conformers, all of which must come together at the same time, or (ii) the nucleus is formed from still molten conformers that must be free to associate with each other and cannot be constrained by other interactions (e.g., bound to chaperones).
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Molecular Hydrogen as an Energy Source for *Helicobacter pylori*

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The gastric pathogen *Helicobacter pylori* is known to be able to use molecular hydrogen as a respiratory substrate when grown in the laboratory. We found that hydrogen is available in the gastric mucosa of mice and that its use greatly increased the stomach colonization by *H. pylori*. Hydrogenase activity in *H. pylori* is constitutive but increased fivefold upon incubation with hydrogen. Hydrogen concentrations measured in the stomachs of live mice were found to be 10 to 50 times as high as the *H. pylori* affinity for hydrogen. A hydrogenase mutant strain is much less efficient in its colonization of mice. Therefore, hydrogen present in animals as a consequence of normal colonic flora is an energy-yielding substrate that can facilitate the maintenance of a pathogenic bacterium.

The bacterial oxidation of molecular H_2 commonly occurs in nature, as hydrogen gas released by other bacteria represents a useable high-energy reductant (*1*). Once H_2 is bound and "split" by a membrane-associated hydrogenase, further oxidation-reduction and energy-generating steps are facilitated by a series of membrane-bound heme-containing electron carriers. Hydrogen is a by-product of colonic fermentation (*2*), and hydrogen has been reported to be produced (measured as excreted gas) in the gastrointestinal tract of both rodents (*3*) and humans (*4*). However, whether molecular hydrogen is used as an energy reservoir for pathogenic bacteria residing in animals is not known. To help understand the microbial communities associated with digestion, H_2 levels were determined in the termite hind-gut (*5*) and recently from the cockroach midgut (*6*), but H_2 levels in tissues of vertebrate animal hosts has not been assessed. *Helicobacter pylori* is a pathogen that solely colonizes the mucosal surfaces of the human stomach, where it gives rise to gastritis and peptic ulcers and is correlated with the development of certain types of gastric cancer (*7*). We previously reported that lab-grown *H. pylori* can express a membrane-bound "uptake-type" hydrogenase (*8*). H_2 use by *H. pylori* was accompanied by changes to other electron-carrying cell proteins that are related to energy-producing processes within cells to carry out a myriad of cell-building functions. Here we show that the mucous lining of the stomach contains ample amounts of molecular H_2. Combined with our measurements of the binding affinity of these bacteria for H_2, we conclude that hydrogenase is saturated with H_2 in the host tissues. A mutant *H. pylori* strain unable to oxidize hydrogen is severely impaired in its ability to colonize in mice. Therefore, H_2 availability and its use as an energy source is a formerly unrecognized factor in understanding how a human pathogen grows and persists in an animal host.

One hallmark of the energy-conserving uptake hydrogenases is the ability to respond positively to exogenously supplied hydrogen (*9*, *10*). Hydrogenase activity (*11*) in *H. pylori* is constitutive under all conditions we have tested, but in a chemically defined media (*12*) amperometrically determined hydrogenase activity (*13*) increases from a baseline value of 0.7 nmol H_2 oxidized/min/10^8 cells in cultures grown under micro-aerobic conditions (12% O_2, 5% CO_2, balance N_2) to 3.1 nmol H_2 oxidized/min/10^8 cells when supplemented with 10% H_2. A much milder stimulation of hydrogenase activity occurs when the cultures are grown in rich media or on blood-containing plates (BA plates) (*13*), in which hydrogenase activity is stimulated approximately twofold by the addition of 10% hydrogen (*14*). To characterize hydrogenase regulation, we used promoter fusions with the reporter gene *xylE* (*15*) from *Pseudomonas putida* to generate catechol 2,3-dioxygenase, which can be easily assayed spectrophotometrically (*13*). We assayed XylE activity in *H. pylori* strains carrying plasmids with hydrogenase structural gene promoter-*xylE* fusion (*phyd:xylE*), a nonhydrogenase related promoter-*xylE* fusion (pHP0630:*xylE*), and a promoterless *xylE* gene (pHel:*xylE*). The results (Table 1) show that hydrogenase is regulated at the transcriptional level. The gene directly adjacent to hydrogenase (designated HP0630 and annotated as conserved; no known function in the sequenced strain 26695) (*16*) is not regulated by hydrogen,

Table 1. XylE activities [expressed as XylE units/10^8 cells (*13*)] of *H. pylori* harboring *xylE* reporter plasmids grown under different growth conditions.

