The Hyb Hydrogenase Permits Hydrogen-Dependent Respiratory Growth of *Salmonella enterica* Serovar Typhimurium

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**ABSTRACT** *Salmonella enterica* serovar Typhimurium contains three distinct respiratory hydrogenases, all of which contribute to virulence. Addition of H₂ significantly enhanced the growth rate and yield of *S.* Typhimurium in an amino acid-containing medium; this occurred with three different terminal respiratory electron acceptors. Based on studies with site-specific double-hydrogenase mutant strains, most of this H₂-dependent growth increase was attributed to the Hyb hydrogenase, rather than to the Hya or Hyd respiratory H₂-oxidizing enzymes. The wild type strain with H₂ had 4.0-fold greater uptake of ¹⁴C-labeled amino acids over a period of minutes than did cells incubated without H₂. The double-uptake hydrogenase mutant containing only the Hyb hydrogenase transported amino acids H₂ dependently like the wild type. The Hyb-only-containing strain produced a membrane potential comparable to that of the wild type. The H₂-stimulated amino acid uptake of the wild type and the Hyb-only strain was inhibited by the protonophore carbonyl cyanide m-chlorophenylhydrazone but was less affected by the ATP synthase inhibitor sodium orthovanadate. In the wild type, proteins TonB and ExbD, which are known to couple proton motive force (PMF) to transport processes, were induced by H₂ exposure, as were the genes corresponding to these periplasmic PMF-coupling factors. However, studies on *tonB* and *exbD* single mutant strains could not confirm a major role for these proteins in amino acid transport. The results link H₂ oxidation via the Hyb enzyme to growth, amino acid transport, and expression of periplasmic proteins that facilitate PMF-mediated transport across the outer membrane.

**IMPORTANCE** Complex carbohydrates consumed by animals are fermented by intestinal microflora, and this leads to molecular hydrogen production. *Salmonella enterica* serovar Typhimurium can utilize this gas via three distinct respiratory hydrogenases, all of which contribute to virulence. Since H₂ oxidation can be used to conserve energy, we predicted that its use may augment bacterial growth in nutrient-poor media or in competitive environments within H₂-containing host tissues. We thus investigated the effect of added H₂ on the growth of *Salmonella Typhimurium* in carbon-poor media with various terminal respiratory electron acceptors. The positive effects of H₂ on growth led to the realization that *Salmonella* has mechanisms to increase carbon acquisition when oxidizing H₂. We found that H₂ oxidation via one of the respiration-linked enzymes, the Hyb hydrogenase, led to increased growth, amino acid transport, and expression of periplasmic proteins that facilitate proton motive force-mediated transport across the outer membrane.

When an animal consumes complex sugars that are not absorbed or are difficult to metabolize, these sugars reach the intestinal flora and are anaerobically fermented by resident microbes (1, 2). One result is production of molecular hydrogen (H₂), and it is well established that such H₂ production can vary with the animal’s diet (3), including that of humans (4–6). The colonically produced gas can be distributed to many tissues where pathogens reside (7, 8). Some pathogens capitalize on this, using the high-energy reductant as an energy source to facilitate their growth (8). One of these is *Salmonella enterica* serovar Typhimurium, in which H₂ has been shown to be an important energy source for virulence during host colonization. Study of *Salmonella* hydrogenase mutants has shown that each of the three uptake hydrogenases contributes to virulence, and a triple uptake mutant lacking all respiratory H₂-oxidizing ability was avirulent in a mouse model (9).

