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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 csubunits 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 membranebound 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 3254⁶⁾ 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 dehydrogenase¹⁰⁾ and D-gluconate dehydrogenase, yielding 5-keto D-gluconate¹¹⁾ using molecular biological techniques. The reaction product of this enzyme from Dsorbitol 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 isomerase¹³⁾ 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-CaCO₃ agar slant, which was prepared by adding 2% agar and 0.5% CaCO₃ 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 \times 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 Dsorbitol, 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 μ mol 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 MgCl₂ (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 Dsorbitol, and 5 mM MgCl₂ (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 dialvzed against 5 mM MES-NaOH buffer (pH 6.0) containing 0.1% Triton X-100, 25 mM Dsorbitol, and 5 mM MgCl₂ (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 hemecatalyzed 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 midexponential 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 $50 \mu g/ml$ 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 Lsorbose 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 Dfructose 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





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, SSNSFSADV-VIVGSGV; cytochrome c subunit, EDQATTIXRXA-YXA (X: unidentified); small subunit, EETKPPLASR-DEYERFFEV.

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 myoinositol, were inert. The $K_{\rm m}$ and $V_{\rm max}$ values for Dsorbitol 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 a 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 Acetobacter²²⁾ 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 Dfructose 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 Dsorbitol 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 Dsorbitol 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 Lsorbose 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 HindIII 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 regulator²³⁾ 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 socalled 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

1000	1100	1110	1120	1120	1140	1160	1160	1170	22.00	21.20	2100	2100
1090	1100	1110	1120	1130	1140	1120	1160	1170	3160	3170	3180	3130
TGCGACTGATACI	TTCCTGTGTC1	GACACATAGGA	ATCAGTCATG	STCTAAGGG	ATAATACAAGO	STAATCACCAA	ATAAGTGAGT	TCTATTTA	GCATTGCCGACC	CCGACAACCGG	TGACACTGA	GCAAAACACAT.
1180	1190	1200	1210	1220	1230	1240	1250	1260	GIAD	PDNR	LTL	SKTH
GTAGTTTACTACC	ATGACAAATG	GTGTCTAAAAC	ACAACTAAAG	TTCGTAAC	CAGAATAGAAC	ACACCTTAAA	ATGACTATT	ATTCGAAT	3250	3260	3270	3280
1270	1280	1290	1300	1310	1320	1330	1340	1350	CCGAGTCAACAG	TOBAGAGTTOT	ACCATACCA	AGGAGCTGTTC
