

Host Hydrogen Rather than That Produced by the Pathogen Is Important for *Salmonella enterica* Serovar Typhimurium Virulence

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Salmonella enterica serovar Typhimurium utilizes molecular hydrogen as a substrate in various respiratory pathways, via H₂-uptake enzymes termed Hya, Hyb, and Hyd. A different hydrogenase, the hydrogen-evolving Hyc enzyme, removes excess reductant during fermentative growth. Virulence phenotypes conferred by mutations in *hyc* genes, either alone or in combination with mutations in the H₂-uptake enzyme genes, are addressed. Anaerobically grown $\Delta hycB$ or $\Delta hycC$ single-deletion strains were more sensitive to acid than the wild-type strain, but the Δhyc strains were like the virulent parent strain with respect to both mouse morbidity and mortality and in organ burden numbers. Even fecal-recovery numbers for both mutant strains at several time points prior to the animals succumbing to salmonellosis were like those seen with the parent. Neither hydrogen uptake nor evolution of the gas was detected in a hydrogenase quadruple-mutant strain containing deletions in the *hya*, *hyb*, *hyd*, and *hyc* genes. As previously described, a strain lacking all H₂-uptake ability was severely attenuated in its virulence characteristics, and the quadruple-mutant strain had the same (greatly attenuated) phenotype. While H₂ levels were greatly reduced in ceca of mice treated with antibiotics, both the $\Delta hycB$ and $\Delta hycC$ strains were still like the parent in their ability to cause typhoid salmonellosis. It seems that the level of H₂ produced by the pathogen (through formate hydrogen lyase [FHL] and Hyc) is insignificant in terms of providing respiratory reductant to facilitate either organ colonization or contributions to gut growth leading to pathogenesis.

The enteric bacterium *Salmonella enterica* serovar Typhimurium, like many other enteric bacteria, including *Escherichia coli*, contains genes required for both hydrogen (H₂) production and H₂ oxidation. Both *E. coli* and *S. Typhimurium* possess the H₂-oxidizing respiratory hydrogenases Hya and Hyb, also referred to as Hyd-1 and Hyd-2 (1). *S. Typhimurium* (but not *E. coli*) also possesses a third H₂-oxidizing hydrogenase, termed Hyd or Hyd-5, that is coupled to aerobic respiration (2–7). The multi-subunit H₂-evolving hydrogenase is termed Hyc and is associated with the formate hydrogen lyase (FHL) system (8–10). Yet another (potential) hydrogenase termed Hyf may contribute to a second formate hydrogen lyase system (FHL-2) uniquely in *E. coli* (11, 12). Sequence homology searches using the National Center for Biotechnology Information (NCBI) (ncbi.nlm.nih.gov) and J. Craig Venter Institute (JCVI) Comprehensive Microbial Resource (CMR) (cmr.jcvi.org) BLAST tools and the BioCyC (biocyc.org) cross-species comparisons reveal no orthologs of the *E. coli* *hyf* genes in *S. Typhimurium*.

During anaerobic growth with glycolytic carbon sources, *E. coli* carries out mixed-acid fermentation resulting in the conversion of one-third of this carbon to strongly acidic formic acid. The endogenously produced formate is further metabolized to H₂ and CO₂ by the combined activity of the Hyc hydrogenase and the FDH-H enzymes (6, 10, 13). This FHL system (including H₂ evolution via Hyc) thus functions in relieving cells of the toxic effects of acid accumulation (14). In *S. Typhimurium*, a portion of the fermentatively produced H₂ can be recycled primarily via the respiratory hydrogenase termed Hya (12). The physiological connection of Hya to the acid-combating FHL was discovered by studying an *S. Typhimurium* *hya* deletion strain; its phenotype supports the idea that Hya-mediated H₂ oxidation also contributes to the acid resistance of *S. Typhimurium* (15).

Uptake hydrogenases are important enzymes enabling H₂ respiration by many bacteria, and they are also involved in conferring

virulence to some H₂-utilizing pathogens (16). These NiFe hydrogenases have been shown or predicted to enable a number of pathogens to use molecular H₂ within the host as a respiratory energy-yielding substrate (16–24). Previous studies in our laboratory have shown that each of the membrane-associated uptake-type respiratory hydrogenases contributes to the survival and growth of *S. Typhimurium* within the host. As expected, triple mutants (lacking the three respiratory enzymes Hya, Hyb, and Hyd) are deficient in colonizing mice, as shown in our laboratory and in those of others (16, 25). This finding is supported by a very recent study on *Salmonella* competition for H₂ within limited-microbiota animals, in which the triple-H₂-uptake hydrogenase mutant was present at 100-fold-lower numbers than the parent in the stool following intragastric inoculation (26).

