Expression Systems for Soluble Metal-Dependent Formate Dehydrogenase

Masaki Ihara^{1,2*}, Yusuke Kawano³, Yusuke Fujiwara¹, Tetsuya Kodo¹, Manami Mizuguchi¹, Keisuke Mochiduki¹, Kai Kodoh¹, Ayako Okabe¹, Izumi Matsuno¹

Faculty of Agriculture, Shinshu University, 8304 Minamiminowa, Nagano 399-4511, Japan,¹ JST, PRESTO, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan,² and Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan³

*Corresponding author.

Telephone/Fax: +81-265-77-0508, email address: m_ihara@shinshu-u.ac.jp

Abstract

Molybdenum or tungsten-dependent formate dehydrogenases (FDH) can reduce carbon dioxide (CO₂) to formate under ordinary conditions, and therefore, are considered promising catalysts for CO₂ fixation. However, to our knowledge, no study on the modification of metal-dependent FDHs has been published, likely because of a lack of convenient expression systems for the recombinant enzymes. We attempted to establish an methodology for the preparation and modification of soluble oxygen-tolerant metal-dependent FDHs, for the following three strategies: (1) *Escherichia coli* FDH is converted from a membrane bound protein into a soluble protein by deleting the C-terminal membrane-anchor of small subunit (FdoH) and the whole membrane subunit (FdoI) and expressed homologously in *E. coli*; (2) originally soluble FDHs from *Desulfovibrio* are expressed heterologously in *E. coli*; and (3) *Desulfovibrio* FDHs are genetically engineered by the homologous gene recombination method and expressed homologously in *Desulfovibrio*. We successfully established the expression systems for (1) and (3), and succeeded in purification of the soluble FDHs with a tag by using affinity columns.

Introduction

- 1 Formate dehydrogenase (FDH) is an enzyme that can reduce carbon dioxide (CO₂) to formate. Two classes
- 2 of FDHs have been discovered thus far: metal-independent nicotinamide adenine dinucleotide (NADH)-
- 3 based FDHs [1, 2] and metal-dependent molybdenum (Mo)-based or tungsten (W)-based FDHs [3-5]. We

had earlier created several NADPH-dependent type FDHs and combined them with photosystem I (PSI), ferredoxin, and ferredoxin-NADP⁺-reductase to establish an artificial light-driven carbon dioxide reduction system (Figure 1A). The system successfully produced formate in vitro and in vivo [6]. However, the reduction of carbon dioxide by NADPH ($\text{CO}_2 + \text{NADPH} \rightarrow \text{HCOO}^- + \text{NADP}^+$) is thermodynamically unfavorable. To create a more efficient system, it is necessary to use a metal-dependent FDH that can directly accept electrons from a low potential electron donor, such as PSI (Figure 1B).

Metal-dependent FDHs consist of a catalytic subunit and electron transfer subunits. The catalytic subunit possesses a Mo or W coordinated by a selenocysteine (SeCys) residue, two molybdopterin guanine dinucleotides (MGDs), and [4Fe-4S] clusters [7-10]. *Escherichia coli* has three metal-dependent FDHs; formate dehydrogenase H (EcFDH-H), formate dehydrogenase N (EcFDH-N) and formate dehydrogenase O (EcFDH-O). EcFDH-H is a part of the multifunctional membrane-bound protein, the formate hydrogenlyase complex, responsible for the anaerobic oxidation of formate to CO₂ and molecular hydrogen. It can be isolated as a soluble monomeric enzyme [7, 11-13]. EcFDH-N is a component of an anaerobic respiratory chain, formate-nitrate oxidoreductase, which is also a membrane-bound protein complex and catalyzes oxidation of formate coupled to nitrate respiration [14]. It was purified and crystallized as a trimeric enzyme containing a catalytic subunit (FdnG), an electron transfer subunit (FdnH), and a transmembrane subunit with type-b heme (FdnI) (Figure 2A). EcFDH-O, an isoenzyme of EcFDH-N, is a membrane-bound enzyme composed of three subunits, FdoG, FdoH, and FdoI, which exhibit high sequence similarity to the corresponding subunits of EcFDH-N. The expression of EcFDH-O is induced under micro-aerobic and aerobic conditions. It is proposed that a physiological role of EcFDH-O is to ensure rapid adaptation during a sudden shift from aerobiosis to anaerobiosis [15]. The dimer of FdoG and FdoH is directed toward the cytoplasmic side, and with FdoH anchored to the periplasmic membrane and the FdoI subunit by a C-terminal hydrophobic domain [16]. Among these three metal-dependent FDHs from *E. coli*, EcFDH-H has been well-studied due to its structural simplicity and solubility [3], but it is intractable for practical applications due to its extremely high oxygen-sensitivity.

In contrast to *E. coli* FDHs, the metal-dependent FDHs from some anaerobic bacteria, such as sulfate reducing bacteria and *Syntrophobacter fumaroxidans*, are expressed in a soluble form and are thought to transfer electrons via several types of periplasmic electron carrier proteins by forming a complex in the

periplasmic space [4, 17-19]. Their subunit structures are relatively simple, containing a catalytic subunit and one or two electron transfer subunit(s) (Figure 2B). Therefore, they are considered suitable for bioengineering applications and have been used as catalysts for CO₂/formate interconversion on electrodes [4, 20]. However, the electron transfer between the FDHs and electrodes remains to be improved. In addition, the FDHs are easily inactivated, which is a critical obstacle for practical applications. The manipulation of various properties and stability of enzymes is usually accomplished by alteration of the amino acid sequence. However, such protein engineering attempts have not been carried out because of the difficulty in gene-manipulation of anaerobic bacteria and lack of heterologous expression systems.

To overcome these setbacks with regard to metal-dependent FDHs, we tried to establish expression systems for the recombinant soluble enzymes. For our expression systems, we chose to produce EcFDH-O and metal-dependent FDHs from *Desulfovibrio vulgaris* Miyazaki F and *Desulfovibrio gigas*, since they are oxygen-tolerant and can be purified under aerobic conditions. We adopted three strategies: (1) Conversion of EcFDH-O into a soluble protein by deleting the C-terminal membrane-anchor of FdoH and the FdoI subunit and homologous expression in *E. coli*; (2) Heterologous expression of the soluble FDHs from *Desulfovibrio* in *E. coli*; and (3) Homologous expression of the modified *Desulfovibrio* FDHs in genetically engineered (by homologous gene recombination) *D. vulgaris*.

Materials and methods

Strain, Culture medium and growth conditions

All bacterial strains used in this study are listed in Table 1. *D. vulgaris* (DSM19637) and *D. gigas* (DSM1382) were purchased from Leibniz-Institut DSMZ-German Collection of Microorganisms and Cell Cultures. *E. coli* strains, BW25113 and the derivatives, JW3865 ($\Delta fdoG::Kan^R$) and JW3863 ($\Delta fdoI::Kan^R$), were obtained from *E. coli* Keio Knockout Collection.

