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Molecular Properties of Membrane-Bound FAD-Containing D-Sorbitol Dehydrogenase from Thermotolerant *Gluconobacter frateurii* Isolated from Thailand

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There are two types of membrane-bound D-sorbitol dehydrogenase (SLDH) reported: PQQ–SLDH, having pyrroloquinoline quinone (PQQ), and FAD–SLDH, containing FAD and heme *c* as the prosthetic groups. FAD–SLDH was purified and characterized from the PQQ–SLDH mutant strain of a thermotolerant *Gluconobacter frateurii*, having molecular mass of 61.5 kDa, 52 kDa, and 22 kDa. The enzyme properties were quite similar to those of the enzyme from mesophilic *G. oxydans* IFO 3254. This enzyme was shown to be inducible by D-sorbitol, but not PQQ–SLDH. The oxidation product of FAD–SLDH from D-sorbitol was identified as L-sorbose. The cloned gene of FAD–SLDH had three open reading frames (*sldSLC*) corresponding to the small, the large, and cytochrome *c* subunits of FAD–SLDH respectively. The deduced amino acid sequences showed high identity to those from *G. oxydans* IFO 3254: SldL showed to other FAD-enzymes, and SldC having three heme *c* binding motives to cytochrome *c* subunits of other membrane-bound dehydrogenases.

Key words: FAD; sorbitol dehydrogenase; acetic acid bacteria; *Gluconobacter*; cytochrome *c*

Gluconobacter strains are strict aerobes belonging to the group of acetic acid bacteria able to oxidize many compounds incompletely to accumulate the oxidized products in the culture medium by using membrane-bound dehydrogenases localized on the outer surface of the cytoplasmic membrane.[1,2] Such 'incomplete' oxidation is called oxidative fermentation and is carried out by membrane-bound enzymes, like alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) in vinegar fermentation. This character of the microorganism leads to applications in industry for fermentation of valuable products such as L-sorbose, dihydroxyacetone, D-gluconate, and keto-D-gluconates.[3,4]

L-Sorbose is an important intermediate in the industrial production of vitamin C. The membrane-bound D-sorbitol dehydrogenase (SLDH) has been considered to play a main role in L-sorbose fermentation.[5] In *Gluconobacter* strains, two types of membrane-bound D-sorbitol dehydrogenases have been purified and well characterized. One was purified from *G. suboxydans var* α IFO 3254[6] as flavohemoprotein, containing a covalently bound FAD having three subunits with molecular mass of 63 kDa (flavoprotein), 51 kDa (cytochrome *c*), and 17 kDa (unknown), designated FAD–SLDH. In addition, another membrane-bound SLDH (PQQ–SLDH) was recently purified from *G. suboxydans* var α IFO 3255,[5] having one subunit of 80 kDa and pyrroloquinoline quinone (PQQ) as the prosthetic group. Recently, its gene, *sldA*, composing an operon with *sldB*, was cloned,[7,8] and the enzyme was shown to be identical to glycerol dehydrogenase,[9] found ubiquitously in *Gluconobacter* strains, and also to arabitol dehydrogenase[10] and D-gluconate dehydrogenase, yielding 5-keto D-gluconate[11] using molecular biological techniques. The reaction product of this enzyme from D-sorbitol was determined to be L-sorbose, but that of FAD–SLDH has not been determined. In *Gluconobacter* strains, two membrane-bound D-gluconate dehydrogenases have been found. One is a flavocytochrome producing 2-keto-D-gluconate similar to FAD–SLDH,[12] and the other is a quinoprotein identical to PQQ–SLDH that produces 5-keto-D-gluconate, as described above. Analogically, it is possible that FAD–SLDH might produce not L-sorbose but D-fructose, differently from

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Abbreviations: SLDH, D-solbitol dehydrogenase; PQQ, pyrroloquinoline quinone; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; FDH, D-fructose dehydrogenase; AcB, sodium acetate buffer; *tat*, twin-arginin translocation; 2KGDH, 2-keto-D-gluconate dehydrogenase; GADH, D-gluconate dehydrogenase

PQQ–SLDH.[4] Since nowadays D-fructose is produced industrially from D-glucose by glucose isomerase[13] as a reversible reaction, the theoretical yield of D-fructose never exceeds 50% of the starting material without separation of the product. Hence it is advantageous if the enzyme which oxidizes D-sorbitol irreversibly to produce D-fructose is found and applied in oxidative fermentation.[4]

In this study, screening of D-fructose-producing microorganisms in *Gluconobacter* IFO strains and thermotolerant strains from Thailand was performed with both growing and resting cells. Thermotolerant strains were included because of their potential advantage in industry.[14] Furthermore, EDTA treatment was performed with the membrane fraction to distinguish FAD–SLDH from PQQ–SLDH.[10] Among these strains, the isolated strain THD32 classified as *Gluconobacter frateurii* appeared to have high activity of FAD–SLDH, and hence this enzyme was purified from the PQQ–SLDH disrupted mutant of the THD32 strain. Then molecular cloning of the gene of FAD–SLDH was done using primers designed from N-terminal amino acid sequences of the subunits.

Materials and Methods

Materials. All other chemicals were obtained from commercial sources. Yeast extract was a kind gift from the Oriental Yeast Co., Ltd.