Growth condition	Plasmid		
	phyd:xylE	pHP0630:*xylE*	pHel:*xylE*
− Hydrogen	1.6	9.1	<0.1
+ Hydrogen	6.6	10.4	<0.1

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and no XylE activity was seen in the strain harboring a promoterless *xylE* gene (Table 1). Hydrogenase transcription was not affected by other environmental conditions such as pH or oxygen concentration (*14*), and proper regulation of the hydrogenase operon (as measured by p*hyd:xylE*) is retained in the hydrogenase structural gene mutant Hyd:cm (*14*), indicating that hydrogenase is not self-regulated. That the enzyme expression responds to molecular hydrogen availability supports our previous proposal (*8*) that the role of hydrogenase is in respiratory hydrogen oxidation.

A whole-cell Michaelis constant (apparent K_M) for hydrogen was determined to be 1.8 μM, indicating a very high affinity for hydrogen, and a value similar to the whole-cell affinities of other hydrogen-oxidizing bacteria (*17*). The method used (*17*) to determine this K_M uses live, intact cells with O_2 available as the only terminal electron acceptor in the H_2 oxidizing respiratory chain. Therefore, our measured apparent K_M is for the entire hydrogen oxidizing system. We have previously shown that hydrogen oxidation in *H. pylori* grown in an H_2-containing atmosphere is linked to cytochrome reduction, with the heme-containing components functioning as intermediate electron carriers before reduction of the terminal (O_2-binding) oxidases (*8*).

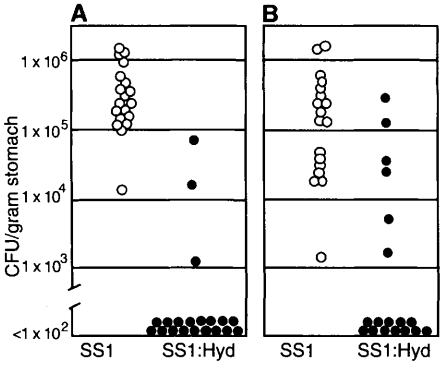

Fig. 1. Mouse colonization assay of *H. pylori* SS1 and Hyd:cm (SS1). Data are presented as a scatter plot of colony forming units per gram of stomach as determined by plate counts (*13*). Mice were considered positive if more than 1×10^3 colony-forming units (CFU) of *H. pylori* per gram of stomach were recovered. (**A**) Results from experiment 1, in which mice were inoculated two times with a dose of 2×10^8 cells. (**B**) Results from experiment 2, in which mice were inoculated two times with a dose of 1×10^9 cells. For both panels, open symbols represent SS1 inoculated mice, and closed symbols represent Hyd:cm inoculated mice. According to Student's *t* -distribution test (*21*), the parent strain results (both experiments) are significantly greater than the mutant at the 99% degree of confidence (α' equals 0.01, for a one-tailed test). This conclusion was so even if the undetectable CFU's (most of the data points for the mutant) were assumed to be 1×10^3 CFU of *H. pylori* per gram of stomach.

Hydrogenase mutants in the SS1 (mouse colonization strain) background (SS1:Hyd) are deficient in their ability to colonize in mice. From two separate mouse colonization studies, only 24% (9 of 38) of the hydrogenase mutant-inoculated mice were colonized, as compared to 100% (37 of 37) colonization when inoculated with the parent strain (Fig. 1). The colonization efficiency of the mutant strain correlates with the inoculum dosage, with only 15% colonization at a inoculation dose of 2×10^8 (Fig. 1A) but 33% colonization at a inoculation dose of 1×10^9 (Fig 1B). SS1-inoculated mice were 100% colonized (the strain was able to colonize every mouse) at both inoculation doses when we used an initial "two-dose" regimen for inoculation (*13*).

