Engineering Hydrogen Gas Production from Formate in a Hyperthermophile by Heterologous Production of an 18-Subunit Membrane-bound Complex*

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Background: Biohydrogen production from formate may aid in efficient chemical and fuel production using H₂ as the energy carrier.

Results: The hyperthermophile Pyrococcus furiosus was engineered to convert formate to H₂.

Conclusion: An 18-gene cluster can be inserted into the P. furiosus chromosome for active production of a membrane-bound system.

Significance: This work demonstrates the versatility of this model organism for metabolic engineering purposes.

Biohydrogen gas has enormous potential as a source of redox for the microbial production of biofuels, but its low solubility and poor gas mass transfer rates are limiting factors. These limitations could be circumvented by engineering biofuel production in microorganisms that are also capable of generating H₂ from highly soluble chemicals such as formate, which can function as an electron donor. Herein, the model hyperthermophile, Pyrococcus furiosus, which grows optimally near 100 °C by fermenting sugars to produce H₂, has been engineered to also efficiently convert formate to H₂. Using a bacterial artificial chromosome vector, the 16.9-kb 18-gene cluster encoding the membrane-bound formate hydrogen lyase complex of Thermococcus onnurineus was inserted into the P. furiosus chromosome and expressed as a functional unit. This enabled P. furiosus to utilize formate as well as sugars as an H₂ source and to do so at both 80° and 95 °C, near the optimum growth temperature of the donor (T. onnurineus) and engineered host (P. furiosus), respectively. This accomplishment also demonstrates the versatility of P. furiosus for metabolic engineering applications.

Hydrogen gas (H₂) can be generated from a variety of feedstocks and has great potential as a renewable energy carrier (1, 2). There is currently great interest in engineering microorganisms for efficient H₂ production (3, 4), particularly from abundant one-carbon compound feedstocks such as formate (5). In addition, H₂ is proposed as the energy carrier for converting carbon dioxide into industrial chemicals and liquid biofuels or so-called “electrofuels.” Although gas mass transfer and the rapid supply of sparingly soluble H₂ to the site of carbon fixation within a microbe is a major challenge (6), this could be circumvented by the production of biohydrogen from highly soluble H₂ donors such as formate, which simultaneously generates CO₂. The hyperthermophile Thermococcus onnurineus is capable of formate oxidation coupled to H₂ evolution (7). The related organism, Pyrococcus furiosus, cannot utilize formate as a source of H₂ (8), but it has many desirable features as a metabolic engineering host for the H₂-dependent reduction of CO₂ to chemicals and electrofuels (6, 9).

P. furiosus and T. onnurineus are members of the archaeal order Thermococcales, with optimal growth temperatures ranging from 75 to 100 °C. They grow heterotrophically by the fermentation of sugars or peptides with formation of either H₂ or H₂S, and most require S₀ as an electron acceptor. A hallmark of this group is the presence of a respiratory membrane-bound [NiFe]hydrogenase (Mbh)² complex that is responsible for evolving H₂ as a means for disposing of reductant from fermentation. A very close homolog of Mbh, known as Mbx, is involved in reducing S₀ to H₂S (8). The Mbh and Mbx complexes each occur in conjunction with a Na⁺/H⁺ antiporter (Mrp) module, such that H₂ and H₂S evolution are coupled to the formation of a Na⁺ ion gradient that allows energy conservation via Na⁺-dependent ATP synthase (10, 11).

In some Thermococcales species, there are additional modules that function in conjunction with the Mrp-Mbh complexes, such as the formate dehydrogenase (Fdh) of T. onnurineus. This Fdh, together with an Mrp-Mbh, forms a multiprotein membrane-bound formate hydrogen lyase (FHL) system that allows oxidation of formate to generate H₂ and CO₂, where the CO₂ is predominantly bicarbonate (7). The standard reduction potentials of the formate/bicarbonate and H₂/H⁺ couples are very similar, but the thermodynamics of the

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2 The abbreviations used are: Mbh, membrane-bound hydrogenase; Mbp, Na⁺/H⁺ antiporter; Fdh, formate dehydrogenase; FHL, formate hydrogen lyase; BAC, bacterial artificial chromosome; POR, pyruvate ferredoxin oxidoreductase.
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**EXPERIMENTAL PROCEDURES**

Vector Construction—PCR products of the mbh1 promoter region (180 b starting immediately upstream of the PF1423 gene start), the T. onnurineus FHL operon (TON1563–1580, 16.9 kb), homologous flanking regions targeting a locus termed genome region 5 (between convergent genes PF1232-PF1233, −0.5 kb each), the pyrF pop-out marker cassette (9), and bacterial artificial chromosome (BAC) vector backbone (amplified from pBELOBac11, obtained from New England Biolabs) were assembled into a single vector, pGL054 (Fig. 2A), using Gibson Assembly (New England Biolabs) (23). pGL054 was sequence-verified.