Electrons generated from H₂ splitting are passed along metabolically versatile bacterial electron transport chains to a variety of acceptors, including fumarate, nitrate, sulfate, CO₂, or O₂ (10), depending on the inherent terminal oxidase content of the particular microorganism. Such respiratory chains conserve energy in the form of ATP. The three H₂-consuming hydrogenases known as Hyb, Hyd, and Hya in *Salmonella Typhimurium* (11) are membrane bound and contain NiFe centers. The enterobacterial uptake-type (H₂-oxidizing) hydrogenases are viewed as auxiliary energy input providers contributing to the proton gradient across the cell membrane (10, 12). Metabolically flexible H₂-utilizing bacteria (e.g., the facultative
The strains used are shown in Table 1. We used CR-Hyd medium (14, 15) with the modification that no glucose was added, so the peptone and casein hydrolysate served as the carbon sources for the growth of the strains. The condition used was such that only the H₂ uptake enzymes were produced and no H₂ was produced, so only the effects of exogenously added H₂ were being observed. Growth was not significantly higher in cells grown with nitrate. Growth yields with H₂ were increased 1.8-fold and about 3-fold for cells grown with TMAO and DMSO, respectively, compared to those under H₂-lacking conditions (Table 2). Growth was not significantly higher in cells grown with nitrate. When grown with fumarate, the triple mutant (strain ALZ43; Table 1), lacking all H₂ uptake ability, never responded to H₂ addition. The wild type (WT) growth rate (doubling time) with H₂ was 1.5 h, whereas without H₂ it was about 5 h (Fig. 1). Less pronounced but significant growth rate differences (in comparing bacteria with H₂ added versus those with no H₂ added) were observed for the WT on either TMAO or DMSO (data not shown).

To assign specific hydrogenase activity to growth effects, endpoint growth yields were determined for uptake-type hydrogenase double and triple mutant strains. The strain containing only the Hyb hydrogenase (ALZ42; Table 1) had increased growth yield in the presence of H₂ (more than 3.0-fold greater in fumarate-

| Strain/plasmid      | Genotype/description                                      | Reference |
|---------------------|-----------------------------------------------------------|-----------|
| S. enterica serovar Typhimurium strains |                                                           |           |
| JSG 210             | 14028s (WT)                                               | ATCC      |
| ALZ36               | JSG210 with Δhyb::FRTΔhyd::FRT (Hya only)                 | 29        |
| ALZ37               | JSG210 with Δhyb::FRTΔhyd::FRT (Hyd only)                 | 29        |
| ALZ42               | JSG210 with Δhyd::FRTΔhyd::FRT (Hyb only)                 | 29        |
| ALZ43               | JSG210 with Δhyd::FRTΔhyd::FRTΔhyd::FRT (triple mutant)   | 29        |
| RLK1                | JSG210 ΔexbD::FRTΔexbD                                        | This study |
| RLK2                | JSG210 ΔtonB::FRTΔtonB                                        | This study |
| Plasmids            |                                                           |           |
| pCP20               | Amp<sup>a</sup>; contains flippase gene for λ Red mutagenesis | 30        |
| pKD46               | Amp<sup>a</sup>; contains λ Red genes γ, β, andexo          | 30        |
| pKD4                | Kan<sup>a</sup>; contains kan cassette                     | 30        |

<sup>a</sup> FRT, flippase recombinase recognition target.

**RESULTS AND DISCUSSION**

**Effects of exogenous H₂ on growth.** The ability to use hydrogen is important for *S. enterica* serovar Typhimurium survival within the animal host (9). To address possible growth-stimulating effects of H₂, the growth parameters of the parent strain and various double mutants (thus, each mutant strain contains only one of the three uptake-type enzymes) were compared in cultures with and without added H₂. This was done anaerobically with four different terminal electron acceptors: trimethylamine-N-oxide (TMAO); dimethyl sulfoxide (DMSO); sodium fumarate; and sodium nitrate. The strains used are shown in Table 1. We used CR-Hyd medium (14, 15) with the modification that no glucose was added, so the peptone and casein hydrolysate served as the carbon sources for the growth of the strains. The condition used was such that only the H₂ uptake enzymes were produced and no H₂ was produced, so only the effects of exogenously added H₂ were being observed. Growth was not significantly higher in cells grown with nitrate. Growth yields with H₂ were increased 1.8-fold and about 3-fold for cells grown with TMAO and DMSO, respectively, compared to those under H₂-lacking conditions (Table 2). Growth was not significantly higher in cells grown with nitrate. When grown with fumarate, the triple mutant (strain ALZ43; Table 1), lacking all H₂ uptake ability, never responded to H₂ addition. The wild type (WT) growth rate (doubling time) with H₂ was 1.5 h, whereas without H₂ it was about 5 h (Fig. 1). Less pronounced but significant growth rate differences (in comparing bacteria with H₂ added versus those with no H₂ added) were observed for the WT on either TMAO or DMSO (data not shown).