Bacterial strains and growth conditions. Gluconobacter IFO strains and thermotolerant strains (some are in Ref. 14) newly isolated from Thailand were used in this experiment. These strains were maintained on potato-$CaCO_3$ agar slant, which was prepared by adding 2% agar and 0.5% $CaCO_3$ to a potato medium consisting of 5 g of D-glucose, 10 g of yeast extract, 10 g of polypeptone, 20 g of glycerol, and 100 ml of potato extract, filled to 1-liter with tap water. Preculture was done in 5 ml of potato medium with shaking for 24 h at 30 °C, and transferred to 100 ml of the appropriate medium in a 500 ml Erlenmeyer flask, and then cultured for another 24 h. Bacterial growth was monitored with a Klett-Summerson photoelectric colorimeter with a red filter.

Screening of D-fructose producing strains in Gluconobacter. Screening of D-fructose producing strains was performed using growing cells, resting cells, and membrane fractions of *Gluconobacter* strains. Production of D-fructose was determined by the enzymatic method with D-fructose dehydrogenase (FDH),[15] which was a kind gift from Toyobo, after removal of cells or membrane by centrifugation.

Preparation of membrane fraction. Cells were harvested by centrifugation at 9,000 rpm for 10 min and washed once with distilled water. The washed cells were resuspended with 10 mM sodium acetate buffer (AcB, pH 5.0) at a concentration of 3 ml/g-wet cells, and passed twice through a French pressure cell press (American Instruments Co., Silver Spring, MD., U.S.A.) at 16,000 psi. After centrifugation at 6,000 rpm for 10 min to remove the intact cells, the supernatants were ultracentrifuged at 40,000 rpm for 60 min. The resultant precipitate was resuspended with McIlvaine buffer (McB, pH 5.0) and used as a membrane fraction. EDTA treatment of the membrane fraction was done as described previously,[10] and the treated membrane was resuspended with McB, pH 5.0.

Qualitative and quantitative analyses of ketohexose. D-Fructose concentration was measured by FDH as described above. Qualitative analysis of ketohexose was performed using thin layer chromatography (TLC) analysis. Samples were spotted on a silica gel plate 60 (Merck) and developed with a solvent reagent containing ethyl acetate:acetic acid:methanol:deionized water (6:1.5:1.5:1). After the silica gel plate was dried, phenol sulfuric reagent was spread to visualize color development. Identification of the reaction product was also done by HPLC equipped with Aminex HPX-87P (7.8 × 300 mm, Bio-Rad). The mobile phase was deionized water and the column temperature was 66 °C. Elution was monitored by absorption at 210 nm. When analyzed at a flow rate at 0.6 ml/min, L-sorbose and D-fructose were detected at 14.9 and 17.9 min respectively. For quantitative measurement of the total amount of ketohexose, resorcinol was used, as described previously.[10]

Enzyme assay. Dehydrogenase activities with D-sorbitol, glycerol, D-mannitol, D-arabitol, ethanol, and acetaldehyde were measured by the ability to reduce potassium ferricyanide, as described previously.[6] One unit of enzyme activity was defined as the amount of enzyme which catalyzed 1 μmol of substrate oxidation per min under the above conditions, which was equivalent to 4.0 absorbance at 660 nm.

Determination of protein concentration. Protein concentration was measured by a modified version of the Lowry method.[16] Bovine serum albumin was used as the standard protein.

Purification of FAD–SLDH from G. frateurii THD 32. All steps of purification were carried out at 4 °C. The membrane fraction was suspended in 10 mM AcB (pH 5.0), and was adjusted to 10 mg of protein/ml. To solubilize the membrane proteins, Triton X-100, KCl, and D-sorbitol were added at final concentrations of 1%, 0.1 M, and 0.1 M, respectively, and gently stirred in an ice bath for 90 min. Solubilized enzyme obtained by centrifugation at 40,000 rpm for 90 min was dialyzed against 10 mM AcB (pH 5.0) containing 0.1% Triton X-100, 25 mM D-sorbitol, and 5 mM $MgCl_2$ (buffer A) for 6 h, by exchanging the buffer twice. Precipitate was removed by

centrifugation at 9,000 rpm for 20 min, and the supernatant was put onto a CM-cellulose column equilibrated with buffer A. The enzyme activity was passed though the column, whereas large portions of alcohol dehydrogenase and aldehyde dehydrogenase were adsorbed on this column. The active fractions were combined and put into a dialysis tube, and dialyzed against 10 mM AcB (pH 4.5) containing 0.1% Triton X-100, 25 mM D-sorbitol, and 5 mM $MgCl_2$ (buffer B) for 6 h, and this was repeated by changing the dialysis buffer. The dialysate was put onto a CM-cellulose column equilibrated with buffer B. The column was washed with buffer B and major impurities were removed, and then elution of the enzyme was done with a linear gradient of KCl (0–0.1 M). Enzyme activity was eluted at about 30 mM KCl. The active fractions were put into a dialysis tube and concentrated by embedding it in sucrose powder, and then dialyzed against 5 mM MES–NaOH buffer (pH 6.0) containing 0.1% Triton X-100, 25 mM D-sorbitol, and 5 mM $MgCl_2$ (Buffer C) for 8 h, by exchanging the buffer twice. The dialysate was put onto a DEAE-cellulose column equilibrated with buffer C. Aldehyde dehydrogenase activity was removed by washing with the same buffer while the FAD–SLDH was eluted at about 30 mM KCl in the linear gradient (0–0.1 M).

