Harnessing *Escherichia coli* for Bio-Based Production of Formate under Pressurized H₂ and CO₂ Gases

Magali Roger, Thomas C. P. Reed, Frank Sargent

School of Natural and Environmental Sciences, Newcastle University, Newcastle upon Tyne, England, United Kingdom

**ABSTRACT** *Escherichia coli* is a Gram-negative bacterium that is a workhorse for biotechnology. The organism naturally performs a mixed-acid fermentation under anaerobic conditions where it synthesizes formate hydrogenlyase (FHL-1). The physiological role of the enzyme is the disproportionation of formate into H₂ and CO₂. However, the enzyme has been observed to catalyze hydrogenation of CO₂ given the correct conditions, and so it has possibilities in bio-based carbon capture and storage if it can be harnessed as a hydrogen-dependent CO₂ reductase (HDCR). In this study, an *E. coli* host strain was engineered for the continuous production of formic acid from H₂ and CO₂ during bacterial growth in a pressurized batch bioreactor. Incorporation of tungsten, in place of molybdenum, in FHL-1 helped to impose a degree of catalytic bias on the enzyme. This work demonstrates that it is possible to couple cell growth to simultaneous, unidirectional formate production from carbon dioxide and develops a process for growth under pressurized gases.

**IMPORTANCE** Greenhouse gas emissions, including waste carbon dioxide, are contributing to global climate change. A basket of solutions is needed to steadily reduce emissions, and one approach is bio-based carbon capture and storage. Here, we present our latest work on harnessing a novel biological solution for carbon capture. The *Escherichia coli* formate hydrogenlyase (FHL-1) was engineered to be constitutively expressed. Anaerobic growth under pressurized H₂ and CO₂ gases was established, and aqueous formic acid was produced as a result. Incorporation of tungsten into the enzyme in place of molybdenum proved useful in poising FHL-1 as a hydrogen-dependent CO₂ reductase (HDCR).

**KEYWORDS** *Escherichia coli*, mixed-acid fermentation, bioengineering, formate hydrogenlyase, hydrogen-dependent carbon dioxide reductase, carbon dioxide hydrogenation, pressurized bioreactor, carbon capture, fermentation, genetic engineering
which, under anaerobic conditions when using glucose as sole carbon and energy source, can perform a “mixed-acid fermentation” that produces acetate, lactate, succinate, and ethanol as end products. Mixed-acid fermentation also produces formate, which is often further disproportionated to H₂ and CO₂ (8). The enzyme responsible for this is the formate hydrogenlyase (FHL-1) complex (9, 10), which comprises a soluble catalytic domain containing a molybdenum- and selenocysteine (SeCys)-dependent formate dehydrogenase (FDH-H) module (encoded by the fdhF gene) linked by two [Fe-S] cluster-containing proteins (encoded by hycB and hycF) to a nickel-dependent hydrogenase module (Hyd-3, encoded by hycE and hycG) (9, 10). The soluble catalytic domain of FHL-1 is anchored to the cytoplasmic membrane via two integral membrane subunits encoded by the hycCD genes, and under physiological fermentative conditions, the FHL-1 forward reaction serves to detoxify formic acid accumulation and regulate environmental pH (9). Molecular hydrogen gas (H₂) and carbon dioxide are generated as products (9).

FHL-1 can also operate in reverse as an H₂-dependent CO₂ reductase, or HDCR (11). The yield of formate produced by the HDCR reverse reaction was initially low when carried out at ambient gas pressures (11); however, the design of a laboratory-scale stirred tank reactor, which could be operated at precisely controlled elevated gas pressures (up to 10 atmospheres), improved substrate solubility and gas transfer rates and led to a concomitant increase in the yield of the formate product (12).

The original HDCR experiments using pressurized gaseous substrates were conducted with pregrown cell paste (i.e., nongrowing cells) and carried out in the absence of any carbon or energy sources save for H₂ and CO₂ (12). In order to harness the HDCR activity for practical applications, it would be desirable to enable E. coli to perform hydrogen-dependent CO₂ reduction, not only under pressure but also during all active growth phases. In this study, we used a multiscale bioengineering approach to tackle this issue by (i) optimizing the host strain to produce FHL-1 under any growth regimen, (ii) attempting to remove bottlenecks in maturation and biosynthesis of FHL-1, and (iii) chemically engineering a catalytic bias in favor of HDCR activity. To characterize the new bacterial strains and growth conditions, a laboratory-scale bioreactor designed for batch fermentation under pressurized H₂ and CO₂ was used. A final strain was designed, built, and characterized that constitutively produced an engineered fusion protein and was shown to perform both the FHL forward reaction and HDCR reverse reaction similar to the native enzyme. Incorporation of tungsten, in place of the native molybdenum, was shown to poise the engineered enzyme in the direction of hydrogen-dependent carbon dioxide reduction. This work demonstrates that CO₂ can be continuously captured by FHL-1 in actively growing E. coli cells, providing the basis for a new pressurized platform for renewable biotechnology.

RESULTS AND DISCUSSION

Harnessing FHL-1 expression by genetic engineering. The first obstacle to overcome for exploiting E. coli FHL-1 as a carbon fixing technology was the natural expression regime of the enzyme, which is geared naturally toward environmental conditions favoring the forward reaction. Thus, physiological FHL-1 biosynthesis is controlled by the presence of formate and acidic pH (13–15). The expression of the fdhF gene and hyc operon is coordinated and regulated by a formate-responsive transcriptional regulator FhlA (16) and the repressor HycA (17). Thus, it is clear that strategies to remove native, especially formate-dependent, control of FHL-1 biosynthesis are needed in order to produce active FHL-1 under all growth regimens.