1270	ADCTARTCA	A DCAR BCCARC	a a concerne	CTCTCTCTC	ABB TACACAE?	TRACACACCCC	CCDDCEBBB	CORCECTO			D 17 m	K P I P
AIACAIACIAACE	MAC DATION	MUTCHANGORANC		GIGICANG	MAIACAGAIA	IIACACAGECO	CONTERNAM	GCAGCCIC	P E S I	V K S Ç	DRI	N L L F
		3	Subunit III	MSR	(NTD	IHSP	срьк	. Q P	3340	3350	3360	3370
1360	1370	1380	1390	1400	1410	1420	1430	1440	AGGGTTACTTCC	CGCAGTGCCAT	CGTCGGGCA	GCACGATCATG
TTCTCCGGGTCTC	ACGCCGTGGG	ATATTGGCGGG	TGGAATCAGT	CTTTTGAC	GGCGACTTCAC	TACGCTTACA	TGCAGAGGA	AACGAAGC	KGYF	РОСН	PSG	STIM
LLRVS	5 8 8 G	ILAG	GIS	L L T	ATS	LRLH	IAEE	тк	3430	3440	3450	3460
1450	1460	1470	1490	1490	1500	1510	1520	1520	ATCACCACCAAA	ACCTCTTTCTTC	CCACTTCAC	CCCTCTTCTCT
1450	1400	1970 magaalogomg	1400	1990	1500	1010	1020	2550	HIGHCCHCGANA		NCCAGI ICAG	scoole lie le
CACCICICGCANO	CCGGGACGAC	TATGAACGCTT	TTTCGAAGTG	nerestes	CUTCATGGATC	GOGHOMMOT	CUACULICI	TATUGGAC	нрны	NDEV	ASS	AVES
PPLAS	RDE	YERF	FEV	SRR	LL M D	REKV	инрі	IG	3520	3530	3540	3550
1540	1550	1560	1570	1580	1590	1600	1610	1620	TTCGCGTTGCAG	CATCCCTGAAA	AGGAGATGC	TTCATGCGTGA
AGGCCCTTTACGA	CACTCTTTTC	TCTCAAAGAGC	AGCTTACCGA	AGTGAGAT	CAGTCAGCTGO	CATGATCTTCT	CACTACAAA	AAAGTTTA	LRVA	ASLK	KEM	LHA*
QALYD	TLL	SORA	AYR	SEI	SOL	HDLI	. Т Т К	KF			Subunit	II M R E
1630	1640	1650	1660	1670	1680	1690	1700	1710	3610	3620	3630	3640
CTTCACCCCCCC	CREECCCCCC	Checcechera	TTCTCACAA	CCCCTCNN	ACACACOMPTO	Three to be	concecere	CTRCCCCC	COTTOCCCTCTC	CTCTTCCCCCCC	CACTORCOC	ACCCCRCRCCR
GITCAGCCGCCGA		GAGGCCGAGCA	CITCIONCAMO	SCGCI GMM	MOMONCONTIC	AIGCICIONI	GCACGGC1G	GIACCOGG	derredddidie		CAGICAGEG	NGGGCAGAGA
SSAAE	L P A R	EAEH	ISD K	ALK	LTI	HALM	нси	I R	ASGV	LFGA	Q S A	RAED
1720	1/30	1/40	1750	1/60	1770	1780	1/90	1800	3700	3710	3720	3730
GCGTCGTCGGCCA	GATAGTCGTC	GTCTACCGTGC	GGCCACCATG	STITGCTCI	GACTGATGATO	CGGTCTTTCC	CAAGACTTA	TGCGACAG	GACTGCGTTGCC	TGCCATACGAA	ACCAGGTGGG	GCTCCCTTTGC
GVVGQ	Q I V V	VYRA	ATM	FAL	TDD	AVFF	Y K T Y	АТ	DCVA	СНТК	PGG	APFA
1810	1820	1830	1840	1850	1860	1870	1880	1890	3790	3800	3810	3820
CGAGACCCTTCTA	CTGGACTGAR	AAGCCACCAGT	CGTTGAGACG	CCARCAGO	aaccoccaaca	TGTCTCCATC	GGAATATGT	CGCAGAAT	AACATTACACCC	SATCCGGATAC	CONTRACO	ADDROCOCCA
A B B F N		K D D U	0011010100			t c p c		2 5	N T T D	D D D T	C T C	V V T F
A K F F I	1010	1000	1020	1010	1000	1000	1070	1000	N 1 1 F		GIG	K I I E
1900	1910	1920	1930	1940	1950	1960	1970	1980	3880	3890	3900	3910
CCCAGTAAGAAAC	GGATGTTATT	TCAATGAGTTC	TTCGAATTCC	TTTTCGGC	CAGATGTTGTGA	ATCGTGGGATC	CGGTGTCGC	AGGGGCCA	GCTCATCTCTT	CCAGCCATGCC	2TACACGGCC	TATTCGGAGAT
SQ *	Subuni	<u>ti m s s</u>	SNS	FSA	DVV	IVGS	<u> </u>	GA	AHLF	PAMP	ΥΤΑ	YSEI
1990	2000	2010	2020	2030	2040	2050	2060	2070	3970	3980	3990	4000
GTATCGCCAACGA	ACTTGCGAGE	GCCGGCCTCTC	CGTCATCGTT	CTTGAAGC	TOCCCGAGO	TOBACCOCCA	GCATATTCT	TGAAAATT	GTGGCCCCCTG	CGGCAGGATAA	CCGAAGACC	GAGTTGAAATT
C T D N E	T 7 9	D C T C	17 T 17	IEZ	CDD	TDDC	. U T 1	E M	V A V I.	RODN	PKT	F L K F
J I A A E		A G L 3	0110	0100		1 0 6 0		0160	1000	4070	4080	4000
2080	2090	2100	2110	2120	2130	2140	2120	2160	4060	4070	4080	4090
TCCGTACCACGGA	AAACAAGGGA	GCATACCAGCI	TCCCTACCCI	CCCGTGCC	TTGGGCGATGC	ATCCACCAGA	ATCAGGGCTC	TCCGAATG	GCAGGACCTCCG	SCCGCAAAGGG	IGATCCTCAG	ACCTATTCCAA.