SDS–Polyacrylamide gel electrophoresis (SDS–PAGE). SDS–PAGE was done on 12.5% (w/v) acrylamide slab gel by the method described by Laemmli.[17] The following calibration proteins with the indicated molecular masses were used as references for measurement of molecular mass: phosphorylase b (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), and lysozyme (14.4 kDa). Proteins were stained with 0.1% Coomassie Brilliant Blue R-250. Cytochrome *c* was stained by heme-catalyzed peroxidase activity.[18]

Determination of N-terminal amino acid sequences of the purified FAD–SLDH. After SDS–PAGE, the proteins in the gel were transferred electrophoretically onto a polyvinylidene difluoride membrane at 100 mA for 4 h. The proteins were stained with Coomassie Brilliant Blue G-250, destained with 50% methanol, and dried, and then the stained bands were cut off. The N-terminal amino acid sequence was analyzed with a protein sequencer PPSQ-21 (Shimadzu).

Electroporation method. Competent cells of *Gluconobacter* strains were obtained by the following method: A preculture (1 ml) was transferred into 100 ml of 1% D-sorbitol medium in a 500 ml Erlenmeyer flask with shaking at 30 °C for 12 h. Cells were chilled on ice for 30 min before harvesting by centrifugation at 4,000 rpm for 10 min at 4 °C, and washed twice with ice-cold 10% (w/v) glycerol. The final cell pellet was resuspended with 0.5–1 ml of ice-cold 10% (w/v) glycerol. The cell suspension (40 µl each) was frozen in an Eppendorf tube with liquid nitrogen and stored at −80 °C. The competent cells were thawed on ice just before use and mixed with a DNA solution, and then transferred into a cold gene pulser cuvette. Introduction of DNAs into the competent cells were achieved by applying 1.8 kV electric pulse to the cuvette with a Gene Pulser (Bio-Rad). After pulsing, 1 ml of ice-cold potato medium was immediately added to the cuvette. Then the suspension was transferred into a test tube and incubated for 6 h at 30 °C with shaking. After incubation, 100 µl of the cell suspension was spread on an agar plate medium containing 1% glycerol, 1% sorbitol, 0.1% yeast extract, 0.1% polypeptone, and kanamycin (50 µg/ml), and incubated at 30 °C.

DNA techniques. Restriction enzyme digestion, DNA ligation, and other DNA modifications were performed according to the vendor's recommendations. Preparation of plasmid DNA from *E. coli* strains and other general molecular biology techniques were done as described by Sambrook *et al.*[19] Genomic DNA of *Gluconobacter* strains was isolated from cells grown to a mid-exponential phase in D-sorbitol medium by a method of Marmur[20] with some modifications. PCR reaction was performed using the Ready.To.Go/PCR Bead Kit (Amersham Biosciences). Disruption of PQQ–SLDH was confirmed by PCR, as described previously.[11] To obtain the gene fragment of FAD–SLDH, the primers SLDH1 (TCG(C)GCCGAT(C)GTCGTCATC(G)GT) and SLDH2 (TGG(A)CAG(C)GCT(C)TCGCAA(G)TC-G(A)CC) were used. PCR was performed with 25 cycles under the following conditions: denaturation for 2 min at 94 °C, annealing for 2 min at 55 °C, and extension for 1 min at 72 °C. DNA fragments obtained by PCR were isolated by agarose gel eletrophoresis and purified with QIA Quick Gel Extraction Kit (Qiagen), and then cloned into pGEM-T Easy™ vector (Promega).

Southern hybridization. Chromosomal DNA was digested with suitable restriction enzymes and electrophoresed in agarose gel, and then transferred to a Hybond N^+ membrane (Amersham Biosciences) by capillary blotting. The DNA bands were then fixed to the membrane by exposure to UV light for 5 min. Hybridization and detection was carried out with the ECL Direct Nucleotide Labeling System (Amersham Biosciences) according to the protocol provided by the supplier.

Colony hybridization. Colonies of the gene library constructed from the chromosomal DNA of *G. frateurii* THD 32 in pUC119 were grown on an LB agar plate containing 50 µg/ml of ampicillin, and transfered to a Hybond-N^+ membrane and lysed. Hybridization and detection were performed using the DIG System (Roche Diagnostics) according to the protocol provided by the supplier.

Nucleotide sequence analysis. Plasmids for sequencing were prepared with a QIA Prep Spin Miniprep Kit (Qiagen). Sequencing was performed using ABI Prism 310 (Applied Biosystems). Sequence data were analyzed using Genetyx-Mac (Software Development) and Clone Manager (Scientific and Educational Software). Homology search analysis and alignment were performed with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The sequence described in this study has been deposited with DNA Data Bank of Japan (DDBJ) under accession no. AB192961.

