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Enhanced Hydrogen Production from Formic Acid by Formate Hydrogen Lyase-Overexpressing *Escherichia coli* Strains

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Genetic recombination of *Escherichia coli* in conjunction with process manipulation was employed to elevate the efficiency of hydrogen production in the resultant strain SR13 2 orders of magnitude above that of conventional methods. The formate hydrogen lyase (FHL)-overexpressing strain SR13 was constructed by combining FHL repressor (*hycA*) inactivation with FHL activator (*fhlA*) overexpression. Transcription of large-subunit formate dehydrogenase, *fdhF*, and large-subunit hydrogenase, *hycE*, in strain SR13 increased 6.5- and 7.0-fold, respectively, compared to the wild-type strain. On its own, this genetic modification effectively resulted in a 2.8-fold increase in hydrogen productivity of SR13 compared to the wild-type strain. Further enhancement of productivity was attained by using a novel method involving the induction of the FHL complex with high-cell-density filling of a reactor under anaerobic conditions. Continuous hydrogen production was achieved by maintaining the reactor concentration of the substrate (free formic acid) under 25 mM. An initial productivity of 23.6 g hydrogen h^{-1} $liter^{-1}$ (300 liters h^{-1} $liter^{-1}$ at 37°C) was achieved using strain SR13 at a cell density of 93 g (dry weight) cells/liter. The hydrogen productivity reported in this work has great potential for practical application.

In recent years, much attention has been paid to hydrogen as a renewable energy source as a result of the projected decrease in fossil fuel reserves on the one hand and improvements in hydrogen fuel cell technology on the other (3). A wide range of applications of hydrogen, from cars to small devices, is anticipated. The well-established method for hydrogen production in which oil or natural gas is chemically refined occurs at high temperatures and pressures. In contrast, the less well-established biological methods have the merit of obviating the production of carbon monoxide, which is extremely harmful to the electrodes of hydrogen fuel cells. In addition, biological reactions occur at ambient temperatures and pressures, thus lowering the energy requirements of the production process.

Microorganisms produce hydrogen via two main pathways: photosynthesis and fermentation. Oxygenic photosynthetic microorganisms include *Chlamydomonas reinhardtii*, while anoxygenic photosynthetic microorganisms include *Rhodobacter sphaeroides*. On the other hand, fermentation is the pathway used by facultative anaerobes, such as *Escherichia coli* and *Enterobacter* species, and by strict anaerobes, such as *Clostridium* species (6, 13, 18, 19, 25). In general, the fermentative hydrogen productivity per cell is higher than the productivity achieved by photosynthetic organisms. Biohydrogen productivities of 151.2 mg hydrogen h^{-1} $liter^{-1}$ by *Enterobacter cloacae* IIT-BT 08 and 605 mg hydrogen h^{-1} $liter^{-1}$ (7.4 liters h^{-1} $liter^{-1}$ at 25°C) by an undefined consortium of mesophilic bacteria, the highest productivities reported to date, are still not commercially effective (13, 14). For commercially viable biohydrogen production, it is necessary to overcome two main limiting factors, namely, a low hydrogen yield from raw materials and a low volumetric hydrogen production rate. In previous studies, improvements in hydrogen yield up to 11.6 mol hydrogen generated from 1 mol glucose 6-phosphate have been demonstrated (27). Low volumetric productivity was attributed to a low hydrogen production rate per cell and low cell density, which resulted from a low growth rate under anaerobic conditions (2). In order to overcome the problem, it is necessary to improve the specific hydrogen production rate by genetic modification and to increase the cell density in the reactor, where the cells would behave as an effective "catalyst." In contrast to previous studies, in which cell growth and hydrogen production were coupled, this study tackles the problem by first growing the cells and then subsequently using the biomass as a catalyst for the conversion of formate into hydrogen.

Besides glucose, several substrates for hydrogen production by fermentative microbes have been identified: the cofactors NADH and NADPH are produced in the course of degradation of sugars, such as glucose, and need to be oxidized to enable a new cycle. Similarly, ferredoxin and cytochrome, via their roles in electron transfer, or formate, which is the end point of the catabolism of many organic compounds, have been used to promote hydrogen production. Among these alternatives, formate was shown to enable the highest hydrogen productivity. Furthermore, formic acid can be derived from inexpensive materials, such as biomass (1, 20). Biological hydrogen production from formate is catalyzed by the formate hydrogen lyase (FHL) complex. The complex exists in various microbial genera, including *Enterobacter*, *Methanogenes*, and photosynthetic bacteria (7, 12, 24). Among these, the FHL complex of