First, an E. coli strain (MR87.5) was constructed in which other hydrogenases (ΔhyaB, ΔhyaC) and potential formate production and utilization pathways (ΔpfIA, ΔfdhE) were inactivated (Table 1). As previously observed in the context of bio-H₂ production, mutant strains unable to synthesize active pyruvate formate lyase (ΔpfIA), which generates formate from pyruvate during fermentation, should only produce FHL-1 when exogenous formate is supplemented to the growth medium (18). This phenotype was observed here for strain MR87.5 (hyaE disruptions, ΔhyaB, ΔhyaC, ΔpfIA, and ΔfdhE) where H₂-dependent
CO₂ reductase (HDCR, reverse FHL-1) activity was only observed in MR87.5 after the cells had been pregrown in exogenous formate at 0.2% (wt/vol) final concentration (Fig. 1B). Next, the FhlA regulator was specifically targeting for mutagenesis. It has been shown that FhlA variant E183K exhibits a constitutively active phenotype on hyc trancription, maintaining high expression levels even in the complete absence of externally added formate (19). In our work, the MR87.5 strain was modified by the inclusion of the FhlAE183K allele on the chromosome to give E. coli strain MR93.25 (Table 1). This new strain demonstrated some improved HDCR activity when initially cultured in the

---

**TABLE 1 Strains produced in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG1655</td>
<td>E. coli K-12 F- ΔlviG rfb-50 rph-1</td>
<td>58</td>
</tr>
<tr>
<td>MG059e1</td>
<td>MG1655 hycE586</td>
<td></td>
</tr>
<tr>
<td>MR87.5</td>
<td>MG059e1 ΔhyaB ΔhybC ΔpfIA ΔfdhE</td>
<td>This work</td>
</tr>
<tr>
<td>MR93.25</td>
<td>MR87.5 fhlAE183K</td>
<td>This work</td>
</tr>
<tr>
<td>MR40</td>
<td>MG059e1 ΔhyaB ΔhybC ΔpfIA ΔfdhE ΔhyaA ΔfdhF ΔfadhF ΔfadhF(_{HA})::hycB</td>
<td>This work</td>
</tr>
<tr>
<td>MR60</td>
<td>MG059e1 ΔhyaB ΔhybC ΔpfIA ΔfdhE ΔhycA ΔfdhF P(<em>{pfl}) ΔfadhF(</em>{HA})::hycB</td>
<td>This work</td>
</tr>
<tr>
<td>MR36</td>
<td>MG059e1 ΔhyaB ΔhybC ΔpfIA ΔfdhE ΔhycA ΔfdhF P(<em>{pfl}) ΔfadhF(</em>{HA})::hycB</td>
<td>This work</td>
</tr>
<tr>
<td>MR41</td>
<td>MG059e1 ΔhyaB ΔhybC ΔpfIA ΔfdhE ΔhycA ΔfdhF P(<em>{pfl}) ΔfadhF(</em>{HA})::hycB</td>
<td>This work</td>
</tr>
<tr>
<td>MR72</td>
<td>MG059e1 ΔhyaB ΔhybC ΔpfIA ΔfdhE ΔhycA ΔfdhF P(<em>{pfl}) ΔfadhF(</em>{HA})::hycB</td>
<td>This work</td>
</tr>
<tr>
<td>MR62</td>
<td>MR60 ΔiscR</td>
<td>This work</td>
</tr>
<tr>
<td>MR94.5</td>
<td>MR60 ΔiscR ΔmetU</td>
<td>This work</td>
</tr>
<tr>
<td>TOM001</td>
<td>MR60 ΔhycCD</td>
<td>This work</td>
</tr>
</tbody>
</table>

---

**FIG 1** Formate-independent production of HDCR activity by genetic engineering. (A) Genetics of the E. coli FHL system in parental and engineered (e.g., MR60) strains. The hyc operon is normally encoded at 61 min on the chromosome, while fdhF is normally located at 91 min. For the construction of the engineered hyc operon, the fdhF gene was deleted, and hycA was replaced by a version of fdhF fused with hycB via an HA epitope tag. (B) Hydrogen-dependent carbon dioxide reduction (HDCR) assays using cells pregrown under anaerobic fermentative conditions with or without additional formate where indicated. Production of formic acid from gaseous H₂ and CO₂ was assayed by HPLC (n = 6). Washed cells were suspended in MOPS buffer and incubated under a CO₂ and H₂ atmosphere. Following incubation at 37°C for 20 h, aliquots were taken, cells removed by filtration, and the formate content of the clarified supernatants analyzed by HPLC. The activity of the MR60 strain bearing the T5 promoter is highlighted. Other strains included in the experiment were the control strain TOM001 (MR60 ΔhycCD) and MR87.5 and MR93.25, which contain native FHL-1 but require addition of exogenous formate to induce expression because of a ΔpflA mutation. MR93.25 also carries an E183K allele in the gene encoding the formate regulator FhlA. MR40 carries a genetic fusion of formate dehydrogenase (FdhF) with its redox partner (HycB) under the control of the native promoter (P\(_{hyc}\)). Derivatives of MR40 include MR60 (with the fusion under the control of the T5 promoter), MR36 (with the fusion under the control of the P\(_{pfl}\) promoter), MR41 (P\(_{pfl}\)), and MR72 (P\(_{pfl}\)). (C) The production of the FdhF\(_{HA}\)-HycB fusion protein was observed by Western immunoblotting using an antibody against the HA epitope in the fusion linker sequence. Whole-cell samples from strain MR40 (P\(_{hyc}\)) and its promoter-substituted derivatives, MR60 (P\(_{pfl}\)), MR36 (P\(_{pfl}\)), MR41 (P\(_{pfl}\)), and MR72 (P\(_{pfl}\)), were prepared from anaerobic cultures grown with or without extra formate in the growth medium where indicated. The behavior of the MR60 strain, which is the major focus of this study, is highlighted.
absence of exogenous formate (Fig. 1B); however, surprisingly, HDCR activity in the FhIA E183K variant remained strongly inducible by pregrowth in external formate (Fig. 1B). This strain was deemed not suitable for further engineering.

