Role of the Hya Hydrogenase in Recycling of Anaerobically Produced H_2 in *Salmonella enterica* Serovar Typhimurium[▽]

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Double and triple uptake-type hydrogenase mutants were used to determine which hydrogenase recycles fermentatively produced hydrogen. The $\Delta hyb \Delta hya$ and $\Delta hyd \Delta hya$ double mutants evolved H_2 at rates similar to that of the triple mutant strain, so Hya alone oxidizes the bulk of H_2 produced during fermentation. When only Hya was present, no hydrogen production was observed in nutrient-limited medium. H_2 uptake assays showed that Hya can oxidize both exogenously added H_2 and formate hydrogen lyase-evolved H_2 anaerobically. Even after anaerobic growth, all three uptake-type hydrogenases could function in the presence of oxygen, including using O_2 as a terminal acceptor.

Due to the anticipated scarcity of fossil fuels, there has been a surge of interest in H_2 production for alternative energy means. Numerous studies have attempted to engineer H_2 -producing organisms, such as photosynthetic bacteria, cyanobacteria, and *Escherichia coli*, to produce maximal amounts of H_2 while minimizing the H_2 -oxidizing capability of the organism (4, 5, 9, 10, 19). Hydrogenase expression and activity are controlled by multiple regulatory pathways and respond to fluctuations in pH, oxygen levels, and availability of metabolites and metal cofactors (17). In addition, the presence of hydrogen uptake hydrogenases decreases the net H_2 yield even under conditions that favor H_2 production. It is therefore important to understand the interactions between H_2 -oxidizing enzymes (i.e., respiratory hydrogenases) and H_2 -producing enzymes.

Gene sequence analysis has revealed that many enteric bacteria contain the genes necessary for hydrogen production and oxidation. The *E. coli* hydrogenases have been studied extensively, while *Salmonella enterica* serovar Typhimurium hydrogenases have been studied to a lesser extent. Both *E. coli* and *Salmonella* serovar Typhimurium contain the hydrogen-oxidizing hydrogenases Hya and Hyb. *Salmonella* serovar Typhimurium also contains Hyd, which is another hydrogen-oxidizing hydrogenase (2, 13, 15). Hyc and Hyf are hydrogen-evolving hydrogenases that are present in both *E. coli* and *Salmonella* serovar Typhimurium, although it is unknown whether Hyf is functional (1).

The *Salmonella* serovar Typhimurium hydrogenases are important for cellular metabolism. Hyc produces H_2 in order to remove excess reductant generated during mixed-acid fermentation. Hyc and formate dehydrogenase constitute the formate hydrogen lyase (FHL) complex (16), which oxidizes formate to produce CO_2 and H_2 (12). The *hyb* genes in *E. coli* and *Salmonella* serovar Typhimurium are expressed at high levels under anaerobic respiration conditions, and Hyb probably contributes to energy conservation (11, 15, 20). Hyb oxidizes H_2 and generates electrons, which are passed through the electron

transport chain to terminal acceptors such as fumarate. The protons generated contribute to the proton-motive force. The role of Hya is not as well characterized. Hya may be used to recycle Hyc-produced H_2 , since the *hya* operon is expressed at high levels during fermentative growth, or it may play a role in acid stress resistance (6, 14, 20, 21). The *hyb* genes are expressed at high levels under aerobic conditions in *Salmonella* serovar Typhimurium, and Hyb may couple H_2 oxidation to O_2 reduction (20).

Redwood et al. recently examined the roles of uptake-type hydrogenases on net hydrogen production in *E. coli* (10). Cells were pregrown aerobically or anaerobically with formate and then allowed to ferment in anaerobic bottles. H_2 gas was collected, and other fermentation products were measured. They found that H_2 production increased by 37% in an *hya hyb* double mutant (compared to that in the wild type) that was grown overnight aerobically with formate. This increase in production was associated with the loss of *hyb* and not *hya*. Therefore, in *E. coli*, Hyb may be responsible for recycling fermentatively produced H_2 .

In this study, we measured the effect of uptake-type hydrogenase mutations on H_2 production in *Salmonella* serovar Typhimurium. We found that the majority of H_2 -recycling activity in fermenting cells was dependent on the presence of *hya*, and having only Hya was sufficient to prevent any detectable H_2 evolution. These results demonstrate yet another difference between H_2 metabolism in *E. coli* and H_2 metabolism in *Salmonella* serovar Typhimurium.