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| ALZ42               | JSG210 with Δhyd::FRTΔhyd::FRT (Hyb only)                 | 29        |
| ALZ43               | JSG210 with Δhyd::FRTΔhyd::FRTΔhyd::FRT (triple mutant)   | 29        |
| RLK1                | JSG210 ΔexbD::FRTΔexbD                                        | This study |
| RLK2                | JSG210 ΔtonB::FRTΔtonB                                        | This study |
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**TABLE 1** Strains and plasmids used in this study

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containing medium) compared to that of cells grown without added H$_2$. This H$_2$-facilitated growth occurred when ALZ42 was grown with fumarate, nitrate, TMAO, or DMSO (Table 2). The growth rate of ALZ42 was clearly also H$_2$ stimulated (Fig. 1). The strain that contained only Hya (ALZ36; Table 1) did not respond significantly to the presence of H$_2$ and had growth yields similar (in nitrate, TMAO, or DMSO) to those of the WT in those same media when grown without H$_2$ (Table 2). The strain that contained only Hyd (ALZ37; Table 1) had increased growth yield with H$_2$ when TMAO was provided as an electron acceptor; still, its final yield was much less than those of ALZ42 and the WT. Addition of H$_2$ had no effect on growth or amino acid uptake of the triple mutant strain ALZ43 that lacks all H$_2$-oxidizing ability, and this strain had the lowest growth yield among the strains used.

In the growth experiments, the effects of exogenously added H$_2$ were addressed. This is appropriate, as organs colonized by Salmonella were shown to contain significant levels of H$_2$ (8). Sawers et al. demonstrated that H$_2$ evolution is low (between 0.016 and 0.001 μmol of H$_2$ evolved per min) when S. enterica serovar Typhimurium is grown under anaerobic respiration with fumarate (16). We wanted to determine whether cells were producing H$_2$ (which likely would affect the growth yield) under the growth conditions used in our study. One milliliter of headspace gas from 8-h stationary-phase cultures of the triple uptake mutant (ALZ43) grown with fumarate, DMSO, nitrate, or TMAO was assayed for the presence of H$_2$ using an amperometric Clark-type electrode (17). There was no detectable H$_2$ (less than 10 nmol) present in headspace gas from these cultures. This result indicates that the cells were not producing appreciable H$_2$ in the medium and under the atmosphere conditions used in the study herein.

Collectively, our results indicate that addition of exogenous H$_2$ to the headspace greatly enhances the growth rate and yield on fumarate and some other anaerobic respiratory electron acceptors. The bulk of this growth rate increase can be attributed to the Hyb enzyme. Yamamoto and Ishimoto (18) reported that Escherichia coli cells continuously bubbled with hydrogen grew with nitrate, fumarate, or TMAO provided as an electron acceptor and that both H$_2$ uptake and H$_2$ evolution activities were the greatest with fumarate. Hydrogenase activity staining bands from gels indicated the expression of multiple forms of the E. coli enzymes in the fumarate-containing medium, and from growth yields, they suggested that 1 mol of ATP is produced per mol of H$_2$ oxidized.

### Amino acid uptake.

The growth studies described above were performed in an amino acid-containing medium, so we measured the uptake of $^{14}$C-labeled amino acids when cells were in an atmosphere containing H$_2$ and in one without the gas. Both the WT strain and Hyb-only-containing strain ALZ42 demonstrated significantly increased amino acid uptake ability in the presence of H$_2$. Although the uptake by ALZ42 was initially one-half that of the WT, both strains had 4-fold greater uptake in the first 5 min with H$_2$ than without H$_2$ added (Table 3). After 5 min, the amino acid accumulation continued at a lower rate, but at all points, the uptake was greater for both strains when H$_2$ was provided. The results indicate that the energy for uptake/transport of amino acids in these strains is provided via oxidation of H$_2$, akin to what was observed in Helicobacter hepaticus by Mehta et al. (19). The similar results for ALZ42 and the WT, which contains three distinct H$_2$-oxidizing enzymes, indicate that the Hyb hydrogenase is important for amino acid accumulation. The activity of the Hyb hydrogenase probably suffices to allow the organism to glean energy from H$_2$ for significant solute transport and thus for growth and survival of S. Typhimurium under anoxic and nutrient-limiting conditions. To confirm that the Hyb enzyme plays the largest role in amino acid uptake in nutrient-limited medium, the other mutant strains, each containing a single uptake-type hydrogenase, were assessed for H$_2$-dependent amino acid uptake (Table 4). The strains containing Hya or Hyd as the only uptake hydrogenase were capable of much less uptake of the $^{14}$C-labeled amino acid pool than the WT, and H$_2$ had little effect on their amino acid accumulation abilities. Still, the accumulation by Hyd-only-containing strain ALZ37 was slightly stimulated by H$_2$. The

