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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 H_2 as the energy carrier.
Results: The hyperthermophile *Pyrococcus furiosus* was engineered to convert formate to H_2.
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 reductant 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 H_2 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 H_2, has been engineered to also efficiently convert formate to H_2. Using a bacterial artificial chromosome vector, the 16.9-kb 18-gene cluster encoding the membrane-bound, respiratory 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 H_2 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 (H_2) 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 H_2 production (3, 4), particularly from abundant one-carbon compound feedstocks such as formate (5). In addition, H_2 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 H_2 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 H_2 donors such as formate, which simultaneously generates CO_2. The hyperthermophile *Thermococcus onnurineus* is capable of formate oxidation coupled to H_2 evolution (7). The related organism, *Pyrococcus furiosus*, cannot utilize formate as a source of H_2 (8), but it has many desirable features as a metabolic engineering host for the H_2-dependent reduction of CO_2 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 H_2 or H_2S, and most require S^0 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 H_2 as a means for disposing of reductant from fermentation. A very close homolog of Mbh, known as Mbx, is involved in reducing S^0 to H_2S (8). The Mbh and Mbx complexes each occur in conjunction with a Na^+/H^+ antiporter (Mrp) module, such that H_2 and H_2S 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 H_2 and CO_2, where the CO_2 is predominantly bicarbonate (7). The standard reduction potentials of the formate/bicarbonate and H_2/H^+ couples are very similar, but the thermodynamics of the

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

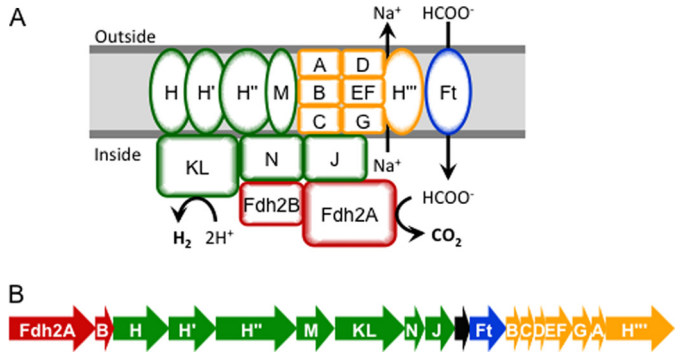

FIGURE 1. **The *T. onnurineus* FHL complex and gene cluster.** *A*, a schematic representation of the *T. onnurineus* FHL complex (encoded by TON_1563–1580) based on operon structure and homology with *P. furiosus* Mrp-Mbh, showing subunits of formate dehydrogenase (Fdh2) in *red*, membrane-bound hydrogenase (*Mbh*) in *green*, formate transporter (*Ft*) in *blue*, and Na^+/H^+ transporter (*Mrp*) 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*; Mrp, TON_1574–80, *yellow*).

FHL reaction become favorable ($\Delta G = -8$ to -20 kJ mol^{-1}) at high temperature, high formate concentrations and low partial pressures of H_2 (7). Thus, in *T. onnurineus*, cell growth can be supported by formate-driven ATP synthesis. *T. onnurineus* contains three different Fdh-containing gene clusters, two of which also encode Mbh and Mrp modules, and it has been shown that only one of these (Mrp-Mbh-Fdh2) is required for formate-dependent growth and H_2 evolution (7). This FHL complex is encoded by an 18-gene cluster (TON_1563-1580), which also includes a formate transporter of the FocA formate channel type (12), although the mechanism of formate uptake is unclear. A schematic representation of the membrane-associated *T. onnurineus* FHL complex is presented in Fig. 1*A*.

