A whole-cell, high-throughput hydrogenase assay to identify factors that modulate [NiFe]-hydrogenase activity

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[FeFe]-hydrogenases have attracted attention as potential therapeutic targets or components of a hydrogen-based economy. [FeFe]-hydrogenase production is a complicated process that requires many associated accessory proteins that supply the requisite cofactors and substrates. Current methods for measuring hydrogenase activity have low throughput and often require specialized conditions and reagents. In this work, we developed a whole-cell high-throughput hydrogenase assay based on the colorimetric reduction of benzyl viologen to explore the biological networks of these enzymes in Escherichia coli. We utilized this assay to screen the Keio collection, a set of nonlethal single-gene knockouts in E. coli BW25113. The results of this screen highlighted the assay’s specificity and revealed known components of the intricate network of systems that underwrite [FeFe]-hydrogenase activity, including nickel homeostasis and formate dehydrogenase activities as well as molybdopterin and selenocysteine biosynthetic pathways. The screen also helped identify several new genetic components that modulate hydrogenase activity. We examined one E. coli strain with undetectable hydrogenase activity in more detail (ΔeutK), finding that nickel delivery to the enzyme active site was completely abrogated, and tracked this effect to an ancillary and unannotated lack of the fumarate and nitrate reduction (FNR) anaerobic regulatory protein. Collectively, these results demonstrate that the whole-cell assay developed here can be used to uncover new information about bacterial [FeFe]-hydrogenase production and to probe the cellular components of microbial nickel homeostasis.

[FeFe]-hydrogenase enzymes catalyze the reversible formation of hydrogen gas from protons and electrons at an intricate bimetallic active site (1, 2). These enzymes are found in many microorganisms, including several pathogens (1), and because the enzymes and the dedicated maturation machinery required for production are orthogonal to human systems, these pathways are attractive targets for the development of new antibiotic strategies (1, 3, 4). Furthermore, hydrogenases are the subject of extensive research because of potential applications as catalysts in a hydrogen-fueled economy (2). Despite ongoing efforts to characterize these enzymes and their associated pathways, progress is hindered because current methods to measure hydrogenase activity in cells or extracts are laborious, are not amenable to high-throughput methods, and require specialized equipment (5–17). A rapid low-cost hydrogenase activity screening method would allow for the collection of system-level data and facilitate studies of the mechanisms of production, optimization of activity, and identification of inhibitors.

There are four [FeFe]-hydrogenases encoded in the genome of the facultative anaerobe Escherichia coli that serve several metabolic functions (1, 18). Hyd-1 and Hyd-2 are predominately hydrogen-oxidizing enzymes that contribute to proton gradients across the inner membrane and are thought to operate under different reductive potentials (18). Hyd-3 is a component of the formate hydrogenlyase complex (FHL)3 that produces H2 upon the oxidation of formate (19). Finally, Hyd-4 putatively reduces protons, but its biochemical role and the growth conditions under which it is expressed are not clearly defined (18, 20). Expression of Hyd-1 and Hyd-2 is activated under anaerobic growth conditions (18), whereas expression of Hyd-3, and possibly Hyd-4, is activated in part by FhlA, which responds to changes in the levels of formate (18, 21).

[FeFe]-hydrogenases are composed of two subunits: a large subunit containing the nickel–iron catalytic metalocenter, which includes cyanide and carbon monoxide ligands, and a small subunit bearing iron–sulfur clusters (1, 2, 22). These enzymes are typically coupled to auxiliary proteins and other enzymes within large metabolic complexes, such as formate hydrogenlyase, that link the hydrogenase activity to other cellular functions (18, 19). In addition, the biosynthesis of these...
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enzymes requires a suite of dedicated maturation proteins that synthesize the metal clusters, and these systems are in turn supported by networks of metal homeostasis and metabolic factors that ensure the supply of the individual components (4, 23, 24).

One of the essential cofactors of these enzymes is nickel, which is toxic at elevated concentrations (25, 26). For this reason, the acquisition and distribution of intracellular nickel ions is a highly controlled process (27). The nickel homeostasis network in E. coli includes a nickel importer and an exporter, NikABCDE and RcnA, respectively (28); nickel-dependent transcription regulators RcnR and NikR (29); and the nickel metallochaperones HypA/HybF, HypB, and SlyD, which deliver nickel to the hydrogenase enzyme precursor proteins (4). The only other nickel enzyme identified in this bacteria, glyoxalase I, may employ alternative metal ions, and the mechanism of metal loading is undefined (30). Thus, the activity of [NiFe]-hydrogenase serves as a reporter of the integrity of nickel homeostasis systems and the function of the nickel metallochaperones.

A variety of methods to monitor hydrogenase activity have been reported. These include spectrophotometric analysis of small-molecule dyes that are oxidized or reduced by hydrogenases, including benzyl viologen and methyl viologen (5–10, 17); electrochemical methods that measure the reduction potentials or molecular hydrogen concentrations (11–14); and GC to detect changes in the levels of hydrogen gas (15, 16). The growth conditions, sample processing, and specialized equipment required for these methods are not amenable to high-throughput screening, which is typically performed on multiwell plates by robotic equipment. For example, hydrogenase activity is often analyzed in cell lysates or with purified proteins prepared using multistep protocols from bacteria grown under anaerobic conditions. Similarly, recently described bacterial assays involve a centrifugation step and/or buffers saturated with externally sourced hydrogen gas and artificial exclusion of oxygen (11, 31).

In this work, we present the development of a whole-cell hydrogenase assay amenable to high-throughput screening and demonstrate that it reports on the function of several pathways that support enzyme production, including nickel homeostasis and biosynthesis of molybdopterin and selenocysteine. To probe the utility of this assay, we screened the Keio collection for loss of activity and characterized a selection of those identified strains that are not currently associated with [NiFe]-hydrogenases. Several strains with enhanced activity had deficient cell wall biosynthesis, revealing the unexpected application of this assay to study factors that modulate cell wall or outer membrane integrity. One of the strains with deficient hydrogenase activity, ΔeutK, was unable to produce any active hydrogenases due to defective nickel incorporation into the [NiFe]-hydrogenase precursor proteins, which was traced to an unexpected ancillary genetic deficiency in the fur gene. This assay will be valuable for the future collection of “omic” data to characterize the microbial components required for [NiFe]-hydrogenase maturation, to optimize production for biotechnology applications, and for the discovery of small-molecule inhibitors to be used as research tools or therapeutics.