We determined the average hydrogen content of the mucus layer of the mouse stomach to be 43 μM, over 20 times as much as that of the apparent whole-cell K_M for hydrogen. This concentration represents the average of 31 measurements taken from different regions of stomachs from four live, anesthetized mice (*13*) (Table 2). These measurements were taken on different days and at different times during the day and ranged in concentrations from 17 to 93 μM, indicating that under most conditions the hydrogen oxidizing system in *H. pylori* would be saturated. It may be expected that the type of diet of the animal would affect the colonic flora fermentation responses; diet would then affect the hydrogen concentrations in tissues, but was not studied here.

A wide range of characteristics attributed to infectious bacteria are classified as virulence determinants to successfully combat inherent host protection mechanisms. However, the primary sources of energy used by infectious bacteria to sustain their growth, once they are established in an animal host, remain largely unknown (*18*). The use of molecular H_2, a high-energy, diffusible reductant produced by colonic fermentations from other host-residing bacteria, thus represents a useful tool for understanding how a human pathogen grows and persists in an animal host. Hydrogen use may play an especially important role in setting up the stable infection required for the most serious of the pathologies associated with *H. pylori* infection, gastric ulceration, and cancer. Blood- or serum-containing media is commonly used for routine (laboratory) culture of *H. pylori*, and the nature of the carbon and energy sources used in the host are unknown. *Helicobacter pylori* is very limited in its use of oxidizable carbon substrates (*19*), and the primary environment for *H. pylori* colonization is within the complex and viscous mixture of glycoproteins known as mucin. This is expected to provide little nutritional value for the pathogen. Fermentation reactions in the colon include the hydrogen-producing reactions accompanied by acetate and butyrate production by bacteria of the anaerobic large intestine (*2*). This colonic H_2 must move into other tissues, presumably by a combination of cross-epithelial diffusion (*6*) and vascular-based transport processes (*20*). Indeed, it has been estimated that 14% of all the intestinal-produced hydrogen is excreted through the breath (of humans), and the authors speculate that the hydrogen is carried to the lungs via the bloodstream (*4*). The proportion of exhaled gas as H_2 can vary considerably among individuals (*2*, *4*), so it may be possible to correlate *H. pylori* infection with inherent host H_2-production characteristics. From our studies, H_2 use must represent a large energy boost for a bacterium living in an energy-poor environment (such as gastric mucosa). H_2 is an energy substrate not used by the host, so competition for this high-energy substrate in the gastric environment is not a factor. Also, some other human pathogens contain uptake-type hydrogenases, so H_2 utilization within animal hosts may extend beyond just *H. pylori* and gastric infections.

Table 2. Hydrogen concentrations in mouse stomachs. A 50-μm size microelectrode probe was used to measure H_2 in the mucus lining area of the stomach of live (anesthetized) mice. For assay details see (*13*).

Mouse no.	H_2 range (μM)	Sites measured
1	25–93	8
2	35–88	8
3	17–29	7
4	19–77	8

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N-Linked Glycosylation in *Campylobacter jejuni* and Its Functional Transfer into *E. coli*

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N-linked protein glycosylation is the most abundant posttranslation modification of secretory proteins in eukaryotes. A wide range of functions are attributed to glycan structures covalently linked to asparagine residues within the asparagine-X-serine/threonine consensus sequence (Asn-Xaa-Ser/Thr). We found an N-linked glycosylation system in the bacterium *Campylobacter jejuni* and demonstrate that a functional N-linked glycosylation pathway could be transferred into *Escherichia coli*. Although the bacterial N-glycan differs structurally from its eukaryotic counterparts, the cloning of a universal N-linked glycosylation cassette in *E. coli* opens up the possibility of engineering permutations of recombinant glycan structures for research and industrial applications.