The primary goal of this research was to convert *P. furiosus* into a formate-oxidizing, H_2-evolving microorganism by inserting the 16.9-kb 18-gene FHL operon into its chromosome (Fig. 1*B*). An efficient genetic system has been developed for *P. furiosus*, the first for a microorganism that can grow at, or even close to, 100 °C (13). The genetically tractable *P. furiosus* strain, COM1, has been used for making gene deletions (14–16), overproducing its proteins (17, 18), and for metabolic engineering (9, 19). The COM1 strain is remarkably efficient at both DNA uptake and recombination (13, 20, 21), although the factors responsible are not clear (22). It is also not known how large of a DNA fragment can be functionally inserted into the *P. furiosus* chromosome. Herein, we demonstrate chromosomal insertion of a DNA fragment of almost 19 kb for heterologous expression of the *T. onnurineus* FHL operon in *P. furiosus*. As a member of the Thermococcales, *P. furiosus* contains an Mbh-Mrp gene cluster that is used for fermentative H_2 production during growth on sugars (10, 15). Successful engineering of the FHL operon into *P. furiosus* enabled this organism the capacity to utilize both sugars and formate as sources of H_2 at 80° to 95 °C, at or near the optimum growth temperatures of *T. onnurineus* (80 °C) and *P. furiosus* (100 °C).

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 pBELOBac-11, obtained from New England Biolabs) were assembled into a single vector, pGL054 (Fig. 2*A*), 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.

TABLE 1
Strains used and constructed in this study

Strain	Parent	Description	Ref.
COM1	DSM 3638	Δ*pyrF*	13
MW144	COM1	Δ*pyrF* P_{gdh}*pyrF* P_{mbh1} (TON1563-TON1580)	This study
MW004	COM1	Δ*pyrF*::*pyrF*	16

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 ~3×10^6 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.

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 stoppered 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 $MgCl_2$) 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, Alltech Molecular Sieve column 5A 80/100). Membrane bound hydrogenase (Mrp-Mbh) activity was measured by the production of H_2 from pyruvate via reduced ferredoxin (regenerated by pyruvate ferredoxin oxidoreductase, POR), and total hydrogenase activity was measured using sodium dithionite with methyl viologen (10). Care was taken to maintain strict anaerobic conditions during extract preparation and enzyme assays.

H_2 Quantitation—H_2 was measured by sampling the headspace of closed bottle cultures with a pressure-lock syringe. Samples from identical bottles of medium (without cells) containing known amounts of H_2 were used as standards. H_2 content from headspace samples was analyzed by gas chromatography (see above). We assumed H_2 solubility in the liquid phase as negligible; therefore, H_2 production is expressed as moles produced per liquid phase, using the ideal gas law (assuming 1 mole of gas at 25 °C and 1 atmosphere equals 24.5 liters).

Formate Quantitation—Formate was quantified via HPLC either by derivatization with 2,4-dibromoacetophenone (25) or direct analysis on an Aminex 87H column (Bio-Rad). For derivatization, spent media samples of 20 μl were diluted 5- to 10-fold with water, and pH was adjusted to ~9. Subsequently 100 μl acetonitrile, 200 μl of 50 mM 2,4-dibromoacetophenone (in acetonitrile), and 50 μl of 1 M 15-crown-5 ether (in acetonitrile) were added, the mixture was incubated for 45 min at 80 °C, and 10-μl aliquots were analyzed by HPLC (Agilent 1260 Infinity; Poroshell 120 EC-C18 column; solvents, 0.05% (v/v) trifluoroacetic acid and acetonitrile; gradient, 90/10 to 40/60 in 8 min at 2 ml min^{-1}). The Aminex 87H column was run with an isocratic mobile phase of 5 mM H_2SO_4 for 30 min, and organic acids were detected by UV absorbance at 210 nm.

RNA Extraction and Quantitative RT-PCR—RNA was extracted from 10 ml samples of culture using a phenol: chloroform extraction method as previously described (26). Genomic DNA was digested using TURBO DNase (Ambion). Synthesis of cDNA was performed with 1 μg of purified RNA using the Affinity Script QPCR cDNA synthesis kit (Agilent). The Brilliant II SYBR® Green QPCR Master Mix (Agilent) was used for quantitative RT-PCR experiments with primers designed to amplify a ~150 b product within the target gene: PF0971 (PORγ gene), PF1423 (*mbh1*), TON1563, TON1567, TON1573, TON1580.

