A New Function of the *Desulfovibrio vulgaris* Hildenborough [Fe] Hydrogenase in the Protection against Oxidative Stress*

Marjorie Fournier, Zorah Dermoun, Marie-Claire Durand, and Alain Dolla‡

From the Laboratoire de Bioénergétique et Ingénierie des Protéines, CNRS 31, Chemin Joseph Aiguier, 13402 Marseille, cedex 20, France

Sulfate-reducing bacteria, like *Desulfovibrio vulgaris* Hildenborough, have developed a set of reactions allowing them to survive in oxic environments and even to reduce molecular oxygen to water. *D. vulgaris* contains a cytoplasmic superoxide reductase (SOR) and a periplasmic superoxide dismutase (SOD) involved in the elimination of superoxide anions. To assign the function of SOD, the periplasmic [Fe] hydrogenase activity was followed in both wild-type and sod deletant strains. This activity was lower in the strain lacking the SOD than in the wild-type when the cells were exposed to oxygen for a short time. The periplasmic SOD is thus involved in the protection of sensitive iron-sulfur-containing enzyme against superoxide-induced damages. Surprisingly, production of the periplasmic [Fe] hydrogenase was higher in the cells exposed to oxygen than in those kept in anaerobic conditions. A similar increase in the amount of [Fe] hydrogenase was observed when an increase in the redox potential was induced by addition of chromate. Viability of the strain lacking the gene encoding [Fe] hydrogenase after exposure to oxygen for 1 h was lower than that of the wild-type. These data reveal for the first time that production of the periplasmic [Fe] hydrogenase is up-regulated in response to an oxidative stress. A new function of the periplasmic [Fe] hydrogenase in the protective mechanisms of *D. vulgaris* Hildenborough toward an oxidative stress is proposed.

Microbial sulfate reduction has been reported to be an ancient process as old as 3.47 gigayears. Sulfate reduction thus represents an early specific metabolic pathway, allowing time calibration of a deep node on the tree of life (1). Sulfate-reducing bacteria (SRB) are universally distributed in marine and microbial mats where sulfate reduction is the dominant anaerobic biomineralization pathway. They constitute a group of anaerobic prokaryotes, which are unified by sharing the capacity to carry out dissimilatory sulfate reduction to sulfide as a major component of their bioenergetic processes. Although sulfate reduction is generally considered to be an anaerobic process, the abundance and metabolic activity of SRB in oxic zones is frequently evaluated as higher than those in neighboring anoxic zones in numerous biotopes (2, 3). In situ detection of sulfate reducing activity and SRB populations in these biotopes showed that SRB species did not have the same capability of resisting oxygen. Analysis of the photoxic zone of cyanobacterial mats revealed a well-defined SRB distribution. Although Desulfobacter and Desulfbacterium were restricted to the deepest levels in the mats, Desulfococcus were predominant in the photoxic zone with *Desulfovibrio* also present. This distribution suggests that both groups contain oxygen-tolerant members (4). It is thus clear that SRB exhibit a differentiated set of reactions to oxygen: first, many SRB form aggregates (5), resulting in a higher tolerance to oxygen exposure; second, at least *Desulfovibrio* species migrate in response to the oxygen concentration in their environment (5); and third, many species respire with oxygen (6). However, although it has been demonstrated that aerobic respiration is coupled with proton translocation and ATP conservation, aerobic growth in pure culture has never been proved (7). The high respiration rate merely seems to have a protective function. In an oxygen gradient, bacteria form bands at the outer edge of the oxic zone, suggesting both negative and positive aerotaxis responses (8). The capability of SRB to move toward oxygen and reduce it might play an ecological role in the zone of transition from an oxic to an anoxic environment, protecting themselves and other obligate anaerobes from the toxic effects of oxygen and creating an optimal redox environment for bacterial growth.

