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Protein Engineering of the Transcriptional Activator FhlA To Enhance Hydrogen Production in *Escherichia coli*▽†

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***Escherichia coli* produces H_2 from formate via the formate hydrogenlyase (FHL) complex during mixed acid fermentation; the FHL complex consists of formate dehydrogenase H (encoded by *fdhF*) for forming $2H^+$, $2e^-$, and CO_2 from formate and hydrogenase 3 (encoded by *hycGE*) for synthesizing H_2 from $2H^+$ and $2e^-$. FHL protein production is activated by the σ^{54} transcriptional activator FhlA, which activates transcription of *fdhF* and the *hyc*, *hyp*, and *hydN-hypF* operons. Here, through random mutagenesis using error-prone PCR over the whole gene, as well as over the *fhlA* region encoding the first 388 amino acids of the 692-amino-acid protein, we evolved FhlA to increase H_2 production. The amino acid replacements in FhlA133 (Q11H, L14V, Y177F, K245R, M288K, and I342F) increased hydrogen production ninefold, and the replacements in FhlA1157 (M6T, S35T, L113P, S146C, and E363K) increased hydrogen production fourfold. Saturation mutagenesis at the codons corresponding to the amino acid replacements in FhlA133 and at position E363 identified the importance of position L14 and of E363 for the increased activity; FhlA with replacements L14G and E363G increased hydrogen production (fourfold and sixfold, respectively) compared to FhlA. Whole-transcriptome and promoter reporter constructs revealed that the mechanism by which the FhlA133 changes increase hydrogen production is by increasing transcription of all of the genes activated by FhlA (the FHL complex). With FhlA133, transcription of P_{fdhF} and P_{hyc} is less sensitive to formate regulation, and with FhlA363 (E363G), P_{hyc} transcription increases but P_{hyp} transcription decreases and hydrogen production is less affected by the repressor HycA.**

Hydrogen is a promising fuel, since it can be produced from renewable sources (16) and its combustion does not produce pollutants, such as CO, CO_2, and SO_2, like conventional fossil fuels (34). To create a sustainable energy system based on hydrogen, improvements in hydrogen production are required to make it competitive with fossil fuels (34). It is important to note that the cost of new infrastructure to transport hydrogen may be avoided if hydrogen can be generated at the end user's location rather than at a central production facility (65).

Microbial fermentation is a potential method for large-scale hydrogen production (11), and there are two primary means of microbial hydrogen production: photosynthesis and fermentation. Fermentative reactors have the advantage that waste biomass (20) may be used as a feedstock. In addition, reactors with fermentative bacteria are considered more practical than those with photosynthetic bacteria, as photosynthetic systems require reactors with large surface areas (7) and have hydrogen production rates orders of magnitude lower than those of fermentative bacteria (25).

The hydrogen required to power a home using a 1-kW hydrogen fuel cell is 24 mol H_2/h (25). If hydrogen is produced by fermentation of glucose, the annual cost of the glucose is approximately $6,400 (59). To decrease the cost, it is necessary to increase the yield or use less expensive feedstocks (59). The hydrogen yield may be increased by utilizing additional fermentation end products, such as acetate, succinate, and lactate, to produce hydrogen. To power a home, the required size of the reactor for hydrogen production by fermentation of glucose or formate is approximately 500 liters. This size may be reduced by increasing the hydrogen production rate (59).

Escherichia coli is an attractive fermentative microorganism to engineer for hydrogen production because the majority of enzymes and genes related to hydrogen production are known (9) and it is easy to manipulate genetically (13). Under anaerobic conditions, *E. coli* produces hydrogen from formate through the reaction $HCOO^- + H_2O \leftrightarrow H_2 + HCO_3^-$, which is catalyzed by the formate hydrogenlyase (FHL) complex (63). The structural components of the FHL complex are formate dehydrogenase H, encoded by *fdhF* (3), which converts formate to $2H^+$, $2e^-$, and CO_2; hydrogenase 3 (Hyd-3), encoded by *hycE* (large subunit) and *hycG* (small subunit), which is reported to be a NiFe hydrogenase (49) that synthesizes molecular hydrogen from $2H^+$ and $2e^-$ (48); and the electron transfer proteins encoded by *hycBCDF*, which are thought to shuttle electrons between formate dehydrogenase H and Hyd-3 (49). An active FHL complex also requires the protease HycI (43), the putative electron carrier HydN (33), and the maturation proteins HycH (48), HypF (33), and HypABCDE (19).

The FHL complex has at least two regulators, FhlA and HycA. FhlA, the product of the last gene of the *hyp* operon