Harnessing production of the entire FHL-1 enzyme is further complicated by the fact that the formate dehydrogenase and hydrogenase genes are located at separate loci on the chromosome. In an initial attempt to stabilize coproduction of the entire FHL-1 complex, a previous study engineered an *E. coli* strain in which the FDH-H moiety was physically tethered to HycB, resulting in the production of a fully assembled and functional complex (20). Keeping with this strategy here, the *fdhF* gene was first deleted from the parental strains before the *hycA* gene was replaced by a version of the *fdhF* gene fused to *hycB* using a hemagglutinin (HA) epitope tag (Fig. 1A). This new strain (MR40) was then further modified by the inclusion of alternative transcriptional promoter regions upstream of the *ΔfdhF::hycB* fusion allele (Table 1). The promoters T5, proD, *tatA*, and *ynfE* were chosen as various examples of strong, constitutive or anaerobically induced promoter sequences. The four new strains carrying these promoters (Table 1) were then analyzed for the production of the FdhFHA-HycB fusion protein by Western immunoblotting against the HA tag (Fig. 1C) and for HDCR activity using intact whole cells (Fig. 1B). As shown in Fig. 1B, when the expression of FHL-1 was left under the control of what remains of the native *hyc* promoter (P_hyc) in MR40, the cells exhibited no HDCR activity when cultivated in the absence of formate, and no protein could be detected by Western blot analysis using an antibody raised against the HA epitope tag. Moreover, the MR40 strain yielded only low levels of the FdhFHA-HycB fusion protein when cells were grown with extra formate in the medium (Fig. 1C). As a result, the HDCR activity was only partially restored when formate was supplemented in the growth medium (Fig. 1B).

Next, the synthetic promoter constructs were tested. Among the promoters screened, T5 is a strong promoter used in plasmid-based expression systems (21), and the *ynfE* promoter was proposed to be highly inducible under anaerobic conditions (22). Another promoter, termed proD, which is constitutive (23), was also tested. Of these, the MR60 strain, which contained the T5 promoter upstream of the *ΔfdhF::hycB* allele fusion, showed the most convincing protein production yield in the absence of exogenous formate (Fig. 1C). The MR60 strain also displayed strong HDCR activity when grown in the complete absence of exogenous formate (Fig. 1B). In order to demonstrate that the observed HDCR activity was dependent upon active FHL-1, a further control strain was constructed. MR60 was modified by the addition of a Δ*hycCD* allele that would remove the membrane arm of FHL-1, thus rendering the enzyme inactive (11). The new strain, TOM001 (Table 1), displayed negligible HDCR activity in the small-scale assays (Fig. 1B). Taken together, it is clear that the strategy of coproducing a formate dehydrogenase-hydrogenase fusion protein under a single constitutive promoter, and by removing any requirement for formate for expression, has been successful in generating an *E. coli* strain harboring active HDCR.

**Exploring enhancement of cofactor biosynthesis and insertion.** It is notable that the HDCR/formate production yield in the engineered strain MR60 matched, but never exceeded, that observed using the parental strain (Fig. 1B). This suggested that the expression regime was ultimately not the limiting factor in formate production activity. Further genetic engineering was employed to further boost HDCR activity. Maturation of FHL-1 is a multistep process that depends on accessory proteins involved in the biosynthesis of the [Fe-S] clusters, the molybdenum cofactor (MoCo) of the formate dehydrogenase, and the [NiFe] active site cluster of the hydrogenase (9). Previous strategies to stimulate hydrogenase expression and activity have involved the deletion of the *iscR* gene (24, 25). Here, a version of the MR60 strain carrying a Δ*iscR* allele (E. coli strain MR62) was constructed (Table 1). Attention was also given here to the MoCo biosynthesis pathway, which is highly conserved and involves a series of accessory proteins and cosubstrates (26). Here, we focused on the deregulation of synthesis of the 5'-adenosyl methionine (SAM) radical, which plays a critical role in the first step of the pathway (27). Increasing cellular availability of SAM may remove a potential bottleneck.
in this highly complex biosynthetic pathway. To do this, the MR62 strain was further engineered by the inclusion of a ΔmetJ deletion to yield strain MR94.5 (Table 1). Finally, it was considered worthwhile to attempt to boost the [NiFe] cofactor biosynthesis capability in the strains, and to do this, these cells were transformed with a multicopy vector carrying a synthetic version of the hypA1-B1C1D1E1-X operon from Ralstonia eutropha (Cupriavidus necator) (28, 29).

Strains with engineered cofactor biosynthesis pathways were analyzed for H2 production activity catalyzed by the FHL-1 forward reaction. As shown in Fig. 2, H2 production activity initially decreased in the MR40 parent strain carrying the fdhFHA::hycB allele fusion, while the incorporation of a T5 promoter upstream of the fusion in the MR60 strain restored the activity to beyond native levels. The ΔhycCD derivative of MR60 (TOM001) was found to be devoid of hydrogen production activity (Fig. 2). This mirrored the behavior of all three strains in the HDCR assay (Fig. 1B). Subsequent deletions of the iscR or metJ or inclusion of extra [NiFe] cofactor accessory genes added no material improvements to FHL-1 activity (Fig. 2). This clearly shows that, under these growth conditions, the metal cofactor biosynthesis, insertion, and maturation pathways of the enzyme were not a limiting factor.