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TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Genotype, description, or sequence	Reference or source
Strains		
W3110	F^- λ^- IN(*rrnD-rrnE*)1 *rph-1*	ATCC 27325
SR11	W3110 Δ*hycA*	This work
SR12	W3110/pSR301	This work
SR13	SR11/pSR301	This work
JM109	*endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) *recA1* F′[*traD*36 *proAB*$^+$ *lac1*^{q} *lacZ*ΔM15]	ATCC 53323
Plasmids		
pTH18ks1	rep-ts Km^r	8
pMV5	*sacB* $Spec^r$	26
pSTK1	pTH18ks1; *sacB*	This work
pSTK101	pSTK1; 267-bp deletion in *hycA*	This work
pHSG398	Cm^r; high-copy-number plasmid	TaKaRa
pSR201	pHSG398; *hycA*	This work
pSR202	pHSG398; 267-bp deletion in *hycA*	This work
pMW118	Amp^r; low-copy-number plasmid	Nippon Gene
pSR301	pMW118; *fhlA*	This work
Primers		
hycA-Fw	CTCTGGATCCATTTTCATCTTCGGGCGTGC	This work
hycA-Rv	CTCTGAGCTCAAAGGTCACATTTGACGGCG	This work
sacB-Fw	CTCTGCATGCAACCCATCACATATACCTGC	This work
sacB-Rv	CTCTGCATGCATCGATCCTCTAGAGTATCG	This work
fhlA-Fw	GGGGTACCTAAAATTCTAAATCTCCTATATGTTAG	This work
fhlA-Rv	CGGGATCCTGCGTCATCTCATCGATGACAA	This work
fdhF-Fw	CAACCTGGTCGTCGATAACG	This work
fdhF-Rv	CGGATCTTTCACGTACTGGT	This work
hycE-Fw	ACCACGCCTGGCAGACCAAA	This work
hycE-Rv	GGAGTGCTCAGCAGCACATC	This work
fhlART-Fw	GCGATCCATAATCTCAGTGGG	This work
fhlART-Rv	ATCGCTTTCCAGCAATCCG	This work
fdhFRT-Fw	TGAAATCGCCACCCGTATG	This work
fdhFRT-Rv	AGAAATCCGGGCACAGATGAC	This work
hycART-Fw	AGCCACACCATCGAGTATTACGTC	This work
hycART-Rv	AATCATGCCGTCAATGCTCAG	This work
hycERT-Fw	GGTAACGACGAACGCAAACTG	This work
hycERT-Rv	GCGAACCGTAATCCAACACTTAG	This work

E. coli has been the most extensively characterized at both the physiological and genetic levels.

The FHL complex of *E. coli* consists of formate dehydrogenase (FDH-H), hydrogenase (Hyd-3), and electron transfer mediators. Together, these form a membrane protein complex (4, 22, 28). Electron acceptors, like oxygen or nitrate, generally inhibit the expression of the FHL complex, whereas its biosynthesis is controlled by the concentration of formate in the cell (21). The oligoelements selenium and molybdenum are necessary at the active site of FDH-H, and nickel is necessary at the active site of Hyd-3 (5, 11). Additionally, the FHL complex can effectively function at a pH lower than 7.0 (16). The current hypothesis is that controlling these factors by cultivating cells anaerobically in the presence of formate and metal ions and in slightly acidic pH effectively induces the cellular expression of the FHL complex.

Transcription of the FHL complex is under the control of several genes, including *fhlA*, which codes for the FHL activator protein FHLA, a tetramer that binds to the upstream region of the DNA encoding the FHL complex and that promotes the transcription of the FHL complex (15, 23). Moreover, *hycA* codes for the FHL repressor protein HYCA, which binds to FHLA or to the FHLA-formate complex. The FHLA-HYCA complex seems to repress the transcription of the FHL complex (16, 22). Since *fhlA* and *hycA* control the transcription of the FHL complex, it is theoretically possible to control the specific FHL activity and the specific hydrogen production rate by manipulating these genes or their genetic controls.

In this study, we report on a process for hydrogen production that uses FHL-overexpressing strains of *E. coli* at high cell density to utilize formic acid. This novel method has the potential to be applied at medium scale for the generation of electricity, and thus to enable the construction of biofuel-powered small appliances.

MATERIALS AND METHODS

Construction of recombinant strains. The strains, plasmids, and primers used in this study are shown in Table 1. SR11, a *hycA* disruption strain, was constructed by using a modification of the method of Sauter et al. (22). pSR201 was obtained by amplifying the *hycA* gene of *E. coli* K-12 strain W3110 using primers hycA-Fw and hycA-Rv, digesting the product with BamHI and SacI, and inserting the resulting digest within the BamHI and SacI sites of pHSG398. pSR202 was constructed from pSR201 by digestion with AvaII and XmnI, followed by blunting and religating with the 8-bp EcoRI linker, GGAATTCC. pSTK1 was obtained by amplifying the *sacB* region of pMV5 using primers sacB-Fw and sacB-Rv, digesting the product with SphI, and inserting the digest into pTH18ks1. pSTK101 was constructed by inserting the BamHI- and SacI-digested