#### TABLE 3 $^{14}$C-labeled amino acid uptake by the WT and ALZ42 strains

| Strain and condition | $^{14}$C-labeled amino acid uptake (cpm [10$^3$]/10$^8$ cells)$^a$ at: | 5 min | 10 min | 20 min |
|---------------------|---------------------------------------------------------------|-------|-------|-------|
| WT                  |                                                               |       |       |       |
| +H$_2$              |                                                               | 31.0 ± 3.8$^b$ | 37.8 ± 2.3$^b$ | 35.0 ± 1.9$^b$ |
| −H$_2$              |                                                               | 7.8 ± 1.3   | 9.3 ± 0.9 | 9.9 ± 0.8 |
| ALZ42 (Hyb only)    |                                                               |       |       |       |
| +H$_2$              |                                                               | 14.0 ± 1.6$^b$ | 14.9 ± 1.4$^b$ | 20.4 ± 3.5$^b$ |
| −H$_2$              |                                                               | 3.1 ± 0.7   | 3.4 ± 0.5 | 3.5 ± 0.5 |

$^a$ Values represent $^{14}$C-labeled amino acid uptake by $10^8$ cells ± standard deviation (n = 4).

$^b$ Significantly higher uptake level than without H$_2$ (P < 0.005 [Student’s t test]).
ALZ43 (triple mutant) upon treatment of the cells with CCCP before the addition of the activities of both the WT and ALZ42 strains markedly decreased. The amino acid uptake was significantly lower than without inhibitor (Table 4). Pretreatment of the cells with sodium orthovanadate also resulted in reduced amino acid uptake activity of both the WT and ALZ42 strains. However, considerable uptake activity remained; the uptake rate with inhibitor was still about 50% of the uninhibited rate for the WT at both 5 and 10 min. ALZ42 had 39% and 52% of the uninhibited rate at 5 min and 10 min, respectively. These results indicate that H₂-dependent amino acid transport in these strains is driven by both PMF and ATP but that the PMF likely plays the larger role in H₂-facilitated amino acid uptake.

**Hydrogenase activity and membrane potential (ΔΨ).** Hyb-only-containing strain ALZ42 demonstrated H₂ uptake hydrogenase activity that was 65% of that of the WT (42.7 ± 8.1 nmol H₂ uptake/min/10⁹ cells), while ALZ36 (containing Hya only) and ALZ37 (containing Hyd only) showed 10.8% and 2.0% of the uptake hydrogenase activity of the WT, respectively. ALZ43 did not show any uptake hydrogenase activity. Therefore, under the conditions used in this study, the bulk of the H₂ uptake activity in *Salmonella* Typhimurium is accomplished by the activity of the Hyb hydrogenase.

We utilized the fluorescence ratio imaging technique to measure the membrane potential component (ΔΨ) of PMF in the WT and ALZ42 strains by using a cationic dye, JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; Molecular Probes, Invitrogen). A shift in the emission spectrum of the JC-1 dye from red (590 nm) to green (530 nm) indicates a decrease in membrane potential and hence an increased green (530 nm)/red (590 nm) ratio. ALZ42 showed a green/red fluorescence ratio comparable to that of the WT (Fig. 2), indicating similar PMF levels in the two strains. The membrane polarization of ALZ42 (Hyb-only-containing strain) was 75% of that of the WT (red/green ratios, 2.56 ± 0.41 and 1.91 ± 0.28 in the WT and ALZ42 strains, respectively), and the difference between the two strains was statistically insignificant at a 99% confidence level. ALZ43 (triple mutant lacking Hya, Hyb, and Hyd) showed significantly decreased membrane potential compared to that of the WT (Fig. 2). The WT treated with CCCP was

| Strain and condition | ¹⁴C-labeled amino acid uptake (cpm [10⁶]/10⁶ cells) at: |
|----------------------|--------------------------------------------------|
|                      | 5 min                                            |
|                      | 10 min                                           |
| ALZ36 (Hya only)     | +H₂ 2.3 ± 0.7                                    |
|                      | −H₂ 1.9 ± 0.3                                    |
| ALZ37 (Hyd only)     | +H₂ 3.0 ± 0.6                                    |
|                      | −H₂ 2.0 ± 0.2                                    |
| ALZ43 (triple mutant)| +H₂ 0.7 ± 0.2                                    |
|                      | −H₂ 0.6 ± 0.1                                    |

* Values represent ¹⁴C-labeled amino acid uptake by 10⁶ cells ± standard deviation (n = 4).