Results

Isolation of thermotolerant strains which produce D-fructose and EDTA-tolerant SLDH

Screening of D-fructose-producing strains was performed in *Gluconobacter* species including IFO strains and isolated thermotolerant strains from Thailand. All the strains examined (more than 100 strains), accumulated only small amounts of D-fructose in the medium, less than 5% of the D-sorbitol used, that is, <0.5 g/l, in both growing- and resting-cell conditions. The remaining part of D-sorbitol appeared to be converted to L-sorbose in all strains tested, because large amounts of ketohexoses were detected by resorcinol test (data not shown). Perhaps the oxidation rate from D-sorbitol to D-fructose was much slower than that to L-sorbose by PQQ–SLDH. Hence, several strains producing relatively high amounts of D-fructose were selected to examine FAD– or PQQ–SLDH activity at the membrane level. With the selected strains, EDTA treatment of the membrane fraction was performed to eliminate PQQ–SLDH activity. Most of the strains showed SLDH activity sensitive to EDTA treatment, while some strains such as THD32 were resistant, like *G. suboxydans* var α IFO 3254, which previously was used to purify FAD–SLDH (data not shown). Even with the membrane fraction of strain THD32 treated with EDTA, D-fructose was not produced by D-sorbitol oxidation; instead, a substantial amount of L-sorbose was detected (data not shown). For further analysis of this 'EDTA-resistant' enzyme, which is assumed to be FAD–SLDH, purification of this enzyme was addressed from strain THD32. This strain grew at 37 °C at which mesophilic strains cannot grow.[14] According to the 16S rDNA analysis, this strain is classified as *G. frateurii* (S. Moonmangmee, personal communication).

Purification of FAD–SLDH from G. frateurii THD32

From the membrane fraction of strain THD32, purification of SLDH was attempted. Partially purified SLDH, having three main bands of 61.5, 52, and 22 kDa on SDS–PAGE, was obtained, but its purity was not improved by further column chromatographies (data not shown). Hence we decided to purify the enzyme from the PQQ–SLDH mutant constructed using a plasmid pSUP202-*sldA*::Km, a suicide plasmid with the gene of the large subunit of PQQ–SLDH from *G. suboxydans* IFO 3255 disrupted by the kanamycin-resistant gene.[11] Disruption and absence of the wild-type *sldA* gene was confirmed by PCR (data not shown).

The mutant strain grew at almost the same rate as the wild-type strain; the growth rate was slightly slower on the medium with D-sorbitol plus D-gluconate or glycerol, but better on the medium with D-sorbitol alone, when each carbon source was added at a concentration of 1% (w/v). Among the three media used, growth yield of both the wild-type and the mutant strain was best on the medium with D-sorbitol plus D-gluconate (data not shown). SLDH activity (the sum of PQQ–SLDH and FAD–SLDH activities) was highest in both wild-type and mutant cells grown on the medium with D-sorbitol alone, while enzyme activities with other substrates did not significantly change among four media tested (Fig. 1), indicating that D-sorbitol is an inducer for FAD–SLDH. Activity with D-arabitol disappeared in the *sldA* mutant and was unchanged in the wild-type strain grown on different carbon sources (Fig. 1), indicating that PQQ–SLDH was constitutively expressed. When the concentration of D-sorbitol in the growth medium

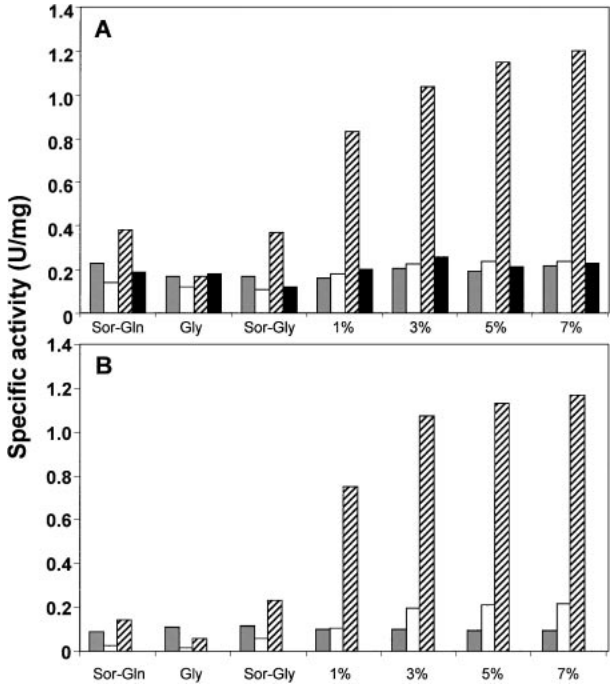

Fig. 1. Enzyme Activities in the Membrane Fractions of the Wild-Type and PQQ–SLDH Mutant Strains of *G. frateurii* THD32 Grown on Different Media.

Wild-type (A) and the PQQ–SLDH-disrupted mutant (B) of *G. frateurii* THD32 were grown to late exponential phase on each medium containing 0.3% polypeptone, 0.3% yeast extract and different concentration of sugar alcohol [1.0% each of D-sorbitol and D-gluconate (Sor-Gln), 1.0% each of D-sorbitol and glycerol (Sor-Gly), 1.0% glycerol (Gly), or 1.0–7.0% D-sorbitol (1%, 3%, 5%, and 7%)]. Dehydrogenase activities were measured with ferricyanide as the electron acceptor, as described in "Materials and Methods", with glycerol (gray), D-mannitol (white), D-sorbitol (hatched), and D-arabitol (black) as substrate.

Table 1. Purification Summary of FAD–SLDH from the PQQ–SLDH Mutant of *G. frateurii* THD32

Step	Total protein (mg)	Protein (mg/ml)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification Fold
Membrane	1512	10.43	1228	0.81	100	1
Solubilization	291	1.73	1278	4.40	104	5
CM-cellulose (1)	80	0.42	851	10.7	69	13
CM-cellulose (2)	21	0.15	679	32.8	55	40
DEAE-cellulose	3	0.06	326	109	27	134

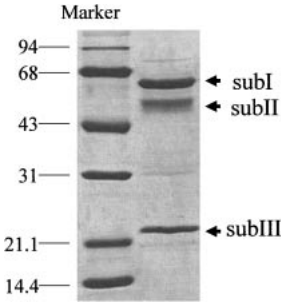

Fig. 2. SDS–PAGE Analysis of the Purified FAD–SLDH from the PQQ–SLDH Mutant of *G. frateurii* THD32.