Biasing HDCR activity by biochemical engineering of the formate dehydrogenase. One major obstacle to consider is the thermodynamics and reversibility of the FHL-1 system. The standard redox potentials of the two half-reactions of FHL-1 are very close together; thus, the directionality of the enzyme is very strongly influenced by environmental conditions, with low-pH/high-formate/low-H2 partial pressure favoring FHL activity and with high-pH/low-formate/high-H2 partial pressure favoring HDCR activity. Clearly, it would be desirable, if possible, to minimize any tendency toward the FHL forward reaction while HDCR activity is ongoing.

The activities of metal-dependent formate dehydrogenases for formate oxidation and CO2 reduction vary greatly depending on the originating biological system, the composition of the active-site metal (molybdenum or tungsten), and the nature of its coordinating ligand (either cysteine or selenocysteine amino acid side chains) (30–32). Molybdenum and tungsten are closely related and share an identical organic cofactor when found in enzymes. Overall, tungsten-containing formate dehydrogenases have

![FIG 2 Metallocofactors are not a limiting factor in FHL-1 H2 production activity. E. coli strains were grown under anaerobic fermentative conditions in rich medium supplemented with 0.4% (wt/vol) glucose and 0.2% (wt/vol) formate for 20 h incubation at 37°C. Addition of exogenous formate is required, as all strains carry a Δpf4 allele that blocks the main pathway of anaerobic formate production. The MR87.5 strain contains the native FHL-1 complex, while MR40 produces an FdhFHA-HycB fusion protein. The MR60 strain has the fusion protein under the control of the T5 promoter sequence. Strain TOM001 is a direct derivative of MR60 that carries a ΔhycCD allele that specifically inactivates FHL-1 (11). Strains MR62 (MR60 ΔiscR) and MR62.5 (MR60 ΔiscR ΔmetJ) were assayed either alone (−) or when transformed with an empty vector (pSU-PROM) or a vector encoding the R. eutropha [NiFe] cofactor biosynthesis pathway (hypA1-X), which has been shown to be functional in E. coli (28). The formate-dependent H2 evolution activity was assayed by measuring H2 content in the gas phase by GC after growth at 37°C for 20 h (n ≥ 3 and error bars indicate SEM). A one-tailed t test was used to determine statistical significance (*, P < 0.01; **, P < 0.05).]
been suggested to be more efficient at reducing CO₂ because of the lower midpoint potential of the active site metal (33). Thus, it was considered here whether *E. coli* FHL-1 could be produced as a variant containing tungsten.

One simple approach used to substitute molybdenum for tungsten in enzymes is the growth of *E. coli* in the presence of increasing amounts of tungsten salts (34, 35). Here, *E. coli MR60* cells were first grown under anaerobic conditions using a rich medium without any further supplementation. The metal content of the FHL-1 enzyme was then analyzed by inductively coupled plasma mass spectrometry (ICP-MS) (Table 2). In theory, a perfectly assembled FHL-1 should contain one molybdenum atom and one nickel atom per mol of enzyme (10). The FHL-1 fusion protein complex was purified via a His tag present on the hydrogenase subunit as previously described (20, 36), and, as shown Table 2, the isolated protein was found to contain clearly detectable amounts of nickel ions in a ratio of 1:0.4 with molybdenum. Under these conditions, the enzyme contained essentially no tungsten (Table 2). Clearly, this experiment does not return a perfect 1:1 ratio for molybdenum to nickel. The suggestion is that, under this growth and purification regimen, half of the formate dehydrogenase component is either not stably bound to the complex or not correctly assembled. Note, however, that the fusion protein approach does confer some extra stability on the enzyme, as a similar purification of native FHL-1 resulted in a ratio of only 0.27 mol molybdenum for every mole of nickel (36).

Next, the growth medium was supplemented with tungstate salts before the FHL-1 was again purified and analyzed. In this instance, the ICP-MS data revealed the ratio of molybdenum to nickel in the enzyme had dropped to 0.01 (Table 2). However, the growth in the presence of tungstate salts had simultaneously boosted the tungsten present in the enzyme to a ratio of 0.7 for every nickel atom (Table 2). This demonstrates that tungsten can be incorporated into the FHL-1 enzyme in place of molybdenum when supplied in the growth medium as a tungstate salt.

Next, the MR60 cells growing in the presence of increasing quantities of tungsten salts were analyzed for both forward and reverse reactions of the FHL-1 enzyme (Fig. 3). Both FHL forward and HDCR reverse activities tended to decrease as the concentration of tungstate ions increased in the growth medium. However, the trend profiles of the inhibitions were strikingly different (Fig. 3). Notably, at 1 μM tungstate in the growth medium, a 50% loss of FHL forward (H₂ production) activity was observed, while the same cells retained full HDCR (CO₂ reduction) activity under these conditions (Fig. 3). This result strongly suggests that the substitution of the molybdenum atom at the active site of FDH-H by a tungsten atom can either subtly shift the catalytic bias toward CO₂ reduction or simply inhibit the forward reaction. This simple way to change the kinetic properties of the enzyme could be a useful discovery if FHL-1 is ever going to be exploited fully as a carbon capture technology. Note that, however, both forward and reverse activities were lost when cells were grown with the highest concentration of tungstate ions (Fig. 3). It has been shown that the expression of *fdhF* and *hyc* is normally regulated by molybdate concentration in the cell through the action of the transcriptional regulator ModE (37), but this is unlikely to be an issue in our engineered strain. However, there could be wider, global effects of tungstate on cofactor biosynthesis, especially through expression of the biosynthetic genes themselves, which are controlled by a riboswitch in *E. coli* (38). Indeed, previous studies showed that the incorporation of molybdenum or tungsten at the active site of

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Ratio of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>⁹⁸Mo to ⁵⁹Ni</td>
</tr>
<tr>
<td>LB medium</td>
<td>0.4049</td>
</tr>
<tr>
<td>LB plus 1 mmol liter⁻¹ Na₂WO₄</td>
<td>0.0126</td>
</tr>
</tbody>
</table>

TABLE 2 Ratio of active site metal content in isolated FHL-1 as determined by ICP-MS

December 2021 Volume 87 Issue 21 e00299-21 aem.asm.org
formate dehydrogenases in *Desulfovibrio* species is regulated not only by different selectivities in metal incorporation but also at the level of gene expression (32, 39, 40).