In particular, the Hyb enzyme permits H₂-dependent growth of *S. Typhimurium* under nutrient-limited conditions (24) and aids transport processes via generation of a proton motive force. An unbiased competitive-infection-deficiency assay wherein a set

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype/description ^a	Reference
Strains		
<i>S. Typhimurium</i> JSG210	ATCC 14028s (WT)	12
<i>S. Typhimurium</i> ALZ43	JSG210 Δ <i>hyb</i> ::FRT Δ <i>hyd</i> ::FRT Δ <i>hya</i> ::FRT (triple mutant)	12
<i>S. Typhimurium</i> RLK5	JSG210 Δ <i>hycB</i> ::FRT (Δ <i>hycB</i>)	This study
<i>S. Typhimurium</i> RLK7	JSG210 Δ <i>hycC</i> ::FRT (Δ <i>hycC</i>)	This study
<i>S. Typhimurium</i> RLK9	ALZ43 Δ <i>hycC</i> ::FRT (quadruple mutant)	This study
Plasmids		
pCP20	Contains flippase gene for λ Red mutagenesis; Amp ^r	30
pKD46	Contains λ Red genes γ , β , and <i>exo</i> ; Amp ^r	30
pKD4	Contains <i>aphA3</i> cassette; Kan ^r	30

^a Amp, ampicillin; FRT, flippase recombinase recognition target; Kan, kanamycin; WT, wild type.

of 500 individual transposon insertion strains were screened in mice revealed that the Hyb hydrogenase significantly aids *S. Typhimurium* growth in early stages of host infection (27). Mice lacking commensal microbiota lacked cecal H₂ production, and the Hyb-hydrogenase mutant strain was then like the parent with respect to gut luminal colonization. Interestingly, the purposeful colonization of mice containing low microbiota levels with a H₂ consumer resulted in inhibition of Hyb-dependent growth of *S. Typhimurium* in that host (27). Still, H₂-dependent pathogenesis of *S. Typhimurium* is likely not limited to H₂ providing a competitive edge in the gut, as H₂ levels far exceeding those corresponding to the affinity of *Salmonella* hydrogenases for H₂ (16) were measured in other areas colonized by the pathogen (28). Also, each H₂-utilizing hydrogenase is capable of coupling H₂ oxidation to O₂ reduction (16), and aerobic respiration is important for systemic virulence (25).

The importance of H₂ in support of early stages of *S. Typhimurium* gut colonization means that any source of H₂ would aid the pathogen's survival. One such source is the FHL system. Also, other *in vivo* roles of Hyc, such as acid resistance and excess electron disposal, have never been addressed. To address these issues, we assessed the effects of mutations in the Hyc H₂-evolving hydrogenase on the virulence capabilities of *S. Typhimurium*. There is no doubt that the amount of hydrogen available in animals is determined by the interplay between H₂-producing fermentative bacteria and those that are the H₂ consumers (29). Still, for a bacterium that can do both and can establish itself in a host niche primarily free of other microorganisms, the contribution of the H₂-evolving system for subsequent respiratory system-based H₂ utilization was not known. Our results suggest that during *in vivo* growth and pathogenesis of *S. Typhimurium*, the H₂ produced by the Hyc and the FHL system is far less important than the H₂ originating from the H₂-producing gut community. Even an artificial reduction in animal H₂ levels via prior antibiotic treatment does not reveal a role for H₂ evolution in *S. Typhimurium* metabolism *in vivo*.