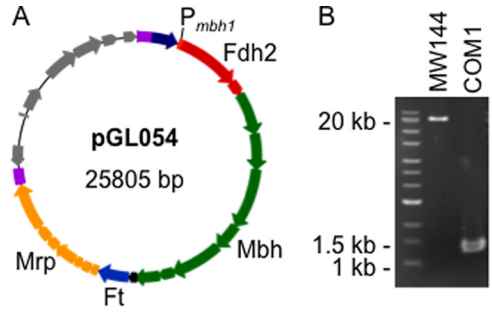

FIGURE 2. **Insertion of the *T. onnurineus* FHL gene cluster into the *P. furiosus* chromosome.** *A*, the BAC-based vector pGL054 containing the FHL operon (with genes color-coded according to Fig. 1) under the control of the *mbh1* promoter (P_{mbh1}, *orange*), with the *pyrF* marker cassette (*indigo*) and 5′ and 3′ 0.5-kb homologous recombination regions (*purple*) used for targeted insertion into the *P. furiosus* chromosome. The BAC vector backbone features are shown in *gray* (from *left* to *right*: *cat* marker, *oriS*, *repE*, *sopA*, *sopB*, sopC, and cos). *B*, PCR confirmation with genomic DNA of MW144 *versus* COM1 parent with primers amplifying from ~100 b outside each homologous flanking region of the target insertion locus.

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^0, 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. 2*A*). 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. 2*B*).

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^0 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 *P. furiosus* Mbh to evolve H_2 (Fig. 3*A*). 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-

trol strains exhibited comparable pyruvate-dependent H_2 evolving activity, which is a measure of the activity of POR and the Mbh complex, but not of the FHL complex (Fig. 3*B*). 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. 3*B*). 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.

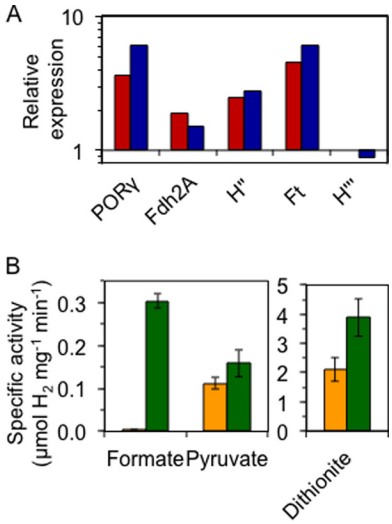

FIGURE 3. **The *T. onnurineus* FHL complex is actively expressed in *P. furiosus*.** *A*, quantitative RT-PCR expression of select FHL genes in MW144, relative to *mbh1* expression, at both 98 °C (*red*) and 80 °C (*blue*). Relative expression of the *P. furiosus* PORγ gene is shown for comparison.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

Analysis of in Vivo H_2 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. 4*A*). As can be seen in Fig. 4*B*, compared with the control strain and no formate conditions, MW144 grown in the presence of formate produced almost twice as much H_2 after 20 h (37 *versus* 22 mM), reaching a maximum of 62 mM H_2 by 32 h (although by 54 h, high gas pressures caused difficulty in accurate headspace sampling of MW144 with formate). Additionally, MW144 con-