**Developing a bioprocess for CO₂ hydrogenation by *E. coli* throughout bacterial growth: ambient gas pressure.** The ultimate goal of this research was to generate a host strain, and define some growth conditions, that will perform HDCR throughout the growth phase. Next, we employed a bioXplorer P400 laboratory-scale bioreactor with a gas sparging system to allow a constant and efficient delivery of H₂, CO₂, and/or N₂ to the culture. The engineered MR60 strain, which can only generate formate via engineered FHL-1, was chosen and grown in the presence of tungstate salts to maintain unidirectional HDCR activity. Following inoculation, the oxygen present in the growth medium was observed to be rapidly consumed by the bacteria. When the residual dissolved oxygen had reduced to 0%, only then were H₂ and CO₂ sparged through the cell culture at 50 ml min⁻¹. In this first experiment, no overpressure was applied. Initially, a concomitant drop of pH in the growth medium was observed as CO₂ was added. Hence, sodium hydroxide was automatically pumped into the growth medium to maintain the pH at 7.0 (Fig. S1 in the supplemental material). Bacterial growth was observed in the first 8 h of the run, until the glucose was fully consumed, reaching a maximum turbidity optical density at 600 nm (OD₆₀₀) of 1.8 (i.e., 0.45 g cell dry weight [gCDW] liter⁻¹) (Fig. 4). Under these conditions, a maximum of ~8 mmol liter⁻¹ formate was produced after 24 h, with a maximum rate of formate production of 2.4 mmol liter⁻¹ h⁻¹.

These results were considered promising since, even at ambient pressure, the performances of the bioprocess using the optimized *E. coli* MR60 strain were considered to outcompete comparable systems in which microorganisms naturally produce formate, such as *Desulfovibrio* sp. (41). Indeed, while formate produced by the MR60-optimized strain of *E. coli* is in the same range as that produced by *Desulfovibrio desulfuricans*, the maximum rate of formate production is 4 times higher in this *E. coli* system. Furthermore, while formate production in *D. desulfuricans* was observed to begin upon entry into stationary phase, in this system, formate production started immediately upon H₂ and CO₂ sparging in the cell culture. Moreover, as formate production is deregulated in this genetic background, formate production continued even after cells entered stationary phase (Fig. 4). This clearly emphasizes the potential of an *E. coli*
Developing a bioprocess for CO₂ hydrogenation by *E. coli* throughout bacterial growth: controlled, elevated gas pressure. To investigate the effect of elevated pressure on *E. coli* MR60 cell growth, glucose consumption, and formate production, the bioreactor was next pressurized at 2, 4, or 6 bar with H₂ and CO₂ at a flow rate of 50 ml min⁻¹. Under these conditions, formate production yield (amount of formate produced per unit of cell density) could be increased with gas partial pressure up to 4 bar pressure (Fig. 5); however, no further enhancement of yield of formate produced was
observed above 4 bar pressure (Fig. 5). Strikingly, however, above 2 bar pressure, the absolute formate content in the bioreactor was seen to decrease drastically (Fig. 6C). To determine whether the elevated pressure per se or the composition of the gas mixture itself was detrimental to the cells, the E. coli MR60 strain was subsequently placed in the pressurized bioreactor under 10 bar pressure of 100% nitrogen gas (Fig. 7A). Strikingly, neither cell growth rate nor the final cell density was impacted negatively by elevated N₂ pressure (Fig. 7A). The cells also produced normal levels of lactate during fermentation (Fig. 6D) but were unable to generate formate (Fig. 6D). This strongly suggests that inhibition of cell growth under elevated H₂/CO₂ pressure is either linked to increasing concentrations of molecular hydrogen and carbon dioxide themselves, or perhaps due to the FHL-1-catalyzed hydrogen-dependent CO₂ reductase activity the cell is being forced to carry out. Indeed, increasing CO₂ concentrations could conceivably induce reversal of certain central metabolic reactions (42) or perhaps interfere with the function of the endogenous carbonic anhydrases (43).

In order to address these questions directly, the TOM001 strain (MR60 ΔhyccD) was chosen for further experimentation. This strain has no FHL (forward) activity (Fig. 2) nor any HDCR (reverse) activity (Fig. 1). To investigate the effect of elevated pressure on E. coli TOM001 growth, the strain was introduced into the pressurized bioreactor set at 6 barG (absolute pressure) with H₂ and CO₂ (Fig. 7B). In the absence of active FHL-1, the TOM001 strain exhibited no significant growth defects when growing under pressurized CO₂/H₂ (Fig. 7B).

These data suggest that reverse FHL-1 activity (maximally forced in our engineered strains when growing under pressurized CO₂/H₂) induces a growth defect. This is most likely related to the complex membrane biology of FHL-1. Sequence analysis suggests the enzyme activity could be coupled to proton or ion transport across the membrane, although the experimental evidence for this in studies of E. coli FHL-1 is not strong (11). Likewise, formate itself must be secreted from the cell after production, and the activity of the channel involved could also be intimately linked with that of the transmembrane proton motive force (44). One solution to overcoming the observed growth defects could involve engineering a water-soluble version of FHL-1 that is not dependent upon attachment to the cytoplasmic membrane for activity.

