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REVIEW

# Molecular Hydrogen Metabolism: a Widespread Trait of Pathogenic Bacteria and Protists

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**SUMMARY** ..... 1
**INTRODUCTION** ..... 2
**OVERVIEW OF $H_2$ METABOLISM AND HYDROGENASES** ..... 3
Biochemistry: Two Distinct Hydrogenases in Pathogens ..... 3
Physiology: Integration and Regulation of $H_2$ Metabolism in Pathogens ..... 5
Ecology: Subversion of Gastrointestinal Microbiota ..... 8
Distribution: $H_2$ Metabolism as a Widespread Trait in Pathogens ..... 10
**$H_2$ CONSUMPTION IN PATHOGENS** ..... 16
*Campylobacterales* ..... 16
*Helicobacter pylori*: $H_2$-dependent PMF generation in the gastric mucosa ..... 16
*Campylobacter jejuni*: niche expansion through hydrogenotrophic aerobic and anaerobic respiration ..... 21
*Campylobacter concisus*: essentiality of uptake hydrogenases for growth ..... 22
Other *Campylobacterales* ..... 23
*Enterobacteriales* ..... 24
*Escherichia coli*: insights from a metabolically flexible model organism ..... 24
*Salmonella* Typhimurium: differential roles of hydrogenases during infection ..... 25
*Shigella flexneri*: conditional essentiality of an uptake hydrogenase ..... 27
Other *Enterobacteriales* ..... 28
Other Bacteria ..... 28
**$H_2$ PRODUCTION IN PATHOGENS** ..... 29
*Bacteria* ..... 29
*Escherichia coli* and *Salmonella* Typhimurium: formate-dependent $H_2$ production by [NiFe]-hydrogenases ..... 29
*Clostridium perfringens* and *Clostridioides difficile*: obligate fermenters with multiple [FeFe]-hydrogenases ..... 32
Other $H_2$-producing bacteria ..... 33
*Eukarya* ..... 34
*Trichomonas vaginalis*: fermentation within hydrogenosome organelles ..... 34
*Giardia intestinalis* and *Entamoeba histolytica*: evidence for $H_2$ production in parasites lacking hydrogenosomes ..... 35
*Acanthamoeba castellanii* and *Naegleria fowleri*: flexibility dependent on respiration versus fermentation ..... 36
**POTENTIAL OF $H_2$ METABOLISM AS A THERAPEUTIC TARGET SPACE** ..... 36
Promises and Challenges of Inhibitor Development ..... 36
Strategies for Inhibitor Development ..... 37
Other Intervention Strategies ..... 38
**CONCLUSIONS** ..... 39
**ACKNOWLEDGMENTS** ..... 39
**REFERENCES** ..... 39

**SUMMARY** Pathogenic microorganisms use various mechanisms to conserve energy in host tissues and environmental reservoirs. One widespread but often overlooked means of energy conservation is through the consumption or production of molecular hydrogen ($H_2$). Here, we comprehensively review the distribution, biochemistry, and physiology of $H_2$ metabolism in pathogens. Over 200 pathogens and pathobionts

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carry genes for hydrogenases, the enzymes responsible for $H_2$ oxidation and/or production. Furthermore, at least 46 of these species have been experimentally shown to consume or produce $H_2$. Several major human pathogens use the large amounts of $H_2$ produced by colonic microbiota as an energy source for aerobic or anaerobic respiration. This process has been shown to be critical for growth and virulence of the gastrointestinal bacteria *Salmonella enterica* serovar Typhimurium, *Campylobacter jejuni*, *Campylobacter concisus*, and *Helicobacter pylori* (including carcinogenic strains). $H_2$ oxidation is generally a facultative trait controlled by central regulators in response to energy and oxidant availability. Other bacterial and protist pathogens produce $H_2$ as a diffusible end product of fermentation processes. These include facultative anaerobes such as *Escherichia coli*, *S.* Typhimurium, and *Giardia intestinalis*, which persist by fermentation when limited for respiratory electron acceptors, as well as obligate anaerobes, such as *Clostridium perfringens*, *Clostridioides difficile*, and *Trichomonas vaginalis*, that produce large amounts of $H_2$ during growth. Overall, there is a rich literature on hydrogenases in growth, survival, and virulence in some pathogens. However, we lack a detailed understanding of $H_2$ metabolism in most pathogens, especially obligately anaerobic bacteria, as well as a holistic understanding of gastrointestinal $H_2$ transactions overall. Based on these findings, we also evaluate $H_2$ metabolism as a possible target for drug development or other therapies.

**KEYWORDS** *Campylobacter*, *Clostridium*, *Helicobacter*, *Mycobacterium*, *Salmonella*, *Trichomonas*, bioenergetics, hydrogenase, pathogens, protists

## INTRODUCTION

Pathogens can conserve energy through a wide range of mechanisms. Many conserve energy primarily through respiration, in which electron transfer generates a proton-motive force (PMF), enabling ATP synthesis through oxidative phosphorylation. A variety of electron donors (e.g., NADH, succinate, formate, and $H_2$) and electron acceptors (e.g., $O_2$, fumarate, nitrate, and nitrite) have been shown to support their respiration (reviewed in reference 1). Other pathogens primarily adopt a fermentative lifestyle, in which organic carbon is incompletely oxidized and ATP is synthesized by substrate-level phosphorylation. This results in the production of both organic and inorganic end products (e.g., acetate, butyrate, $H_2$, and $CO_2$; reviewed in reference 2). Studying pathogen bioenergetics is critical for resolving how pathogens grow and survive in host tissues and other reservoirs. Hence, bioenergetics has emerged as a promising target space, and new antibiotics targeting this particular field have even been approved recently (1, 3). $H_2$ metabolism by pathogens is a particularly important, but relatively underexplored, area.

$H_2$ is a desirable respiratory energy source for pathogens for two reasons. First, reflecting its highly negative standard redox potential (−414 mV) (2), its oxidation is highly exergonic and can be coupled to the reduction of all major physiological electron acceptors (for a review, see reference 4). Second, this diffusible gas is abundant and accessible in host tissues as a result of fermentative $H_2$ production by colonic microbiota (5–7). $H_2$ is present in concentrations of ~168 $\mu$M in the small intestine and ~43 $\mu$M in the stomach of mice (8, 9), with similar levels predicted in humans (5). This amount far exceeds the apparent affinities of most pathogens for $H_2$ (apparent $K_m$, 1.8 to 2.5 $\mu$M) (8–10); hence, pathogens are thought to be saturated with $H_2$ within host tissues (11). Genetic studies have shown that the virulence of several major human pathogens depends on $H_2$ oxidation, notably *Helicobacter pylori* (9), *Campylobacter jejuni* (12), and *Salmonella enterica* serovar Typhimurium (8, 13). These organisms use specialized enzymes called hydrogenases to cleave $H_2$ heterolytically into electrons and protons; the derived protons contribute to PMF generation, whereas the electrons enter aerobic or anaerobic respiratory chains. While these bacteria primarily assimilate carbon heterotrophically (1), their ability to release energy through $H_2$ oxidation gives them a critical competitive advantage during colonization of the gastrointestinal tract (9, 13). Moreover, we hypothesize that the flexibility conferred by $H_2$ metabolism

facilitates pathogen persistence within different host tissues and environmental reservoirs.

Many bacterial and protist pathogens also produce $H_2$ in anoxic environments. The production of this diffusible gas provides an efficient way to dispose of reductant. This is especially beneficial in environments such as gastrointestinal tracts, where the availability of fermentable carbon sources generally exceeds that of respiratory electron acceptors (14). Obligate anaerobes such as *Clostridium perfringens* (15) and *Trichomonas vaginalis* (16) can grow efficiently through hydrogenogenic fermentation. In contrast, facultative anaerobes such as *Escherichia coli* (reviewed in reference 17) and *Giardia intestinalis* (18) produce $H_2$ as a strategy to survive electron acceptor limitation. Depending on the organism, hydrogenases oxidize the formate, NADH, and reduced ferredoxin produced during carbohydrate oxidation and use the electrons derived to reduce protons to $H_2$ (15, 17, 19, 20). Microorganisms extensively regulate their $H_2$-metabolizing pathways to adapt to environmental change (21). Some bacteria with particularly flexible metabolism, such as *S.* Typhimurium and *E. coli*, even switch between net production and consumption of $H_2$ depending on resource availability (22, 23). It is highly likely that, in the environment of the human or animal body, the availability of $H_2$ is a determinant that can influence the composition and distribution of the body's microbiota and in turn has a major impact on pathogenesis.

Expanding on these principles, the following sections provide a comprehensive assessment of $H_2$ metabolism in pathogens. We use genome surveys to demonstrate that the determinants of $H_2$ metabolism are widespread among pathogens and are universally distributed among gastrointestinal bacteria. Hydrogenase classes and gene names are used per the hydrogenase database (HydDB) (24) system throughout the review; this system predicts structural (e.g., subunit composition) and functional (e.g., $H_2$-oxidizing versus $H_2$-evolving) features of hydrogenases based on their primary sequence. Thereafter, we explore the basis, role, and importance of $H_2$ metabolism in specific pathogens. Reflecting past and current literature, much of the review focuses on well-studied bacteria within the *Campylobacterales* (*H. pylori*, *C. jejuni*, and *Campylobacter concisus*), *Enterobacteriales* (*E. coli*, *S.* Typhimurium, and *Shigella flexneri*), and *Clostridiales* (*C. perfringens* and *Clostridioides difficile*). It also summarizes our knowledge regarding $H_2$ metabolism in other established and emerging pathogens, including $H_2$ production by protist parasites. We also consider how metabolic interactions between $H_2$-metabolizing pathogens and microbiota influence infection while acknowledging considerable further research is needed in this area. Integrating these findings, we evaluate the pros and cons of inhibiting pathogen $H_2$ metabolism through future development of small-molecule inhibitors or manipulation of the microbiota.

## OVERVIEW OF $H_2$ METABOLISM AND HYDROGENASES

### Biochemistry: Two Distinct Hydrogenases in Pathogens

Hydrogenases are the enzymes that catalyze oxidation and production of $H_2$ in microorganisms. They catalyze the most fundamental chemical reaction:

$$H_2 \rightleftharpoons \left[H^+ + H^-\right]^{\ddagger} \rightleftharpoons 2H^+ + 2e^-$$

Despite the apparent simplicity of this reaction, hydrogenases are highly complex in structure and mechanism. This reflects the fact that, under cellular conditions, the oxidation of $H_2$ is thermodynamically favorable but kinetically challenging. Moreover, sophisticated enzymatic mechanisms are required to use the electrons and protons derived from this oxidation for chemiosmosis, i.e., the process of generating an ion gradient (4). Bacteria use two distinct classes of hydrogenases to overcome these challenges, the [NiFe]-hydrogenases and [FeFe]-hydrogenases, both of which are found in pathogens (25, 26). In a remarkable example of convergent evolution, these hydrogenases exhibit structural and mechanistic similarities despite their distinct phylogenetic origins. As detailed in a recent review (27), the bimetallic centers of both hydrogenases coordinate $H_2$ and catalyze its heterolytic cleavage (by increasing its acidity in the presence of the base) into a proton ($H^+$), which is released, and a hydride anion ($H^-$).

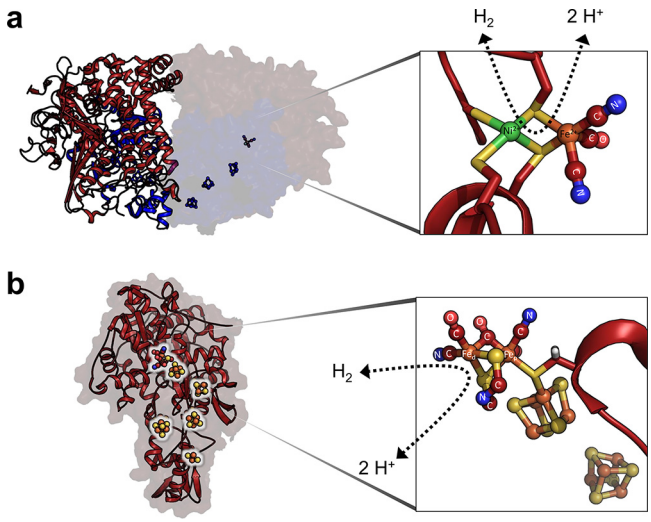


**FIG 1** Architecture and mechanism of [NiFe]- and [FeFe]-hydrogenases. (a) Dimer-of-dimer structure of the group 1d [NiFe]-hydrogenase Hyd from *S.* Typhimurium (PDB entry 4C3O). The catalytic subunits are shown in red, and the small electron-transferring subunits are in blue. One dimer of the large and small subunit is shown as a partially transparent protein surface to aid clarity. The metal ions of the [NiFe] cofactor and the iron-sulfur clusters are shown as sticks and spheres, respectively. A magnification of the active-site $NiFe(CN)_2CO$ cofactor on the right shows the Ni ion in green, the Fe ion in orange, and the $CN^-$ and CO diatomic ligands as red/blue and red/magenta spheres, respectively. The sulfurs of the cysteinyl residues coordinating the cofactor are shown in yellow. (b) [FeFe]-hydrogenase from *Clostridium pasteurianum* CpI (PDB entry 4XDC) with a partially transparent protein surface to highlight the location of the active-site H-cluster cofactor and the iron-sulfur clusters. The atoms of the cofactors are represented using the same colors as those mentioned above. The iron ions of the H-cluster cofactor (expanded on the right) are labeled $Fe_d$ and $Fe_p$ to indicate that they are distal and proximal, respectively, to the attached iron-sulfur cluster. In contrast to the [NiFe] cofactor, the H cluster has 2 $CN^-$ and 3 CO diatomic ligands, as well as an azadithiolate ligand (-S-$CH_2$-NH-$CH_2$-S-) group bridging the iron ions. Note that the heterodimer of [NiFe]-hydrogenase and the monomer of [FeFe]-hydrogenase can interact with different protein modules, depending on the bacterium. This determines whether the enzyme functions in respiration ($H_2$ oxidation), fermentation ($H_2$ evolution), or electron bifurcation.

The proton is initially accepted by a base associated with the catalytic center (28, 29) and is subsequently transferred to the aqueous exterior through a series of amino acids (28, 30, 31). In contrast, the two electrons on the hydride anion (28, 32) are relayed through iron-sulfur clusters to downstream acceptors (e.g., respiratory cytochromes), and the resultant proton is also released from the enzyme (25, 26). This reaction is reversible, and many hydrogenases primarily catalyze the reduction of protons to dihydrogen (4). Ultimately, the directionality of hydrogenases is determined by a combination of their intrinsic redox chemistry (i.e., catalytic bias) and their coupling to extrinsic electron donors or acceptors (33–35).

The [NiFe]-hydrogenases are widespread enzymes in bacteria (36). Broadly distributed among aerobic and anaerobic pathogens, they have been shown to have key roles in the pathogenesis of *H. pylori* (9), *C. jejuni* (12), and *S.* Typhimurium (8). These enzymes are relatively well understood as a result of extensive biochemical, structural, spectroscopic, and electrochemical studies (4, 27). As depicted in Fig. 1a, the large subunit of these enzymes binds a nickel-iron catalytic center that is ligated by two CN, one CO, and four cysteine ligands (25). The diatomic ligands (CN and CO) facilitate the cleavage of $H_2$ by modulating the redox chemistry of the two metals at this site (28). All [NiFe]-hydrogenases also contain a small subunit containing up to three iron-sulfur clusters (36); depending on the directionality of the enzymes, electrons are transferred between the active site and the physiological electron donor or acceptor via these clusters (25, 37). As elaborated below, there is an enormous diversity of these hydrogenases in terms of features, such as catalytic parameters, subunit composition, and redox partners (25, 37–43). This enables them to support a range of roles in bacterial respiration and fermentation, among other processes (4, 36). While the active site of

[NiFe]-hydrogenases is generally inhibited by oxygen, various classes have been discovered that function under ambient conditions, some of which support aerobic respiration (36, 44, 45). Several mechanisms enable these enzymes to either exclude or remove $O_2$ from the active site, as described in several independent studies (37, 39–41, 44).

The [FeFe]-hydrogenases are typically associated with obligate anaerobes (36, 46, 47). They are distributed in numerous fermentative bacterial pathogens (e.g., *C. perfringens*) (15) and have also been acquired by many protist species (e.g., *T. vaginalis*) (47). Based on structural studies (Fig. 1b), the main components of this hydrogenase class are a conserved core domain that binds the catalytic cofactor (the H cluster) and a variable C-terminal domain that binds two to four iron-sulfur clusters. The di-iron catalytic center is ligated by two CN ligands, three CO ligands, an azadithiolate bridge, and a [4Fe4S] cluster via a bridging cysteine (26, 29, 48). This site mediates heterolytic cleavage of $H_2$, with the hydride bound at the metal center and the proton accepted by the azadithiolate nitrogen (29, 32). [FeFe]-hydrogenases are generally catalytically biased toward $H_2$ production and typically use reduced ferredoxin as their electron donor; they are particularly desirable for obligate fermenters such as clostridia, given their very high turnover rates ($\sim$10,000 $s^{-1}$) (49). However, many bacteria also encode trimeric [FeFe]-hydrogenases that reversibly bifurcate electrons from $H_2$ to ferredoxin (20, 50, 51). Several respiratory uptake [FeFe]-hydrogenases have also been characterized, notably fast-acting periplasmic hydrogenases in sulfate-reducing bacteria (48, 52). In contrast to the [NiFe]-hydrogenase, [FeFe]-hydrogenases generally only function under anoxic conditions and are irreversibly damaged by $O_2$ exposure (53, 54).

For both types of hydrogenase, the $H_2$-binding sites are complex structures comprising metal ions coordinated by unusual ligands. These are synthesized through dedicated pathways. It is well established that maturation of [NiFe]-hydrogenases is a multistage process that requires the concerted activity of at least seven factors (HypA, HypB, HypC, HypD, HypE, HypF, and HupD) (55–57). More recently, the maturation pathway of the [FeFe]-hydrogenase has largely been resolved and involves three key enzymes (HydE, HydF, and HydG) (33, 58, 59). The details of the maturation of these enzymes are beyond the scope of this review and have been extensively reviewed elsewhere (27, 33, 56). Other proteins are also necessary for hydrogenase function, including those involved in the transport of nickel and iron, the biosynthesis of iron-sulfur clusters, and the translocation of hydrogenases to different cellular compartments (reviewed in references 60 and 61).

## Physiology: Integration and Regulation of $H_2$ Metabolism in Pathogens

Pathogens use hydrogenases in a wide range of physiological contexts. As summarized in Table 1, the [NiFe]- and [FeFe]-hydrogenases have both extensively diversified into multiple functionally and phylogenetically distinct groups. These groups differ in key traits, such as catalytic behavior, genetic organization, redox partners, cellular localization, and oxygen tolerance. At least 13 [NiFe]-hydrogenase subgroups and 5 [FeFe]-hydrogenase subgroups are known to be found in pathogens.

