Biocatalysis

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Application to Photocatalytic H_2 Production of a Whole-Cell Reaction by Recombinant *Escherichia coli* Cells Expressing [FeFe]-Hydrogenase and Maturases Genes

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Abstract: *A photocatalytic H_2 production system using an inorganic–bio hybrid photocatalyst could contribute to the efficient utilization of solar energy, but would require the development of a new approach for preparing a H_2-forming biocatalyst. In the present study, we constructed a recombinant strain of Escherichia coli expressing the genes encoding the [FeFe]-hydrogenase and relevant maturases from Clostridium acetobutylicum NBRC 13948 for use as a biocatalyst. We investigated the direct application of a whole-cell of the recombinant E. coli. The combination of TiO_2, methylviologen, and the recombinant E. coli formed H_2 under light irradiation, demonstrating that whole cells of the recombinant E. coli could be employed for photocatalytic H_2 production without any time-consuming and costly manipulations (for example, enzyme purification). This is the first report of the direct application of a whole-cell reaction of recombinant E. coli to photocatalytic H_2 production.*

Inorganic–bio hybrid photocatalysts, which consist of inorganic semiconductors in combination with a H_2-forming biocatalyst, have attracted interest for clean H_2 production system by photocatalytic water splitting. Such system would be of great value in the efficient use of solar energy for hydrogen production. One type of known H_2-forming biocatalysts, the hydrogenases, catalyzes both oxidation of H_2 into protons and reduction of protons to H_2 when combined with an electron acceptor/donor. The hydrogenase enzymes are classified into three groups according to the composition of metals in their catalytic centers: [Fe]-hydrogenase, [NiFe]-hydrogenase, and [FeFe]-hydrogenase.[1] One approach for developing inorganic–bio hybrid photocatalysts for H_2 production is to combine an inorganic semiconductor with a purified hydrogenase, for example, by combining TiO_2 with a bacterial hydrogenase[2] or with a thermophilic archaeal [NiFe]-hydrogenase.[3] More recently, visible light-driven H_2 production has been achieved with dye-sensitized TiO_2 and [NiFeSe]-hydrogenase,[4] and with mercaptopropionic acid-capped CdTe nanocrystals or CdS nanorods in combination with recombinant clostridial [FeFe]-hydrogenase.[5] These semiconductor/enzyme systems demonstrated successful photocatalytic H_2 production; however, the low yield of the natural hydrogenase isolated from the original organisms or the difficulty of purification manipulation due to the O_2-sensitivity of the enzymes generally restricts practical use of the system.

An alternative approach for photocatalytic H_2 production by an inorganic–bio hybrid photocatalyst is the use of whole cells as a biocatalyst in place of purified hydrogenases. The advantage of this approach is not only that whole cell catalyst is easier to obtain, but also that its stability is much higher than the purified enzymes. Some laboratories have achieved H_2 production by using TiO_2 and *Clostridium butylicum* cells in combination with the electron mediator methylviologen (MV^{2+}),[6] or by using Bi_2O_3 or dye-sensitized TiO_2 and a photosynthetic bacterium *Rhodopseudomonas capulata* in combination with MV^{2+}.[7] The semiconductor/MV^{2+}/bacterial cell system has the advantage that the cells that serve as biocatalysts can be easily prepared by harvesting cells from culture without the need for manipulations such as cell disruption and protein purification. However, the low growth rates of the relevant microorganisms are a disadvantage in preparing the biocatalyst. Therefore, there is a need for a biocatalyst that shows the high H_2-forming activity and can be obtained through simple manipulations; such a biocatalyst would facilitate the development of a practical semiconductor/biocatalyst system for photocatalytic H_2 production. The use of recombinant *Escherichia coli* cells as a biocatalyst is one of promising methods.