FRTTE	Z N K G	AYQI	, P Y P	P V P	W A M	HPPE) Q G S	P N	AGPP	AAKG	DPQ	TYSK
2170	2180	2190	2200	2210	2220	2230	2240	2250	4150	4160	4170	4180
GCTATCTGCATAC	GACCGGACCT	GACGGTGCTGC	GTATCAGCAG	GGCTATCT	GCGTGTTGTTC	GGGGAACGAC	CTESCACTE	GGCAGGAT	ACCTGTCATACA	CCCCGCAATTT	CTGATGGGC	GAACGCAGCAG
CYLUT	TCD	DCA	V O O	C Y I	p v v	C C 7 1	- W E W	B C	TCHT	PRNF	T. M. G	ERSS
0 1 0 11 1	2220	2300		3700		0 0 1 1		2340	4340	4250	40.00	4270
2260	2210	2460	2290	2300	2310	2320	2330	2340	4240	42.30	42.00	4270
GIGCUIGGCGGIA	rereccerei	GACITCGAGCI	TCATTCCCGA	TATGGCGT	TGGUCGUGACI	GGGCCATCAP	IGTACGATGA	TCTGGAAC	ANCATCACACCE	ACCALGAATAG	JUGUAT COUC	GACTOGAGCGA
CAWRY	LPS	DFEI	, H S R	YGV	GRD	WAIP	K Y D D	LE	NITP	SMNS	GIG	DWSE
2350	2360	2370	2380	2390	2400	2410	2420	2430	4330	4340	4350	4360
CATTCTACTATCA	GGCCGAAGTC	ATGATGGGCGT	GGCAGGCCCC	AACATGGA	CGTCGATGACO	TGGGATCTCC	ACGATCTCA	CGATTACC	GCTCAGGCGGCA	GCATGATGGG	GAAGCTGTT	GAACATAGCTT
PFYYO	AEV	MMGV	AGP	NMT	IV D D	LGSE	RSH	DY	AOAA	GMMG	EAV	EHSF
2440	2450	2460	2470	2480	2490	2500	2510	2520	4420	4430	4440	4450
CONCORCONCE	100000000000000000000000000000000000000	mancecees	TCLCTTTCCC	BBBCCCB	CCATCACAAC	CORDERACCO	CCTCCTTC	CORCERC	CONCRETECCOR	NACAPCCACCA	DACCORDCOR	AAACACCCCCCC
CONTONNOGANGI	ACCCCIGICC	INIGGCGCGGA	TCAGITICGC	ANACIGAI	CCATGAGAAGA	CGAMITACCO	CGICGIICA	CGAGCCAC	CONCHONICCON	MAGATCONGON	.noconnocn	MANAGECOCO.
PMKEV	PLS	YGAU) Q F R	кті	ньк	TNYF	суун	EP	RUIP	KILD	SQA	корк
2530	2540	2550	2560	2570	2580	2590	2600	2610	4510	4520	4530	4540
AGGCCCGTAACAC	TCGCCCTTAT	'GACAAGCGCCC	AACCTGTGAG	GGCAACAA	CAACTGCATGO	CGATCTGTCC	GATCGGGGC	GATGTACA	CCAAAACTTGAT	CGTGAAGATGAC	CTGTTTCCG	ATGGACGGGGA
QARNI	RPY	DKRP	TCE	GNN	IN CM	PICF	IGA	M Y	PKLD	REDD	LFP	MDGE
2620	2630	2640	2650	2660	2670	2680	2690	2700	4600	4610	4620	4630
ACCOBATTORCTO	GGTCARTCRT	GCGGAAGCACC	ACCCCCCCCC	ATTATCCC	CABTCCCCTTC	TCTACCCACT	CCACACCCA	CCCCACCA	GCAGGAGCGGCC	SATCACTTCAC	SCOOTCTCCC	TOCTOCARTOC
Accountiteacte	COOTCANTCAL		Addcaccear	ATTAICCO	SAMIGCOUTIC	I V D I		COCCAGEA	3 C 3 3	DHET	D 0 1	C C N N
NGIHS	V N H	AEAR	GAR	1 1 1	NAV	VIRL		A S	AOAA	D n r I	F 3 L	0 0 N A
2710	2720	2730	2740	2750	2760	2770	2780	2790	4690	4700	4/10	4720
ACAAGAAGGTCGI	GGCCGTAAA1	TATTACGATCC	CGACAAGAAI	TCTCATCG	TGTCACCGGT/	AGTICTICGI	GGTCGCTGC	GCACTGCA	AACGGCGTTGAT	CGCACGACGAA	GGTCATCAC	GTTCTGATGCC