Pathogens that mediate hydrogenotrophic growth (as defined by the ability to use molecular hydrogen as an energy source during growth) do so by coupling the oxidation of $H_2$ to the reduction of respiratory electron acceptors, such as $O_2$ or fumarate (1). This depends on the generation of a PMF across the cell membrane to generate a proton gradient and, thus, energize ATP synthesis via the $F_1F_o$-ATPase (i.e., oxidative phosphorylation); the PMF is the sum of two components generated by proton translocation, the pH gradient ($\Delta$pH; the difference in concentration of $H^+$ across the membrane) and the membrane potential ($\Delta\psi$; the difference in electrical potential across the membrane) (62). Most hydrogenotrophic pathogens use membrane-bound, periplasmically oriented hydrogenases to catalyze $H_2$ oxidation (i.e., group 1b, 1c, and 1d [NiFe]-hydrogenases). The periplasmic protons derived from $H_2$ oxidation directly contribute to PMF generation. In parallel, the low-potential electrons released are transferred through respiratory chains to terminal electron acceptors (4, 63). Several

**TABLE 1** List of hydrogenase classes present in the genomes of pathogens[e]

| Enzyme | Operon | Proposed function in pathogens | Example pathogen(s) | Reference(s) |
|---|---|---|---|---|
| $H_2$-consuming [NiFe]-hydrogenases | | | | |
| Group 1a | *hysAB* | $H_2$-dependent sulfite respiration | *Bilophila wadsworthia*, *Clostridium botulinum*[a] | 66 |
| Group 1b | *hynABC*[b] | $H_2$-dependent aerobic and fumarate respiration | *Helicobacter pylori*, *Campylobacter jejuni* | 9, 12, 64, 200 |
| Group 1c | *hybOABC* | $H_2$-dependent fumarate respiration; may be proton motive | *Escherichia coli*, *Salmonella* Typhimurium | 13, 206, 213 |
| Group 1d | *hyaABC* | $H_2$-dependent aerobic and fumarate respiration; oxygen tolerant | *Escherichia coli*, *Salmonella* Typhimurium | 22, 215, 243 |
| Group 1f | *hyoSLE* | Unknown; may support $H_2$-dependent aerobic respiration | *Corynebacterium diphtheriae*[a] | 450 |
| Group 1h | *hhySLE* | $H_2$-dependent aerobic respiration; oxygen tolerant, high affinity | *Mycobacterium gordonae*, *Rhodococcus equi* | 45, 283 |
| Group 1i | *vhzGAC* | Unknown; may support $H_2$-dependent anaerobic respiration | *Eggerthella lenta*[a] | 24 |
| Group 2a | *hucLSAEB* | $H_2$-dependent aerobic respiration; oxygen tolerant, high affinity | *Mycobacterium gordonae* | 45, 68 |
| Group 2d | *huaSL* | Unknown; may support $H_2$-dependent aerobic respiration | *Arcobacter butzleri*[a] | 451 |
| $H_2$-producing [NiFe]-hydrogenases | | | | |
| Group 3b | *hyhBGSL* | NADPH-coupled $H_2$ production; may be physiologically reversible | *Mycobacterium gordonae*, *Rhodococcus equi*[a] | 19, 275 |
| Group 4a | *hycBCDEFG*[c] | Formate-dependent $H_2$ production; may be proton motive | *Escherichia coli*, *Salmonella* Typhimurium | 17, 84 |
| Group 4c | *cooMKLXUH* | CO-coupled $H_2$ production; may be proton motive | *Bilophila wadsworthia*[a] | 452 |
| Group 4e | *echABCDEF* | Ferredoxin-coupled $H_2$ production; reversible and proton motive | *Eggerthella lenta*[a] | 286 |
| [FeFe]-hydrogenases | | | | |
| Group A1 | *hydA*[d] | Ferredoxin-coupled $H_2$ production; some act in reverse direction | *Trichomonas vaginalis*, *Clostridium perfringens* | 15, 344 |
| Group A2 | *hydAgltB* | Unknown; may be functionally linked to glutamate synthase | *Treponema denticola*[a] | 117 |
| Group A3 | *hydABC* | Reversible, bifurcates electrons from $H_2$ to NAD and ferredoxin | *Clostridioides difficile*[a], *Brachyspira pilosicoli*[a] | 20, 70 |
| Group A4 | *hytAE1E2* | Formate-dependent $H_2$ production; physiologically reversible | *Clostridioides difficile*[a] | 356 |
| Group B | *hydM*[d] | Ferredoxin-coupled $H_2$ production | *Entamoeba histolytica*, *Bacteroides fragilis* | 273, 407 |

[a]Activity of the listed hydrogenase has not been formally demonstrated in these pathogens.
[b]The group 1b [NiFe]-hydrogenases of *Campylobacterales* are traditionally called *hydABC* (9), but (according to HydDB) this group should be annotated *hynABC* to avoid confusion with the group A3 [FeFe]-hydrogenases.
[c]Variants of the group 4a [NiFe]-hydrogenase, called Hyf (*hyfABCDEFGHI*), are also known (209).
[d]There is considerable functionally relevant variation in the domain organization of group A1 and B [FeFe]-hydrogenases (7, 366).
[e]Hydrogenase classes and gene names are used per the hydrogenase database (HydDB) (24). General references are used where the hydrogenase class has not been explicitly studied in pathogens.

terminal electron acceptors are known to support hydrogenotrophic respiration in pathogens, including $O_2$ (e.g., *H. pylori*) (64), fumarate (e.g., *S*. Typhimurium) (13), nitrate (e.g., *Campylobacter rectus*) (65), and sulfite (e.g., *Bilophila wadsworthia*) (66). Depending on the respiratory chain components, electron flow energizes the translocation of protons from the cytosol to the periplasm through either vectorial (i.e., direct pumping) or scalar (i.e., redox loop) mechanisms (67). As elaborated below, certain pathogens also depend on $H_2$ oxidation to mediate carbon fixation (68–70) and to energize demanding processes, such as substrate uptake (71–73), protein secretion (74), and swarming motility (75).

An equally important role of hydrogenases is to dissipate reductant in the form of $H_2$ during fermentation. In this process, the energy derived from the incomplete oxidation of carbohydrates to organic and gaseous end products is used to generate ATP through substrate-level phosphorylation (2). This process sustains growth of obligate fermentative pathogens such as clostridia and trichomonads. In these organisms, cytosolic [FeFe]-hydrogenases (A1, A3, and B subtypes) mediate the reoxidation of ferredoxin and nicotinamides reduced during carbon breakdown (35, 46; reviewed in reference 75) (Table 1). Some protists, such as *T. vaginalis*, compartmentalize this process within specialized organelles called hydrogenosomes (16, 76). A distinct system operates in facultative fermenters such as *E. coli* (77) and *C. concisus* (78). It is thought that these bacteria switch to use fermentation to survive insufficiency of their preferred respiratory electron donors. They use specialized membrane-bound, potentially ion-motive complexes (formate hydrogenlyases containing group 4a [NiFe]-hydrogenases) to decompose the fermentation product formate into $H_2$ and $CO_2$ (17). This process is thought to maintain redox homeostasis, regulate cytoplasmic pH, and potentially generate PMF (23, 79).

In general, $H_2$ metabolism is tightly regulated. Some obligate fermentative pathogens are thought to generate $H_2$ throughout their life cycle and, hence, constitutively synthesize their hydrogenases. However, for most other bacteria, $H_2$ metabolism is a facultative trait that is induced in response to cellular and environmental cues (4). A good example in this regard is the production of multiple hydrogenases by *S*. Typhimurium (see "*Salmonella* Typhimurium: differential roles of hydrogenases during infection" below). This bacterium switches between three major modes of $H_2$ metabolism, which are each mediated by a different hydrogenase (80, 81): (i) growth by aerobic hydrogenotrophic respiration (group 1d [NiFe]-hydrogenase) (82); (ii) growth by anaerobic hydrogenotrophic respiration (group 1c [NiFe]-hydrogenase) (83); and (iii) persistence by hydrogenogenic fermentation (group 4a [NiFe]-hydrogenase) (84). *S*. Typhimurium hierarchically regulates the three hydrogenases by sensing levels of exogenous electron acceptors. This allows maximization of ATP generation relative to resource availability. It is proposed that this flexibility allows the bacterium to persist in environments deficient in oxidants and rapidly invade host tissues when respiratory electron acceptors are available (23).

The genes controlling $H_2$ metabolism are usually clustered together to enable their coordinated expression. A straightforward example of this is the *hyn* and *hyp* operons of *C. jejuni*. The *hyn* operon of this pathogen (*hynABCD*) encodes the three structural subunits and a maturation endopeptidase of its group 1b [NiFe]-hydrogenase. A second operon, *hyp* (*hypFBCDEA*), encodes the six proteins required for the synthesis of the [NiFe] cofactor (12, 85). Both operons are induced during infection, with the maturation genes being expressed at lower levels than the structural genes (86). A range of activators and repressors can bind the promoters of hydrogenase operons in response to internal and external signals. Indeed, various signals and regulators have been shown to control hydrogenase operon expression across different bacteria, including redox state (e.g., ArcA) (87), oxygen levels (e.g., FNR) (88), energy availability (e.g., CRP) (89), metal availability (e.g., Fur and NikR) (90), and developmental stage (e.g., Spo0A) (91). The cellular and molecular basis of this regulation is detailed elsewhere (4, 21, 23). Some environmental organisms also regulate hydrogenase gene expression in re-

sponse to hydrogen partial pressures ($pH_2$), but this is less relevant for most pathogens, given that $H_2$ is usually abundant in host tissues (21).

**Ecology: Subversion of Gastrointestinal Microbiota**

It is becoming increasingly apparent that many pathogens, especially those of the gastrointestinal tract (GIT), do not act in isolation. Their pathogenesis often involves metabolic interactions, including $H_2$ exchange, with nonpathogenic microbiota (92). GIT microbiota generally protect against colonization from intestinal pathogens through a range of mechanisms; for instance, competitive exclusion of energy sources has been well documented (93, 94). However, many pathogens are adept at subverting microbiota to obtain resources for their expansion. This is best exemplified by *S.* Typhimurium, which uses sophisticated mechanisms to acquire respiratory electron acceptors, including from the host and other microbial cells, during gut invasion (92, 95). Impairment of GIT microbiota and their associated metabolisms, for example, due to antibiotic treatment, dietary factors, and inflammatory conditions, is often associated with increased susceptibility to pathogen invasions. For example, pathogens often cause antibiotic-induced diarrhea by exploiting microbiota-derived sugars released by microbial lysis (96, 97). It is also thought that pathogens can orchestrate remodeling of the microbiota toward a dysbiotic state, which favors their growth or persistence (98). While these concepts have been studied mostly concerning carbon source acquisition, they are also relevant for understanding the pathogenesis of $H_2$-metabolizing bacteria.

For hydrogenotrophic pathogens, substrate availability is governed by the dynamics and ecology of $H_2$ metabolism in the GIT (Fig. 2). Despite recent interest in the microbiota, relatively little is known about the processes and organisms that control $H_2$ cycling (6, 7, 99). Genomic and metagenomic studies indicate that most gastrointestinal microorganisms can metabolize $H_2$, including members of all five dominant phyla (*Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia*) (7). It is thought that most $H_2$ is produced by carbohydrate-fermenting *Clostridia* and potentially *Bacteroidia* via ferredoxin-dependent and electron-bifurcating [FeFe]-hydrogenases (7, 100, 101). While some of the $H_2$ produced is excreted in breath and flatus, much is reoxidized by hydrogenotrophic microorganisms within the colon (5, 102). The human colon is known to harbor hydrogenotrophic acetogenic, sulfate-reducing, and fumarate-reducing bacteria, as well as methanogenic archaea; these bacteria produce the end products acetate, hydrogen sulfide, succinate, and methane, respectively (7, 103–106) (Fig. 2). The presence, abundance, and activities of these microorganisms vary greatly between individuals (102, 103, 107). During hydrogenotrophic growth, for instance, intestinal respiratory bacteria outcompete methanogens and acetogens because of their higher substrate affinity and higher growth yield (104, 108). However, it is thought that bacterial growth is oftentimes restricted by the low availability of respiratory electron acceptors; hence, these bacteria rarely become dominant members of the microbiota (109). These factors may explain why multiple hydrogenotrophic groups typically coexist in the human GIT (7, 110).

Nevertheless, it is thought that hydrogen availability does not generally limit growth of hydrogenotrophic pathogens. Microsensor studies of live anesthetized mice have detected high levels of dissolved $H_2$ in intestines (168 $\mu$M) (8). As a result of diffusion from the colon, $H_2$ is also abundant in other organs that pathogens colonize, such as the stomach (43 $\mu$M), liver (43 $\mu$M), and spleen (55 $\mu$M) (9, 10). Although equivalent measurements have not been performed in humans, various lines of evidence suggest $H_2$ is present at similarly high concentrations in the body (5, 111) (Fig. 3). The concentrations reported in mice are between 20 and 80 times higher than reported for the apparent $K_m$ of $H_2$-oxidizing hydrogenase in the various pathogens (1.8 to 2.5 $\mu$M) (8–10). To our knowledge, no study has investigated substrate competition between pathogenic and commensal hydrogenotrophic microorganisms. Based on thermodynamic theory, pathogens are likely to outcompete other microflora, given they can monopolize the saturating levels of substrate and can generate large amounts of ATP through hydrogenotrophic respiration (23, 61). However, as stated above, limitation for

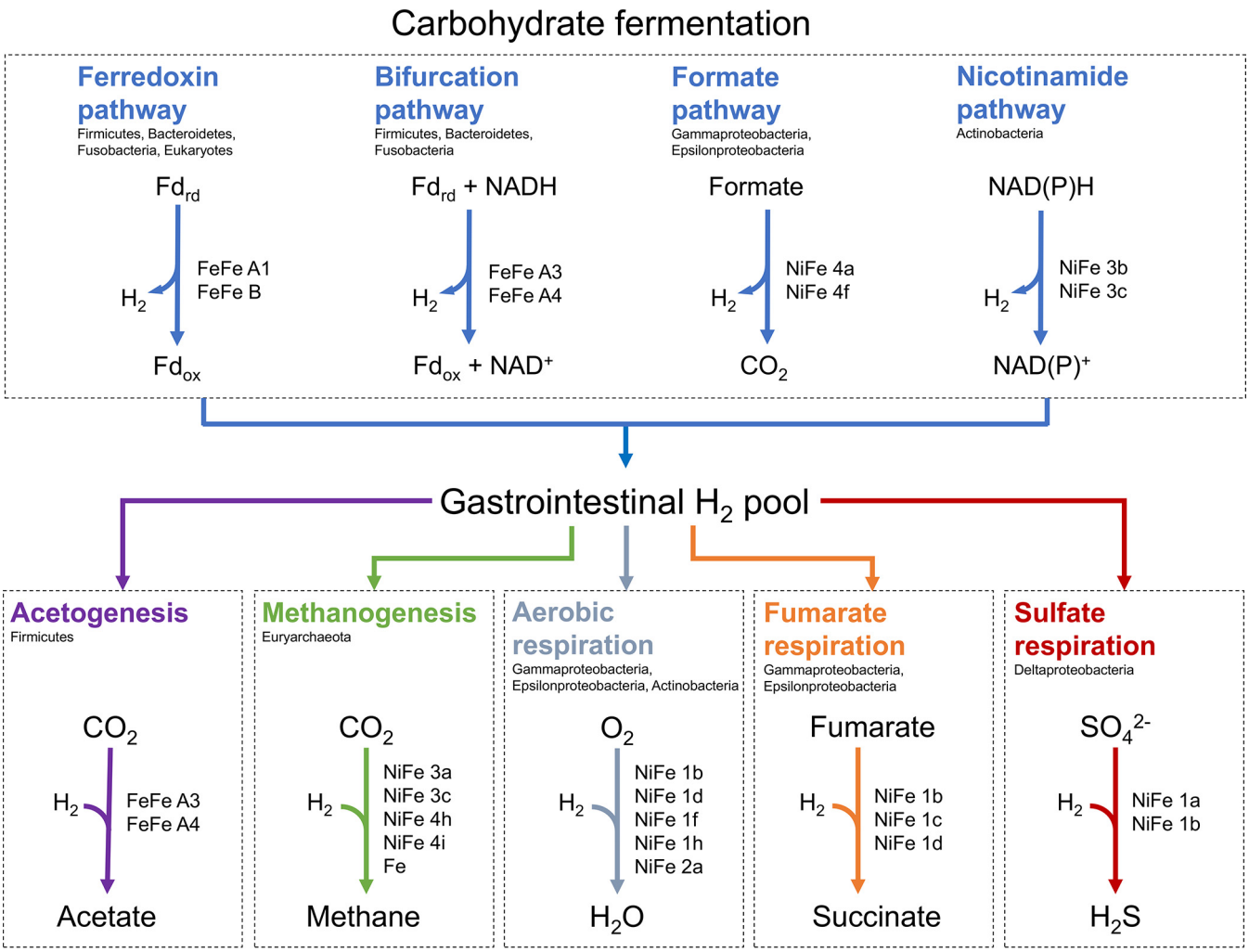


**FIG 2** Outline of molecular hydrogen transactions in the human gastrointestinal tract. Diverse fermentative bacteria and eukaryotes produce $H_2$ during carbohydrate fermentation (hydrogenogenesis). These organisms couple the reoxidation of ferredoxin, nicotinamides, and formate to the reduction of protons. Pathogens are known that can mediate all four $H_2$ production pathways shown here, namely, the ferredoxin pathway, electron-bifurcation pathway, formate pathway, and nicotinamide pathway. The $H_2$ produced can be consumed by hydrogenotrophic bacteria and archaea, diffused to other tissues, or excreted to the atmosphere. $H_2$ oxidation can be used to support acetogenesis, methanogenesis, fumarate reduction, sulfate reduction, and, in oxic sites, aerobic respiration. Pathogens are known that can mediate hydrogenotrophic aerobic respiration, fumarate respiration, sulfate reduction, and potentially acetogenesis. Different classes of [NiFe]-hydrogenase and [FeFe]-hydrogenase mediate each of the outlined pathways.

other resources (electron acceptors) is likely to restrict their growth. Hydrogenotrophs that can generate respiratory electron acceptors, for example, *Salmonella* (13) and *Campylobacter* (112) species, which can produce fumarate through the tricarboxylic acid and urea cycles, therefore are likely to be especially competitive in the $H_2$-rich, oxidant-limited environment of the GIT. It is generally assumed that pathogens acquire $H_2$ from the bulk dissolved pool, although local variability in $H_2$ concentrations in tissues and the GIT is likely to occur. Consequently, it is plausible that some pathogens engage in specific interactions with hydrogenogenic fermenters, which facilitates interspecies hydrogen transfer, as has been described previously for certain methanogens (113) and sulfate reducers (114).

In contrast, fermentative pathogens face a major thermodynamic challenge in $H_2$-rich host tissues. Taking into account Le Chatelier's principle, $H_2$ production only remains favorable if $H_2$ is continuously removed (2). Thus, bacterial and eukaryotic fermentative pathogens likely benefit from direct or indirect metabolic interactions with hydrogenotrophic microbiota that maintain the local $H_2$ concentration below threshold levels, facilitating otherwise thermodynamically unfavorable reactions. Some

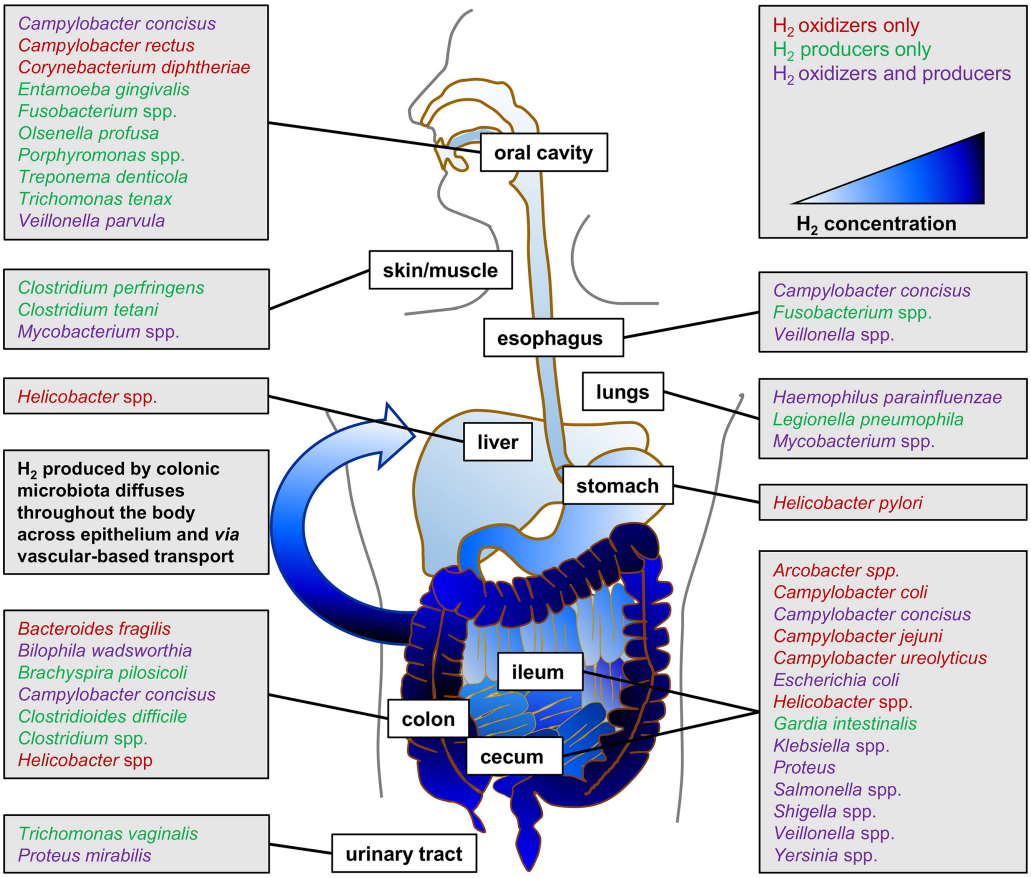


**FIG 3** Human niches for $H_2$-metabolizing bacteria and protists. Shown are the relative concentrations of $H_2$ in the gastrointestinal tract and other organs. Also shown are selected pathogens known to consume and/or produce $H_2$ in each organ.

pathogens have evolved mechanisms to recycle endogenously produced $H_2$, for example, *Salmonella* (22). This notwithstanding, there is evidence that several pathogens can rapidly produce $H_2$ even at high concentrations of the gas. For example, hydrogen can reach millimolar concentrations during growth of *C. perfringens* both *in vitro* and *in vivo* (115, 116). It is conceivable that some pathogens remodel their fermentation pathways to maintain $H_2$ production at high partial pressures of $H_2$, albeit with the compromise of reduced ATP synthesis. This is exemplified by the saccharolytic rumen bacterium *Ruminococcus albus*, which switches from using electron-bifurcating hydrogenases at low $pH_2$ (producing 4 ATP) to using a ferredoxin-dependent hydrogenase at high $pH_2$ (producing 3.3 ATP) by sensing and responding to $H_2$ partial pressures (51, 100). Similar metabolic remodeling might explain why most hydrogenogenic pathogens, including *C. perfringens*, *C. difficile*, and *T. vaginalis*, encode multiple hydrogenases (36, 117).