The cultivation of *D. vulgaris* in liquid medium was performed under anaerobic conditions as described previously [21]. The liquid medium contained 0.5 g K₂HPO₄, 2.0 g MgSO₄·7H₂O, 1.0 g Na₂SO₄, 1.0 g NH₄Cl, 0.1 g CaCl₂·2H₂O, 1.0 g yeast extract, 1.0 mg sodium resazurin, 3.3 g 60% sodium lactate and 0.1 g L-ascorbic acid in 1 L water, with pH adjusted to 7.8 using NaOH. For the solid plate culture, the cell suspension and melted top agar were mixed under aerobic conditions and then quickly poured on the bottom

60 agar medium. The top agar contained 3.0 g tryptic soy broth, 7.5 g agar powder, 2.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4.2 g
61 60% sodium lactate, 0.9 g $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 0.5 g sodium resazurin, 0.2 g sodium thioglycolate and 0.2
62 g, L-ascorbic acid in 1 L water, pH adjusted to 7.8 with NaOH. The composition of bottom agar medium was
63 the same as that of the top agar, except that tryptic soy broth was excluded and 20 g/L agar power was added
64 to the medium. The plate cultures inoculated with *D. vulgaris* were incubated under strict anaerobic
65 conditions at 30°C.

66 *E. coli* was cultivated using LB broth, Lennox. In the microaerobic and aerobic cultures, cells were added to
67 Erlenmeyer flasks filled with LB medium up to 50% and 10% of the height, respectively, flasks were loosely
68 capped with aluminum foil and incubated at 30°C with shake speeds of 100 and 130 rpm, respectively.
69 Deletions in the *fdnGHI* and *fdoGHI* genes were introduced into *E. coli* BW25113 strain using temperature-
70 dependent suicide vectors, pKO3- Δ fdo and pKO3- Δ fdn, producing the BW $\Delta\Delta$ strain that harbors deletions in
71 EcFDH-O and EcFDH-N.

72 When required, ampicillin was added to a final concentration of 100 $\mu\text{g/ml}$, and chloramphenicol to a final
73 concentration of 10 or 30 $\mu\text{g/ml}$ to the cultures of *D. vulgaris* or *E. coli*, respectively.

74

75 ***Genetic engineering***

76 The cloning vectors, pUC19, pBR322 and pACYC184, were obtained from TaKaRa Bio (Otsu, Japan). For
77 genetic manipulations, FastDigest restriction enzymes (Thermo Fisher Scientific, Massachusetts, USA),
78 PrimeStar GXL DNA polymerase (TaKaRa Bio) or KOD FX DNA polymerase (TOYOBO, Osaka, Japan)
79 and In-Fusion HD Cloning kit (TaKaRa Bio) were used for the digestion, amplification and ligation of DNA
80 fragments, respectively. The extraction of genomic DNA, plasmid DNA and total RNA were carried out
81 using Genomic DNA Extraction Kit (RBC Bioscience, New Taipei City, Taiwan), Hi Yield Plasmid Mini
82 Kit (RBC Bioscience) and High Pure RNA Isolation Kit ver.12 (Roche Applied Science, Penzberg, Upper
83 Bavaria, Germany). The total RNA samples were reverse transcribed to cDNA using Rever Tra Ace[®] qPCR
84 RT Master Mix with gDNA remover (TOYOBO) and random hexamer primer. Quantitative PCR was
85 conducted using an ABI Step One (Applied Biosystems; Carlsbad, CA), with THUNDERBIRD[®] SYBR[®]
86 qPCR Mix (TOYOBO).

87

88 **Plasmid construction**

89 The primer sequences are listed in Table 2, and the plasmid structures are shown in Figure 3. A temperature
90 dependent suicide plasmid for the *fdo* gene destruction, pKO3- Δ fdo, was prepared by combining the *NotI*
91 digested pKO3 [22] with two DNA fragments containing partial sequences of *fdoG* and *fdoI*, which were
92 amplified from *E. coli* genomic DNA using the primer sets 1 and 2, respectively. A plasmid for the *fdn* gene
93 destruction, pKO3- Δ fdn, was synthesized in the same manner as pKO3- Δ fdo, using the primer sets 3 and 4.
94 pBR322 was digested with *EcoRI* and *SalI* and inserted with a fragment containing the promoter region of
95 the *fdo* operon, *fdoG*, *fdoH* and *fdoI*, amplified from *E. coli* genomic DNA with the primer set 5, generating
96 the plasmid pBR-fdoGHI, for the expression of the wild-type membrane bound EcFDH-O. The expression
97 plasmids for the solubilized EcFDH-O—pBR-fdoGH, pBR-fdoGHst and pBR-fdoGHtst, were prepared in
98 the same manner as pBR-fdoGHI, by using the primer sets 6, 7 and 8, respectively, instead of the primer set
99 5.

100 pUC-based expression plasmids were prepared as follows. The linearized pUC19 was amplified with the
101 primer set 9 by inverse PCR and ligated to a fragment amplified from pBR-fdoGHst with the primer set 10,
102 resulting in pUC-fdoGHtst. pUC-fdoGHtHis was obtained by inverse PCR using pUC-fdoGHtst as a
103 template, with the primer set 11. pUC-fdoGHtst and pUC-fdoGHtHis contain the gene coding for EcFDH-O,
104 with deletion in the membrane subunit and membrane anchor domain and tagged with Strep-tagII and
105 histidine tag at the C-terminal, respectively.

106 The plasmids for the expression of *D. vulgaris* FDHs and *D. gigas* FDH were prepared using pBR322 or
107 pUC19 and *E. coli fdo* promoter. pBR322 was digested with *EcoRI* and *SalI* and ligated to the *E. coli fdo*
108 promoter (amplified from *E. coli* genomic DNA using the primer set 12), and the *D. vulgaris* FDH genes
109 (*DvMF643* and 644) (amplified using the primer set 13), yielding pBR-DvMF643. pBR-DvMF646, pBR-
110 DvMF1217, pBR-DvMF2533, pBR-DvMF643st, pBR-DvMF646st, pBR-DvMF1217st, and pBR-
111 DvMF2533st were prepared in the same manner as pBR-DvMF643, by using primer sets 14, 15, 16, 17, 18,
112 19, and 20, respectively, instead of 13. pBR-DgFDH and pBR-DgFDHst were prepared using pBR322
113 digested with *EcoRI* and *SalI*, the *E. coli fdo* promoter, and the *D. gigas* FDH genes (*DgfdhA* and *DgfdhB*)
114 amplified using primer sets 21 and 22. pUC-DvMF1217st and pUC-DgFDHst were obtained using the

115 inverse PCR product amplified from pUC19 with the primer set 9, and the fragments amplified from pBR-
116 DvMF1217st and pBR-DgFDHst, respectively, with the primer set 23.

117 A plasmid for the chimera FDH, pUC-fdoG/DgigHst, composed of the catalytic subunit of EcFDH-O and the
118 electron transfer subunit of *D. gigas* FDH, was synthesized by combining the inverse PCR product of
119 pUC19, the fragment amplified from pBR-fdoGH, with the primer set 24 and the fragment amplified from
120 pBR-DgFDHst with the primer set 25.

121 Plasmids for the co-expression of accessory genes were prepared as follows: pACYC-FdhE645 was prepared
122 by inserting the fragment amplified from *E. coli* genomic DNA using the primer set 26 into the *Xba*I and *Sal*I
123 sites of pACYC184. pACYC-FdhE648 and pACYC-FdhDE1233 were prepared in a similar manner as
124 pACYC-FdhE645, by using the primer sets 27 and 28, respectively.