Ten μg of purified FAD–SLDH was applied and stained with Coomassie Brilliant Blue R-250.

was increased (1–7%), the activity with D-sorbitol increased, although no significant difference in growth was observed, whereas the other enzyme activities did not change very much (Fig. 1). Thus it is clear that FAD–SLDH is induced with D-sorbitol in the medium.

The membrane fractions were prepared from mutant cells grown on 1% D-sorbitol alone, and purification was carried out as described in "Materials and Methods". A summary of purification is shown in Table 1. The enzyme was purified 134-fold with 27% recovery, and the purified enzyme exhibited a specific activity of 109 U/mg of protein. The purified enzyme showed a typical spectrum with absorption maxima at 415, 521, and 551 nm, corresponding to a reduced cytochrome *c*, which is consistent with the fact that the enzyme was purified in the presence of D-sorbitol. Three protein bands with molecular masses of 61.5 kDa, 52 kDa, and 22 kDa were found on SDS–PAGE (Fig. 2). Under UV light, the large and middle protein bands showed fluorescence on SDS–PAGE gel before staining, and the former protein band showed much more intense fluorescence after the gel was treated with performic acid, suggesting that the large subunit contains covalently bound flavin and that the covalent linkage might be on a cystein residue.[21] The 52 kDa protein band was found to be stained by heme staining after SDS–PAGE (data not shown), indicating that it is a cytochrome *c*. The N-terminal amino acid sequences were determined as follows: large subunit, SSNSFSADVVIVGSGV; cytochrome *c* subunit, EDQATTIXRXAYXA (X: unidentified); small subunit, EETKPPLASRDEYERFFEV.

Catalytic properties of the purified enzyme

The substrate specificity of the purified enzyme was examined. Among the substrates tested, D-sorbitol was the best and D-mannitol was oxidized at 5% of the reaction rate of D-sorbitol. The other polyols, such as glycerol, ribitol, D- and L-arabitol, dulcitol, and *myo*-inositol, were inert. The K_m and V_{max} values for D-sorbitol were calculated to be 20.4 mM and 93.5 U/mg respectively when ferricyanide was used as an electron acceptor. The optimum pH and temperature for the reaction of the purified enzyme was about pH 4.5 and at 25 °C. These characters are quite similar to the enzyme from *G. suboxydans* var α IFO 3254.[6] The enzyme appeared to be less active and stable at higher temperatures even though it was obtained from a thermotolerant strain (data not shown). We prepared the PQQ–SLDH mutant of *G. suboxydans* var α IFO 3254 using pSUP202-*sldA*::Km. Its thermostability as to SLDH activity in the membrane fraction was found to be similar to that of the mutant of the THD32 strain (data not shown), indicating no significant difference between enzymes from the two strains.

Identification of the oxidation product from D-sorbitol by purified FAD–SLDH

The oxidation of D-sorbitol with purified FAD–SLDH was carried out by coupling with purified ubiquinol oxidase of *Acetobacter*[22] in the presence of quinol (Q_2H_2) for 8 h at 25 °C with gentle shaking. Only a small amounts of D-fructose (less than 0.1% of the initial amount of D-sorbitol) was detected by FDH assay, while a resorcinol test showed a high amount of ketohexose (about 7% of the initial D-sorbitol). The reacted product had similar mobility to L-sorbose on thin-layer chromatography, after it was developed with L-sorbose and D-fructose as standards. The product was also analyzed by HPLC with an Aminex HPX-87P column. The peak of the reaction product appeared at the same retention time as that of L-sorbose, but not that of D-fructose. These results clearly indicate that the oxidation product of D-sorbitol by the purified FAD–SLDH from *G. frateurii* THD32 was not D-fructose but L-sorbose.

Furthermore, the membrane fraction from the PQQ–SLDH disrupted strain was used to confirm the oxidation product from D-sorbitol. The reaction product of D-sorbitol with the membrane fraction was compared to the standard of L-sorbose and D-fructose by TLC. Only one oxidation product, corresponding to L-sorbose, was

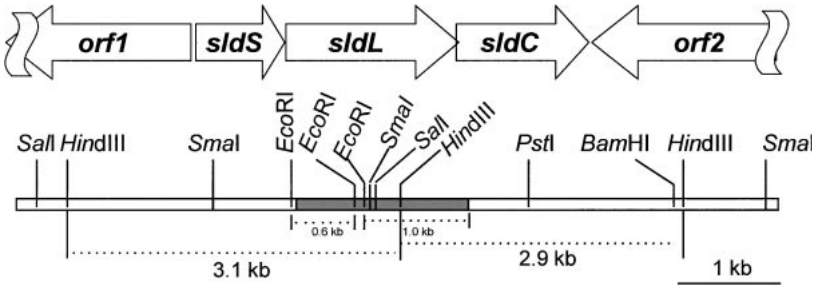

Fig. 3. Schematic Representation of the Gene Fragment Obtained in This Study.
The gray box represents the gene fragment obtained by PCR with SLDH1 and SLDH2 as primers. Orf1 and Orf2 are not complete.

detected (data not shown), indicating that FAD–SLDH is also involved in D-sorbitol oxidation to produce L-sorbose in the cytoplasmic membrane. It was also confirmed that little activity if any to produce D-fructose is present in the membrane fraction.