The responses of SRB to the presence of oxygen have been most thoroughly studied in *Desulfovibrio* species. Various mechanisms of oxygen reduction in *Desulfovibrio* species have been described. *Desulfovibrio gigas* is able to generate ATP from internal polyglucose reserves by full reduction of oxygen directly to water. This reduction is linked to NADH oxidation and involves several soluble enzymes and electron transport proteins, the key enzyme being the rubredoxin-oxygen oxidoreductase (9, 10). An oxygen-reducing respiratory chain involving a membrane-bound cytochrome *bd* terminal oxygen reductase has been purified in the same organism (11), and genes for a membrane-bound cytochrome c oxidase have been found in *D. vulgaris* Miyazaki (12). Genome analysis of *D. vulgaris* Hildenborough (www.tigr.org) and *D. sulfuricans* G20 (www.jgi.doe.gov) revealed the presence of both genes for cytochrome c oxidase and cytochrome *bd*. The involvement of hydrogenase and *c*-type cytochromes in periplasmic oxygen reduction by *Desulfovibrio* species was recently proposed (13). Although these two classes of metalloproteins have been shown to be the key enzymes of the energy-generating metabolism in anaerobic sulfate respiration (14), oxygen reduction constitutes a new mechanism in which these two proteins might be involved.

Before photosynthetic bacteria, which appeared 2.7 gigayears ago, little or no oxygen was expected in the atmosphere. However, several models are proposed that describe the release of low amounts of oxidative molecules (hydrogen peroxide or molecular oxygen) (15, 16), forcing anaerobic life forms to develop defenses. Because of the ancestral character of microbial
sulfate reduction, earlier than the cyanobacterial activity, the mechanism by which sulfate-reducing bacteria resist oxidative stress would represent an ancient critical system developed during earliest life forms.

Because *Desulfovibrio* cells could be in contact with oxygen and even reduce it, they must have developed mechanisms of defense against the reactive oxygen species that are derived from partial oxygen reduction. As a matter of fact, cells contain a number of potential generators of superoxide anion, such as cytochrome, flavodoxin, and ferredoxin. Superoxide dismutase and catalase, which are enzymes well known to eliminate superoxide and hydrogen peroxide in aerobic organisms, have also been characterized in some *Desulfovibrio* species (17, 18).

In addition to these enzymes, a superoxide reductase (SOR) and a rubrerythrin, which has an NADH-dependent H₂O₂ reductase activity, have also been found in sulfate-reducing bacteria (19–22). SOR and rubrerythrin reduce superoxide and hydrogen peroxide, respectively, without regeneration of oxygen, a feature that might be important for oxygen detoxification in anaerobes (23). The multiplicity of these enzymes renders the function of each one difficult to assign. *Desulfovibrio vulgaris* Hildenborough contains both SOD and SOR for eliminating superoxide ions (18, 19, 24). Following gene deletion, it was proposed that SOR might play a key role in oxygen defense (18, 24). Because of its periplasmic location, SOD is thought to remove the periplasmic-generated superoxide (25) that could induce oxidative damage on sensitive iron-sulfur enzymes.

To assign a function to the periplasmic SOD, we report here the effect of oxygen on the periplasmic hydrogenase of *D. vulgaris* Hildenborough wild-type and *sod* mutant strains. The physiological protective effect of SOD is described as well as a response mechanism of *D. vulgaris* cells to the presence of oxygen. Evidence is given for a peculiar function of periplasmic [Fe] hydrogenase in the protection against oxidative stress.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—*D. vulgaris* Hildenborough (26), *D. vulgaris* SOD100 (SOD100) (23), and *D. vulgaris* HYD100 (HYD100) (27) were cultured in medium C and on solid medium E (26) at 32 °C in an anaerobic chamber (COY) filled with a 5% H₂/5% CO₂/90% N₂ mixed gas atmosphere. Growth medium was supplemented with kanamycin (50 μg/ml) and chloramphenicol (10 μg/ml) when appropriate.

**Exposure to Pure Oxygen or Chromate**— Cultures (50 ml) of wild-type and mutant strains were grown anaerobically in medium C with kanamycin until A₅₆₀ was equal to 0.8. These cultures (20 ml) were used to inoculate 200 ml of medium C that was in turn incubated in the anaerobic COY chamber at 32 °C for 24 h. An aliquot of the culture (100 ml) was removed from the anaerobic chamber for pure oxygen flushing at room temperature while the remaining culture was kept in anaerobic conditions in the COY chamber at room temperature. Aliquots (25 ml) of oxidized aerobically and anaerobically cultured were collected after 15, 30, and 60 min, and protein extracts were prepared. Alternatively, aliquots (100 μl) of cultures exposed to oxygen for 60 min were collected and transferred to the anaerobic chamber, serially diluted, and plated on solid medium E. Colonies were counted after 6 days of anaerobic incubation at 32 °C as the number of surviving colony-forming units/ml and compared with the surviving colony-forming units/ml obtained from cells that were not exposed to oxygen.