Results

Assay development

Optimization—To design a whole-cell hydrogenase activity assay suitable for high-throughput infrastructure, the reduction of benzyl viologen was assessed as an indicator. This dye is an established hydrogenase substrate and affords colorimetric detection of activity (5–10). The BW25113 strain of E. coli was used for assay development because it is the parent strain of the Keio collection (32) and has been previously used in studies of [NiFe]-hydrogenase (33). As a negative control, a strain of E. coli lacking the large subunit of Hyd-3, ΔhycE, was chosen. This strain is a conservative choice for assay optimization because there are several other active hydrogenase enzymes in E. coli, and under the screening growth conditions Hyd-3 did not supply all of the benzyl viologen reduction capacity (see below). The optimization of the assay was assessed by monitoring the Z’-score (34), a statistical parameter that measures the difference between positive and negative responses (see Equation 1).

Hydrogenase enzymes are typically expressed under rigorous anaerobic growth conditions (18); however, we found that active hydrogenase was produced in E. coli grown in LB medium in a 96-well plate with the lid on, without shaking, in a standard ambient incubator (Fig. 1A). Higher levels of activity,
as well as larger statistical separation between the control strains, were generated by using TYET medium supplemented with sodium molybdate and sodium selenite, which are incorporated into the cofactors of the enzymes coupled to Hyd-3 in the formate hydrogenlyase complex, as well as formate for gene activation (18) and glucose to permit rapid growth and drive mixed acid fermentation (33). The growth of BW25113 and ΔhyeC E. coli in supplemented TYET medium was monitored over time, and both strains achieved stationary phase after 6 h (Fig. S1). Potential edge effects were analyzed by comparing the benzyl viologen reduction activities of the cells grown at the edge of the plate versus those grown at interior positions. Although the difference was small and the normalized activities for the edge (1.03 ± 0.07) and interior position (0.98 ± 0.05) were within one S.D., the difference was significant (p < 0.005) (Figs. S2 and S3), so a corresponding correction was applied in subsequent experiments. The benzyl viologen reduction activity was measured after 6, 8, or 10 h of growth, and the best Z’-scores were observed at 6 or 8 h (Fig. 1A). Finally, 10 mg/ml benzyl viologen and 250 mM sodium formate were selected as the final concentrations in the developing solution for an end-point assay (Fig. S4).

Assay specificity—The E. coli genome encodes four different hydrogenase enzyme systems, but the expression of each enzyme varies with growth conditions (1, 18). To assess which enzyme contributes to the activity measured in this assay, benzyl viologen reduction was evaluated with E. coli strains that do not express the large subunits of Hyd-1 (ΔhyaB), Hyd-2 (ΔhybC), or Hyd-3 (ΔhyeC). The activity of a Hyd-4 – deficient strain was not measured during method development because Hyd-4 is likely not expressed in WT E. coli under our growth conditions (18), consistent with the observation that disruption of the large subunit gene hyfG did not affect activity in the Keio collection screen (see below). The results reveal that Hyd-2 and Hyd-3 are required for optimal benzyl viologen reduction activity in this whole-cell assay, whereas Hyd-1 is a minor contributor (Fig. 1C). In addition, given that benzyl viologen is a small-molecule electron acceptor, it was possible that other constituents of living bacteria could also reduce this dye. To test for hydrogenase-independent activity, strains deficient in the nickel metallochaperone (ΔhypB) and the nickel importer (ΔnikA) were analyzed as negative controls because these factors have a comprehensive impact on [NiFe]-hydrogenase production (35, 36). These strains produced even lower activity compared with strains lacking either hydrogenase 2 or 3, demonstrating that the assay is primarily reporting on [NiFe]-hydrogenase activity.

For hydrogenase-dependent reduction of benzyl viologen, the electrons are provided by the molecular hydrogen substrate. In E. coli, hydrogen gas is produced during the oxidation of formate to CO₂ by the formate hydrogenlyase complex (19). This complex includes Hyd-3 as well as the enzyme formate dehydrogenase (FDH), which requires a molybdopterin cofactor (19, 37). To explore the importance of this activity, Na₂WO₄ was added to the hydrogenase growth medium. Tungstate serves as a molybdic mimic that prevents the incorporation of molybdenum into FDH, thereby preventing the production of active enzyme (37). We found that addition of Na₂WO₄ resulted in a dose-dependent inhibition of the whole-cell benzyl viologen reduction activity (Fig. 1D) with a minimal effect on growth (Fig. S5). This observation is consistent with a decrease in active formate dehydrogenase (38) and the requirement for the production of molecular hydrogen through the formate hydrogenlyase complex to reduce benzyl viologen.

Robotic Keio collection screen

To establish that the whole-cell hydrogenase assay presented here is amenable to robotic high-throughput analysis and to validate its utility to identify factors that impact hydrogenase production, it was used to screen the Keio collection of all non-essential single-gene deletions in E. coli (32). Each mutant of the Keio collection was grown in duplicate in supplemented TYET medium in 384-well plates for 6 h before the benzyl viologen reduction activity was measured. The activities were normalized for growth, plate, and well position and were highly reproducible (Figs. 2A and S6 and Table S2). Examination of the activities of strains with deletions in established hydrogenase components revealed that the switch to the automated high-throughput conditions of the screening facility resulted in a greater dependence on Hyd-2 (ΔhybC) for the benzyl viologen reduction, and as expected the impact on activity was more substantial upon deletion of the upstream pleiotropic maturaton factors, such as nikA and hypB, than upon deletion of any of the individual large subunits (Fig. 2B).