## Distribution: $H_2$ Metabolism as a Widespread Trait in Pathogens

We performed comprehensive genomic surveys and literature searches to gain a broader understanding of the distribution of $H_2$ metabolism in pathogens. In total, genomes of 204 pathogens surveyed harbored the catalytic subunits of one or more hydrogenases. There is published experimental evidence from cellular or biochemical studies that at least 46 of these species can consume or produce $H_2$. These findings are reported in Table 2. Phylogenetic trees of the [NiFe]-hydrogenases and [FeFe]-hydrogenases in key pathogens are also provided in Fig. 4.

The hydrogenase-positive pathogens are highly diverse in terms of their taxonomic affiliation, the host tissues they target, and their broader metabolic traits. Hydrogenases

**TABLE 2** Distribution of hydrogen metabolism in pathogens and pathobionts[a]

| Organism | [NiFe]-hydrogenase(s) | [FeFe]-hydrogenase(s) | Predicted activity | Key reference(s) |
|---|---|---|---|---|
| *Bacteria* | | | | |
| *Actinobacteria* | | | | |
| *Coriobacteriaceae* | | | | |
| *Cryptobacterium curtum* | 1i, 4a, 4e | | U + P | |
| *Eggerthella lenta* | 1i, 3b, 4e | | U + P | 286 |
| *Olsenella profusa* | | A2, B | P? | |
| *Slackia exigua* | 1i, 4a, 4e | A1, A2 | U + P | |
| *Corynebacteriaceae* | | | | |
| *Corynebacterium amycolatum* | 1f | | U | |
| *Corynebacterium diphtheriae* | 1f | | U | |
| *Corynebacterium durum* | 1f | | U | |
| *Corynebacterium freiburgense* | 1f | | U | |
| *Mycobacteriaceae* | | | | |
| *Mycobacterium avium* | 1h | | U | |
| *Mycobacterium chelonae* | 1h | | U | |
| *Mycobacterium colombiense* | 1h | | U | |
| *Mycobacterium fortuitum* | 1h | | U | |
| *Mycobacterium gordonae* | 1h, 2a, 3b | | U + P | 68, 285 |
| *Mycobacterium haemophilum* | 3b | | P | |
| *Mycobacterium kansasii* | 1h, 3b | | U + P | |
| *Mycobacterium iranicum* | 2a, 3b | | U + P | |
| *Mycobacterium liflandii* | 1h | | U | |
| *Mycobacterium marinum* | 1h, 3b | | U + P | |
| *Mycobacterium parascrofulaceum* | 3b | | P | |
| *Mycobacterium phlei* | 2a | | U | |
| *Mycobacterium smegmatis* | 1h, 2a, 3b | | U + P | 19, 45 |
| *Mycobacterium tusciae* | 1h, 2a | | U | |
| *Mycobacterium ulcerans* | 3b | | P | |
| *Mycobacterium xenopi* | 1h, 3b | | U + P | |
| *Mycobacterium yongonense* | 1h | | U | |
| *Nocardiaceae* | | | | |
| *Rhodococcus equi* | 1h, 3b | | U + P | 283 |
| *Bacteroidetes* | | | | |
| *Bacteroidaceae* | | | | |
| *Bacteroides fragilis* | | B | U? | 101, 273 |
| *Odoribacteraceae* | | | | |
| *Butyricimonas virosa* | | A3, B, C | P? | |
| *Porphyromonadaceae* | | | | |
| *Porphyromonas asaccharolytica* | | B | P? | |
| *Porphyromonas gingivalis* | | B | P? | |
| *Porphyromonas levii* | | B | P? | |
| *Porphyromonas macacae* | | B | P? | |
| *Betaproteobacteria* | | | | |
| *Neisseriaceae* | | | | |
| *Laribacter hongkongensis* | | A2 | P? | |
| *Deltaproteobacteria* | | | | |
| *Desulfovibrionaceae* | | | | |
| *Bilophila wadsworthia* | 1a × 2, 1b, 1d, 4c | A1 | U + P | 66 |
| *Desulfovibrio desulfuricans* | 1a, 1b, 4c, 4e × 2 | A1 | U + P | 48, 453 |
| *Lawsonia intracellularis* | 1d | | U | 274 |
| *Epsilonproteobacteria* | | | | |
| *Helicobacteraceae* | | | | |
| *Helicobacter bizzozeronii* | 1b | | U | |
| *Helicobacter canadensis* | 1b | | U | |
| *Helicobacter canis* | 1b | | U | |
| *Helicobacter cinaedi* | 1b | | U | |
| *Helicobacter felis* | 1b | | U | |
| *Helicobacter fennelliae* | 1b | | U | |
| *Helicobacter heilmannii* | 1b | | U | |
| *Helicobacter hepaticus* | 1b | | U | 10, 71 |
| *Helicobacter pullorum* | 1b | | U | |
| *Helicobacter pylori* | 1b | | U | 9, 64, 69, 74 |
| *Helicobacter suis* | 1b | | U | |
| *Helicobacter wighamensis* | 1b | | U | |
| *Campylobacteraceae* | | | | |
| *Arcobacter butzleri* | 1b × 2, 2d | | U | |
| *Arcobacter cryaerophilus* | 1b | | U | |
| *Arcobacter skirrowii* | 1b | | U | |

(Continued on next page)

**TABLE 2** (Continued)

| Organism | [NiFe]-hydrogenase(s) | [FeFe]-hydrogenase(s) | Predicted activity | Key reference(s) |
|---|---|---|---|---|
| *Campylobacter coli* | 1b | | U | |
| *Campylobacter concisus* | 1b, 4a | | U + P | 78, 188 |
| *Campylobacter curvus* | 1b, 4a | | U + P | |
| *Campylobacter fetus* | 1b, 4a | A1 | U + P | |
| *Campylobacter gracilis* | 1b | | U | |
| *Campylobacter helveticus* | 1b | | U | |
| *Campylobacter hyointestinalis* | 1b, 4a | A1 | U + P | |
| *Campylobacter jejuni* | 1b | | U | 12, 169, 172 |
| *Campylobacter lanienae* | 1b | | U | |
| *Campylobacter lari* | 1b | | U | |
| *Campylobacter mucosalis* | 1b | | U | |
| *Campylobacter rectus* | | A1 | U | 65, 201 |
| *Campylobacter showae* | 1b, 4a | | U + P | |
| *Campylobacter sputorum* | 1b, 4a | | U + P | 200 |
| *Campylobacter upsaliensis* | 1b, 4a | | U + P | |
| *Campylobacter ureolyticus* | 1b | A1 | U | |
| *Firmicutes* | | | | |
| *Clostridiaceae* | | | | |
| *Clostridium botulinum* | 1a | A3, B × 2 | U + P | |
| *Clostridium cadaveris* | 1a | A1 × 2, A3, B × 2, C | U + P | |
| *Clostridium chauvoei* | | A1, B | P | |
| *Clostridium intestinale* | | A1 × 2, A3, B × 2 | P | |
| *Clostridium perfringens* | | A1 × 2, B × 2 | P | 15, 344, 345 |
| *Clostridium septicum* | | A1 × 2, B × 2 | P | 101, 340 |
| *Clostridium tetani* | | B | P? | |
| *Enterococcaceae* | | | | |
| *Enterococcus avium* | | A3 | P? | |
| *Enterococcus gilvus* | | A3 | P? | |
| *Enterococcus raffinosus* | | A3 | P? | |
| *Erysipelotrichaceae* | | | | |
| *Bulleidia extructa* | | A1 | P | |
| *Erysipelatoclostridium innocuum* | | A1 | P | |
| *Erysipelatoclostridium spiroforme* | | A3, B | P? | |
| *Eubacteriaceae* | | | | |
| *Eubacterium yurii* | | A3, B | P? | |
| *Pseudoramibacter alactolyticus* | | A2, B | P? | |
| *Lachnospiraceae* | | | | |
| *Anaerostipes caccae* | | A1, A2, A3, B | P | |
| *Lachnoclostridium bolteae* | | A1, A3 × 2, B × 2, C × 2 | P | |
| *Lachnoclostridium citroniae* | | A1, A3 × 2, B × 2, C × 2 | P | |
| *Lachnoclostridium clostrioforme* | | A1, A3, B × 2, C × 2 | P | |
| *Lachnoclostridium hathewayi* | | A1, A3, B, C × 2 | P | |
| *Lachnoclostridium gnavus* | | A3, B | P? | |
| *Peptoniphilaceae* | | | | |
| *Peptoniphilus duerdenii* | | A2, B | P? | |
| *Peptostreptococcaceae* | | | | |
| *Clostridioides difficile* | | A3, A4, B × 2 | U + P | 70, 101, 117 |
| *Filifactor alocis* | | B | P? | |
| *Peptostreptococcus anaerobius* | | A2, B × 2 | P? | |
| *Terrisporobacter glycolicus* | | A1, A3, A4, C | P | |
| *Ruminococcaceae* | | | | |
| *Anaerotruncus colihominis* | | A3, B × 2, C | P | |
| *Veillonellaceae* | | | | |
| *Centipeda periodontii* | 1d | | U | |
| *Megasphaera micronuciformis* | | A1, B | P | |
| *Selenomonas artemidis* | 1d | | U | |
| *Selenomonas infelix* | 1d | | U | |
| *Selenomonas sputigena* | 1d | A3, B | U + P | |
| *Veillonella dispar* | 1d | A1 × 2 | U + P | |
| *Veillonella montpellierensis* | 1d | A1 | U + P | |
| *Veillonella parvula* | 1d | A1 | U + P | 454 |
| *Fusobacteria* | | | | |
| *Fusobacteriaceae* | | | | |
| *Fusobacterium gonidiaformans* | | A1, A3 | P | |
| *Fusobacterium necrophorum* | | A1, A3 | P | |
| *Fusobacterium ulcerans* | | A1, A3 | P | |
| *Fusobacterium varium* | | A1, A3 | P | |
| *Gammaproteobacteria* | | | | |

(Continued on next page)

**TABLE 2** (Continued)

| Organism | [NiFe]-hydrogenase(s) | [FeFe]-hydrogenase(s) | Predicted activity | Key reference(s) |
|---|---|---|---|---|
| *Aeromonadaceae* | | | | |
| *Aeromonas caviae* | 1c, 4a | | U + P | |
| *Aeromonas hydrophila* | 1c, 4a | | U + P | |
| *Aeromonas veronii* | 1c, 4a | | U + P | |
| *Enterobacteriaceae* | | | | |
| *Budvicia aquatica* | 1c, 4a | | U + P | |
| *Cedecea davisae* | 4a | | P | |
| *Citrobacter freundii* | 1c, 1d, 4a | | U + P | 303 |
| *Citrobacter koseri* | 1c, 4a | | U + P | |
| *Citrobacter rodentium* | 1c, 4a | | U + P | |
| *Cronobacter dublinensis* | 4a | | P | |
| *Cronobacter malonaticus* | 4a | | P | |
| *Cronobacter pulveris* | 4a | | P | |
| *Cronobacter sakazakii* | 4a | | P | |
| *Cronobacter turicensis* | 4a | | P | |
| *Edwardsiella tarda* | 1c, 1d, 4a | | U + P | 258 |
| *Enterobacter aerogenes* | 4a | | P | 296, 297 |
| *Enterobacter agglomerans* | 4a | | P | 258 |
| *Enterobacter cloacae* | 1c, 4a | | U + P | |
| *Enterobacter gergovia* | 4a | | P | |
| *Escherichia albertii* | 1c, 1d, 4a | | U + P | |
| *Escherichia coli* | 1c, 1d, 4a × 2 | | U + P | 17, 77, 206, 212, 213 |
| *Escherichia fergusonii* | 1c, 1d, 4a | | U + P | |
| *Escherichia hermannii* | 1c, 4a | | U + P | |
| *Hafnia alvei* | 1c, 4a × 2 | | U + P | |
| *Klebsiella oxytoca* | 4a | | P | 302 |
| *Klebsiella pneumoniae* | 1c, 4a × 2 | | U + P | 254, 300 |
| *Leminorella grimontii* | 1c, 4a | | U + P | |
| *Morganella morganii* | 1c, 4a | | U + P | |
| *Pleisomonas shigelloides* | 1c, 1d | | U | |
| *Proteus hauseri* | 1c | | U | |
| *Proteus mirabilis* | 1c, 4a | | U + P | 75, 256, 262 |
| *Proteus penneri* | 1c, 4a | | U + P | |
| *Proteus vulgaris* | 1c, 4a | | U + P | 257, 263, 264 |
| *Providencia alcalifaciens* | 1c, 4a | | U + P | |
| *Providencia heimbachae* | 1c, 4a | | U + P | |
| *Providencia rettgeri* | 1c | | U | |
| *Providencia rustigianii* | 1c, 4a | | U + P | |
| *Providencia stuartii* | 1c | | U | |
| *Rahnella aquatilis* | 4a | | P | |
| *Salmonella bongori* | 1c, 1d, 4a | | U + P | |
| *Salmonella enterica* | 1c, 1d × 2, 4a | | U + P | 8, 13, 81, 238, 243 |
| *Serratia fonticola* | 1c, 4a | | U + P | |
| *Serratia liquefaciens* | 4a | | P | |
| *Serratia marcescens* | 4a | | P | 258 |
| *Serratia plymuthica* | 4a | | P | |
| *Shigella boydii* | 1c, 1d, 4a × 2 | | U + P | |
| *Shigella dysenteriae* | 1c, 1d, 4a | | U + P | |
| *Shigella flexneri* | 1c, 1d, 4a × 2 | | U + P | 251 |
| *Shigella sonnei* | 1c, 1d, 4a | | U + P | |
| *Yersinia aldovae* | 1c, 4a | | U + P | |
| *Yersinia bercovieri* | 1c, 4a | | U + P | |
| *Yersinia christensenii* | 1c, 4a | | U + P | |
| *Yersinia enterocolitica* | 1c, 4a | | U + P | |
| *Yersinia frederiksenii* | 1c, 4a | | U + P | |
| *Yersinia intermedia* | 1c, 4a | | U + P | |
| *Yersinia mollarotii* | 1c, 4a | | U + P | |
| *Yersinia rohdei* | 1c, 4a | | U + P | |
| *Yersinia ruckeri* | 4a | | P | |
| *Yokenella regensburgei* | 1c, 4a | | U + P | |
| *Francisellaceae* | | | | |
| *Francisella philomiragia* | 3b | | P? | |
| *Legionellaceae* | | | | |
| *Legionella pneumophila* | 3b | | P? | |
| *Pasteurellaceae* | | | | |
| *Actinobacillus pleuropneumoniae* | 1c | | U | 269, 270 |
| *Aggregatibacter actinomycetemcomitans* | 1c, 4a | | U + P | |
| *Aggregatibacter aphrophilus* | 1c, 4a | | U + P | |

(Continued on next page)

**TABLE 2** (Continued)

| Organism | [NiFe]-hydrogenase(s) | [FeFe]-hydrogenase(s) | Predicted activity | Key reference(s) |
|---|---|---|---|---|
| *Haemophilus haemolyticus* | 1c, 4a | | U + P | |
| *Haemophilus parainfluenzae* | 1c, 4a | | U + P | |
| *Haemophilus pittmaniae* | 1c, 4a | | U + P | |
| *Pasteurella bettyae* | 1c, 4a | | U + P | |
| *Pasteurella dagmatis* | 4a | | P | |
| *Pseudomonadaceae* | | | | |
| *Pseudomonas aeruginosa* | 1d | | U | |
| *Vibrionaceae* | | | | |
| *Grimontia hollisae* | 2c, 3d | | U? | |
| *Photobacterium damselae* | 1b | | U | |
| *Vibrio furnissii* | 4a | | P | |
| *Spirochaetes* | | | | |
| *Brachyspiraceae* | | | | |
| *Brachyspira alvinipulli* | | A3, B | P | |
| *Brachyspira hampsonii* | | A3, B | P | |
| *Brachyspira hyodysenteriae* | | A3, B | P | |
| *Brachyspira intermedia* | | A3, B | P | |
| *Brachyspira murdochii* | | A3, B | P | |
| *Brachyspira pilosicoli* | | B | P | |
| *Spirochaetaceae* | | | | |
| *Treponema brennaborense* | | A3, C × 2 | P | |
| *Treponema denticola* | | A2, B | P | |
| *Treponema pedis* | | A3 | P | |
| *Eukarya* | | | | |
| *Blastocystida* | | | | |
| *Blastocystis* sp. strain ST1 | | A1 × 2 | ? | 420 |
| *Blastocystis* sp. strain ST4 | | A1 | ? | 420 |
| *Blastocystis* sp. strain ST7 | | A1 | ? | 420, 421 |
| *Centramoebida* | | | | |
| *Acanthamoeba castellanii* | | A1 | P | 416 |
| *Diplomonadida* | | | | |
| *Giardia intestinalis* | | A1 | P | 18, 407, 409 |
| *Spironucleus barkhanus* | | A1 × 6 | P | |
| *Spironucleus salmonicida* | | A1 × 7 | P | 382, 384 |
| *Spironucleus vortens* | | A1 × 20 | P | 381, 383 |
| *Entamoebidae* | | | | |
| *Entamoeba dispar* | | A1 × 2, B | P | |
| *Entamoeba histolytica* | | A1 × 2, B | P | 407 |
| *Entamoeba invadens* | | A1 × 3, B | P | |
| *Entamoeba nuttallii* | | A1 × 2, B | P | |
| *Schizopyrenida* | | | | |
| *Naegleria fowleri* | | A1 | P | 418 |
| *Trichomonadida* | | | | |
| *Dientamoeba fragilis* | | A1 × 3 | P | 373, 374 |
| *Histomonas meleagridis* | | A1 × 6 | P | 376, 378 |
| *Pentatrichomonas hominis* | | A1 × 14 | P | 375 |
| *Tetratrichomonas gallinarum* | | A1 × 35 | P | |
| *Trichomonas gallinae* | | A1 × 5 | P | |
| *Trichomonas stableri* | | A1 × 3 | P | |
| *Trichomonas tenax* | | A1 × ? | P | 372 |
| *Trichomonas vaginalis* | | A1 × 13 | P | 363, 371, 393 |
| *Tritrichomonas foetus* | | A1 × 9, B | P | 76, 370, 455 |

[a]The list shows the phylogenetic lineages of the [NiFe]-hydrogenases and [FeFe]-hydrogenases encoded by the genomes based on the classification scheme shown in Table 1. These data are primarily derived from the hydrogenase database (HydDB) (24) but are also expanded with information from newly sequenced genomes. Based on this information and the wider literature, we can predict whether the organisms can mediate $H_2$ uptake (U), $H_2$ production (P), or both (U + P). Multiplication signs (×) are used when more than one copy of a hydrogenase subgroup is encoded in a genome. Question marks are used where activity cannot be predicted. References are only provided where hydrogenase activity has been experimentally demonstrated through physiological or biochemical studies. Note that listed microorganisms include both common human pathogens and occasional opportunistic pathogens. Strain-level differences are not accounted for, and organisms are classified based on NCBI taxonomy.

are a universal trait of bacterial and protist pathogens known to colonize the GIT, but they are also present in pathogens that target other niches (Fig. 3). The next two sections of the review provide deeper insights into $H_2$ metabolism of the referenced pathogens. As well as those with at least partially characterized $H_2$ metabolism, numerous bacterial pathogens harbor hydrogenase genes but that have not been studied concerning $H_2$ metabolism, at least based on our current knowledge. These