In the case of exposure to ammonium chromate (VI), cells were cultured as described above until A₅₆₀ was equal to 0.8. 100 μM (NH₄)₂CrO₄ (final concentration) was added to 100 ml of cultures, and aliquots (25 ml) were periodically removed for protein extraction. Change of the redox potential of the medium after either chromium addition or oxygen flushing was measured using a combined redox electrode.

**Preparations of TE Extracts and Crude Extracts**—Cells were harvested by centrifugation (5600 × g, 20 min at 4 °C) and washed once with 1 ml of 0.1 M Tris-HCl/0.15 M NaCl (pH 7.6). The cell pellets were then resuspended in 0.25 ml of 0.1 M Tris-HCl/0.1 M EDTA (pH 9) and incubated 30 min at 37 °C. After centrifugation (2000 × g, 5 min at 4 °C), the red supernatant (TE extract) was transferred into a clean tube. To prepare crude extracts, the washed cells were resuspended in 5 ml of 5 mM Tris-HCl (pH 7.6) and lysed by two passages through a Constant Cell Disruption System (2 kilohars). After centrifugation at 5600 × g for 30 min at 4 °C, the supernatant (crude extract) was collected. All buffers used for the extracts preparation were flushed with argon before use. Proteins were quantified using the Bio-Rad DC software.

**Hydrogenase Activity on Native Polyacrylamide Gel Electrophoresis (Native PAGE)**—10 μg of proteins from either TE extract or crude extract were dried, resuspended in 10 μl of native protein loading sample buffer (0.1 M Tris-HCl (pH 6.8), 20% glycerol (w/v), 0.2% bromphenol blue (w/v)), and loaded onto 12% (w/v) nondenaturing polyacrylamide gel. After electrophoresis in 0.02 M Tris-HCl/0.2 M glycine buffer (pH 8.3), the gel was incubated in 100 mM Hepes buffer (pH 7.6) and bubbled under argon for 20 min. Methyl viologen (0.1 mM final concentration) was added, and the solution was flushed with pure hydrogen until the hydrogenase band activities were revealed. The activity staining was fixed by addition of 3,3',5,5'-tetramethylbenzimidazoline. Stained gels were scanned using the ImageScanner system from APBiotech, and densitometric analyses were performed using the ImageMaster 2D software (APBiotech).

**Spectrophotometric Assays for Hydrogenase Activities**—Quantification of hydrogenase activities on the various fractions was performed spectrophotometrically. Hydrogen was used as electron donor and methyl viologen as electron acceptor. The reduction of methyl viologen (1 mM) with 15,600 m⁻¹s⁻¹ was followed at 894 nm in anaerobic cuvettes filled with an oxygen-scavenging system (0.5 units/ml glucose oxidase, 250 units/ml catalase, and 2.5 mM glucose) in 50 mM Hepes (pH 7). Before starting the reaction, the assay mixture was first bubbled for 10 min with argon then 10 min with hydrogen. Appropriate amounts of protein extracts (from 2.5 to 20 μg) were added, and the kinetics of reduction was recorded. One enzymatic unit of hydrogenase was defined as the amount of enzyme required for the reduction of 1 μmol of methyl viologen per minute. The specific activity was defined as the number of enzymatic units per milligram of proteins.

**Immunoblotting**—Hydrogenases content was followed by immunoblotting using rabbit polyclonal antibodies against either [Fe] hydrogenases from *Desulfovibrio desulfuricans ATCC 7757* and *Desulfovibrio fructosovorans*, respectively. After SDS-PAGE, proteins were transferred onto a nitrocellulose membrane. Immunoblotting and detection were performed as already described (28). Quantification of immunodetected bands was performed by densitometric analysis after scanning of the blots using the ImageScanner system of APBiotech.

**Cytochrome c Content**—The total amount of cytochrome c in TE extracts was determined spectrophotometrically. Spectra from 260 to 600 nm were recorded on a Kontron Uvicon 922 spectrophotometer. Relative amounts of cytochrome were quantified on the basis of the absorbance of the Soret peak (400 nm) in the oxidized state.