NKKVV	AVN	YYDF	DKN	SHR	VTG	KFFV	VAA	нс	NGVD	RTTN	GHH	VLMP
2800	2810	2820	2830	2840	2850	2860	2870	2880	4780	4790	4800	4810
TTGAGAGTGCCAR	GCTGCTCCTC	CTGTCCGCCGA	TGACAAAAAT	CCCCGGGG	CATTGCCAACA	GTTCAGATCA	GGTTGGTCG	GAACATGA	GIGGCGAAACTT	ACCAACTATGT	TCCGGGACA	TTTGGAAGTGG
TEGNE	T. L. T	LSAF	DKN	D D C	TEN	SSD	V G T	N M	VAK 1	TNYV	5 6 7	FGSG
- E U A P		L	2020	2020	2040	0 0 0 0	2 4 6 8	2020		4990	4990	
5893	2300	2910	2920	2930	2940	2950	2960	2970	4870	4000	4090	4900
TGGATCACACGGO	SCGTACAGCTC	TCGTTTATGAG	CGGAAACGAC	CTCTCTGTG	GCCGGGCCGT	GICCICIGCI	GACCAGCAT	TATCGACT	GGCCCTCTGCCA	GCACTAGTGAAG	GATATGCCG	GCCTTAATTGG
MDHTO	S V Q L	SFMS	GND	SLW	PGR	GPLL	TSI	I D	GPLP	A L V K	DMP	ALIG
2980	2990	3000	3010	3020	3030	3040	3050	3060	4960	4970	4980	4990
CGTTTCGTGACGO	CCCATGGCGC	AGCGAACGTGG	TGCGTATCTT	GTGCATAT	GGTTGACGATA	ATCAGGTCG	CTTCGCAAC	GGGTCTGG	TEGTTCAGACGG	CGCACTCAAAAA	ACAGAAATAA	TCAAATATTAT
S F B D C	: p w p	SED	a v r	V H M	r v n n	NOVT	FAT	GL	WFRP	RTOK	ок*	
3 F R D 6	3 E W K	JERG	, A I L	v n M		N Q V L	, , A 1	G 1.		Q R	- 020 ·	1000
3070	3080	3090	3100	3110	3120	3130	3140	3150	5050	0060	3070	0080
CGATTGCCAAGGC	SCTATGTCGGC	AAAGAGCTGGA	AGAGCAGATC	CGTTATGG	SCTCCTCTCATO	SCCGTTCGTCI	CTTCAGTCA	TAACGAAG	AAACAATATCTT	TCAGTATTAAAJ	VTCGAATATA	TATTTATATAT
AIAKO	S Y V G	KELE	EQI	RYG	S S H	AVRI	, F S H	NE				

		3160		3170		3180		3190		3200		3210		322	0		3230		3240
GC7	TTC	GCCGA	cccci	SACAAC	CGGC	TGACAC	EGAG	CAAAAC	ACATI	AAAGAC	GTTC	TGGGCA	TTCC	TCACC	CCG	AAGI	TCTAT	INCA	AGCTTC
G	T	A D 2250	Ъ	D N	к	L T I	. S	K T	н	K D	v	L G 3300	1 P	H 221	PI	EN	/ Y	Ŷ	K L 2220
~~~	act	3230	anci	3200	TOTO	3270 ACCATA		3280	COPCI	3290	erer	3300	TONT	0301 CBCTC	U CTA		332U		3330 CC3C33
P	F .	S T	V	K S	C 1010	D F '	P K	F L	F	KF	T.	M B	L M	guore	6 1	T T	) P	0	W T
	2	3340		3350	Ŷ	3360		3370		3380	-	3390		340	0	• •	3410	×	3420
AGO	GTT	TACTT	CCG	CAGTGC	CATC	CGTCGG	GCAG	CACGAT	CATG	GAACA	GACO	CCACCA	ATTC	GGTCC	TTG	ACGO	STGAG	rgee	GCACCO
к	G	ΥF	₽	Q C	н	PSO	5 5	TI	м	G T	D	РТ	N S	v	vi	DO	5 E	С	RT
		3430		3440		3450		3460		3470		3480		349	0		3500		3510
ATC	ACC	CACGA	AAACI	CTGTTT	GTTG	CCAGTTO	CAGCO	GTCTT	CTCT	TCGGTC	GGTA	CAGGCA	ATAT	CACCO	TGA	CCAI	TGCC	SCAC	TGGCGC
н	D	ΗE	N	L F	v	AS S	5 A	VF	S	S V	G	TG	N I	Т	L '	T 1	L A	А	LA