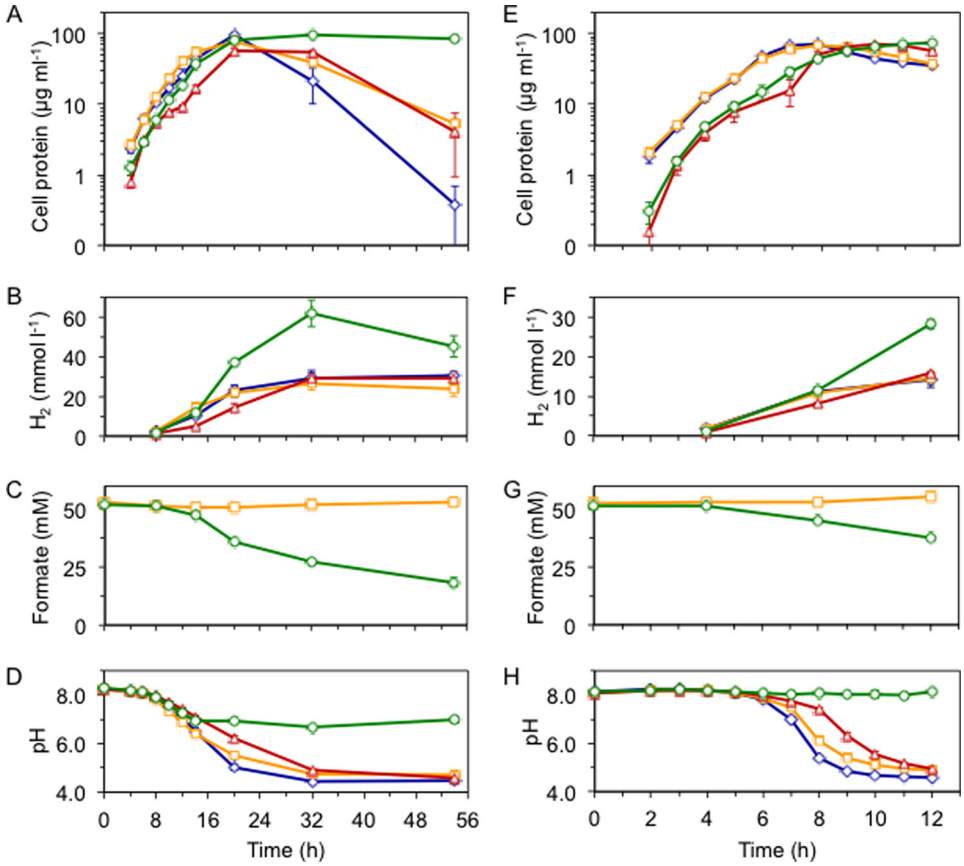

FIGURE 4. **The *T. onnurineus* FHL complex is active *in vivo* in *P. furiosus*.** Growth (as measured by soluble cell protein, *A* and *E*), H_2 produced per liter of culture (*B* and *F*), formate in spent medium (*C* and *G*), and culture pH (*D* and *H*) of MW004 cultured in the absence (*blue diamonds*) 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. ($n = 3$).

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. 4*C*). These data clearly show that the FHL complex is functionally expressed in *P. furiosus* and is able to efficiently oxidize formate with concomitant H_2 evolution. Interestingly, even though the optimal growth temperature of *T. onnurineus* is 80 °C, the FHL complex was still produced and active in *P. furiosus* at 95 °C. After 12 h of growth at 95 °C (Fig. 4*E*), MW144 produced >2-fold more H_2 in the presence of formate rather than in its absence (28 mM *versus* 16 mM; Fig. 4*F*) and consumed a corresponding amount of formate (~14 mM; Fig. 4*G*). The maximum rate of H_2 production for MW144 grown with formate was the same at 95 and 80 °C (4.2 mmol H_2 liter^{-1} h^{-1}) and was at least 2-fold higher than growth without formate.

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 subculturing exponential and stationary phase cells and monitoring growth (data not shown). The pH stabilizing effect of the *T. onnurineus* FHL complex in *P. furiosus* is akin to that of the analogous FHL system found in *Escherichia coli* and related microorganisms (31). The *E. coli* FHL is important for cell survival under anaerobic acid stress conditions whereby FHL increases intracellular pH by converting endogenously produced formate to H_2 and CO_2 ($HCOO^- + H^+ \rightarrow CO_2 + H_2$).