**RESULTS**

Because of the periplasmic location of the *D. vulgaris* Hildenborough [Fe] superoxide dismutase, it has been proposed that this enzyme is involved in the protection of periplasmic iron-sulfur proteins against superoxide-induced damage (25). One of these sensitive enzymes could be the periplasmic [Fe] hydrogenase. To confirm this hypothesis, hydrogenase activities of periplasmic fractions of wild-type and SOD100 strains were compared. Hydrogenase activity was directly visualized on native PAGE (Fig. 1). When cells were kept in anaerobic conditions, the intensity of the hydrogenase activity-stained bands detected in both SOD100 and wild-type TE extracts was similar (Fig. 1A, lanes 1 and 2). On the other hand, when cells were exposed for 15 min to pure oxygen, the band intensity was higher in the wild-type TE extract than in that of SOD100 (Fig. 1A, lanes 3 and 4). Densitometric analysis of these bands allowed quantification of about 1.5 times more hydrogenase activity in wild-type than in SOD100 extracts (Fig. 1B). The periplasmic hydrogenase activity appeared to be higher in wild-type than in SOD100 when cells were exposed to oxygen, suggesting that superoxide dismutase protects the hydrogenase from damage induced by superoxide anions.

Interestingly, Fig. 1 shows that the hydrogenase activity-stained band was more intense when cells were exposed for 15
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A specific hydrogenase activity rise is related to an increase in protein synthesis and not to an enzyme activation phenomenon.

To relate the [Fe] hydrogenase activity with the capability of the cell to survive oxygen, viability of *D. vulgaris* wild-type and HYD100 after exposure for 1 h to pure oxygen were compared. While, in the absence of oxygen, both wild-type and HYD100 had the same viability in lactate-sulfate medium, the viability of the two strains differed largely when the cells were exposed to pure oxygen. Colonies counting on plates showed that 5% of HYD100 were able to survive 1 h of exposure to oxygen, whereas 23.3% survivors were found for the wild-type in the same conditions (Table 1). The [Fe] hydrogenase appears to be highly involved in the cellular mechanisms allowing cells to survive exposure to oxygen.

Two factors must be considered when cells are exposed to oxygen: first, the direct effect of oxygen that might inactivate some enzymes and, second, an increase in the redox potential of the medium. One way of distinguishing between these two effects is to use chemical oxidant compounds instead of oxygen. The redox potentials $E^\circ$ of the couples $\frac{1}{2}O_2/H_2O$ and $Cr(VI)O_2^+/Cr(III)(OH)_3$ are very similar (820 and 800 mV, respectively). It is thus expected that, in terms of redox potential effects, oxygen and chromium would have almost the same consequences on the redox potential of the cell environment.

When 100 µM ammonium chromate was added to 100 ml of *D. vulgaris* Hildenborough culture in medium C, the redox potential quickly increased and then decreased to its initial value. The increase of the redox potential is of the same order as when the culture is flushed with pure oxygen as described above (Fig. 5A). An increase of the hydrogenase activity in the TE extracts was observed (Fig. 5B). Hydrogenase activity increased to reach a maximum 30 min after chromate addition

min to pure oxygen when than when they were kept in anaerobic conditions. This increase in intensity was detected in both wild-type and SOD100 cells. Periplasmic hydrogenase activity thus appeared to be higher when cells were briefly exposed to pure oxygen than when they were kept in anaerobic conditions.

To determine whether this hydrogenase activity increase was specific to the periplasmic [Fe] hydrogenase, the same experiment was conducted on crude extracts (Fig. 2A). Although the intensity of the hydrogenase activity band corresponding to the [Fe] hydrogenase increased over the time of exposure to pure oxygen, no significant difference was observed on the upper activity bands that could be attributed to the other hydrogenases ([NiFe] and [NiFeSe] hydrogenases) (Fig. 2A, lanes 3, 5, and 7). As a control, no significant intensity increase was observed during this time period when the cells were kept in anaerobic conditions (Fig. 2A, lanes 1, 2, 4, and 6). Intensity measurement was performed by densitometric analysis, and variations are shown in Fig. 2B. An averaged intensity increase in 1.64 times was determined for the [Fe] hydrogenase activity band when cells were exposed to pure oxygen for 60 min.