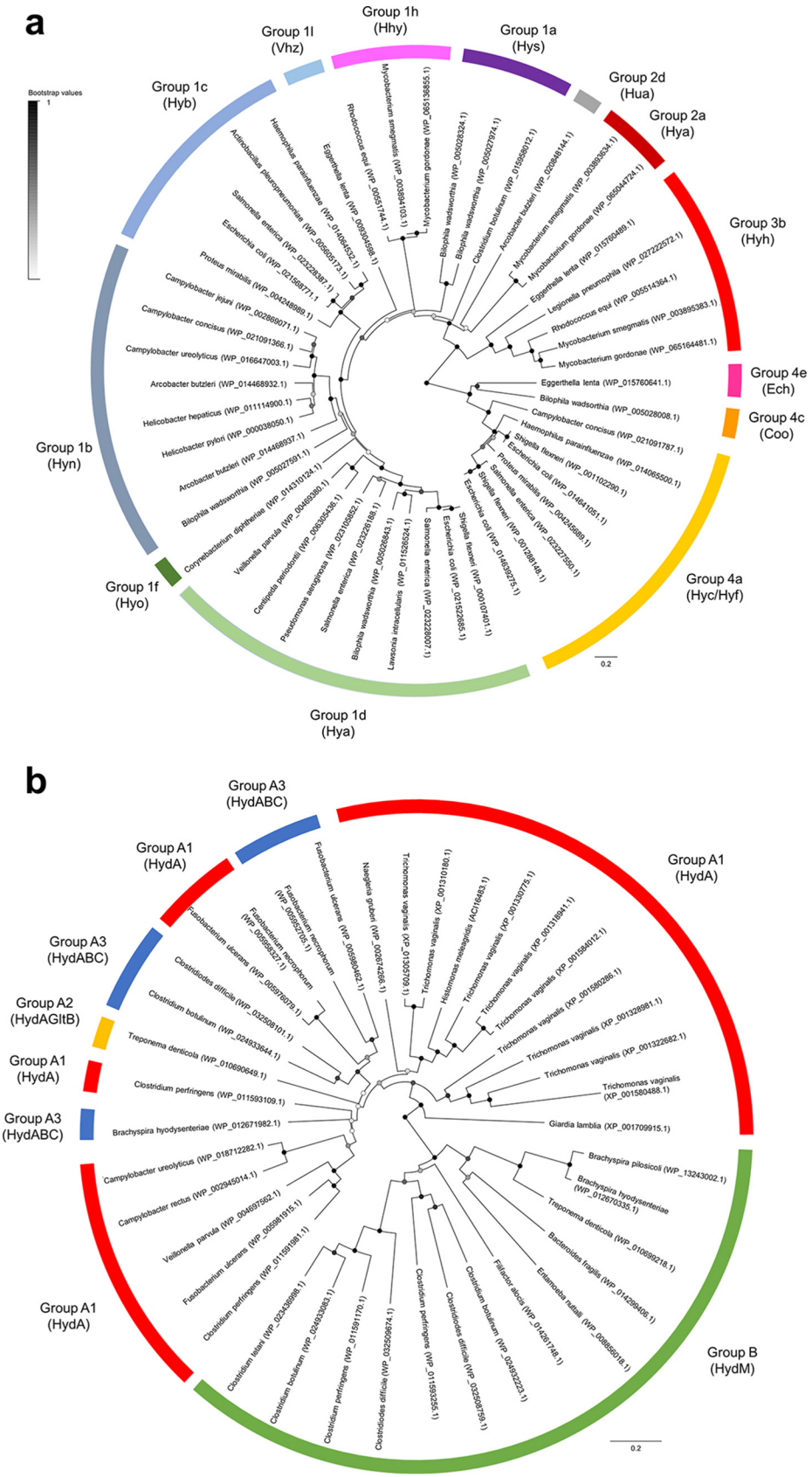


**FIG 4** Phylogenetic tree of hydrogenases from selected pathogens. The trees are based on the amino acid sequences of the catalytic subunits of [NiFe]-hydrogenases (a) and [FeFe]-hydrogenases (b). The trees were

(Continued on next page)

include the major pathogens *Corynebacterium diphtheriae*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Legionella pneumophila*, and *Brachyspira pilosicoli*. There is also a distinct paucity of literature relating to anaerobic bacterial pathogens, especially from the phyla *Spirochaetes*, *Fusobacteria*, *Bacteroidetes*, and *Firmicutes*, where $H_2$ metabolism is often assumed to occur but has been rarely measured or studied.

Based on our surveys, many major pathogens nevertheless lack hydrogenases. These include most pathogens that adopt an intracellular lifestyle, notably *Mycobacterium tuberculosis*, *Mycobacterium leprae*, *Chlamydia trachomatis*, and *Rickettsia rickettsii*, which have reduced genomes and, in turn, limited metabolic flexibility. Hydrogenases are also absent from major pathogens, such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Listeria monocytogenes*, *Bacillus anthracis*, *Bordetella pertussis*, *Neisseria meningitidis*, and *Mycoplasma pneumoniae*. The reason why these species lack hydrogenases is not clear. However, it is worth noting that several of them invade niches, such as the human respiratory mucosa, epidermis, or the brain, where $H_2$ levels might be more scarce than in other parts of the human body (Fig. 3). Among eukaryotes, hydrogenase genes are absent from the genomes of pathogenic fungi, trypanosomads, apicomplexans, and helminths. It should be noted that we did not incorporate the findings of a large-scale study reporting hydrogenase activity in anaerobic pathogens, given it is potentially unreliable (118); the assay used to detect hydrogenase activity is nonspecific, and potential false-positive activity has been detected in several organisms that do not encode hydrogenases (e.g., *S. aureus*). In the next sections, we focus on these findings and consider the broader distribution of $H_2$ metabolism, suggesting opportunities for further research to address these gaps in our knowledge.

## $H_2$ CONSUMPTION IN PATHOGENS

### *Campylobacterales*

*Helicobacteraceae* and *Campylobacteraceae* are the two families within the order *Campylobacterales*. An apparent ancestral trait of these families is the capacity to oxidize $H_2$ via membrane-bound respiratory hydrogenases (group 1b [NiFe]-hydrogenases). Note that the *Campylobacterales* hydrogenases belonging to group 1b are traditionally referred to as HydABC (9); however, according to HydDB, this group should be annotated HynABC to avoid confusion with an unrelated group of enzymes (group A3 [FeFe]-hydrogenases) (24). These Hyn enzymes, in addition to being encoded by various commensal and environmental strains, have been retained in various pathogens within these families (36). These include the major human pathogens *H. pylori* and *C. jejuni*. Nevertheless, the role of these enzymes significantly differs between species and even among different strains. Some species have also acquired additional hydrogenases, including those that support fermentative $H_2$ production (65, 78) (see "Other $H_2$-producing bacteria" below).

***Helicobacter pylori*: $H_2$-dependent PMF generation in the gastric mucosa.** *Helicobacter pylori* was the first pathogen to be shown to use $H_2$ during infection (9). This bacterium primarily colonizes the human gastric mucosa and is a major causative agent of gastric ulcers, chronic gastritis, and gastric cancers (119–121). As a microaerophilic bacterium, *H. pylori* is usually cultured in the presence of $CO_2$ (5 to 10%) and limited amounts of $O_2$ (2 to 10%). While $H_2$ is rarely added to gas mixtures, its addition causes an approximate doubling in growth yields in both complex and defined liquid media (69). Hydrogenase activity was first detected in *H. pylori* in 1996, a year before the genome sequence was released (64). Maier and colleagues detected $H_2$-uptake activity

**FIG 4** Legend (Continued)
constructed using the neighbor-joining method using 500 bootstrap replicates. For each sequence, the organism and protein accession number are shown. The colored rings show the subgroup that each hydrogenase affiliates with. Note that, for group A [FeFe]-hydrogenases, subgroup designation (A1, A2, and A3) is based on genetic organization rather than phylogeny; hence, there are multiple radiations of some subgroups.

**TABLE 3** Rates and affinities of $H_2$ uptake among various pathogenic bacteria[a]

| Organism (strain) | Uptake activity (nmol $H_2$ $min^{-1}$ [$10^9$ cells]$^{-1}$) | Apparent $K_m$ ($\mu$M) | Key reference |
|---|---|---|---|
| *Campylobacter concisus* (13826) | 113 ± 6 | NA | 78 |
| *Campylobacter concisus* (51562) | 199 ± 9 | NA | 78 |
| *Helicobacter pylori* (26695) | 33 ± 4 | 1.8 | 9 |
| *Helicobacter pylori* (43505) | 37 ± 2 | 1.8 | 9 |
| *Helicobacter hepaticus* (51449) | 3.2 ± 0.2 | 2.5 | 10 |
| *Salmonella* Typhimurium (14028s) | 12 ± 2 | 2.1 | 8 |
| *Shigella flexneri* | 68 ± 12 | NA | 251 |

[a]$H_2$ uptake activities are expressed as means ± standard deviations. All activities reported in this table were determined amperometrically with whole cells using $O_2$ provided as the terminal electron acceptor. NA, affinity not measured.

in whole cells of microaerobically grown *H. pylori* using oxygen as the terminal electron acceptor (Table 3). This activity was subsequently shown to be specifically associated with membrane fractions (64).

The genome sequence confirms the presence of a single group 1b [NiFe]-hydrogenase in *H. pylori* (36, 122). The three structural subunits of the hydrogenase (HynABC) are transcribed as part of the *hyn* operon (*hynABCDE*) (123) (Fig. 5). While the hydrogenase has not yet been purified, we can predict aspects of its interaction with the aerobic respiratory chain based on its behavior in whole cells and homology (~70% identity) with the well-characterized hydrogenase from the phylogenetically related species *Wolinella succinogenes* (124–126). As summarized in Fig. 6a, it is probable that the catalytic subunits of the hydrogenase are oriented toward the periplasm; thus, the oxidation of $H_2$ to protons generates PMF. Electrons derived from $H_2$ oxidation are transferred from the [NiFe] cofactor at the catalytic center of the large subunit (HynB) through the three [FeS] clusters of the small subunit (HynA) and to the heme of the membrane-bound cytochrome *b* subunit (HynC). It is predicted that the electrons are subsequently relayed from the cytochrome *b* subunit to the menaquinone pool. This model is consistent with the potent inhibition of hydrogenase activity by the quinone analog 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) (74). The electrons ultimately are used by the proton-pumping cytochrome $cbb_3$ oxidase to reduce $O_2$ (127–129). On this basis, aerobic hydrogenotrophic respiration by *H. pylori* should result in the net translocation of eight protons per $H_2$ molecule oxidized, although this remains to be proven. An outstanding question is how the *H. pylori* hydrogenase tolerates poisoning by $O_2$. Based on studies on related bacteria, the group 1b [NiFe]-hydrogenases are typically highly sensitive to oxygen (130, 131). However, *H. pylori* appears to have evolved cellular or molecular mechanisms to protect the enzyme from oxygen exposure and, hence, can use it under microoxic conditions.

Multiple proteins are required for the synthesis of the redox centers of the *H. pylori* hydrogenase. In common with other $H_2$-metabolizing bacteria, the genome has a six-cistron operon (*hypABCDEF*) whose gene products mediate synthesis of the [NiFe] cofactor. Genetic studies have shown that each protein is required for the manifestation of *H. pylori* hydrogenase activity (132, 133); unexpectedly, two of these gene products (HypA and HypB) were found to be involved in nickel mobilization for both hydrogenase and urease; hence, disruption of either gene causes pleiotropic phenotypes (132, 134–139). Two other proteins required for assembly, HynD and HynE, are encoded by the same operon as the structural subunits (133); the former is an endopeptidase specific for the hydrogenase catalytic subunit (i.e., HupD homolog), and the latter is a unique hypothetical protein potentially involved in nickel mobilization or periplasmic targeting (133, 140) (Fig. 5). Other components required for hydrogenase assembly include a series of nickel and iron transporters (133, 141–143) and the Nif system (NifS, NifU, and Nfu), which mediates [FeS] cluster assembly (144, 145). In addition, *H. pylori* possesses three histidine-rich proteins involved in nickel sequestration (146–148). Hpn and Hpn-2, which are both only found in gastric *Helicobacter* species, are multimeric

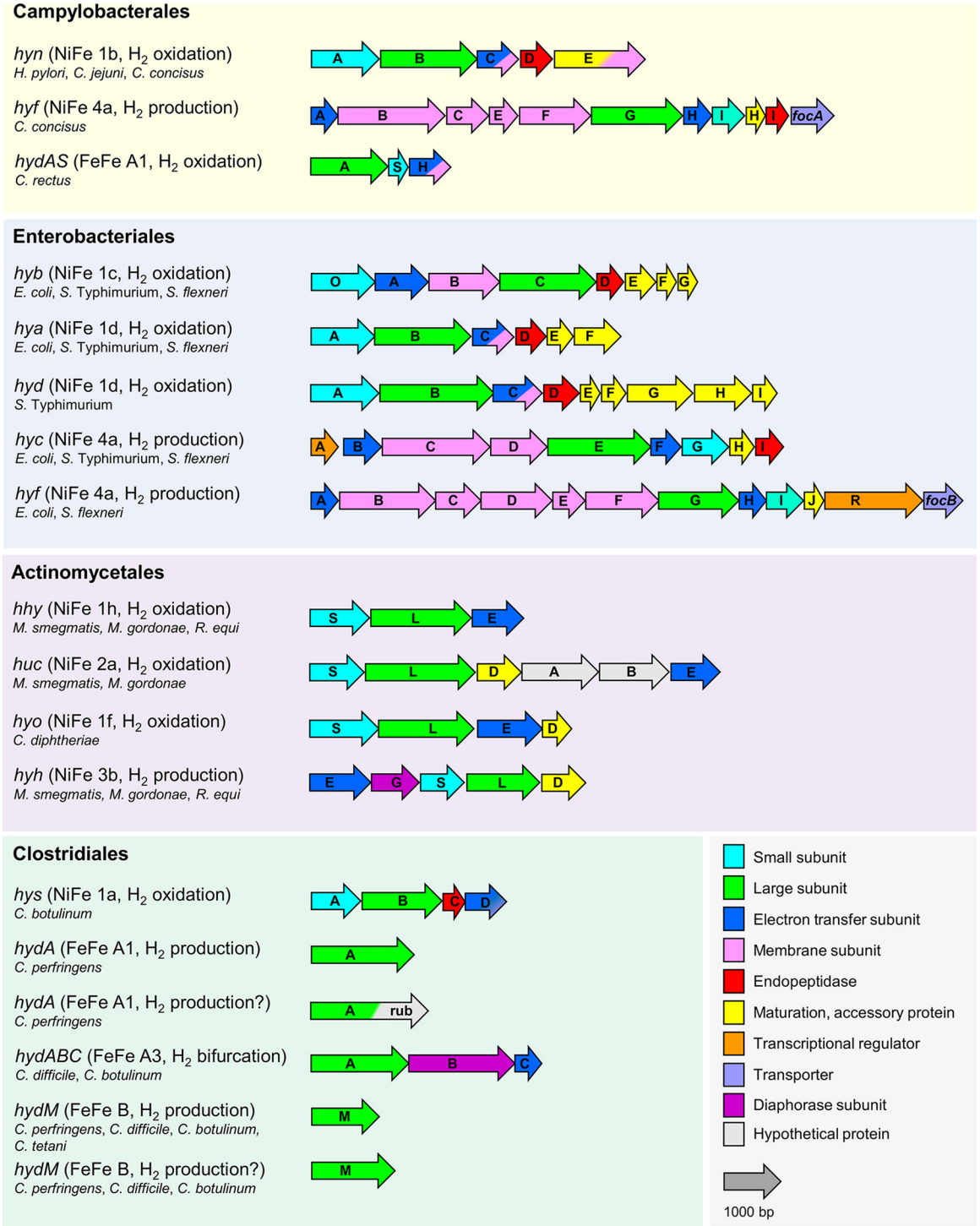


**FIG 5** Structure of the operons encoding hydrogenase structural subunits and associated proteins from selected pathogens. Each gene is shown to scale and is colored based on its predicted function per the legend in the bottom-right corner.

high-affinity nickel-binding proteins. The third nickel-binding protein, HspA, is related to the heat shock protein GroES but has a unique histidine-rich nickel-binding terminus. Knockout studies show all proteins are either required or important for hydrogenase and urease maturation (147, 149, 150). Based on the presence of a Tat (twin-arginine translocase)-dependent signal peptide on HynA, the assembled hydrogenase is thought to be translocated to the membrane (151). While the Tat system appears to be

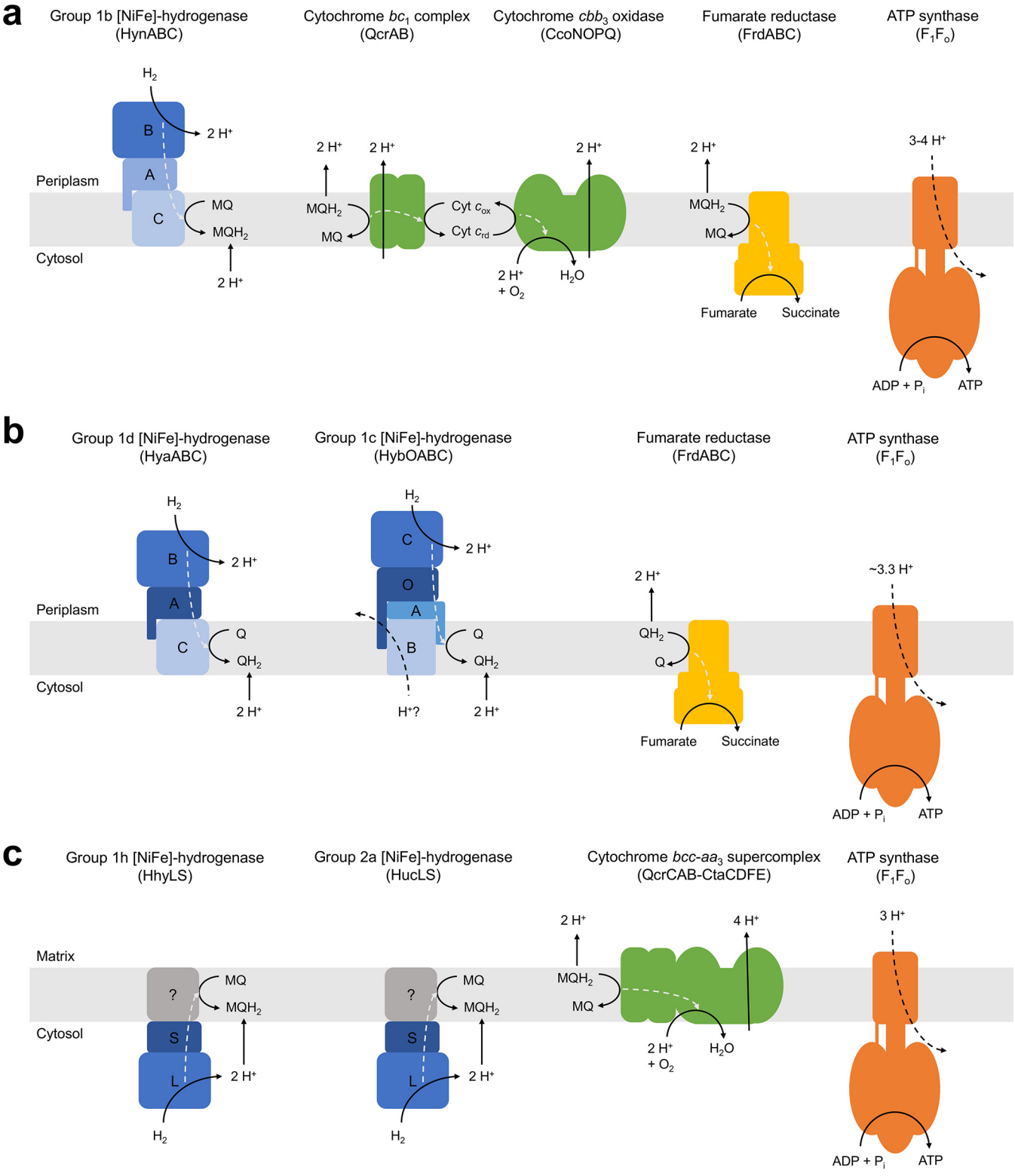


**FIG 6** Integration of hydrogenase into the respiratory chains of selected pathogens. (a) Model of $H_2$ oxidation by the Hyn [NiFe]-hydrogenase in three pathogens within the *Campylobacterales*, *Campylobacter jejuni*, *Helicobacter pylori*, and *Campylobacter concisus*. The [NiFe] center of the hydrogenase large subunit (HynB) oxidizes $H_2$ produced exogenously or, in *C. concisus*, through the formate hydrogenlyase reaction (*C. concisus* only). Electrons are relayed through the small subunit (HynA; via iron-sulfur clusters) and the membrane subunit (HynB; via a *b*-type cytochrome) to the menaquinone (MQ) pool. Electrons are transferred to the terminal electron acceptor $O_2$ (via cytochrome $cbb_3$ oxidase) or fumarate (*C. jejuni* only; via fumarate reductase). These processes theoretically lead to the net translocation of eight and four protons per $H_2$ molecule oxidized, respectively. Note that some pathogens, for example, *Campylobacter rectus*, instead oxidize $H_2$ using the [FeFe]-hydrogenase HydASH. (b) Model of $H_2$ oxidation by the Hya and Hyb [NiFe]-hydrogenases in three pathogens within the order *Enterobacteriales*, *Escherichia coli*, *Salmonella* Typhimurium, and *Shigella flexneri*. These bacteria oxidize $H_2$ produced either exogenously or endogenously

(Continued on next page)

essential in *H. pylori*, conditional *tatC* mutants have greatly reduced hydrogenase activity, supporting this contention (152).