In *T. onnurineus*, formate oxidation is coupled to ATP generation via ion pumping (see Fig. 1*A*), and there is evidence that it is also assimilated into cellular carbon (32). In the absence of S^0, *T. 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). *P. furiosus* also shows very poor growth on peptides (without S^0), although unlike *T. onnurineus*, it can utilize the sugars found in yeast extract (24). To determine whether the FHL complex could supply energy to *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 *P. furiosus* strains grown with and without formate, indicating MW144 could not use formate as an energy source (cell densities of ~5 × 10^7 cells ml^{-1} or cell protein of 10 μg ml^{-1} were obtained, Fig. 5*A*). Nevertheless, although MW144 did not gain energy from formate oxidation, the FHL complex was highly active, with MW144 producing ~6-fold more H_2 (24 *versus* 4 mM, Fig. 5*B*) and consuming a corresponding amount of formate (12 mM, Fig. 5*C*). Although the formate-dependent H_2 production activity of the FHL complex is similar in both the native organism and *P. furiosus*, the energy-conserving feature observed in *T. onnurineus* must result from other factors distinct from the FHL operon but functioning in combination with the FHL complex.

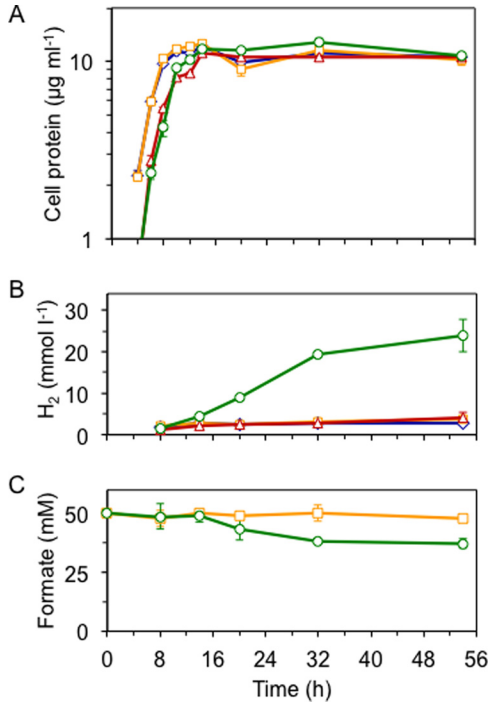

FIGURE 5. **Formate oxidation and H_2 production function independently from primary metabolism.** Growth (as measured by soluble cell protein, *A*), H_2 produced per liter of culture (*B*), formate in spent medium (*C*) of MW004 cultured in the absence (*blue diamonds*) or presence (*yellow squares*) of 50 mM formate compared with MW144 in the absence (*red triangles*) or presence (*green circles*) of 50 mM formate, at 80 °C in medium supplemented with 0.5 g of tryptone and 1× vitamin mixture per liter. *Error bars* represent S.D. ($n = 3$).

DISCUSSION

The *T. onnurineus* FHL complex in *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 H_2. Moreover, the maximum rate (4.2 mmol H_2 liter^{-1} h^{-1}) and amount (~45 mmol H_2 liter^{-1}) of H_2 produced by recombinant *P. furiosus* strain MW144 after 24 h (in the presence of maltose, Fig. 4*B*) is comparable with that reported for *T. onnurineus* (3.8 and ~38, respectively) (5), although the rate and amount of H_2 produced are at least 3-fold lower than *T. onnurineus* when MW144 is grown with formate on peptide-containing medium (without maltose). When maltose was supplied in the *P. furiosus* growth medium, the mole ratio of H_2 evolved to formate utilized was not stoichiometric because sugar fermentation also leads to H_2 production. The ratio of excess 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 H_2 produced per mole of formate consumed.

The functionality of the engineered FHL complex in *P. furiosus* therefore appears to be very similar to that of *T. onnurineus* for production of H_2 from formate. However, *P. furiosus* has the advantage over *T. onnurineus* in that it can also generate H_2 by sugar fermentation. Moreover, this H_2 production occurs by a completely independent pathway (via *P. furiosus* Mbh) that does not interfere with formate to H_2 conversion (via the FHL Mbh). Thus, the engineered *P. furiosus* strain MW144 has the

unique property of being able to independently convert both sugars and formate to H_2 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 H_2 gas significantly improves its prospects for serving as an effective metabolic engineering host for chemical and electrofuel production (6, 9).

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