The results of the screen highlighted 109 knockout strains with reduced hydrogenase activity, referred to as “hits.” The roles of these gene products include metal homeostasis, hydrogenase maturation and regulation, selenocysteine biosynthesis, iron–sulfur (FeS) cluster biosynthesis, molybdopterin biosynthesis, and Hyd-2 and FHL components (Table S3). Based on literature reports, we compiled a list of 43 strains in the Keio collection that have deletions of genes that are established as needed for benzyl viologen reduction by FHL/Hyd-3 or Hyd-2 using either formate or hydrogen as the reductant in E. coli (Table S4). Of these genes, 31 were hits (72% identified), validating the target specificity. In addition, this screen provided conclusive evidence for a role in hydrogenase activity for another 20 genes that have a biological link to [NiFe]-hydrogenase or formate dehydrogenase activities, such as the biosynthesis of molybdopterin or selenocysteine (Table S3), and demonstrated that benzyl viologen reduction in whole cells requires both an active hydrogenase and a mechanism to reduce formate. A separate subset (34 strains) are directly related to general cell metabolism and are connected to hydrogenase activity by affecting carbon metabolism, inner membrane proton gradient, or gene expression, or inhibited growth to a low optical density (OD). The remaining mutant strains (24 strains) do not have a clear link to the maturation or structure of the [NiFe]-hydrogenase enzymes, or they encode for hypothetical proteins of unknown function. This final group may include novel genes with a role in the production of [NiFe]-hydrogenase enzymes, and some possible connections are discussed below.

As a complementary approach, gene ontology (GO) term enrichment (39–41) was performed on the list of genes with reduced activity and uncovered several ontologies related to nickel transport, molybdopterin biosynthesis, and selenocys-
Figure 2. Keio collection screen. A, replica plot of the normalized benzyl viologen reduction activity of the different mutants of the Keio collection. Hits were classified as any strain with a normalized activity < 0.619, which corresponds to 3 times the S.D. of the middle 95% of the data. B, the normalized activity of several strains of the Keio collection relating to hydrogenase large subunits, maturation factors, and formate metabolism. C, the 109 gene deletion mutants that displayed reduced hydrogenase activity were classified based on GO biosynthetic processes, and statistical enrichment was calculated using EcoCyc pathway tools. Enrichment was based on functional overrepresentation of the genes resulting in reduced hydrogenase activity, using a Fisher’s exact test to calculate p value. The six most statistically enriched, nonredundant GO biosynthetic process classifications are shown. a.u., arbitrary units.

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teine biosynthesis (Fig. 2C), indicating that our screen enriched for genes that are involved in metal homeostasis and cofactor production.

The screen also highlighted 105 knockout strains with an increase in benzyl viologen reduction activity. Although the primary directive was to identify factors that inhibit activity (i.e. gene deletions of maturation factors), strains with higher activity were of interest because none of the deleted genes were directly connected to hydrogenase regulation. Furthermore, many of the gene knockouts with increased activity are involved in cell wall or outer membrane biogenesis (e.g. dapF, mltE, alr, and wecE). Of note, GO terms related to biosynthesis of enterobacterial common antigen, which is a glycolipid found at the surface of Enterobacteriaceae, were particularly enriched in this group (p = 7.47 × 10^{-12}).

Hit validation and characterization

We sought to validate a selection of the strains identified in the automatic screen of the Keio collection (referred to as robotic screen) with either high or low activity and with no obvious connection to the production of Hyd-3/FHL and Hyd-2. As described above, 22% of the initial hits (n = 24) that exhibited reduced benzyl viologen reduction activity could not be classified in any known category. Almost half of these strains encode uncharacterized genes, so it was possible that this screen had uncovered novel components of hydrogenase production. We further refined this list because 80% of the genes with an established connection to hydrogenase exhibited an activity less than 0.5 times that of the WT strain. Using this more rigorous condition, the list of uncharacterized genes of interest shrinks to nine entries: ydfI, ycgY, yceO, ygjI, yccE, eutK, mlaC, ydeI, and fadR. We also selected three of the strains with the highest activity and that grew to an OD > 0.4, Δalr, ΔdksA, and ΔmetL, along with ΔompA, which was the most active strain with an OD similar to that of the WT strain. Cell strains ΔnikA and ΔselA were employed as negative controls for the subsequent analysis. These two strains serve to help differentiate between genes essential for the production of the hydrogenase enzymes or the associated formate dehydrogenase components, respectively.

For each strain, we verified the genetic mutation by confirming the location of the kanamycin cassette by using colony PCR (data not shown). The next step in the validation process was to reproduce the robotic screen results by manually assessing each strain with the optimized whole-cell hydrogenase assay. In general, we found a good agreement with a tendency that the robotic screen produced lower normalized activities compared with those observed with the manual whole-cell assay (Figs. 3 and S7). This trend is likely caused by sample selection bias, because we chose strains that had lower activity, and does not represent a systematic difference between the two methodologies. It is also worth noting that the whole-cell assay is a kinetic analysis and the robotic measurement was a single time point, so the manual whole-cell assay would have a wider dynamic range and higher accuracy at the extreme low/high activities.
After confirming the decrease in whole-cell hydrogenase activity, we performed a secondary hydrogenase assay that measures the hydrogen-depandant reduction of benzyl viologen in crude cell lysates. This assay was performed in an atmosphere containing 3–5% H₂ and bulk N₂ and is widely used to measure the total hydrogenase activity independent of other cell machinery such as a functioning formate dehydrogenase (5, 7, 9, 42). Using this secondary screen, we were able to differentiate between strains that had a low whole-cell response due to a deficient hydrogen oxidation activity versus other factors such as faulty formate dehydrogenase or substrate accessibility (Fig. 3). For example, the ΔselA strain has a low whole-cell activity due to the absence of selenocysteine, a required amino acid for the function of the formate dehydrogenase, but this does not impact the maturation of the [NiFe]-hydrogenase active site and the activity measured in the cell lysates. In contrast, the ΔnikA strain exhibited low activity in both assays because it does not support the production of the metal cluster in mature [NiFe]-hydrogenase. Most of the strains analyzed exhibited activities in the lysate assay that were similar or higher than that of the WT strain, indicating a defect in a component other than the hydrogenase enzyme itself, such as in formate dehydrogenase maturation or reduced substrate access. Several of these proteins are uncharacterized and will be of interest for researchers studying molybdenum and selenium biochemistry. This secondary screen highlighted a hit with low activity in both assays: ΔeutK. In addition, we found that strains with an increase in whole-cell activity did not produce higher hydrogenase activity in the lysates.

