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Cloning of Genes Coding for L-Sorbose and L-Sorbosone Dehydrogenases from *Gluconobacter oxydans* and Microbial Production of 2-Keto-L-Gulonate, a Precursor of L-Ascorbic Acid, in a Recombinant *G. oxydans* Strain

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We have purified L-sorbose dehydrogenase (SDH) and L-sorbosone dehydrogenase (SNDH) from *Gluconobacter oxydans* T-100 that showed an ability to convert D-sorbitol to 2-keto-L-gulonate (2-KLGA). A genomic library of *Gluconobacter oxydans* T-100 was screened with a probe, a 180-bp PCR product which was obtained from degenerate oligodeoxyribonucleotides based on the elucidated sequence of the purified SDH (used as primers) and the genomic DNA of *G. oxydans* T-100 (used as a template). From sequencing of the DNA from a clone positive to the probe, the SNDH and the SDH were estimated to be coded in sequential open reading frames with 1,497 and 1,599 nucleotides, respectively, which was confirmed by expression of the DNA in *Escherichia coli* that showed both enzymatic activities. The DNA was introduced to a shuttle vector which was prepared from a plasmid of *G. oxydans* T-100 and pHSG298 to obtain an expression vector designated pSDH155. The production of 2-KLGA by pSDH155 in *G. oxydans* G624, an L-sorbose-accumulating strain, was improved to 230% compared to that of *G. oxydans* T-100. Chemical mutation of the host strain to suppress the L-idonate pathway and replacement of the original promoter with that of *E. coli tufB* resulted in improving the production of 2-KLGA. Consequently, high-level production from D-sorbitol to 2-KLGA (130 mg/ml) was achieved by simple fermentation of the recombinant *Gluconobacter*.

L-Ascorbic acid is widely used in pharmaceutical and food industries as a vitamin and as an antioxidant. The most popular process for synthesizing L-ascorbic acid is Reichstein's method, in which glucose is transformed to 2-keto-L-gulonic acid (2-KLGA), a key intermediate for L-ascorbic acid, in five steps (19). Although one molecule of glucose should be converted to one molecule of L-ascorbic acid, the practical yield of this chemical process, with the recent advances of chemical engineering, is thought to be around 50% (4). On the other hand, the use of microbial processes to produce L-ascorbic acid has recently become attractive, because plant investment costs are lowered and ecological problems of organic-solvent disposal is eliminated. Furthermore, the production yield may be improved still more compared with that of the chemical method. Several bacterial fermentation process for 2-KLGA have been reported (4, 12, 14, 18, 30). Soneyama et al. (24) reported a two-stage fermentation method using *Erwinia* spp. to convert D-glucose to 2,5-diketo-D-gluconic acid (2,5-DKGA) followed by *Corynebacterium* spp. to transform 2,5-DKGA to 2-KLGA. Anderson et al. (3) and Grindley et al. (6) independently developed this method by introducing 2,5-DKGA reductase gene from *Corynebacterium* spp. into *Erwinia* cells to attain direct fermentation of 2-KLGA from D-glucose. However, the yield of 2-KLGA was insufficient, probably because the oxidation reaction from D-glucose to 2,5-DKGA with 2,5-DKGA-forming enzyme and the reduction of 2,5-DKGA with 2,5-DKGA reductase are not fully cooperative in *Erwinia* cells. Another approach is to convert D-sorbitol or L-sorbose to 2-KLGA via L-sorbosone (9, 25). In *Gluconobacter oxydans* UV10, D-sorbitol is transformed to 2-KLGA in sequential oxidation steps, in which L-sorbose dehydrogenase (SDH) and L-sorbosone dehydrogenase (SNDH) are involved (10, 26). In the *Gluconobacter*, SDH is reported to be a 58-kDa protein, located on the surface of the cytoplasmic membrane and containing one molecule of FAD as an electron acceptor (26). The SNDH from *G. oxydans* is a cytoplasmic protein (molecular mass, approximately 50 kDa) that requires NAD(P) as an electron acceptor (10). To carry out the two-step conversion on the membrane of *G. oxydans* cells, Shinjoh et al. cloned the gene for the membrane-bound SNDH from *Acetobacter liquefaciens* and introduced the gene into 2-KLGA-producing *G. oxydans* (23). However, the yield of 2-KLGA in the recombinant strain under fermentation conditions was not improved compared to that by the wild strain *G. oxydans* UV10 (22, 23). Our approach was to construct a recombinant strain that ferments 2-KLGA from D-sorbitol by (i) cloning both genes for SDH and SNDH from *G. oxydans* T-100 that produces 2-KLGA from D-sorbitol and (ii) transforming *G. oxydans* G624, which accumulates L-sorbose by introducing these dehydrogenase genes.

