JB
Journals.ASM.org

Role of the NiFe Hydrogenase Hya in Oxidative Stress Defense in *Geobacter sulfurreducens*

Pier-Luc Tremblay and Derek R. Lovley

Department of Microbiology, University of Massachusetts, Amherst, Massachusetts, USA

***Geobacter sulfurreducens*, an Fe(III)-reducing deltaproteobacterium found in anoxic subsurface environments, contains 4 NiFe hydrogenases. Hyb, a periplasmically oriented membrane-bound NiFe hydrogenase, is essential for hydrogen-dependent growth. The functions of the three other hydrogenases are unknown. We show here that the other periplasmically oriented membrane-bound NiFe hydrogenase, Hya, is necessary for growth after exposure to oxidative stress when hydrogen or a highly limiting concentration of acetate is the electron source. The beneficial impact of Hya on growth was dependent on the presence of H_2 in the atmosphere. Moreover, the Hya-deficient strain was more sensitive to the presence of superoxide or hydrogen peroxide. Hya was also required to safeguard Hyb hydrogen oxidation activity after exposure to O_2. Overexpression studies demonstrated that Hya was more resistant to oxidative stress than Hyb. Overexpression of Hya also resulted in the creation of a recombinant strain better fitted for exposure to oxidative stress than wild-type *G. sulfurreducens*. These results demonstrate that one of the physiological roles of the O_2-resistant Hya is to participate in the oxidative stress defense of *G. sulfurreducens*.**

Hydrogenases are ubiquitous enzymes catalyzing the reversible interconversion of H_2 with $2H^+$ and $2e^-$ (40, 42). Hydrogenases are separated into three classes depending on the metal composition of their active site: NiFe hydrogenases, FeFe hydrogenases, and Fe hydrogenases (40, 42). NiFe and FeFe hydrogenases are involved in hydrogen respiration, hydrogen production during fermentation, methanogenesis, and the recovery of H_2 generated during nitrogen fixation (42). Fe hydrogenases are found in certain methanogenic archaea and catalyze a step in the conversion of carbon dioxide to methane (44).

Geobacteraceae, a family in the *Deltaproteobacteria*, are mostly found in anoxic subsurface environments in which insoluble Fe(III) is the main electron acceptor (3, 4, 11, 19, 35–39). Many *Geobacteraceae* can respire hydrogen, a source of electrons made available in the subsurface by fermentative species (27, 30). There are 4 NiFe hydrogenases encoded in the genome of *Geobacter sulfurreducens*, which has served as a model organism for the study of *Geobacteraceae* (8, 33). Hyb, a periplasmic hydrogenase, was characterized as essential for hydrogen-dependent growth (8, 10). The other periplasmic hydrogenase, Hya, is not essential for this process (10). No function was attributed to the two cytoplasmic hydrogenases, Hox and Mvh (8).

Throughout evolution, anaerobic bacteria have developed defense mechanisms against oxidative stress. Recently, hydrogenases have been implicated in this process (42). In the case of homoacetogenic bacteria isolated from termite guts, hydrogen oxidation was shown to be a way to remove O_2 from the environment (6). A role in a protective mechanism against oxidative stress was proposed for a periplasmic FeFe hydrogenase of the deltaproteobacterium *Desulfovibrio vulgaris* (5, 16).

Like *Desulfovibrio* species (1, 12, 13, 15), *G. sulfurreducens* is able to survive exposure to O_2 and even to respire it at low concentrations (23). In this study, we employed a set of mutants to evaluate whether the periplasmic hydrogenases of *G. sulfurreducens* participate in the defense against oxidative stress. We demonstrate that the NiFe hydrogenase Hya is more resistant to oxidative stress than Hyb. We show that Hya has a function in the overall protection against reactive oxygen species (ROS) and, more specifically, in preventing deactivation of the hydrogenase Hyb after O_2 exposure. We also describe a strategy to make a strict anaerobe more resistant to oxidative stress by creating a recombinant *G. sulfurreducens* strain overexpressing Hya that grows more rapidly than the wild type under oxidative stress with hydrogen as the electron donor.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *G. sulfurreducens* strains were routinely maintained anaerobically (N_2-CO_2, 80:20) at 30°C in NBF medium with acetate (10 mM) as the electron donor and fumarate (40 mM) as the electron acceptor as previously described (9). For hydrogen-dependent growth, 10 ml of hydrogen gas was injected into the headspace of a 27-ml pressure tube containing 10 ml of donor-free NBF medium, and 1 mM acetate was added as a carbon source (10). Cultures grown with a limiting concentration of acetate as the electron donor contained 1 mM acetate. The medium was reduced with 20 μM cysteine. To subject cells to oxidative stress, only 0.4 μM cysteine was added. For cultures exposed to oxygen, 0.15 ml of O_2 was added to the headspace of a 27-ml pressure tube containing 10 ml of medium prior to inoculation. *Escherichia coli* was cultivated in Luria-Bertani medium. Appropriate antibiotics were added when necessary.