hycA-disrupted region of pSR202 within the BamHI and SacI sites of pSTK1. pSTK101 was electroporated into strain W3110, and the resulting transformant strain was cultivated in LB medium containing kanamycin (50 mg/liter) at 43°C. The recombinant strain was cultivated in minimal medium containing sucrose [KH_2PO_4, 2.0 g/liter; K_2HPO_4, 7.0 g/liter; $(NH_4)_2SO_4$, 1.0 g/liter; $MgSO_4 \cdot 7H_2O$, 0.1 g/liter; thiamine-HCl, 20 mg/liter; sucrose, 100 g/liter; agar, 15 g/liter] at 30°C for 24 h, and the *sacB* region was excised to obtain strain SR11. The *fhlA*-overexpressing strain SR12 was obtained as follows. The *fhlA* gene of W3110 was amplified using primers fhlA-Fw and fhlA-Rv. The amplified fragment was digested with KpnI and BamHI and inserted into the KpnI and BamHI sites of the low-copy-number plasmid pMW118, yielding vector pSR301. pSR301 was electroporated into the host strains W3110 and SR11 to yield the *fhlA*-overexpressing strain SR12 and the combined *hycA*-disrupted and *fhlA*-overexpressing strain SR13, respectively. All genetic manipulations were performed using *E. coli* JM109.

Northern blot analysis. *E. coli* K-12 strain W3110 and the recombinant strains (Table 1) were cultivated in defined BC medium [$(NH_4)_2HPO_4$, 10 g/liter; K_2SO_4, 2 g/liter; NaCl, 0.3 g/liter; $MgSO_4 \cdot 7H_2O$, 0.2 g/liter; $FeSO_4 \cdot 7H_2O$, 4 mg/liter; $ZnSO_4 \cdot 7H_2O$, 0.9 mg/liter; $CuSO_4 \cdot 5H_2O$, 0.4 mg/liter; $MnSO_4 \cdot 4\text{-}5H_2O$, 0.2 mg/liter; $CaCl_2 \cdot 2H_2O$, 0.8 mg/liter; $Na_2B_4O_7 \cdot 10H_2O$, 0.09 mg/liter; $(NH_4)_6Mo_7O_{24}$, 0.4 mg/liter; $(NH_4)_2Ni(SO_4)_2 \cdot 6H_2O$, 0.9 mg/liter; $Na_2SeO_3 \cdot 5H_2O$, 0.6 g/liter, pH 7.0, with 6 M HCl] supplemented with 150 mM glucose until the culture reached the exponential phase (optical density at 610 nm [OD_{610}], 0.8). Total RNA was subsequently extracted with an RNeasy Mini Kit (QIAGEN) in accordance with the manufacturer's instructions. Residual DNA was removed by using an RNase-free DNase Set (QIAGEN). Fifty micrograms of total RNA per lane was denatured at 65°C for 10 min in a final volume of 30 μl of RNA sample buffer containing 10 μl formamide, 4 μl 37% formaldehyde, and 3 μl 10× morpholinepropanesulfonic acid (MOPS) buffer. Denatured RNA samples were separated by electrophoresis on a 1% agarose gel in 1× MOPS buffer containing 2.2 M formaldehyde and then transferred to a nylon membrane (Hybond-N^+; Amersham Bioscience) with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Preparation of labeled DNA probes, hybridization, and stringent washes were performed with Gene Images Random Prime Labeling Module (Amersham Bioscience), and detection of specific transcripts on the blots was performed with Gene Images CDP-Star Detection Module (Amersham Bioscience) according to the manufacturer's instructions. DNA probes specific for the individual genes were generated by PCR with the following primer combination: fdhF-Fw and fdhF-Rv for *fdhF* and hycE-Fw and hycE-Rv for *hycE* (Table 1).

Quantitative RT-PCR. The same RNA samples used for Northern blot analysis were used for one-step quantitative reverse transcription (RT)-PCR using the QuantiTect SYBR Green RT-PCR kit (QIAGEN) according to the manufacturer's instructions and the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Fifty nanograms of total RNA per well was incubated for 30 min at 50°C for reverse transcription, heated for 15 min at 95°C for initial PCR activation, and thereafter amplified for 40 cycles, with each cycle consisting of denaturation at 95°C for 15 s, annealing at 57°C for 20 s, and extension at 60°C for 1 min. To check for any nucleic acid contamination, one negative control without template RNA was run. Individual target genes were amplified with the following primers: fhlART-Fw and fhlART-Rv for *fhlA*, fdhFRT-Fw and fdhFRT-Rv for *fdhF*, hycART-Fw and hycART-Rv for *hycA*, and hycERT-Fw and hycERT-Rv for *hycE* (Table 1).