**TABLE 5** Effects of inhibitors on ¹⁴C-labeled amino acid uptake by the WT and ALZ42 strains

| Strain and presence of added H₂ | ¹⁴C-labeled amino acid uptake (cpm [10⁶]/10⁶ cells) at: |
|---------------------------------|--------------------------------------------------|
|                                 | 5 min                                            |
|                                 | 10 min                                           |
| WT                              |                                                   |
| None                            | 29.0 ± 2.0                                       |
| CCCP                            | 0.7 ± 0.5b                                       |
| Orthovanadate                   | 14.8 ± 1.2b                                      |
| ALZ42 (Hyb only)                |                                                   |
| None                            | 14.0 ± 1.6                                       |
| CCCP                            | 0.3 ± 0.1b                                       |
| Orthovanadate                   | 5.5 ± 0.1b                                       |

* Values represent ¹⁴C-labeled amino acid uptake by 10⁶ cells ± standard deviation (n = 4).

b Significantly lower uptake level than without inhibitor (P < 0.005 [Student’s t test]).
included as a control to show the green/red ratio of a disintegrated membrane potential. As expected, CCCP-treated WT cells had the highest green/red ratio among the samples. These results support our hypothesis that the Hyb hydrogenase is involved in carrying out the bulk of the respiratory hydrogen oxidation, and therefore in maintaining the PMF of the cells, under H₂-added conditions.

Involvement of the TonB-ExbD system. In E. coli and other Gram-negative bacteria, the cytoplasmic PMF is utilized by the TonB-ExbB-ExbD system for substrate transport by the TonB-dependent outer membrane transport proteins (TBDTs) (25, 26). Initially shown to be specifically for the uptake of iron complexes and vitamin B₁₂, the role of the TonB-dependent transport has since been expanded to the transport of various other substrates, such as nickel, carbohydrates, cobalt, and copper (27). While the precise mechanism of transport remains unclear, it has been suggested that TonB transduces the PMF to the TBDTs via its periplasmic interaction with ExbD, forming a TonB-ExbB-ExbD complex, and that TonB requires PMF to form the complex with ExbD (28). In an effort to investigate the effect of added H₂ on the PMF-facilitated cross-linking between TonB and ExbD in our strain, we subjected the WT to formaldehyde-mediated cross-linking and visualization of the TonB-ExbD complex using TonB- and ExbD-specific antibodies. We were unable to visualize the ExbD-TonB complex in our strains, although a large ExbD-immunoreactive adduct was observed in the culture growing with H₂. Importantly, a marked increase in the production of the TonB and ExbD proteins under the condition with H₂ added was observed (Fig. 3). Based on densitometry, the increases in expression due to incubation with H₂ were 4.0-fold and 11.0-fold for TonB and ExbD, respectively. Quantitative real-time PCR showed elevated tonB and exbD transcript levels in the WT (about 2-fold- and 4-fold-higher expression of tonB and exbD, respectively) and a 1.8-fold increased exbD transcript level in ALZ42 when the strains were grown under exogenously added H₂. DNA gyrase B (gyrB) was used as an internal control to normalize the expression levels of tonB and exbD, since a microarray analysis (not discussed here) revealed the expression of gyrB to be unaltered under the conditions used in this study. As in other Gram-negative bacteria, the TonB-ExbD system of Salmonella Typhimurium could play a crucial role in fulfilling the increased demand for the delivery of substrates such as iron siderophores, vitamin B₁₂, nickel complexes, and carbohydrates in a nutrient-limited environment.