To get more accurate values, hydrogenase activities were quantified by spectrophotometric assay. A specific hydrogenase activity of 10,101 ± 193 units/mg of protein was found in the TE extract of cells exposed for 60 min to pure oxygen, compared with 7,115 ± 181 units/mg of protein for cells kept under anaerobic conditions. This 1.4-fold increase is of the same order as that calculated from the densitometric analysis. At this point, it was unknown whether this activity rise was due to an increase in specific enzyme activity or to an increase in protein synthesis. To answer this question, the amount of hydrogenases in the cells was followed. Western blot and immunodetection analyses using polyclonal antibodies against either [Fe] hydrogenase or [NiFe] hydrogenase were performed to analyze the hydrogenase content of the cells in the presence or absence of oxygen. Although the intensities of the immunodetected bands corresponding to both [NiFe] and [NiFeSe] hydrogenases did not change over the time period of cell exposure to pure oxygen (Fig. 3A), that of the immunodetected band corresponding to the [Fe] hydrogenase increased. The more intense signal was observed when the cells were exposed for 60 min to oxygen. Again, when cells were kept in anaerobic conditions, no significant increase in the amount of [Fe] hydrogenase was detected (Fig. 3B). This experiment shows that the hydrogenase activity rise is related to an increase in protein synthesis and not to an enzyme activation phenomenon.

Several studies have pointed out that the periplasmic cytochrome c₃ is the physiological electron acceptor of the [Fe] hydrogenase (29–31). The UV-visible spectrum of *D. vulgaris* TE extracts exhibits various absorbance bands, including the Soret peak (Fig. 4A) that are specific to hemoproteins. The evolution of the periplasmic c-type cytochrome content was monitored spectrophotometrically by following the absorbance of the Soret peak (409 nm) in the oxidized state. When the cells were exposed for various times to oxygen, the intensity of the Soret band increased. On the other hand, no change was observed during this time period in the absence of oxygen (Fig. 4B). In a similar way to the [Fe] hydrogenase, the amount of c-type cytochromes in the periplasmic space thus increased in response to the presence of oxygen.

From the experiments described above, it can be deduced that the periplasmic [Fe] hydrogenase is involved in the immediate response of the cell to the presence of oxygen. To specify the importance of this enzyme, the effect of oxygen was also studied on a mutant strain, *D. vulgaris* HYD100, in which the gene encoding for this hydrogenase had been deleted (27). Changes in total hydrogenase activity and cytochrome content of HYD100 cells, induced by the presence of oxygen, were followed. Hydrogenase activity-stained PAGE revealed no change in band intensity corresponding to both [NiFe] and [NiFeSe] hydrogenases (data not shown). Neither production nor activity change in any of the remaining hydrogenases was thus observed when the cells were exposed to oxygen to compensate for the absence of the [Fe] hydrogenase in this strain. On the other hand, an increase in the periplasmic cytochrome content was also observed (Table 1).

![Fig. 1. Native PAGE of *D. vulgaris* Hildenborough wild-type and SOD100 TE extracts. A, hydrogenase-activity stained gel after native PAGE. 10 µg of protein of TE extracts was loaded. Lanes 1 and 2, wild-type and SOD100 TE extracts without cell exposure to oxygen. Lanes 3 and 4, wild-type and SOD100 TE extracts after 15 min cell exposure to oxygen. B, densitometric analysis of the hydrogenase activity-stained bands from A.](image-url)
and then decreased to its initial value. Similar changes in the cytochrome content were observed (data not shown) with a maximum cytochrome content reached 30 min after chromate addition. Chromate addition has thus the same consequences as flushing with pure oxygen on *D. vulgaris* cells concerning both hydrogenase and cytochrome contents.