Construction of the Δ*hyaSLB*::Km^r Δ*hybL*::Cm^r double mutant. To construct a Δ*hyaSLB*::Km^r Δ*hybL*::Cm^r double mutant, the Δ*hybL*::Cm^r mutant allele was introduced into DL7. *hybL*::Cm^r was amplified by PCR with primers MChyd21for (5′-GCACGAACTGTCCGGCATCG-3′) and MChyd26rev (5′-AGTTCTCTTCCAGGTGGTTG-3′) using DL8 genomic DNA as the template. Electroporation of the 2-kb PCR product and double mutant isolation were performed as previously described (9, 26). The double mutant was verified by PCR and Sanger sequencing.

Received 10 January 2012 Accepted 15 February 2012
Published ahead of print 24 February 2012
Address correspondence to Pier-Luc Tremblay, pierluct@cns.umass.edu.

doi:10.1128/JB.00044-12

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli Top10	*recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1* Δ*lacU169*	Invitrogen, Carlsbad, CA
G. sulfurreducens		
PCA (ATCC 51573)	Wild type	7
DL7	Δ*hyaSLB*::Km^r	10
DL8	Δ*hybL*::Cm^r	10
DLMC10	Δ*hyaSLB*::Km^r Δ*hybL*::Cm^r	Maddalena V. Coppi
P*hyb* mutant	ΔP*hya*::P*hyb*; Gm^r	This work
hyb P*hyb* mutant	Δ*hybL*::Cm^r ΔP*hya*::P*hyb*; Gm^r	This work
Plasmids		
pCR2.1-TOPO	PCR cloning vector; Ap^r Km^r	Invitrogen, Carlsbad, CA
pUC19-Gm^r*loxP*	pUC19 carrying Gm^r*loxP*; Ap^r Gm^r	2
pPLT158	pCR2.1 carrying P*hyb* upstream of the 5′ part of *hyaS*; Ap^r Km^r	This work
pPLT159	pPLT158 carrying the 3′ part of Gsul 0124; Ap^r Km^r	This work
pPLT160	pPLT159 carrying a gentamicin resistance cassette between the 3′ part of Gsul 0124 and P*hyb*; Ap^r Km^r Gm^r	This work

Replacement of native *hya* promoter by P*hyb*. A 0.1-kb chromosomal region containing the native promoter of the *hya* operon was replaced by the 0.6-kb *hyb* promoter region. The P*hyb* promoter was fused to the 5′ part of the *hyaS* gene by recombinant PCR with the primers fullhybupAvrII2g (5′-CATCCTAGGCAAGAAGGCGTCTCTGGAAGAGC-3′), recphybhyadn2g (5′-TTGCCATCGTCGTTACCTGCCATAAATCCCCCTGTCGGTATGAT), recphybhyaup (5′-ATCATACCGACAGGGGGATTTATGGCAGGTAACGACGATGGC), and hya500dn (5′-GACGGTGATAGTTGCCATGGCGC-3′). The resulting 1.1-kb PCR product was cloned into pCR2.1-TOPO with a TOPO TA cloning kit (Invitrogen, Carlsbad, CA), resulting in pPLT158. The 3′ part of Gsul 0124 (0.5 kb) was amplified by PCR with primers hyaup500BHI (5′-CTCGGATCCAGTACGGCCACAAGGCTCACCTG-3′) and hyaupAvrII 5′-CTGCCTAGGCTTTCCGCGATGGTCCTGGTGTTG-3′), digested with BamHI-AvrII (NEB, Beverly, MA) and ligated with the T4 DNA ligase (NEB) upstream of P*hyb* into pPLT158, resulting in pPLT159. pUC19-Gm^r*loxP* was cut with XbaI (NEB), and the gentamicin resistance cassette was cloned into the AvrII restriction site located between the 3′ part of Gsul 0124 and P*hyb* of pPLT159, resulting in pPLT160. pPLT160 was linearized with KpnI (NEB) and electroporated into wild-type *G. sulfurreducens* and the DL8 strain as described previously (9, 26). Recombinant strains were verified by PCR and Sanger sequencing.

Quantitative reverse transcription-PCR (qRT-PCR). Total RNA was extracted with an RNeasy Minikit (Qiagen, Valencia, CA) from mid-log-phase cultures grown with hydrogen, 10 or 1 mM acetate as an electron source, 20 or 0.4 μM cysteine, no O_2, or 0.15 ml of O_2 in the headspace. The Enhanced Avian First-Strand Synthesis kit (Sigma-Aldrich, St-Louis, MO) was used to generate cDNA using random primers. *hyaS* (GSU0123) and *hybS* (GSU0782) transcripts were amplified and quantified with the SYBR green PCR master mix (Applied Biosystems, Foster City, CA) and the ABI 7500 real-time PCR system. Primers used for the amplification of *hyaS* were hyaS759f (5′-GGGCCAGTATGTGGAACAAT-3′) and hyaS825r (5′-GCCCATTTTGTAGAGGCAGT-3′). Primers used for the amplification of *hybS* were hybS229f (5′-CTTGACATGATTTCGCTGGA-3′) and hybS386r (5′-TTGCAGTAGATGCCGTTGTC-3′). Expression of these genes was normalized to expression of *proC*, a constitutively expressed gene in *G. sulfurreducens* (18). *proC* was amplified with primers proc1f (5′-ATGCTGAAGGGAAGCACTCT-3′) and proc75r (5′-GGCCAGCAGCCCTTTGAT-3′). Relative levels of expression of the studied genes were calculated by the $2^{-\Delta\Delta CT}$ method (25).