Cloning of the gene encoding membrane-bound FAD–SLDH from G. frateurii THD32

Two oligonucleotide primers were synthesized: a forward primer, named SLDH1, was designed from the N-terminal amino acid sequence of subunit I, while a reverse primer, named SLDH2, was designed according to the result of alignment of amino acid sequences of cytochrome *c* subunits of several membrane-bound dehydrogenases (see Fig. 5). PCR amplification was performed under the conditions described in "Materials and Methods". A band of expected size, about 1.7 kb, was obtained and confirmed to be the part of the gene of SLDH by nucleotide sequencing. The PCR product was used as a probe for Southern hybridization, and 3.1 kb and 2.9 kb of the *Hin*dIII fragments were assumed to contain the whole part of the gene cluster (Fig. 3). These fragments were isolated from the library by colony hybridization. The two plasmids obtained were sequenced, and finally, a 6,183 bp-nuleotide sequence containing the complete structural genes of FAD–SLDH was confirmed.

The nucleotide and amino acid sequences of FAD–SLDH and the flanking regions are shown in Fig. 4. Three open reading frames (ORFs) corresponding to the small, large and cytochrome *c* subunits were found and named *sldSLC*. They might be in the same transcriptional unit. A rho-dependent terminator-like sequence was found at position 4,997–5,034. The upstream of this operon was found to be an AraC-like transcriptional regulator[23] which is possibly involved in transcriptional control of the *sldSLC* gene. The downstream sequences showed identity to transposase, which is responsible for transposon movement.[24]

The GTG (nt 1,305) might be the start codon of *sldS*, and a possible Shine-Dalgarno sequence was found at 12 bp upstream of this start codon. The N-terminal amino acid sequence of this small subunit determined by the protein sequencer was found inside in this ORF, and the signal sequence was confirmed to be 41 amino acids. It has twin-arginine sequence and thus might be used in the twin-arginine translocation (*tat*) system.[25] On the other hand, the N-terminal amino acid sequence of the large subunit was found without any signal sequence, suggesting that the large subunit might be translocated together with the small subunit by the *tat* system. SldS encodes 197 amino acids and the calculated molecular weight of the mature protein is 22,306 Da.

The coding region of the large subunit was started at position 1,917 with the ATG codon. The gene *sldL* encoded a polypeptide of 545 amino acid residues with a calculated molecular mass of 60,075 Da. The deduced amino acid sequence of subunit I was found to have the sequence GSGVAG at a position between the 15th and the 20th residues, corresponding to the binding motif of FAD (GXGXXG).

The ORF corresponding to the cytochrome *c* subunit, *sldC*, started at position 3,547. A possible SD sequence, AGGAGA, was found at 6 nt upstream of the start codon. The gene encoded a 478 amino acid protein with a molecular mass of 51,057 Da. The N-terminal amino acid sequence of the cytochrome *c* subunit was found inside this ORF, and the 31 amino acid residues were confirmed to be a typical *sec*-dependent signal sequence.[26] The deduced amino acid sequence was revealed to have three CXXCH sequence motives serving as heme *c* binding sites.

A protein similarity search on data base by the BLAST program revealed that the large subunit is almost identical to the large subunit of FAD–SLDH from *G. oxydans* IFO 3254 (98% identity), and also similar to many FAD-dependent enzymes of the so-called glucose-methanol-choline oxidoreductase family, including glucose dehydrogenase from *Burkholderia cepacia* (49%), and 2-keto-D-gluconate dehydrogenase (2KGDH) from *Pantoea agglomerans* (formally *Erwinia herbicola*) (44%). The deduced amino acid sequences of the small subunit showed identity to the small subunits of FAD–SLDH from *G. oxydans* IFO 3254 (79%), and the small subunit of *P. agglomerans* 2KGDH (18%). The predicted amino acid sequence of *sldC* showed considerable identity to the cytochrome *c* subunits of SLDH of *G. oxydans* IFO 3254 (98%) 2KGDH of *P. agglomerans* (42%), and GDH of *B. cepacia* (42%), and to those of D-gluconate dehydrogenase (GADH) of

