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Hydrogen-Oxidizing Capabilities of *Helicobacter hepaticus* and In Vivo Availability of the Substrate

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Hydrogen-oxidizing hydrogenase activity was detected in *Helicobacter hepaticus* and compared to the activity in *Helicobacter pylori* for characteristics associated with hydrogen uptake respiratory hydrogenases. Intact whole cells could couple H_2 oxidation to oxygen uptake, and no H_2 uptake was observed without oxygen available to complete the respiratory pathway. The *H. hepaticus* enzyme coupled H_2 oxidation to reduction of many positive potential acceptors, and it underwent anaerobic or reductive activation. *H. hepaticus* had a strong affinity for molecular H_2 (apparent K_m of 2.5 μM), and microelectrode measurements on the livers of live mice demonstrated that H_2 is available in the host tissue at levels 20-fold greater than the apparent whole-cell K_m value.

Although most of the member species of *Helicobacter* are not colonizers of the gastric mucosa, the gastric colonizer (*Helicobacter pylori*) receives the bulk of the research attention. Nevertheless, the enterohepatic types are beginning to be studied as important natural colonizers and emergent pathogens of animals (3, 18). They colonize the intestinal tract (and sometimes the liver) of humans and other mammals, and many have been considered to be part of the normal intestinal flora (see references 17 and 18). One member of this diverse group of helicobacters is *Helicobacter hepaticus*; it was originally isolated from strains of mice with a high incidence of hepatitis and liver tumors (4, 7). Since then, the correlation of this bacterium with liver diseases (chronic active hepatitis, typhlitis, and hepatocellular tumors) and irritable bowel disease-like symptoms has become stronger (see references 3 and 18), but *H. hepaticus* has not been isolated from the human liver. Nevertheless, the fact that hepatic helicobacters are associated with diseased liver tissue in other animals, including primates (5, 6, 8), has sparked increased attention to *H. hepaticus* physiology. Similarly, the reports correlating the presence of *Helicobacter* sp. DNA with (human) patients having primary liver carcinomas have further fueled interest in hepatic helicobacters (3).

Recently members of our group showed that H_2 uptake-type hydrogenase activity is a bacterial characteristic that is important for conferring colonizing ability to *H. pylori* (16). Hydrogen oxidation is carried out by many diverse respiratory bacteria, since it is one of several possible reducing substrates common in nature (10). The low-potential electrons can be coupled to energy-conserving processes, and this ability likely helps *H. pylori* persist in the energy-poor environment of the gastric mucosa. The affinity of the *H. pylori* bacteria for the high-energy diffusible substrate (H_2) combined with microelectrode measurements to assess H_2 levels in the stomachs of live mice allowed us to conclude that the characterized H_2-oxidizing electron transport chain observed in laboratory-grown *H. pylori* (12) is saturated with H_2 while the bacterium is established in the host (16). H_2 has been measured as an excreted product from the intestinal tracts of humans and rodents (due to its production by intestinal flora), and it has been speculated to be carried through the vascular systems of animals (11, 22). Our goal was to determine the availability of H_2 and its potential metabolism as a respiratory substrate for possible energy conservation by *H. hepaticus*. As with our related stomach hydrogen measurement work, we detected ample molecular hydrogen levels in the livers of live mice, and this level far exceeded the estimation of affinity of *H. hepaticus* for H_2 (apparent K_m value). Here an H_2-oxidizing hydrogenase enzyme within *H. hepaticus* was partially characterized and compared to the *H. pylori* H_2-oxidizing system for some primary characteristics associated with the uptake-type hydrogenases.

After we initiated the present study, a partial genomic sequence analysis of *H. hepaticus* was reported; 56 coding regions were identified, and one of these is a partial ortholog (85% identity) of *hydB* of *Wolinella succinogenes* (9). This would indicate that the bacterium contains the large subunit of a NiFe hydrogenase. Hydrogenases can either evolve or consume H_2, and they play diverse roles enzymologically and physiologically (21). To aid in determining the presence of a putative H_2 uptake hydrogenase in *H. hepaticus*, direct amperometric (14) hydrogenase assays were carried out. *H. hepaticus* ATCC 51449 was grown on blood agar plates in anaerobic jars in an atmosphere of 5% CO_2, 10% H_2, and the balance N_2. Bacteria were incubated at 37°C for 5 days and harvested by resuspending cells from cotton swabs into phosphate-buffered saline (PBS) and then centrifuging (10,000 rpm in a Beckman-Coulter Microfuge-18 for 10 min). The pellet was resuspended, and the centrifugation step was repeated. The suspended cells (in 2 to 3 ml of PBS) were adjusted to approximately 2×10^9 cells per ml in PBS. This corresponds to an optical density at 600 nm of about 1.0 and required the initial use of more than 25 blood agar plates to conduct just 6 to 10 independent hydrogenase assays. For comparison be-

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TABLE 1. Hydrogen oxidation activities with oxygen and methylene blue[a]