In the present study, we engineered a recombinant strain of *E. coli* expressing the genes encoding the [FeFe]-hydrogenase and relevant maturases from *Clostridium acetobutylicum* NBRC 13948 (ATCC 824) as a potential biocatalyst. We investigated the direct application of this recombinant strain for photocatalytic H_2 production (Figure 1). The clostridial [FeFe]-hydrogenase is appealing for this application. Notably, among the three classes of hydrogenases, the [FeFe]-hydrogenases show the highest turnover numbers and tend to exhibit superior rates of H_2 formation.[8] Additionally, [FeFe]-hydrogenase has been obtained in active form at good yield based on heterologous expression in *E. coli*.[8] *E. coli* is the most commonly used microorganism for genetic engineering because of its well-characterized genomic and metabolic functions and its rapid cell growth on a wide range of carbon sources. These characteristics of *E. coli* cells are an advantage for the preparation of the whole-cell biocatalyst compared to other microorganisms previously reported, such as *C. butylicum*[6] or *R. capulata*.[7] A whole-cell reaction using genetically

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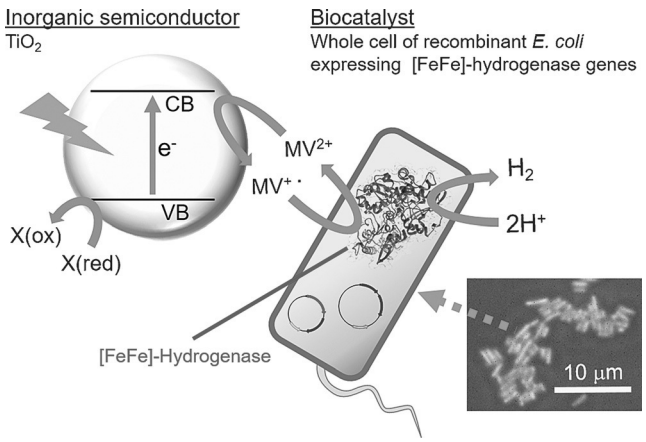

Figure 1. Photocatalytic H_2 production by the combination of TiO_2, methylviologen, and the recombinant *E. coli* expressing [FeFe]-hydrogenase and relevant maturases.

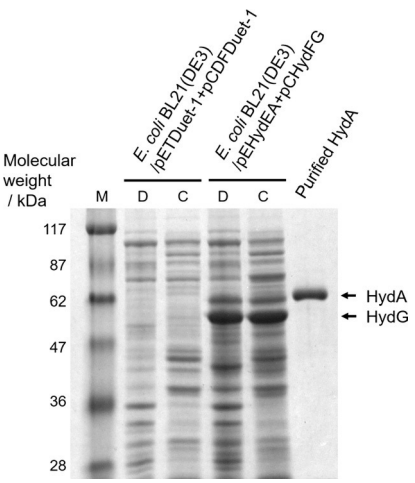

Figure 2. SDS-PAGE analysis of recombinant *E. coli* expressing *hydAEFG*. Lane M: molecular weight marker; D: cell debris; C: cell-free extract.

engineered *E. coli* cells is a promising alternative for the practical inorganic–bio hybrid photocatalyst because the cells, as biocatalyst, can be obtained through quite simple methods without time-consuming and costly manipulations (for example, cell disruption and protein purification) while avoiding the potential environmental impacts that might result from the need for growth at high temperature and pressure.

To the best of our knowledge, this is the first report of the direct application of a recombinant *E. coli* expressing the genes encoding [FeFe]-hydrogenase and the relevant maturases for photocatalytic H_2 production. To demonstrate the applicability of the recombinant *E. coli* cells for photocatalytic H_2 production, the following three experiments were performed.

First, *E. coli* BL21(DE3) cells expressing [FeFe]-hydrogenase genes were constructed. The hydrogenase- and maturase-coding genes were cloned into expression plasmids using previously reported methods[8b] with some modifications (See the Materials and Methods section in the Supporting Information). Specifically, four *C. acetobutylicum* NBRC 13948 genes encoding [FeFe]-hydrogenase (HydA, Gene ID: 1116211) and maturases (HydE, Gene ID: 1117814; HydF, Gene ID: 1117834; HydG, Gene ID: 1117539) were PCR amplified from NBRC 13948 genomic DNA. The resulting *hydA* and *hydE* fragments were cloned into expression plasmid pETDuet-1, while the resulting *hydF* and *hydG* fragments were cloned into expression plasmid pCDFDuet-1. *E. coli* BL21(DE3) was co-transformed with the resulting expression plasmids, pEHydEA and pCHydFG, respectively, to construct *E. coli* BL21(DE3)/pEHydEA + pCHydFG, which served as the experimental strain. Separately, *E. coli* BL21(DE3) was transformed with the two empty vectors to construct *E. coli* BL21(DE3)/pETDuet-1 + pCDFDuet-1, which served as a negative control strain. Gene expression and protein purification via the Strep-II tag system were carried out under anaerobic conditions according to the reported method[8a] with some modifications (see the Supporting Information). Figure 2 shows an SDS-PAGE analysis of gene expression and recombinant protein synthesis. The synthesis of recombinant HydA and HydG was clear, but HydE and HydF were not readily visualized in the cell-free extract from *E. coli* BL21-(DE3)/pEHydEA + pCHydFG. However, further experiments assessing H_2 formation activity using the purified HydA and whole cells clearly confirmed that active HydA was successfully synthesized. Furthermore, the yield of purified HydA was around 400–500 μg from a 200 mL culture, and the yield of the *E. coli* cells was 0.4–0.5 mg-wet cell from a 200 mL culture.