125 A homologous recombination plasmid pDvMF-cmst was constructed using *Hind*III-*Pst*I treated pUC19 and
126 four fragments containing a part of the gene for *D. vulgaris* FDH small subunit, the chloramphenicol
127 cassette, the FDH promoter, and a part of the gene for MobB. The templates and primer sets used to amplify
128 these four fragments were as follows: *D. vulgaris* genomic DNA with the primer set 29; pACYC184 with the
129 primer set 30; *D. vulgaris* genomic DNA with the primer set 31; and *D. vulgaris* genomic DNA with the
130 primer set 32. pDvMF-cmPsaE was prepared in the same manner as pDvMF-cmst, using the primer set 33 to
131 amplify the *D. vulgaris* FDH small subunit, and adding the fragment coding for PsaE gene amplified with the
132 primer set 34 from *Synechocystis* sp. PCC6803 genome. pDvMF-cmst2 was obtained by inserting a SacB
133 gene amplified with the primer set 35 into pDvMF-cmst digested with *Xba*I and *Sac*I.

134 pUC-fdoGHtst'MW and pUC-fdoGHtst'WM were obtained by Quik change method from pUC-fdoGHtst'
135 which was isolated from random library as described in ***Random mutagenesis and screening*** section.

136

137 ***In-gel visualization of enzyme activity and western blotting***

138 All steps were carried out under aerobic conditions. Cells (50 mg wet weight) were resuspended in 1 ml of
139 50 mM Tris-HCl pH 7.5 with 6 U/ml DNase, 250 µg lysozyme, 0.1% Triton X-100 and 5 mM MgCl₂, and
140 incubated for 10 min at room temperature. Non-denaturing polyacrylamide gel electrophoresis was
141 performed in 7.5% gel containing 0.1% Triton X-100 under air atmosphere at 4°C. After electrophoretic
142 separation of the proteins, the gels were incubated in 50 mM Tris-HCl pH 7.5 containing 2 mM benzyl

143 viologen (BV) and 2 mM nitroblue tetrazolium, 80 mM formate and 0.1% Triton X-100 under a nitrogen
144 atmosphere at 30°C. For western blotting, denaturing polyacrylamide gel electrophoresis was performed in
145 7.5% gel containing 0.1% sodium dodecyl sulfate under air atmosphere at room temperature. The proteins in
146 the gel were then transferred to PVDF membrane and immunostained using anti-NWSHPQFEK (Strep-tagII)
147 antibody (GenScript Inc., Piscataway, NJ) and anti-rabbit IgG antibody (Santa Cruz Biotechnology Inc.,
148 Dallas, TX).

149

150 ***Random mutagenesis and screening***

151 Random mutagenesis was performed by amplifying the DNA fragment coding for EcFdoGHt (ca. 4100 bp)
152 using the low fidelity KOD FX DNA polymerase. The DNA fragment was re-inserted into pUC plasmid, and
153 the resulting plasmids were introduced into BWΔΔ, yielding thousands of colonies. Five hundred
154 randomly selected colonies were cultured in 96-well plates, lysed, and centrifuged. The supernatants were
155 individually added to 8-strip PCR tubes containing StrepTactin-Sepharose beads. After incubation for 10 min
156 at room temperature, the beads were washed twice with a buffer containing 50 mM Tris-HCl pH 7.5 and
157 0.1% Triton X-100, and incubated in a buffer containing 50 mM Tris-HCl pH 7.5, 2 mM benzyl viologen
158 (BV) and 2 mM nitroblue tetrazolium, 80 mM formate, and 0.1% Triton X-100 under a nitrogen atmosphere
159 at 30°C.

160

161 ***Protein purification***

162 Cells (10 g wet weight) were resuspended in 50 ml of 50 mM Tris-HCl pH 7.5 with 6 U/ml DNase, 250 µg
163 lysozyme, 0.1% Triton X-100, and 5 mM MgCl₂, and incubated for 20 min at room temperature. After
164 centrifugation, the supernatant was loaded onto a His-accept (Nacalai Tesque, Kyoto, Japan) or a
165 StrepTactin-Sepharose column (3 × 2 cm, IBA GmbH, Goettingen, Germany) equilibrated with buffer A (50
166 mM Tris-HCl pH 7.5, 500 mM NaCl, and 0.1% Triton X-100). Unbound protein was washed from the
167 column with buffer A, and the protein was then eluted with buffer A containing 500 mM imidazole from the
168 His-accept column or buffer A containing 2.5 mM D-desthiobiotin from the StrepTactin-Sepharose column.
169 The fractions containing enzyme activity were dialyzed against 50 mM Tris-HCl pH 7.5 including 0.1%
170 Triton X-100, and concentrated by centrifugation at 3,500 × g in centrifugal filters (Amicon Ultra, 50 K,

171 Millipore, Eschborn, Germany). The protein samples were stored at 4°C and used within 2 days.

172

173 *Enzymatic measurement*

174 FDH activity was routinely determined spectrophotometrically by following the formate-dependent
175 reduction of BV at 30°C in glass cuvettes sealed with a rubber stopper and an aluminum cap under strictly
176 anaerobic conditions. To make the conditions strictly anaerobic, the cuvette and the assay buffer (50mM
177 Tris-HCl buffer pH 7.4 containing 10 mM BV) were flushed with a constant flow of N₂ for 20 min.
178 Reactions were started by adding the FDH sample to the cuvette and increase in absorbance of BV at 600 nm
179 ($\epsilon_{600\text{ nm}} = 8.3\text{ mM}^{-1}\text{ cm}^{-1}$) was monitored. Activity was expressed as units/mg of protein, where one unit (U) is
180 equivalent to oxidation of 1 μmol formate/min.

181

182 **Results and Discussion**

183 *Recombinant and soluble EcFDH-O in E.coli*

184 We started with a feasibility study to determine whether EcFDH-O can be expressed as a soluble protein by
185 deleting the membrane subunit, FdoI. An *E.coli* mutant with a defective *fdoI* gene (JW3863) was cultivated
186 under microaerobic and aerobic conditions, together with the wild type strain (BW25113) and *fdoG* gene-
187 defective mutant (JW3865). Their activities were assessed by the in-gel activity stain method. Although none
188 of the samples from aerobic cultures exhibited any active band (data not shown), all samples from
189 microaerobic cultures resulted in the presence of a band at the top of the separation gel (Figure 4A, Lanes 1,
190 2 and 3). The band intensity in BW25113 was remarkably stronger than that in JW3863 or JW3865. One
191 interpretation of this observation is that the strong band in BW25113 represents intact EcFDH-O and
192 EcFDH-N, and the weak band in two mutants is due to the presence of only EcFDH-N. It is also likely that
193 the intact EcFDH-O and EcFDH-N cannot go through the separation gel because of their insolubility and
194 give rise to the active bands at the top of the gel. In accordance with this hypothesis, the weak band at the top
195 of the gel disappeared in the BW $\Delta\Delta$ strain that harbors deletions in EcFDH-O and EcFDH-N (Figure 4A,
196 Lane4). Interestingly, JW3865 showed a weak band with higher mobility (~ 100 kDa) (Figure 4A, Lane2),
197 suggesting that EcFDH-O can be expressed as a soluble heterodimeric protein consisting of FdoG and FdoH
198 (EcFdoGH) in the absence of FdoI in JW3863.