To investigate whether the TonB-ExbD system is also involved in H₂-stimulated amino acid uptake, we made ΔexbD and ΔtonB single-deletion mutants (strains RLK1 and RLK2, respectively; Table 1) and subjected them to the amino acid uptake assays described previously. Clear phenotypes distinguishable from that of the WT (i.e., decreases in uptake by the mutants) were not observed (data not shown). Bacteria contain a wide variety of trans-membrane amino acid transporters (23), and this includes transporters that are aided by energy-coupling proteins other than TonB-ExbD. Nevertheless, the ΔexbD mutant strain demonstrated 40% reduced nickel uptake compared to that of the WT (63Ni uptake, 20.4 × 10² ± 2.3 × 10² cpm/10⁸ cells in RLK1 and 33.9 × 10² ± 6.7 × 10² cpm/10⁸ cells in the WT), indicating a role for ExbD in nickel uptake in Salmonella. It is possible that in the presence of hydrogen, the bacteria upregulate the expression of ExbD and TonB to transport more nickel into the cells for proper hydrogenase maturation.

Our study shows that Salmonella Typhimurium can grow in an H₂-dependent manner and that most of the H₂ oxidation by nutrient-limited anaerobically growing Salmonella Typhimurium is aided by the activity of the Hyb hydrogenase alone. Growth under anaerobic respiration with terminal electron acceptors was enhanced with H₂, and this H₂-facilitated growth ability was assigned to a specific H₂-using hydrogenase. The energy available from H₂ oxidation is coupled to the uptake of carbon, and the uptake is driven by both PMF and ATP. H₂ increases the expression of genes that encode specific proteins (ExbD and TonB) known to complex PMF to aid solute transport processes. Similarly, immunologically identified TonB and ExbD proteins were significantly induced by incubation with H₂. The increased expression of the PMF-dependent transport proteins (ExbD and TonB) under the H₂-added condition is likely a way for the bacteria to balance energy input with nutrient acquisition. The results herein link H₂ oxidation via the Hyb hydrogenase enzyme to H₂-
dependent growth, solute transport, and expression of periplasmic proteins that facilitate PMF-mediated transport across the outer membrane.

MATERIALS AND METHODS
Strains, growth conditions, and reagents. WT S. enterica serovar Typhimurium ATCC 14028s and hydrogenase mutant strains described by Zbell and Maier (29) were used in this study. All mutant strains were shown to be nonpolar (29). tonB and exbD gene single-deletion mutants were constructed using the lambda Red system as previously described (30). The deletions were confirmed by PCR using primers complementary to the regions flanking the deleted genes and by sequencing across the deletions (Georgia Genomics Facility, University of Georgia). The strains and plasmids used in this study are listed in Table 1, and the primers used are listed in Table 6.

Strains were maintained in Luria-Bertani (LB) broth or on LB plates. Experiments were performed in CR-Hyd medium (14, 15) containing bacteriological peptone (0.5%, wt/vol), casein hydrolysate (0.2%, wt/vol), thiamine (0.001%, wt/vol), MgCl₂ (1 mM), (NH₄)₆Mo7O24 (1 mM), and NaSeO₃ (1 mM). The medium was supplemented with sodium fumarate (0.5%), sodium nitrate (0.5%), TMAO (0.5%), and DMSO (0.25%) where indicated. No carbohydrate was added, but 5% (0.5%), sodium nitrate (0.5%), TMAO (0.5%), and DMSO (0.25%) were injected into the bottles to a final concentration of 0.5 μCi/ml of growth medium. The mixture contains 15 uniformly labeled amino acids (1-Ala, 1-Arg, 1-Asp, 1-Glu, 1-Gly, 1-His, 1-Ile, 1-Leu, 1-Lys, 1-Phe, 1-Pro, 1-Ser, 1-Thr, 1-Tyr, and 1-Val). 14C-labeled amino acid uptake by the cultures was measured at 5, 10, and 20 min by a previously described method (19). For the inhibitor effects, cells were grown as described above and CCCP or sodium orthovanadate (Sigma-Aldrich Co., St. Louis, MO) was added 10 min before the addition of the radiolabeled amino acid mixture, respectively. CCCP was added to a final concentration of 50 μM, and sodium orthovanadate was added to a final concentration of 10 mM. These concentrations have been used for studies of other enteric bacteria (28, 31). The experiments (Tables 1 to 3) were repeated a total of three times with similar results, and the data shown are from four replicate samples from one experiment.