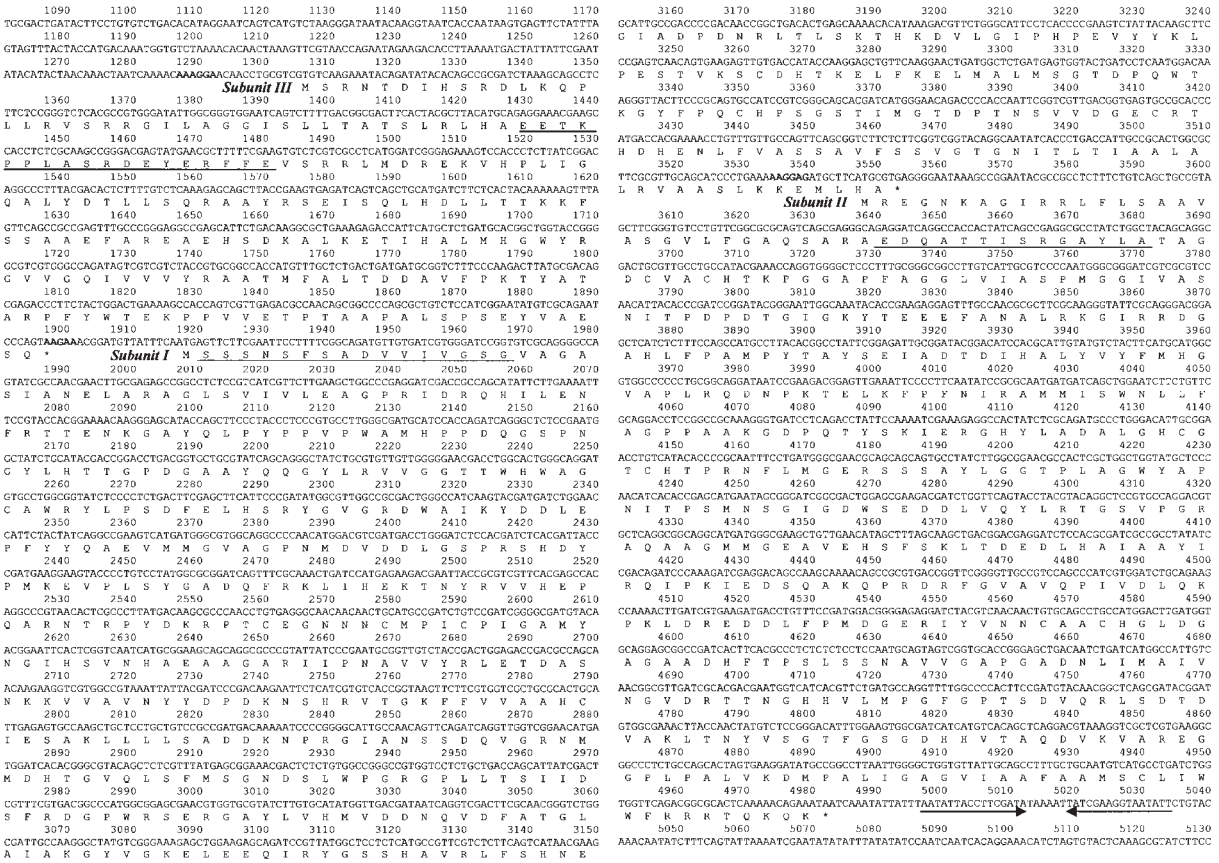

Fig. 4. Nucleotide and Amino Acid Sequences of *sldSLC*.

The nucleotide and amino acid sequences of *sldSLC* are shown. A probable ribosome-binding sequence prior to each gene is shown in boldface. The rho-dependent terminator-like sequence is indicated by arrows. The amino acid sequences from subunits of the purified FAD–SLDH detected by an amino acid sequencer are underlined.

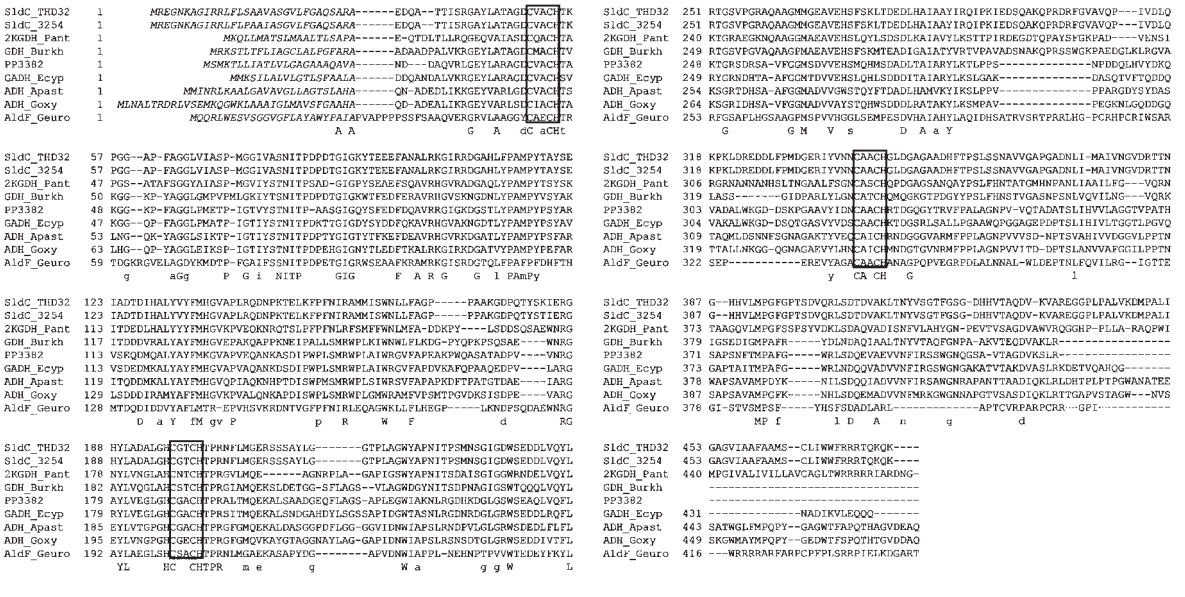

Fig. 5. Alignment of SldC with Cytochrome *c* Subunits from Several Membrane-Bound Dehydrogenases.