199 We next examined host-vector systems with BW $\Delta\Delta$ strain as the host. The plasmids, pBR-fdoGHI, pBR-
 200 fdoGHst, pBR-fdoGHtst, and pUC-fdoGHtst were introduced in BW $\Delta\Delta$ and the resulting transformants were
 201 grown under microaerobic conditions. As shown in Lane 5 of Figure 4A, the transformant pBR-
 202 fdoGHI/BW $\Delta\Delta$ presented a strong band at the same position as BW25113, indicating that the expression
 203 system can produce active recombinant EcFDH-O at the same level as the native system. pBR-
 204 fdoGHst/BW $\Delta\Delta$ also showed a weak band similar to JW3863 (Figure 4A, Lane6), suggesting a successful
 205 production of soluble recombinant EcFdoGH protein. In pBR-fdoGHtst/BW $\Delta\Delta$ that carries the membrane-
 206 anchor truncated EcFdoGH (EcFdoGHt), no band was observed (data not shown). Protein activity was
 207 recovered in pUC-fdoGHtst/BW $\Delta\Delta$, which harbors the same gene and the same promoter as pBR-
 208 fdoGHtst/BW $\Delta\Delta$, but the different plasmid (Figure 4A, Lane7). These observations indicated that EcFdoGH
 209 is destabilized by deletion of the membrane-anchor in EcFdoGHt, but the enhanced gene expression level in
 210 pUC-fdoGHtst/BW $\Delta\Delta$ due to high plasmid copy number results in the the increased accumulation of the
 211 recombinant protein in cell. Indeed, the quantitative PCR analysis revealed that the *fdoG* transcript level in
 212 pUC-fdoGHtst/BW $\Delta\Delta$ was remarkably higher than that in BW25113 and pBR-fdoGHtst/BW $\Delta\Delta$ (Figure 5).
 213 Interestingly, the C-terminal-truncated protein, fdoGHtst, was less migrated than fdoGHst. But, it is
 214 reasonable because the C-terminal truncation results in the decrease in negative charges by 4, which would
 215 significantly affect the mobility in non-denaturing polyacrylamide gel electrophoresis.
 216 In spite of the high expression level, the activity of EcFdoGHt in pUC-fdoGHtst/BW $\Delta\Delta$ was much lower
 217 than that of EcFDH-O in BW25113, indicating a significantly low stability of EcFdoGHt. To get improved
 218 activity of EcFdoGHt, EcFdoGHt genes were subjected to random mutagenesis, and the resultant mutants
 219 (ca. 500 clones) were randomly selected and assessed in 8-strip PCR tubes (Figure 4B). This resulted in the
 220 identification of one clone expressing FDH at a significantly higher level (Figure 4A, Lane8). The plasmid
 221 isolated from the clone was designated as pUC-fdoGHtst'. DNA sequencing analysis confirmed the presence
 222 of two mutations in pUC-fdoGHtst', one in the promoter region (C $\rightarrow$ T, 55 bases upstream of the start codon)
 223 and another in the signal peptide cleavage sites (T $\rightarrow$ C, Lue32 $\rightarrow$ Pro), suggesting that they may alter the
 224 transcript level, the efficiency of the maturation processes or the protein stability. As shown in Figure 5, the
 225 *fdoG* transcript level in pUC-fdoGHtst'/BW $\Delta\Delta$ was slightly lower than that in the pUC-fdoGHtst/BW $\Delta\Delta$, but

226 it seemed to be insufficient to explain the result. To evaluate the effect of each mutation, we prepared two
 227 plasmids, one carrying the single mutation in the promoter region (pUC-fdoGHtst'MW), and the other with
 228 the mutation in the signal peptide cleavage site (pUC-fdoGHtst'WM). As shown in Figure 4C, BW $\Delta\Delta$ strain
 229 transformed with pUC-fdoGHtst'MW (pUC-fdoGHtst'MW/BW $\Delta\Delta$) exhibited a trace level of activity,
 230 whereas the pUC-fdoGHtst'WM transformant (pUC-fdoGHtst'WM/BW $\Delta\Delta$) expressed significantly higher
 231 activity, clearly demonstrating that the mutation Lue32 $\rightarrow$ Pro has a crucial effect on the expression level of
 232 active FDH. Two plausible possibilities are: (1) the mutation at the signal peptide sites may retard the
 233 transportation of EcFdoGHt to periplasm membrane and/or cleavage of the signal peptide, which helps to
 234 complete the maturation process, (2) the mutation may prevent the signal peptide cleavage, which stabilizes
 235 the protein structure.
 236 To purify the EcFdoGHt protein tagged with Strep-tagII, we applied the lysate of pUC-fdoGHtst'/BW $\Delta\Delta$ to
 237 StrepTactin-Sepharose column. However, a lot of active protein was found in the flow-through fractions
 238 (Figure 4D), raising the possibility that the Strep-tagII is not accessible for binding to the column. This may
 239 happen due to digestion of the C-terminal region, or its sequestration inside the protein. Interestingly,
 240 although the expression level of histidine-tagged FDH in pUC-fdoGHtHis'/BW $\Delta\Delta$ was almost the same as
 241 the Strep-tagII version in pUC-fdoGHtst'/BW $\Delta\Delta$ (Figure 4A, Lane 9 and 10), the purification yield of the
 242 histidine-tagged FDH was remarkably higher than the FDH with Strep-tagII (Figure 4D). The western
 243 blotting analysis demonstrated that the purified protein samples showed the expected immune bands (data
 244 not shown), but the purity and purification fold cannot be estimated because of the low amount of the
 245 purified FDH. The specific activity of partially purified EcFdoGHt with histidine tag was 0.2 U/mg.

246

247 *Desulfovibrio* FDH in *E.coli*

248 It is known that *Desulfovibrio* species express soluble metal-dependent FDHs, but isolation of proteins from
 249 these anaerobic bacteria as hosts is time consuming and labor intensive. Therefore, we tried to use the
 250 tractable host, *E.coli*, to produce *Desulfovibrio* FDHs. All heterodimeric FDH genes identified in *D. vulgaris*
 251 (*DvMF643-644*, *DvMF646-647*, *DvMF1217-1216* and *DvMF2533-2534*) and *D. gigas* (*DgfdhA* and *DgfdhB*)
 252 were cloned into pBR322 (pBR-DvMF643st, pBR-DvMF646st, pBR-DvMF1217st, pBR-DvMF2533st, and
 253 pBR-DgFDHst) together with the promoter of EcFDH-O (Figure 3). Unfortunately, BW $\Delta\Delta$ cells transformed

with these plasmids showed no significant FDH activities under anaerobic, microaerobic, and aerobic conditions. Further attempts by deletion of Strep-tagII (pBR-DvMF643, pBR-DvMF646, pBR-DvMF1217, and pBR-DvMF2533), vector exchange to pUC (pUC-DvMF1217st and pUC-DgFDHst), and swapping the catalytic subunit of *D. gigas* FDH (*DgfdhA*) with *E. coli* *fdoG* (pUC-fdoG/DgigHst) exhibited no significant effects. These results indicated that *Desulfovibrio* FDH polypeptides of s cannot be adequately processed properly by *E.coli* maturation system. Therefore, we tried the co-expression of accessory genes from *D. vulgaris* and *Desulfovibrio* FDHs using pACYC-FdhDE1233, pACYC-FdhE645, and pACYC-FdhE648. However, no FDH activity was detected, indicating that the accessory genes we tried in this study are not enough to complete the maturation process of *Desulfovibrio* FDHs.