		3520		3530		3540		3550		3560		3570		356	0		3590		3600
TTUGUGTTGUAGUATUUUTGAAAAAGGAGATGCTTCATGCGTGAGGGGGAATAAAGCCGGAATACGCCGCCTCTTTCTGTCAGCTGCCGTF																			
Suhunit II M REGNKAGI RRLFL SAAV																			
		3610		3620		3630	*** **	3640	K E	3650	R	3660		367	0	E.	3680	~	3690
GCT	TCC	GGTG	FCCT	STTCGG	CGCG	CAGTCA	-	GGCAG	AGGA'	TCAGGC	CACC	ACTATC	AGCC	GAGGO	GCC	TAT	TGGC	IACA	GCAGGC
Α	s	G 1	/ L	FG	А	Q S	A I	RΑ	E D	Q A	т	т і	S	R G	А	Υ	L A	т	A G
		3700		3710		3720		3730		3740		3750		376	0		3770	-	3780
GAC	rGC	GTTG	CCTG	CATAC	GAAA	CCAGGT	GGGG	CTCCCT	TTGC	GGGCGG	CCTI	GTCATT	GCGT	CCCC7	ATG	GGCC	GGAT	GTC	GCGTCC
D	Ċ	vi	A C	нт	к	ΡG	G	ΑP	FΑ	GG	2	V I	A.	S P	м	Ġ	G I	v	AS
		3790		3800		3810		3820		3830		3840		385	0		3860		3870
AAC	AT?	PACACO	CCGA	ICCGGA	TACG	GGAATTO	GCA	AATACA	CCGA	AGAGGA	GTTT	GCCAAC	GCGC	TTCGC	AAG	GGT	ATTCG	CAGG	GACGGA
N	I	TI	P D	P D	т	GI	G	K Y	ТЕ	EE	F	A N	А	LR	ĸ	G	IR	R	DG
		3880		3890		3900		3910		3920		3930		394	U		3950		3960
GCI	CA.	refer	rrçç	AGCCAT	GUUT	TACACG	JUCTJ	ATTCGG	AGAT:	TGCGGA	TACE	GACATC	UACG	CATTO	TAT	GTC.	VE	ATG	CATGGC
м	п	2020	r r	2000	r	2000	M.	1 3	2 1	A 010	1	D 1	л	A D	1	v	1 1	ы	1050
GT0	GCC	3970	raca	3960 CAGGA	TAAT	2220	Vraci	ADOO	D D T T	CCCTT	Caat	arcccc		403 TGATO	ATC	accr	4040	re me	CTGTTC
v 10		D00000.	1000	0 0	M	D K	T 1	C L	K F	p v	N	I P	a	M M	1	s	W N	1.	CIGIIC
		4060		4070		4080	· ·	4090		4100		4110	**	412	۰ <u>۵</u>		4130		4140
GCI	GGJ	ACCTC	agge	GCAAA	GGGT	GATCOT	AGA	CTATT	CCAAJ	AATCGA	AAGA	GGCCAC	TATC	TCGCA	GAT	seco	TGGG	ACAT	TGCGGA
Α	G	PI	ΡA	A K	G	DP	0	гү	S K	ΙE	R	G H	Y	LA	D	A	LG	н	CG
		4150		4160		4170		4180		4190		4200		421	Ð		4220		4230
ACC	TG	CATA	CACCI	CCGCAA	TTTC	CTGATG	GCG	AACGCA	GCAG	CAGTGC	CTAT	CTTGGC	GGAA	CGCCF	CTC	GCTO	GCTG	STAT	GCTCCC
Τ	С	H 1	Г P	R N	F	L M	GI	ER	S S	SΛ	Y	L G	G	T P	L	А	G W	Y	A P
		4240		4250		4260		4270		4280		4290		430	0		4310		4320
AAC	ATC	CACAC	CGAC	CATGAA	TAGC	GGCATCO	30CC	ACTGGA	GCGAJ	AGACGA	TCTC	GTTCAG	TACC	TACGI	ACA	GGC.	recert	CCA	GGACGT
N	I	TI	P S	M N	s	GI	G 1	D W	S E	D D	L	V Q	Y	L R	т	G	s v	Р	GR
-		4330		4340		4350		4360		4370		4380		439	10		4400		4410
601	CAC	100306	AGG	JATGAT	0000	GAAGCT	37763	MCATA	GUIT:	TAGCAA	GCTG	ACGGAC	0466	ATCTO	CAC	200	ATCGC:	.600	TATAIC
~	Ŷ	4420	• •	4420	0	4440	· .	4450	.) r	4460	-	4470	E	0 14		~	1490	А	4500