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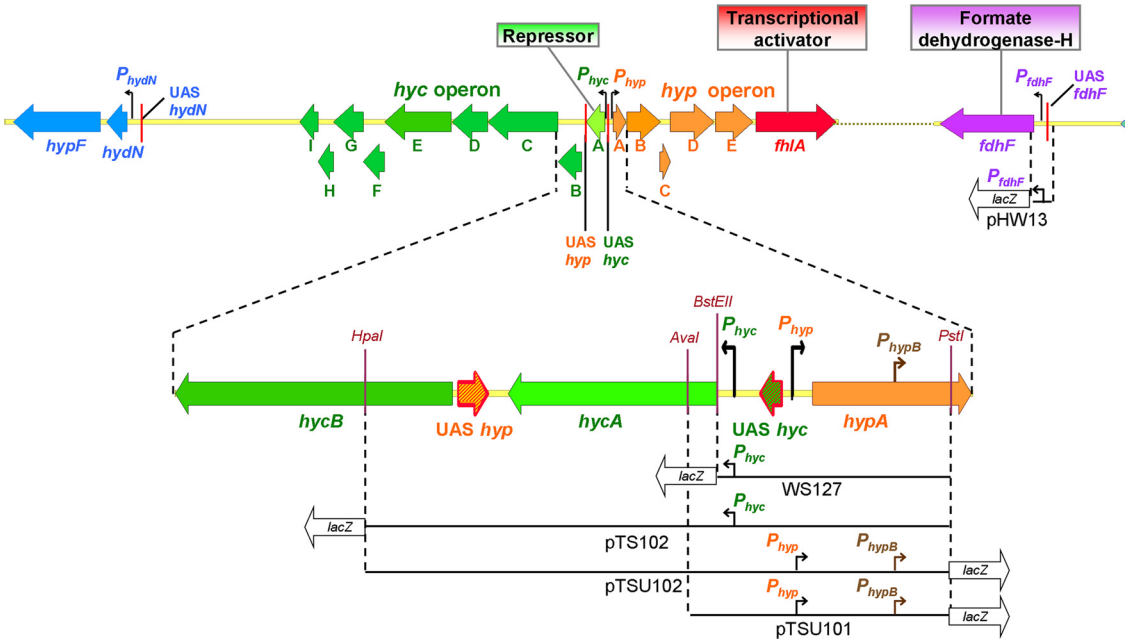

FIG. 1. Physical map of the transcriptional units activated by FhlA. Coding regions are represented by block arrows, −12/−24 promoters are indicated by black right-angled arrows, the FNR-dependent promoter P_{hypB} is indicated by brown right-angled arrows, and the UAS where FhlA binds (8, 33, 51) are shown by a red-hatched arrow and red boxes. The fragments present in the *lacZ* reporter fusions used for the transcriptional studies are indicated in black (the *lacZ* gene is not drawn to scale).

(50) (Fig. 1), is the transcriptional activator of the *fdhF* gene and the *hyc*, *hyp*, and *hydN-hypF* operons, which form the formate regulon (22). In addition to the FhlA-dependent promoter P_{hyp}, *fhlA* is also transcribed by an FNR-dependent promoter located within *hypA* and by its own weak constitutive promoter (44). Translation of FhlA is inhibited by the RNA regulator OxyS, which forms a stable antisense-target complex with *fhlA* mRNA overlapping the ribosome binding site (2). HycA, the product of the first gene of the *hyc* operon, represses *hyc* and *hyp*, possibly by binding FhlA (48).

FhlA requires formate (44) to activate transcription from −12/−24 promoters by the σ^{54}-RNA polymerase complex. FhlA with bound formate (18) binds to the upstream activating sequences (UAS) located about 100 bp upstream of the transcriptional start site of *fdhF* (8) and to *hydN-hypF* (33), in the region between the divergently transcribed *hyp* and *hyc* operons for activation of *hyc*, and in the intergenic region between *hycA* and *hycB* for the activation of *hyp* (51) (Fig. 1). Intracellular molybdate is required for transcription of *fdhF* and *hyc* (42). Also, for maximum transcription of *hyc*, the integration host factor must bind between the UAS and the promoter of the *hyc* operon (17). FhlA, as a member of the σ^{54} family, has a structure composed of three domains (35, 55). The N domain (amino acids 1 to 381) (35) is responsible for binding formate and oligomerization as a tetramer (23); it is very large, and its sequence does not show similarity to other σ^{54} regulators (50). The central domain (amino acids 388 to 617) (35) is responsible for ATP hydrolysis once formate is bound to the N domain; this reaction is essential for the formation of the open complex of RNA polymerase with DNA, which leads to transcription initiation (18). This region is not influenced by formate and is thought to interact with the RNA polymerase-σ^{54} complex (23). The C-terminal domain (amino acids 618 to 692) (35) contains a helix-turn-helix motif responsible for DNA binding (50).

Most of the previous studies to enhance *E. coli* hydrogen production have focused on metabolic engineering (36, 37, 64); for example, we achieved a 141-fold enhancement with the *hyaB*, *hybC*, *hycA*, and *fdoG* mutations coupled with overexpression of $fhlA^+$ using formate as the substrate (30), and a 4.6-fold enhancement was achieved with the *hyaB*, *hybC*, *hycA*, *fdoG*, *frdC*, *ldhA*, and *aceE* mutations using glucose as the substrate (28). In contrast, protein engineering has not been used extensively to increase hydrogen production, although we recently reported that hydrogen production may be increased 30-fold by using error-prone PCR (epPCR), DNA shuffling, and saturation mutagenesis of *hycE* (the large subunit of Hyd-3) (31). In this work, we sought to increase hydrogen production by *E. coli* through epPCR and saturation mutagenesis of *fhlA*.

MATERIALS AND METHODS

Bacterial strains, growth, and total protein. The *E. coli* strains and plasmids used in this study are listed in Table S1 in the supplemental material; all strains were grown at 37°C. Overnight cultures were made from fresh, single colonies using either Luria-Bertani medium (46), modified complex medium (30) without formate, or modified complex-formate medium with 20 mM formate. Antibiotics were used to maintain plasmids, as well as to select the host, and were used at the following concentrations: ampicillin at 100 μg/ml, chloramphenicol (Cm) at 30 μg/ml, kanamycin (Km) at 100 μg/ml, and spectinomycin at 50 μg/ml. The total protein concentration was 0.22 mg ml^{-1} (turbidity at 600 nm)$^{-1}$ (58). JW0098 (Δ*oxyS*) was constructed via P1 transduction (57) by selecting for Cm resistance that was transferred along with the *oxyS* deletion from *E. coli* K-12 GSO35 (1). For each strain from the Keio collection, the deletion of the target gene was verified by two PCRs (see Table S2 in the supplemental material). First, to determine if the wild-type allele was deleted, a PCR using a primer upstream