MATERIALS AND METHODS

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 agar (LBA) plates, and LBA plates with appropriate antibiotics (100 μ g ampicillin ml⁻¹ and 25 μ g kanamycin ml⁻¹) were used when necessary. CR-Hyd medium (3, 31) and LB medium, both supplemented with glucose (0.4%) and sodium formate (20 mM), were used where indicated. Cells were grown at 37°C anaerobically with or without H₂. Anaerobic conditions with H₂ were established by sparging sealed 165-ml bottles with N₂ for 15 min and then with anaerobic mix (10% H₂, 5% CO₂, and 85% N₂) for 20 min; 10% H₂ was then injected to bring the volume of added H₂ to 20% partial pressure. Cells were grown anaerobically without H₂ in 165-ml bottles by sparging with N₂ for 15 min and then injecting the sealed bottles containing cells with CO₂ to 5% partial pressure (24). Acidity resistance experiments were done in LBK medium (LB with 7.5 g KCl liter⁻¹) supplemented with 0.4% glucose, 50 mM sodium formate, or 50 mM MOPS [3-(N-morpholino) propanesulfonic acid] when indicated. The pH of LBK-glucose-formate medium was adjusted to 5.5, and the pH of LBK-glucose-MOPS was adjusted to 7.0. The wild-type, RLK5, and RLK7 strains were inoculated at a dilution of 1:100 (starting concentration, about 10⁷ cells ml⁻¹), cells were grown anaerobically (no headspace, no shaking) for 20 h at 37°C and serially diluted in phosphate-buffered saline (PBS) (pH 7), and 0.1 ml was plated on LBK plates. CFU were counted the next day. Results shown (CFU ml⁻¹) are means and standard deviations of results from three to four independent growth experiments.

Mutant strain construction. Hyc is another term for Hyd-3 of *E. coli*. Strains with single deletions in *hycB* (subunit 2 or iron sulfur subunit; STM2852) and, separately, *hycC* (subunit 3 or membrane subunit; STM2851) were constructed using the lambda Red system as previously described (12, 30). The *hycB*- and *hycC*-deleted mutants were named strain RLK5 and strain RLK7, respectively. The deletions made using this system are nonpolar, and the mutant strains do not contain any antibiotic resistance markers. In addition to the single-deletion strains, a hydrogenase quadruple mutant (RLK9) with deletions of the *hya*, *hyb*, *hyd*, and *hyc* genes was also constructed. For RLK9 construction, the ALZ43 hydrogenase triple mutant (12) (Table 1) was used as the background strain and *hycC* (STM2851) was deleted using the lambda Red system. The deletions were confirmed by PCR using primers complementary to the regions flanking the deleted genes and by sequencing across the deletions (Georgia Genomics Facility, University of Georgia, Athens, GA). The *hycC* and *hycB* mutants showed growth characteristics (growth rates and endpoint growth yields measured as total CFU per ml after 18 h of incubation at 37°C) similar to those of the wild type in both CR-Hyd and LB media (data not shown). The strains are listed in Table 1, and the primers used are listed in Table 2.

Amperometric hydrogenase assays. The hydrogenase activities of the hydrogenase triple- and quadruple-mutant strains (ALZ43 and RLK9, respectively) were compared amperometrically as described previously (12). The strains were grown overnight under anaerobic conditions in bottles containing LB supplemented with glucose (0.4%) and sodium formate (20 mM). Replicates of 2.5-ml samples at cell concentrations of 2 \times 10⁸ CFU per ml were withdrawn from the cultures and assayed for hydrogen evolution. The samples were injected into the sealed and stirred dual-electrode chamber (32), and the evolution of hydrogen over time was recorded.

Mouse experiments. The ability of the mutant strains to cause infection in mice was assessed by using the typhoid fever-mouse model (33). Female BALB/c mice (obtained from the National Cancer Institute, Frederick, MD) were orally inoculated individually with cell suspensions of strains RLK5 and RLK7 and the wild-type strain, following methods described previously (16). Cells were washed and suspended in sterile PBS, and 0.1-ml volumes of the cell suspension containing 10⁶ bacterial cells were introduced orally into each mouse. The mice were observed twice daily, and morbidity was recorded. Postinoculation organ bacterial burdens were determined by euthanizing the mice 4 days (96 h) after inoculation. Immediately after euthanization, the livers and spleens were removed and homogenized in sterile PBS. Dilutions of the homogenate