Glycosylation has generally been considered to be restricted to eukaryotes where the attachment of glycan structures to proteins usually occurs at an Asn-Xaa-Ser/Thr consensus (N-linked) or at Ser/Thr residues (O-linked). It is now evident that protein glycosylation is also abundant in prokaryotes (*1, 2*). N-linked protein glycosylation of S-layer proteins seems to be restricted to the archaeal domain, whereas serine-, threonine-, or tyrosine-linked (O-linked) glycosylation is predominantly found in bacteria. However, specific N-glycoproteins in bacteria have been reported (*3*).

N-linked glycosylation is the most frequent protein modification in eukaryotes. In the central step of the process that takes place at the luminal side of the endoplasmic reticulum (ER) membrane, a preassembled oligosaccharide ($Glc_3Man_9GlcNAc_2$, where Glc is glucose, Man is mannose, and GlcNAc is *N*-acetylglucosamine) is transferred from the lipid carrier dolichyl pyrophosphate to asparagine residues of nascent polypeptide chains by the oligosaccharyltransferase (*4*). Such general glycosylation systems have not been described in prokaryotes, with the exception of the enteropathogenic bacterium *Campylobacter jejuni*, where the *pgl* gene cluster (Fig. 1A) seems to encode a general protein glycosylation system (*5, 6*). A number of *pgl* genes encode proteins with sequence similarity to glycosyltransferases and to enzymes required for sugar biosynthesis, a fact that supports this hypothesis. The *pglB* gene encodes a protein with strong similarity to Stt3p found exclusively in eukaryotes and in archaea but not in any other bacterial species (Fig. 1B). Genetic and biochemical studies in yeast have demonstrated that the Stt3 protein is an essential component of the oligosaccharyltransferase complex (*7–9*), the central enzyme in the process of N-linked protein glycosylation. In eukaryotes, the oligosaccharyltransferase complex consists of at least eight different subunits (*8*), yet, the precise catalytic mechanism of this enzyme is unknown (*10–12*).

To analyze the *pgl* glycosylation system, we constructed a *C. jejuni pglB* mutant strain and probed the membrane protein extract with a polyclonal antiserum raised against *C. jejuni* whole-cell extracts (Fig. 2A) (*13*). The reactivity of this antiserum is markedly reduced by prior treatment of *C. jejuni* proteins with a deglycosylating agent, indicating that the glycan portion of *C. jejuni* glycoproteins is highly immunogenic (*5*). Mutation of the *pglB* gene also resulted in changes to the immunoreactivity of *C. jejuni* membrane proteins (Fig. 2A) suggesting that PglB was involved in the biosynthesis of these putative glycoproteins. This mutant phenotype was partially complemented by introducing the 16-kilobase *pgl* locus on a plasmid. We attributed the partial complementation of the *pglB* mutant phenotype to the instability of the large plasmid in *C. jejuni*. Our experiments confirmed a direct involvement of PglB in the generation of glycoproteins in *C. jejuni* (*5*). One of the immunoreactive proteins (arrow in Fig. 2A) was purified from the *C. jejuni* extract and identified by matrix-assisted laser desorption/ionization (MALDI) mass mapping as the periplasmic AcrA (Cj0367c). An *acrA* deletion mutant of *C. jejuni* was analyzed by immunoblot analysis using the glycoprotein-specific antiserum. The 47-kD immunoreactive protein was absent in *acrA* mutant cells, and this phenotype was complemented by the expression of plasmid-borne *acrA* gene (Fig. 2A). Antiserum raised against recombinant *C. jejuni* AcrA produced in *Escherichia coli* revealed mature protein migrating at an apparent molecular size of 47 kD (Fig. 2B). In contrast to the glycoprotein-specific serum (Fig. 2A), an unglycosylated AcrA with increased mobility was recognized by the AcrA-specific antibodies in a *pglB* mutant strain (Fig. 2B). Partial complementation of the *pglB* phenotype was achieved with the *pgl* locus, as visualized by three distinct AcrA-specific bands. Thus, mature AcrA carries two oligosaccharide modifications, and partial complementation of the *pglB* mutation resulted in diglycosylated, monoglycosylated, and nonglycosylated AcrA protein. Only the two former forms were recognized by the glycosylation-specific serum. Therefore, AcrA was a target for the general glycosylation system and glycosylation required the *pgl* gene cluster, in particular PglB activity.

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