**Bio-based catalysts for carbon capture.** It was recently demonstrated that bacteria from the Desulfovibrio genus can produce formate from H₂ and CO₂ (41, 45), involving a periplasmic HydAB [FeFe]-hydrogenase (in H₂ oxidation mode) and a cytoplasmic molybdenum-dependent formate dehydrogenase enzyme, both most likely connected via the periplasmic tetraheme cytochrome c₃ network (41). Moreover, it was proposed that D. desulfuricans was able to grow during formate production. As a result, the concentration of formate in the bioreactor increased for 64 h before a maximum steady-state value of 30 mM formate production was achieved. However, although this result was outstanding, the whole bioprocess clearly suffered from low biomass yield (41). In contrast, here, we demonstrated that E. coli can be harnessed for formate production by optimizing the native FHL-1 enzyme complex to behave as an HDCR enzyme. At pressures below 6 barG, the optimized strain itself showed comparable performances to D. desulfuricans cells grown in batch reactor. In addition, operating the bioreactor at moderate pressure (e.g., 2 barG) H₂/CO₂ led to a doubling in the formate concentration in the cell suspension. This clearly demonstrates the potential of engineering the E. coli strain as host for bio-based production of formate while fixing CO₂.

New biocatalysts with remarkable kinetic properties for CO₂ reduction have been recently discovered, such as the HDCR from Thermoanaerobacter kivui (46) or the formate dehydrogenase from Rhodobacter aestuarii (47). Indeed, the HDCR enzymes from Gram-positive acetogenic bacteria share many similarities with formate dehydrogenases. The HDCR from Acetobacterium woodii is a soluble cytoplasmic enzyme that, while reversible, has the dedicated physiological role of reducing CO₂ to formate using...
FIG 6 Elevated H₂/CO₂ pressure partially inhibits bacterial growth. A bioXplorer P400 bioreactor was loaded with rich medium containing 0.8% (wt/vol) glucose containing 1 μmol liter⁻¹ tungstate salts and operated at 37°C. The E. coli MR60 strain (carrying the FdhFHA-HycB fusion under the control of the T5 promoter) was used as inoculum, and H₂ and CO₂ gas sparging (flow rate of 50 ml min⁻¹) was initiated with ambient pressure (0 bar; black; n = 2), 2 bar overpressure (blue; n = 2), 4 bar overpressure (purple; n = 2), or 6 bar overpressure (green; n = 2). (A to C) Growth was monitored at OD₆₀₀ (A), while glucose consumption (B) and formate production (C) were followed by HPLC. (D) HPLC data also provide data on the levels of other end products of mixed-acid fermentation. The data from the H₂/CO₂ cultures at ambient, 2, 4, and 6 bars are shown (n = 2), together with the metabolite data from the MR60 strain grown under 10 bar absolute pressure of N₂ only (n = 3). Gas sparging was initiated at the 45-minute mark once all residual dissolved oxygen had been consumed by the culture.
H₂ as reductant (48). Unlike *E. coli*, *A. woodii* can utilize fixed formate as the sole carbon source; however, the organism can also be harnessed to perform as an efficient whole-cell carbon capture system (49). Unlike FHL-1, *A. woodii* HDCR is not membrane attached, which may be considered an advantage in biotechnological applications, but contains an [FeFe]-hydrogenase, which is of a class usually very oxygen sensitive and not naturally found in *E. coli*. Nevertheless, a heterologous expression system has recently been developed to allow production of the active enzyme in *E. coli* (50). Thus, developing whole-cell biocatalysis using engineered *E. coli* remains a very promising option and offers the potential for large-scale and low-cost production (51).

FIG 7 Growth inhibition under H₂/CO₂ is caused by the HDCR activity of FHL-1. (A) *E. coli* MR60, which is positive for FHL-1 activity (FHL⁺), was grown in the bioXplorer 400P bioreactor at 37°C with 100% nitrogen gas at 10 bar overpressure (10 barG). Bacterial growth was monitored at OD₆₀₀ (n = 2). Growth under 10 barG N₂ is compared with the data (also shown in Fig. 6A and panel B) of the growth behavior of MR60 under a H₂/CO₂ gas mixture at 6 barG (n = 2). (B) *E. coli* strain TOM001, which is a direct derivative of MR60 but is negative for FHL-1 activity (FHL⁻), was grown under a H₂/CO₂ gas mixture at 6 barG (n = 2). In this experiment, the bioreactor was initially pressurized from 0 to 5 barG with N₂ at 100 ml min⁻¹ and then reduced to 50 ml min⁻¹ to pressurize from 5 to 6 barG. At 6 barG pressure, the flow rate of N₂ was reduced to 10 ml min⁻¹, and finally, H₂ and CO₂ were introduced at a rate of 5 ml min⁻¹, maintaining 6 barG. This method provides the desired 6 barG CO₂/H₂ mixture but is less wasteful of substrate gases. Growth of TOM001 (FHL⁻) under 6 barG CO₂/H₂ is compared with the data (also shown in Fig. 6A and panel A) of the growth behavior of MR60 (FHL⁺) under an H₂/CO₂ gas mixture at 6 barG (n = 2).
Concluding remarks. In this study, an *E. coli* host strain was designed and built for the bio-based production of formate from H₂ and CO₂ in a batch bioreactor. This involved the rational genetic engineering of formate hydrogenlyase-1, a complex bidirectional metalloenzyme, in its native cellular chassis. Chemical substitution of tungsten for molybdenum in FHL-1 was found to instill a catalytic bias toward H₂-dependent CO₂-reductase activity, which promises to be a key finding in future process development. The engineered strain showed an ability to grow under pressure up to 10 bar N₂. Under carbon capture conditions, the modification of a continuous gas fermentation bioreactor.