Second, the H_2 formation-specific activities of the purified HydA and the whole cells were investigated. Table 1 shows H_2 formation-specific activity as the rate of H_2 formation with reduced MV as an electron donor. Reaction solution (consisting of 100 mM Tris-HCl (pH 7), 150 mM NaCl, 5 mM MV^{2+}, and 25 mM sodium dithionite) was prepared under anaerobic conditions in a 10 mL glass vial sealed with an aluminum cap and rubber septum; headspace gas was replaced by N_2, and the sealed vial was pre-heated to 37 °C. The H_2 formation-specific activity of enzymatic reaction of purified HydA was 411 ± 8.9 μmol(H_2) min^{-1} (mg protein)$^{-1}$. The H_2 formation-specific activity of the whole-cell reaction of *E. coli* BL21-

Table 1: H_2-formation-specific activity of the purified HydA and the whole cells of recombinant *E. coli*.

Biocatalyst	H_2-formation-specific activity[a]	
	μmol min^{-1} (mg protein)$^{-1}$	μmol min^{-1} (mg wet cell)$^{-1}$
Purified HydA[b]	411 ± 8.9	–
E. coli BL21(DE3)/pEHydEA + pCHydFG[c]	–	0.721 ± 0.092
E. coli BL21(DE3)/pETDuet-1 + pCDFDuet-1[c]	–	not detected

[a] Standard reaction conditions: 2 mL of 100 mM Tris-HCl (pH 7), 150 mM NaCl, 5 mM MV^{2+}, 25 mM sodium dithionite, 37 °C. [b] 10 μg purified enzyme. [c] 0.02 g wet cell. The reaction rate was calculated using the amount of H_2 formed at 0, 2, and 4 min, and normalized by the amount of biocatalysts to calculate specific activity. The values represent the average of three (for [b]) or four (for [c]) experiments ± standard deviations.

(DE3)/pEHydEA + pCHydFG was 0.721 ± 0.092 μmol-(H_2) min^{-1} (mg wet cell)$^{-1}$; and no H_2 formation was detected in a reaction using *E. coli* BL21(DE3)/pETDuet-1 + pCDFDuet-1. These results revealed that the transformed *hydAEFG* conferred H_2-forming activity on *E. coli* BL21-(DE3) cells, and that the whole-cell reaction using recombinant *E. coli* BL21(DE3)/pEHydEA + pCHydFG can be used for H_2 production with reduced MV as the electron donor.

Third, to demonstrate the applicability of the recombinant *E. coli* cells in a semiconductor/biocatalyst system, photocatalytic H_2 production was carried out using purified HydA or recombinant *E. coli* cells. Figure 3a shows the amounts of

