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

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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 H₂ 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 O₂. 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 O₂-resistant Hya is to participate in the oxidative stress defense of *G. sulfurreducens*.

Hydrogenases are ubiquitous enzymes catalyzing the reversible interconversion of H₂ 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 H₂ 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 O₂ 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 O₂ 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 O₂ 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 (N₂-CO₂, 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 O₂ 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 ΔhyaSLB::Km^r ΔhybL::Cm^r double mutant. To construct a ΔhyaSLB::Km^r ΔhybL::Cm^r double mutant, the ΔhybL::Cm^r mutant allele was introduced into DL7. *hybL*::Cm^r was amplified by PCR with primers MChyd21for (5'-GCACGAACTGTCCGGCATCG-3') and MChyd26rev (5'-AGTTCTCTCCAGGTGGTTG-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.

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
<i>E. coli</i> Top10	<i>recA1 endA1 gyrA96 thi-1 hsdR17</i> ($r_K^- m_K^+$) <i>supE44 relA1 ΔlacU169</i>	Invitrogen, Carlsbad, CA
<i>G. sulfurreducens</i>		
PCA (ATCC 51573)	Wild type	7
DL7	Δ <i>hyaSLB::Km^r</i>	10
DL8	Δ <i>hybL::Cm^r</i>	10
DLMC10	Δ <i>hyaSLB::Km^r ΔhybL::Cm^r</i>	Maddalena V. Coppi
<i>Phyb</i> mutant	Δ <i>Phya::Phyb; Gm^r</i>	This work
<i>hyb Phyb</i> mutant	Δ <i>hybL::Cm^r ΔPhya::Phyb; Gm^r</i>	This work
Plasmids		
pCR2.1-TOPO	PCR cloning vector; Ap ^r Km ^r	Invitrogen, Carlsbad, CA
pUC19-Gm ^r <i>loxP</i>	pUC19 carrying Gm ^r <i>loxP</i> ; Ap ^r Gm ^r	2
pPLT158	pCR2.1 carrying <i>Phyb</i> upstream of the 5' part of <i>hyaS</i> ; 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 <i>Phyb</i> ; Ap ^r Km ^r Gm ^r	This work

Replacement of native *hya* promoter by *Phyb*. 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 *Phyb* promoter was fused to the 5' part of the *hyaS* gene by recombinant PCR with the primers fullhybupAvrII2g (5'-CATCTAGGCAAGAAGGCGTCTCTGGAAGAGC-3'), recphybhyadn2g (5'-TTGCCATCGTTCGTTACCTGCCATAAA TCCCCTGTCCGGTATGAT), recphybhyaup (5'-ATCATACCGACAGGGGATTTATGGCAGGTAACGACGATGGC), 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'-CTC GGATCCAGTACGGCCACAAGGCTCACCTG-3') and *hyaupAvrII* 5'-CTGCCTAGGCTTTCCGGATGGTCTGCTGGTGTG-3'), digested with BamHI-AvrII (NEB, Beverly, MA) and ligated with the T4 DNA ligase (NEB) upstream of *Phyb* 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 *Phyb* 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 O₂, or 0.15 ml of O₂ 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'-GCCCCATTTGTAGAGGCAGT-3'). Primers used for the amplification of *hybS* were *hybS229f* (5'-CTTGACATGATTCGCTGG A-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'-GGCC AGCAGCCCTTTGAT-3'). Relative levels of expression of the studied genes were calculated by the 2^{-ΔΔCT} 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. O₂-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 H₂-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 O₂⁻ generated by the xanthine oxidase reaction and H₂O₂. 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 H₂. 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 H₂O₂ was divided by the number of CFU obtained from the cell suspension not exposed to H₂O₂.