**Strain Construction—P. furiosus** COM1 was transformed as described previously (13) with pGL054 linearized using the unique PvuI restriction site (positioned between sopA and sopB on the BAC vector backbone). Transformant colonies were cultured anaerobically in defined cellobiose medium (13), genomic DNA was isolated using the ZymoBead™ Genomic DNA kit (Zymo Research), and PCR screens were performed with primers ~100 b outside the homologous flanking regions used to insert the expression construct into the chromosome. PCR-verified isolates were further purified twice on solid defined cellobiose medium prior to saving glycerol stocks. One of the purified pGL054 transformants was designated MW144, and this strain was used for phenotypic analyses. Strains used and constructed in this study are listed in Table 1.

**Growth and Cell Protein Quantitation—P. furiosus** strains were cultured under strict anaerobic conditions in artificial seawater medium containing per liter: 1× base salts (24), 1× trace minerals (24), 0.26 μM sodium tungstate, 0.25 μg of resazurin, 0.5 g of cysteine, 1 g of sodium bicarbonate, and 1 mM potassium phosphate buffer, with pH adjusted to 6.8 prior to bottling. Medium was adjusted to contain equivalent amounts of tungstate and molybdate because it is unknown whether the cofactor utilized by Fdh contains molybdenum or tungsten. Media was aliquoted into serum bottles, and the headspace was replaced with argon after three cycles of vacuum and argon. For growth curves, this medium was supplemented with 0 or 5 g maltose with 0.5 g of casein hydrolysate (USB) and 1× vitamin mix per liter (13). Sodium formate was added at a concentration of 50 mM from an anaerobic stock solution. Medium was inoculated to ~3 × 10⁶ cells ml⁻¹, and cultures were incubated at 98 °C or 80 °C with shaking. For extract preparation, growth medium was supplemented with 5 g of maltose and 5 g of yeast extract per liter, and cultures were incubated at 80 °C with stirring. When necessary, cell growth was monitored by cell counts using a Petroff-Hausser counting chamber. Cell protein was quantified from 1-ml culture samples using the Bradford protein assay kit (Bio-Rad). Briefly, cells were harvested by centrifugation from 1-ml culture samples and lysed by osmotic shock in an equal volume of water, with vortexing and one freeze-thaw cycle. Lysate was centrifuged at 10,000 × g for 1 min to pellet insoluble cell debris prior to quantitation of soluble cell protein.

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**TABLE 1**

| Strain   | Parent  | Description | Ref. |
|----------|---------|-------------|------|
| COM1     | DSM 3638|             |      |
| MW144    | COM1    | ΔpyrF       | 13   |
|          |         | ΔpyrFΔgdp    |      |
|          |         | ΔpyrFΔmbh   |      |
|          |         | (TON1563-TON1580) |       |
|          |         | ΔpyrF::pyrF  | 16   |