TABLE 2 Primers used in this study

Primer	Primer sequence (5' → 3')	Application
<i>hycB</i> del-F	CAAAAATGACAATCACCTGAGGAATGCCTGTGTGTAGGCTGGAGCTGCTTC	<i>hycB</i> deletion
<i>hycB</i> del-R	GACGCCGCTAGTAATCAGTGAAAGTGAATCATATGAATATCCTCCTTA	<i>hycB</i> deletion
<i>hycB</i> -check-F	ACGTTGAAACCAAAGATGGCGA	<i>hycB</i> deletion confirmation
<i>hycB</i> -check-R	AATTTGCAGCATGTGGCCGGTAAA	<i>hycB</i> deletion confirmation
<i>hycC</i> del-F	CTTGTTTCAGCAGGCTCAGAGTGGGGATGCATATGTGTAGGCTGGAGCTGCTTC	<i>hycC</i> deletion
<i>hycC</i> del-R	GCGCTGAATTAACGGATAAAACACACTCATTTTCATATGAATATCCTCCTTA	<i>hycC</i> deletion
<i>hycC</i> -check-F	GTGAGCTGACGTTTAATACCGA	<i>hycC</i> deletion confirmation
<i>hycC</i> -check-R	CGACCGAGCAGTTTGATAATGT	<i>hycC</i> deletion confirmation

were plated on bismuth sulfite agar (BSA; Difco/Becton Dickinson), a selective medium for *Salmonella* species. Colonies were counted after overnight incubation of the plates at 37°C. Two mice inoculated with sterile PBS were included as negative controls in each experiment. For fecal-count comparisons, the BALB/c mice were inoculated with 1×10^7 cells by oral gavage, and fresh fecal pellets were obtained from each mouse at 1.5, 2.5, and 3.0 days later. Two to three fecal pellets were weighed and placed into a Dounce hand-held glass homogenizer, and 2 ml of PBS (containing 0.5% [wt/vol] saponin) was added. The saponin inclusion was verified to not affect *S. Typhimurium* (strain JSG210) viability. After 10 min of incubation at room temperature, the feces samples were suspended, and 0.1 ml of the suspension, as well as 0.1 ml from a 1:10 (PBS) dilution, was plated. *Salmonella* CFU numbers were scored on Brilliant Green agar plates.

For the antibiotic treatment experiment, a total of 14 mice were given (by oral gavage) bacitracin, neomycin, and streptomycin (200 µg of each antibiotic per g of body weight) each morning for 3 days. This regimen was described by Wichmann et al. as greatly decreasing short-chain fatty-acid levels produced by gut commensals in mice (34). On the fourth day, two antibiotic-treated mice as well as two nontreated mice were euthanized and measurements of cecal hydrogen were done as previously described (27). Excised ceca were immobilized and penetrated with a needle as described previously (13), and H₂ levels were determined via amperometric probe measurements at the cecal tip, the midsection, and the cecal connection to the intestinal tract. All values were corrected for H₂S signals as described previously (17, 27). The 12 remaining antibiotic-treated mice were orally inoculated individually with cell suspensions of strain RLK5, strain RLK7, or the wild-type strain ($n = 4$ for each). Mouse morbidity was monitored as described above. IACUC approval was obtained for all animal procedures.

RESULTS AND DISCUSSION

Mutant strains. Based on the literature and available gene annotation databases, the *hyc* operons of both *S. Typhimurium* and *E. coli* consist of nine genes: *hycABCDEFGHI*. Amino acid sequence alignments using the homology search tools mentioned above showed that the *S. enterica* serovar Typhimurium Hyc subunits share 80% to 96% sequence similarity with the Hyc subunits of *E. coli*. HycA is thought to play a regulatory role for the *hyc* operon (for a review, see reference 35), HycB is probably a membrane anchor protein, HycC and HycD are transmembrane proteins, and HycE is the Ni-containing large subunit of Hyc-hydrogenase, while HycG is the membrane-associated small subunit of the enzyme. HycF is probably an electron carrier, and HycI is a specific endopeptidase involved in the maturation of the FHL complex. The function of HycH, though required for the activity of the FHL complex, is unknown. As both HycB and HycC are required for the activity of the FHL complex (8, 9), we hypothesized that deletion of either *hycB* or *hycC* would result in strains incapable of endogenous H₂ evolution. Nonpolar mutations in *hycB* and *hycC* were made here.

Hydrogenase activity. Conclusions on the nature of the roles of H₂ derived from *in vivo* results depend on the availability of strains lacking H₂-evolving ability. Some of the H₂-uptake enzymes are active even when *S. Typhimurium* is producing H₂ (12). To associate loss of H₂ evolution ability with *hyc* deletion, the background strain had to be lacking all H₂-utilizing ability. We thus used a background strain, ALZ43, that lacks all three respiratory hydrogenases (Hya, Hyb, and Hyd) (12) to construct a hydrogenase quadruple mutant (RLK9). The hydrogenase activity of RLK9 was compared with that of ALZ43. Strain RLK9 neither evolved nor took up H₂ (<0.1 nmol/min/10⁹ cells, the detection limit), whereas the ALZ43 triple-mutant strain evolved H₂ at the rate of 10.3 nmol/min/10⁹ cells. Therefore, deletion of a single gene (*hycC*) of the *hyc* operon abolished the H₂-evolving ability of the Hyc hydrogenase. The corresponding quadruple mutant containing a Δ *hycB* deletion was not made.