Transcription of the *hyn* operon is controlled by various regulatory proteins in response to distinct stimuli. In axenic cultures, the structural genes encoding this hydrogenase are among the most strongly upregulated during the transition from the exponential to the stationary phase (153, 154). This suggests that $H_2$ oxidation facilitates persistence of this bacterium when other energy sources are limited. Synthesis of the hydrogenase is also induced following exposure to acidic pH levels equivalent to those found in the gastric mucosa (155). Consistent with the metal composition of the hydrogenase, transcription of the *hyn* operon is differentially regulated in response to both iron and nickel. This is mediated by the ferric uptake regulator (Fur) (156–158) and the nickel uptake regulator (NikR) (90, 159, 160). Indeed, the *hyn* operon is transcriptionally repressed by the apo (iron-free) form of Fur, meaning the presence or addition of $Fe^{2+}$ leads to increased transcription (156). In addition, the *hyn* operon is transcriptionally repressed by the nickel-specific regulator NikR (90, 160). Both the Fur and NikR transcription factors are central hubs of the *H. pylori* regulatory network and, thus, have pleiotropic roles and undergo extensive cross talk. There is also evidence, based on promoter-reporter fusions, that *hyn* transcription is stimulated by $H_2$ (9); however, given that *H. pylori* lacks a regulatory hydrogenase, it is unclear whether this induction is due to direct sensing of $H_2$ or indirect redox effects of this gas on cellular physiology.

Genetic studies have shown that gastric colonization of *H. pylori* depends on the hydrogenase. A mutant of *H. pylori* lacking the gene encoding the hydrogenase catalytic subunit (Δ*hynB* strain) was not nearly as efficient as the parental strain at colonizing the gastric mucosa of mice; only 24% of the mice inoculated with the mutant were colonized (9 of 38 mice) compared to 100% colonization for the wild type (37 of 37 mice) (9). Based on genome sequence analysis and hydrogenase assays, *H. pylori* is unable to produce $H_2$ and therefore must rely solely on exogeneous $H_2$ produced by gastrointestinal microbiota to conserve its energy (64, 122). Nevertheless, *H. pylori* is probably continuously exposed to saturating levels of $H_2$ throughout infection in the human stomach. Indeed, dissolved $H_2$ has been detected at high concentrations (average, 43 $\mu$M; range, 17 to 93 $\mu$M) in the stomach of live, anesthetized mice, and a substantial fraction of the $H_2$ produced by colonic bacteria is known to diffuse to the human stomach (5, 102, 111). Given that the apparent $K_m$ for $H_2$ of the hydrogenase in whole cells is approximately 1.8 $\mu$M, *H. pylori* is likely to be saturated with $H_2$ in host tissues. After colonization, $H_2$ oxidation may also energize persistence of *H. pylori* within the gastric mucosa, but this research area has yet to be systematically explored.

In recent years, $H_2$ oxidation by *H. pylori* has been implicated in the development of gastric cancer (74). CagA-positive *H. pylori* strains are strongly associated with an increased risk of developing adenocarcinoma of the stomach (120, 161). This reflects the fact that the CagA protein (cytotoxin-associated gene A), encoded by the Cag pathogenicity island (PAI), causes biochemical and morphological changes in gastric epithelial cells, which promote carcinogenesis. Briefly, CagA is delivered to gastric

**FIG 6** Legend (Continued)
by the formate hydrogenlyase reaction. Despite being from a distinct phylogenetic subgroup, the Hya hydrogenase has an architecture and mechanism similar to those of the Hyn hydrogenase. For the architecturally distinct Hyb hydrogenase, electrons are thought to be transferred through the large subunit (HybC; containing NiFe center), small subunit (HybO; containing iron-sulfur clusters), and an additional periplasmic subunit (HybA; containing a *b*-type cytochrome) to ubiquinone or menaquinone (Q). The membrane-anchoring subunit, HybB, does not participate in electron transfer, given that it lacks a cofactor; however, evidence suggests that it is proton motive. It is thought that electrons are primarily transferred to fumarate reductase under physiological conditions, but other terminal reductases are also known to support $H_2$ oxidation in laboratory experiments. This leads to the net translocation of at least four protons per $H_2$ molecule oxidized. (c) Model of $H_2$ oxidation by the Hhy and Huc hydrogenases within three pathogens in the *Actinomycetales*, *Mycobacterium smegmatis*, *Mycobacterium gordonae*, and *Rhodococcus equi*. These organisms oxidize $H_2$ available exogenously or endogenously through activity of the Hyh hydrogenase. Electrons are relayed through the cytosolically oriented large (HhyL and HucL) and small (HhyS and HucS) hydrogenase subunits to the menaquinone pool. Electrons then are transferred to the terminal electron acceptor $O_2$ via the proton-translocating cytochrome *bcc-aa*$_3$ supercomplex (6 $H^+$ translocated per $H_2$ molecule oxidized). Electrons can also be transferred from Hhy to the nontranslocating cytochrome *bd* oxidase (2 $H^+$ translocated per $H_2$ molecule oxidized). Note that Huc is absent from *R. equi*, and some pathogens, for example, *Corynebacterium diphtheriae*, encode the distinct hydrogenase HyoLSE. Note that other hydrogenotrophic respiratory chains are known, for example, the sulfite-reducing chains of *Bilophila wadsworthia*, but these are not sufficiently well understood to be depicted here.

epithelial cells by the bacterium's type IV secretion system (162), where it undergoes tyrosine phosphorylation within epithelial cells (163). Upon phosphorylation, it interacts with multiple host signaling molecules, including the pro-oncogenic phosphatase SHP2 (164, 165). The PMF generated through hydrogenotrophic aerobic respiration appears to drive CagA translocation. Wang et al. have shown that a carcinogenic strain with a greater ability to translocate CagA has higher hydrogenase activity than its noncarcinogenic parent (74). Concordantly, a Δ*hynABCDE* hydrogenase deletion mutant was unable to translocate CagA into human gastric epithelial AGS cells and did not induce gastric cancer in gerbils, while 50% of the animals infected with the wild-type strain (hydrogenase positive, CagA translocating) developed gastric cancers (74). In agreement with these results, significantly higher hydrogenase activity was measured in a series of *H. pylori* strains isolated from cancer patients compared to those measured in strains isolated from gastritis patients (74). Nevertheless, a wider sampling of clinical strains is needed to explore the correlations between hydrogenase activity and carcinogenesis.

Additionally, a recent study by Kuhns et al. found a link between $H_2$ utilization and $CO_2$ fixation in *H. pylori* (69). *H. pylori* can assimilate $CO_2$ in an ATP-dependent reaction using acetyl-coenzyme A (CoA) carboxylase (acetyl-CoA + $HCO_3^-$ + ATP → malonyl-CoA + ADP + $P_i$), and this enzyme has been correlated with the growth enhancement of the bacterium on elevated $CO_2$ (166). Proteomic studies revealed that the biotin carboxylase subunit of this enzyme is among the most highly induced proteins when $H_2$ is added to the medium. Likewise, there was a 3-fold increase in acetyl-CoA carboxylase activity and an increased uptake of radiolabeled $HCO_3^-$ in $H_2$-supplemented cultures (69). This indicates that ATP generated by aerobic hydrogenotrophic respiration energizes carbon fixation. Overall, this suggests that *H. pylori* is a mixotroph that can use $H_2$ and organic carbon as energy sources and $CO_2$ and organic compounds as carbon sources.

***Campylobacter jejuni*: niche expansion through hydrogenotrophic aerobic and anaerobic respiration.** *Campylobacter jejuni* is the principal causative agent of human gastroenteritis in developed countries. It resides in the GIT of many wild and domesticated animals but is most frequently transmitted through the handling and consumption of contaminated poultry (167). As recently reviewed (1, 168), this versatile pathogen can use a wide range of respiratory electron donors (e.g., NADH, $H_2$, formate, succinate, and sulfite) and electron acceptors (e.g., $O_2$, fumarate, nitrate, nitrite, and tetrathionate) (112, 169–171). This respiratory flexibility presumably allows the pathogen to maintain a membrane potential and, thus, viability in a range of host and environmental reservoirs. The genome of *C. jejuni* carries a set of genes for respiratory hydrogen oxidation similar to those used by *H. pylori* (85) (Fig. 5). These include the structural subunits of the group 1b [NiFe]-hydrogenase (*hynABC*) (12), a complete set of genes encoding hydrogenase maturation factors (*hynD* and *hypFBCDEA*), and those encoding a Ni-uptake ABC transporter (*nikZYXWV*) (172). Strong benzyl viologen-linked hydrogenase activity has been measured in *C. jejuni* whole cells (173). Consistent with their respective annotation, mutagenesis of the *hydB* structural gene or *nikZ*, which encodes the periplasmic nickel-binding protein, abolished hydrogenase activity in this strain (12, 172). It is also established that the hydrogenase is targeted to the cytoplasmic membrane in a Tat-dependent manner (174).

Several *in vitro* studies have demonstrated that $H_2$ is a major electron donor for *C. jejuni*. In a seminal study, Carlone and Laschelles demonstrated in 1982 that $H_2$ supplementation enhanced growth of *C. jejuni* strain C-61 (169). The strain grew optimally when incubated with agitation under an atmosphere of 30% $H_2$, 5% $O_2$, and 10% $CO_2$, with formate and fumarate also enhancing growth (12, 169). Subsequent studies have verified that this growth stimulation is hydrogenase dependent (12). Furthermore, respirometry studies have shown that $H_2$ oxidation can support both aerobic respiration and fumarate reduction in this strain (169, 175) (Fig. 6). In fact, oxygen consumption in membrane vesicles is 50- to 100-fold higher with $H_2$ or formate as the substrate than with NADH or succinate (175). Little is known about how hydrogenase synthesis is regulated in this organism, but it has been shown that

environmental cues such as oxygen deprivation and acidic shock induce expression (176, 177). The synthesis and activity of the hydrogenase, together with the formate dehydrogenase and alternative terminal reductases, are particularly high under microaerophilic conditions (177, 178). Altogether, this suggests that *C. jejuni* can adapt to a wide range of environments through a combination of hydrogenotrophic aerobic and anaerobic respiration.

Several studies have also indicated that $H_2$ oxidation is important for virulence of *C. jejuni*. Using a galline model of infection, Weerakoon and colleagues showed that strains carrying a mutation in genes encoding hydrogenase (Δ*hydB*) or formate dehydrogenase (Δ*fdhA*) colonized ceca at reduced rates compared to those of wild-type strains. While differences were modest for single mutants, a severe colonization deficiency was observed for the Δ*hydB* Δ*fdhA* double-null mutant (12). Thus, the authors concluded that while the loss of either the hydrogenase or the formate dehydrogenase can be compensated by the presence of the other enzyme, both $H_2$ and formate are important electron donors, and at least one of them needs to be present for normal colonization efficiency. In other work, it was shown that a *hydB* deletion renders *C. jejuni* unable to interact either with human intestinal cell lines (INT-407) or with primary chicken intestinal epithelial cells; cell division and morphology were also affected (179). Transcriptome profiling has confirmed that the structural and maturation genes are expressed during colonization (86), and it was recently observed that certain maturation factors are highly upregulated during human infection (180).

Nevertheless, having hydrogenase among the respiratory repertoire of a pathogen does not necessarily mean better host colonization capacity. For instance, a study by Hiett and colleagues, aimed at comparing genomic and proteomic differences between a robust chicken gastrointestinal colonizer (strain A74/C) and a weak colonizer (reference strain NCTC1168), found that the hydrogenase large subunit was absent from the former (181). Since all results point to the importance of $H_2$ uptake in *C. jejuni* metabolism and virulence, the absence of hydrogenase in the A74/C strain is probably compensated by the presence of other respiratory complexes, as discussed above. In agreement with this hypothesis, A74/C but not NCTC1168 carries genes for a putative dimethyl sulfoxide (DMSO) reductase, which could account for the robust colonizer phenotype (181). The fact that *C. jejuni* can access more respiratory electron donors than *H. pylori* (1) suggests it is less heavily reliant on $H_2$.

***Campylobacter concisus*: essentiality of uptake hydrogenases for growth.** In most pathogens investigated to date, $H_2$ uptake is important but not essential for growth. *C. concisus* was recently reported to be an exception (78). First isolated from a patient with gingivitis (182), this bacterium has since been shown to commonly inhabit the human oral cavity and GIT (183–185). Its presence has been tentatively associated with a range of other diseases and ailments, including periodontitis, enteritis, inflammatory bowel diseases, and Barrett's esophagus syndrome (186, 187). Since its isolation, it has been known that this bacterium grows using $H_2$ as an energy source (182), and it has since become standard practice to isolate and grow *C. concisus* strains on $H_2$-enriched microoxic gas mixtures (183). Interestingly, while the bacterium respires a wide range of electron acceptors (78), $H_2$ is always critical for growth: it is required under microoxic conditions and greatly enhances yields under anoxic conditions (78, 188). Consistent with this, whole-cell hydrogenase assays have revealed that *C. concisus* has the highest $H_2$-uptake hydrogenase activity measured among pathogenic bacteria (Table 3). Under $H_2$-replete conditions, there are higher levels of proteins associated with the growth-related processes of protein synthesis (elongation factor EF-Tu) and nutrient transport (various outer membrane proteins) (78).

The essentiality of $H_2$ uptake has recently been inferred genetically. In contrast to the previously discussed *Campylobacterales* (*H. pylori* and *C. jejuni*), genome sequencing has revealed that *C. concisus* encodes two distinct hydrogenases (36, 189) (Fig. 5). The *hyn* operon encodes an $H_2$-consuming respiratory hydrogenase (group 1b [NiFe]-hydrogenase) closely related to those of *C. jejuni* and *H. pylori*. The *hyf* operon encodes an $H_2$-producing formate hydrogenlyase (FHL) complex (group 4a [NiFe]-hydrogenase)

similar to that of *E. coli*. Whereas *hyf* genes could be deleted, attempts to delete the *hyn* genes failed under a range of growth conditions, suggesting the respiratory hydrogenase is essential. Consistent with this hypothesis, attempts to delete the *hypE* gene required for the synthesis of the catalytic centers of both hydrogenases also failed (78). In conjunction with the growth data, this strongly suggests that $H_2$ uptake is essential for viability of this organism. The ability of the bacterium to endogenously generate $H_2$ through the formate hydrogenlyase complex might explain why exogenous $H_2$ is not required for growth under anoxic conditions (78, 188). Nevertheless, the essentiality of $H_2$ for *C. concisus* is still not well understood and will require further studies, especially given that the pathogen encodes primary dehydrogenases to use alternative electron donors (e.g., formate).

### Other *Campylobacterales*

In addition to *H. pylori*, hydrogenase genes have been widely detected in the genomes of other *Helicobacter* species (Table 2). These include both gastric strains (e.g., *H. suis*, *H. bizzozeronii*, *H. heilmannii*, and *H. felis*) and enterohepatic strains (e.g., *H. cinaedi*, *H. fennelliae*, *H. bilis*, and *H. canis*), primarily of zoonotic origin (190–192). The murine enterohepatic pathogen *H. hepaticus* (193, 194) is the only other species where $H_2$ metabolism has been comprehensively studied. In common with *H. pylori*, whole cells of this bacterium contain a membrane-bound hydrogenase (195–197) that is kinetically adapted to high concentrations of $H_2$ ($K_m = 2.5\ \mu M$) (10). It couples $H_2$ uptake to oxygen (10) or nitrate reduction (R. J. Maier, unpublished data), enhancing growth in an $H_2$-rich atmosphere (71). While mutant strains lacking this hydrogenase (Δ*hyaB*) efficiently colonized the liver and cecum of A/JCr mice, they did not produce the liver lesions (lymphoplasmacytic hepatitis with hepatocytic coagulative necrosis) observed in mice infected with the wild-type strain (71). Labeling experiments demonstrated that the hydrogenase facilitates amino acid transport in this strain by generating a PMF (71). While the role of $H_2$ in other *Helicobacter* species is not yet known, the conservation of $H_2$-uptake hydrogenase genes suggests it is central to metabolism and pathogenesis. Therefore, the use of an $H_2$-enriched atmosphere to increase the likelihood of recovering *Helicobacter* species from biopsy specimens and other samples has become a clinical best practice (198).

Other than *C. concisus* and *C. jejuni*, a wide range of other *Campylobacter* species also encode uptake hydrogenases (Table 2). For example, the zoonotic pathogen *Campylobacter sputorum* grows optimally under $H_2$-enriched microaerophilic conditions (199) and harbors the activity of a respiratory [NiFe]-hydrogenase (200). In an interesting exception among hydrogenotrophs, the periodontal pathogen *Campylobacter rectus* (182) lacks a [NiFe]-hydrogenase and instead harbors an [FeFe]-hydrogenase with an unusual genetic organization (36) (Fig. 5). This pathogen supports growth on $H_2$ and can couple $H_2$ oxidation to the reduction of fumarate, nitrate, and elemental sulfur (65, 201). Despite [FeFe]-hydrogenases typically being associated with $H_2$ production, some are catalytically biased toward $H_2$ oxidation (52, 202). The structural proteins for this hydrogenase include a small subunit with a Tat signal peptide and a cytochrome *b* subunit predicted to relay electrons into the respiratory chain. Several strains, notably *Campylobacter ureolyticus* and *Campylobacter fetus*, encode both [NiFe]- and [FeFe]-hydrogenases. Further studies are required to distinguish these hydrogenases concerning their catalytic activities and physiological functions.

Finally, uptake hydrogenases are widespread in *Arcobacter* species, including the emerging human pathogens *A. butzleri*, *A. cryaerophilus*, and *A. skirrowii* (203). *A. butzleri* encodes two respiratory hydrogenases (group 1b [NiFe]-hydrogenases) and another of unknown function (group 2d [NiFe]-hydrogenase). The uptake hydrogenases are encoded in adjacent loci, suggesting that gene duplications happened at some point in the evolutionary history of this species (204). Although $H_2$ metabolism has yet to be studied in this organism, the presence of multiple $H_2$-uptake enzymes may enable the organism to oxidize $H_2$ efficiently across the range of concentrations encountered in the various niches (animal, human, and environmental) that they inhabit.

### *Enterobacteriales*

The *Enterobacteriales* include $H_2$-metabolizing pathogens from four currently recognized families, *Enterobacteriaceae*, *Aeromonadaceae*, *Pasteurellaceae*, and *Vibrionaceae*. Of these, by far the most intensively studied are *E. coli* and *S.* Typhimurium from the *Enterobacteriaceae*. Both *E. coli* and *S.* Typhimurium have become model systems for studying $H_2$ uptake in general and medical contexts, respectively (23, 205). These organisms, as well as many other members of the *Enterobacteriales*, encode two classes of uptake hydrogenases (group 1c [NiFe]-hydrogenase and group 1d [NiFe]-hydrogenase) with distinct properties and physiological roles (36, 206).

***Escherichia coli*: insights from a metabolically flexible model organism.** A wide range of studies has investigated the genetics, physiology, regulation, maturation, biochemistry, and structural biology of *E. coli* hydrogenases (23, 205). In contrast to *H. pylori* and *C. jejuni*, which each encode a single hydrogenase, *E. coli* encodes four hydrogenases (Fig. 5): two $H_2$-oxidizing enzymes (Hya and Hyb) (207, 208), which are discussed below, and two $H_2$-producing enzymes (Hyc and Hyf) (77, 209), which are discussed in "*Escherichia coli* and *Salmonella* Typhimurium: formate-dependent $H_2$ production by [NiFe]-hydrogenases" below. Although most *E. coli* strains do not cause illness, there are several pathogenic strains (pathotypes) associated with diarrhea, urinary tract infections, bloodstream infections, and meningitis (210, 211). As far as is known, all physiological knowledge gathered on *E. coli* hydrogenases comes from studies on nonpathogenic strains (primarily the laboratory workhorse K-12), and no study has linked $H_2$ metabolism to *E. coli* pathogenicity (23). However, given that hydrogenases are highly conserved in pathogenic strains and closely related *Enterobacteriaceae*, knowledge derived from these studies has proven useful for understanding the role and basis of $H_2$ metabolism in pathogens.

The two uptake hydrogenases of *E. coli* share some similarities but also many differences. In common with the *H. pylori* and *C. jejuni* enzymes, both are periplasmically oriented, membrane-bound enzymes that liberate protons in the periplasm and transfer electrons derived from $H_2$ oxidation into the anaerobic respiratory chain (212, 213) (Fig. 6b). However, the enzymes are divergent at the primary sequence level (~43% sequence identity) and affiliate with distinct [NiFe]-hydrogenase subgroups (group 1d for Hya, also known as Hyd-1; group 1c for Hyb, also known as Hyd-2) (36) (Table 1). Moreover, they differ in subunit composition: whereas Hya is a heterotrimeric enzyme containing a cytochrome *b* anchor (212), Hyb is a tetrameric enzyme with a proton-translocating subunit (213). These differences are reflected in the catalytic behavior of the hydrogenases. Pioneering electrochemical work from Lukey and colleagues shows that Hya operates optimally within a relatively high redox potential range (+50 to +150 mV) in a strictly oxidative direction (206). In contrast, Hyb functions optimally at lower redox potentials (−200 to −100 mV) and even mediates significant $H_2$ production under reducing conditions (206, 214). As elaborated below, this distinct behavior reflects the contrasting structural features of the enzymes and likely is relevant for the adaptation of *E. coli* to different environmental conditions.