Cultivation conditions. *E. coli* K-12 strain W3110 and the recombinant strains (Table 1) were initially cultivated aerobically at 37°C in 10 ml BC medium supplemented with 30 mM glucose and 50 mg/liter ampicillin where necessary. The 24-h cell suspension was centrifuged at 6,500 × *g* for 20 min and washed appropriately for cultivation in BC medium in an anaerobic chamber (model A; Coy). As the FHL induction phase, anaerobic cultures were carried out in an atmosphere comprising 95% nitrogen and 5% hydrogen. Cells were inoculated to an initial OD_{610} of 0.2 in an appropriate volume of BC medium supplemented with 150 mM glucose and 50 mg/liter ampicillin where necessary, maintaining the pH at 6.0 using 5 M NaOH. Following 10 h of incubation under anaerobic conditions, a sample of the cell suspension was collected by centrifugation, washed appropriately, and resuspended to an OD_{610} of 1.0 in 50 ml phosphate buffer, pH 6.5. This suspension was subsequently used to measure the specific hydrogen production rate. For the measurement of volumetric hydrogen production, cells were resuspended to an appropriate cell density in 100 ml phosphate buffer, pH 6.5, with 100 μl antifoam (Antifoam SI; Wako Chemicals).

Measurement of hydrogen production ability. Cell density during cultivation was measured spectrophotometrically (Novaspec II; Amersham Bioscience). An OD_{610} of 1.0 was equivalent to 0.41 g (dry weight) cells/liter. The specific hydrogen production rate was measured as the rate of hydrogen produced at 37°C from a stirred cell suspension at an OD_{610} of 1.0 in 50 ml phosphate buffer in the presence of 100 mM sodium formate. The volumetric hydrogen production rate was measured at 37°C by injecting 25 mM formic acid into a 100-ml cell suspension in a reactor (internal diameter [φ], 85 mm; cylindrical reactor equipped with a mixer). The concentration of hydrogen in the gaseous environment was measured by gas chromatography (GC-14B; Shimadzu), and the hydrogen production rate was detected by gas chromatography and a gas flow meter (model DPM-3; Kofloc) calibrated for the respective compound, including equimolar hydrogen and carbon dioxide.

RESULTS

Improvement of specific hydrogen production rate by genetic modification. In order to improve volumetric hydrogen production, one strategy was to enhance the specific FHL activity by genetic modification. In this study, *hycA* and *fhlA*, the genes coding for the transcriptional repressor and activator of the FHL complex, respectively, were modified in order to enhance expression of FHL enzymes. The *hycA*-disrupted strain SR11, the *fhlA*-overexpressing strain SR12, and the combined *hycA*-disrupted and *fhlA*-overexpressing strain SR13 were constructed (see Materials and Methods).

To evaluate the expression of the transcriptional level of the FHL complex, the main components of the FHL complex, formate dehydrogenase large subunit (FDH-H) and hydrogenase large subunit (Hyd-3), which are encoded by *fdhF* and *hycE*, respectively, were quantified by Northern blot analysis. The samples of total RNA were extracted at the exponential growth phase. As shown in Fig. 1A, transcription of *fdhF* and *hycE* was obviously upregulated in SR12 and dramatically upregulated in SR13. This confirmed the upregulation of some components of the FHL complex by overexpressing *fhlA*.

In order to confirm these results, quantitative RT-PCR was performed on *fhlA* and *hycA*, in addition to *fdhF* and *hycE*. The analysis did not reveal any significant difference in *fdhF* and *hycE* transcription in SR11, whereas *fdhF* and *hycE* were upregulated in SR12 and SR13, respectively (Table 2). Additionally, *fhlA* upregulation in both SR12 and SR13 was more than double the upregulation of other FHL components. However, the fact that *hycA* was upregulated only in the recombinant strain containing intact *hycA* (SR12) implied that *fhlA* and *hycA* shared a regulatory mechanism.

Since upregulation of the FHL complex was confirmed at the transcriptional level, the specific hydrogen production rate was evaluated. The specific hydrogen production rates of 10-h cultures of strains SR11, SR12, and SR13 in the presence of 100 mM sodium formate were 1.2-, 1.7- and 2.8-fold higher, respectively, than that of the wild-type strain (Fig. 1B). In SR12, the hydrogen production rate was higher than in the wild-type strain despite *hycA* being overexpressed. This was possibly due to the transcription of *fhlA* exceeding that of *hycA*. In SR13, marked improvement of the hydrogen production rate was detected. Taken together, these results suggested that the enzymes of the FHL complex were overexpressed in the recombinant strains. Moreover, due to differences in phenotype among recombinant strains, *fhlA*-overexpressing strains SR12 and SR13 exhibited clear-cut higher growth rates under anaerobic conditions, where the maximum specific growth rate (μ_{max}) was about 0.35 h^{-1}, although the growth rate of SR11 was not significantly different from that of the wild-type strain, with a μ_{max} of about 0.28 h^{-1}. As shown above, therefore, we