Strain (electron acceptor)	H_2 uptake activity (nmol/min/10^9 cells)
H. pylori 43504 (O_2)	37 ± 2
H. pylori 26695 (O_2)	33 ± 4
H. hepaticus 51449 (O_2)	3.2 ± 0.2
H. pylori 26695 (MB, aerobic)	23 ± 4
H. pylori 26695 (MB, anaerobic)	176 ± 15
H. hepaticus 51449 (MB, aerobic)	3.9 ± 0.5
H. hepaticus 51449 (MB, anaerobic)	25 ± 2

[a] Hydrogen concentrations were determined directly amperometrically as described previously (12, 14) on 0.5-ml cell aliquots (in argon-sparged PBS containing between 1.2×10^9 and 3.2×10^9 cells per ml) added to a 2.8-ml-volume amperometric chamber equipped to monitor both H_2 and O_2 levels. Live intact cells were used for oxygen-dependent measurements, and Triton X-100-permeabilized cells (19) were used for the methylene blue (MB)-dependent measurements. For permeabilization, 2 ml of cell suspension received 10 μl of 10% Triton X-100 solution, and the suspension-detergent mixture was incubated at room temperature (but in 100% argon atmosphere) for 20 min before assay. Cell numbers were determined by taking the average from direct counting (by microscopy) of 10 replicate fields on slides. The oxygen level for the aerobic assays was approximately 20 μM, and the anaerobic assays contained 50 μM (freshly prepared in argon-sparged PBS) sodium dithionite. Methylene blue was added to saturation of activity, which was up to 250 μM. Other details of procedures are as described previously (12). The data are the means ± standard deviations for four or five independent replicate samples.

TABLE 2. Electron acceptor specificity of hydrogenase[a]

Electron acceptor (concn)	Eo′ (mV)[c]	Relative activity (%)	
		H. pylori	*H. hepaticus*
Methylene blue (200 μM)	11	100	100
Phenazine methosulfate (400 μM)	80	>100	89
DCPIP[b] (200 μM)	217	54	68
Ferricyanide (1 mM)	360	85	68
Cytochrome *c* (100 μM)	250	33	71
Benzyl viologen (1 mM)	−360	<0.5	<1.0

[a] The 100% activities (methylene blue rate) were 382 and 62 nmol/min/mg of protein for *H. pylori* (membranes) and *H. hepaticus* (permeabilized cells), respectively. The *H. pylori* data are from reference 12. The *H. hepaticus* permeabilized cells were prepared as described in footnote *a* of Table 1, and protein was measured with bicinchoninic acid (Pierce Chemical Co) after adding 1% sodium dodecyl sulfate and then heating cells (90°C for 10 min). All electron acceptor activities were determined at saturating levels anaerobically in the presence of 50 μM sodium dithionite.

[b] DCPIP, 2, 6-dichlorophenolindophenol.

[c] Eo′, standard oxidation-reduction potential.

tween *H. hepaticus* and *H. pylori*, the latter bacteria were grown and treated the same way except that 2 days' growth was sufficient for cell harvest (and to avoid appearance of undesired coccoid cells).

H. hepaticus whole cells were able to couple H_2 oxidation to respiratory O_2 consumption. No H_2 uptake was observed anaerobically. However, we immediately noticed that the rate of H_2 oxidation was lower than we routinely observe for *H. pylori*. To address this observation rigorously, H_2 oxidation activity by *H. hepaticus* was compared to those of two common *H. pylori* strains grown in the same conditions (blood agar plates incubated in a 10% H_2-containing atmosphere). Table 1 documents that the H_2-O_2 respiratory pathway for *H. hepaticus* is only about 10% of the rate (on a per-cell basis) for *H. pylori*. The H_2 oxidation by whole cells was dependent on the inclusion of oxygen, since no H_2 uptake (or evolution) was observed under anaerobic conditions.

Uptake-type hydrogenases are reported to undergo an anaerobic activation (sometimes referred to as reductive activation) phenomenon. This is characterized by a much higher enzyme activity when conditions are highly reducing, or anaerobic. When O_2 was present in the reaction mixture, the *H. hepaticus* enzyme had only 16% of the anaerobic H_2-oxidizing rate (Table 1). This amount of anaerobic activation is similar to what we observe for cells of *H. pylori* (Table 1) and what we had observed for membranes obtained from a clinical *H. pylori* isolate previously (12). For the systems in which this anaerobic activation has been studied in detail (2, 20), the achievement of full activity under reducing conditions is related to the rate of electron acceptor reduction (i.e., hydrogenase turnover).