The physiological role of Hya has remained controversial. Somewhat paradoxically, the enzyme is highly tolerant toward oxygen (215) and can even support hydrogen-driven aerobic respiration in membrane preparations (216–220), yet its synthesis is optimal in anoxic stationary-phase cultures (87, 221, 222). The enzyme potentially maintains redox homeostasis in response to changes in energy and oxidant availability during transitions to and from stationary phase (23, 205, 223). As recently reviewed (23), transcription of the *hya* operon (*hyaABCDEF*) (224, 225) is controlled by a network of regulators; it is activated by both the redox-sensing two-component system ArcAB and the stationary-phase sigma factor RpoS (87, 221, 222). The operon encodes the three structural subunits of the enzyme (212), a specific endopeptidase (HyaD), and two hypothetical proteins required for Tat translocation (226) (Fig. 5). While its biological function remains enigmatic, Hya is the best-characterized hydrogenase from a structural perspective among pathogens. Periplasmically oriented large subunits (HyaB) and

small subunits (HyaA) form a 2:1 complex with a membrane-bound cytochrome *b* anchor (HyaC) (Fig. 6b). As with other group 1d [NiFe]-hydrogenases (36, 131), the small subunit contains an unusual proximal [4Fe3S] cluster, coordinated by six cysteinyl residues. This cluster enables reactivation of an $O_2$-inhibited active site of the enzyme (212, 215) through a reverse electron flow mechanism, as detailed elsewhere (44).

The physiological role of Hyb is better understood. The enzyme primarily sustains anaerobic hydrogenotrophic growth of *E. coli* using fumarate as an electron acceptor (77, 214, 219, 227). It is thought that this hydrogenase can also generate PMF by coupling electron transfer to vectorial proton translocation via its transmembrane subunit (213, 214, 228). On some fermentable substrates, this complex can also act in reverse as a PMF-driven quinol-dependent proton reductase in a process thought to counterbalance an overreduced redox state of the quinone pool (206, 214). The transcription of the *hyb* operon (*hybOABCDEFG*) (229) (Fig. 5) is induced in response to carbon limitation and anaerobiosis (87). The enzyme contains four structural components: the large subunit (HybC), the small subunit (HybO), a ferredoxin-like protein (HybA), and the proton-pumping transmembrane subunit (HybB) (213, 230) (Fig. 6b). The crystal structures of the large and small subunits of the hydrogenase were recently solved, but it currently remains unclear how this enzyme couples electron transfer to proton translocation (213). The *hyb* operon also encodes a specific endopeptidase (HybD) (231), a Tat-targeting chaperone (226, 232), and isoforms of the maturation proteins HypA (HybF) (233) and HypC (HybG) (234).

***Salmonella* Typhimurium: differential roles of hydrogenases during infection.** Of all pathogens, we have the most sophisticated understanding of $H_2$ metabolism in the major foodborne enteric pathogen *S.* Typhimurium. This reflects the synergy achieved through *in vitro* and *in vivo* physiological studies, combined with biochemical characterization of purified enzymes. Like *E. coli*, four hydrogenases are encoded in the genome of *S.* Typhimurium (8, 235). Three are homologs of Hya, Hyb, and Hyc (236–238). However, Hyf is absent from the genome and a third uptake hydrogenase, Hyd, is present instead (82) (Fig. 5). Thus, the bacterium contains three respiratory hydrogenases and one fermentative hydrogenase. An equivalent set of genes is also found in the genomes of *Salmonella* Typhi, the causative agent of typhoid fever, among other serotypes (239). Together with collaborators, we have shed some light on the respective roles of the enzymes in *S.* Typhimurium through work with pure cultures and murine models. This was achieved using reporter gene fusions to measure gene expression and by constructing mutant strains to compare activities and phenotypes of the enzymes with those of the wild-type strain (8, 13, 22, 80, 81, 83, 84). A summary of the roles and regulation of each enzyme, based on these studies, is provided in Table 4.

Culture-based studies have provided strong insights into the physiological roles of the uptake hydrogenases in *S.* Typhimurium (23). Genetic dissection shows all three enzymes support hydrogenotrophic respiration (8), and a triple mutant lacking these hydrogenases is devoid of $H_2$-oxidizing activity (8, 84). In contrast to *E. coli*, a clear physiological role can be attributed to Hya: it consumes exogenously available or endogenously produced $H_2$ during fermentative conditions when respiratory electron acceptors are available (22, 84). It also contributes to acid resistance (22). In contrast, Hyb is the dominant enzyme during anaerobic growth and couples to either fumarate, trimethylamine N-oxide (TMAO), or dimethyl sulfoxide (DMSO) as respiratory electron acceptors (83). Consistent with this, $H_2$ supplementation significantly enhances the growth rate and yield of *S.* Typhimurium on low-nutrient media (83). Based on transcriptome studies, the PMF generated from Hyb activity is also thought to energize uptake of various nutrients, including the major serum organic acid glucarate (72, 73). In line with these roles, expression of the genes encoding both Hya and Hyb is induced under anaerobiosis and appears to be regulated either directly or indirectly by the oxygen sensor FNR and redox sensor ArcA (240, 241). Hyb is also subject to catabolite repression by the cyclic AMP (cAMP) receptor protein (CRP) (89), suggesting *S.* Typhi-

**TABLE 4** Summary of the expression and role of the four hydrogenases in *Salmonella* Typhimurium[a]

| Hydrogenase | Feature(s) | Reference(s) |
|---|---|---|
| Hya (Hyd-1) | Expressed during fermentative survival and regulated by FNR, ArcA | 80, 240 |
| | Recycles exogenous and endogenous $H_2$ under fermentative conditions | 81, 84 |
| | Important for acid resistance and macrophage colonization | 81 |
| | Expressed at low levels in liver, spleen, and ileum in mice | 81 |
| Hyb (Hyd-2) | Expressed during anaerobic growth and regulated by FNR, ArcA, CRP | 80, 89, 240 |
| | Supports growth by hydrogenotrophic fumarate respiration | 83, 238 |
| | Expressed in phagocytes and likely gastrointestinal tract | 81 |
| | Uses microbiota-derived $H_2$ to invade gastrointestinal tract in mice | 13, 248, 456 |
| Hyd (Hyd-5) | Expressed under aerobic growth and repressed by ArcA | 8, 81 |
| | Mediates hydrogenotrophic aerobic respiration and is oxygen tolerant | 8, 82, 243 |
| | Strongly expressed in macrophages | 81 |
| | Expressed early in liver and spleen, later in ileum of mice | 81 |
| Hyc (Hyd-3) | Expressed during fermentative survival and regulated by FNR, FhlA | 238, 240 |
| | Forms formate hydrogen lyase complex that produces $H_2$ | 237, 238 |
| | Important for anaerobic acid resistance | 84 |
| | Not required for colonization in murine model | 84 |

[a]Findings are based on studies in pure culture and mouse models.

murium uses $H_2$ to supplement its energetic demand when preferred organic energy sources are limiting (Fig. 7).

The unique hydrogenase in *S.* Typhimurium, traditionally called Hyd or Hyd-5, is strongly linked to supporting aerobic hydrogenotrophic growth. As a group 1d [NiFe]-hydrogenase (36), the enzyme is closely related to Hya, has similar biochemical properties, and can even be matured by the same endopeptidase (242). The overriding factor that differentiates Hyd from Hya, however, is that they are differentially synthesized in oxic and anoxic conditions: *hya* expression is induced during fermentative growth, whereas *hyd* is optimally expressed during oxic growth and is subject to anoxic repression by ArcA (80) (Fig. 7). Biochemical and electrochemical characterization of purified Hyd confirms that it is a highly $O_2$-tolerant uptake hydrogenase (82). Moreover, structural characterization confirms that it contains various adaptations associated with oxygen tolerance, including the characteristic proximal [4Fe3S] cluster coordinated by six cysteinyl residues in its small, electron transfer subunit (243) (Fig. 1a). The operon encoding this enzyme (*hydABCDEFGHI*) encodes several accessory proteins essential for hydrogenase maturation (244); these include two proteins implicated in synthesizing

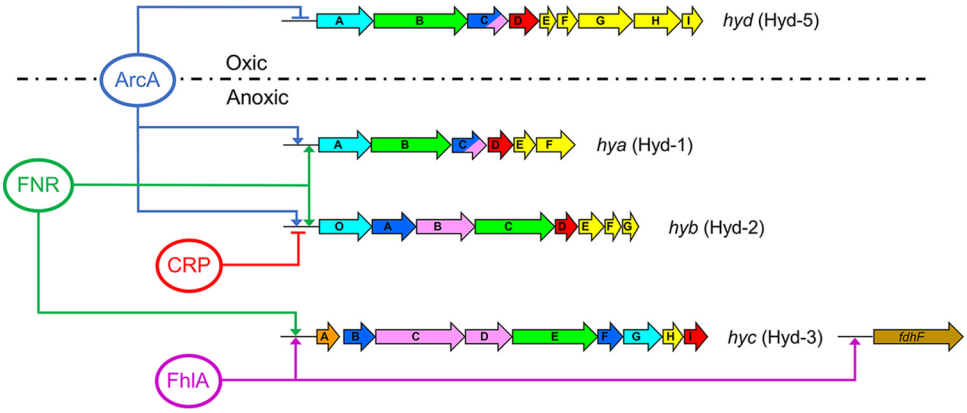


**FIG 7** Regulation of hydrogenase operon expression in *Salmonella* Typhimurium in response to $O_2$. The four hydrogenase operons are shown and have the same color coding as that shown in Fig. 5. Four regulators are shown: the redox sensor ArcA, the oxygen sensor FNR, the cAMP-binding protein CRP, and the formate sensor FhlA. Positive regulation by the FNR, ArcA, or FhlA transcription factor is indicated by arrows, while negative regulation by CRP and ArcA is indicated by lines ending in a turnstile (T). The horizontal dashed line depicts the aerobic-anaerobic interface.

the [4Fe3S] cluster under oxic conditions (244–246) (Fig. 5). Thus, whereas Hya is an oxygen-tolerant enzyme operating under anoxic conditions, the activity of Hyd is both oxygen tolerant and oxygen dependent.

Four research groups have independently demonstrated that hydrogen uptake is central to the virulence of *S*. Typhimurium (8, 13, 247, 248). In 2004, a study found that double and triple mutants of the uptake hydrogenases had reduced virulence in mice. In fact, the triple mutant was completely avirulent and was rapidly cleared from tissues (8). Craig et al. also observed a severe attenuation of the triple mutant (247). Also supporting these findings, it has been observed through resolvase *in vivo* expression technology (RIVET) that *hya* and *hyd* are differentially expressed in organs during mouse infection (81). Reflecting their distinct but overlapping roles, single hydrogenase mutants are also profoundly impaired in survival under some conditions. The Δ*hya* strain is unable to colonize murine macrophages, perhaps reflecting its importance for acid tolerance (81). In contrast, the Δ*hyb* strain is highly defective in colonization of mice. During competitive infection experiments, this strain grew 100-fold more slowly than the wild type and had considerably reduced bacterial loads in the cecum, spleen, and liver (13, 248). Moreover, hydrogenase mutants are highly defective in distal gut invasion and fecal shedding, thereby limiting host-to-host transmission (248). Altogether, these findings suggest that *S*. Typhimurium coutilizes organic compounds with hydrogen to meet its energy demands during colonization.

It is now recognized that gastrointestinal colonization of *S*. Typhimurium depends on interactions with $H_2$-metabolizing commensal microbiota. In mouse models, this bacterium primarily consumes $H_2$ from exogenous sources (i.e., commensal microbiota) rather than from endogenous sources (i.e., FHL reaction) (13, 84). This requires that the bacterium simultaneously exploits $H_2$ producers and outcompetes other $H_2$ consumers in the intestinal tract (elaborated in "Ecology: subversion of gastrointestinal microbiota" above) (Fig. 2). Consistent with these findings, this strain fails to colonize mice if the $H_2$ supply is disrupted either by antibiotic treatment (presumably removing hydrogenogens) or through inoculation of a nonpathogenic hydrogenotrophic strain (possibly through competitive exclusion) (13). This is part of a wider array of approaches that *S*. Typhimurium uses to acquire electron donors and acceptors from the host and the microbiota for expansion within the mammalian intestine (95). These findings emphasize that unraveling microbiota-pathogen metabolic interactions is critical for understanding pathogenesis and may provide options for preventing or treating infections.

***Shigella flexneri*: conditional essentiality of an uptake hydrogenase.** *S. flexneri*, a major cause of diarrhea, especially in the developing world (249), encodes the same set of four hydrogenases as *E. coli* (Hya, Hyb, Hyc, and Hyf) (36, 250). However, knockout studies have revealed that the physiological roles of the uptake enzymes differ between the two organisms. McNorton and Maier showed that the Hya enzyme is the dominant $H_2$-uptake enzyme in *S. flexneri*. Following anaerobic growth, a Δ*hya* mutant did not consume $H_2$, whereas the Δ*hyb* mutant strain rapidly consumed $H_2$ at levels indistinguishable from that of the wild-type strain (251). Loss of $H_2$ oxidation profoundly affects the bioenergetics of *S. flexneri*. Based on fluorescence measurements, the membrane potential of the Δ*hya* strain is approximately 15 times lower than that of the wild type and similar to that of cells treated with the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (251).

Consistent with this profound difference in energetic parameters, the hydrogenase mutant fails to persist under stressful conditions. Following acid shock (pH 2.5), CFU counts for the Δ*hya* strain decreased by 7 orders of magnitude within 6 h. In contrast, the wild type was highly tolerant of acid shock and increased rates of $H_2$ oxidation to compensate (251). Under anaerobic conditions, the hydrogenase-negative mutant was even more acid sensitive than mutants of the acid-combating glutamate-dependent acid resistance (GDAR) pathway involved in removing intracellular protons (252). While the mechanism underlying this phenotype is unclear, the authors proposed that the periplasmic deposition of protons by the hydrogenase ($H_2 \rightarrow 2\ H^+$) helps to resist proton influx from outside the cell or maintain a membrane potential between the

periplasm and cytosol (251). Performing this acid-combating function is critical for *S. flexneri*, as the bacterium encounters extreme acid conditions after ingestion by macrophages in the colon (253). These observations are also consistent with the increased acid sensitivity observed for the *S.* Typhimurium Δ*hya* mutant strain (81).

### Other *Enterobacteriales*

As summarized in Table 1, a wide range of other *Enterobacteriaceae* encode uptake hydrogenases. Whereas Hyb is distributed in most of these pathogens, Hya is only encoded in six genera and always together with Hyb. This is consistent with the finding that Hyb is the major uptake hydrogenase in both *E. coli* and *S.* Typhimurium (23). Several other species have been experimentally shown to support hydrogenotrophic anaerobic growth, including *Klebsiella pneumoniae* (254), *Citrobacter freundii* (255), *Proteus mirabilis* (256), and *Proteus vulgaris* (257). There is also indirect evidence that $H_2$ metabolism is important for the zoonotic pathogen *Edwardsiella tarda* (258, 259); deletion of a putative hydrogenase maturation factor, Sip2, caused loss of virulence, acid resistance, serum survival, and intracellular replication (259).

The hydrogenases of *Proteus*, a genus frequently associated with nosocomial urinary tract infections (260), are of special interest. The genomes of these pathogens each encode just two hydrogenases (261), namely, a Hyb-type uptake hydrogenase and a Hyf-type $H_2$-evolving hydrogenase (36), both of which have been shown to be active in whole cells (75, 257, 262). Both *P. mirabilis* and *P. vulgaris* support hydrogenotrophic growth using fumarate derived from either the tricarboxylic acid or urea cycles (75, 257). In both species, the $H_2$-uptake hydrogenases responsible have been purified from membrane fractions and biochemically characterized (256, 263, 264). More recently, it was demonstrated that hydrogenotrophic fumarate respiration supports the energetically demanding process of swarming motility. Transposon mutants in the hydrogenase subunit *hybB*, together with genes involved in fumarate production, were defective in motility. This suggests that hydrogenase activity maintains a sufficient PMF to drive the flagellar motor (75). These findings are likely to be clinically important, given swarming underlies the ability of *P. mirabilis* to colonize medical devices (e.g., catheters) and invade the human urinary tract (265). Although the presence of $H_2$ in the urinary tract has not been formally established to our knowledge, it is likely that some of the gas produced by the colonic microbiota can diffuse to this niche (Fig. 3), thereby being available for consumption by *P. mirabilis*.

In common with species from the *Enterobacteriaceae* family, many pathogens within the *Pasteurellaceae* and *Aeromonadaceae* also encode group 1c [NiFe]-hydrogenases, including *Actinobacillus pleuropneumoniae*, *Aggregatibacter actinomycetemcomitans*, *Haemophilus haemolyticus*, *Pasteurella bettyae*, and *Aeromonas hydrophila* (36). To our knowledge, they have only been studied in the major porcine pathogen *A. pleuropneumoniae*. The hydrogenase is expressed in cell culture under anoxic conditions in an FNR-dependent manner (266) and has also been detected during acute infection (267, 268), although mutational studies indicate the enzyme is dispensable for virulence (269, 270).

### Other Bacteria

As summarized in Table 2, a wide range of other pathogens beyond the *Enterobacteriales* and *Campylobacterales* also encode putative $H_2$-uptake hydrogenases. Several obligately anaerobic pathogens have been shown to mediate $H_2$ oxidation, notably the opportunistic colonic agents *Bilophila wadsworthia* (family *Desulfovibrionaceae*) (271) and *Bacteroides fragilis* (family *Bacteroidaceae*) (272). $H_2$ supports rapid growth of *B. wadsworthia* using taurine-derived sulfite as the terminal electron acceptor (66). Its genome encodes some seven hydrogenases (24) that are differentially active depending on the growth conditions (66). *B. fragilis* cultures have also been reported to possess soluble hydrogenase activity and mediate hydrogenotrophic fumarate reduction (273). However, these findings should be treated with caution given that it is mechanistically unclear how $H_2$-derived electrons are transferred into the respiratory chain. The organ-

ism encodes a group B [FeFe]-hydrogenase typically associated with $H_2$ production (7), and indeed other studies have reported low-level $H_2$ production by this species (101). The intracellular animal pathogen *Lawsonia intracellularis* (family *Desulfovibrionaceae*) has also been reported to require $H_2$ for growth under both oxic and anoxic conditions (274).

A range of aerobic bacteria can also oxidize $H_2$. Opportunistic pathogens within the genus *Mycobacterium* (family *Mycobacteriaceae*) encode oxygen-tolerant, high-affinity uptake hydrogenases (group 1h and 2a [NiFe]-hydrogenases) associated with the aerobic respiratory chain (275, 276) (Fig. 5 and 6c). Based on genetic studies in *Mycobacterium smegmatis*, the expression of their genes is induced during starvation and hypoxia, and they enhance survival by oxidizing $H_2$ to subatmospheric levels (19, 45, 277, 278). While *M. smegmatis* is rarely pathogenic (279), the hydrogenases are also conserved in more serious pathogens, such as *M. avium*, *M. marinum*, *M. fortuitum*, and *M. gordonae* (280); they have apparently been lost, however, during evolution of the obligate human pathogens *M. tuberculosis* and *M. leprae*. It is probable that $H_2$ facilitates persistence of these organisms in environmental reservoirs, but its role within host tissues is unclear. It should be noted that many mycobacteria, including *M. tuberculosis*, also oxidize the reduced gas carbon monoxide (280–282), and this process has recently been linked to persistence (282). Among other aerobes, *Rhodococcus equi* (family *Nocardiaceae*) also scavenges atmospheric $H_2$ during persistence (283). Hydrogenase lineages that support aerobic respiration are also encoded by the major pathogens *Corynebacterium diphtheriae* (group 1f [NiFe]-hydrogenase) (284) and *Pseudomonas aeruginosa* (some strains only; group 1d [NiFe]-hydrogenase) (36), but their roles have yet to be investigated.

The sections above demonstrate that a wide variety of pathogens can use $H_2$ to support aerobic or anaerobic respiration. However, there is growing evidence that some pathogens can grow chemolithoautotrophically by using electrons derived from $H_2$ to support carbon fixation. A subset of mycobacteria containing ribulose 1,5-bisphosphate carboxylase (RuBisCO), including *M. gordonae* (68, 285), can grow chemolithoautotrophically and mixotrophically on $H_2/CO_2$ under oxic conditions. Among anaerobes, preliminary evidence suggests the colonic bacteria *Eggerthella lenta* (family *Coriobacteriaceae*) (286, 287) and *C. difficile* (family *Peptostreptococcaceae*) (70) are capable of hydrogenotrophic acetogenesis through the Wood-Ljungdahl pathway. However, systematic studies are ultimately needed to confirm the biochemical activity, physiological role, and medical significance of these enzymes. Indeed, a role for autotrophic pathways of pathogens within host tissues or environmental reservoirs has yet to be convincingly demonstrated.

