A small enzyme showing diaphorase activity was purified from culture supernatant of *Clostridium kluyveri* and its N-terminal amino acid sequence was determined. This sequence identified a gene (*diaA*) encoding a protein (DiaA) of 229 amino acids with a predicted molecular weight of 24,981 in the genomic DNA sequence database of *C. kluyveri* constructed by the Research Institute of Innovative Technology for the Earth. The predicted protein was composed of a flavin reductase-like domain and a rubredoxin-like domain from its N-terminus. The *diaA* gene was cloned into an expression vector and expressed in an *Escherichia coli* recombinant. Recombinant enzyme rDiaA showed NADH/NADPH diaphorase activity with 2,6-dichlorophenolindophenol and nitro blue tetrazolium. The enzyme was most active at pH 8.0 at 40°C. The UV-visible absorption spectrum and thin layer chromatography (TLC) analyses indicated that one rDiaA molecule contained a tightly bound FMN molecule as a prosthetic group. An iron molecule was also detected in an enzyme molecule.

**Key words:** *Clostridium kluyveri*; diaphorase; rubredoxin; NADH dehydrogenase

The term "diaphorase" refers to a ubiquitous class of flavin-bound enzymes that show NADH dehydrogenase activity, where the electron acceptor is generically an oxidized synthetic dye such as methylene blue or 2,6-dichlorophenolindophenol (DCPIP). On the basis of the original functions of the enzymes, they are classified into various enzyme categories: NAD(P)H dehydrogenase (quinone) (EC 1.6.5.2), NADPH dehydrogenase (EC 1.6.99.1), NADH dehydrogenase (EC 1.6.99.3), dihydrolipoyl dehydrogenase (EC 1.8.1.4), or nitric-oxide synthase (EC 1.14.13.39) (homepage of Enzyme Nomenclature IUBMB: [http://www.chem.qmul.ac.uk/iubmb/enzyme/](http://www.chem.qmul.ac.uk/iubmb/enzyme/)). NAD(P)H dehydrogenase (quinone) catalyzes a two-electron reduction of substrates with a preference for short-chain acceptor quinones such as ubiquinone and benzoquinone. NADPH and NADH dehydrogenases are a group of oxidoreductases acting on NADH or NADPH with other electron or hydrogen acceptors such as ferric chloride and O2. Dihydrolipoyl dehydrogenase is a component of the multienzyme 2-oxo-acid dehydrogenase complexes, such as the pyruvate dehydrogenase complex, responsible for the conversion of pyruvate to acetyl-CoA. Nitric-oxide synthase catalyzes the synthesis of nitric oxide (NO) from the terminal nitrogen atom of L-arginine in the presence of NADPH and O2 in the body, contributing to transmission from one neuron to another, to the immune system, and to the dilation of blood vessels. Some of these enzymes are known to have diaphorase activity. Diaphorases are useful in the colorimetric determination of NAD(P)H, and thus various dehydrogenase activities can be assayed when coupled with various dyes that act as hydrogen acceptors from NAD(P)H and hence several kinds of diaphorases are commercially available. NADH diaphorase histochemistry has been used extensively to detect nitric oxide synthase activity in various cell types, including neuronal cell bodies, vascular endothelium, cells of the immune system, and epithelial cells, and thus has gained great importance as a diagnostic chemical.

*Clostridium kluyveri* was originally isolated from an enrichment culture containing ethanol and 20% (wt/vol) yeast extract as carbon sources. Recently, this bacterium has drawn the attention of the scientific community for its ability to produce long-chain carbon compounds such as butyrate and caproate as fermentation products from short chain carbon compounds such as acetate and ethanol as fermentation substrates. *C. kluyveri* has also been found to be a source of bacterial diaphorase. A diaphorase smaller than dihydrolipoyl dehydrogenase has been partially purified from the culture supernatant of *C. kluyveri* and biochemically characterized. Although the small size of the purified enzyme was
ascribed to proteolysis of the parental dihydrolipoyl dehydrogenase at that time, no further investigation of this protein or its gene have been carried out since then.

In this study, we purified a small diaphorase from the culture supernatant of C. kluyveri and identified the diaA gene encoding this small enzyme (DiaA) in the unpublicized genomic DNA sequence database of C. kluyveri constructed by Research Institute of Innovative Technology for the Earth (RITE) on the basis of the N-terminal amino acid sequence of the purified enzyme. The diaA gene was amplified by PCR from C. kluyveri genomic DNA, cloned in expression vector pET-28a, and expressed in Escherichia coli. The recombinant enzyme, purified as a 6xHis-tagged protein, was purified and examined for its enzyme properties.