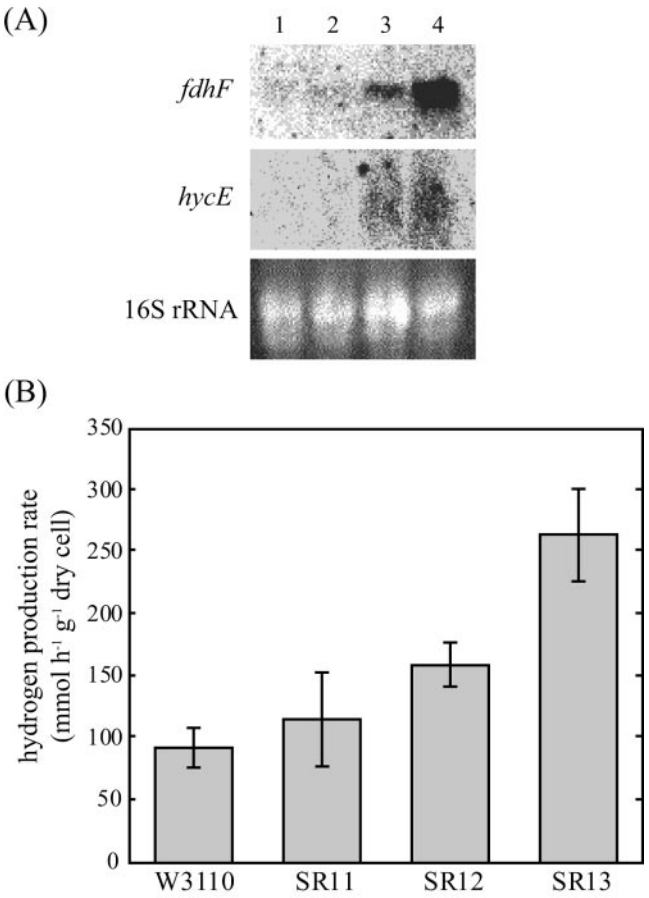

FIG. 1. FHL expression of the strains SR11, SR12, and SR13 and hydrogen productivity. (A) Northern blot of the strains (lane 1, W3110; lane 2, SR11; lane 3, SR12; lane 4, SR13) whose total RNA was extracted at exponential growth phase. Probes were assigned to formate dehydrogenase large subunit, *fdhF*, and hydrogenase large subunit, *hycE*. The images of 16S rRNA are shown as internal controls. (B) Specific hydrogen production rates of 10-h anaerobic cultures resuspended in 50 ml phosphate buffer at an OD_{610} of 1.0 in the presence of 100 mM sodium formate. The error bars indicate standard deviations.

successfully developed the highly active "catalyst" for hydrogen production by overexpressing the transcriptional activator *fhlA* and disrupting the transcriptional repressor *hycA*.

Optimization of reaction conditions for hydrogen production from formate. Apart from the improvement of the FHL activity shown above, optimization of reaction conditions was a necessary prerequisite in order to accomplish efficient hydrogen production. Changes in the specific hydrogen production rate with temperature and pH were determined by comparing the initial rates of hydrogen produced in the presence of 100 mM sodium formate at different temperature and pH values by using wild-type strain W3110 (Fig. 2). Maximum productivity was observed around 42°C, while below and above this temperature a gradual decrease in productivity was observed (Fig. 2A). At 27°C and 57°C, productivity was almost half the maximum value. Likewise, peak productivity was observed around pH 6.5, while below and above this pH, productivity gradually decreased. Hydrogen production was detected even at alkaline pH (Fig. 2B).

In addition to optimization of environmental conditions for the reactions, substrate optimization is important in order to achieve efficient and continuous hydrogen production. Therefore, the rate of hydrogen production from sodium formate and free formic acid was investigated by using wild-type strain W3110. The change in productivity occasioned by adding increasing concentrations of sodium formate to cells suspended in phosphate buffer to an OD_{610} of 1.0 was determined by measuring the initial rate of hydrogen production (Fig. 3). A maximum initial hydrogen production rate of 99 mmol h^{-1} g (dry weight) $cells^{-1}$ was obtained in the presence of 100 mM sodium formate. However, although the initial hydrogen production rate increased with increasing formate concentration, both the concentration of sodium ions and the pH increased upon conversion of formate to hydrogen and carbon dioxide (data not shown). Increased pH and/or sodium ion concentration led to a decrease in the hydrogen production rate (Fig. 2B). For this reason, sodium formate was suboptimal for continuous hydrogen production. On the other hand, when free formic acid replaced sodium formate as the substrate, an initial productivity peak of 82 mmol h^{-1} g (dry weight) $cells^{-1}$ was obtained at 25 mM formic acid concentration, and hydrogen was not formed when the formic acid concentration exceeded 50 mM (Fig. 3). The peak was attributed to the balance between the pH and the formate concentration. The pH decreased temporarily just after free formic acid was added to the cell suspension but reverted to its original value with the complete conversion of formic acid to hydrogen and carbon dioxide (data not shown). Continuous hydrogen production was thus possible when the free formic acid concentration was maintained under 25 mM. Moreover, control of the hydrogen production rate was possible by varying the formate concentration in the suspension.