Bacterial strains, growth conditions, and reagents. The strains and plasmids used in this study are listed in Table 1. Strains were maintained in Luria-Bertani (LB) broth or on LB plates with appropriate antibiotics (50 μg ampicillin ml^{-1} and 25 μg kanamycin ml^{-1}). CR-Hyd medium (15) with glucose (0.4%) and sodium formate (20 mM) was used where indicated. Cells were grown at 37°C either anaerobically or aerobically. Anaerobic conditions were established by sparging sealed 165-ml bottles with N_2 for 15 min and then anaerobic mix (10% H_2 , 5% CO_2 , and 85% N_2) for 20 min. Although overnight cultures were used for assays (see Table 2), cell densities were no more than 5×10^8 to 6×10^8 cells/ml at harvest.

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TABLE 1. Strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
<i>S. enterica</i> serovar Typhimurium strains		
JSG210	14028s (wild type)	ATCC
ALZ7	JSG210 with $\Delta hyb::FRT^a$	20
ALZ8	JSG210 with $\Delta hyd::FRT$	20
ALZ9	JSG210 with $\Delta hya::FRT$	20
ALZ36	JSG210 with $\Delta hyb::FRT$ $\Delta hyd::FRT$	This work
ALZ37	JSG210 with $\Delta hyb::FRT$ $\Delta hya::FRT$	This work
ALZ42	JSG210 with $\Delta hyd::FRT$ $\Delta hya::FRT$	This work
ALZ43	JSG210 with $\Delta hyb::FRT$ $\Delta hyd::FRT$ $\Delta hya::FRT$	This work
Plasmids		
pCP20	Amp ^r ; contains flippase gene for λ Red mutagenesis	3
pKD46	Amp ^r ; contains λ Red genes γ , β , and <i>exo</i>	3
pKD4	Kan ^r ; contains <i>kan</i> cassette	3

^a FRT, flippase recombinase recognition target.

Mutant strain construction. Mutants with deletions of the *hyb*, *hyd*, and *hya* genes were constructed using the lambda Red system, as reported previously (7, 21). Hydrogenase structural gene deletions made by this system were shown to be nonpolar (see reference 7), and the strains do not contain any antibiotic resistance markers. The double mutants and triple mutant were constructed using each hydrogenase single deletion mutant and a hydrogenase mutant in which the kanamycin resistance cassette replaced the hydrogenase gene (7). Phage P22Htint (J. Gunn, The Ohio State University, Columbus, OH) was used to transduce the antibiotic marker from the $\Delta hyb::Kan$, $\Delta hyd::Kan$, or $\Delta hya::Kan$ strain into the *hya*, *hyb*, or *hyd* single mutant, making double mutant strains. The kanamycin resistance cassette was removed by transforming with pCP20 and then growing at 37°C. The triple mutant was constructed similarly by transducing the antibiotic resistance cassette from a hydrogenase single mutant strain into a hydrogenase double mutant. The double mutants and triple mutant were confirmed by PCR. The resulting strains are listed in Table 1.

Amperometric hydrogenase assays. Wild-type, *hya* *hyb*, *hyb* *hyd*, or *hya* *hyd* double mutant, or *hya* *hyb* *hyd* triple mutant cells were grown overnight under anaerobic conditions in bottles containing CR-Hyd medium with glucose and formate. Cells were removed from the closed bottle with a syringe after overnight growth. The cell suspension was injected into a stirred and sealed chamber containing a Clark type probe. Hydrogen evolution was recorded by amperometry (8). In order to measure hydrogen uptake, 100 μ l of H₂-saturated phosphate-buffered saline was added to the chamber and the disappearance of H₂ was recorded.