Sensitivity to acid. Since *S. Typhimurium* Hyc enzymes are hypothesized to remove excess reductant generated during fermentative growth by working with formate dehydrogenase H, as part of the FHL system, *hyc* mutants were expected to be more (formic) acid sensitive than the wild-type strain. Therefore, the wild-type (JSG210), RLK5 (*hycB*), and RLK7 (*hycC*) strains were grown for 20 h under anaerobic conditions at pH 5.5 or pH 7.0 before the final cell yield was assessed (Fig. 1). Both *hyc* mutants were significantly more affected than the wild-type strain when cells were grown in the presence of 50 mM formate at pH 5.5 (Fig. 1). Cell growth levels and final yields at pH 7 were similar in all three strains (Fig. 1). These results confirm the importance of the *S. Typhimurium* Hyc hydrogenase in anaerobic acid resistance.

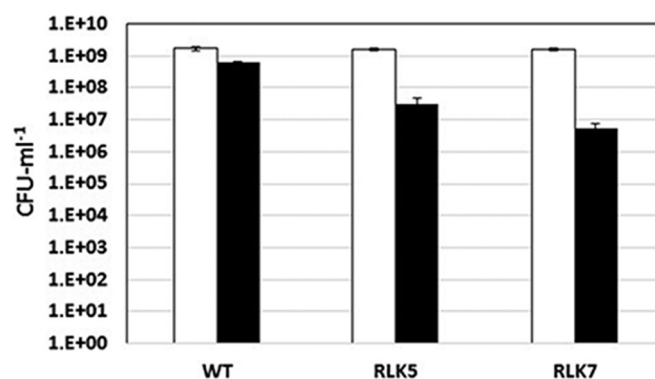


FIG 1 Acid sensitivity of *S. enterica* serovar Typhimurium strains JSG210 (wild type [WT]), RLK5 (Δ *hycB*), and RLK7 (Δ *hycC*). Cells were grown for 20 h in LBK medium supplemented with glucose and either MOPS (pH 7; black bars) or formate (pH 5.5; white bars), diluted, and spread.

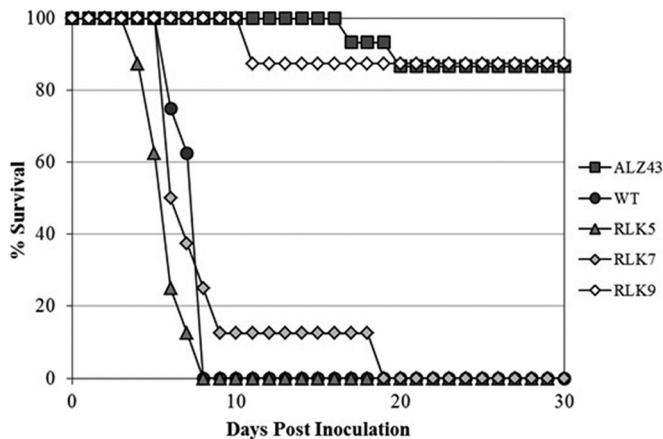


FIG 2 Virulence of *S. enterica* serovar Typhimurium strains JSG210 (WT), RLK5 ($\Delta hycB$), RLK7 ($\Delta hycC$), RLK9 (quadruple mutant), and ALZ43 (triple mutant) on BALB/c mice. The results shown are for 15 mice infected as described for the ALZ43 strain and 8 mice each as described for the WT, RLK5, RLK7, and RLK9 strains. Another experiment yielded a similar result.