**DISCUSSION**

It has been proposed that *D. vulgaris* Hildenborough superoxide reductase (SOR) is involved in the elimination of cytoplasmic superoxide anions produced subsequent to partial oxygen reduction by either soluble or membrane-bound oxygen reductases (18, 24). In contrast, [Fe] SOD is a periplasmic protein. Periplasmic [CuZn] superoxide dismutases of several Gram-negative pathogens can protect against superoxide-radical-mediated host defenses and thus contribute to virulence (32). Because *D. vulgaris* Hildenborough is not a pathogen, this function is improbable. The periplasmic space of *D. vulgaris* contains a lot of low redox potential enzymes that might generate superoxide anions in the presence of oxygen. If not scavenged, superoxide anions would induce damage in the forms of oxidation and destabilization of iron-sulfur clusters of some enzymes and lead to their inactivation (33). The [Fe] superoxide dismutase could thus be involved in the elimination of periplasmic-generated superoxides, preventing sensitive enzymes from inactivation. In fact, *Desulfovibrio* periplasm contains several iron-sulfur-containing enzymes (hydrogenases or formate-dehydrogenases) involved in the energetic metabolism of the cell (27, 34–35) that might be sensitive to superoxide anions. [Fe] hydrogenase is one of the most abundant iron-sulfur enzymes in the *D. vulgaris* periplasm. This enzyme is composed of two subunits that coordinate two [4Fe-4S] clusters in a ferredoxin-like domain and an H-cluster composed of a typical [4Fe-4S] cubane bridged to a binuclear active site iron center (36). This high content of iron-sulfur clusters renders this hydrogenase a good model of a superoxide-sensitive enzyme. To experimentally assign a function to the periplasmic [Fe] SOD of *D. vulgaris*, sensitivity of the [Fe] hydrogenase was thus followed in vivo in both *D. vulgaris* Hildenborough wild-type and the sod-deleted strains (SOD100). Our results show that hydrogenase activity is higher in wild-type than in SOD100 strains when cells are exposed to pure oxygen for a short time. Sensitivity of hydrogenase is thus higher in the SOD100 strain than in the wild-type. This result is consistent with the hypothesis that the periplasmic superoxide dismutase is involved in the protection of sensitive enzymes against superoxide-induced damage. An intriguing point is that in both strains (i.e. wild-type and SOD100), total hydrogenase activity in the periplasmic space increased when the cells were exposed to oxygen. This hydrogenase activity rise was related to an increase in the

**FIG. 2.** Native PAGE of *D. vulgaris* wild-type crude extracts from cells exposed to oxygen for various times. A: lanes 3, 5, and 7, 10 μg of protein of crude extracts of cells exposed to oxygen for 15, 30, and 60 min, respectively. Lanes 1, 2, 4, and 6, 10 μg of protein of crude extract from cells kept in anaerobic conditions at time 0 and after 15, 30, and 60 min, respectively. Positions of the [Fe] hydrogenase and the [NiFe] hydrogenases are indicated. B, densitometric analysis of the [Fe] hydrogenase activity-stained band. •, lanes 3, 5, and 7; •, lanes 1, 2, 4, and 6.

**FIG. 3.** Immunoblot of gel as in Fig. 2. Polyclonal antibodies against [NiFe] (A) and [Fe] (B) hydrogenases were used as primary antibodies.
amount of protein as shown by immunoblotting. On the other hand, this phenomenon was only observed for the periplasmic [Fe] hydrogenase, because no difference was detected concerning the other hydrogenases. Our data show that *D. vulgaris* [Fe] hydrogenase synthesis is induced when the cells are exposed to pure oxygen. Up-regulation of the [Fe] hydrogenase synthesis by oxygen is a new mechanism indicated here for the first time. [Fe] hydrogenase is a key enzyme for the dissimilatory sulfate reduction. Its physiological function has been shown to be hydrogen uptake when both hydrogen and lactate are electron donors for sulfate reduction (27) and hydrogen production with pyruvate and lactate as electron donors in the absence of sulfate (37). *In vitro* experiments have shown that hydrogenases are oxygen-sensitive enzymes (38, 39). It is thus surprising that the presence of oxygen up-regulates the synthesis of the enzyme. In the same way, the periplasmic cytochrome content increases when cells are exposed to oxygen. This phenomenon has already been described for *D. desulfuricans*. The authors reported that, under continuous culture conditions with low oxygen partial pressures, the cytochrome c content increased by about 20% (40). Although our experimental conditions were different the same effect, namely the presence of oxygen on the cytochrome c content, was observed.

![Soret peak](image)

**FIG. 4.** Periplasmic cytochrome content. A, UV-visible spectra of *D. vulgaris* TE extract in both oxidized and dithionite-reduced states. B, absorbance of the Soret peak from *D. vulgaris* wild-type TE extract is reported as a function of cell exposure to pure oxygen from 0 to 60 min (dashed bars). Black bars constitute the control (i.e. without oxygen exposure for the same time).