**Characterization of strains with more activity**

The GO ontologies and the results of the lysate assay suggested that the increase in whole-cell activity observed in some strains could be related to substrate accessibility. A survey of the disrupted genes with known functions indicated that they all play a role in cell wall biosynthesis, so it is reasonable to suggest that these defects increase the accessibility of benzyl viologen to hydrogenase. To establish whether this model is plausible, we measured the hydrogenase activity of cells grown in the presence of antibiotics that inhibit cell wall biosynthesis: ampicillin, a β-lactam antibiotic that targets penicillin-binding proteins (43), and D-cycloserine, which inhibits alanine racemase (44). This latter enzyme is the product of the gene alr, which was identified as a more active strain in the robotic screen. Kanamycin was used as a control to verify that the increase in activity was not due to general cell stress. We measured the whole-cell hydrogenase activity of the WT *E. coli* following growth in the presence of antibiotic concentrations that resulted in approximately half the expected growth rate (Fig. S8). This experiment revealed a 7-to-8-fold increase (p < 0.01) in whole-cell benzyl viologen reduction activity in the presence of either ampicillin or D-cycloserine versus only an insignificant (p = 0.90) change upon the addition of kanamycin (Fig. 4).

To further characterize the selected strains, we performed an assay to monitor 1-N-phenylnaphthylamine (NPN) uptake. NPN is a small hydrophobic molecule that is used to study bacterial permeability because the fluorescence intensity increases upon passage of NPN into the inner membrane of *E. coli* (45). We found a linear relationship between NPN uptake and whole-cell benzyl viologen reduction activity (Fig. 4), further supporting the model that an increase in the benzyl viologen reduction activity is related to substrate availability. The one exception was the ΔdksA strain, which exhibited WT levels of whole-cell hydrogenase activity, lysate activity, and NPN uptake. Furthermore, dksA is annotated as a transcription factor and does not fit the profile of the other strains with increased activity. Taken together, it appears that this particular strain was a false positive from the initial robotic Keio screen.

**Characterization of ΔeutK**

The observation that ΔeutK *E. coli* exhibited severely reduced activity in both the whole-cell and lysate hydrogenase activity assays suggested a deficiency in hydrogenase maturation and prompted us to further characterize this strain. The final step in the maturation of the large hydrogenase subunit is cleavage of a short peptide from the C terminus (4), which serves as a checkpoint confirming that the catalytic metal clus-
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**Figure 4. Cell permeability and increased whole-cell hydrogenase assay.** A, whole-cell hydrogenase activity of *E. coli* grown in the presence of the indicated concentrations of antibiotics, which decreased growth by 50%. Error bars represent one S.D. of three biological replicates. B, normalized NPN uptake versus normalized whole-cell hydrogenase activity. Error bars represent S.E. Data were collected from five biological replicates. BW, BW25113.

To explore the underlying cause of the failure of ΔeutK to generate active hydrogenase, we took advantage of the whole-cell assay to rapidly screen for conditions that could restore hydrogenase activity. We screened biologically relevant divalent metals, carbon monoxide, citrulline, and ethanolamine (Fig. S9). Exogenous carbon monoxide has been used to complement a deficiency in generating the carbon monoxide ligand (46), and citrulline restores a deficiency in cyanide production caused by deletion of *carB* (47). Of these supplements, only exogenous nickel complemented the deficiency in hydrogenase activity (Figs. S9 and 5B), and Western blot analysis revealed an increase in the amount of processed HycE upon nickel supplementation of the ΔeutK strain (Fig. 5A). These results indicate that abrogated hydrogenase maturation in this strain is due to disruption of nickel homeostasis, a model supported by the decrease in total nickel content of ΔeutK cells compared with WT bacteria as measured by inductively coupled plasma MS (ICP-MS; Fig. 5C).

To generate more information about theeutK strain, we sequenced the genome, confirming that the kanamycin gene was inserted at the expected location and that the operons and genes associated with nickel homeostasis, hydrogenase activity, and hydrogenase maturation (*hya, hyb, hyc, hfy, hyp, nik*, and *slyD*) shared 100% sequence identity with those of the WT BW25113 strain (48). However, the ΔeutK genome also lacked an ~77-kbp segment (CP009273.1: 1,385,098–1,462,167) that includes the gene encoding the fumarate and nitrate reduction (FNR) protein, an anaerobic regulator that contributes to hydrogenase maturation and can serve as a substrate for all three of the actively expressed [NiFe]-hydrogenases 2 or 3 in *E. coli* (36, 49, 50). To test whether the lack of FNR was impacting hydrogenase production in the ΔeutK strain, a pBAD24 vector bearing *ftr* was constructed, and full hydrogenase activity was restored in ΔeutK upon induction with arabinose, consistent with a key role of FNR in hydrogenase biosynthesis in this bacteria (Fig. 5D). Complementation of ΔeutK with pBAD24-εutK, however, did not restore any hydrogenase activity (data not shown), further confirming that the hydrogenase deficiency was due to insufficient production of FNR. One explanation for how FNR contributes to hydrogenase maturation is through regulation of the expression of the Nik uptake transporter (36, 50). To test this possibility, we examined expression from the *nik* promoter by using a β-gal reporter, which revealed substantial activity in the WT strain under the anaerobic growth conditions used (Fig. 5E), as well as the expected inhibition upon nickel supplementation, due to the activity of the nickel-responsive repressor NIKR (29, 51). In contrast, no LacZ activity was detectable in the ΔeutK strain under any of the growth conditions examined, indicating that this mutation completely blocked expression of the Nik transporter. As with the hydrogenase activity, preliminary experiments demonstrated that the LacZ activity was restored in the ΔeutK strain upon expression of *ftr* from the pBAD plasmid (data not shown).