Virulence. *S. Typhimurium* is highly virulent in mice, causing typhoid fever-like disease and death within a few days of inoculation. The JSG210 wild-type strain caused infection and death of all mice within 10 days of oral inoculation (Fig. 2). Two mice of 15 infected with the less-virulent triple-mutant strain (ALZ43) became nearly immobile and were euthanized, one on day 17 post-inoculation and the other on day 19 postinoculation. The remaining mice survived until the termination of the experiment on day 30. These findings are reasonably consistent with the observations in our previous *S. Typhimurium* virulence study (16); although some mice inoculated with the triple mutant looked ill in that previous study, no animals became immobile and so none were euthanized. The mice inoculated with RLK5 and RLK7 showed a survival pattern similar to that seen with the mice inoculated with the wild-type strain (Fig. 2). Similar results were obtained from experiments repeated at later times that compared the levels of survival of mice infected with RLK5, RLK7, and the wild type (data not shown).

We also compared the virulence level of the hydrogenase quadruple mutant (RLK9, lacking all four hydrogenases) to those of the wild-type strain and the ALZ43 triple mutant (lacking three uptake-type hydrogenases but containing only the Hyc hydrogenase) as the positive and negative controls, respectively. The quadruple-deletion strain showed virulence comparable to that of the ALZ43 triple-mutant strain; of the 8 mice injected with the RLK9 strain, 1 died within 30 days of inoculation (at day 11; see Fig. 2). This indicated that *S. Typhimurium* requires the uptake-type respiratory hydrogenases but not the H₂-evolving hydrogenase for full virulence.

Organ colonization. The numbers of *Salmonella* bacteria associated with liver and spleen of infected mice were determined 96 h after oral inoculation. These were determined from four individual mice per strain, as previously described (16). Two mice injected with sterile PBS were included in each experiment as negative controls. The mouse colonization numbers of *S. Typhimurium* for the wild-type strain and the *hyc* mutants RLK5 and RLK7 ranged from 0.8×10^6 to 2.8×10^6 in the livers and from 1.0×10^6 to 2.9×10^6 in the spleens (Table 3). No viable bacteria were recovered from the organs of the mice inoculated with PBS.

TABLE 3 Organ colonization numbers of *S. enterica* serovar Typhimurium strains JSG210, RLK5, and RLK7 in the livers and spleens of infected mice^a

Strain	CFU/liver ($\times 10^6$ cells)	CFU/spleen ($\times 10^6$ cells)
WT	1.2–2.7	1.5–2.3
RLK5	1.1–3.4	1.4–3.7
RLK7	0.8–1.7	1.0–2.9

^a Numbers indicate ranges of CFU per organ among four mice inoculated with each strain ($P > 0.01$, $n = 4$). WT, strain JSG210.

Recovery in feces. Hyc and FHL are most often associated with anaerobic metabolism, when the need for eliminating reducing equivalents is great. Due to the anaerobic nature of the intestinal tract, it seemed plausible that strains RLK5 and RLK7 would be deficient in fecal recovery numbers compared to the parent strain. However, at all three times tested (days 1.5, 2.5, and 3.0 postinoculation), there were no detectable differences (data for day 2.5 are shown in Fig. 3) among the three strains. The day 1.5 and day 3.0 experiments were repeated with similar results (data not shown). It seems that the Hyc enzymes do not have a large effect on *Salmonella* survival in this host.

Microbiota-produced hydrogen. Most hydrogen present in the animals is believed to come from the breakdown of saccharides by the commensal intestinal microbiota (36). It was possible the Hya, Hyb, and Hyd enzymes are always saturated with this “host H₂” *in vivo*, such that the H₂ produced by Hyc is insignificant for the pathogen’s metabolism. Therefore, we treated mice with antibiotics to reduce the commensal microflora, a procedure known to markedly decrease production of short-chain fatty acids (34), which are commensal-produced fermentation products often-times associated with H₂ production. Antibiotic-treated mice indeed contained far less H₂ in their ceca than non-antibiotic-treated animals: H₂ levels in ceca of mice given normal diets and without antibiotic treatment were $61 \pm 14 \mu\text{M}$ ($n = 12$), whereas H₂ levels in ceca of antibiotic-treated animals were all measured to be below $5 \mu\text{M}$ ($n = 8$). Still, the pathogenesis of the two *hyc* strains was like that of the parent strain on the low-H₂ animals. Of the 4 mice inoculated with the wild-type strain, the 4 mice inoculated with the $\Delta hycB$ strain, and the 4 mice inoculated with the $\Delta hycC$ strain, all 12 succumbed to salmonellosis between days 6 and 8.