## $H_2$ PRODUCTION IN PATHOGENS

### *Bacteria*

$H_2$-producing bacterial pathogens fall broadly into two groups. The facultative anaerobes, typified by *Enterobacteriales* such as *E. coli* (77) and *S.* Typhimurium (238), survive limitation for electron acceptors by dissipating excess reductant as $H_2$. This process is mediated by a unique set of [NiFe]-hydrogenases that form formate hydrogenlyases (FHL). In contrast, the obligate anaerobes, typified by members of the order *Clostridiales* such as *C. perfringens* (15) and *C. difficile* (117), grow by fermenting organic carbon to $H_2$. This process depends on kinetically highly active, albeit oxygen-labile, [FeFe]-hydrogenases.

***Escherichia coli* and *Salmonella* Typhimurium: formate-dependent $H_2$ production by [NiFe]-hydrogenases.** At times and in places where respiratory electron acceptors are scarce, *Enterobacteriaceae* survive by activating mixed-acid fermentation. During this process, formate is produced from glycolytically derived pyruvate (pyruvate formate-lyase, or PFL) and is eventually disproportionated to $H_2$ and $CO_2$ by the FHL complex (23, 288–290) (Fig. 8a). The determinants of this process are encoded by the vast majority of pathogenic enterobacteria (Table 2). Formate-dependent $H_2$ production has been most comprehensively studied in *E. coli* (17, 77, 291–293) and *S.*

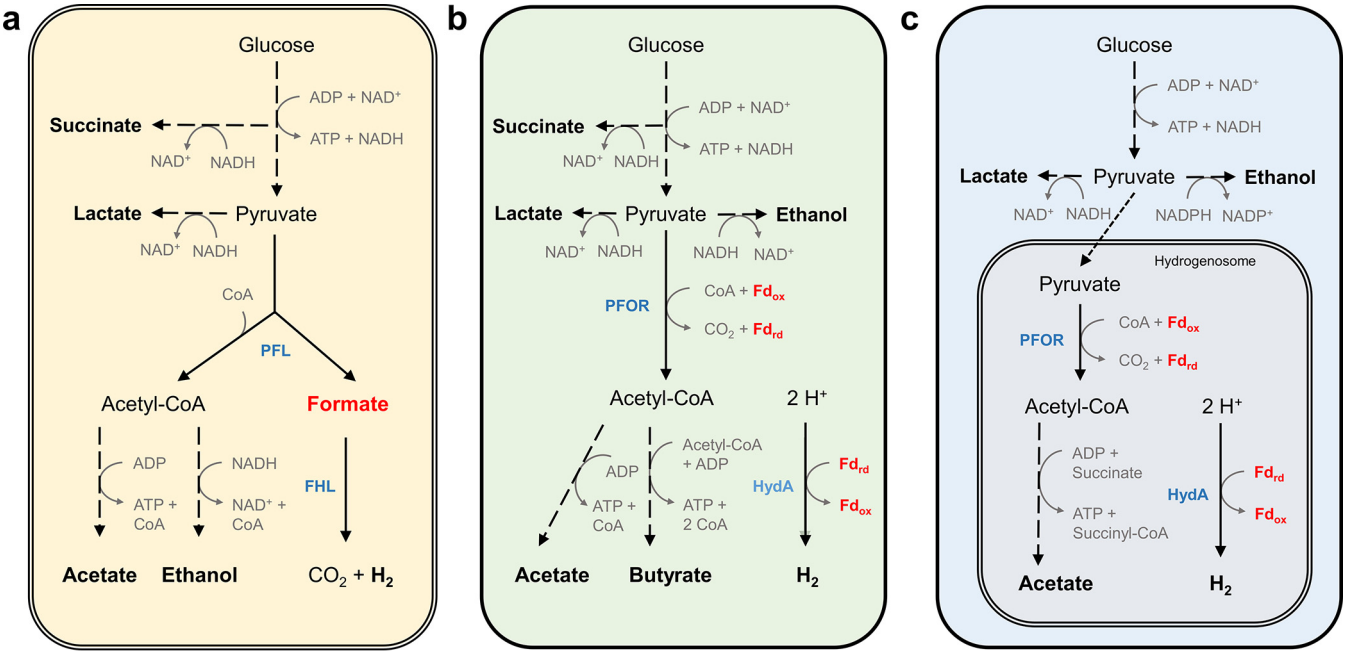


**FIG 8** Metabolic processes resulting in fermentative hydrogen production in key bacterial pathogens. The schemes show the key fermentation processes in *Salmonella* Typhimurium (a), *Clostridium perfringens* (b), and *Trichomonas vaginalis* (c). The fermentation products are boldfaced, the enzymes responsible for $H_2$ production are colored blue, and the electron donors for $H_2$ production are colored red. PFL, pyruvate-formate lyase; FHL, formate hydrogenlyase (containing group 4a [NiFe]-hydrogenase); PFOR, pyruvate:ferredoxin oxidoreductase; HydA, group A [FeFe]-hydrogenase; and Fd, ferredoxin. Note that other fermentation pathways are known, for example, the NADPH- or NADH-coupled hydrogenase of *Mycobacterium smegmatis*, but they are insufficiently understood to be depicted here.

Typhimurium (84, 237, 238, 294, 295). However, this process has also been experimentally observed in other pathogenic *Enterobacteriaceae*, notably *Enterobacter aerogenes* (296–299), *K. pneumoniae* (254, 258, 300, 301), *K. oxytoca* (302), and *Citrobacter freundii* (258, 303, 304).

Formate disproportionation is mediated by the membrane-bound enzyme complex FHL (17, 77, 305). The purified enzyme complex from *E. coli*, Hyc (also known as Hyd-3 and FHL-1), contains four core components: a molybdenum-dependent formate dehydrogenase-H that catalyzes formate oxidation (FdhF), a group 4a [NiFe]-hydrogenase that catalyzes proton reduction (HycE), three iron-sulfur cluster subunits that relay electrons between the catalytic centers (HycBFG), and two subunits that anchor the complex to the membrane (HycCD) (17) (Fig. 5). While the purified enzyme is physiologically reversible (77, 306–308), it is strongly biased toward $H_2$ production and maintains this activity even under high partial pressures of $H_2$ (17). While $H_2$-uptake hydrogenases of the *Enterobacteriaceae* can theoretically act in the reverse direction (206, 214), knockout studies have validated that FHL complexes are solely responsible for $H_2$ production under most physiologically relevant conditions (84, 309).

In *E. coli*, the FHL complex is only synthesized when carbon sources are available but respiratory electron acceptors are absent (291, 310, 311). There are two reasons for this regulation. First, *E. coli* hierarchically regulates use of its electron acceptors to maximize ATP generation in the following order of preference: aerobic respiration, nitrate respiration, fumarate respiration, and finally fermentation (312). Second, the reaction is only thermodynamically favorable under fermentative conditions when formate accumulates and the pH decreases (292). To facilitate this control, the nine-gene *hyc* operon (encoding the hydrogenase structural subunits), the five-gene *hyp* operon and separately encoded *hypF* gene (maturation factors), and the *fdhF* gene (formate dehydrogenase component) are tightly transcriptionally coupled (313, 314). Genetic studies have demonstrated that hydrogenogenic fermentation occurs when the following three conditions are met: (i) $O_2$ is absent (FNR induced; signals absence of electron

acceptors for aerobic respiration) (77, 292, 315); (ii) nitrate is absent (Nar system repressed; signals the absence of electron acceptors for nitrate respiration) (292, 316); and (iii) formate is present (FhlA induced; signals absence of other electron acceptors) (317–319). In common with the $H_2$-uptake hydrogenases, synthesis of the FHL complex is also regulated through the Hyp maturation factors (55, 88, 320, 321). Likewise, the FHL of *S*. Typhimurium was shown to be regulated by anaerobiosis, nitrate, and formate (294) (Fig. 7).

Accumulating evidence suggests that FHL complexes have a multifaceted role in the physiology of *Enterobacteriaceae*. The apparent primary role of the enzyme complexes is to dissipate reductant and detoxify formate during persistence under anoxia. However, three independent studies have indicated that FHL complexes are also critical for acid tolerance in *E. coli* and *S*. Typhimurium (79, 84, 322). These complexes mediate the net consumption of protons from the cytosol ($HCOO^- + H^+ \rightarrow CO_2 + H_2$) and, hence, may provide a simple but elegant mechanism to regulate internal pH. It has also been proposed that FHL complexes generate a PMF through a chemiosmotic mechanism (17, 323, 324). Multiple lines of evidence suggest this, most notably their transmembrane localization (17, 77), their uncoupler sensitivity (325, 326), their ATP synthase dependence (327), and their conservation with ion-motive hydrogenases (36, 328). Generation of a PMF would only be thermodynamically feasible under specific conditions, given that the standard redox potentials of the formate/$CO_2$ and $H_2/2H^+$ couples are similar (17). In addition to a possible direct role of FHL in chemiosmotic energy coupling, the $H_2$ generated from this reaction can be recycled through nitrate or fumarate respiration when electron acceptors become available (22, 262).

While the *in vitro* role of FHL has been established in pathogens, it is less clear what role these enzymes play *in vivo*. The only insights have come from genetic dissection of the four hydrogenases in *S*. Typhimurium (Table 4). As expected, a Δ*hya* Δ*hyb* Δ*hyd* triple mutant lacking the three uptake hydrogenases produces, but does not oxidize, $H_2$ (8, 84), whereas no $H_2$ production occurs in a Δ*hya* Δ*hyb* Δ*hyd* Δ*hyc* quadruple mutant also lacking the FHL complex (84). In a murine model, single mutants lacking Hyc structural subunits behaved identically with respect to organ colonization, morbidity, or mortality (84). This suggests that the organism either does not produce $H_2$ during infection or compensates for loss of this process. These findings also support the prevailing model that the pathogen primarily oxidizes $H_2$ derived from exogenous sources (i.e., gut microbiota) rather than endogenous sources (i.e., FHL) during infection (13, 22, 84). However, given the multifaceted physiological role and wide conservation of FHL complexes, it nevertheless seems probable that these enzymes confer a significant competitive advantage on *Enterobacteriaceae*. Most plausibly, they likely confer the capability to survive oxidant limitation or acidic pH in host or environmental reservoirs.

Many *Enterobacteriaceae* encode a distinctive FHL complex, Hyf (also known as Hyd-4 or FHL-2). This enzyme complex differs from the Hyc-based FHL-1 concerning the presence of three additional transmembrane subunits (HyfDEF) present in FHL-2 (209) (Fig. 5). These subunits are homologous to the proton-translocating subunits of complex I (NADH dehydrogenase), ND2, ND4, and ND5; this suggests the enzyme serves as a formate-driven proton pump, but this is unlikely to be thermodynamically favorable under physiological conditions (209, 329). Phylogenetic analysis suggests that Hyf (FHL-2) is the ancestral complex and that Hyc (FHL-1) evolved through the loss of these additional subunits (36). FHL complexes are variably conserved in the genomes of pathogenic enterobacteria (Table 1) (36). Many species encode both (e.g., *Citrobacter* spp. and *Escherichia* spp.), others encode either Hyc (e.g., *Salmonella* spp., *Enterobacter* spp., and *Klebsiella* spp.) or Hyf (e.g., *Proteus* spp., *Morganella morganii*, and *Yersinia enterocolitica*), and a few lack both (e.g., *Yersinia pestis* and *Providencia stuartii*) (36, 330). Most *Shigella* species also do not produce $H_2$ and have lost the capacity to synthesize FHL; the reported exceptions are strains of *S. boydii* serotypes 13 and 16 and *S. flexneri* serotype 6 (251).

As recently reviewed, it remains controversial as to whether Hyf is a fossil or a

functional enzyme in *E. coli* (205). Under most conditions, its expression is silent (331, 332) and its activity is negligible compared to that of Hyc (333). However, formate-dependent $H_2$ production by Hyf has been observed under alkaline conditions (334–336). Nevertheless, some pathogens that encode only Hyf can mediate formate-coupled $H_2$ evolution, including *P. mirabilis* (262) and potentially *Serratia marcescens* (258). Thus, Hyf enzymes are active under physiological conditions in some pathogens and may contribute to transmission or infection.

***Clostridium perfringens* and *Clostridioides difficile*: obligate fermenters with multiple [FeFe]-hydrogenases.** As outlined in Table 2, a wide range of obligately anaerobic pathogens also have the coding capacity for hydrogenogenic fermentation. The most notable of these are the human pathogens within the order *Clostridiales*. These include *C. difficile* (pseudomembranous colitis), *C. perfringens* (gas gangrene), *Clostridium tetani* (tetanus), and *Clostridium botulinum* (botulism) (101). Clostridial fermentation has also been linked to necrotizing enterocolitis (337). It is thought that these pathogens adopt an obligately fermentative lifestyle in which carbohydrates and proteins are degraded to organic acids (e.g., butyrate) and molecular hydrogen (Fig. 8b), with ATP being generated through substrate-level phosphorylation (15, 338). *C. perfringens* is a particularly efficient $H_2$ producer and sustains doubling times of less than ten minutes in pure culture through fermentation alone (339). In a dramatic example of this, $H_2$ can accumulate to millimolar levels during advanced gas gangrene infection (116, 340). However, while $H_2$ metabolism has been comprehensively studied in several environmental clostridia, surprisingly little dedicated research has been performed on the metabolism of these pathogens.

Some insights into hydrogen metabolism in clostridia come from genome sequencing (Table 2). Whereas facultative anaerobes produce $H_2$ using formate- or nicotinamide-coupled [NiFe]-hydrogenases, obligate anaerobes primarily use ferredoxin-dependent [FeFe]-hydrogenases. In an important study, Calusinska and colleagues showed that both pathogenic and environmental clostridia encode multiple [FeFe]-hydrogenases (117). These enzymes vary in terms of their phylogenetic grouping, domain architecture, and the presence of additional subunits (36, 117) (Fig. 5). A feature common to all pathogenic clostridia appears to be the presence of one or more group B [FeFe]-hydrogenases; these can be present in either a short form containing two [4Fe4S] clusters (*C. perfringens*, *C. difficile*, *C. botulinum*, and *C. tetani*) or a long form containing one [2Fe2S] and three [4Fe4S] clusters (*C. difficile*, *C. perfringens*, and *C. botulinum*) (36, 117). Hydrogenases from this group have yet to be purified but are thought primarily to couple ferredoxin oxidation to $H_2$ production (7, 36). Other hydrogenases can also be present. *C. perfringens* contains two group A1 [FeFe]-hydrogenases, one standard and one atypical (117, 341). *C. difficile* and *C. botulinum* both encode trimeric electron-bifurcating group A3 [FeFe]-hydrogenases, which are predicted to couple ferredoxin and NADH reoxidation to $H_2$ production (117, 342). Finally, *C. difficile* encodes group A4 [FeFe]-hydrogenases that are predicted to relay electrons between formate and $H_2$ (343).

One hydrogenase of pathogenic clostridia, the standard group A1 [FeFe]-hydrogenase of *C. perfringens*, has been investigated through genetic and biochemical studies (15). In axenic cultures, the genes encoding this hydrogenase are transcribed as part of an operon along with a gene encoding butyrate kinase, and expression is highly induced during growth on carbohydrates. Genetic deletion of the hydrogenase structural genes eliminated $H_2$ production and caused a 3-fold reduction in growth yield. In addition, the hydrogenase has been recombinantly synthesized, purified, and characterized (344). The enzyme mediates rapid and efficient $H_2$ production in both colorimetric and electrochemical assays (344, 345). Altogether, this indicates the enzyme is the primary a hydrogenase involved in saccharolytic fermentation to butyrate and $H_2$ (15) (Fig. 8b). The high activity of this enzyme makes it ideally suited to support rapid fermentative growth. There is currently no information, however, regarding the physiological roles of the other three hydrogenases of this organism.

Transcriptomic and proteomic studies have shown that clostridial hydrogenases are

differentially synthesized both *in vitro* and *in vivo*. In *C. perfringens*, [FeFe]-hydrogenases are differentially regulated during necrotic enteritis of the chicken intestine (346). In *C. difficile*, hydrogenase gene expression is linked to both sporulation and nutrient availability (91, 347–349). A proteomic analysis identified approximately 300 core proteins in *C. difficile* endospores, including a short-form group B [FeFe]-hydrogenase (347). Other studies indicate that the formate-coupled hydrogenase is also activated by the key sporulation entry regulator Spo0A (91), while the long-form group B [FeFe]-hydrogenase is regulated by the catabolite control protein CcpA (348). Hydrogenases are also differentially expressed during infection in murine and porcine models (350–352). In the murine model, formate dehydrogenase and the short-form group B hydrogenase are also among the induced enzymes during infection, concomitant with production of short-chain fatty acids (Fig. 1) (351). In further support of their importance for virulence, the *C. difficile* hydrogenases are highly conserved across clinical isolates (353). While these findings suggest clostridial pathogenesis involves $H_2$ metabolism, there are numerous unanswered questions regarding the role, regulation, and importance of the hydrogenases involved.

While it is assumed that the clostridial hydrogenases are primarily involved in $H_2$ production, some may have an oxidative role. For *C. perfringens*, the strongest candidate for an uptake enzyme is its atypical group A1 [FeFe]-hydrogenase. Its N-terminal domain shares more than 60% amino acid sequence identity to a *C. pasteurianum* hydrogenase (CpII), which is catalytically biased toward $H_2$ oxidation (202). Its C-terminal domain is homologous to rubredoxins, which mediate deactivation of reactive oxygen species and anaerobic respiration in *C. perfringens* (354, 355). Hence, a conceivable role for this enzyme is the use of $H_2$-derived electrons to reduce peroxide species, thereby contributing to the relative aerotolerance of this species. With respect to *C. difficile*, formate dehydrogenase-linked hydrogenases and electron-bifurcating [FeFe]-hydrogenases are both known to be physiologically reversible in other species (20, 343, 356, 357). Given recent reports that this species is a facultative autotroph (70), these hydrogenases may support $CO_2$ fixation via the Wood-Ljungdahl pathway. Ultimately, dedicated physiological and biochemical studies are needed to understand the specific roles of the multiple hydrogenases in pathogenic clostridia.

**Other $H_2$-producing bacteria.** Several other facultative anaerobic pathogens encode Hyf-type FHL complexes (Table 2). These complexes are present in the gammaproteobacterial pathogens *Aggregatibacter actinomycetemcomitans*, *Haemophilus haemolyticus*, and *Pasteurella bettyae*. Studies in the former organism indicate that these enzymes are also under the control of carbon- and oxygen-sensing regulators (358, 359). However, no study to our knowledge has reported formate-coupled $H_2$ production in these organisms. Several strains within the *Campylobacteraceae* also encode these enzymes, most notably *C. concisus* (36). The operon encoding FHL in *C. concisus* is similar to the *hyf* operon of *E. coli*, although the *hyfD* gene (encoding the ND2-like subunit) is absent (78). Genetic and biochemical studies have shown that this organism indeed mediates $H_2$ production using this enzyme under anoxic conditions; however, it is still not clear whether formate or another organic acid is the electron donor (78). By analogy with *E. coli* and *S.* Typhimurium, we hypothesize that *C. concisus* can also recycle endogenous $H_2$ using its uptake hydrogenase under anoxic conditions. In turn, the ability of this pathogen to switch between aerobic respiration, anaerobic respiration, and fermentation may enable it to adapt to various niches within the human body, for example, in response to changes in electron acceptor availability.

A wide range of obligately anaerobic pathogens are also predicted to mediate hydrogenogenic fermentation. Putative [FeFe]-hydrogenases are encoded in opportunistic pathogens from the phyla *Spirochaetes* (e.g., *Brachyspira pilosicoli* and *Treponema denticola*), *Fusobacteria* (e.g., *Fusobacterium nucleatum*), *Firmicutes* (e.g., *Veillonella dispar*), and possibly *Bacteroidetes* (e.g., *B. fragilis*) (Table 2). In common with clostridial pathogens, these organisms generally encode the electron-bifurcating group A3 [FeFe]-hydrogenases in concert with a ferredoxin-dependent group A1 or B [FeFe]-hydrogenase

(36). However, to our knowledge, $H_2$ production has yet to be investigated in these organisms.