Benzyl viologen assay. *G. sulfurreducens* hydrogen-fumarate cultures reduced with 20 μM cysteine were grown to an optical density (OD) at 600 nm of 0.1 (mid-log phase). Cells were collected by centrifugation and washed twice with anaerobic buffer A (50 mM Tris-Cl [pH 8.0], 150 μg/ml chloramphenicol). Cells were resuspended in buffer A to concentrate them 7.5-fold. O_2-exposed cell suspensions were maintained for 5 min in the presence of atmospheric oxygen just before starting the assay. Reduction of benzyl viologen was assayed under anaerobic conditions as previously described (21). At time zero, 500 μl of cell suspension was added to 9.5 ml of H_2-saturated buffer A containing 2 mM benzyl viologen. Reduction of benzyl viologen was followed at room temperature by OD at 550 nm. At this wavelength, the extinction coefficient of reduced benzyl viologen is 9 mM^{-1} cm^{-1} (21). Protein concentrations were determined with the bicinchoninic acid assay (Sigma-Aldrich). Hydrogen oxidation activity was expressed as nmol of reduced benzyl viologen per mg of proteins.

Resistance of *G. sulfurreducens* strains to exogenous O_2^- generated by the xanthine oxidase reaction and H_2O_2. Mid-log-phase *G. sulfurreducens* hydrogen-fumarate cultures reduced with 20 μM cysteine were collected by centrifugation, washed twice, and resuspended in 4 ml of anaerobic phosphate-buffered saline (PBS). The cell suspension was separated in two sealed pressure tubes filled with H_2. In order to evaluate the impact of hydrogen peroxide exposure, 1 mM was added to one of cell suspensions, which were then incubated for 2 h at 30°C. The cell suspensions were then serially diluted in PBS and plated on acetate (10 mM)-fumarate (40 mM) medium. To calculate the survival rate, number of CFU obtained from the cell suspension exposed to H_2O_2 was divided by the number of CFU obtained from the cell suspension not exposed to H_2O_2.

The same experimental design was adopted to establish the ability of *G. sulfurreducens* strains to survive superoxide exposure. O_2^- was generated in the cell suspensions under H_2 as previously described (17). Briefly, O_2 (10 ml) and 1 mM xanthine were added along with 1000 U/ml of bovine liver catalase to eliminate H_2O_2. Xanthine oxidase was added to initiate superoxide production.

RESULTS AND DISCUSSION

The NiFe hydrogenase Hya protects *G. sulfurreducens* against oxidative stress. As previously reported (10), strains in which *hyb* was deleted grew poorly in medium in which hydrogen was provided as the primary electron donor, whereas a strain in which only *hya* was deleted grew well (Fig. 1A). Slow growth of the *hyb*-deficient strains was observed over time (Fig. 1A), but this was