Hydrogen-oxidizing hydrogenases are known for having high affinities for their substrate. To ascertain the usefulness of the *H. hepaticus* enzyme to possible in vivo growth of the bacterium, a whole-cell Michaelis constant (apparent K_m) for hydrogen was determined. The whole-cell K_m for molecular hydrogen was determined using a Lineweaver-Burk plot of whole-cell hydrogenase activities at limiting hydrogen concentrations as described previously (13). This apparent K_m was found to be 2.5 μM (average for three experiments), indicating a high affinity for hydrogen. This apparent K_m was determined on live intact whole cells with O_2 available as the only terminal electron acceptor in the H_2-oxidizing respiratory chain. Therefore, our reported apparent K_m is for the entire functioning hydrogen-oxidizing system.

The characteristics of hydrogenase in *H. hepaticus* are similar to what was reported for *H. pylori*, so it is likely the *H. hepaticus* system is also membrane bound and involved in energy conservation. It likely involves reduction of other membrane-associated electron transport proteins after the "H_2 splitting" step. Therefore, the dye-mediated redox potential range at which this H_2-oxidizing system functions was determined. Typically, H_2 uptake hydrogenases function with redox acceptors of positive potential but not with negative redox potential acceptors (15). As for *H. pylori*, the *H. hepaticus* enzyme was able to couple H_2 oxidation to reduction of the positive acceptors (Table 2) but not the negative potential acceptor benzyl viologen. These experiments were conducted in the absence of oxygen. An interesting difference between the *H. pylori* and *H. hepaticus* systems is the suitability of cytochrome *c* as an acceptor of hydrogenase. The midrange redox potential cytochrome *c* functioned as a very good acceptor for the *H. hepaticus* hydrogenase; this may mean that the in vivo acceptor of electrons from hydrogenase in *H. hepaticus* is poised at a higher redox potential (quinone or cytochrome) than the initial acceptor in the membrane of *H. pylori*. We have previously shown that hydrogen oxidation in *H. pylori* is linked to cytochrome reduction, with these heme-containing components functioning as intermediate electron carriers prior to reduction of the terminal (O_2-binding) oxidases (12). However, performance of these difference spectral experiments was not possible for *H. hepaticus*, since H_2-oxidizing membrane particles could not be obtained. The membrane isolation was done in a way that restricted oxygen (use of argon-sparged buffers during and after cell disruption, and transfer of extracts via an argon-sparged syringe), but was not done under strictly anaerobic conditions, so it is possible that O_2 labile factors

TABLE 3. Microelectrode-determined hydrogen concentrations in mouse livers

Mouse no.	H_2 range (μM)	Mean ± SD	No. of sites measured[a]
1	43–63	54 ± 9	10
2	29–89	53 ± 18	12
3	43–68	57 ± 11	12

[a] The sites measured included all lobes of the liver, and measurement was accomplished by insertion of a 50-μm H_2 sensing probe (16) into live (but anesthetized) mice. The mice were female strain C57BL (Jackson Labs, Bar Harbor, Maine) and were anesthetized with halothane.

exist in the H_2-O_2 respiratory chain. Nevertheless, since H_2 oxidation is coupled to O_2 uptake, and it is coupled to reduction of dyes of positive potential there may be (as yet unidentified) heme-containing components in the *H. hepaticus* membrane that are involved in conserving the energy from electrons initially provided from H_2.

We determined hydrogen concentrations in the liver of live adult mice amperometrically, using a modified Clark-type microelectrode model H_2-50 (Unisense A/S, Aarhus, Denmark) (Table 3). The electrode had a tip diameter of 50 μm. Female C57BL mice (Jackson Labs, Bar Harbor, ME) were anesthetized with halothane. Mice were kept alive but fully anesthetized for the entire procedure and euthanatized immediately after the last measurement. The liver was surgically exposed and the microelectrode was inserted into the liver tissue; most measurements were taken upon passing the probe less than 1.0 mm into the liver, so as not to damage the (fragile) probe. Indeed probe damage was encountered in some initial measurements in attempts to insert the probe deeper into the tissue. The hydrogen readings were recorded after the signal had stabilized (about 5 to 7 s). The procedure was repeated at up to 12 sites per mouse liver. Standard curves of H_2 were obtained as described by the manufacturer by use of their calibration chamber. The average hydrogen levels were over 50 μM, thus exceeding the apparent K_m by 20-fold, and slightly exceeding the levels we reported for mouse stomach H_2 levels (16). The tissue H_2 measurements together would support a hypothesis that H_2 is available in many tissues within the animal and warrants further considering the availability of hydrogen as an energy reservoir for use by infectious bacteria within the host animals.

The source of the microelectrode-determined H_2 is likely the vascular system transporting H_2 that originated from colonic fermentations, and the large variation in measured values is not surprising considering the mouse liver is (surgically) partially exposed to the air atmosphere. This air exposure would presumably result in lower measured levels than occur in the intact animal. Hydrogen excretion from the intestinal tract of rodents has been documented (1), but the levels of the gas we found associated with the specific tissues (stomach and liver) of live mice were not expected. Of course, the usefulness of this small and diffusible, but highly energetic, substrate for *H. hepaticus* liver colonization must await critical animal studies using gene targeted *H. hepaticus* mutants lacking H_2 utilizing ability.

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