TABLE 1. Hydrogen production by JW2701-1 expressing the *fhlA* alleles via pCA24N after 0.5 h of anaerobic incubation in modified complex 20 mM formate medium

Strain	*fhlA* allele	FhlA amino acid change(s)	n^a	H_2 production rate (μmol mg protein^{-1} h^{-1})[e]	Relative H_2 production rate
JW2701-1(pASKA2701)	*fhlA*		24	0.8 ± 0.3	1
JW2701-1(pVSC133)	*fhlA133*[b]	Q11H, L14V, Y177F, K245R, M288K, I342F	5	7 ± 2	9
JW2701-1(pVSC14)	*fhlA14*[c]	L14G	4	3.5 ± 0.5	4
JW2701-1(pVSC1157)	*fhlA1157*[d]	M6T, S35T, L113P, S146C, E363K	4	2.9 ± 0.5	4
JW2701-1(pVSC363)	*fhlA363*[c]	E363G	5	5 ± 1	6

[a] *n*, number of independent cultures.
[b] Obtained via epPCR of whole *fhlA*.
[c] Obtained via saturation mutagenesis.
[d] Obtained via epPCR of the *fhlA* region encoding the N domain of FhlA.
[e] The values are averages ± standard deviations.

of the target gene and a primer inside the coding region of the target gene was performed. Second, to verify that the Km resistance gene was inserted at the target locus, a PCR using a primer upstream of the target gene and a primer inside the coding region of the Km resistance gene was performed. The deletions in strains JW0098 and MW1002 were also verified by PCR.

Random mutagenesis of *fhlA*. The *fhlA* gene from plasmid pASKA2701 (21) under the control of the *p*T5-*lac* promoter was mutated by epPCR as described previously (14) using 50 pmol of each primer (FhlAfront and FhlArev) (see Table S2 in the supplemental material), 0.5 mM $MnCl_2$, and a 3-min extension time. The epPCR product was cloned into pASKA2701 using the MfeI and HindIII restriction enzymes with Antarctic phosphatase (New England Biolabs, Beverly, MA) treatment of the vector; the ligation mixture was electroporated (24) into strain JW2701-1 (Δ*fhlA*) (4) (complementation of the *fhlA* deletion by pASKA2701 was reported by us previously [29]). For epPCR of the *fhlA* region encoding the N domain of FhlA, the conditions were the same as described above, but primers FhlAfront and FhlAN (see Table S2 in the supplemental material) were used with a 2-min extension time. The enzymes used for the cloning were MfeI and BsrGI.

Saturation mutagenesis. A QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to perform saturation mutagenesis of *fhlA* at all of the mutated codons of *fhlA133* (Table 1) and at the codon corresponding to E363, which is mutated in *fhlA1157* (Table 1), using pASKA2701 as a template. The DNA primers (see Table S2 in the supplemental material) contained NNS at the target codon (N is A, G, C, or T, and S is G or C) to allow the substitution of all 20 amino acids using the 32 possible codons (10); the constructed plasmids were electroporated (24) into JW2701-1, and at least 300 colonies (45) were screened for enhanced hydrogen production using chemochromic membranes.

Hydrogen screening. Chemochromic membranes (GVD Corp., Cambridge, MA) formed by a thin film of WO_3 covered with a catalytic layer of palladium, were used to detect hydrogen gas, by a colorimetric response, from colonies grown anaerobically (52). These membranes were used to identify mutants with enhanced hydrogen production due to mutations in *fhlA* generated by epPCR and saturation mutagenesis as described previously (31). Modified complex (30) agar plates containing 20 mM formate and Cm were used for screening; isopropyl β-D-1-thiogalactopyranoside (IPTG) was not added because overexpression of *fhlA* by adding IPTG is not beneficial for hydrogen production (28).

Hydrogen assay. For all of the mutants with enhanced hydrogen production that were identified with the chemochromic membranes, hydrogen production was quantified using anaerobic cells. Overnight aerobic cultures (25 ml) in modified complex medium (30) supplemented with 20 mM formate and Cm, as well as uninoculated modified complex medium supplemented with 20 mM formate and Cm, were sparged for 5 min with nitrogen to remove oxygen. Sealed crimped-top vials (27 ml) were also sparged for 2 min with nitrogen. Inside an anaerobic glove box, 9 ml of sparged uninoculated modified complex medium and 1 ml of sparged overnight culture were added to each vial. The amount of hydrogen generated in the headspace was measured after 0.5 h of anaerobic incubation at 37°C by gas chromatography using a 6890N gas chromatograph (Agilent Technologies Inc., Santa Clara, CA) as described previously (32). The work in the anaerobic glove box took about 36 min; therefore, at the time of analysis (listed as 0.5 h of incubation), the cells had been anaerobic for over 1 h.

Cloning of *fhlA* alleles. To study the impacts of the beneficial mutations on the transcription of the FhlA-controlled loci using compatible plasmids, the *fhlA*, *fhlA133*, and *fhlA363* alleles were cloned from plasmids pASKA2701, pVSC133, and pVSC363 into plasmid pVLT35 (12). The plasmids harboring the *fhlA* alleles were digested with XhoI and HindIII, and pVLT35 was digested with SalI and HindIII (New England Biolabs, Beverly, MA). The DNA fragments were ligated after the digested pVLT35 was treated with Antarctic phosphatase (New England Biolabs, Beverly, MA) and were electroporated into JW2701-1 (4).