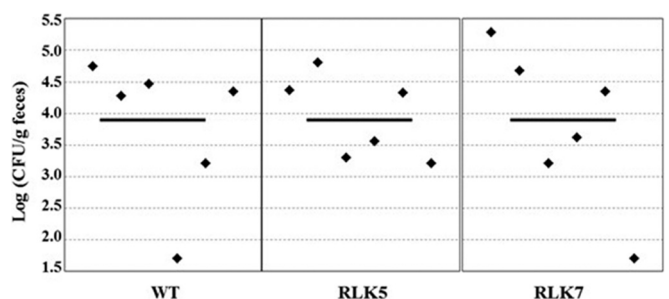


FIG 3 Recovery of *S. enterica* serovar Typhimurium from feces of mice infected with strains JSG210 (WT), RLK5 ($\Delta hycB$), and RLK7 ($\Delta hycC$). Fresh fecal pellets were obtained from each mouse ($n = 6$ per strain) at 2.5 days after inoculation. Fecal pellets were homogenized, diluted, and plated on Brilliant Green agar plates, and CFU were counted. Each point represents the CFU count from the feces of one mouse, and the solid lines represent the geometric means of the colonization numbers for each group.

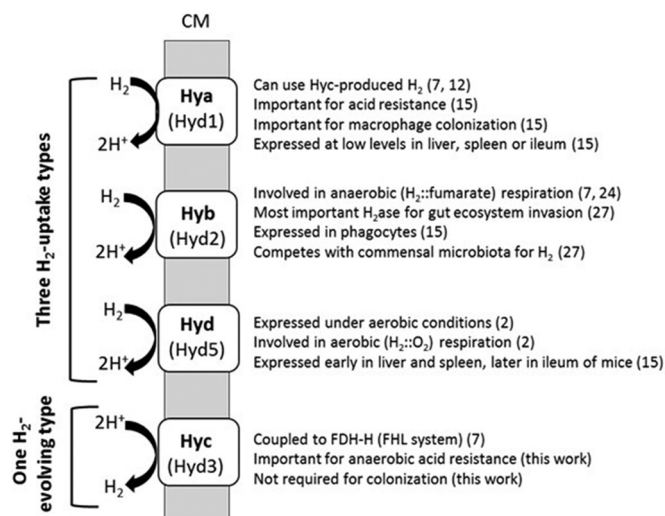


FIG 4 Putative and known roles of the four hydrogenases in *S. enterica* serovar Typhimurium. Numbers in parentheses represent reference citations.

Conclusion. Our study showed the importance of *S. Typhimurium* Hyc hydrogenase for anaerobic acid resistance *in vitro*. There was no significant difference in the mouse-colonizing abilities or organ burdens of the two *hyc* mutants and the wild type. The mouse mortality experiments showed that the hydrogenase triple and quadruple mutants are clearly less virulent than the wild type and are similar in their morbidity effects. As a facultative anaerobe, *S. Typhimurium* can utilize both aerobic and anaerobic metabolic pathways, depending on the physiological environment, and it has been suggested that during infection of the host, the bacteria have mechanisms to grow and survive under unfavorable growth conditions within the host, a very important one being their ability to utilize molecular H₂ produced within the host (24, 28).

While *S. Typhimurium* *hyb* and *hya* genes were expressed *in vivo* in infected mice (15, 37) and in the bloodstream of bacteremic *S. enterica* serovar Typhi-infected humans (23), respectively, *hyc*-related genes were not described as expressed in either study. While it is possible that the FHL system might enable the bacteria to survive and grow under fermentative conditions within the host intestinal tract, this idea was not supported by the recovery numbers from fresh feces (this study). Recent results from Craig and coworkers (25) highlight the importance of aerobic respiration for host colonization; this would explain why a strictly anaerobic process such as the FHL/H₂ evolution is not important *in vivo*. FHL and associated H₂ production could be useful in other environments or within other hosts, as the metabolic flexibility of *Salmonella* is becoming more appreciated (37–40). Our results suggest that evolution of H₂ by the activity of Hyc is not a requirement for either the virulence or the gut survival or fecal transmission of *S. Typhimurium*. Host microbiota-produced H₂ is an important component of *S. Typhimurium* gut colonization as an early step toward more-robust infection (27). The results presented here support the idea that H₂ respiration to aid pathogen colonization, including organ colonization and morbidity, must largely originate in H₂-evolving anaerobes residing among the members of the commensal intestinal community. Current knowledge on the respective roles of all four *S. Typhimurium* hydrogenases is summarized in Fig. 4.

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