Finally, it has recently been recognized that some obligately aerobic bacteria switch to hydrogenogenic fermentation as a last resort. Some mycobacteria, after entering stationary phase due to oxygen deprivation, maintain redox balance by producing large amounts of $H_2$. This process is mediated by a cytosolic group 3b [NiFe]-hydrogenase that is predicted to directly transfer electrons from NAD(P)H to protons (19) (Fig. 5); this is only thermodynamically favorable if the NAD(P)H/NAD(P)$^+$ ratio is high (e.g., due to the absence of respiratory electron acceptors) and $H_2$ levels remain low (e.g., due to reoxidation or dissipation). The enzyme responsible is activated under low oxygen and redox states by the well-characterized response regulator DosR (19, 275, 277). Deletion of the genes encoding the enzymes responsible results in impaired redox homeostasis and reduced hypoxic survival. In common with *E. coli* and *S.* Typhimurium, this $H_2$ is recycled by uptake hydrogenases when electron acceptors for aerobic or anaerobic respiration are available (19). Such hydrogenases are present in a range of nontuberculous mycobacteria, including *M. marinum*, *M. gordonae*, *M. kansasii*, and some *M. ulcerans* isolates (36), as well as *Legionella pneumophila* and *Rhodococcus equi* (Table 2). It is tempting to speculate that fermentation contributes to the persistence of these pathogens within natural and constructed environments. A further area to be explored is whether facultative fermentation contributes to the persistence of mycobacteria in response to new antimycobacterial drugs targeting aerobic respiration (360, 361). While *M. tuberculosis* lacks these hydrogenases, it expresses a complex related to FHL in a DosR-dependent manner (362); however, it is unlikely that this enzyme can produce $H_2$ given that the subunit homologous to hydrogenase catalytic subunits lacks cysteine residues to bind a [NiFe] center (275).

### *Eukarya*

Various human and animal infections are also caused by protists (i.e., unicellular eukaryotes). A phylogenetically and physiologically diverse subset of these pathogens grows or survives by mediating the hydrogenogenic fermentation of organic carbon compounds. [FeFe]-hydrogenase activity has been detected in several major human parasites, including *T. vaginalis* (363), *G. intestinalis* (18), and *Naegleria* (364) (Table 2).

***Trichomonas vaginalis*: fermentation within hydrogenosome organelles.** Various fermentative eukaryotes contain $H_2$-producing organelles known as hydrogenosomes (365). These organelles are now thought to have evolved multiple times from a mitochondrial ancestor across diverse eukaryotic lineages (366–369). Research on the bovine parabasalid pathogen *Tritrichomonas foetus* led to the landmark discoveries of eukaryotic $H_2$ production in 1957 (370) and the hydrogenosome in 1973 (76). Since then, equivalent organelles have been reported in other pathogenic parabasalids, such as *T. vaginalis* (16, 371), *Trichomonas tenax* (372), *Dientamoeba fragilis* (373, 374), *Pentatrichomonas hominis* (375), and *Histomonas meleagridis* (376–378). Group A1 [FeFe]-hydrogenases and their maturation factors are localized in these organelles, where they mediate $H_2$ production (379, 380). It has also been shown that diplomonads from fish pathogens within the genus *Spironucleus* also contain hydrogenosomes and mediate rapid $H_2$ production under microaerophilic conditions (381–384).

Most of our understanding of hydrogenosomal metabolism comes from studies on the human sexually transmitted parasite *T. vaginalis* (reviewed in references 365 and 368). In this organism, pyruvate produced during glycolysis is imported into the hydrogenosome, oxidized to acetyl-CoA via pyruvate-ferredoxin oxidoreductase (PFO), and converted to the fermentative end product acetate (Fig. 7c). The ferredoxin reduced by the PFO reaction (385, 386) is then reoxidized via a group A1 [FeFe]-hydrogenase (363, 387), resulting in formation of $H_2$. In addition, ferredoxin can be reduced by NADH dehydrogenase subunits (NuoE and NuoF) in *T. vaginalis* (388, 389), possibly through an electron-bifurcating mechanism (390). It has also been proposed that one or more hydrogenases in *T. vaginalis* form a complex with the NADH dehydrogenase subunits, directly accepting electrons from NADH oxidation (389, 391).

This proposal is consistent with the observation that *T. vaginalis* mutants lacking ferredoxin retain some hydrogenase activity (392). A ferredoxin-dependent hydrogenase has also been purified from *T. vaginalis* and exhibits features similar to those of bacterial group A1 [FeFe]-hydrogenases, including significant activity, sensitivity to carbon monoxide inhibition, and the spectroscopic signatures of an H cluster (393).

Nevertheless, it remains unclear whether hydrogenases are essential for viability of *T. vaginalis*. To our knowledge, no studies to date have investigated the effects of deleting the genes encoding hydrogenases or their maturation factors on *T. vaginalis* pathogenesis, although some insights into their essentiality have come from investigations on the effects of pharmaceuticals on hydrogenosomal metabolism. It has been reported that resveratrol is a specific inhibitor of hydrogenase activity and causes cytotoxicity at high concentrations (394). However, a more nuanced picture has emerged from studies into the development of resistance to metronidazole, still the first-line treatment for trichomoniasis. Metronidazole is a nitroimidazole prodrug that is reductively activated by the hydrogenosomal ferredoxin and effectively competes for electrons with hydrogenase (395, 396). However, metronidazole-resistant strains of trichomonads have been characterized with reduced levels of hydrogenase synthesis or activity (397–399). One way this is achieved is through rewiring metabolic flux away from $H_2$ and acetate production and toward ethanol production (397). Hence, while hydrogen production is a core feature of *T. vaginalis* metabolism, the pathogen may harbor sufficient metabolic flexibility to bypass it.

An unexpected revelation from the *T. vaginalis* genome is that it encodes up to 13 [FeFe]-hydrogenases (400–402). Similar findings have been made from the genomes of *Tritrichomonas*, *Histomonas*, and *Spironucleus* species (384, 403) (Table 2). It is currently unclear whether some of these hydrogenases are functionally redundant or whether they all have unique physiological roles in the cell. However, results of proteomics studies indicate at least five of them are simultaneously present in the hydrogenosomal proteome (402). Possible factors that may differentiate them include synthesis patterns, subcellular localization, enzyme kinetics, and redox partners. While all eukaryotic hydrogenases described to date produce $H_2$, it cannot be ruled out that some also act in the oxidative direction, as was recently proposed for *T. vaginalis* (404). Consistent with having distinct physiological roles, these hydrogenases show considerable differences in the structure of the domains flanking the catalytic H-cluster: some are of a short form with two [4Fe4S] clusters at the N terminus; others are of a long form with three [4Fe4S] clusters and one [2Fe2S] cluster at the N terminus; and yet others are fusion proteins with C-terminal domains homologous to CysJ (36, 403, 405, 406). The functional significance of these differences remains unclear, highlighting the need for further biochemical and physiological studies on this fascinating system.

***Giardia intestinalis* and *Entamoeba histolytica*: evidence for $H_2$ production in parasites lacking hydrogenosomes.** Hydrogenases are also present in some parasitic protists that lack hydrogenosomes. Their presence was first documented in the prevalent diarrheal pathogens *G. intestinalis* (synonym *Giardia lamblia*) and *Entamoeba histolytica*, to the considerable surprise of researchers in the field (18, 407). Both organisms lack mitochondria and hydrogenosomes, although they possess remnant organelles, called mitosomes, that do not participate in ATP production (408, 409). In *Giardia*, it has been shown that the single group A1 [FeFe]-hydrogenase encoded by this organism is primarily localized to the cytosol rather than the mitosome (409). Hydrogenase activity in this organism is induced under anoxic conditions and is highly sensitive to oxygen poisoning (18). On this basis, it has been proposed that $H_2$ production enables the organism to dissipate excess reductant under anaerobic conditions (18).

In contrast, the genome of *E. histolytica* and related species contains three hydrogenases: two group A1 [FeFe]-hydrogenases and a group B [FeFe]-hydrogenase (407, 410, 411) (Table 2). Two have been shown to be synthesized, and a group A1 [FeFe]-hydrogenase has been shown to be active in recombinant systems

(407). Transcriptome profiling has revealed that hydrogenase gene expression varies between *Entamoeba* strains and is sometimes correlated with increased virulence (412–415). Comparative transcriptome studies indicated that hydrogenase gene expression is higher in the virulent *E. histolytica* than in the avirulent *E. dispar* (412). Likewise, in a gerbil model, increased expression of the group B [FeFe]-hydrogenase genes is associated with increased pathogenicity (413). However, for both *Giardia* and *Entamoeba* spp., genetic studies are required to provide unequivocal evidence for the roles of these hydrogenases in growth, survival, and virulence.

***Acanthamoeba castellanii* and *Naegleria fowleri*: flexibility dependent on respiration versus fermentation.** Some pathogenic amoebas also contain mitochondria with apparent dual capabilities for aerobic respiration and hydrogenogenic fermentation (368). For example, the nuclear genome of the opportunistic pathogen *Acanthamoeba castellanii* encodes a complete pathway for hydrogenogenic fermentation, including a mitochondrially targeted [FeFe]-hydrogenase, its maturases, and pyruvate-ferredoxin oxidoreductases (47, 416). Proteomic and antibody-staining studies have confirmed these enzymes are preferentially localized to the mitochondria (416). *Naegleria* species, including the deadly pathogen *N. fowleri* (causing primary amoebic meningoencephalitis), have a similar genetic capacity. The nuclear genome of the nonpathogenic species *N. gruberi* encodes genes for aerobic respiration together with an [FeFe]-hydrogenase with a mitochondrial import signal (417). Surprisingly, however, the genes encoding the hydrogenase apparently are expressed under aerobic conditions and the enzyme is reportedly localized to, and active in, the cytosol (364). A similar hydrogenase has also been detected in the genome and proteome of *N. fowleri* (418). Altogether, these findings suggest that both *Naegleria* and *Acanthamoeba* switch from respiration to fermentation depending on oxygen partial pressures in different environmental reservoirs and host tissues. However, more in-depth studies are required to systematically test these hypotheses.

Stramenophiles such as *Blastocystis* species are among the most prevalent enteric protists, although their actual pathogenicity continues to be a source of debate (419), as does their capacity to metabolize $H_2$. *Blastocystis* contains mitochondrion-related organelles (MRO) with features resembling both hydrogenosomes and mitochondria, including the presence of an organellar genome (368, 420). Through an analysis of three different isolates, Stechmann and colleagues demonstrated that putative [FeFe]-hydrogenases and pyruvate-ferredoxin oxidoreductases are synthesized and function in the MRO. The localization of a putative hydrogenase within the MRO was also confirmed by epifluorescence microscopy (420). However, the activity of these enzymes was not detected in whole-cell biochemical assays in cultures of subtype 7 (421). Moreover, *Blastocystis* genomes lack two of the maturation factors required for [FeFe]-hydrogenase assembly (HydF and HydG) (390). Together, these findings have led to speculation that the putative hydrogenases in fact have functions distinct from $H_2$ production (422).

## POTENTIAL OF $H_2$ METABOLISM AS A THERAPEUTIC TARGET SPACE

### Promises and Challenges of Inhibitor Development

The above sections demonstrate that the consumption and production of $H_2$ are critical for growth, survival, and virulence of several major pathogens. This suggests that there is clinical value in developing small-molecule inhibitors targeting hydrogenases or their maturation factors. However, both considerable promise and significant limitations are associated with this potential target space. Based on their physiological roles, inhibition of respiratory hydrogenases would theoretically cause membrane depolarization and ATP depletion, whereas inhibiting fermentative hydrogenases may cause reductive stress. Inhibiting hydrogenases may also affect intracellular pH homeostasis (22, 79, 222, 251). It has previously been speculated that hydrogenases are a promising drug target (1, 61, 423), and this area is subject to patents (424). A range of compounds, such as carbon monoxide, acetylene, and formaldehyde, competitively inhibit the active sites of hydrogenases

(425–427). However, with the possible exception of studies using resveratrol (394), to our knowledge no dedicated research has been performed to develop or test drug-like inhibitors of these enzymes.

Two developments suggest that hydrogenases are worth exploring as drug targets. The first is the emergence of drug-resistant pathogens. Several antibiotic-resistant bacteria designated priority pathogens by WHO harbor hydrogenases, notably carbapenem- and cephalosporin-resistant *Enterobacteriaceae* (critical priority) and clarithromycin-resistant *H. pylori*, fluoroquinolone-resistant *Campylobacter* spp., and fluoroquinolone-resistant *Salmonella* spp. (all high priority) (428). Drugs with novel targets and modes of action therefore are urgently required to treat multidrug-resistant infections. The second is the recent validation that energetics is a fruitful target space for antibiotic development (1, 3). This is reflected by the landmark FDA approval of the ATP synthase inhibitor bedaquiline and the clinical development of respiratory chain inhibitors for tuberculosis treatment (360, 429, 430). Whereas most drugs target growth-related processes, inhibitors of energy metabolism can be bactericidal for pathogens during growth and persistence (429, 431).

However, inhibiting $H_2$ metabolism still is likely to be a challenge for several reasons. First, given $H_2$ oxidation is a facultative process in most pathogens, hydrogenase inhibition may not exert effects as severe as those targeting core oxidative phosphorylation complexes. Hydrogenase inhibitors are likely to be most effective against $H_2$-dependent respiratory pathogens with limited metabolic flexibility, for example, *H. pylori* (9) and *C. concisus* (78), as well as obligate fermenters, such as *C. perfringens* (15) and *T. vaginalis* (16). However, as evidenced by metronidazole-resistant mutants of *T. vaginalis* (399), even organisms that obligately metabolize $H_2$ potentially can bypass this pathway when subject to intense selection. Hence, therapies solely reliant on hydrogenase inhibition may fail, but there is promise in therapies that inhibit hydrogenases together with other targets (either through combination therapies or monotherapies with pleiotropic effects). Inhibitor development is also complicated by the presence of multiple hydrogenases in many pathogens that can potentially cross-compensate, for example, in *S.* Typhimurium and *T. vaginalis* (Table 2). This may be overcome by the products of single-copy genes required for hydrogenase function, such as certain maturation factors and nickel importers.

Finally, administration of hydrogenase inhibitors is likely to lead to some off-target effects. While hydrogenases are absent from human cells, most gut bacteria encode hydrogenases (particularly [FeFe]-hydrogenases) (7), and disruption of normal $H_2$ cycling is strongly linked to gastrointestinal dysbiosis (6). Such effects theoretically could be alleviated by specifically targeting hydrogenase classes primarily associated with pathogens (e.g., group 1b [NiFe]-hydrogenases). Moreover, off-target effects are also likely to be justified under certain circumstances, for example, in patients with severe infections or those already exhibiting significant gut dysbiosis.

### Strategies for Inhibitor Development

Several options exist to develop hydrogenase inhibitors. The most promising is to screen purify enzymes or whole cells using colorimetric hydrogenase assays (e.g., viologen assays) (432, 433). Structure-based drug design is also an option in some cases, given X-ray crystal structures are now available for the three different uptake hydrogenases of *Enterobacteriaceae* (212, 213, 215, 243). There may also be value in exploring synergies of hydrogenase inhibitors in combination therapies, given $H_2$ metabolism is strongly linked to energy conservation during persistence in multiple pathogens (9, 18, 275, 294). In addition to their clinical potential, the availability of specific hydrogenase inhibitors would also provide much insight into the biochemistry, physiology, and ecology of $H_2$ metabolism for researchers within and outside biomedical disciplines.

Another avenue to explore is the use of quinone analogs to inhibit the quinone reductase site of uptake hydrogenases. Such inhibitors are likely to have pleiotropic effects. However, a range of studies suggests that such hydrogenases are dispropor-

tionately more sensitive to quinone analogues than other primary dehydrogenases (e.g., complex I) (125, 434–436). This is particularly reflected by both laboratory and clinical strains of *H. pylori*, which are highly sensitive to inhibition by HQNO (74). As many quinone reductase inhibitors and quinone analogs are already available, screening them for inhibition of $H_2$-oxidizing activity and inhibition of $H_2$-dependent cell growth (32) is likely to yield hydrogenase inhibitors and reveal promising leads for antibiotic development. In this regard, it is notable that coenzyme Q and some of its analogs are already considered safe by the FDA for use as health supplements. Furthermore, other quinone analogs are currently used as antibiotics, although it is worth noting that their complete, or even primary, mode of action is not known (437–440). Hence, there are precedents for successful drug development and regulatory approval in this space.

Another potentially fruitful approach is to target one of the specific metals required for hydrogenase activity, namely, nickel. Targeting iron is not a valid option, given the ubiquitous importance of the metal for both microbial and mammalian cellular processes. In contrast, nickel is an attractive target, given no major effect on host cells is expected, because mammals are not known to synthesize Ni-dependent enzymes. Several groups, including our own, have previously proposed that nickel sequestration is a possible therapeutic approach (148, 441–443). Sequestering nickel from cells is predicted to prevent the maturation of [NiFe]-hydrogenases. We fully acknowledge this approach will not uniquely inhibit the hydrogenases; nickel inhibitors would also pleiotropically act to prevent the synthesis and/or activity of a range of other Ni-requiring enzymes. Other than [NiFe]-hydrogenases, urease- and nickel-dependent superoxide dismutase, acireductone dioxygenase, and glyoxalase I are among the bacterial enzymes that use nickel as cofactors. In fact, it is estimated that Ni-requiring enzymes are important for the virulence of at least 40 bacterial and nine eukaryotic pathogenic species (443). At least five of these species contain more than one nickel enzyme that is important in pathogenesis, for example, *H. pylori*, which depends on both [NiFe]-hydrogenase and urease for virulence (9, 444). Nickel-chelating chemicals, as well as histidine-rich peptides, are among candidate chelators to explore; the challenge is to use those that are highly specific for nickel over other metals.

The Ni chelation approach has already been tested against *S.* Typhimurium. Besides [NiFe]-hydrogenases, nickel-dependent acireductone dioxygenase and glyoxalase I are present in the pathogen, although urease is missing. A recent study from our laboratory revealed that the nickel-specific chelator dimethylglyoxime (DMG) (i) inhibits $H_2$-uptake activity in *S.* Typhimurium; (ii) is safe, even at high (millimolar) levels both in mammalian (mouse) and insect (wax moth larva) models; and (iii) protects against *S.* Typhimurium infection. Indeed, DMG treatment led to reduced mouse colonization (decreased bacterial burden in the spleen and liver of DMG-treated mice), as well as reduced mortality in both mice (50% survival) and wax moth larvae (60% survival) compared to that of the control group (100% mortality in both mice and insects) (445). While DMG-mediated hydrogenase inhibition might only partially account for the observed reduced virulence, these findings nevertheless validate that a nickel chelation strategy is a promising approach against hydrogenase-containing pathogens. One caveat is that beneficial Ni-requiring prokaryotic and eukaryotic microorganisms may be an important component of a healthy human gut, so multiple aspects of host physiology could be affected by nonspecific disruption of nickel homeostasis.

### Other Intervention Strategies

Beyond small-molecule inhibitors, other experimental approaches are available to prevent or treat infections of $H_2$-metabolizing pathogens. One is to manipulate micronutrient availability through dietary modulation or chelation therapy. For example, there is evidence from animal and human studies that low-nickel diets help to prevent *H. pylori* infection, which reflects the critical roles of hydrogenase and urease in this pathogen (148, 446, 447). Manipulating $H_2$ metabolism by gastrointestinal microbiota

may also help to prevent or treat infections (Fig. 2). Although an underexplored area, $H_2$ metabolism in pathogens can be both positively and negatively influenced by interactions with other $H_2$ consumers or producers. This is best reflected by the hydrogenotroph *S.* Typhimurium, which depends on hydrogenogenic bacteria but competes with hydrogenotrophs to invade the GIT (see "*Salmonella* Typhimurium: differential roles of hydrogenases during infection" above). Likewise, $H_2$ producers depend on interactions with hydrogenotrophs to maintain $H_2$ partial pressure at sufficiently low concentrations for secondary fermentations to remain thermodynamically favorable (7, 99, 100, 107). A range of strategies is possible to influence $H_2$ dynamics in the GIT, including dietary manipulation, probiotic intake, and fecal transplants (6). However, the development of sophisticated interventions depends on advancing our currently rudimentary understanding of the physiology and ecology of $H_2$ metabolism in the human GIT.

## CONCLUSIONS

$H_2$ metabolism is still oftentimes referred to as a side metabolism characteristically associated with anaerobes. We hope that this broad perspective, in a manner similar to that of our recent work highlighting the environmental importance of $H_2$ metabolism (36, 100, 448, 449), will help to change this narrative by emphasizing the central role of $H_2$ metabolism in pathogenesis. Here, we definitively show that $H_2$ metabolism is a widespread and important feature of pathogens. This metabolism is critical for the expansion of facultative anaerobes into different niches and is central to the energy conservation of many obligate anaerobes. Indeed, $H_2$ metabolism is proving to be important for the pathogenesis of the main cellular agents of enteritis, gastritis, and gastric cancer and also supports pathogens of the urinary tract, oral cavity, and muscle tissue. In turn, there is promise that these challenges will be met through developing new antibiotics or other therapies targeting this space.

This review equally reflects that there are many gaps in our knowledge of $H_2$ metabolism. This includes major specific questions, for example, regarding how obligately anaerobic bacterial pathogens metabolize hydrogen and why they have multiple hydrogenases. Perhaps more of a concern, however, is the fact that we lack a holistic understanding of the gastrointestinal $H_2$ economy and in turn how this influences infection dynamics. Amid the current gut microbiota revolution, while much attention has been given over to carbon and nitrogen transactions (92, 96, 97), the role of $H_2$ exchange is still underappreciated. Further studies are also warranted to explore whether metabolic flexibility, particularly with regard to $H_2$ metabolism, contributes to the persistence of pathogens in environmental reservoirs.

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