Hydrogen production with overexpression of *hycA* and with isogenic mutants. The hydrogen production of the JW2701-1 strains harboring the pVLT35-derived plasmids pVSV2701, pVSV133, and pVSV363 (carrying the *fhlA*, *fhlA133*, and *fhlA363* alleles, respectively) with and without pASKA2695 (*hycA*$^+$) was evaluated with a hydrogen assay (*hycA* and *fhlA* were expressed constitutively). Hydrogen production by JW0833-1 (Δ*grxA*), JW0599-1 (Δ*ahpF*), and JW0098 (Δ*oxyS*) (see Table S1 in the supplemental material) was also evaluated with a hydrogen assay. At least three independent cultures of each strain were assayed.

Hydrogen uptake assay. pVSC133 and pASKA2701 were electroporated into MW1002, a strain that lacks activity of the uptake hydrogenases Hyd-1 and Hyd-2, as well as chromosomal *fhlA*. Hydrogen uptake activity by Hyd-3 was assayed in modified complex medium with 20 mM formate, as described previously (32), by measuring the increase in absorbance that results from reducing colorless, oxidized methyl viologen to a purple product ($MV^{2+} + 1/2H_2 \rightarrow MV^+ + H^+$). Two independent cultures of each strain were evaluated.

Transcription of the *fdhF* gene and *hyc* and *hyp* operons. To explore the mechanism by which the FhlA variants enhance hydrogen production, transcription of the *hyc*, *hyp*, and *fdhF* promoters was evaluated using a β-galactosidase assay in strains lacking *fhlA* in the chromosome. For the *hyc* promoter (P_{hyc}) and the *hyp* promoter (P_{hyp}), two *lacZ* fusion systems were studied: one set included the *hyc* UAS (strain WS127 [54] for P_{hyc} and pTSU101 [51] for P_{hyp}) (see Table S1 in the supplemental material), and the other set included the *hyc* UAS, *hycA*, and the *hyp* UAS (pTS102 [51] for P_{hyc} and pTSU102 [51] for P_{hyp}) (see Table S1 in the supplemental material). Thus, the transcriptional activation levels of *hyp* in the presence of one or two FhlA binding regions could be compared. pHW13 (60), which harbors a P_{fdhF}::*lacZ* fusion, was used for the *fdhF* promoter (P_{fdhF}). The DNA fragments in these *lacZ* fusion systems are shown in Fig. 1.

Plasmids pASKA2701, pVSC133, and pVSC363 (harboring the *fhlA* alleles) were electroporated into strain WS127, which lacks *fhlA* and contains the chromosomal *lacZ* reporter harboring the *hyc* UAS and P_{hyc}, whereas the *lacZ* reporter plasmids to study the P_{hyc}, P_{hyp}, and P_{fdhF} promoters were electroporated into JW2701-1 strains harboring plasmids pVSV2701, pVSV133, and pVSV363. For the β-galactosidase assay, cells were prepared in the same manner as for the hydrogen assay using appropriate antibiotics, and enzyme activity was conducted as described previously (62).

Whole-transcriptome analysis. To investigate why strains with FhlA133 produce more hydrogen, whole-transcriptome analysis was performed. JW2701-1(pVSC133) and JW2701-1(pASKA2701) were cultured as for the hydrogen assay, and total RNA was isolated with the RNeasy kit (Qiagen, Inc., Valencia, CA) as described previously (41) using a bead beater. *E. coli* GeneChip Genome 2.0 arrays (part no. 511302; Affymetrix, Inc., Santa Clara, CA) were used; they contained 10,208 probe sets for open reading frames, rRNA, tRNA, and 1,350 intergenic regions for four *E. coli* strains (MG1655, CFT073, O157:H7-Sakai, and O157:H7-EDL933). cDNA synthesis, fragmentation, end terminus biotin labeling, and hybridization were performed as described previously (15). Background values, noise values, and scaling factors for the two arrays were comparable, and the intensities of polyadenosine RNA controls were used to monitor the labeling process. For each binary microarray comparison of differential gene expression, if the gene with the higher transcription rate did not have a consistent transcription rate based on the 11 probe pairs (a detection *P* value of less than 0.05), the genes were discarded. A gene was considered differentially expressed

when the *P* value for comparing two chips was less than 0.05 (to ensure that the change in gene expression was statistically significant and that false positives arose at less than 5%) and when the expression ratio was higher (1.2-fold) than the standard deviation for all K-12 genes of the microarrays (1.2-fold) (40).

qRT-PCR. To validate the whole-transcriptome analysis data, the transcription of *grxA*, *ahpF*, *hycE*, *hypD*, *fdhF*, and *hydN* was quantified using quantitative real-time reverse transcription PCR (qRT-PCR) (5) with the RNA samples used for the whole-transcriptome analysis. The housekeeping gene *rrsG* (16S rRNA) was used to normalize the expression data. Three technical replicates were performed for each gene using the StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA) and the Power SYBR green RNA-to-C_T 1-Step Kit (Applied Biosystems, Foster City, CA). The primers for qRT-PCR are given in Table S2 in the supplemental material. The expression ratios for the genes analyzed were calculated according to the $2^{-\Delta\Delta CT}$ method (26).

Plasmid isolation, SDS-PAGE, and DNA sequencing. Plasmids were isolated using the QIAprep Spin Miniprep kit (Qiagen, Inc., Valencia, CA). The formation of recombinant proteins under the conditions used for the hydrogen assay was analyzed with standard Laemmli discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12%) (46). A dideoxy chain termination method (47) with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Wellesley, MA) was used to determine the nucleotide changes in the *fhlA* alleles; the primers used for sequencing are given in Table S2 in the supplemental material.