The nickel uptake assays, cultures were grown anaerobically without H₂ as described above. After the cultures reached an A₀₉₀₀ of 0.1, 20% (vol/vol of headspace) H₂ was injected into the bottles. After 60 min, 10³Ni (Amersham Biosciences, Sweden) was injected into the bottles to a final concentration of 0.5 μCi/ml of growth medium and the uptake activity was measured at 1- and 5-min intervals.

Real-time quantitative PCR. RNA was isolated from the test (20% H₂ added to the medium) and control (no added H₂) cultures (A₀₉₀₀ = 0.4) of the WT and ALZ42 strains using the RNA extraction kit from Qiagen (Qiagen Inc., Valencia, CA) by following the manufacturer’s instructions. First-strand cDNA was synthesized from 200-ng purified RNA samples using random hexamers and Moloney murine leukemia virus SuperScript III reverse transcriptase (Invitrogen) at 42°C for 50 min. The iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) and iQ Sybr Green Supermix (Bio-Rad) were utilized for real-time PCR of control and test cDNAs. The expression level (threshold cycle) of each sample was normalized using DNA gyrase B (gyrB) as an internal control. The relative n-fold change in gene expression for each sample was determined using the 2⁻ΔΔCT method as previously described (32). The gene-specific primers used for real-time PCR are listed in Table 6.

Western blot assays. To identify the effect of H₂ on the expression levels of the ExbD and TonB proteins under the condition provided, overnight cultures of the WT and ALZ42 strains grown anaerobically in the presence or absence of 20% H₂ (in CR-Hyd medium without glucose and supplemented with 0.5% sodium fumarate and 5 μM NiCl₂) were subjected to the m vitro formaldehyde cross-linking method previously described (28). The ExbD and TonB proteins and their cross-linked complexes were detected by immunoblotting using ExbD-specific polyclonal

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**TABLE 6 Primers used in this study**

| Primer | Sequence (5’→3’) | Application |
|--------|------------------|-------------|
| exbD del-F | GTCATGGCAATTCGGTCTTAAAGGAACTGAAACGATTATGACTTCATGTGTAGGCTGGAGCTGCTTC | exbD deletion |
| exbD del-R | CITACCGGCGCCTACAGCGTCAGCAGAATACCATATGAATATCCTCCTTA | exbD deletion |
| exbD-check-F | TGCAATTTCCGGCGGTCAAA | exbD deletion confirmation |
| exbD-check-R | TATTTGCTTTCGGCCTCTTCCTG | exbD deletion confirmation |
| exbD-F | CGGTGAAGGCGGATAAACACCATGT | exbD real-time PCR |
| exbD-R | CTTACCCGGCCTACAGCGTCAGCAGAATACCATATGAATATCCTCCTTA | exbD real-time PCR |
| tonB del-F | TGGTTTTTACCACTGAAACGATTATGACTTCATGTGTAGGCTGGAGCTGCTTC | tonB deletion |
| tonB del-R | TGGCGATGTCGTATGCTGCTAC | tonB deletion |
| tonB-check-F | CGGTATCGCGAATGCTATT | tonB deletion confirmation |
| tonB-check-R | TGCGATCGGTGTAGCTGTGCTTAC | tonB deletion confirmation |
| tonB-F | TTCACCTTACCGCGGCTCTTAATA | tonB real-time PCR |
| tonB-R | AAGAATTGAAGACACCGGAAGC | tonB real-time PCR |
| gyrB-F | CGGGTTCAATTCACCCAGACCTTT | Real-time PCR internal control |
| gyrB-R | TAGGATGGGCGGCTGATCCGATTA | Real-time PCR internal control |
antibodies and TonB-specific monoclonal antibodies (33). The antibodies were kindly provided by Kathleen Postle, Pennsylvania State University, University Park. The entire cross-linking experiment was repeated three times with the same results, as shown in Fig. 3.

**Hydrogenase assay.** The H₂ uptake hydrogenase activity of the WT and ALZ42 strains was assayed in whole cells by following the reduction of methylene blue at 570 nm and is expressed as nmol H₂ taken up/min/10⁹ cells. Gen uptake activity was determined by measuring the reduction of methylene blue at 570 nm (red) was observed. The cationic dye JC-1 forms red fluorescent aggregates at higher potential and remains as green fluorescent monomers.

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