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**FIGURE 1.** The T. onnurineus FHL complex and gene cluster. **A**, a schematic representation of the T. onnurineus FHL complex (encoded by TON_1563–1580) based on operon structure and homology with P. furiosus, showing subunits of formate dehydrogenase (Fdh2) in red, membrane-bound hydrogenase (Mbh) in green, formate transporter (Ft) in blue, and Na⁺/H⁺ transporter (Mrt) in yellow. The hypothetical protein encoded by TON_1572 is not shown. **B**, the TON_1563–1580 gene cluster (Fdh2, TON_1563–64, red; Mbh, TON_1565–71, green; hypothetical gene, TON_1572, black; formate transporter, TON_1573, blue; Mrt, TON_1574–80, yellow).
Cell-free Extract Preparation and Enzyme Assays—Cell-free extracts of *P. furiosus* cell pellets were prepared as follows using strict anaerobic conditions in an anaerobic chamber (Coy Laboratory Products). Cells were suspended in 50 mM MOPS, pH 7.5 (3 ml of buffer per gram of cells) and lysed by osmotic shock and sonication (Qsonica model Q55, 1 min at amplitude 30). To prepare membrane extracts, the cell-free extract was centrifuged at 100,000 × g for 1 h. The resulting pellet was suspended in 9 ml of buffer and centrifuged again, and the pellet was suspended in 0.5 ml of buffer and stored at 4 °C in stopped glass vials until assayed. Formate hydrogen lyase activity was measured by the production of *H*₂ from formate. A buffer (50 mM MOPS, pH 7.5, 20 mM NaCl, 2 mM MgCl₂) containing 25 mM sodium formate was preheated to 80 °C, and the reaction was initiated by the addition of extracts (~0.5 mg ml⁻¹), and gas samples were analyzed by gas chromatography (Shimadzu GC8A with TCD detector, oven 70 °C, injector/detector 120 °C.

**RESULTS**

**Insertion of the *T. onnurineus* FHL Operon into the *P. furiosus* Chromosome**—The FHL cluster was assumed to be a single operon as all but one of its intergenic spaces are less than 12 bp in size (the exception is 42 bp). The native promoter of FHL was replaced with that of the *P. furiosus* Mbh operon (P*mbh1*), the transcription of which is activated by the SurR regulator during growth in the absence of *S*₇, thereby allowing Mbh to catalyze *H*₂ evolution during sugar fermentation (27, 28). A BAC vector was used to facilitate cloning of the construct for chromosomal insertion of the FHL operon (Fig. 2A). Insertion of the expression construct was targeted to a region of the *P. furiosus* chromosome (between convergent genes PF1232-PF1233) having little to no transcriptional activity as determined by analysis of tiling array data (29). Using 0.5-kb homologous recombination regions, the resulting ~19-kb fragment of DNA containing the FHL operon along with the *pyrF* selectable marker was inserted into the chromosome in one transformation event, generating the strain MW144 (Fig. 2B).

**In Vitro Evaluation of FHL Complex Activity**—To examine the functionality of the *T. onnurineus* FHL system in *P. furiosus*, strain MW144 was grown on the disaccharide maltose in the absence of *S*₇ to activate expression of genes controlled by the *mbh1* promoter, which included both the FHL and Mbh operons. Although *P. furiosus* grows optimally near 100 °C, the MW144 strain was grown at 80 °C, the optimal growth temperature of *T. onnurineus*, as our previous work has demonstrated that heterologously-produced enzymes in *P. furiosus* have the highest specific activities when produced near the optimum growth temperature of their organism of origin (9). Quantitative RT-PCR indicated that expression of the FHL operon in MW144 was at or above the level of *mbh1* gene expression, and similar to that of the major sugar metabolism enzyme pyruvate ferredoxin oxidoreductase (POR), which generates the reduced ferredoxin that is oxidized by Mbh to evolve *H*₂ (Fig. 3). Furthermore, formate-dependent *H*₂ evolution activity (0.3 ± 0.02 units mg⁻¹) was present in membrane preparations of MW144 cells but not in the *P. furiosus* parental control strain MW004 (<0.01 unit mg⁻¹). However, both MW144 and con-
Control strains exhibited comparable pyruvate-dependent H₂ evolving activity, which is a measure of the activity of POR and the Mbh complex, but not of the FHL complex (Fig. 3B). In contrast, total hydrogenase activity, as measured using the artificial electron donor dithionite-reduced methyl viologen, was almost 2-fold higher in MW144 membrane preparations compared with MW004 (Fig. 3B). Taken together, these data indicate that the T. onnurineus FHL complex neither replaces nor reduces the activity of the native Mbh, and that in contrast to Mbh, the FHL complex does not efficiently use reduced ferredoxin as an electron donor. Hence, the MW144 strain has twice the Mbh activity (P. furiosus Mbh plus T. onnurineus FHL) but similar pyruvate-dependent ferredoxin-linked Mbh activity (Mbh only) as the parent strain.