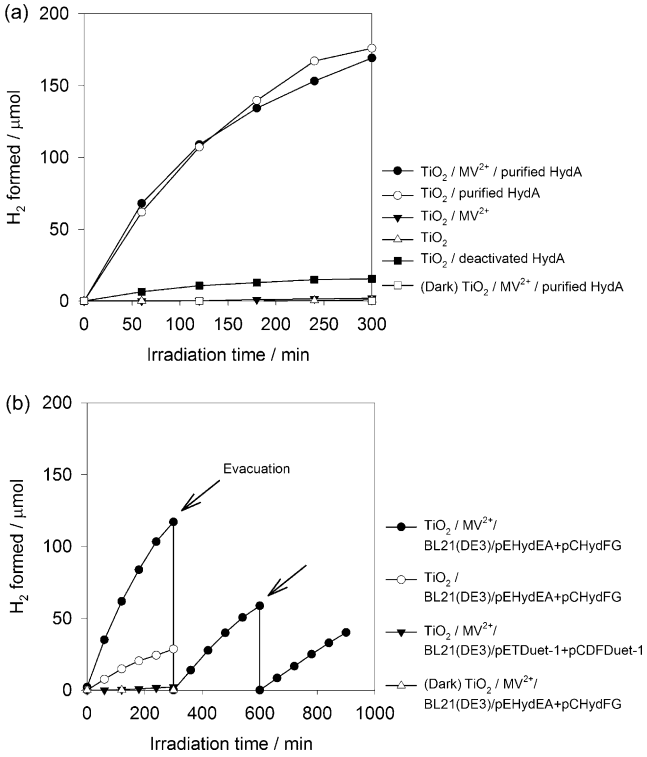

Figure 3. Amount of H_2 formed by reactions a) by the combination of TiO_2 and purified HydA and b) by the combination of TiO_2 and recombinant *E. coli* cells. (Dark) indicates the reaction without light irradiation.

H_2 formed by the semiconductor/enzyme reaction system under light irradiation conditions using 50 mg TiO_2 (anatase form) and 50 μg purified HydA (0.77 nmol) in 20 mL of reaction solution containing 100 mM Tris-HCl (pH 7), 150 mM NaCl, 5% (v/v) glycerol, and 100 mM ascorbic acid. Ascorbic acid was used as the sacrificial reagent. MV reduction by TiO_2 was confirmed under the conditions (Supporting Information, Figure S1). H_2 was formed from the reactions containing active purified HydA (TiO_2/HydA or TiO_2/MV^{2+}/HydA), whereas little or no H_2 was formed from a reaction containing deactivated (by exposure to air at 100 °C for 5 min) HydA or in a reaction lacking HydA (containing TiO_2/MV^{2+} or TiO_2 alone). Notably, H_2 formation was observed in the TiO_2/HydA reaction even in absence of MV^{2+}, indicating that electrons were being transferred directly from the conduction band of TiO_2 to HydA in this reaction system. Direct electron transfer between a TiO_2 electrode and recombinant clostridial HydA, and H_2 formation from a TiO_2/recombinant-HydA electrode with an electric potential, has been previously reported.[9] The main focus was the development of a TiO_2 bioelectrode with immobilized HydA that can be used as H_2-evolving cathode. Therefore, the objective is fundamentally different from our photocatalyst research. Our experiment is the first report of photocatalytic production of hydrogen from the combination of TiO_2 anatase powder and recombinant HydA. In the TiO_2/MV^{2+}/HydA and TiO_2/HydA system, the turnover numbers (TON, expressed in units of mol-(H_2) mol purified HydA)$^{-1}$) for 5 h of photocatalytic reaction were 222000 and 231000, respectively. Turnover frequencies (TOF, expressed in units of TON s^{-1}) for a reaction period of 60 min were 247 and 225, respectively. Furthermore, the apparent quantum yield of photocatalytic reaction using 300 nm monochromatic light irradiation in the TiO_2/MV^{2+}/HydA was 1.57% (see the Materials and Methods section in the Supporting Information).