The same experimental design was adopted to establish the ability of *G. sulfurreducens* strains to survive superoxide exposure. O₂⁻ was generated in the cell suspensions under H₂ as previously described (17). Briefly, O₂ (10 ml) and 1 mM xanthine were added along with 1000 U/ml of bovine liver catalase to eliminate H₂O₂. 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

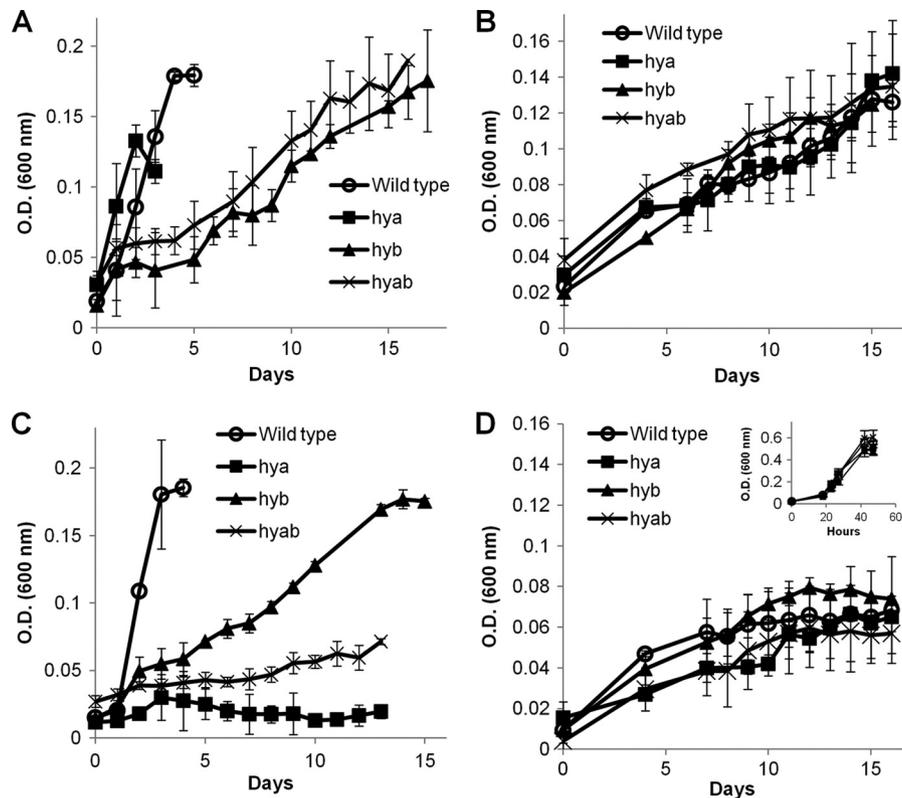


FIG 1 Growth of *G. sulfurreducens* hydrogenase mutants exposed to oxidative stress. The wild-type strain and *hya*, *hyb*, and Δ *hyaSLB::Km^r Δ hybL::Cm^r* (*hyab*) mutants were cultivated with a high (A and B) or low (C and D) cysteine concentration. Hydrogen (A and C) or highly limiting acetate (B 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 sub-

stantial 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-

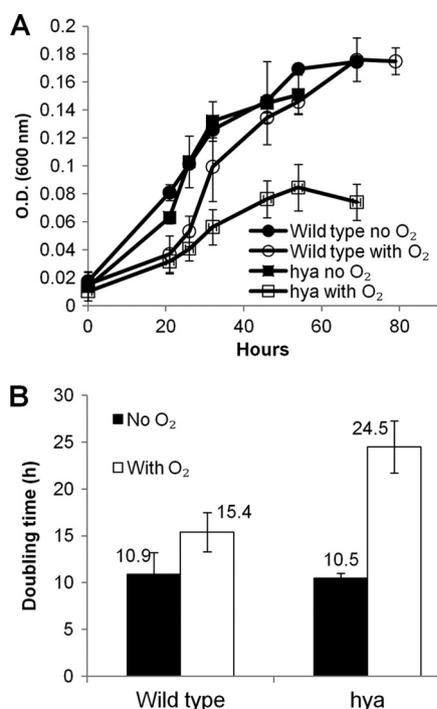


FIG 2 Growth of the *G. sulfurreducens* *hya* mutant exposed to O₂. The wild type and the *hya* mutant were grown with hydrogen as the electron donor and fumarate as the electron acceptor. Where indicated, 0.15 ml of O₂ was introduced into the headspace prior to inoculation. Growth curves (A) and doubling times (B) are the means from at least three independent replicate cultures.