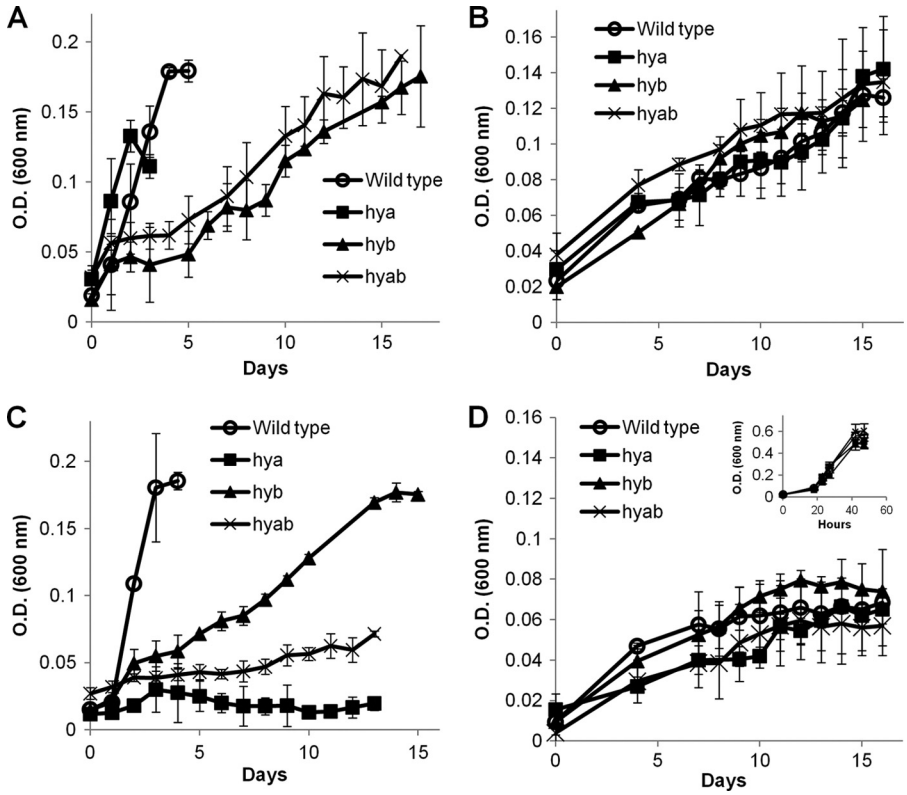

FIG 1 Growth of *G. sulfurreducens* hydrogenase mutants exposed to oxidative stress. The wild-type strain and *hya*, *hyb*, and Δ*hyaSLB*::Km^r Δ*hybL*::Cm^r (*hyab*) mutants were cultivated with a high (A and B) or low (C and D) cysteine concentration. Hydrogen (A and C) or highly limiting acetate (B and D) was the electron donor. Growth with low cysteine and nonlimiting acetate is shown in an inset in panel D. Each curve is the mean from at least three independent replicate cultures. Bars designate one standard deviation of the mean.

attributed to growth with the low concentrations of acetate (1 mM) that were included in the medium as a carbon source, because the rate of growth was comparable to that of cells in medium with acetate alone (Fig. 1B). When the cysteine concentration in the medium was decreased 50-fold, growth at these low acetate concentrations was substantially diminished (Fig. 1D). When hydrogen was also provided as an electron donor in the low-cysteine medium, the growth of wild-type cells and the *hyb*-deficient strain was comparable to growth in the presence of hydrogen with the higher concentration of reductant (Fig. 1C). However, strains in which *hya* was deleted grew much more poorly in the low-cysteine medium than in the medium with the higher concentration of cysteine. In contrast, if the cultures were grown with a higher concentration of acetate (10 mM) that supported rapid growth, deleting *hya* had no impact on growth in low-cysteine medium (Fig. 1D, inset).

These results demonstrated that when sufficient electron donor was present, in the form of either hydrogen or high concentrations of acetate, wild-type cells did not require the higher concentrations of medium reductant. Increased electron donor availability might provide more electrons to combat oxidative stress. The surprising finding was the relative importance of *hya* and *hyb* in overcoming oxidative stress with hydrogen. Whereas *hya*, which is not required for growth on hydrogen and has been reported to encode almost no hydrogenase uptake capacity (10), was required in order for the cells to overcome the stress, deleting *hyb*, which is required for growth on hydrogen and encodes substantial hydrogen uptake capacity, had no impact. In contrast to the substantial impact of deleting *hya* on growth in low-cysteine medium in the presence of hydrogen, the *hya* deletion had had only a slight negative impact, if any, during growth with low acetate and had no impact in high-acetate medium. These results suggested that *hya* might be specifically responsible for providing electrons to aid cells in combating oxidative stress when hydrogen is available as an electron donor.

In order to further evaluate the potential role of Hya in oxidative stress defense, *G. sulfurreducens* growth with hydrogen in the presence of oxygen in the headspace was evaluated. Oxygen had a slight effect on the growth of wild-type cells (Fig. 2A), increasing the doubling time 1.4-fold (Fig. 2B). The presence of oxygen had a more substantial impact on growth of the *hya* mutant (Fig. 2A), increasing the doubling time 2.3-fold (Fig. 2B).

Sensitivity of *G. sulfurreducens hya* to exogenous O_2^- and H_2O_2. Enzymes expected to contribute to the defense against oxidative stress encoded in the *G. sulfurreducens* genome (33) include those that degrade superoxide (superoxide dismutase [GSU1158] and superoxide reductase [GSU0720]), as well as those that are involved in hydrogen peroxide degradation, such as cytochrome *c* peroxidase (GSU2813), catalase (GSU2100), various peroxiredoxins (GSU0066, GSU0352, GSU0893, GSU3246, and GSU3447), and rubrerythrins (GSU2612 and GSU2814) (33, 34). In order to determine if Hya might be directly or indirectly involved in the promotion of these activities, the impact of deleting *hya* on the response to superoxide or hydrogen peroxide ex-

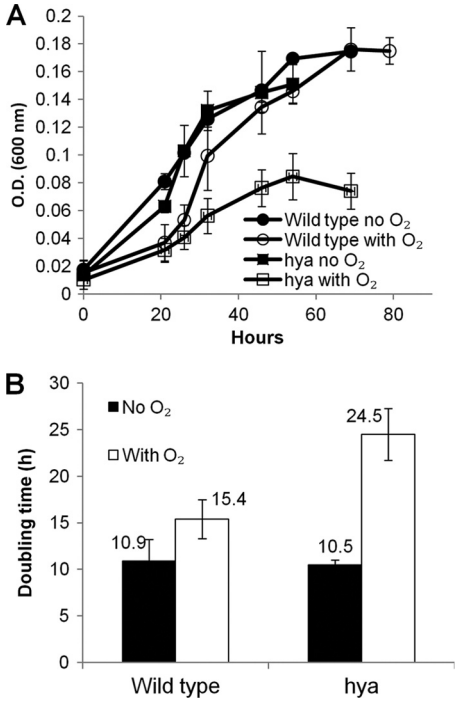

FIG 2 Growth of the *G. sulfurreducens hya* mutant exposed to O_2. The wild type and the *hya* mutant were grown with hydrogen as the electron donor and fumarate as the electron acceptor. Where indicated, 0.15 ml of O_2 was introduced into the headspace prior to inoculation. Growth curves (A) and doubling times (B) are the means from at least three independent replicate cultures.