Microarray data accession number. The expression data for the whole-transcriptome analysis of JW2701-1(pVSC133) and JW2701-1(pASKA2701) have been deposited in the NCBI Gene Expression Omnibus (GEO) (6) and are accessible as GSE13902.

RESULTS

Isolation of mutants with enhanced H_2 production. To increase hydrogen production and to better understand the transcription activation of the genes of FHL by FhlA, epPCR was used to construct a random-mutagenesis library of *fhlA*. We screened 2,200 colonies using the chemochromic membranes in a host that lacks *fhlA* in the chromosome (JW2701-1). Using the hydrogen assay, we identified variant FhlA133, which allows JW2701-1(pVSC133) to have a ninefold-higher hydrogen production rate than JW2701-1(pASKA2701) (Table 1).

The FhlA133 variant has six amino acid changes (Q11H, L14V, Y177F, K245R, M288K, and I342F) in the N-terminal domain, which motivated us to focus on mutagenesis of *fhlA* only in the N-terminal domain (the *fhlA* region coding for the first 388 amino acids of FhlA). Thus, a second epPCR library was constructed targeting only this region, and an additional 4,400 colonies were screened from this new library. From this screening, variant FhlA1157 (expressed via pVSC1157), which has five amino acid changes (M6T, S35T, L113P, S146C, and E363K), was identified as causing a fourfold increase in hydrogen production (Table 1).

Saturation mutagenesis. To identify which amino acid replacements in FhlA133 are important for enhanced hydrogen production, saturation mutagenesis of *fhlA* was performed on each of the six mutated codons in *fhlA133* that corresponded to Q11, L14, Y177, K245, M288, and I342. For each position, at least 300 colonies were screened to ensure, with a probability of 99.99%, that all possible codons were utilized (45). Only the mutation encoding L14G (expressed in pVSC14) resulted in an increase in the hydrogen production rate (fourfold) (Table 1); therefore, position L14 of FhlA is important for controlling hydrogen production.

Saturation mutagenesis was also performed at the codon corresponding to E363 of FhlA, since the replacement E363K was identified in FhlA1157 and because the E363K amino acid replacement increases P_{hyc} transcription approximately 3-fold in the presence of 30 mM formate (34-fold without formate) and decreases the impact of formate (22). FhlA363 (E363G) was identified from the hydrogen screen with JW2701-1, and this replacement caused hydrogen production rates sixfold higher than that of the strain with FhlA.

Plasmids harboring each of four mutated *fhlA* alleles found through epPCR and saturation mutagenesis of *fhlA* (*fhlA133*, *fhlA1157*, *fhlA14*, and *fhlA363*) were isolated and reelectroporated into JW2701-1; hydrogen production was assayed to confirm that the mutations in the plasmid were responsible for the higher hydrogen production rates (Table 1). In addition, enhanced hydrogen production by the mutants harboring *fhlA133* and *fhlA363* was confirmed for a third time with the hydrogen assay after the *fhlA*, *fhlA133*, and *fhlA363* alleles were cloned into pVLT35 (data not shown). Hence, consistent data were obtained demonstrating that the beneficial mutations in *fhlA* were directly related to enhanced hydrogen production. SDS-PAGE of the cell lysates from JW2701-1 expressing the *fhlA* alleles from pASKA2701, pVSC133, pVSC1157, pVSC14, and pVSC363 indicated that the higher hydrogen production rates were not due to changes in the amount of FhlA (data not shown).

To confirm that the hydrogen produced by the strains studied came from the formate added to the medium (rather than from other medium components), we compared hydrogen production by JW2701-1(pVSC133) and JW2701-1(pASKA2701) with and without formate. Hydrogen production in the absence of formate was 0.5 ± 0.1 μmol H_2 mg protein^{-1} h^{-1} for JW2701-1(pVSC133) and 0.290 ± 0.005 μmol H_2 mg protein^{-1} h^{-1} for JW2701-1(pASKA2701). The hydrogen production of JW2701-1(pVSC133) in modified complex medium with 20 mM formate was 14- ± 5-fold higher than the hydrogen production without formate; for JW2701-1(pASKA2701), hydrogen production with 20 mM formate was 3- ± 1-fold higher than without formate. Since the amount of hydrogen produced in the medium without formate was very small relative to the amount produced with 20 mM formate, we concluded that the hydrogen produced by the strains studied came predominantly from the formate added to the medium.

Transcription of *fdhF*, the *hyc* operon, and the *hyp* operon. Since FhlA is the transcriptional activator of the genes of the FHL complex, the impacts of formate (20 mM) on the transcription of *fdhF* (which encodes formate dehydrogenase H), the *hyc* operon (which encodes the structural proteins of the FHL complex), and the *hyp* operon (which encodes maturation proteins) (Fig. 1) were evaluated by the β-galactosidase assay. With *fhlA133* expressed from pVSC133, P_{hyc} transcription in strain WS127 (including the *hyc* UAS and P_{hyc}) was increased 2.3-fold with 20 mM formate and 8-fold in the absence of formate (Fig. 2A). Using plasmid pTS102, which contains the *hyc* UAS, *hycA*, and the *hyp* UAS, P_{hyc} transcription in the strain harboring *fhlA133* was 1.7-fold higher than in the strain with *fhlA* with and without formate (Fig. 2A). Using pTSU101 (including the *hyc* UAS and P_{hyp}), transcription rates of P_{hyp} in the strains with *fhlA133* and *fhlA* were very similar (Fig. 2B); for pTSU102 (including the *hyp* UAS), P_{hyp} transcription increased 1.5-fold in the strain with *fhlA133* relative to the strain with *fhlA* (Fig. 2B). Increased transcription due to the mutations in *fhlA133* was also observed for P_{fdhF}; transcription of this promoter was 1.7-fold higher with 20 mM formate and