**Discussion**

**Assay specificity**

This assay monitors the reduction of benzyl viologen, a redox dye that has been used in hydrogenase research since 1957 (6). Benzyl viologen changes from colorless to violet upon reduction and can serve as a substrate for all three of the actively expressed *E. coli* enzymes (19, 52, 53). Theoretically, benzyl viologen could also be reduced by other electron sources, particularly in the context of the intact membranes of the whole-cell system. However, examination of the activities of several mutant strains of *E. coli*, along with the results of the Keio collection screen, revealed that even in the background of the complete cellular metabolism of the living bacteria, benzyl viologen reduction requires active [NiFe]-hydrogenases 2 or 3 in *E. coli*. It is not clear whether Hyd-1 or Hyd-4 cannot reduce benzyl viologen under these assay conditions or whether they are not well-expressed. However, it is worth noting that the results of the assay are sensitive to experimental conditions. For example, edge locations on the plates resulted in higher activity but lower growth. Furthermore, replacing LB medium with supplemented TYET medium resulted in a 2.5-fold increase in activity, and switching from the manual assay to the robotic system
altered the relative contributions from the different hydrogenases. These observations suggest that variables in the growth conditions, including the primary carbon source, could be tweaked to prompt a response from Hyd-1 if desired (18).

Although many of the genes known to contribute to benzyl viologen reduction were identified in the screen of the Keio collection, 12 of the 43 expected genes did not come up as hits. It is likely that disruption of many of these latter genes might not lead to low activity under the specific conditions used in this assay. For example, in previous work, deletion of five of the genes (*slyD*, *hycH*, *hycI*, *feoB*, and *iscU*) did not completely abolish hydrogenase activity of BW25113 and ΔeutK *E. coli* strains grown according to the whole-cell assay protocol. The 60Ni counts were normalized to 24Mg counts to account for variations in the total number of cells, and that ratio was normalized to WT content. The ΔeutK strain displays a decrease in nickel content ($p < 0.01$). D, the normalized benzyl viologen reduction activity of BW25113 and ΔeutK *E. coli* with or without transformation with the pBAD24-frm plasmid, grown in TYET medium supplemented with 2.5 mM arabinose ($n = 3$). E, expression of *Pnik* in anaerobically grown BW25113 and ΔeutK *E. coli* measured using the $\beta$-gal activity. **represents activities below the detection limit. The data represent the averages of three biological replicates, and the error bars represent one S.D.

Figure 5. Complementation of the ΔeutK strain hydrogenase deficiency. A, representative Western blots of HycE maturation. Following nickel incorporation, a 32-residue peptide is removed from HycE, resulting in faster migration of the processed form. B, the normalized benzyl viologen reduction activity of BW25113, ΔselA, ΔnikA, and ΔeutK *E. coli* grown in either TYET medium or TYET medium supplemented with 1 mM NiSO$_4$ ($n = 3$). C, ICP-MS was performed on BW25113 and ΔeutK *E. coli* strains grown according to the whole-cell assay protocol. The 60Ni counts were normalized to 24Mg counts to account for variations in the total number of cells, and that ratio was normalized to WT content. The ΔeutK strain displays a decrease in nickel content ($p < 0.01$). D, the normalized benzyl viologen reduction activity of BW25113 and ΔeutK *E. coli* with or without transformation with the pBAD24-frm plasmid, grown in TYET medium supplemented with 2.5 mM arabinose ($n = 3$). E, expression of *Pnik* in anaerobically grown BW25113 and ΔeutK *E. coli* measured using the $\beta$-gal activity. **represents activities below the detection limit. The data represent the averages of three biological replicates, and the error bars represent one S.D.

The only enzyme that contains both molybdopterin and selenocysteine and that was classified as a hit was FdhF, a subunit of the formate hydrogenlyase complex along with Hyd-3. However, the ΔfdhF strain retained over half of the WT activity, whereas the ΔselA and ΔmoaA strains had $<15\%$ of WT activity. Given that the other selenocysteine-containing proteins in *E. coli* are also formate dehydrogenases, this result suggests that there is metabolic cross-talk between the various formate dehydrogenases and/or functional replacement of formate dehydrogenase in the formate hydrogenlyase complex in *E. coli*. Consistent with the model that formate reduction is required, the addition of Na$_2$WO$_4$ into the growth medium also inhibited the activity in the whole-cell assay. An alternative explanation is that benzyl viologen reduction activity is depressed because a functional formate dehydrogenase is necessary for the expression of active hydrogenase, either through indirect regulation or effects on maturation or stability; however, Western blot
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analysis of HycE expression in the ΔselA strain suggests that protein expression is not impacted in this case.

Highly active strains

In addition to the low-activity hits, the screen of the Keio collection also identified strains that exhibited higher hydrogenase activity than WT E. coli. This increase in benzyl viologen reduction activity was not driven by an increase in production of the hydrogenase enzyme itself but increased substrate access for the strains examined. These results suggest that the whole-cell hydrogenase assay could be used to screen for mutations or small molecules that increase the permeability of cells.

Hydrogenase deficiency in ΔeutK, an fnr defect

Of the strains with deficient hydrogenase activity, only one of the new strains, ΔeutK, exhibited abrogated metallocenter assembly. Through chemical complementation experimentation, we discovered that exogenous NiSO₄ partially restored the activity of the mutant bacteria and activated processing of the HycE protein. This phenotype is reminiscent of other E. coli mutants deficient in known nickel homeostasis components. However, eutK is part of the ethanolamine utilization (eut) operon that enables the conversion of ethanolamine to ammonia and acetaldehyde, which is further processed to ethanol, acetyl-CoA, acetate, or acetyl phosphate (59). None of the other eut components were classified as hits in the screen of the Keio collection, and there is no established connection between ethanolamine usage and metal metabolism. Instead, subsequent experiments revealed an additional modification of the ΔeutK strain genome and demonstrated that the hydrogenase deficiency is due to the lack of the fnr gene.