263

264 ***Modification of Desulfovibrio FDH in Desulfovibrio***

One of the techniques for modification of the *Desulfovibrio* FDHs is gene manipulation by homologous recombination. We first optimized gene introduction method into *D. vulgaris* by electroporation and found that the highest efficiency (10–20 colonies per electroporation) was obtained by the following procedure: (1) *D. vulgaris* cells were washed twice with cold water, (2) plasmid DNA was added up to a final concentration of 20 ng/ml, (3) electroporation voltage of 1750 V was charged, (4) the electroporated cells were cured for over 12 h under an N₂ atmosphere, and (5) the cells were spread onto the selection medium containing 10 µg/ml chloramphenicol. We then used quantitative PCR to show that the FDH composed of large and small subunits coded by *DvMF1217* and *1216*, respectively, exhibited the highest expression level in four *D. vulgaris* FDHs (data not shown). Therefore, we chose C-terminus of small subunit of FDH (*DvMF1216*) as the insertion site and constructed two recombination vectors, pDvMF-cmSt and pDvMF-cmPsaE (Figure 3). pDvMF-cmSt was designed to isolate a strain producing a soluble dimeric FDH with Strep-tagII (DvFDHst) by direct marker exchange (without the isolation step for single recombinants). On the other hand, pDvMF-cmPsaE was designed to isolate a strain generating a FDH fused with PsaE, one of the subunits of PSI complex (DvFDH-PsaE) by direct marker exchange. The two plasmids were then introduced into cells by the optimized electroporation conditions, yielding 81 independent clones. From these clones, a recombinant derived from pDvMF-cmPsaE was successfully isolated, but no recombinant from pDvMF-cmSt could be found. The majority of these clones (62 of 81 clones) were single cross-over candidates, and several clones

(18 of 81 clones) were unknown chloramphenicol-tolerant strains, indicating that the simultaneous double cross-over recombination event was rather rare (1 in 81 clones).

To introduce a selection pressure for the second recombination, the sucrose dependent lethal gene, *sacB*, was inserted into pDvMF-cmSt, generating pDvMF-cmSt' (Figure 3). pDvMF-cmSt' was introduced into the *D. vulgaris* cells by electroporation, and then dozens of the single recombinant clones were isolated, in which the plasmid was incorporated into the genome. Several of these clones were grown in the presence of 10% sucrose to select for the deletion of *sacB* gene by second recombination. Of the resultant sucrose-tolerant clones, 61 clones were investigated for the genome sequence around the insertion site, revealing that only one clone possessed the insertion mutation of the Strep-tagII sequence into the designed site and others having partially deleted the *sacB* gene.

For the protein extraction from *D. vulgaris* cells, we optimized the extraction conditions. *D. vulgaris* cells are surrounded by black iron sulfide powder, which protect against cell disruptions. Although sonication treatment is usually used for the disruption of *D. vulgaris* cells, we found that the FDH activity is easily inactivated by sonication. We, therefore, employed the lysozyme-DNase treatment for the cell lysis. The supernatant containing soluble FDHs was prepared by centrifuging the lysate in the presence of 0.1% Triton X-100. The addition of Triton X-100 was not indispensable in this procedure, but had an effect to stabilize the active form of FDH, improving the yield.

The cell lysates prepared as described above were analyzed by western blotting assay. As shown in Figure 6A, a band with an expected molecular mass (~30 kDa) on the western blotting was observed in DvFDHst sample, indicating that DvFDHst was successfully produced, but no band was seen for pDvMF-cmPsaE (data not shown). The supernatants of the lysate samples were subjected to the in-gel activity assay, and showed several active bands, which is due to the presence of several FDH isozymes and makes it difficult to assess the expression of DvFDHst and DvFDH-PsaE (Figure 6B). DvFDHst and DvFDH-PsaE were isolated on StrepTactin beads from the lysate supernatants, and stained by incubating in the presence of 2 mM BV, 2 mM nitroblue tetrazolium, 80 mM formate, and 0.1% Triton X-100 under a nitrogen atmosphere at 30°C. As shown in Figure 6C, DvFDHst on StrepTactin beads was remarkably stained, whereas DvFDH-PsaE sample was not obviously stained. These results indicated that the soluble recombinant DvFDHst can be expressed

309 as an active form in the *D. vulgaris* recombinant strain, and successfully isolated from the lysate using
310 StrepTactin beads, and that the expression of DvFDH-PsaE is controversial.

311

312 **Conclusions**

313 We successfully showed that EcFdoGHt tagged with histidine tag can be expressed in *E. coli* and purified.
314 The specific activity of partially purified EcFdoGHt (0.2 U/mg) was relatively lower than that of native
315 FDH. However, we demonstrated the first example of the improvement of metal-dependent FDHs by a
316 directed evolution procedure, which will open the door to engineering studies on FDHs. The attempts to
317 express *Desulfovibrio* FDHs in *E. coli* were hampered by the lack of knowledge of the maturation processes.
318 We should study the prerequisites for the complete maturation of metal-dependent FDHs. Generation of the
319 recombinant FDH by homologous recombination was also intractable because of the difficulty in the
320 isolation of the recombinants. However, we succeeded in the preparation of the soluble DvFDH tagged with
321 Strep-tagII, and successfully isolated using StrepTactin beads. Our demonstration of the preparation of
322 tagged DvFDH is especially important in the development of FDH electrode.

323

324 **ACKNOWLEDGMENTS**

325 This research was supported by JST PRESTO “Light Energy and Chemical Conversion”.

326

327 **References**

- 328 [1] Ferry JG. Formate Dehydrogenase. *Fems Microbiol Lett.* 1990;87:377-82.
329 [2] Tishkov VI, Popov VO. Protein engineering of formate dehydrogenase. *Biomol Eng.*
330 2006;23:89-110.
331 [3] Bassegoda A, Madden C, Wakerley DW, Reisner E, Hirst J. Reversible Interconversion
332 of CO₂ and Formate by a Molybdenum-Containing Formate Dehydrogenase. *J Am Chem*
333 *Soc.* 2014;136:15473-6.
334 [4] Reda T, Plugge CM, Abram NJ, Hirst J. Reversible interconversion of carbon dioxide
335 and formate by an electroactive enzyme. *P Natl Acad Sci USA.* 2008;105:10654-8.
336 [5] Leopoldini M, Chiodo SG, Toscano M, Russo N. Reaction Mechanism of
337 Molybdoenzyme Formate Dehydrogenase. *Chem-Eur J.* 2008;14:8674-81.
338 [6] Ihara M, Kawano Y, Urano M, Okabe A. Light Driven CO₂ Fixation by Using
339 Cyanobacterial Photosystem I and NADPH-Dependent Formate Dehydrogenase. *Plos*
340 *One.* 2013;8.