Materials and Methods

Bacterial strains, plasmids, and cultivation. C. kluyveri ATCC8527 was obtained from the American Type Culture Collection (ATCC). This bacterium was inoculated in a 120-ml anaerobic pressure bottle containing 100 ml of Broth 1120 specified by ATCC with an N2 gas head space, and cultivated at 37 °C for 5 d. E. coli strains DH5α and BL21(DE3) respectively were used in the cloning and expression of the diaA gene. Plasmid pCR2.1-TOPO (Invitrogen, Carlsbad, CA) was used in TA cloning. Plasmid pET-28a(+), used in the production of the recombinant enzyme was obtained from Novagen (Madison, WI). E. coli BL21(DE3), carrying a derivative of pET-28a(+), was grown in Lenox Broth (LB) medium supplemented with kanamycin sulfate (40 μg/ml). LB Broth EZMix (Sigma-Aldrich, St. Louis, Mo.) was also used for better expression of the recombinant protein.

Purification of a diaphorase (DiaA) from culture supernatant of C. kluyveri. Total proteins in 5-d-old culture supernatant of C. kluyveri were precipitated by adding solid ammonium sulfate to 90% saturation. The resulting precipitate was dissolved in 5 mM potassium phosphate buffer (pH 7.2) containing 0.1 mM flavin mononucleotide (FMN), and dialyzed against the same buffer. Purification of a diaphorase from the dialysate was carried out with a DEAE-Toyopearl column (3 × 20 cm; Tosoh, Tokyo) and a TSK-GEL G-3000 column (Tosoh). The active fractions were concentrated with an ultrafiltration membrane.

Enzyme assay. Diaphorase activity against DCPIP (Merck, Darmstadt, Germany) was determined by measuring the decrease in absorbance at 600 nm using spectrophotometer BioSpec-1600 (Shimadzu, Kyoto, Japan). The reaction mixture contained 2.6 ml of 0.2 M Tris–HCl (pH 8.5), 0.1 ml of 0.12 M ZnCl2, 0.1 ml of 6 mM NADH, and 0.1 ml of 2.4 mM DCPIP. The reaction was started with 0.1 ml of enzyme. One unit of enzyme activity was defined as the amount of enzyme that reduces 1 micromole of DCPIP per min at 25 °C at pH 8.5 under the specified conditions. Enzyme activity against 0.5 mM nitro blue tetrazolium (NBT) was assayed by measuring the increase in absorbance at 585 nm with 9 mM NADPH in 0.3 ml of 50 mM Tris–HCl (pH 8.0) at 37 °C.16 Protein concentrations were determined by the Bradford method, with bovine serum albumin as the standard.17

The effect of pH on diaphorase activity was investigated using Britton and Robinson’s universal buffer (50 mM phosphoric acid, 50 mM boric acid, 50 mM acetic acid; the pH was adjusted to 3–11 using 1 M NaOH). For determination of pH stability, recombinant DiaA was incubated overnight at 4 °C in Britton and Robinson’s buffer at different pHs, and then the residual activities were measured under the standard assay conditions. To determine the optimum temperature, the reaction mixtures were incubated at different temperatures (30 to 70 °C) using a Shimadzu UV-1200 spectrophotometer with a temperature control system. Thermal stability was measured by incubating the enzyme at various temperatures (30 to 70 °C) for 30 min, followed by measurement of residual enzyme activities under standard assay conditions.

Cloning of the diaA gene in E. coli. The Chromosomal DNA was isolated from C. kluyveri using a QIAamp mini spin column (Qiagen, Valencia, CA), and was used as a template for PCR amplification. PCR primers 5′-CCCCGATCCATGGATTGATAATAAAGCA-3′ and 5′-CCCCGACATTATCC-AAAGGTTTAAATTTGC-3′, including BamHI and SalI sites (underlined), were designed to generate a 690-bp fragment of the diaA gene. PCR amplification was carried out using KOD DASH DNA polymerase (Toyobo, Osaka, Japan) on a thermal cycler under the following conditions: 1 cycle of initial denaturation at 98 °C for 3 min, 30 amplification cycles consisting of a denaturation step at 92 °C for 30 s, annealing at 60 °C for 30 s, and an elongation step at 72 °C for 1 min, and a final extension at 72 °C for 15 min. The amplified DNA fragments were purified from an agarose gel by GFX PCR DNA and a gel band purification kit (Amersham Biosciences, Buckinghamshire, UK), ligated into TA cloning vector pCR 2.1 and introduced into E. coli DH5α by the standard transformation protocol. The insert DNA was recovered from the plasmid by digestion with BamHI and SalI, and ligated in to pET-28a(+), yielding pET-diaA.