Effect of hydrogenase deletions on H₂ production. Redwood et al. found that in *E. coli*, Hyb is important for recycling Hyc-produced H₂ during fermentative growth (10). Whether Hyb had the capacity for recycling additional (exogenous) H₂

TABLE 2. Hydrogenase activity in wild-type *Salmonella* serovar Typhimurium or in hydrogenase mutant strains containing a single uptake enzyme

Strain or genotype	Enzyme(s)	Hydrogenase activity (nmol H ₂ /min/10 ⁹ cells) ^a		
		H ₂ evolution	H ₂ uptake with endogenous acceptors ^b	H ₂ uptake with oxygen ^c
Wild type	Hya, Hyb, Hyd	<0.02 ^d	27 $\pm$ 9	47 $\pm$ 8
Δhyb Δhyd	Hya	<0.02 ^d	5.2 $\pm$ 2.8	18 $\pm$ 4
Δhyb Δhya	Hyd	18 $\pm$ 8	ND	5.2 $\pm$ 1.5
Δhyd Δhya	Hyb	17 $\pm$ 7	ND	14 $\pm$ 5
Triple mutant	None	14 $\pm$ 6	ND	<0.02 ^d

^a Cells were grown overnight anaerobically in bottles containing CR-HYD media with 0.4% glucose and 20 mM sodium formate. Results are the means $\pm$ standard deviations for at least three independent experiments.

^b H₂ uptake was first assayed anaerobically with overnight cultures in media, with no exogenous electron acceptor added as indicated. In this case (i.e., anaerobic assays), H₂ uptake could not be assessed (ND, not done) for three of the strains due to ample H₂ evolution.

^c H₂ uptake was assayed with low levels (final concentration, 45 to 85 μ M) of oxygen added via syringe into the amperometric chamber from a 100% O₂-saturated solution. H₂ (75.4 nmol) from a 100% saturated solution in phosphate-buffered saline was injected into the chamber to initiate H₂ uptake assays.

^d This level was deemed to be the minimum detectable level by the amperometric assay. H₂ evolution was assayed anaerobically, sometimes after a lag (see the text).

was not addressed. However, it has also been hypothesized that *E. coli* Hya may function to oxidize H₂ during fermentation (14). Our previous study revealed that in *Salmonella* serovar Typhimurium, *hya* gene expression was maximal during fermentative growth (20). We constructed hydrogenase double and triple mutants in *Salmonella* serovar Typhimurium to determine which hydrogenase was important in recycling H₂ produced via fermentation in this organism.

Wild-type *Salmonella* serovar Typhimurium and hydrogenase double and triple mutants were grown anaerobically overnight in sealed bottles containing CR-Hyd medium with glucose and formate. This medium was designed for optimal expression and maturation of the FHL complex. Hydrogen evolution was measured by injecting cells into a sealed chamber containing a Clark-type electrode. H₂ production was observed after an approximately 5-min lag time, during which the cells respired any O₂ which may have entered the chamber. The presence of O₂ would inhibit Hyc function as part of the FHL complex (16, 18). We assume that the O₂ exposure placed this H₂-evolving enzyme into the "ready" but oxidatively inactivated state (see reference 18). Consistent with this, we observed that when sodium dithionite (50 μ M) was added to the chamber to make it anaerobic, the lag time to reach maximal activities was only about 10 s (the likely time for complete dithionite mixing/oxygen removal).

H₂ production was not observed for wild-type cells (Table 2). This is undoubtedly due to the activity of the H₂-oxidizing enzymes. As Hyc produces molecular hydrogen, the uptake-type hydrogenases oxidize it, resulting in a lack of any observable H₂ uptake or evolution. Interestingly, we did not observe H₂ production in the Δhyb Δhyd mutant, either. This result suggests that Hya has sufficient H₂ uptake activity to use all of the hydrogen produced by Hyc. Alternatively, it is possible that Hya somehow inhibits FHL activity in wild-type cells exposed to H₂ so that when Hya is missing, this negative regulation is

relieved. The result could be like that illustrated by the results in Table 2, i.e., the wild type does not produce H_2 but strains lacking Hya do so. However, this possibility is minimal, as when wild-type cells were grown to early exponential phase in medium (LB with glucose [see reference 20]) in which H_2 production is abundant compared to H_2 uptake reactions, both H_2 evolution and H_2 uptake could be detected. In such cells (in which some Hya is still synthesized [20]) assayed anaerobically, 34.5 nmol H_2 /min/ 10^9 cells was produced (average of two independent determinations), whereas when oxygen was added, H_2 uptake was observed at 19.6 nmol H_2 /min/ 10^9 cells. This result also shows that Hya is not at sufficient levels to recycle all fermentative H_2 production in some (common) culture conditions.