Figure 3b shows the amount of H_2 formed by the semiconductor/recombinant *E. coli* cell reaction system using 50 mg TiO_2 and 0.1 g-wet cell of *E. coli* BL21(DE3)/pEHydEA + pCHydFG or *E. coli* BL21(DE3)/pETDuet-1 + pCDFDuet-1 in 20 mL of reaction solution. Under light irradiation conditions, H_2 was formed from reactions incorporating TiO_2/MV^{2+}/*E. coli* BL21(DE3)/pEHydEA + pCHydFG or TiO_2/*E. coli* BL21(DE3)/pEHydEA + pCHydFG, but little H_2 was formed from reactions containing TiO_2/MV^{2+}/*E. coli* BL21(DE3)/pETDuet-1 + pCDFDuet-1. The total amounts of H_2 formed in 5 h reactions were 117 μmol and 28.6 μmol with TiO_2/MV^{2+}/*E. coli* BL21(DE3)/pEHydEA + pCHydFG and TiO_2/*E. coli* BL21(DE3)/pEHydEA + pCHydFG, respectively. When the recombinant cells were used, the addition of MV^{2+} enhanced the H_2 formation rate as a redox mediator (Supporting Information, Table S1). In the case of the TiO_2/MV^{2+}/*E. coli* BL21(DE3)/pEHydEA + pCHydFG system, the rate of H_2 formation decreased compared to the initial rate, but H_2 formation continued throughout the whole examination period (15 h), with total amount for the 15 h reaction reaching 216 μmol. In comparison with the TiO_2/MV^{2+}/purified HydA system in Figure 3a, the initial rate of H_2 formation is low; however, the stability of the biocatalyst and total amount of H_2 formed will be higher for the TiO_2/MV^{2+}/*E. coli* BL21(DE3)/pEHydEA + pCHydFG. The stability is also higher when compared to the use of CdS nanorods in combination with purified HydA, capable of producing H_2 for only 4 h.[5b] The advantage of the use of whole cell in the stability of biocatalysts is then clearly shown. The apparent quantum yield of photocatalytic reaction using 300 nm monochromatic light irradiation in the system was 0.31%. This value is less than the value of TiO_2/MV^{2+}/purified HydA system. Microscopic observation and β-galactosidase assay, which is often used as a marker of cell lysis,[10] suggest that the recombinant *E. coli* cells did not lyse under photocatalytic reaction conditions (Supporting Information, Figures S2 and S3 and Results and discussion therein). Therefore, the electron-transfer steps from the reduced MV extracellularly added to intracellular HydA might be rate-limiting steps in the whole-cell system.

Generally, most polar molecules cannot cross the cell membrane easily and, indeed, MV^{2+} has been reported as an impermeant cation for *E. coli* cell membrane.[11] On the other hand, it is known that redox mediators, including MV^{2+}, can affect the intracellular NADH level and alter the NADH-relating metabolic pathways.[12] In *E. coli* cells, the extracellularly added redox mediator and the oxidoreduction potential can also affect the intracellular metabolism and the NADH/NAD^+ ratio.[11,12e,13] We confirmed that H_2 was formed from the mixture of NADH and CFE of the recombinant *E. coli* cells, whereas H_2 was not formed from the mixture of NADH and the whole cell or purified HydA (Supporting Information, Figure S4). These results suggest that the direct contact between reduced MV and HydA is not necessary for H_2 production in intracellular condition. The mechanism of the electron transfer from the reduced MV extracellularly added to the intracellular HydA remains unclear. Nevertheless, H_2 production by HydA should be possible by altering the intracellular NADH levels with the reduced MV extracellularly added.

In conclusion, our results clearly demonstrate that whole-cell recombinant *E. coli* cells expressing the genes encoding the [FeFe]-hydrogenase and maturases can serve as a H_2-forming biocatalyst as part of an inorganic-bio hybrid photocatalytic H_2 production system. This semiconductor/recombinant cell system is a promising approach for the development of a practical and clean photocatalytic H_2 production system. The application of dye-sensitized TiO_2 to our semiconductor/recombinant *E. coli* cell system is under investigation and will be reported in future work.

Experimental Section

The genomic DNA of *C. acetobutylicum* NBRC 13948 (ATCC 824) was obtained from the NITE Biological Research Center. Photocatalytic H_2 production was performed using a closed gas circulating system and an external light source (300 W Xe lamp). In the glovebox, 20 mL of reaction mixture containing 50 mg TiO_2 anatase (Wako), 20 mL of 100 mM Tris-HCl (pH 7), 150 mM NaCl, 5% glycerol, 100 mM ascorbic acid, 5 mM methylviologen, and biocatalyst (50 μg of purified HydA or 0.1 g-wet cell of recombinant *E. coli*) was prepared in a quartz reaction cell with vacuum valves to protect biocatalysts from O_2 exposure. After the quartz reaction cell was connected to the gas circulating system, the atmosphere of the system was evacuated and changed with Ar. The amount of H_2 formed was analyzed using a gas chromatograph (GC-8A, Shimadzu Corp., Japan) equipped with a column Molecular Sieve 5A (GL Sciences Inc., Japan) and an integrator C-R6A (Shimadzu Corp.). A gas sampler was directly connected to the reaction system and Ar was used as a carrier gas for GC analysis. Experimental details for plasmid construction, gene expression and protein purification, activity assay, photocatalytic H_2 production, and calculation of the quantum yields are described in the Supporting Information.

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