**TABLE I**

|                  | Periplasmic cytochrome content | Cell viability |
|------------------|-------------------------------|----------------|
|                  | Anaerobic conditions          | After 1 h of exposure to O₂ | Anaerobic conditions          | After 1 h of exposure to O₂ |
| Wild-type        | 100                           | 233            | 100                           | 23.3            |
| HYD100           | 100                           | 183            | 100                           | 5               |

* Determined from the absorbance of the Soret peak (409 nm).
* Determined as the number of colony-forming units/ml on medium E.
Several deleterious effects of oxygen on SRB must be considered. When oxygen penetrates into the cell it can inhibit sulfate reduction, probably because of the inactivation of some key enzymes. Moreover, reactive oxygen species are generated leading to various damage to proteins, lipids, and nucleic acids (41). One response of the cell for eliminating oxygen is to reduce it. It has been shown that oxygen reduction by *D. vulgaris* Marburg mainly occurred in the periplasm and that this reduction was hydrogenase/cytochrome-dependent (13). Our experiments suggest that both *D. vulgaris* Hildenborough periplasmic [Fe] hydrogenase and cytochromes are involved in oxygen reduction as well. In response to the presence of oxygen, cells increase the amount of both proteins in the periplasm to increase oxygen reduction rate and thus eliminate it. The fact that, after exposure to oxygen, viability of the strain lacking the gene encoding [Fe] hydrogenase (HYD100) is about four times lower than that of wild-type is in complete agreement with an important function of [Fe] hydrogenase in the protection against oxygen stress.

On the other hand, the presence of oxygen strongly modifies the physicochemical parameters of the environment. First, it causes a chemical oxidation of hydrogen sulfide produced by the cell from sulfate respiration. Second, the redox potential of the culture increases with increasing oxygen concentration (40). This redox environment change might cause a stress to the cells. Ammonium chromate (VI) is a strong oxidizer, and its addition to the medium induces a rapid redox potential increase. In our experimental conditions, the addition of chromate to the culture induces a similar redox potential increase as that observed when the culture is flushed with pure oxygen. It is noteworthy that, in the presence of chromate, both cytochrome and [Fe] hydrogenase synthesis increase. The response of the cells is the same as in the case of the presence of oxygen. It appears that the [Fe] hydrogenase is involved in the response to an oxidative stress induced by either the presence of oxygen or a strong redox potential increase.

What might be the role of the [Fe] hydrogenase in the protection against oxidative stress? As discussed above, this enzyme, coupled to cytochromes, could be directly involved in the elimination of oxygen by reduction to water. The [Fe] hydrogenase could also be involved in a mechanism devoted to the decrease of the environmental redox potential to obtain optimal conditions for cell growth. A few years ago, we reported that, in addition to the transmembrane electron transport from hydrogen to sulfate, which normally occurs during dissimilatory sulfate reduction, the redox reactions driven by the Hmc complex are crucial for the establishment of the required low redox niche necessary for cell development (42). One can thus imagine that the periplasmic [Fe] hydrogenase participates in these mechanisms by supplying electrons to the Hmc complex. On the other hand, electrons derived from hydrogen uptake by the [Fe] hydrogenase could be driven to other electron transport...
systems that have to be characterized. For example, one of these systems could be the outer membrane-bound cytochromes (43) that might drive low potential electrons to the cell surface. A third hypothesis that cannot be ruled out is that an oxidative stress might induce a modification of the hydrogen metabolism leading to an increase of hydrogenase activity, either uptake or production. Such a modification has recently been reported for another obligate anaerobe, namely Thermotoga neapolitana. The authors reported that the rate of hydrogen production increased when low levels of oxygen were provided (44).

Our results clearly demonstrate that [$Fe$] hydrogenase is up-regulated in response to an oxidative stress in the anaerobic sulfate-reducing bacterium *D. vulgaris* Hildenborough. This is the first time that involvement of [$Fe$] hydrogenase in the protection against oxidative stress has been pointed out. Because of the ancestral character of sulfate reduction (1), such a function of [$Fe$] hydrogenase might represent one of the earliest mechanisms to protect the cells against an oxidative stress. Further studies are necessary to obtain a better understanding of the function of this enzyme during oxidative stress and to indicate other original systems involved in this stress.

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