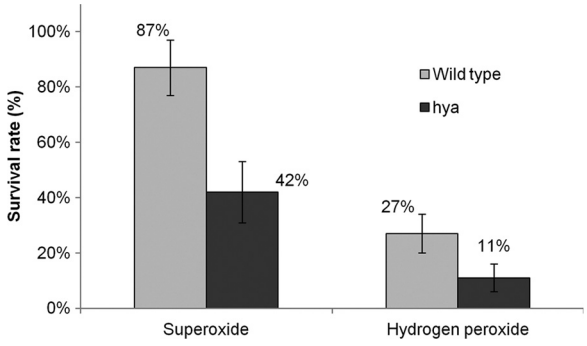

FIG 3 Survival of wild-type and Hya-deficient strains of *G. sulfurreducens* following superoxide and hydrogen peroxide exposure for 2 h. Survival rates are the means from at least three independent experiments.

posure was evaluated. Sensitivity to both was higher in the absence of Hya (Fig. 3).

Overexpression of *hya*. qRT-PCR revealed that transcripts for the gene for the small subunit of Hyb, *hybS*, were 71- to 143-fold more abundant than transcripts for *hyaS* under all of the tested conditions. The difference in the expression of *hyb* and *hya* might explain the difference between the hydrogen oxidation activities of the mutants observed previously (10). There was no significant variation in *hyaS* or *hybS* transcript levels when cells were grown with hydrogen or acetate as the electron donor, at a high concentration versus a low concentration of cysteine, or in the presence or absence of O_2.

Replacement of the native *hya* promoter by the stronger *hyb* promoter increased the transcript level of *hyaS* by two orders of magnitude to a level comparable to that for *hybS* (Fig. 4A). Overexpression of *hya* permitted a *hyb*-deficient mutant to grow with hydrogen as the sole electron donor at low as well as high cysteine concentrations (Fig. 4B). These results demonstrate that Hya can support hydrogen-dependent growth when expressed at higher levels. The facts that cells relying on Hya for hydrogen uptake can grow at low cysteine concentrations but that cells that only have Hyb cannot suggest that Hya is more resistant to oxidative stress than Hyb.

Compared to that of wild-type cells, the growth on hydrogen of the *hyb*-deficient mutant overexpressing *hya* was 4 times slower with high cysteine concentrations and 2 times slower with low cysteine concentrations (Fig. 4C). These results indicate that even when expression levels are comparable, Hyb is more competent than Hya to support growth with hydrogen. The doubling time on hydrogen of a strain overexpressing *hya* with a functional Hyb was comparable to that of the wild type with high cysteine concentrations. However, this strain grew 1.8 times faster than the wild type with low cysteine concentrations. These results demonstrate that genetically modifying *G. sulfurreducens* to overexpress Hya while conserving the Hyb hydrogen oxidation capacity created a strain better suited to grow under oxidative stress when hydrogen is the electron donor.

Hydrogen uptake attributable to Hya. A role of Hya in consuming hydrogen was not clearly established in previous studies in which hydrogen-dependent reduction of anthraquinone-2,6-disulfonate (AQDS), Fe(III)-nitrilotriacetic acid [Fe(III)-NTA], or fumarate was investigated, because the *hya*-deficient mutant reduced AQDS and Fe(III) as well as the wild type with only a marginal diminution of the fumarate reduction rate (10). In order to investigate hydrogenase activity in a more sensitive manner, benzyl viologen reduction assays in the presence of chloramphenicol to block *de novo* protein synthesis (24) were conducted with concentrated cell suspensions.

The bulk of the hydrogen oxidation activity in the wild type could be attributed to Hyb (Table 2), but there was detectable hydrogenase activity in the *hyb*-deficient mutant that was not found in the *hya hyb* double mutant, suggesting that Hya could account for low rates of hydrogen uptake. Since participation of Hya in oxidative stress defense is dependent on the presence of H_2, it is possible that this hydrogen oxidation by Hya is central to its newly discovered function.

Hya prevents inactivation of Hyb caused by O_2. Exposure of cells to atmospheric O_2 for 5 min partially reduced the hydrogenase activity in the wild type, which can be attributed to Hyb (Table 2). The marginal hydrogenase activity detected in the *hyb* mutant was still there after exposure to O_2. In the *hya* mutant, the hydrogenase activity attributable to Hyb was completely inhibited, illustrating the protective effect of Hya on Hyb activity. The strain which lacked *hyb* and in which *hya* was overexpressed had comparable hydrogenase activity before and after oxygen exposure, further indicating that Hya is more resistant to oxidative stress than Hyb.

