observed under all conditions, compared to the WT level (Fig. 5A), suggesting that the HynAB hydrogenase is involved in the regulation of Ech expression. These results indicate that during hydrogen-sulfate respiration and pyruvate fermentation, the Ech hydrogenase is poorly expressed and thus cannot compensate for the absence of the HynAB hydrogenase, preventing growth under these conditions.

**DISCUSSION**

Previous studies have addressed the role of hydrogenases in *Desulfovibrio* spp. In a *D. vulgaris* strain with the periplasmic Hyn1 [NiFe] hydrogenase deleted, cells were able to grow to almost the same level as the WT in lactate-sulfate medium (17). Similar results were obtained with a *D. fructosovorans* periplasmic hynABC and cytoplasmic hnd hydrogenase double mutant that was able to grow under all of the conditions tested (14). A further triple mutant lacking all described periplasmic hydrogenases was also shown to grow in fructose-sulfate medium (16). However, in these studies, there were additional hydrogenases present that could compensate for the absence of the missing genes.

*D. gigas* is uniquely positioned among *Desulfovibrio* spp. for use in testing the function of hydrogenases and the importance of hydrogen cycling in energy conservation, since it contains only two enzymes, one periplasmic and one cytoplasmic. Furthermore, the genomic analyses revealed no sequences related to the pyruvate-formate lyase gene in *D. gigas*, and therefore, formate cycling should not be participating in the energy conservation processes as an alternative to hydrogen cycling.

The results obtained in this work demonstrate that neither of the two hydrogenases is essential for the growth of *D. gigas* under respiratory conditions on an organic substrate (lactate or pyruvate) in the presence of sulfate. In addition, the absence of the hynAB or echBC genes also did not lead to any significant accumulation of molecular hydrogen. Since no other hydrogenase, either periplasmic or cytoplasmic, is present in the genome of *D. gigas* and formate is not participating in the energy conservation process, this suggests that the hydrogen cycling pathway does not play a major part in the bioenergetics of *D. gigas*. The cell yield coefficients calculated for sulfate respiration with lactate and pyruvate are almost identical for the WT and hydrogenase mutant strains. Nevertheless, a slightly lower growth rate is observed for the ∆echBC mutant strain growing on lactate-sulfate and for the ∆hynAB mutant strain in both lactate-sulfate and pyruvate-sulfate, suggesting that a small fraction of the electron flow to sulfate may involve the production of H₂. In *D. vulgaris*, a mathematical model of metabolism, supported by experimental results, indicated that two simultaneous pathways for electron flow during growth in lactate-sulfate coexist, one requiring the obligate cycling of H₂ and the other not requiring it (4). The model estimated that 48% of the electron flow from lactate to sulfate involved H₂ production. Our results suggest that this value may be significantly lower for *D. gigas*. This would be in line with the reduced number of hydrogenases in this organism compared to *D. vulgaris*, which has seven hydrogenases. Nevertheless, we have been unable to date to generate a double hydrogenase mutant of *D. gigas*. However, given the difficulty in transforming this organism, we cannot clearly conclude that this is due to the required presence of at least one hydrogenase.

Thus, as suggested before, the mechanism of hydrogen cycling does not seem to be strictly essential for the *Desulfovibrio* genus (9) and may make different contributions to the overall electron flow in different organisms. This is not entirely surprising since it was previously shown that some SRB do not have any hydrogenases at all (e.g., *Desulfococcus oleovorans*) or have no cytoplasmic hydrogenases (like *Desulfovibrinobacterium baculatum*, which is closely related to *Desulfovibrionaceae*) (6).

Thus, H₂ is not an obligatory intermediate in the oxidation of organic compounds by *D. gigas* since deletion of the hydrogenases does not affect the ability of cells to grow. This agrees with the observation by Lupton et al. that H₂ added to lactate-sulfate medium did not competitively inhibit the oxidation of lactate or increase growth or substrate utilization (3). However, we cannot rule out the possibility that other electron transfer pathways substitute for hydrogen cycling when one of the hydrogenases is miss-
ing. It is plausible that the cells can reroute electron flow if one of several pathways is impaired. This would be the advantage of having dual or multiple pathways for electron flow, and sulfate reducers are recognized for their metabolic flexibility.

Chemiosmotic models of energy conservation have also been proposed in which electrons generated from substrate oxidation are transported through membrane-bound electron carriers to sulfate reduction (vectorial electron transport) and in the process translocate protons to the periplasm (vectorial proton transport) (6, 35). Two membrane complexes that were identified in the D. gigas genome, the Qmo and Dsr complexes, were proposed to perform this function (21, 36). An energy conservation process may result from electron transfer from the quinone pool to AprAB through the Qmo complex (19, 37), and also the Dsr complex may be involved in menaquinol oxidation with reduction of DsrC, associated with proton translocation (36, 38). Furthermore, other electron transfer pathways to sulfate may involveHdr-related proteins that could provide a soluble pathway from different donors (such as lactate, pyruvate, ferredoxin, or H2) to the reduction of DsrC in a flavin-based electron bifurcation mechanism for energy conservation (6). Such a system may involve the HdrABC/FloxFABCD proteins that are also encoded in the D. gigas genome.

Regarding the physiological role of hydrogenases, the periplasmic enzymes are generally presumed to be involved in H2 oxidation. Previous deletions of periplasmic hydrogenases in D. vulgaris demonstrated that in lactate-sulfate or H2-sulfate, the absence of Qrc, and pyruvate demonstrated that this periplasmic hydrogenase is likely to be regulated in the absence of H2-sulfate compared to lactate-sulfate (5, 7). How- ever, the ech mutant strain of D. gigas was able to reach a final cell density similar to that of the WT with a shorter doubling time when growing in H2-sulfate.

Overall, it seems that the Ech hydrogenase does not play a central role in energy metabolism under the conditions tested, which agrees with the fact that no Ech hydrogenase has been identified in the genomes of many SRB, including Desulfovibrio spp. such as D. alaskensis G20 and Desulfovibrio piger (6).

Our results also provide compelling evidence of the importance of obtaining expression data to complement studies of gene deletions. Indeed, the expression of each hydrogenase was significantly altered in the mutants. In particular, the expression of the Ech hydrogenase was almost completely abolished upon deletion of the HynAB enzyme, regardless of the energy source or type of growth. This suggests that HynAB may be somehow involved in the regulation of the ech genes. One interesting observation was that the two hydrogenases have somewhat complementary expression, as the HynAB hydrogenase was expressed more during growth in H2-sulfate and pyruvate fermentation, whereas the Ech hydrogenase was expressed more during growth in lactate-sulfate and pyruvate-sulfate.

In conclusion, the HynAB hydrogenase appears to have a more predominant role in the metabolism of D. gigas and is essential for growth with H2-sulfate and pyruvate fermentation.

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Discussions.