MATERIALS AND METHODS

Construction of bacterial strains. This work was based on *E. coli* K-12 MG059e1, which carries an hycC*tm* allele on the chromosome (36). Strains constructed and employed in this study are listed in Table 1. Gene deletions were carried out by transduction with bacteriophage P1 (52) combined with the lambda red protocol (53) or by homologous recombination using the pMAK705 protocol (54). For those constructed using lambda red, strains from the “Keio” collection of kanamycin-marked mutations in nonessential genes of the *E. coli* K-12 (BW25113) were used as deletion allele donors (55). Deleted genes were hycB, encoding the catalytic subunit of Hyd-1; hyBC, encoding the catalytic subunit of Hyd-2; pfdE, encoding pyruvate formate lyase-activating protein; fdhF, encoding an accessory protein specific for Tat-dependent respiratory formate dehydrogenases; iscR, encoding a transcriptional repressor of the *isc* operon; and metJ, encoding a transcriptional repressor of the *met* regulon. Once target genes were replaced with a kanamycin-marked mutation, the *fdhF* gene was amplified by PCR and assembled in pMAK705 (54). The mutant allele was then incorporated by QuikChange PCR (Agilent) and then transferred onto the chromosome by homologous recombination (54).

Strains producing FdhF as an N-terminal fusion protein to HycB, joined by a linker sequence containing a hemagglutinin (HA) tag flanked by three glutamines on each side, were assembled using a gene fusion construct in pMAK705 that has been described previously (20). The first strain made here using this construct was MR40 (Table 1). In attempts to upregulate expression of ∆*fdhF* and *hycB*, alternative promoter regions were used to modify MR40. First, an existing construct bearing the synthetic T5 promoter region (20) was used to assemble strain MR60 (Table 1). Note that MR60 differs from strain FZBup (pREP4) (20) in that it does not harbor the pREP4 plasmid needed to repress transcription from the engineered T5 promoter. Next, the *E. coli* proD promoter and the promoter of the *E. coli* tatA and *ynfE* genes were amplified and cloned separately into pMAK705 as EcoRI-BamHI DNA fragment (oligonucleotide sequences shown in Table 3). Then, ~500 bp of sequence upstream of *hycA* and ~500 bp of sequence downstream of the 5’ end of the ∆*fdhF* and *hycB* allele were amplified and cloned in the same vectors as the KpnI-EcoRI and BamHI-HindIII DNA fragments, respectively. The promoter alleles were then

### TABLE 3 Oligonucleotide primers used to construct promoter fusion strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Function</th>
<th>Primer name</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR40</td>
<td>Removal of <em>fdhF</em></td>
<td>fhlA QC-Fwd</td>
<td>AAACCGGATAAACTCCGCATC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fhlA QC-Rev</td>
<td>CCGCATAAACAATCATAGTG</td>
</tr>
<tr>
<td>MR36</td>
<td>Engineering the <em>tatA</em> promoter</td>
<td>hycAup-P<em>fdhF</em>-Fwd</td>
<td>AAGGGTAAAGAACATTGCTGGTGCAGAAAAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hycAup-P<em>fdhF</em>-Rev</td>
<td>CCAACGACAGAATTGCTTACCGTGTAAAAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P</em>prom-fdhF-down-Fwd</td>
<td>CCGAGGAGGATACATGAAAGATCTGTCAGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P</em>prom-fdhF-down-Rev</td>
<td>CTTTTTTCATGATCTTCTGGTGAGTAGATGTATAAAC</td>
</tr>
<tr>
<td>MR41</td>
<td>Engineering the <em>ynfE</em> promoter</td>
<td>hycAup-P<em>ynf</em>-Fwd</td>
<td>AAGGGTAAAGAACATTGCTGGTGCAGAAAAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hycAup-P<em>ynf</em>-Rev</td>
<td>CGATAATGGAATTGCTAACCAGTTAAGCAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P</em>prom-<em>fdhF</em>-Fwd</td>
<td>GGAGTGGATTGGATACATGAAAGATCTGTCAGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P</em>prom-<em>fdhF</em>-Rev</td>
<td>CTTTTTTCATGATCTTCTGGTGAGTAGATGTATAAAC</td>
</tr>
<tr>
<td>MR72</td>
<td>Engineering of the <em>proD</em> promoter</td>
<td>hycAup-P<em>proD</em>-Fwd</td>
<td>AAGGGTAAAGAACATTGCTGGTGCAGAAAAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hycAup-P<em>proD</em>-Rev</td>
<td>TGTGCTCTGAGAAGATTGCTACCCTGTTAAACAGAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P</em>proD-<em>fdhF</em>-Fwd</td>
<td>GTTTAACCTTTACTAGAAGTCAACAGAAAGATCTAGATCATGAAAGATCTGTCAGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>P</em>proD-<em>fdhF</em>-Rev</td>
<td>CCTGTGTGACCTCTAGTAAAAAGAGATATTTGTAGAGG</td>
</tr>
</tbody>
</table>
transferred to the chromosome of MR40 as described (20, S4), resulting in three new strains, MR36, MR41, and MR72 (Table 1). Finally, strain TOM001 lacking the membrane arm of FHL-1 (MR60 ΔhycCD; Table 1) was prepared using a previously described pMAK705 plasmid carrying an unmarked ΔhycCD allele (11).