coz	(CB)	ATCC	-222	CATCOL	ceae	ACCORD	icaai	anaco	nana	TGACCO	CTTC	COCOTT	occo	14000	inceres	атсо	TCGA	ста	CAGAAG
R	0	TI	PK	TE	D	S 0	AI	K O	P R	D R	F	G V	A	v o	P	Т	V D	1.	ОК
	-	4510		4520		4530		4540		4550		4560		457	0		4580		4590
CCF	LAAJ	CTTG	ATCG	IGAAGA	TGAC	CTGTTT	CGA	IGGACG	GGGA	GAGGAT	CTAC	GTCAAC	AACT	GTGC	GCC.	TGCC	CATGG.	ACTT	GATGGT
Ρ	к	LI	R	E D	D	L F	P I	4 D	G E	RI	Y	V N	N	C A	А	С	H G	L	D G
		4600		4610		4620		4630		4640		4650		468	0		4670		4680
GCA	GGZ	AGCGG	CCGA	ICACTI	CACG	CCCTCTC	TOTO	CTCCA	ATGC	AGTÀGT	CGGI	GCACCG	GGAG	CTGAC	AAT	CTG	ATCAT	GCC	ATTGTC
А	G	A i	A D	ΗF	Т	P S	L	5 S	N A	v v	G	A P	G	A D	N	L	ΙМ	A	ΙV
		4690		4700		4710		4720		4730		4740		475	0		4760		4770
AAC	GGG	GTTG	ATCG	CACGAC	GAAT	GGTCAT	CACG	TTCTGA	TGCC	AGGTTT	TGGC	CCCACT	TCCG	ATGTA	CAA	CGGG	TCAG	GAT	ACGGAT
N	G	1700	) R	TT	N	G H	н	V L	мр	G F	G	PT	5	U V	Q.	к	1 5	D	T D
		4780		4/90		4800		4810	-	4820		4830	0.000	484	0		4850		4800
GIG	00U(	www.	LINC:	NXX	TGIC	TCCGGG	TOAT:	FTGGAA	6166	CGATCA	TCA1	U TUNCA	SCTC 2	AGGAC	U U	nnGC V	V N	rcGr p	CAAGGC
*	~	4970	u 1	4000		2000		4900	3 6	4010	п	4920	n	V 103	· · ·	n	1910	K	1050
GGC		TCTGC(	age	ACTACT	GAAG	GATATO	TOP	адттэг адттэг	TTGC	SOCTOR	TGTT	ATTGCA	GCCT	TTGCT	GCA-	arc	9990 10760	CTG	ATCTCC
G	P	L I	р р	L V	K	DM	p 1	Α T.	TG	A G	v	T P	A	FA	A	M	SC	L	τ.w
		4960		4970		4980		4990		5000		5010		507	0		5030	-	5040
TGG	STT	CAGACO	GCG	CACTCA	алал	CAGAAA	TAAT	CAAATA	TTAT	TTAATA	TTAC	CTTCGA	тата	AAATT	ATC	GAA	GTAA	TTAT	CTGTAC
W	F	R I	R R	TQ	к	QK	*						۳ 🕈		-			_	
		5050		5060		5070		5080		5090		5100		511	0		5120		5130
537	CD 2	\TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	PPTC:	ACT ATT	הההה	TCCDBT	5 T A T -	0.TTTDT	27270	CONSTC	8 8 TC	acacca	0.000	TOTAC		acry	70000	0.000	TOTTOO

#### 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. frateurii* 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 Dsorbitol,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. cypripedii³²⁾ and P. agglomerans or P. citrea³³⁾ 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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#### References

- Adachi, O., Moonmangmee, D., Toyama, H., Yamada, M., Shinagawa, E., and Matsushita, K., New developments in oxidative fermentation. *Appl. Microbiol. Biotechnol.*, **60**, 643–653 (2003).