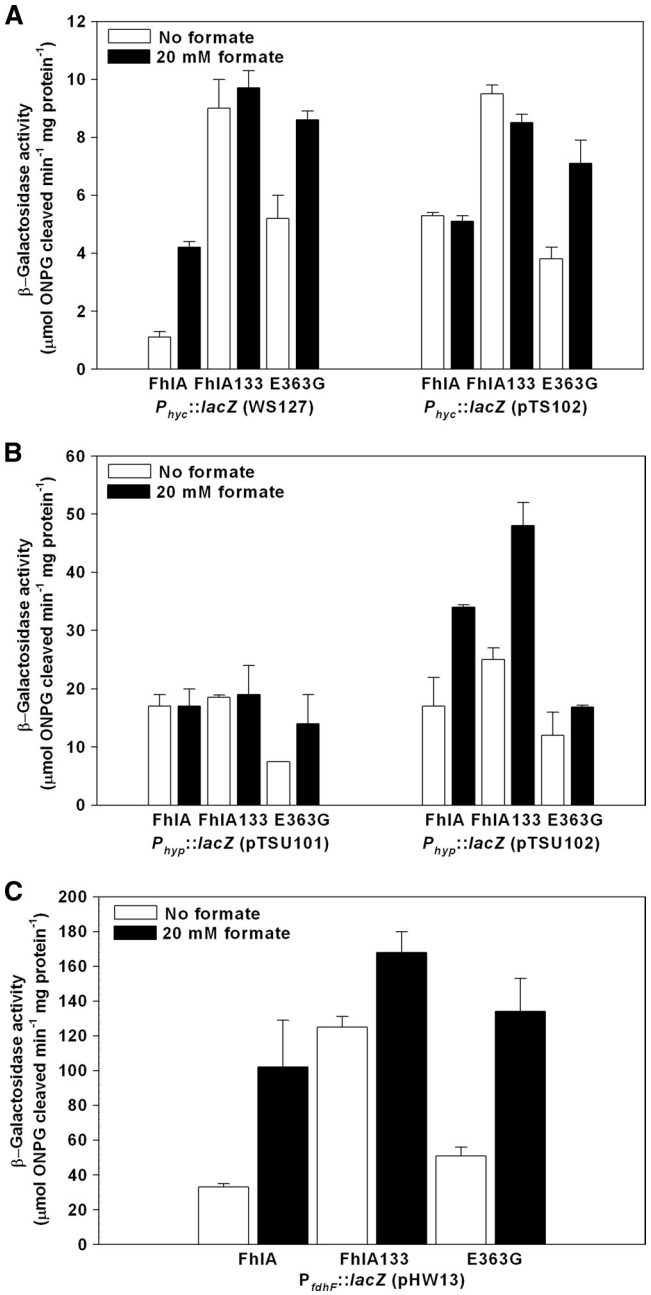

FIG. 2. Transcriptional activation of *hyc*, *hyp*, and *fdhF* by FhlA variants. Cells were cultured anaerobically in modified complex medium without formate or with 20 mM formate for 40 min. The results are the averages of two independent cultures. (A) Transcription of P_{hyc}::*lacZ* using *E. coli* WS127 (the *fhlA* alleles were expressed via pCA24N) and *E. coli* JW2701-1 (Δ*fhlA*) harboring plasmid pTS102 (the *fhlA* alleles were expressed via pVLT35). (B) Transcription of P_{hyp}::*lacZ* using plasmids pTSU101 and pTSU102 with JW2701-1 (the *fhlA* alleles were expressed via pVLT35). (C) Transcription of P_{fdhF}::*lacZ* using plasmid pHW13 with JW2701-1 (the *fhlA* alleles were expressed via pVLT35). ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

3.8-fold higher without formate (Fig. 2C). In addition, P_{hyc} and P_{fdhF} transcriptions were less affected by formate addition in the strains with *fhlA133* than in the strains with *fhlA* (Fig. 2A and C). Hence, the ninefold-higher hydrogen production attained by JW2701-1(pVSC133) appears to be due to an increase in transcription of all three promoters of *hyc*, *hyp*, and *fdhF*.

In the presence of 20 mM formate, the replacement E363G in FhlA increased P_{hyc} transcription; WS127(pVSC363) had 2.1-fold-higher P_{hyc} transcription than WS127(pASKA2701), and using the pTS102 reporter, the strain with *fhlA363* had 1.4-fold-higher P_{hyc} transcription than the wild-type strain. A higher P_{hyc} transcription rate was also observed in the absence of formate for WS127(pVSC363) (fivefold) (Fig. 2A). The transcription of P_{fdhF} was slightly higher in the strain with *fhlA363* than in the strain with *fhlA* (1.5-fold without formate and 1.3-fold with 20 mM formate) (Fig. 2C), while the transcription of P_{hyp} decreased (Fig. 2B). Therefore, the E363G replacement confers a higher transcription rate for *hyc* and *fdhF* that appears to lead to increased hydrogen production.