The hydrogenase activity of the *hyb*-deficient mutant overexpressing *hya*, although substantial, is lower than that of the wild type, demonstrating that Hya is less effective than Hyb at oxidizing H_2 but can do so under oxidative stress. Hydrogen oxidation ac-

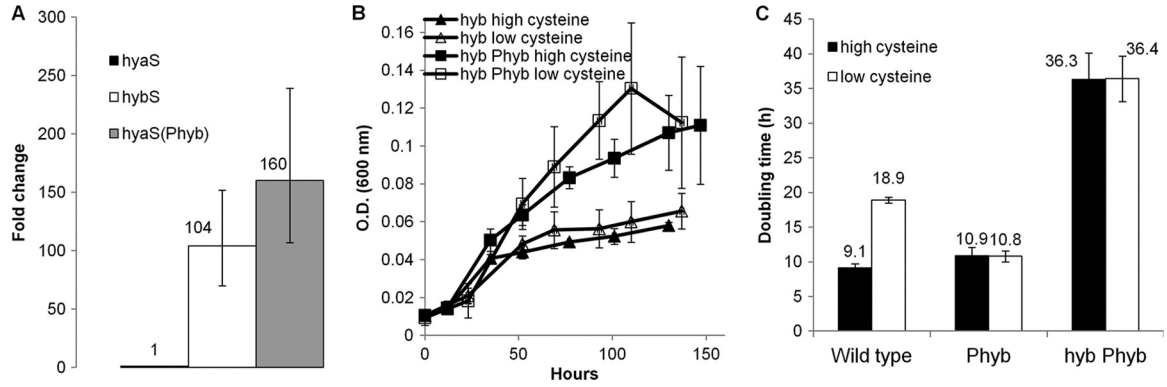

FIG 4 Overexpression of the hydrogenase Hya from the *hyb* promoter. (A) Quantitative RT-PCR assay of *hyaS* and *hybS* under the control of their respective native promoters and of *hyaS* under the control of P*hyb*. (B) Growth on hydrogen of the *G. sulfurreducens hyb* mutant with *hya* under the control of its native promoter or P*hyb* with a high or low cysteine concentration. (C) Doubling times on hydrogen of the wild type, a recombinant strain with *hya* under the control of P*hyb*, and the *hyb* mutant with *hya* under the control of P*hyb* (C). Each value or curve is the mean from at least three replicates.

tivity of a strain overexpressing Hya with a functional Hyb was similar before and higher after exposure to O_2 compared to that of the wild type. Therefore, the observed improvement in the doubling time of this recombinant strain under oxidative stress compared to that of the wild type is most probably due to its increased hydrogen oxidation capacity.

Implications. The O_2 tolerance of the NiFe hydrogenases Hyd-1 and Hyd-2 differs considerably in *E. coli* (21, 22, 32). Hyd-1 is active at higher redox potential (32), and Hyd-2 is more sensitive to O_2 (21, 22, 32). The results presented here suggest a similar difference in the oxygen sensitivities of hydrogenases in *G. sulfurreducens*. Two of the three Hya subunits have high homology (HyaL, 49%; HyaB, 34%) to the respective *E. coli* Hyd-1 subunits, and the Hya accessory protein has high homology (HyaP, 41%) to the accessory protein of *E. coli*. The Hya small subunit, HyaS, has only slightly higher homology to the small subunit of *E. coli* Hyd-2 (48% identity) than to the Hyd-1 small subunit (43% identity). All the Hyb subunit and accessory proteins have high homology (between 33% and 55%) to the respective *E. coli* Hyd-2 subunits. Our results suggest that Hya is less oxygen sensitive than Hyb and that even though Hya is expressed at relatively low levels, its activity helps protect Hyb from oxygen inactivation.

Fe(III) is generally most abundant near the oxic-anoxic interface, where physical perturbations are likely to result in oxygen incursions into the anoxic habitat of *Geobacteraceae* (41, 43), and in most of these environments metabolism is likely to be limited by electron donor availability (14, 28, 29, 31). Hya might aid in survival under such conditions. Homologs of Hya are found in multiple anaerobic bacteria (8, 42) and might have a similar protective function in other species. The finding that it was possible to enhance hydrogen-dependent growth under oxygen stress by overexpressing Hya suggests a strategy for genetic engineering *Geobacter* species for bioremediation or other applications in which oxidative stress may be a concern.