Purification of recombinant DiaA (rDiaA). E. coli BL21 (DE3), harboring pET-diaA, was grown overnight at 37 °C in 500 ml of LB broth containing 40 μg/ml of kanamycin. When the absorbance of the culture reached 0.6 at 600 nm, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the culture was incubated for a further 5 h at 37 °C.
The cells were harvested by centrifugation at 4,000 × g for 20 min, and resuspended in 30 ml of lysis buffer (pH 7.4) containing 50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole. The cells were disrupted by sonication, and the soluble fraction of recombinant protein was collected by centrifugation at 4,500 × g for 25 min. The supernatant was used as the crude enzyme preparation, with diaphorase activity, showed a single band with a molecular size of about 18 kDa on SDS–PAGE. The molecular size of the native rDiaA was determined by gel filtration on a Superdex 200 HR 10/30 column (Amersham Bioscience). The column was eluted with 50 mM 2-(N-morpholino)ethanesulfonic acid buffer (pH 7.5), containing 0.15 M NaCl, at a flow rate of 30 ml/h and a fraction size of 1.25 ml per tube. Horse spleen apoferritin (440 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and vitamin B₁₂ (1.355 kDa) were used as molecular size standards.

To determine the amount of iron in rDiaA, the purified DiaA was pyrolyzed with nitric acid and subjected to a Horiba inductively coupled plasma (ICP) atomic emission spectrometer JY-238 (Kyoto, Japan). Other procedures. SDS–PAGE was carried out by the method of Laemmli (1970). N-terminal amino acid sequence analysis of DiaA was carried out using an Applied Biosystems protein sequencer ABI470A (Foster City, CA) after electrotransfer of the protein onto a polyvinylidene difluoride (PVDF) membrane from an SDS–PAGE gel.

**Results**

**Purification of a diaphorase form C. kluyveri**

The maximum amount of diaphorase activity was found in the culture supernatant of *C. kluyveri* in the stationary phase, probably due to cell lysis. Hence the proteins in the culture supernatant were recovered by ammonium sulfate precipitation. They were used for purification of diaphorase by DEAE-Toyopearl and TSK-GEL G3000 column chromatographies. The final preparation, with diaphorase activity, showed a single band with a molecular size of about 18 kDa on SDS–PAGE (Fig. 1A). This is not consistent with the earlier work of Kaplan et al. (1969), the molecular size of the purified diaphorase to be about 24 kDa. The N-terminal amino acid sequence of 18-kDa protein was determined to be Met-Ile-Asp-Asn-Lys-Ala-Phe-Tyr-Lys.

**DNA sequence of the diaA gene and predicted amino acid sequence of DiaA**

DNA sequencing of the *C. kluyveri* NBRC12016 (identical to ATCC8527 and DSM555) genome was carried out in RITE and the whole genome sequence were determined, although the sequence data have not yet been publicized in the DNA sequence database.

We found a gene encoding a protein whose predicted N-terminal amino acid sequence is completely consistent with the determined sequence of the purified enzyme. This gene, encoding a putative diaphorase, was named diaA, and the predicted amino acid sequence of DiaA is shown in Fig. 2. DiaA consists of 229 amino acids with a molecular weight of 24,981 and it is composed of two domains, viz., an N-terminal flavin reductase-like (Flavin,Reduct) domain and a C-terminal Fe-containing rubredoxin-like domain. *C. kluyveri* DiaA
showed overall sequence homology with certain proteins, e.g., 51% sequence identity with hypothetical ferric-chelate reductase/rubredoxin of *Syntrophus aciditrophicus* (DDBJ accession no. CP000252), 48% sequence identity with nitric oxide reductase of *Moorella thermoacetica* (AF202316), 36% sequence identity with hypothetical protein of *Clostridium leptum* (ABCB02000020), and 37% sequence identity with hypothetical protein of *Clostridium thermocellum* (CP000568). In all the rubredoxin-like domains, four cysteine residues, by which the iron atom is coordinated as \([\text{Fe(S-Cys)}_4]\), were completely conserved (Fig. 2).