Hydrogen uptake assays indicated that Hya also has some limited capacity to use exogenous H_2 beyond what is evolved from the FHL system (Table 2). This aspect may be important within the animal tissues where colonically produced molecular hydrogen resides. The $\Delta hyb \Delta hya$ and $\Delta hyd \Delta hya$ double mutants produced hydrogen at rates comparable to the hydrogen production rates of the triple mutant (Table 2). Whenever *hya* was absent, hydrogen production was observed. This result indicates that Hyb and Hyd are not important for appreciable anaerobic recycling of H_2 during fermentative growth.

When hydrogenase assays are conducted aerobically, the Hyc-associated hydrogen evolving (FHL) system (16, 18) is inoperative (see the text above). Therefore, we tested the ability of the strains to use H_2 with O_2 present as both an inhibitor of FHL (H_2 evolution) and a terminal respiratory acceptor. Although grown anaerobically, all double mutant strains, each containing a single uptake enzyme, were capable of H_2 oxidation (Table 2, rightmost column) with O_2 present. The strain with Hya had only about three times more uptake activity with O_2 present than the same strain did via the endogenous acceptor-mediated H_2 oxidation activity. This means that the Hya hydrogenase is likely well coupled to acceptors that accumulate anaerobically, but it can in addition carry out some H_2/O_2 respiration. In strains grown anaerobically in LB-plus-glucose medium, in which H_2 evolution is abundant, the same trend shown in Table 2 occurred for the Hyb-only and Hyd-only strains; these strains evolved more (about two times more) H_2 than the Hya strain. Also, the Hyd-only strain oxidized the smallest amount of H_2 in assays conducted aerobically from these anaerobic LB medium-grown cultures, whereas the Hya-only strain was able to take up the most H_2 (of the mutant strains) in the oxygen-coupled assays.

Our previous study showed that *hyb* and *hyd* are expressed at low levels during fermentative growth with formate (six times and three times less, respectively, than at their optimal growth conditions. The optimal conditions were anaerobic plus glycerol and fumarate (for *hyb*) and aerobic plus glucose (for *hyd*) (20). However, the high rates of hydrogen production in double mutants (that contain only Hyb or Hyd) indicate that either these protein levels are too low to oxidize FHL-produced H_2 or these hydrogenases are not properly localized to couple H_2 oxidation to the FHL system. Curiously, in our previous study, *hya* expression was repressed when cells were grown with formate compared to expression when cells were grown with glucose alone. Hya gene expression levels were 45.7 ± 3.2 Miller

units when cells were grown in CR-Hyd medium with glucose and 9.9 ± 1.6 Miller units when cells were grown with glucose and sodium formate (20). This level of *hya* expression must be sufficient for the recycling of H_2 under this condition. A previous study by Sawers et al. (15) used rocket immunoelectrophoresis to measure levels of *Salmonella* serovar Typhimurium hydrogenase isoenzyme under different growth conditions. They found that the cellular content of isoenzyme 1 (presumably Hya) was higher in cells grown with formate than in cells grown with glucose alone (15).

It has been shown that Hyb (but not Hya) is important for hydrogen uptake during fermentation in *E. coli* (10). In contrast, Hya is responsible for hydrogen recycling under these conditions in *Salmonella* serovar Typhimurium. Also, Hya can still function aerobically with exogenous H_2 . This study further defines the roles of the *Salmonella* serovar Typhimurium uptake-type hydrogenases. Hya is used during fermentation and has also been shown to be important in acid resistance (21). Nevertheless, it has been shown that *hyd* is expressed at high levels during aerobic growth and while *Salmonella* serovar Typhimurium resides in phagocytes (20, 21). Hyd is probably used primarily for efficient O_2 -coupled energy conservation/respiration at a time of external stress. On the other hand, the *hyb* genes are maximally expressed during anaerobic fermentation (20), yet the Hyb enzyme may not be well coupled to anaerobically accumulating acceptors or to the FHL system. The multiple hydrogenases appear to play important roles in increasing the fitness of the bacterium in order to survive a variety of fluctuating growth environments.

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