- 2) Matsushita, K., Toyama, H., and Adachi, O., Respiratory chain and bioenergetics of acetic acid bacteria. In

"Advances in Microbial Physiology" Vol. 36, eds. Rose, A. H., and Tempest, D. W., Academic Press, Ltd., London, pp. 247–301 (1994).

- Adachi, O., Moonmungmee, D., Shinagawa, E., Toyama, H., Yamada, M., and Matsushita, K., New quinoprotein in oxidative fermentation. *Biochim. Biophys. Acta*, 1647, 10–17 (2003).
- Adachi, O., Moonmangmee, D., Toyama, H., Yamada, M., Shinagawa, E., and Matsushita, K., New developments in oxidative fermentation. *Appl. Microbiol. Biotechnol.*, **60**, 643–653 (2003).
- Sugisawa, T., and Hoshino, T., Purification and properties of membrane-bound D-sorbitol dehydrogenase from *Gluconobacter suboxydans* IFO 3255. *Biosci. Biotechnol. Biochem.*, 66, 57–64 (2002).
- Shinagawa, E., Matsushita, K., Adachi, O., and Ameyama, M., Purification and characterization of Dsorbitol dehydrogenase from membrane of *Gluconobacter suboxydans* var. α. *Agric. Biol. Chem.*, **46**, 135–141 (1982).
- Miyazaki, T., Tomiyama, N., Shinjoh, M., and Hoshino, T., Molecular cloning and functional expression of Dsorbitol dehydrogenase from *Gluconobacter suboxydans* IFO 3255, which requires pyrroloquinoline quinone and hydrophobic protein SldB for acivity development in *E. coli. Biosci. Biotechnol. Biochem.*, 66, 262–270 (2002).
- Shinjoh, M., Tomiyama, N., Miyazaki, T., and Hoshino, T., Main polyol dehydrogenase of *Gluconobacter sub*oxydans IFO 3255, membrane-bound D-sorbitol dehydrogenase, that needs product of upstream gene, *sldB*, for activity. *Biosci. Biotechnol. Biochem.*, 66, 2314–2322 (2002).
- Ameyama, M., Shinagawa, E., Matsushita, K., and Adachi, O., Solubilization, purification and properties of membrane-bound glycerol dehydrogenase from *Gluconobacter industrius. Agric. Biol. Chem.*, 49, 1001–1010 (1985).
- 10) Adachi, O., Fujii, Y., Ghaly, M., Toyama, H., Shinagawa, E., and Matsushita, K., Membrane-bound quinoprotein D-arabitol dehydrogenase of *Gluconobacter* suboydans IFO 3257: a versatile enzyme for the oxidative fermentation of various ketoses. *Biosci. Bio*technol. Biochem., 65, 2755–2762 (2001).
- Matsushita, K., Fujii, Y., Ano, Y., Toyama, H., Shinjoh, M., Tomiyama, N., Miyazaki, T., Sugisawa, T., Hoshino, T., and Adachi, O., 5-Keto-D-guluconate production is catalyzed by a quinoprotein glycerol dehydrogenase, major polyol dehydrogenase, in *Gluconobacter* species. *Appl. Environ. Microbiol.*, **69**, 1959–1966 (2003).
- Shinagawa, E., Matsushita, K., Adachi, O., and Ameyama, M., Purification and characterization of 2keto-D-gluconate dehydrogenase from *Gluconobacter melanogenus*. *Agric. Biol. Chem.*, **45**, 1079–1085 (1981).
- Bhosale, S. H., Rao, M. B., and Deshpade, V. V., Molecular and industrial aspects of glucose isomerase. *Microbiol. Rev.*, 60, 280–300 (1996).
- 14) Moonmangmee, D., Adachi, O., Ano, Y., Shinagawa, E., Toyama, H., Theeragool, G., Lotong, N., and Matsushita, K., Isolation and characterization of thermotolerant *Gluconobacter* strains catalyzing oxidative fermentation at higher temperatures. *Biosci. Biotechnol. Biochem.*, 65, 115–125 (2000).