**Protein purification.** Cells that were grown under anaerobic fermentative conditions (5 liters) were harvested by centrifugation and suspended with lysis buffer containing 50 mM Tris HCl, pH 8.0, with 10 μg ml⁻¹ DNase I (Sigma), 50 μg ml⁻¹ lysozyme (Sigma), and a protease inhibitor cocktail (Roche) at 1 g wet cell weight per 10 ml of buffer. Cells were lysed using a high-pressure cell homogenizer (Homogenising Systems Ltd.) at 1,000 bar before unbroken cells and debris were removed by centrifugation. Membrane proteins were solubilized for 1.5 h at room temperature by adding N-dodecyl-β-D-maltopyranoside (DDM) 1% (wt/vol) directly to the crude extract. Then, the solubilized fraction was loaded onto a 5 ml HisTrap HP column (GE Healthcare) that had been equilibrated in 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 50 mM imidazole, and 0.02% (wt/vol) DDM. Bound proteins were eluted with a 6-column-volume linear gradient of the same buffer containing 500 mM imidazole. Fractions were analyzed by SDS-PAGE (56), and those containing FHL-1 were pooled and concentrated in a Vivaspin (Millipore Inc.) filtration device (50-kDa-molecular-weight cutoff). Metal content was determined by inductively coupled plasma mass spectrometry (ICP-MS) performed as a service by the University of Edinburgh, United Kingdom. For Western immunoblotting, samples were first separated by SDS-PAGE before transfer to nitrocellulose (57). Blots were developed using a mouse anti-HA monoclonal antibody (Invitrogen) and a goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad) and visualized with an ImageQuant LAS-4000 imager (GE Healthcare).

**Whole-cell catalysis of H₂/formate production at ambient pressure.** Cells were grown under anaerobic fermentative conditions in rich LB medium containing 0.4% (wt/vol) glucose. When mentioned, the medium was supplemented with 0.2% (wt/vol) sodium formate and/or 1 μmol liter⁻¹ sodium tungstate. The culture was harvested by centrifugation (Beckman J6-MI centrifuge) for 30 min at 5,000 × g and 4°C. The cell paste was washed twice in 20 mmol liter⁻¹ sodium N-(morpholino)-propanesulfonic acid (MOPS) buffer, pH 7.4, before the cell pellet was suspended in the same buffer, and volume was adjusted to an OD₅₆₅ of 0.5 (~0.125 gCDW). For the FHL forward activity, 5-ml aliquots of washed whole cells were transferred into Hungate tubes before 0.2% (wt/vol) sodium formate was added. The tubes were flushed with nitrogen for 5 min before being incubated at 37°C for 20 h. H₄ content in the headspace was quantified by gas chromatography (GC). For the HDCR reverse reaction, 3 ml of washed whole cells was transferred into Hungate tubes. Tubes were flushed with N₂ for 5 min and then with H₂ for another 5 min before 5 ml CO₂ was added to the tubes. The cells were incubated at 37°C for 20 h. The cell suspension was then passed through a 0.2-μm filter syringe (Sartorius), and the formate content of the cell-free supernatant determined by high-performance liquid chromatography (HPLC).

**Pressurized bioreactor culture conditions.** Growth under pressure was performed in a commercially available bioXplorer 400P system (HEL Ltd., UK). The working culture volume was 250 ml. The bioreactor was set with three gas inlets, H₂, CO₂, and N₂. Each gas inlet was controlled by a gas mass flow controller. The bioreactor was equipped with pH, dissolved oxygen, temperature, and pressure controllers. The WinISO software handled the on-line monitoring and control systems of the reactor. The pH was maintained at 7.0 by addition of a 5-mol liter⁻¹ NaOH solution. The steel bioreactor chamber containing LB-rich medium was first autoclaved before 0.8% (wt/vol) glucose and 1 μmol liter⁻¹ sodium tungstate were added prior to inoculation. A 10% (vol/vol) culture grown in LB-rich medium prepared under aerobic conditions for at least 16 h was used to inoculate the bioreactor. After inoculation, O₂ present in the medium was observed to be rapidly consumed by the culture via the in-line O₂ electrode. The time taken to deplete the residual oxygen content was typically 30 min. After this point, the H₂, CO₂, and/or N₂ gases were sparged through the medium at a gas flow rate of 50 ml min⁻¹. Once the desired back pressure had been reached, the system was maintained at 37°C for 16 h. Aliquots were removed at regular time intervals where the OD₅₆₅ was recorded before cells were removed by passage through a 0.2-μm filter syringe. The resultant supernatants were analyzed for glucose, formate, and other fermentation products by HPLC.

**Analytical methods.** Bacterial growth was monitored by following the OD₅₆₅, and the biomass yield was estimated from the OD₅₆₅ of the culture and the assumption that 1 liter culture with an OD₅₆₅ of 1 contains 0.25 gCDW. Formate and fermentation metabolite analysis and quantification were determined by HPLC using an Ultimate 3000 uHPLC system (Thermo Fisher Scientific) equipped with an Aminex HPX-87H column (Bio-Rad) using a RefractoMax521 refractive index detector and a VWD-3100 variable-wavelength detector set at A₂₁₀. Typically, samples of 10 μl that were previously clarified through 0.2-μm filters were applied to the column equilibrated in 5 mmol liter⁻¹ H₂SO₄ with a flow rate of 0.8 ml min⁻¹ at 50°C.

Hydrogen gas in the culture headspace was quantified using a GC-2014 gas chromatograph (Shimadzu) equipped with a molecular sieve 5Å capillary column and thermal conductivity detector. Nitrogen was used as carrier gas with a 25 ml min⁻¹ flow rate. Typically, 1-ml samples were collected using a gas-tight syringe with Luer lock valve (SGE) and used to manually fill a 500-μl loop.

**SUPPLEMENTAL MATERIAL**
Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.6 MB.
REFERENCES