TABLE 2 Hydrogen oxidation activity

Strain	Reduced benzyl viologen (nmol/min/mg protein) in cells[a]:	
	Not exposed to O_2	Exposed to O_2[b]
Wild type	7.2 ± 0.3	4.7 ± 0.5
hyb mutant	0.1 ± 0.0	0.1 ± 0.0
hya mutant	8.4 ± 0.7	ND[c]
hya hyb mutant	ND	ND
P*hyb* mutant[d]	7.7 ± 2.3	7.5 ± 0.4
hyb P*hyb* mutant	2.5 ± 0.4	2.7 ± 0.1

[a] Each value is the mean and standard deviation of at least three replicates. Chloramphenicol (150 μg/ml) was added to block protein synthesis.
[b] Cells were exposed to atmospheric oxygen for 5 min.
[c] ND, not detected.
[d] The native *hya* promoter was replaced by the *hyb* promoter (P*hyb*).

ACKNOWLEDGMENTS

We thank Maddalena V. Coppi for the construction of strain DLMC10.

This research was supported by the Office of Science (BER), U.S. Department of Energy, Cooperative Agreement no. DE-FC02-02ER63446.

REFERENCES

1. **Abdollahi H, Wimpenny JWT.** 1990. Effects of oxygen on the growth of *Desulfovibrio desulfuricans*. J. Gen. Microbiol. **136**:1025–1030.
2. **Aklujkar M, Lovley DR.** 2010. Interference with histidyl-tRNA synthetase by a CRISPR spacer sequence as a factor in the evolution of *Pelobacter carbinolicus*. BMC Evol. Biol. **10**:230.
3. **Anderson RT, Lovley DR.** 1999. Naphthalene and benzene degradation under Fe(III)-reducing conditions in petroleum-contaminated aquifers. Bioremediation J. **3**:121–135.
4. **Anderson RT, et al.** 2003. Stimulating the in situ activity of *Geobacter* species to remove uranium from the groundwater of a uranium-contaminated aquifer. Appl. Environ. Microbiol. **69**:5884–5891.
5. **Baumgarten A, Redenius I, Kranczoch J, Cypionka H.** 2001. Periplasmic oxygen reduction by *Desulfovibrio* species. Arch. Microbiol. **176**:306–309.
6. **Boga HI, Brune A.** 2003. Hydrogen-dependent oxygen reduction by homoacetogenic bacteria isolated from termite guts. Appl. Environ. Microbiol. **69**:779–786.
7. **Caccavo F, Jr, et al.** 1994. *Geobacter sulfurreducens* sp. nov., a hydrogen- and acetate-oxidizing dissimilatory metal-reducing microorganism. Appl. Environ. Microbiol. **60**:3752–3759.
8. **Coppi MV.** 2005. The hydrogenases of *Geobacter sulfurreducens*: a comparative genomic perspective. Microbiology **151**:1239–1254.
9. **Coppi MV, Leang C, Sandler SJ, Lovley DR.** 2001. Development of a genetic system for *Geobacter sulfurreducens*. Appl. Environ. Microbiol. **67**:3180–3187.
10. **Coppi MV, O'Neil RA, Lovley DR.** 2004. Identification of an uptake hydrogenase required for hydrogen-dependent reduction of Fe(III) and other electron acceptors by *Geobacter sulfurreducens*. J. Bacteriol. **186**:3022–3028.
11. **Cummings DE, et al.** 2003. Diversity of *Geobacteraceae* species inhabiting metal-polluted freshwater lake sediments ascertained by 16S rDNA analyses. Microb. Ecol. **46**:257–269.