FNR is an O₂-sensing protein that controls the expression of multiple genes required for anaerobic growth (49), and previous studies demonstrated that mutation of fnr results in diminished hydrogenase activity in E. coli as well as low cellular nickel content (36, 50). FNR has been implicated as a regulator for several components of hydrogenase enzyme production, including the hyp operon encoding for the maturation factors (60, 61), and the nickel uptake transporter, disrupted in the initially named hydC mutants (36, 62). However, the hydrogenase-deficient phenotype is partially complemented by growing the bacteria in medium supplemented with high concentrations of excess nickel (this work and Ref. 36), which would not be the case if production of the iron center of the enzyme was abrogated by disruption of HypDEF protein production. So, it is likely that the main impact of fnr deletion on hydrogenase production is through nickel uptake. Furthermore, the experiments with the Pnik reporter assay are consistent with direct regulation of this promoter by FNR and indicate that FNR is absolutely required for activation of specific nickel uptake, providing a means to rigorously limit nickel homeostasis and hydrogenase activity to anaerobic metabolism. In addition, the substantial amounts of nickel remaining in the knockout strain, likely imported nonspecifically through other metal transporters, highlights the direct funneling of nickel brought into the cell by the Nik transporter to supply the hydrogenase enzymes. Finally, the fact that no new components of [NiFe]-hydrogenase maturation were identified suggests that the activities that have not been identified, such as biosynthesis of the carbon monoxide ligand, are incorporated into the known factors.

Applications to microbial systems

The assay was optimized in E. coli because this organism serves as a model bacterial system with extensive information available about the cellular components, regulation, and auxiliary metabolic pathways. Furthermore, the ability of E. coli to both produce and consume hydrogen gas allows the bacteria to make the substrate for this assay, molecular hydrogen, in situ without rigorous atmospheric control. In fact, the assay was recently applied to a model pathogenic strain of E. coli, UTI89, in a study that revealed yersiniabactin-mediated nickel import (63). It is reasonable to suggest that this assay could be rapidly applied to other types of bacteria that make hydrogen gas, such as Salmonella enterica serovar Typhimurium, which employs hydrogenase during early stages of infection (64, 65). However, it may not be directly applicable to all bacteria. For example, preliminary attempts to apply this assay to Helicobacter pylori were unsuccessful (data not shown), which may stem from an inability of HpHyd to reduce benzyl viologen (17), the lack of an intimate source of molecular hydrogen, or higher oxygen sensitivity.

This assay provides the necessary tool to screen for small-molecule inhibitors of hydrogenase activity. A literature search uncovered only nonselective small-molecule inhibitors such as carbon monoxide (66) and an NADPH oxidase inhibitor, diphenylidencyanodionium (67), neither of which are suitable antimicrobials. Future work will apply this assay to serve this purpose. In addition, this assay will provide a means to explore associated auxiliary systems that might prove to be excellent targets, such as the nickel homeostasis pathways that are unique to microbial systems or molybdopterin biosynthesis. For example, a recent study demonstrated that gut dysbiosis caused by the growth of Enterobacteriaceae microbes in a murine model is mitigated by dosing with tungstate to disrupt molybdenum-dependent enzymes (68). The observation that the whole-cell hydrogenase assay can report on tungstate-mediated inhibition of formate dehydrogenase indicates that it could be used to find clinically relevant inhibitors for these bacterial pathways as well.

Conclusions

In this work, we present the design, optimization, and use of a whole-cell hydrogenase assay in E. coli. This assay can be readily applied to collect high-volume data that uncover the details of several aspects of microbial metabolism, and in the future it could be used in the search for molecules that target [NiFe]-hydrogenase production or enzymatic activity and in the optimization of bioengineering of hydrogen gas production. We screened the Keio collection to validate the assay and to demonstrate the utility of the assay in the study of [NiFe]-hydrogenase maturation. We found several strains that were less active in the whole-cell screen, and subsequent characterization of these strains indicated that they could be involved in maturation of formate dehydrogenase, such as through selenium or molybdenum biochemistry. Furthermore, strains with enhanced activity revealed that this method could be used to detect phenotypic defects in cell permeability. Finally, the
characterization of ΔeutK E. coli revealed an inability to mature [NiFe]-hydrogenase related to disrupted nickel delivery and FNR-mediated regulation, demonstrating the value of this assay in identifying factors that impact hydrogenase production.

**Experimental procedures**

All resources were sourced as described in Table S1. Strains of E. coli used in these experiments are either described in previous publications (7, 69, 70) or were strains chosen from the Keio collection, some of which were generously provided by the lab of Prof. K. Maxwell (University of Toronto) (32). Plasmids used in these experiments are either described in previous publications (7, 70, 71) or created from the appropriate parent vector using QuickChange or Phusion mutagenesis using primers purchased from Integrated DNA Technologies (a description of each plasmid is in Table S1). The plasmid bearing hypB with both C166A and C198T mutations was created by introducing the C166A mutation into a plasmid already bearing C198T mutation. All mutations were verified by sequencing (ACGT, Toronto, Canada). Cell strains from the Keio collection, except for strains used in the screen itself, were verified using PCR amplification of the kanamycin resistance gene with primers that flank the insertion site. All results are presented unless otherwise noted, and p values were calculated with two-tailed normal distributions of heteroscedastic sample sets.