Whole-transcriptome analysis. To investigate why the mutant JW2701-1(pVSC133) produced more hydrogen than the wild-type strain, we performed a whole-transcriptome analysis. The amino acid changes in FhlA133 induced all four of the transcriptional units regulated by FhlA: the *hyc* operon (1.6-fold), the *hyp* operon (1.5-fold), *fdhF* (1.7-fold), and *hydN-hypF* (1.7-fold) (see Table S3 in the supplemental material). These results corroborate those found using the promoter reporters (1.7-fold for the *hyc* operon, 1.5-fold for the *hyp* operon, and 1.6-fold for *fdhF*) and demonstrate that the *hydN-hypF* operon is also induced by this protein variant.

Surprisingly, the genes of the FHL complex were not the most induced genes. Instead, the highest induction was observed for genes activated by OxyR under conditions of oxidative stress (66): *grxA* (2.8-fold), *ahpF* (2.8-fold), and *ahpC* (2.5-fold) (see Table S4 in the supplemental material). Other stress-related genes were also induced, such as the *psp* operon, which is transcribed by σ^{54}-RNA polymerase (61).

qRT-PCR. qRT-PCR was used to verify the expression of the most induced genes (*grxA* and *ahpF*) and of some genes activated by FhlA (*hycE*, *hypD*, *fdhF*, and *hydN*) (see Table S5 in the supplemental material). The differential changes in expression were comparable to those in the whole-transcriptome analysis: *grxA*, 3.0-fold versus 2.8-fold; *ahpF*, 3.9-fold versus 2.8-fold; *hycE*, 1.2-fold versus 1.5-fold; *hypD*, 1.4-fold versus 1.5-fold; *fdhF*, 1.2-fold versus 1.7-fold; and *hydN*, 1.5-fold versus 1.7-fold.

***grxA*, *ahpF*, and *oxyS* mutations and hydrogen production.** To explore whether the oxidative-stress genes induced in the whole-transcriptome analysis were related to hydrogen production, we analyzed the effects of deleting *grxA*, *ahpF*, and *oxyS* on hydrogen production by BW25113. The rate of hydrogen production by BW25113 was slightly reduced upon deletion of *grxA* (1.6- ± 0.2-fold) and *ahpF* (1.4- ± 0.4-fold); however, the *oxyS* deletion increased hydrogen production by 1.7- ± 0.4-fold.

Hydrogen uptake assay. Hydrogen uptake was assayed directly to determine if the increase in hydrogen production by JW2701-1(pVSC133) was due to a decrease in Hyd-3-mediated hydrogen uptake. MW1002 was used because it lacks *fhlA* and the large subunits of uptake hydrogenases Hyd-1 and Hyd-2; thus, only uptake by Hyd-3, a reversible enzyme capable of hydrogen uptake (29) that is activated by FhlA, is possible. There was no significant difference in the hydrogen uptake

activity of MW1002(pVSC133) (0.53 nmol min^{-1} mg $protein^{-1}$) relative to that of MW1002(pASKA2701) (0.55 nmol min^{-1} mg $protein^{-1}$); therefore, the increase in hydrogen production by JW2701-1(pVSC133) was not due to a change in hydrogen uptake.

Hydrogen production with overexpression of *hycA*. To ascertain if the mutations in *fhlA133* and *fhlA363* alter the HycA-mediated repression of genes encoding FHL (48), we evaluated the effect of *hycA* overexpression on hydrogen production. pASKA2695 (expressing *hycA*) was electroporated into JW2701-1(pVSV2701) and the derived strains harboring *fhlA133* and *fhlA363*. As expected, overexpression of *hycA* reduced hydrogen production; however, the strain with *fhlA363* was repressed less by HycA (1.4- ± 0.7-fold) than the strain with *fhlA* (3.7- ± 0.9-fold) and the strain with *fhlA133* (5- ± 1-fold). These results indicate that E363 of FhlA may be involved in the HycA-mediated inhibition of transcription of the genes encoding FHL.

DISCUSSION

Random mutagenesis of *fhlA* had been conducted previously by Korsa and Böck (22) to find FhlA variants that activate P_{hyc} transcription independently of formate; however, mutagenesis was not conducted to increase hydrogen production, as these variants were used to study the kinetics of ATP hydrolysis in the presence and absence of formate. They found that FhlA with amino acid replacements E358K and E363K activated *hyc* transcription with reduced dependence on formate, whereas E183K conferred a constitutive phenotype (22). Similarly, Self and Shanmugam (54) found several FhlA variants that activated *hyc* transcription without molybdate. Here, using direct screening for hydrogen production, we identified four mutants with increased hydrogen production obtained through epPCR and saturation mutagenesis (Table 1) of *fhlA* and discovered the importance of position L14 of FhlA.

The N-terminal domains of some σ^{54} regulators (e.g., DmpR [56] and XylR [38]) inhibit transcription activation in the absence of their corresponding effectors. FhlA-C, an N-terminally truncated FhlA protein lacking the first 378 amino acids, is active independently of formate and is not affected by the repressor HycA (23). Similarly, FhlA165, which has a deletion from amino acids 5 to 374, activates *hyc* transcription independently of formate, but unlike FhlA-C, its activity was reduced by HycA (53). FhlA-N, a C-terminally and central-domain-truncated protein lacking the last 314 amino acids, repressed transcriptional activation of the *hyc* operon by FhlA in the presence and absence of formate (23). Hence, the N domain of FhlA inhibits FhlA transcriptional activation and is influenced by formate and HycA (53).