Putative signal sequences are italicized. In the bottom line, completely conserved amino acids among the sequences aligned are shown by capital letters, and the conserved amino acids among them with one exception are shown by small letters. From top to bottom, accession numbers to DDBJ are: AB192961 for SldC_THD32 from *G. fraterii* THD32; AB039821-3 for SldC_3254 from *G. oxydans* IFO3254; AF068066-3 for 2KGDH_Pant from *P. agglomerans*; AF493970-1 for GDH_Burkh from *B. cepacia*; AE016786-183 for PP3382 from *P. putida* KT2440; U97665-3 for GADH_Ecyp from *P. cypripedii*; AB086012-2 for ADH_Apast from *A. pasteurianus*; M58760-1 for ADH_Goxy from *G. suboxydans*, and Y08696-2 for AldF_Geuro from *G. europaeus*.

E. cypripedii (36%), PP3382 of *Pseudomonas putida* KT2440 (35%), ADH of *A. pasteurianus* (34%), ADH of *G. oxydans* (34%), and ALDH of *A. pasteurianus* (31%) (Fig. 5).

Discussion

We failed to find a membrane-bound enzyme oxidizing D-sorbitol to D-fructose. Our first assumption was that FAD–SLDH might produce D-fructose from D-sorbitol,[4] but it came out wrong and it is clear that this enzyme produces L-sorbose from D-sorbitol. Since both PQQ–SLDH and FAD–SLDH produce the same oxidized product from D-sorbitol and pass electrons to ubiquinone in the cytoplasmic membrane, it appears that they have no functional differences physiologically. Furthermore, it is obvious that all *Gluconobacter* strains have PQQ–SLDH, which is responsible for producing dihydroxyacetone from glycerol, and this character is used for classical identification of *Gluconobacter* strains, whereas FAD–SLDH is found only in certain *Gluconobacter* strains like THD32. This raises the question of the significance and rare existence of FAD–SLDH. We found a transposase-like gene next to the *sldSLC* gene in *G. frateurii* THD32. Perhaps the gene cluster encoding FAD–SLDH and the regulator gene might be transferred from one microorganism to others in a certain environment, leading to the appearance of this enzyme only in some *Gluconobacter* strains. It is clear that FAD–SLDH is inducible and has higher specific activity, while expression of PQQ–SLDH appears constitutive, as shown in Fig. 2. No significant difference was found in growth on D-sorbitol between the wild-type and the PQQ–SLDH mutant strains. Hence it would be interesting further to analyze the advantage of having FAD–SLDH in addition to PQQ–SLDH.

There is an NAD-dependent enzyme that catalyzes reversible oxidoreduction between D-sorbitol and D-fructose. This enzyme exists in *Gluconobacter* strains and has been purified from *G. suoxydans* IFO3257.[27] The small amount of D-fructose detected in the culture supernatant of several strains might be provided by this enzyme, although this ought to be confirmed. If this is the case, NAD–SLDH might be applicable for D-fructose fermentation. There is a report about another type of membrane-bound SLDH having three subunits similar to ADH with PQQ as the prosthetic group.[28] But this enzyme appears not to exist in strain THD32, judging from the substrate specificities of the membrane fractions in wild-type and the PQQ–SLDH mutant strains in Fig. 2.

The deduced amino acid sequence of the cytochrome *c* subunit has a typical *sec*-dependent signal sequence. On the other hand, the large subunit does not have such a signal sequence but the small subunit has a *tat*-dependent signal sequence. Therefore, the large subunit appears to be translocated together with the small subunit by a 'hitchhiker' mechanism after the prosthetic group, FAD, is incorporated in the cytoplasm.[29] The mechanism of covalent attachment of FAD is not certain; it is probably a spontaneous reaction after incorporation into the apoprotein.[21] SldS showed similarity to the small subunits of GADH and 2KGDH, of which the function is also unknown. Kondo *et al.* have suggested that the 20 kDa small subunit of ADH from *Acetobacter pasteurianus* has a role in the stability of the dehydrogenase subunit and functions as a molecular coupler to the cytochrome *c* subunit on the cytoplasmic membrane,[30] but the small subunit has no similarity to SldS in amino acid sequence. Although the small subunit of ALDH from acetic acid bacteria contains iron-sulfur clusters,[31] the amino acid sequence of SldS does not have such a binding motif.

Interestingly, many of membrane-bound enzymes found in *Gluconobacter* strains, including FDH, GADH, and 2KGDH, are known to contain cytochrome *c* subunits similar to those of ADH and ALDH.[2] Thus, the cytoplasmic membrane of *Gluconobacter* strains contains high amounts of cytochrome *c*, and these enzymes are also assumed to pass electrons to ubiquinone. From bacteria other than acetic acid bacteria, membrane-bound GADH and 2KGDH from *E. cypripedii*[32] and *P. agglomerans* or *P. citrea*[33] respectively have been reported to contain similar cytochrome *c* subunits. These cytochrome *c* subunits show high identity to each other in amino acid sequence (Fig. 5). Moreover, many similar cytochrome *c* genes are found in the genome sequences of several bacteria (data not shown). According to the data of kinetic and structural analyses of ADH, the cytochrome *c* subunit is believed to contain membrane-binding and ubiquinone- and ubiquinol-reacting sites,[34] although no segments with successive hydrophobic amino acid residues are found in the sequence. It is interesting to consider the evolution of these cytochromes *c*, since cytochromes *c* found in respiratory chains in many aerobic organisms, in mitochondria for example, have high redox potential and cannot easily reduce ubiquinone energetically.

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