**Whole-cell hydrogenase assay**

Whole-cell hydrogenase assays were performed as follows unless otherwise noted. E. coli from glycerol stocks were plated on LB agar and grown overnight at 37 °C. A single colony was selected and grown to saturation in LB medium overnight at 37 °C. Modified TYET was prepared by supplementing 10 g/liter tryptone, 5 g/liter yeast extract, and 50 mM Tris, pH 7.5, with 0.4% glucose, 30 mM sodium formate, 1 μM sodium selenite, and 1 μM sodium molybdate and sterilized using a 0.22-μm polyethersulfone membrane Stericup filtration apparatus (EMD Millipore). Phosphate-based media were avoided because of the formation of insoluble phosphate salts upon addition of transition metal ions. Additional medium components (including citrulline, ethanolamine, metal salts, antibiotics, etc.) were added when required. Supplemented TYET was inoculated with 0.8% (v/v) from the overnight culture, and 200 μl was added to the wells of a Corning Costar round-bottom polystyrene 96-well plate (catalog number 3799). The plates were covered with the supplied lid and incubated for 6 h at 37 °C. Plates were cooled to room temperature, and the OD at 630 nm was recorded. A developing solution (10 mg/ml benzyl viologen and 250 mM sodium formate in 20 mM Tris buffer, pH 7.5) was added to each well (20 μl), and the change in absorbance at 630 nm, caused by the reduction of benzyl viologen, was recorded on an ELx808 (BioTek) 96-well plate reader every 30 s for 5 min. Data were converted to ΔAbs·min⁻¹ divided by the OD to adjust for growth and normalized to the average activity of the WT strain.

**Screening hydrogenase activity factors**

The optimal assay conditions were evaluated by calculating the Z’-score (Equation 1), a statistical measurement that describes the separation between the positive and negative controls (34), after correction for edge effects. The Z’-score was calculated using the average and S.D. of the activities from BW25113 as the positive control strain and ΔhycE as the negative control strain (n = 96). A Z’-score of 0.5–1 would result from what is considered an “excellent” assay (34). Assay selectivity experiments were performed in biological triplicate.

\[
Z' = 1 - \frac{3\sigma_c - 3\sigma_\bar{c}}{|\mu_c - \mu_\bar{c}|} \quad \text{(Eq. 1)}
\]

The term σ represents the S.D., and μ represents the mean. The terms +c and −c are used to denote the values for the positive and negative controls, respectively.

**Robotic Keio collection screen**

Overnight cultures were grown in 50 μl of LB media at 37 °C on a Corning clear 384-well tissue culture–treated sterile plates (catalog number 3701), inoculated from a single colony using a ROTOR HDA instrument (Singer Instruments). An aliquot of 50 μl of TYET media was inoculated with ~5 μl of overnight culture using the ROTOR HDA and allowed to grow for 6 h at 37 °C in a 384-well plate without shaking and with the lid on. The plates were cooled to room temperature, and the growth (absorption at 600 nm) was recorded on a Tecan Infinite M1000 Pro, and 5 μl of developing solution was added to each well using a Tecan Freedom EVO liquid handler. After 5 min, the change in absorbance at 500 nm was recorded. The data were corrected for edge and plates effects as described elsewhere (72), normalized to the average activity of the middle 95% of the data (2.5–97.5 percentile), and divided by strain growth. For further analysis, the replicate data were averaged.

**Cell lysate benzyl viologen reduction assay**

Overnight E. coli cultures were grown in LB medium, without shaking, aerobically at 37 °C. Modified TYET was supplemented with 50 mg/liter kanamycin where appropriate. Cultures were inoculated with 0.8% (v/v) of overnight culture, sealed, and grown anaerobically in a 50-ml tube at 37 °C for 6 h. Strain BW25113 was used as a WT control. Cells were harvested by centrifugation at 4000 rpm for 20 min; washed with ice-cold 50 mM Tris buffer, pH 7.5; and resuspended in ice-cold buffer containing 200 mM phenylmethylsulfonyl fluoride and 1 mM DTT. The cells were sonicated on ice and centrifuged at 14,800 rpm for 20 min at 4 °C to separate the lysate from the cell debris. The lysates were frozen on liquid nitrogen and stored at −80 °C if not used immediately.

The samples were prepared in plastic cuvettes containing 1.5 ml of 100 mM potassium phosphate, pH 7.6, 4 mM benzyl viologen dichloride, between 10 and 30 μl of sodium dithionite, and between 1.5 and 7.5 μl of lysate, to ensure linear benzyl viologen reduction rates, in an anaerobic glovebox (95% N₂ and 5% H₂). Hydrogenase activities of the cell lysates were determined by monitoring the reduction of benzyl viologen by electronic absorption spectroscopy on a Cintra 404 spectrometer in the
Screening hydrogenase activity factors

glovebox using an extinction coefficient of 7400 m$^{-1}$ cm$^{-1}$ at 600 nm. The activity was calculated from the rate at which benzyl viologen is reduced (nmol/min) divided by total protein (mg). The total protein concentration of the lysates was measured using a BCA protein assay (Pierce) with BSA as a standard.

NPN uptake cell permeability assay

Bacteria were grown according to the whole-cell assay, 4 wells (800 µl) of culture were collected, and the OD$_{600}$ was recorded. The cell suspension was diluted to an OD of 0.5 with phosphate-buffered saline (PBS) composed of 137 mM NaCl, 10 mM Na$_2$HPO$_4$, 2.7 mM KCl, and 1.76 mM KH$_2$PO$_4$, pH 7.4. The diluted culture (1 ml) was then incubated with 500 µl of PBS saturated with NPN. The mixture was left to incubate for 5 min at room temperature, and cells were collected by centrifugation, spinning at 5 × g for 5 min. The pellet was washed once with 500 µl of PBS and resuspended in 500 µl of PBS. The cell suspension was divided into three 50-µl aliquots on a 96-well half-area black fluorescence plate (Corning). The fluorescence emission at 420 nm upon excitation at 350 nm was recorded using a CLARIOstar plate reader (BMG Labtech). The signal from a control sample without bacteria was used to remove background fluorescence, and the resulting fluorescence intensity was normalized to the WT strain.