341 [7] Boyington JC, Gladyshev VN, Khangulov SV, Stadtman TC, Sun PD. Crystal structure
 342 of formate dehydrogenase H: Catalysis involving Mo, molybdopterin, selenocysteine, and
 343 an Fe₄S₄ cluster. *Science*. 1997;275:1305-8.
 344 [8] Teixeira S, Dias JM, Carvalho AL, Bourenkov G, Bartunik H, Almendra MJ, et al.
 345 Crystallographic studies on a tungsten-containing formate dehydrogenase from
 346 *Desulfovibrio gigas*. *J Inorg Biochem*. 1999;74:89-.
 347 [9] Raaijmakers H, Macieira S, Dias JM, Teixeira S, Bursakov S, Huber R, et al. Gene
 348 sequence and the 1.8 angstrom crystal structure of the tungsten-containing formate
 349 dehydrogenase from *Desulfovibrio gigas*. *Structure*. 2002;10:1261-72.
 350 [10] Jormakka M, Tornroth S, Abramson J, Byrne B, Iwata S. Purification and
 351 crystallization of the respiratory complex formate dehydrogenase-N from *Escherichia coli*.
 352 *Acta Crystallogr D*. 2002;58:160-2.
 353 [11] Gladyshev VN, Khangulov SV, Axley MJ, Stadtman TC. Coordination of Selenium to
 354 Molybdenum in Formate Dehydrogenase-H from *Escherichia-Coli*. *P Natl Acad Sci USA*.
 355 1994;91:7708-11.
 356 [12] Axley MJ, Grahame DA. Kinetics for Formate Dehydrogenase of *Escherichia-Coli*
 357 Formate-Hydrogenlyase. *J Biol Chem*. 1991;266:13731-6.
 358 [13] Axley MJ, Grahame DA, Stadtman TC. *Escherichia-Coli* Formate-Hydrogen Lyase -
 359 Purification and Properties of the Selenium-Dependent Formate Dehydrogenase
 360 Component. *J Biol Chem*. 1990;265:18213-8.
 361 [14] Berg BL, Stewart V. Structural Genes for Nitrate-Inducible Formate Dehydrogenase in
 362 *Escherichia-Coli* K-12. *Genetics*. 1990;125:691-702.
 363 [15] Abaibou H, Pommier J, Benoit S, Giordano G, Mandrandberthelot MA. Expression
 364 and Characterization of the *Escherichia-Coli* Fdo Locus and a Possible Physiological-Role
 365 for Aerobic Formate Dehydrogenase. *J Bacteriol*. 1995;177:7141-9.
 366 [16] Benoit S, Abaibou H, Mandrand-Berthelot MA. Topological analysis of the aerobic
 367 membrane-bound formate dehydrogenase of *Escherichia coli*. *J Bacteriol*. 1998;180:6625-
 368 34.
 369 [17] Costa C, Teixeira M, LeGall J, Moura JJG, Moura I. Formate dehydrogenase from
 370 *Desulfovibrio desulfuricans* ATCC 27774: Isolation and spectroscopic characterization of
 371 the active sites (heme, iron-sulfur centers and molybdenum). *J Biol Inorg Chem*.
 372 1997;2:198-208.
 373 [18] Sebban C, Blanchard L, Bruschi M, Guerlesquin F. Purification and Characterization
 374 of the Formate Dehydrogenase from *Desulfovibrio-Vulgaris* Hildenborough. *Fems*
 375 *Microbiol Lett*. 1995;133:143-9.
 376 [19] Brondino CD, Passeggi MCG, Caldeira J, Almendra MJ, Feio MJ, Moura JJG, et al.
 377 Incorporation of either molybdenum or tungsten into formate dehydrogenase from
 378 *Desulfovibrio alaskensis* NCIMB 13491; EPR assignment of the proximal iron-sulfur cluster
 379 to the pterin cofactor in formate dehydrogenases from sulfate-reducing bacteria. *J Biol*
 380 *Inorg Chem*. 2004;9:145-51.
 381 [20] da Silva SM, Pimentel C, Valente FMA, Rodrigues-Pousada C, Pereira IAC. Tungsten
 382 and Molybdenum Regulation of Formate Dehydrogenase Expression in *Desulfovibrio*
 383 *vulgaris* Hildenborough. *J Bacteriol*. 2011;193:2909-16.
 384 [21] Ihara M, Nakamoto H, Kamachi T, Okura I, Maeda M. Photoinduced hydrogen
 385 production by direct electron transfer from photosystem I cross-linked with cytochrome c(3)
 386 to [NiFe]-hydrogenase. *Photochem Photobiol*. 2006;82:1677-85.
 387 [22] Link AJ, Phillips D, Church GM. Methods for generating precise deletions and
 388 insertions in the genome of wild-type *Escherichia coli*: Application to open reading frame
 389 characterization. *J Bacteriol*. 1997;179:6228-37.
 390