**Construction of pET-diaA and production of rDiaA in *E. coli***

Plasmid pET-diaA was constructed and used in the production of rDiaA by *E. coli*, as described in “Materials and Methods.” Expression was found to be best in LB Broth EZMix (Sigma), as compared to normal LB and Super broth (3.5% Bacto Tryptone, 2% yeast extract, and 0.5% NaCl). Purification of rDiaA was carried out as described in “Materials and Methods,” and the final preparation of the enzyme showed a single band on SDS–PAGE. The molecular mass of the purified protein (about 28 kDa) was in good agreement with the calculated molecular weight of rDiaA, including a 6xHis-tag (28,555). The molecular size of the native form of rDiaA was determined to be 86 kDa by gel filtration on a Superdex 200 HR 10/30 column, suggesting that native rDiaA exists as a trimer. The purified rDiaA was yellowish, suggesting that this protein contained a flavin molecule as a prosthetic group, as predicted from its expected domain organization.

**Biochemical and physicochemical characterization of rDiaA***

It was expected that rDiaA is a flavoprotein on the basis of amino acid sequence homologies and the yellowish color of the enzyme, as described above. The UV-visible absorption spectrum of rDiaA (Fig. 3) showed absorption peaks at 283, 384, and 457 nm, which is characteristic of flavin-bound proteins. From the UV-visible spectrum, however, it was not possible to discriminate FMN or FAD as a cofactor. To determine the cofactor, TLC analysis was carried out with FMN and FAD as standards. As shown in Fig. 4, TLC analysis indicated that rDiaA contained FMN but not FAD as a prosthetic group. The amount of FMN released from rDiaA upon boiling was calculated to be 0.70 per rDiaA molecule, suggesting that one rDiaA molecule essentially includes one FMN molecule, as reported for the native enzyme purified from *C. kluyveri*, although the amount of FMN was less than expected, probably due to inefficient biosynthesis of FMN.

Amino acid sequence homology indicated the presence of a Fe-containing rubredoxin-like domain. The amount of iron was determined to be 1.1 molecule per rDiaA molecule by ICP atomic emission spectroscopy.

The requirement of reduced pyridine nucleotides for diaphorase activity was assayed using different concentrations of NADH or NADPH with 0.05 mM DCPIP as another substrate (electron acceptor). The \(K_m\) values of rDiaA for NADH and NADPH were estimated to be 0.15 and 0.35 mM respectively. The \(K_m\) value of rDiaA for NADH was comparable to that of native diaphorase, reported previously (0.09 mM). The \(V_{max}\) value of
rDiaA was 3,500 U/mg-protein for DCPIP when 0.5 mM NADH was used as the electron donor. The Km value of rDiaA for NBT was calculated to be about 0.5 mM.

When the effect of temperature on diaphorase activity was measured by assaying the activity with NADH and DCPIP at different temperatures at pH 8.0, rDiaA showed highest activity at 40°C. The recombinant enzyme was stable at 30°C upon 30 min of incubation without substrates, but incubation at 40°C and higher temperatures inactivated enzyme activity. The optimum pH for the enzyme was found to be pH 8.0, and the enzyme showed relatively high activity (more than 80% of the highest activity) over a pH range of 6.0 to 9.0. When rDiaA was incubated overnight in Britton and Robinson’s universal buffer solutions at various pHs at 4°C, the enzyme was stable at pH 8.0, but lost enzyme activity completely below pH 6 and above pH 11. When a small diaphorase was first purified from C. kluyveri, it was thought to be a proteolysis product of the parental dihydrolipoyl dehydrogenase, but rDiaA showed no dihydrolipoyl dehydrogenase activity.

**Discussion**

The term “diaphorase” is a synonym for certain functionally characterized enzymes having NADH/NADPH dehydrogenase activity, such as dihydrolipoyl dehydrogenase. Dihydrolipoyl dehydrogenase, a component of the pyruvate dehydrogenase complex responsible for the conversion of pyruvate to acetyl-CoA, is the first enzyme shown to catalyze the oxidation of NADH by methylene blue. The amino acid sequences of dihydrolipoyl dehydrogenases are highly conserved among enzymes of different origins, e.g., an E. coli enzyme (DBJ accession no ABE05639) consists of 475 amino acids, including a Pyr_redox_dim domain and a Pyr_redox domain, according to the Pfam database. When a 24-kDa diaphorase was first purified from C. kluyveri by Kaplan et al., it was expected to be a proteolysis product of the parental dihydrolipoyl dehydrogenase.