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 O₂.

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

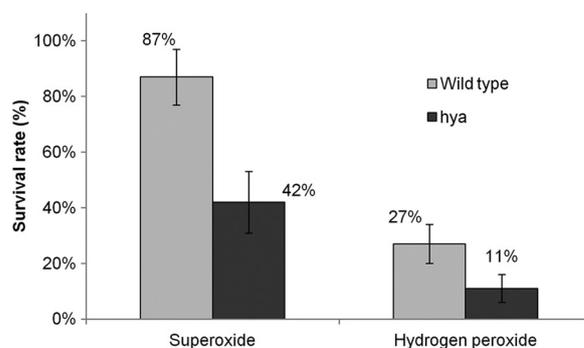


FIG 3 Survival of wild-type and Hya-deficient strains of *G. sulfurreducens* following superoxide and hydrogen peroxide exposure for 2 h. Survival rates are the means from at least three independent experiments.

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 H₂, it is possible that this hydrogen oxidation by Hya is central to its newly discovered function.

Hya prevents inactivation of Hyb caused by O₂. Exposure of cells to atmospheric O₂ 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 O₂. 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 H₂ but can do so under oxidative stress. Hydrogen oxidation ac-

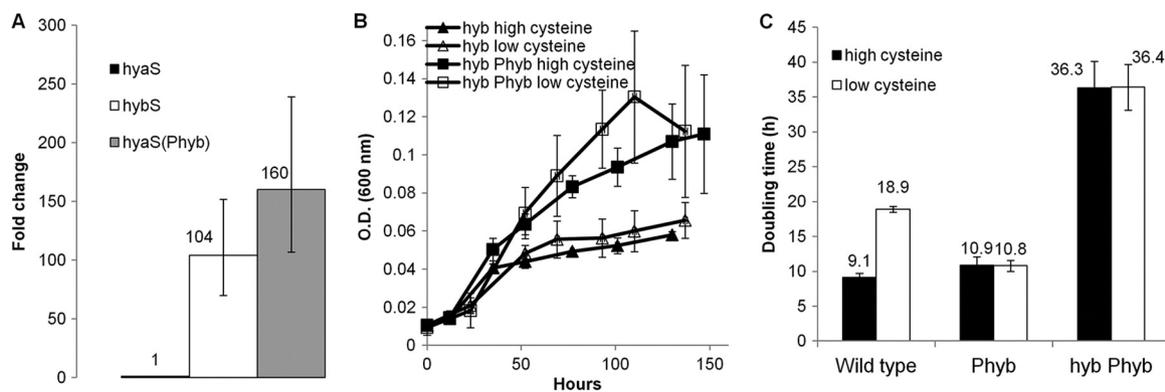


FIG 4 Overexpression of the hydrogenase Hya from the *hyb* promoter. (A) Quantitative RT-PCR assay of *hyaS* and *hybS* under the control of their respective native promoters and of *hyaS* under the control of *Phyb*. (B) Growth on hydrogen of the *G. sulfurreducens* *hyb* mutant with *hya* under the control of its native promoter or *Phyb* 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 *Phyb*, and the *hyb* mutant with *hya* under the control of *Phyb*. 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 O₂ 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 O₂ 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 O₂ (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.

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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
<i>hyb</i> mutant	0.1 ± 0.0	0.1 ± 0.0
<i>hya</i> mutant	8.4 ± 0.7	ND ^c
<i>hya hyb</i> mutant	ND	ND
<i>Phyb</i> mutant ^d	7.7 ± 2.3	7.5 ± 0.4
<i>hyb Phyb</i> 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 (*Phyb*).

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