Construction of a biohydrogen reactor. In order to improve volumetric hydrogen productivity, the effect of increased reactor cell density was investigated by using wild-type strain W3110 (Fig. 4). At the cell density of 74 g (dry weight) cells/liter, the reaction commenced immediately after addition of free formic acid. Approximately half of the added formic acid was degraded in 10 seconds, and 2.5 mmol formic acid was completely degraded into hydrogen and carbon dioxide in 50 s. The initial hydrogen production rate was 9.4 g hydrogen h^{-1} $liter^{-1}$. Gas chromatography measurements confirmed that 2.5 mmol hydrogen was produced from 2.5 mmol free formic acid. Thereafter, in order to evaluate the reaction in detail, the

TABLE 2. Real-time RT-PCR relative quantification of some FHL components of recombinant strains

Probe	Value[a]		
	SR11	SR12	SR13
fhlA	1.5 ± 0.6	8.5 ± 5.0	16.3 ± 9.0
fdhF	1.1 ± 0.2	1.8 ± 0.3	6.5 ± 1.2
hycE	1.1 ± 0.2	3.0 ± 0.8	7.0 ± 2.3
hycA	Not detectable	Not detectable	Not detectable

[a] The values indicated are the ratios of the amounts of transcripts relative to the wild-type strain. The values are averages of triplicate experiments.

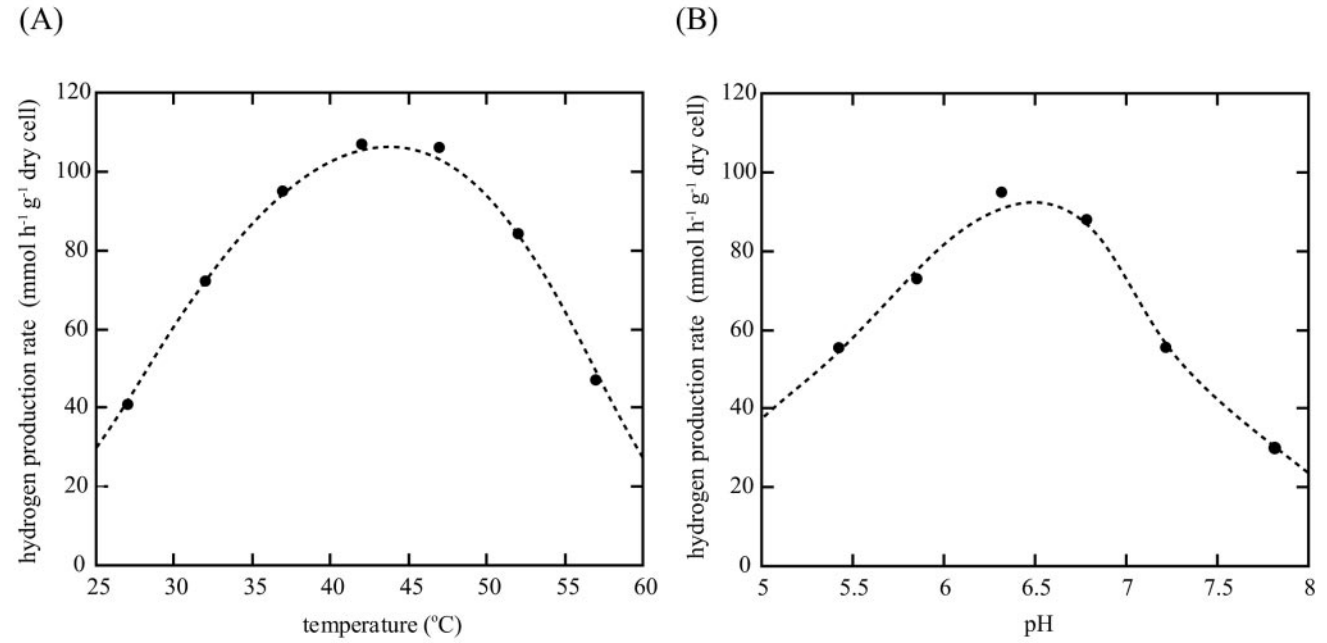

FIG. 2. Temperature and pH dependence of hydrogen production using W3110. A 50-ml cell suspension was mixed with a stirrer, and hydrogen production was initiated by adding 100 mM sodium formate to the reactor. The hydrogen production rates were plotted from the results of the initial hydrogen production rate. (A) Temperature dependence between 25°C and 60°C. (B) pH dependence between pH 5.0 and 8.0.

volumetric hydrogen production rates at various cell densities were determined. At less than 20 g (dry weight) cells/liter, the hydrogen production rate increased linearly with increasing cell density. On the other hand, above 20 g (dry weight) cells/liter, the increase in the hydrogen production rate was no longer linear. As a result, specific hydrogen productivity at 74 g (dry weight) cells/liter was about two-thirds of that at 20 g (dry weight) cells/liter (Fig. 4).