Table 1, Strains and Plasmids

Strains	Genotype
<i>D. gigas</i>	DSM1382
<i>D. vulgaris</i>	DSM19637
1216st	strep-tagged DvMF1217, chloramphenicol resistant cassette
1216-PsaEst	PsaE-fused DvMF1217, chloramphenicol resistant cassette
BW25113	
JW3865	BW25113 $\Delta fdoG::KanR$
JW3863	BW25113 $\Delta fdol::KanR$
BW $\Delta\Delta$	BW25113 $\Delta fdoGHI$, $\Delta fdnGHI$
Plasmids	
pKO- $\Delta fdnGHI$	<i>E. coli fdnG</i> fragment and <i>E. coli fdnI</i> fragment in pKO3
pKO- $\Delta fdoGHI$	<i>E. coli fdoG</i> fragment and <i>E. coli fdol</i> fragment in pKO3
pBR-fdoGHI	<i>E. coli fdo</i> Promoter, <i>E. coli fdoG</i> , <i>E. coli fdoH</i> and <i>E. coli fdol</i> in pBR322
pBR-fdoGH	<i>E. coli fdo</i> Promoter, <i>E. coli fdoG</i> and <i>E. coli fdoH</i> in pBR322
pBR-fdoGHst	<i>E. coli fdo</i> Promoter, <i>E. coli fdoG</i> and strep-tagged <i>E. coli fdoH</i> in pBR322
pBR-fdoGHtst	<i>E. coli fdo</i> Promoter, <i>E. coli fdoG</i> and membrane anchor-truncated and strep-tagged <i>E. coli fdoH</i> in pBR322
pUC-fdoGHtst	<i>E. coli fdo</i> Promoter, <i>E. coli fdoG</i> and membrane anchor-truncated and strep-tagged <i>E. coli fdoH</i> in pUC19
pUC-fdoGHtHis	<i>E. coli fdo</i> Promoter, <i>E. coli fdoG</i> and membrane anchor-truncated and His-tagged <i>E. coli fdoH</i> in pUC19
pBR-DvMF643	<i>E. coli fdo</i> Promoter, <i>DvMF643</i> and <i>DvMF644</i> in pBR322
pBR-DvMF646	<i>E. coli fdo</i> Promoter, <i>DvMF646</i> and <i>DvMF647</i> in pBR322
pBR-DvMF1217	<i>E. coli fdo</i> Promoter, <i>DvMF1216</i> and <i>DvMF1217</i> in pBR322
pBR-DvMF2533	<i>E. coli fdo</i> Promoter, <i>DvMF2533</i> and <i>DvMF2534</i> in pBR322
pBR-DvMF643st	<i>E. coli fdo</i> Promoter, <i>DvMF643</i> and strep-tagged <i>DvMF644</i> in pBR322
pBR-DvMF646st	<i>E. coli fdo</i> Promoter, <i>DvMF646</i> and strep-tagged <i>DvMF647</i> in pBR322
pBR-DvMF1217st	<i>E. coli fdo</i> Promoter, <i>DvMF1216</i> and strep-tagged <i>DvMF1217</i> in pBR322
pBR-DvMF2533st	<i>E. coli fdo</i> Promoter, <i>DvMF2533</i> and strep-tagged <i>DvMF2534</i> in pBR322
pBR-DgFDH	<i>E. coli fdo</i> Promoter, <i>DgfdhA</i> and <i>DgfdhB</i> in pBR322
pUC-DvMF1217st	<i>E. coli fdo</i> Promoter, <i>DvMF1216</i> and strep-tagged <i>DvMF1217</i> in pUC19
pUC-DgFDHst	<i>E. coli fdo</i> Promoter, <i>DgfdhA</i> and <i>DgfdhB</i> in pUC19
pUC-fdoGH/Dgig	<i>E. coli fdo</i> Promoter, <i>DgfdhA</i> and chemera gene of <i>E. coli fdoH</i> and <i>DgfdhB</i> in pUC19
pACYC-FdhDE1233	<i>E. coli fdo</i> Promoter, <i>DvMF1232(FdhD)</i> and <i>DvMF1233(fdhE)</i> in pACYC184
pACYC-FdhE645	<i>E. coli fdo</i> Promoter and <i>DvMF645</i> in pACYC184
pACYC-FdhE648	<i>E. coli fdo</i> Promoter and <i>DvMF648(FdhE)</i> in pACYC184
pDvMF-cmSt	<i>DvMF1215(MobB)</i> fragment, strep-tagged <i>DvMF1216</i> , <i>DvMF1217</i> fragment, chloramphenicol resistant cassette and <i>DvMF1216</i> promoter in pUC19
pDvMF-cmSt'	<i>SacB</i> in pDvMF-cmSt
pDvMF-cmPsaE	<i>DvMF1215(MobB)</i> fragment, PsaE-fused <i>DvMF1216</i> , <i>DvMF1217</i> fragment, chloramphenicol resistant cassette and <i>DvMF1216</i> promoter in pUC19

Table 2, Primer sequences

Primer set	Sequence	
1	5'-CCGGGGATCGCGGCCAAGATCTGCGCTGGCG-3'	5'-GACGACGATCGCACGGTATTCCGGAAAC-3'
2	5'-ATACCGTGCGATCGTCGTCAACGAGG-3'	5'-TAGAGGATCGCGGCCGCGCTGGTAACCCATCC-3'
3	5'-CCGGGGATCGCGGCCTGGACGTCAGTCGCAGAC-3'	5'-CCGCCACTTGGGTAGCGCAGACGGT-3'
4	5'-GCGCTACCCAAGTGGCGGATGTCGG-3'	5'-TAGAGGATCGCGGCCGCCGCTTCTGCCTTCTCG-3'
5	5'-CTTTCGTCTTCAAGAATTCGTTACAAACCCCTTCCTGG-3'	5'-CAAGGGCATCGGTCGACTGCGAATACTCATTACAGC-3'
6	5'-CTTTCGTCTTCAAGAATTCGTTACAAACCCCTTCCTGG-3'	5'-CAAGGGCATCGGTCGACTCATTTGCGCTCCTC-3'
7	5'-CTTTCGTCTTCAAGAATTCGTTACAAACCCCTTCCTGG-3'	5'-CAAGGGCATCGGTCGACTTATTTTTCGAACTGCGGGTGGCTCCAAGCAGATTGCGCTCCTCGTC-3'
8	5'-CTTTCGTCTTCAAGAATTCGTTACAAACCCCTTCCTGG-3'	5'-CAAGGGCATCGGTCGACTTATTTTTCGAACTGCGGGTGGCTCCAAGCAGATTCAGCTTCTTTTCCG-3'
9	5'-TGC GTTGCGCTCACTGCC-3'	5'-TCTGCTTGAGCCACCCGCAGTTCGAAAAATAATTAAGCAGCCCCGA-3'
10	5'-AGTGAGCGCAACGCAGTTACAAACCCCTTCCTGG-3'	5'-GTGGCTCCAAGCAGATTCCGGGTTCTCCGGC-3'
11	5'-GAATCCAGATCTCACCACCACCACCACCTAATTAAG-3'	5'-GGTGGTGAGATCTGGATTCCGGGTTCTCCGGC-3'
12	5'-CTTTCGTCTTCAAGAATTCGTTACAAACCCCTTCCTGG-3'	5'-CATGGATTGCTCCTTGTTTC-3'
13	5'-CAAGGAGCAATCCATGCGAAGACGCCAA-3'	5'-CAAGGGCATCGGTCGACCTACATGAAACCGGCG-3'
14	5'-CAAGGAGCAATCCATGAACACTACGCGGC-3'	5'-CAAGGGCATCGGTCGACTTACACGCCCGCCGC-3'
15	5'-CAAGGAGCAATCCATGACTGTCAATCGCAGG-3'	5'-CAAGGGCATCGGTCGACCTACGCGCGCGCCCG-3'
16	5'-CAAGGAGCAATCCATGAAAATGAACCGGC-3'	5'-CAAGGGCATCGGTCGACCTACCCCGTGCGCAG-3'
17	5'-CAAGGAGCAATCCATGCGAAGACGCCAA-3'	5'-CAAGGGCATCGGTCGACTTATTTTTCGAACTGCGGGTGCTCCAAGCAGACACGCCCGCCGCGTA-3'
18	5'-CAAGGAGCAATCCATGAACACTACGCGGC-3'	5'-CAAGGGCATCGGTCGACTTATTTTTCGAACTGCGGGTGGCTCCAAGCAGACATGAAACCGGCGTA-3'
19	5'-CAAGGAGCAATCCATGACTGTCAATCGCAGG-3'	5'-CAAGGGCATCGGTCGACTTATTTTTCGAACTGCGGGTGGCTCCAAGCAGACGCGCGCGCCCGGAA-3'
20	5'-CAAGGAGCAATCCATGAAAATGAACCGGC-3')	5'-CAAGGGCATCGGTCGACTTATTTTTCGAACTGCGGGTGGCTCCAAGCAGACCCCGTGCGCAGCGG-3'
21	5'-CAAGGAGCAATCCATGCTGATCAAACGTCG-3'	5'-CAAGGGCATCGGTCGACNGCCACNGCGTATTCG-3'
22	5'-CAAGGAGCAATCCATGCTGATCAAACGTCG-3'	5'-CAAGGGCATCGGTCGACTTATTTTTCGAACTGCGGGTGGCTCCAAGCAGACNGCCACNGCGTATTCG-3'
23	5'-AGTGAGCGCAACGCAGTTACAAACCCCTTCCTGG-3'	5'-GCGGGTGGCTCCAAGCAGA-3'
24	5'-AGTGAGCGCAACGCAGTTACAAACCCCTTCCTGG-3'	5'-ATGTCGTCTCCGTTACACCTTTTCC-3'
25	5'-TAACGGAGACGACATATGTCAAAGGCTTCTTCG-3'	5'-GCGGGTGGCTCCAAGCAGA-3'
26	5'-GAAACAAGGAGCAATCCATGCCCGACGCTGCC-3'	5'-GTTATTGGTGCGTCGACTCAGGCTGAGGTTTTTGCG-3'
27	5'-GAAACAAGGAGCAATCCATGGCCGCCAAACACC-3'	5'-GTTATTGGTGCGTCGACTCAATACATATTCCAGAAGGAA GG-3'
28	5'-GAAACAAGGAGCAATCCATGACCTTCGATTCGACC-3'	5'-GTTATTGGTGCGTCGACTCATTCCAGTACCCTGCC-3'
29	5'-TGATTACGCCAAGCTATGGGAAAGATGTTCTTCATC-3'	5'-CTGCGGGTGGCTCCACACCGCGTGCTTGAAGTAG-3'
30	5'-TGGAGCCACCCGCAGTTCGAAAAATAGCGCTGATGTCCGGCG-3'	5'-TTACGCCCCGCCCTGCCACTC-3'
31	5'-CAGGGCGGGGCGTAAGAGAGTAATGCTGTCGTG-3'	5'-GGTAGTCCCCTTTCCTG-3'
32	5'-GGAAAGGGGACTACCCGTAGAACCGCATTGCAAC-3'	5'-CCTCTAGAGTCGACCCTACTCCGAAGGCGG-3'
33	5'-TGATTACGCCAAGCTATGGGAAAGATGTTCTTCATC-3'	5'-ACCTCCAGAACCTCCCACCGCGTGCTTGAAGTAG-3'
34	5'-GGAGGTTCTGGAGGTGTACAACGTG-3'	5'-CTGCGGGTGGCTCCAGGATTACCTTTTTTGGGGGG-3'
35	5'-TAGGGTCGACTCTAGCTGCCGTTCACTATTATTAG-3'	5'-CGGCCAGTGAATTCGTTATTTGTTAACTGTTAATTG-3'