Even though the entire *fhlA* gene was mutated here, all six amino acid replacements in FhlA133 were in the N domain. This suggests that these replacements may decrease the repressive effect of the N domain. Saturation mutagenesis at each codon affected by the mutations in *fhlA133* led only to the discovery of replacement L14G, which stimulates hydrogen production fourfold in a medium supplemented with 20 mM formate. L14 is in the region between amino acids 7 and 37 of FhlA, and Self et al. (53) showed that a truncation of this region abolishes transcriptional activation of the *hyc* operon; therefore, this region is important for hydrogen production. Since FhlA is a transcriptional activator of four loci (33, 50, 51), the increase in hydrogen production due to the mutations in *fhlA* should be related to changes in the transcription of the units activated by FhlA; therefore, we studied the transcriptional activation of the *fdhF* gene and the *hyc* and *hyp* operons by strains harboring *fhlA*, *fhlA133*, and *fhlA363*.

The results of the β-galactosidase transcription assay for strains with *fhlA* (Fig. 2) agree with the data reported previously in which formate induced an increase in P_{fdhF} transcription (60) and an increase in P_{hyp} when the *hyc* UAS and *hyp* UAS were present (using pTSU102) (51). Also, as reported by Schlensog et al. (51), P_{hyc} transcription was not induced by formate using the pTS102 reporter plasmid (Fig. 2A) and P_{hyp} transcription was not induced by formate using pTSU101. From these transcription reporter results, the replacements in FhlA133 led to increased transcription of all three of the promoters studied (P_{hyc}, P_{hyp}, and P_{fdhF}) with and without formate (Fig. 2). Moreover, transcription from P_{hyc} and P_{fdhF} in strains with FhlA133 was less dependent on formate. This is reflected in the hydrogen production rate in the absence of formate [JW2701-1(pVSC133) had 1.7- ± 0.3-fold-higher hydrogen production than JW2701-1(pASKA2701)]. Since the intracellular level of formate determines the transcription rate of the FHL genes by FhlA (44), FhlA133 may be able to activate transcription with a smaller internal concentration of formate than FhlA.

Strain WS127, which was used to measure P_{hyc} transcription, has a deletion of all of the genes of the formate regulon except *fdhF* (54). Thus, we studied P_{hyc} transcription in the absence of the repressor HycA and with only the *hyc* UAS and the *fdhF* UAS present, since the other UAS were deleted. Using this strain, replacements in FhlA133 led to an 8-fold increase in P_{hyc} transcription in the absence of formate and a 2.3-fold increase in P_{hyc} transcription in the presence of 20 mM formate.

Along with indicating that all four of the known FhlA-controlled operons are induced in JW2701-1(pVSC133) versus JW2701-1(pASKA2701), the whole-transcriptome analysis indicated that the replacements in FhlA133 also induced eight genes related to oxidative stress (see Table S4 in the supplemental material). A role for oxidative-stress proteins during anaerobic fermentations is surprising. However, removal of OxyS inhibition of FhlA translation by deleting *oxyS* from BW25113 was expected to provide a small beneficial effect on hydrogen production, and a nearly twofold increase was measured. OxyS RNA forms a stable antisense-target complex with *fhlA* mRNA by binding to a sequence overlapping the ribosome binding site and to a sequence located in the *fhlA* coding region; mutations at either site decrease the stability of the complex (2). For the JW2701-1(pASKA2701) derivatives, deletion of *oxyS* should have less impact on hydrogen production, since the ribosome binding sequence from plasmid pASKA2701 differs from the native sequence where OxyS binds to *fhlA* mRNA. In addition, for *fhlA133* and *fhlA14*, the replacement at position L14 is located in one of the OxyS binding regions (2). Another 12 stress-related genes were also induced (see Table S4 in the supplemental material), which suggests that the increased hydrogen production affects cell physiology and that increases in hydrogen production may be

facilitated by increasing the production of proteins that alleviate stress.

Among these stress-related genes is the *psp* operon; transcription of this operon, as well as that of the operons activated by FhlA, depends on σ^{54}. Among the other 16 σ^{54}-dependent promoters (39), the promoter with the highest similarity to the *psp* promoter is P_{hyc} (66.7% identity). Therefore, FhlA133 may increase transcription of the *psp* operon because of its similarity to the promoters controlling the expression of the genes of the FHL complex.

Mutagenesis in the *fhlA* region coding for the N domain of FhlA produced variant FhlA1157 with replacements M6T, S35T, L113P, S146C, and E363K. In the absence of formate, FhlAE363K has kinetic parameters (K_m and V_{max}) for ATP hydrolysis similar to those of FhlA with bound formate; therefore, mutation E363K renders FhlA less sensitive to formate (22). Here, saturation mutagenesis at position E363 produced the replacement E363G, which increased hydrogen production sixfold. E363G, like E363K, increased transcription of P_{hyc} with formate; E363G also slightly increased P_{fdhF} transcription (50% without formate and 30% with 20 mM formate).

Transcription of P_{hyp} in the presence of *fhlA363* decreased for the two systems studied, pTSU101 (*hyc* UAS) and pTSU102 (*hyc* and *hyp* UAS). Transcription of *hypBCDE* in the presence of only the *hyc* UAS (pTSU101) is due to the FNR-dependent promoter located within *hypA* (27) and is not due to the FhlA-dependent promoter P_{hyp} (Fig. 2B); this promoter did not show significant induction with formate (51). However, using pTSU101, the E363G mutation led to a twofold induction of P_{hyp} transcription by formate (Fig. 2B). Hence, the mechanism for increasing hydrogen production of strains harboring *fhlA133* is different than that of strains harboring *fhlA363*. Strains with *fhlA133* have increased transcription of all of the genes of the FHL complex and have P_{fdhF} and P_{hyc} transcription that is less sensitive to formate regulation, whereas strains harboring *fhlA363* have increased P_{hyc} and P_{fdhF} transcription and less P_{hyp} transcription and have hydrogen production that is less affected by the repressor HycA.

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