Using strain SR13, which had the highest productivity of all the recombinant strains, in a high-cell-density 100-ml reaction in the presence of 25 mM free formic acid, the relationship between cell density and productivity was determined (Fig. 4). The volumetric hydrogen productivity of both wild-type and SR13 strains improved with increasing cell density, but the specific productivity decreased. In the wild-type strain, the productivity of 65 mmol h^{-1} g (dry weight) $cells^{-1}$ at a cell

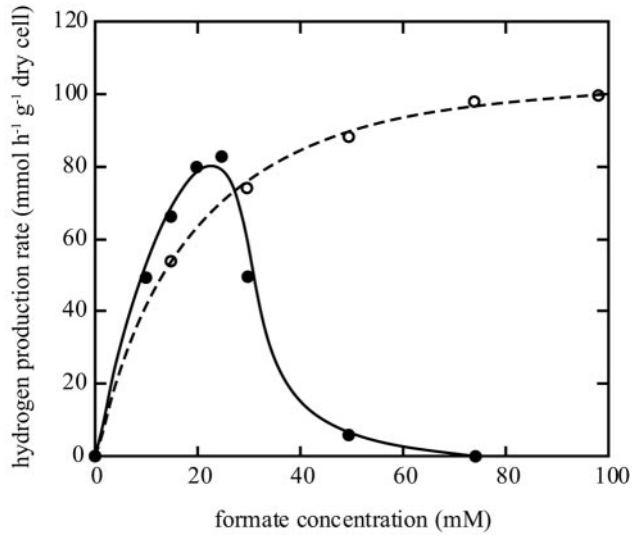

FIG. 3. Changes in initial hydrogen production rates from sodium formate (open circles) and free formic acid (closed circles) with increasing concentrations of formate using W3110. The peak observed with free formic acid was possibly due to the balance between the pH and the formate concentration.

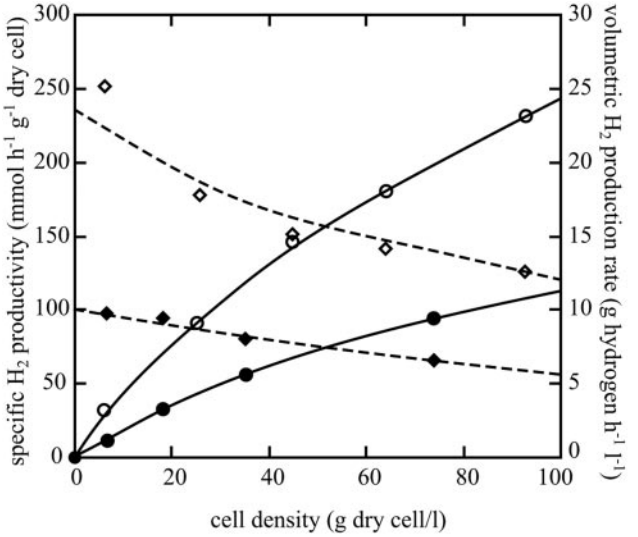

FIG. 4. Effects of cell density on the volumetric hydrogen production rates of SR13 (open circles) and W3110 (closed circles) and the specific hydrogen production rates of SR13 (open diamonds) and W3110 (closed diamonds). Each value represents the initial hydrogen production rate.

density of 74 g (dry weight) cells/liter was 67% that achieved at approximately 0.41 g (dry weight) cells/liter. On the other hand, in the recombinant strain SR13, the specific productivity of 125 mmol h^{-1} g (dry weight) $cells^{-1}$ at the high cell density of 93 g (dry weight) cells/liter was 50% that achieved under the low cell density of 6.2 g (dry weight) cells/liter. Nevertheless, the volumetric hydrogen production rate exhibited by SR13 reached 23.6 g hydrogen h^{-1} $liter^{-1}$ at a cell density of 93 g (dry weight) cells/liter, which was about 2.5-fold higher than the maximum of the wild-type strain. In addition, continuous hydrogen production was realized by maintaining the formic acid concentration under 25 mM in the reactor (data not shown), with no appreciable change in the molar yield of hydrogen from formic acid.

DISCUSSION

In this study, a new process, distinct from conventional methods, for hydrogen production by a high-cell-density reaction using *E. coli* was established. High productivity of the process was dependent upon the efficiency of cellular expression of FHL enzymes combined with the use of a high cell density for the hydrogen-producing reaction step.