- 15) Ameyama, M., Shinagawa, E., Matsushita, K., and Adachi, O., D-Fructose dehydrogenase of *Gluconobacter suboxydans*: purification, characterization, and application to enzymatic microdetermination of D-fructose. *J. Bacteriol.*, **145**, 814–823 (1981).
- 16) Dully, J. R., and Grieve, P. A., A simple technique for eliminating interference by detergents in the Lowry method of protein determination. *Anal. Biochem.*, 64, 136–141 (1975).
- Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriphage T4. *Nature* (London), **227**, 680–685 (1970).
- 18) Thomas, P. E., Ryan, D., and Levin, W., An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. *Anal. Biochem.*, **75**, 168–176 (1976).
- Sambrook, J., Fritsch, E. F., and Maniatis, T., Molecular cloning: a laboratory manual, 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989).
- Marmur, J., A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol., 3, 208–218 (1961).
- Scrutton, N. S., Identification of covalent flavoproteins and analysis of the covalent link. *Methods Mol. Biol.*, 131, 181–193 (1999).
- 22) Matsushita, K., Ebisuya, H., and Adachi, O., Homology in the structure and the prosthetic groups between two different terminal ubiquinol oxidases, cytochrome *a*₁ and cytochrome *o*, of *Acetobacter aceti. J. Biol. Chem.*, 267, 24748–24753 (1992).
- 23) Rhee, S., Martin, R. G., Rosner, J. L., and Davies, D. R., A novel DNA-binding motive in MarA: the first structure for an AraC family transcriptional activator. *Proc. Natl. Acad. Sci. U.S.A.*, 18, 10413–10418 (1998).
- 24) Goryshin, I. Y., and Rezninoff, W. S., Tn5 *in vitro* transposition. *J. Biol. Chem.*, **273**, 7367–7374 (1998).
- Berks, B. C., Sargent, F., and Palmer, T., The Tat protein export pathway. *Mol. Microbiol.*, 35, 260–274 (2000).
- 26) Pugsley, A. P., The complete general secretory pathway in Gram-negative bacteria. *Microbiol. Rev.*, **57**, 50–108 (1993).
- Adachi, O., Toyama, H., Theeragool, G., Lotong, N., and Matsushita, K., Crystallization and properties of NADdependent D-sorbitol dehydrogenase from *Gluconobacter suboxydans* IFO 3257. *Biosci. Biotechnol. Biochem.*, 63, 1589–1595 (1999).
- Choi, E.-S., Lee, E.-H., and Rhee, S.-K., Purification of a membrane-bound sorbitol dehydrogenase from *Gluconobacter suboxydans*. *FEMS Microbiol. Lett.*, **125**, 45– 50 (1995).
- 29) Choi, J. H., and Lee, Y. S., Secretory and extracellular production of recombinant proteins using *Escherichia coli. Appl. Microbiol. Biotechnol.*, **64**, 625–635 (2004).
- 30) Kondo, K., Beppu, T., and Horinouchi, S., Cloning, sequencing, and characterization of the gene encoding the smallest subunit of the three components membranebound alcohol dehydrogenase from *Acetobacter pasteurianus. J. Bacteriol.*, **177**, 5048–5055 (1995).
- 31) Thurner, C., Vela, C., Thony-Meyer, L., Meile, L., and Teuber, M., Biochemical and genetic characterization of the acetaldehyde dehydrogenase complex from *Acetobacter europaeus*. *Arch. Microbiol.*, **168**, 81–91 (1997).

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- 32) Yum, D.-Y., Lee, Y.-P., and Pan, J.-G., Cloning and expression of a gene cluster encoding three subunits of membrane-bound gluconate dehydrogenase from *Erwinia cypripedii* ATCC 29267 in *Escherichia coli*. J. Bacteriol., **179**, 6566–6572 (1997).
- 33) Pujol, C. J., and Kado, C. I., Genetic and biochemical characterization of the pathway in *Pantoea citrea* leading to pink disease of pineapple. J. Bacteriol., 182,

2230-2237 (2000).

34) Matsushita, K., Yakushi, T., Toyama, H., Adachi, O., Miyoshi, H., Tagami, E., and Sakamoto, K., The quinohemoprotein alcohol dehydrogenase of *Glucono-bacter suboxydans* has ubiquinol oxidation activity at a site different from the ubiquinone reduction site. *Biochim. Biophys. Acta*, 1409, 154–164 (1999).