Figure 1. (A) The artificial light-driven carbon dioxide reduction system using NADPH-dependent FDH and photo-electron relay system consisting of plastocyanin (Pc), PSI, ferredoxin (Fd), ferredoxin-NADP⁺ reductase (FNR) and NADP(H). (B) An example of direct light-driven carbon dioxide reduction using metal-dependent FDH and PSI.

Figure 2. (A) Crystal structure of *E. coli* FDH-N (Jormakka, Tornroth et al. 2002). FDH-O exhibits high homology with FDH-N, and they are thought to have essentially the same structure. *E. coli* FDH-N and FDH-O contain a catalytic subunit, an electron transfer subunit and a transmembrane subunit. *E. coli* FDHs are tethered to membrane via the C-terminal domain of the electron transfer subunit and the transmembrane subunit. (B) Crystal structure of *D. gigas* FDH (Teixeira, Dias et al. 1999). *D. gigas* FDH is composed of a catalytic subunit and an electron transfer subunit.

Figure 3. Schematic presentation of plasmid constructs. Open squares represent genes or gene fragments. The orientation of *fdo* promoter and *Dv1216 promoter* are given by arrows. Strep-tagII and histidine tag are marked by closed squares. The asterisks indicate two mutation sites, 55 bases upstream of the start codon (C→T) and the signal peptide cleavage sites (T→C, Lue32→Pro).

Figure 4. (A) In-gel activity stain analysis. The lysates (10 μ l of 40 mg wet cell pellet/ml in each well) from BW25113 (Lane 1), JW3863 (Lane 2), JW3865 (Lane 3), BW $\Delta\Delta$ (Lane 4), pBR-fdoGHI/BW $\Delta\Delta$ (Lane 5), pBR-fdoGHst/BW $\Delta\Delta$ (Lane 6), pUC-fdoGHtst/BW $\Delta\Delta$ (Lane 7), pUC-fdoGHtst'/BW $\Delta\Delta$ (Lane 8 and 9) and pUC-fdoGHtHis'/BW $\Delta\Delta$ (Lane 10) were electrophoresed in a non-denaturing polyacrylamide gel and stained by incubating in the presence of 2 mM BV, 2 mM Nitroblue tetrazolium, 80 mM formate, and 0.1% Triton X-100 under a nitrogen atmosphere at 30°C. Only a 75 kD band in molecular weight markers was identified. Pictures of Lanes 1–8 and Lane 9 and 10 were obtained from two independent experiments. (B) Screening for the highly active clones on StrepTactin-bead in 8-strip PCR tubes. Circles indicate the candidates with highly active clones. (C) In-gel activity stain analysis. The lysates from pUC-fdoGHtst'/BW $\Delta\Delta$ (Lane 1), pUC-fdoGHtst'MW/BW $\Delta\Delta$ (Lane 2) and pUC-fdoGHtst'WM/BW $\Delta\Delta$ were analyzed. (D) In-gel activity stain analysis of the supernatant (Lane 1), flow-through (Lane 2) and eluate (Lane 3) of pUC-fdoGHtst'/BW $\Delta\Delta$ and supernatant (Lane 4), flow-through (Lane 5) and eluate (Lane 6) of pUC-fdoGHtHis'/BW $\Delta\Delta$.

Figure 5. Relative transcript levels of *fdoG* gene against internal standard gene 16srRNA. BW25113 (1 and 5), pUC-*fdoGHtst'*/BW $\Delta\Delta$ (2 and 6), pUC-*fdoGHtst*/BW $\Delta\Delta$ (3 and 7), and pBR-*fdoGHtst*/BW $\Delta\Delta$ (4 and 8) were cultivated under microaerobic (1–4) and aerobic (5–8) conditions, and subjected to RNA extraction and quantitative PCR analysis.

Figure 6. (A) Western blotting analysis. The lysates from wild type *DvMF* strain (Lane 1) and the recombinant strain from pDvMF-cmSt (Lane 2) were analyzed. (B) In-gel activity stain analysis. The supernatants of the lysates from wild type *DvMF* strain (Lane 1), the recombinant strain from pDvMF-cmSt (Lane 2) and the recombinant strain from pDvMF-cmPsaE (Lane 3) were separated on a non-denaturing polyacrylamide gel and stained. (C) Activity stain on StreptTactin-beads in PCR tubes. The StrepTactin-beads were incubated with the supernatants of the lysate from wild type *DvMF* strain (Tube 1), the recombinant strain from pDvMF-cmSt (Tube 2) and the recombinant strain from pDvMF-cmPsaE (Tube 3), washed and stained with formate, BV and nitroblue under nitrogen gas.