12. **Cypionka H.** 2000. Oxygen respiration by *Desulfovibrio* species. Annu. Rev. Microbiol. **54**:827–848.
13. **Dilling W, Cypionka H.** 1990. Aerobic respiration in sulfate-reducing bacteria. FEMS Microbiol. Lett. **71**:123–128.
14. **Esteve-Nunez A, Rothermich M, Sharma M, Lovley D.** 2005. Growth of *Geobacter sulfurreducens* under nutrient-limiting conditions in continuous culture. Environ. Microbiol. **7**:641–648.
15. **Fareleira P, et al.** 2003. Response of a strict anaerobe to oxygen: survival strategies in *Desulfovibrio gigas*. Microbiology **149**:1513–1522.
16. **Fournier M, Dermoun Z, Durand MC, Dolla A.** 2004. A new function of the *Desulfovibrio vulgaris* Hildenborough [Fe] hydrogenase in the protection against oxidative stress. J. Biol. Chem. **279**:1787–1793.
17. **Gee JM, et al.** 2005. *The Brucella abortus* Cu,Zn superoxide dismutase is required for optimal resistance to oxidative killing by murine macrophages and wild-type virulence in experimentally infected mice. Infect. Immun. **73**:2873–2880.
18. **Holmes DE, et al.** 2005. Potential for quantifying expression of the *Geobacteraceae* citrate synthase gene to assess the activity of *Geobacteraceae* in the subsurface and on current-harvesting electrodes. Appl. Environ. Microbiol. **71**:6870–6877.
19. **Ikenaga M, Asakawa S, Muraoka Y, Kimura M.** 2003. Bacterial communities associated with nodal roots of rice plants along with the growth stages: estimation by PCR-DGGE and sequence analyses. Soil Sci. Plant Nutr. **49**:591–602.
20. Reference deleted.
21. **Laurinavichene TV, Chanal A, Wu LF, Tsygankov AA.** 2001. Effect of O_2, H_2 and redox potential on the activity and synthesis of hydrogenase 2 in *Escherichia coli*. Res. Microbiol. **152**:793–798.
22. **Laurinavichene TV, Tsygankov AA.** 2001. H_2 consumption by *Escherichia coli* coupled via hydrogenase 1 or hydrogenase 2 to different terminal electron acceptors. FEMS Microbiol. Lett. **202**:121–124.
23. **Lin WC, Coppi MV, Lovley DR.** 2004. *Geobacter sulfurreducens* can grow with oxygen as a terminal electron acceptor. Appl. Environ. Microbiol. **70**:2525–2528.
24. **Lin B, Westerhoff HV, Röling WFM.** 2009. How *Geobacteraceae* may dominate subsurface biodegradation: physiology of *Geobacter metallireducens* in slow-growth habitat-simulating retentostats. Environ. Microbiol. **11**:2425–2433.
25. **Livak KJ, Schmittgen TD.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods **25**:402–408.
26. **Lloyd JR, et al.** 2003. Biochemical and genetic characterization of PpcA, a periplasmic c-type cytochrome in *Geobacter sulfurreducens*. Biochem. J. **369**:153–161.
27. **Lovley DR.** 2000. Anaerobic benzene degradation. Biodegradation **11**: 107–116.
28. **Lovley DR, Chapelle FH.** 1995. Deep subsurface microbial processes. Rev. Geophys. **33**:365–381.
29. **Lovley DR, Goodwin S.** 1988. Hydrogen concentrations as an indicator of the predominant terminal electron-accepting reactions in aquatic sediments. Geochim. Cosmochim. Acta **52**:2993–3000.
30. **Lovley DR, Holmes DE, Nevin KP.** 2004. Dissimilatory Fe(III) and Mn(IV) reduction. Adv. Microb. Physiol. **49**:219–286.
31. **Lovley DR, Phillips EJ.** 1987. Competitive mechanisms for inhibition of sulfate reduction and methane production in the zone of ferric iron reduction in sediments. Appl. Environ. Microbiol. **53**:2636–2641.
32. **Lukey MJ, et al.** 2010. How *Escherichia coli* is equipped to oxidize hydrogen under different redox conditions. J. Biol. Chem. **285**:3928–3938.
33. **Methe BA, et al.** 2003. Genome of *Geobacter sulfurreducens*: metal reduction in subsurface environments. Science **302**:1967–1969.
34. **Mouser PJ, et al.** 2009. Quantifying expression of *Geobacter* spp. oxidative stress genes in pure culture and during in situ uranium bioremediation. ISME J. **3**:454–465.
35. **Petrie L, North NN, Dollhopf SL, Balkwill DL, Kostka JE.** 2003. Enumeration and characterization of iron(III)-reducing microbial communities from acidic subsurface sediments contaminated with uranium(VI). Appl. Environ. Microbiol. **69**:7467–7479.
36. **Roling WF, van Breukelen BM, Braster M, Lin B, van Verseveld HW.** 2001. Relationships between microbial community structure and hydrochemistry in a landfill leachate-polluted aquifer. Appl. Environ. Microbiol. **67**:4619–4629.
37. **Rooney-Varga JN, Anderson RT, Fraga JL, Ringelberg D, Lovley DR.** 1999. Microbial communities associated with anaerobic benzene degradation in a petroleum-contaminated aquifer. Appl. Environ. Microbiol. **65**:3056–3063.
38. **Snoeyenbos-West OL, Nevin KP, Anderson RT, Lovley DR.** 2000. Enrichment of *Geobacter* species in response to stimulation of Fe(III) reduction in sandy aquifer sediments. Microb. Ecol. **39**:153–167.
39. **Stein LY, La Duc MT, Grundl TJ, Nealson KH.** 2001. Bacterial and archaeal populations associated with freshwater ferromanganous micronodules and sediments. Environ. Microbiol. **3**:10–18.
40. **Tard C, Pickett CJ.** 2009. Structural and functional analogues of the active sites of the [Fe]-, [NiFe]-, and [FeFe]-hydrogenases. Chem. Rev. **109**: 2245–2274.
41. **Thamdrup B.** 2000. Bacterial manganese and iron reduction in aquatic sediments. Adv. Microb. Ecol. **16**:41–84.
42. **Vignais PM, Billoud B.** 2007. Occurrence, classification, and biological function of hydrogenases: an overview. Chem. Rev. **107**:4206–4272.
43. **Vroblesky DA, Chapelle FH.** 1994. Temporal and spatial changes of terminal electron accepting processes in a petroleum hydrocarbon contaminated aquifer and the significance for contaminant biodegradation. Water Resour. Res. **30**:1561–1570.
44. **Zirngibl C, et al.** 1992. H_2-forming methylenetetrahydromethanopterin dehydrogenase, a novel type of hydrogenase without iron-sulfur clusters in methanogenic archaea. Eur. J. Biochem. **208**:511–520.