A *hycA* disruption strain (SR11), an *fhlA*-overexpressing strain (SR12), and a combined *hycA* disruption and *fhlA*-overexpressing strain (SR13) were constructed in order to enhance the expression of FHL enzymes. *hycA* and *fhlA* are the repressor and the activator of the FHL complex, respectively. Augmentation of FHL activity by disrupting *hycA* or overexpressing *fhlA* has previously been reported (22, 23). In this study, the augmentation of the hydrogen productivity was evident in SR11 and SR12 (Fig. 1B). With SR13, markedly higher productivity than with SR11 and SR12 was attained. Based on the overexpression of the FHL components as determined by Northern blot analysis and real-time RT-PCR, it is possible that a synergistic effect on *hycA* and *fhlA* expression occurred. Control of *hycA* expression by FHLA due to the existence of *hycA* within the *hyc* operon has previously been demonstrated (16). Presumably, the overexpression of the *fhlA* gene resulted in a concomitant increase in the amount of the *hycA* transcripts in SR12. The view that a synergistic effect had taken place is corroborated by the observation that, in contrast to strains SR11 and SR12, the overexpressed *hyc* operon in SR13 was not under the control of the repressor HYCA. Additionally, although the transcription of the FHL complex and productivities of SR11 and SR12 were largely on the same order, transcription in SR13 was more than double its productivity. This could be ascribed to mechanistic hindrance for the assembly of the FHL enzyme complex into the membrane when overexpressed, given their membrane-associated nature (22).

SR12 and SR13 strains in which *fhlA* was overexpressed grew at higher rates than the wild-type strain under anaerobic conditions. Considering that the increased productivity of SR12 and SR13 was also due, to a large extent, to enhanced *fhlA* expression, we suggest that the observed high growth rate proceeds from the anaerobic metabolism of formate from glucose to hydrogen and carbon dioxide, which is faster when the FHL complex is overexpressed concomitantly with an increased flux from pyruvate to formate and acetyl-coenzyme A, thus leading to an increased glucose consumption rate (10). On the other hand, there was no striking difference in growth rate between SR11 and the wild-type strain, although the hydrogen production rate of SR11 was slightly higher than that of the wild-type strain. It is necessary that further genetic analysis using techniques such as transcriptome analysis be conducted in order to clarify the detailed functions of *hycA*, *fhlA*, and the FHL complex.

An initial hydrogen production rate of 23.6 g hydrogen h^{-1} $liter^{-1}$ was attained by SR13 at a cell density of 93 g (dry weight) cells/liter. The reaction started immediately after substrate addition. Continuous hydrogen production was maintained at a high rate by maintaining a constant formate concentration of 25 mM in the reaction medium. No cellular growth could be detected during the continuous reaction, though formic acid was promptly metabolized by the action of the FHL complex. A likely explanation for this observation is that growth was limited in high-cell-density culture under anaerobic conditions, and the FHL complex is a means of maintaining pH homeostasis (9, 21). It is worth noting that the higher the reactor cell density, the lower the specific hydrogen production rate (Fig. 4). This phenomenon could be explained by limited substrate dispersion in the reaction medium due to the high cell density, thereby limiting possible interactions with the FHL enzymatic complex. As a result, a possible mechanism to circumvent a decrease in the hydrogen production rate is to expand the gas-liquid interface in the reactor or to mix the suspension more effectively. It should also be noted that production of hydrogen by this approach concomitantly generates an equimolar quantity of carbon dioxide.

It was estimated that 23.9 mol/h hydrogen is necessary in order to activate a 1-kW proton exchange membrane fuel cell, where the efficiency of the fuel cell is 50%, the hydrogen consumption efficiency is 95%, and the average voltage is 0.779 V (17). Furthermore, it was calculated that a 198-liter culture is necessary to power a 1-kW fuel cell by using an undefined consortium of mesophilic bacteria, the productivity of which is 242.0 mg hydrogen h^{-1} $liter^{-1}$ (17). Moreover, we calculated that approximately 80 liters of culture is necessary by using the method of a report in which the hydrogen production rate was 605 mg hydrogen h^{-1} $liter^{-1}$ (14). Based on the initial hydrogen production rate of about 23.6 g hydrogen h^{-1} $liter^{-1}$ attained in this study, only 2 liters of cell suspension would be sufficient to provide 1 kW of electric power, assuming continuous hydrogen production could be performed at a similar rate of 23.6 g hydrogen h^{-1} $liter^{-1}$.

In conclusion, the specific hydrogen production rate was increased 2.8 times by disrupting the gene *hycA*, which represses the expression of the FHL complex, and enhancing the gene *fhlA*, which promotes the expression of the FHL complex. The initial volumetric hydrogen production rate increased up to 23.6 g hydrogen h^{-1} $liter^{-1}$ (300 liters h^{-1} $liter^{-1}$ at 37°C), which surpasses the productivity of conventional hydrogen production by 2 orders of magnitude. Formic acid was completely degraded to hydrogen and carbon dioxide, and continuous hydrogen production was achieved by maintaining the formate concentration under 25 mM.

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