Although we obtained sequence information on the C. kluyveri diaA gene from RITE, recently we found that the same gene from C. kluyveri DSM 5557 is registered as a hypothetical high-molecular weight rubredoxin gene (hrb) in DNA databases under accession no CP000673 (protein id, YP_001396175.1). The diaA sequence was centile identical to the sequence in CP000673. DiaA consisting of two domains, a flavin reductase-like domain and a rubredoxin-like domain, is quite different in structure from the dihydrolipoyl dehydrogenases, as described above. The flavin reductase-like domain consists of about 150 amino acids and is found in flavin reductases with various oxidoreductase and monooxygenase components. VlmR of Streptomyces viridifaciens is the first flavin reductase in this family. It is involved in the synthesis of valaminycin in association with an isobutylamine hydroxylase.

Rubredoxin is a low molecular weight nonheme iron-containing bacterial protein involved in electron transfer, sometimes replacing ferredoxin as an electron carrier. The comparison of DiaA sequence with entries in DNA/protein databases showed the presence of only a small number of proteins with overall sequence similarity, e.g., proteins from S. aciditrophicus, M. thermoacetica, C. leptum, and C. thermocellum. Among these proteins, only Hrb of M. thermoacetica has been genetically and biochemically characterized. In M. thermoacetica, Hrb and FprA (an A-type flavoprotein) were found to be expressed under normal anaerobic growth conditions, and a combination of these two proteins showed nitric acid oxidase activity. Furthermore, Hrb was identified as NADH:FprA oxidoreductase, i.e., a substrate for Hrb was FprA protein. Figure 5 shows some genes around the diaA and hrb genes in M. thermoacetica, C. kluyveri, C. thermocellum, C. leptum, and S. aciditrophicus. Although the diaA and hrb genes are homologous among them, the other genes around diaA and hrb have no relevance to each other. The C. kluyveri diaA gene is flanked by a hypothetical integral membrane protein gene and a hypothetical methionine aminopeptidase gene. The true function of DiaA is unfortunately unknown at present.

Several bacterial species contain enzymes that utilize either NADH or NADPH to reduce a tightly or noncovalently bound FMN or FAD cofactor in their active sites. We found that FMN but not FAD was present in the recombinant enzyme. When we tried to use a MonoQ column (Amersham Bioscience) in the purification of rDiaA, even in the presence of FMN all fractions obtained were colorless and inactive, suggest-
ing that removal of FMN from the enzyme inactivated its activity. Tight binding of FMN to the enzyme is essential for the function of the enzyme.

The active sites of rubredoxins consist of a single iron tetrahedrally associated with four sulfurs of four conserved Cys residues.27) In M. thermoacetica Hrb, it was assumed that the electron flow was NADH ⇒ Hrb-FMN ⇒ Hrb-[Fe(SCys)₄].25) Although the same electron flow was expected for C. kluyveri DiaA, the true electron acceptor in natural reaction was not predictable, as described above.

The molecular mass (18 kDa) of the enzyme purified from C. kluyveri was smaller than the sizes of previously purified C. kluyveri diaphorase (24 kDa) and DiaA (a calculated molecular weight of 24,981) specified in this study. It is most likely that the full-length DiaA corresponds to the previously reported C. kluyveri diaphorase, and that the 18-kDa enzyme is a proteolysis product of the parental diaphorase. When C. kluyveri ATCC8527 was cultivated in Broth 1120 for 5 d, the amount of diaphorase activity produced in the culture medium was about 0.15 U/ml of culture. On the other hand, rDiaA was produced as an intracellular enzyme at high concentrations, about 70 U/ml of culture, indicating that the diaphorase productivity of the recombinant E. coli was much higher than that of C. kluyveri. For practical use of diaphorase, e.g., for colorimetric determination of NAD(P)H, stable enzymes with strong activity are desirable. Although C. kluyveri DiaA was not thermostable, its enzyme properties might be improved by genetic and protein engineering strategies. The small size of this enzyme should be advantageous for its artificial modifications. High expression of diaA did not affect the growth of host strain of E. coli, whereas it is possible that high production of physiologically important enzymes such as dihydrodipicolinate dehydrogenase causes growth inhibition due to disturbance of normal metabolic balance. Since genes homologous with diaA are found in certain bacteria, including thermophilic bacteria such as M. thermoacetica and C. thermocellum, these bacteria should be good sources for thermophilic and/or thermostable diaphorases. In conclusion, small diaphorases structurally related to DiaA are useful in practical applications, e.g., as diagnostic enzymes.

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