Analysis of in Vivo H₂ Production from Formate—To determine the physiological effects of expression of the T. onnurineus FHL complex in P. furiosus, strain MW144 and the control strain MW004 were grown on maltose at 80 °C in the presence and absence of 50 mM formate. According to previously reported work, the doubling time of wild type P. furiosus increases by ~2-fold at 80 °C compared with 100 °C (~80 min versus ~40 min, respectively) (30). Both strains grew similarly with and without formate, reaching stationary phase after ~20 h of growth (as measured by soluble cell protein; Fig. 4A). As can be seen in Fig. 4B, compared with the control strain and no formate conditions, MW144 grown in the presence of formate produced almost twice as much H₂ after 20 h (37 versus 22 mM), reaching a maximum of 62 mM H₂ by 32 h (although by 54 h, high gas pressures caused difficulty in accurate headspace sampling of MW144 with formate). Additionally, MW144 con-

FIGURE 3. The T. onnurineus FHL complex is actively expressed in P. furiosus. A, quantitative RT-PCR expression of select FHL genes in MW144, relative to mbh1 expression, at both 98 °C (red) and 80 °C (blue). Relative expression of the P. furiosus POR gene is shown for comparison. B, specific activities from membrane extracts of MW004 (yellow) and MW144 (green) using the indicated electron donor. Error bars represent S.D. (n = 2).

FIGURE 4. The T. onnurineus FHL complex is active in vivo in P. furiosus. Growth (as measured by soluble cell protein, A and E), H₂ produced per liter of culture (B and F), formate in spent medium (C and G), and culture pH (D and H) of MW004 cultured in the absence (blue diamonds) or presence (yellow squares) of 50 mM formate compared with MW144 in the absence (red triangles) or presence (green circles) of 50 mM formate, at 80 °C (A–D) or 95 °C (E–H) in medium supplemented with 5 g of maltose, 0.5 g of tryptone, and 1 × vitamin mixture per liter. Error bars represent S.D. (n = 3).
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sumed formate as shown by the decrease in the measured concentration of formate in the culture medium from 50 mM to <20 mM after 54 h (Fig. 4C). These data clearly show that the FHL complex is functionally expressed in \( \text{P. furiosus} \) and is able to efficiently oxidize formate with concomitant \( \text{H}_2 \) evolution. Interestingly, even though the optimal growth temperature of \( T. \text{onnurineus} \) is 80 °C, the FHL complex was still produced and active in \( \text{P. furiosus} \) at 95 °C. After 12 h of growth at 95 °C (Fig. 4E), MW144 produced >2-fold more \( \text{H}_2 \) in the presence of formate rather than in its absence (28 mm versus 16 mm; Fig. 4F) and consumed a corresponding amount of formate (≈14 mm; Fig. 4G). The maximum rate of \( \text{H}_2 \) production for MW144 grown with formate was the same at 95 and 80 °C (4.2 mmol \( \text{H}_2 \) liter\(^{-1}\) h\(^{-1}\)) and was at least 2-fold higher than growth without formate.

\( \text{P. furiosus} \) produces acetate during growth, causing a decrease in growth medium pH (to near pH 4); however, the pH medium remained near neutral for MW144 grown with formate (Fig. 4, D and H). The drop in growth medium pH for the control strain and MW144 in the absence of formate caused a decrease in soluble cell protein (presumably due to protein aggregation resulting from low intracellular pH) and a subsequent loss in cell viability, as measured by sub culturing exponential and stationary phase cells and monitoring growth (data not shown). The pH stabilizing effect of the \( T. \text{onnurineus} \) FHL complex in \( \text{P. furiosus} \) is akin to that of the analogous FHL system found in \( \text{Escherichia coli} \) and related microorganisms (31). The \( \text{E. coli} \) FHL is important for cell survival under anaerobic acid stress conditions whereby FHL increases intracellular pH by converting endogenously produced formate to \( \text{H}_2 \) and \( \text{CO}_2 \) (HCOO\(^{-}\) + \( \text{H}^+ \) → \( \text{CO}_2 \) + \( \text{H}_2 \)).