HycE Western blotting

Mutant strains of E. coli were grown according to the whole-cell assay protocol. The cultures were collected (600 µl), and the cells were pelleted and frozen at −80 °C until use. The frozen pellets were thawed, resuspended in lysis buffer (50 mM Tris, 100 mM NaCl, 1 mg/ml lysozyme (BioShop), and 0.5 mM EDTA, pH 7.5), and incubated at 37 °C for 30 min. The cells were then treated to five freeze–thaw cycles using liquid nitrogen and a 37 °C heat block. One unit of DNase (Thermo Scientific) was added and allowed to digest DNA for 10 min at room temperature. The intact cells and insoluble aggregates were removed by centrifugation, and the clarified crude cell lysates were frozen overnight at −20 °C. Proteins were resolved on an 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel along with the MagicMark XP protein standard (ThermoFisher Scientific), and transferred to polyvinylidene difluoride membranes (Millipore). The blots were probed with polyclonal anti-HycE anti-bodies (70) at a 1:1000 dilution, and the secondary goat anti-rabbit antibody (Bio-Rad) was used at a dilution of 1:30,000. SuperSignal West Pico Chemiluminescent Substrate (Thermo) was used for detection.

Cellular metal content

The total metal content of the bacteria was analyzed by ICP-MS. The bacteria were grown according to the whole-cell assay, and 800 µl of culture was collected. The bacteria were pelleted and washed twice with buffer containing 20 mM Tris, 100 mM NaCl, and 1 mM EDTA at pH 7.6. The pellet was frozen and digested with 230 µl of 35% trace-metal free HNO$_3$ (AriStar Ultra, BDH) overnight at 50 °C and diluted to 2 ml with double distilled H$_2$O followed by ICP-MS as described previously (73). Raw isotope counts were background-subtracted using a control sample that did not contain bacteria and normalized to the total amount of 24Mg in each sample and are presented relative to the amount of nickel in the WT strain grown without a nickel supplement. Each data point represents the average of three biological replicates.

Clone verification

An isolated colony was sampled using a sterilized pipette tip and resuspended in 100 µl of PCR reaction mixture containing OneTaq PCR buffer (New England Biolabs), 200 µM dNTPs (Thermo), and 4 nM upstream and downstream primers. DNA primers for each Keio mutant (Table S1) were designed by modifying the primers used in the development of the Keio collection (32) to amplify either the gene in question or kanamycin cassette. OneTaq HotStart DNA polymerase (2.5 units; New England Biolabs) was added, and the mixture was separated into four 25-µl aliquots to perform an annealing temperature gradient during the PCR (50–60 °C) and run according to the published supplier protocol. The PCR products were analyzed on a 1% agarose gel alongside a 1-kb DNA ladder for size determination. Each strain was validated to contain a kanamycin insert cassette (~1550 bp) and compared with the intact gene length observed upon amplifying the gene in the WT BW25113 strain.

Genome sequencing was completed by first isolating the genomic DNA using the GeneJET Genomic DNA Purification kit (Thermo, catalog number K0721). The prepared genomic DNA was analyzed at the Centre for Applied Genomics Next Generation Sequencing Facility (Toronto, Ontario, Canada) and assembled de novo into contigs using Velvet v1.2 (74) on the Shared Hierarchical Academic Research Computing Network (SHARCNET). Specific regions on the genomic DNA were compared with the published BW25113 genome sequence (48) using BLAST v2.6.0 (75). The genome was also mapped to the published genome sequence using Burrows–Wheeler Alignment tool (BWA) (76).

β-Galactosidase activity assays

β-Galactosidase activity assays were conducted with the BW25113 and ΔeutK E. coli strains transformed with the pPC181 plasmid, which contains lacZ under control of the nik promoter, similarly to previously published methods (51, 77). Single colonies were inoculated into LB medium with 20 µg/ml chloramphenicol and grown overnight at 37 °C. The culture was then diluted into TYET medium (0.8%, v/v) and grown for 16 h at 37 °C anaerobically in sealed 15-ml centrifuge tubes with 5 µg/ml chloramphenicol. The cells (2 ml of culture) were collected by centrifugation, resuspended in 4 ml of Z-buffer (60 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 1 mM MgSO$_4$, and 50 mM β-mercaptoethanol), and permeabilized with 50 µl of 0.1% SDS and 100 µl of chloroform. The β-gal reactions were initiated by the addition of 200 µl of 4 mg/ml o-nitrophenyl β-D-galactopyranoside dissolved in sodium phosphate buffer (60 mM Na$_2$HPO$_4$ and 40 mM NaH$_2$PO$_4$) and terminated by the addition of 500 µl of 0.5 mM Na$_2$CO$_3$. Three time points, between 3 and 15 min, for each of three biological replicates were recorded to ensure linearity. Debris was removed by centrifugation, and the amount of free o-nitrophenol was detected by measuring
the electronic absorption at 420 nm. LacZ activity was reported in Miller units (1 Miller unit is equal to 1 nmol of o-nitrophenol produced/min): Miller units = \( 1000 \times \frac{([\text{Abs}_{420}] - (1.75 \times \text{Abs}_{550})/v \times \text{Abs}_{6000})}{t} \) where \( \text{Abs}_{420} \) is the absorbance of o-nitrophenol; \( \text{Abs}_{550} \) is the light scatter from cell debris, which when multiplied by 1.75 is approximately the light scatter observed at 420 nm; \( t \) is the reaction time in minutes; \( v \) is the volume of cell extract assayed in milliliters; and \( \text{Abs}_{6000} \) is the cell density measured after resuspension in the Z-buffer.

**pBAD24-**fnr** and -eutK plasmids and complementation**

Exponential megapriming PCR (EMP) was used to clone FNR into the arabinose-inducible pBAD24 vector (78). The EMP megaprimer was amplified from BW25113 *E. coli* genomic DNA using the *ftr* F1 primer and *ftr* R1 primer (Table S1). The second round of PCR included the megaprimer, the pBAD24-*hypB* plasmid (71), the *ftr* F1 primer, and the R2 primer. The final plasmid was verified by DNA sequencing. The pBAD24-*eutK* plasmid was prepared in the same manner except with the *eutK* F1 primer and *eutK* R1 primer (Table S1). The ΔeutK strain of *E. coli* was transformed with the pBAD24-**fnr** or the pBAD24-**eutK** plasmid. Cultures were grown, and the whole-cell hydrogenase activities were measured as described above except that the medium included 100 μg/ml ampicillin, and 2.5 mM arabinose was added to the supplemented TYET medium.

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