In \( T. \text{onnurineus} \), formate oxidation is coupled to ATP generation via ion pumping (see Fig. 1A), and there is evidence that it is also assimilated into cellular carbon (32). In the absence of \( \text{S}^0 \), \( T. \text{onnurineus} \) displays no growth on peptides (supplied as 0.5–1 g of yeast extract per liter); however, cells do grow when formate is supplied (7). \( \text{P. furiosus} \) also shows very poor growth on peptides (without \( \text{S}^0 \)), although unlike \( T. \text{onnurineus} \), it can utilize the sugars found in yeast extract (24). To determine whether the FHL complex could supply energy to \( \text{P. furiosus} \) via formate oxidation, the MW144 strain was grown at 80 °C solely on peptides (supplied as 0.5 g of tryptone per liter). Cell growth was similarly poor for both the MW144 and control \( \text{P. furiosus} \) strains grown with and without formate, indicating MW144 could not use formate as an energy source (cell densities of \( \approx 5 \times 10^7 \) cells ml\(^{-1}\) or cell protein of 10 \( \mu \)g ml\(^{-1}\) were obtained, Fig. 5A). Nevertheless, although MW144 did not gain energy from formate oxidation, the FHL complex was highly active, with MW144 producing ~6-fold more \( \text{H}_2 \) (24 versus 4 mm, Fig. 5B) and consuming a corresponding amount of formate (12 mm, Fig. 5C). Although the formate-dependent \( \text{H}_2 \) production activity of the FHL complex is similar in both the native organism and \( \text{P. furiosus} \), the energy-conserving feature observed in \( T. \text{onnurineus} \) must result from other factors distinct from the FHL operon but functioning in combination with the FHL complex.

**DISCUSSION**

The \( T. \text{onnurineus} \) FHL complex in \( \text{P. furiosus} \) is a self-contained unit that functions independently of cellular metabolism when formate is present, displaying close to stoichiometric conversion of formate to \( \text{H}_2 \). Moreover, the maximum rate (4.2 mmol \( \text{H}_2 \) liter\(^{-1}\) h\(^{-1}\)) and amount (~45 mmol \( \text{H}_2 \) liter\(^{-1}\)) of \( \text{H}_2 \) produced by recombinant \( \text{P. furiosus} \) strain MW144 after 24 h (in the presence of maltose, Fig. 4B) is comparable with that reported for \( T. \text{onnurineus} \) (3.8 and ~38, respectively) (5), although the rate and amount of \( \text{H}_2 \) produced are at least 3-fold lower than \( T. \text{onnurineus} \) when MW144 is grown with formate on peptide-containing medium (without maltose). When maltose was supplied in the \( \text{P. furiosus} \) growth medium, the mole ratio of \( \text{H}_2 \) evolved to formate utilized was not stoichiometric because sugar fermentation also leads to \( \text{H}_2 \) production. The ratio of excess \( \text{H}_2 \) produced by MW144 when formate is present compared with the amount of formate utilized ranges from 1 to 1.5 during growth (with or without maltose), which is close to the theoretical rate of one mole of \( \text{H}_2 \) produced per mole of formate consumed.

The functionality of the engineered FHL complex in \( \text{P. furiosus} \) therefore appears to be very similar to that of \( T. \text{onnurineus} \) for production of \( \text{H}_2 \) from formate. However, \( \text{P. furiosus} \) has the advantage over \( T. \text{onnurineus} \) in that it can also generate \( \text{H}_2 \) by sugar fermentation. Moreover, this \( \text{H}_2 \) production occurs by a completely independent pathway (via \( \text{P. furiosus} \) Mbh) that does not interfere with formate to \( \text{H}_2 \) conversion (via the FHL Mbb). Thus, the engineered \( \text{P. furiosus} \) strain MW144 has the
unique property of being able to independently convert both sugars and formate to H₂ at temperatures ranging from 80 to near 100 °C. Moreover, this was achieved by the stable insertion of a large DNA element into the P. furiosus chromosome via use of a BAC vector. Indeed, the ability to recombine almost 20 kb of DNA into the genome in a single step with relative ease is a remarkable feature of the P. furiosus genetic system and rivals technologies available for chromosomal integration of foreign DNA in model microorganisms such as E. coli (33, 34). This new genetic tool will enable sophisticated genetic engineering of heterologous metabolic pathways in this model hyperthermophile. Furthermore, enabling P. furiosus with the capacity to use formate in place of exogenously supplied H₂ gas significantly improves its prospects for serving as an effective metabolic engineering host for chemical and electrofuel production (6, 9).

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