In this paper, we describe the isolation and purification of SDH and SNDH from *G. oxydans* T-100, the cloning of these genes, and the construction of expression vectors for recombinant *Gluconobacter* that produces 2-KLGA from D-sorbitol. We also report further investigation on the optimization of productivity of 2-KLGA by means of improving the host strain as well as the promoter sequence.

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MATERIALS AND METHODS

Bacterial strains and materials. *G. oxydans* T-100 (FERM BP-4188), a 2-KLGA-producing strain, and *G. oxydans* G624 (FERM BP-4415), an L-sorbose-accumulating strain, were isolated from a Japanese persimmon (kaki) and from a Japanese peach, respectively. L-Sorbosone and L-idonic acid were prepared by Chemical Products Laboratories of Fujisawa Pharmaceutical Co., Ltd. L-Sorbose and 2,6-dichlorophenolindophenol (2,6-DCIP) were purchased from E. Merck (Darmstadt, Germany). Restriction and modification enzymes were purchased from Takara Shuzo (Kyoto, Japan), Toyobo (Osaka, Japan), or New England Biolabs (Beverly, Mass.). *Escherichia coli* JM109 was from Nippon Gene (Tokyo, Japan).

Purification of SDH and SNDH. *G. oxydans* T-100 was cultivated in 20 liters of a broth containing 5% L-sorbitol, 0.5% glycerol, 0.5% yeast extract, and 1.0% $CaCO_3$ at 30°C for 42 h. The cells (17.7 g [wet weight]) were sonicated in 40 ml of 10 mM phosphate buffer (pH 7.0) and centrifuged (4°C, 6,000 × *g*, 10 min). After ultracentrifugation (4°C, 100,000 × *g*, 60 min) of the resultant supernatant, the membrane and the cytosolic fractions were obtained. The membrane fraction was solubilized in 50 ml of 10 mM phosphate buffer containing 0.3% Triton X-100 and 200 mM L-sorbose according to the previously described method. The fraction was purified by performing the following steps: (i) anion-exchange high-performance liquid chromatography (HPLC) on a TSKgel DEAE-5PW column (inner diameter [i.d.] 7.5 mm; length, 75 mm; Tosoh, Tokyo, Japan), and elution with a linear gradient from 0 to 0.5 M NaCl in 10 mM phosphate buffer containing 0.3% Triton X-100 and 200 mM L-sorbose over 60 min at a flow rate of 1.0 ml/min; (ii) anion-exchange chromatography on a DEAE-Toyopearl 650M column (i.d., 7.0 mm; length, 17 mm; Tosoh) with 10 mM phosphate buffer containing 0.3% Triton X-100 and 200 mM L-sorbose as the equilibration and loading buffer and elution with 0.2 M NaCl in the equilibration buffer with isocratic mode at a flow rate of 0.5 ml/min; and (iii) gel-filtration chromatography on a Superose 12 HR10/30 column (i.d., 10 mm; length, 30 cm; Pharmacia, Uppsala, Sweden) and elution with 0.3% Triton X-100 and 200 mM L-sorbose. The molecular mass of the purified SDH was approximately 58 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The SNDH from the cytosolic fraction was purified by anion-exchange chromatography on a QAE-Toyopearl column (i.d., 16 mm; length, 30 cm; Tosoh) with elution with a linear gradient from 0 to 0.4 M NaCl in 10 mM phosphate buffer (pH 7.0) over 260 min at a flow rate of 1.2 ml/min and then by affinity chromatography on a Blue-Sepharose column (i.d., 10 mm; length, 7 cm; Pharmacia) and elution with a linear gradient from 0 to 0.6 M NaCl in 10 mM phosphate buffer (pH 7.0) over 130 min at a flow rate of 0.5 ml/min. The molecular mass of the purified SNDH was approximately 50 kDa as determined by SDS-PAGE analysis.

Enzyme assay. The SDH activity was assayed according to the method of Sugisawa et al. (26), using L-sorbose as a substrate and 2,6-DCIP as an electron acceptor. The SNDH activity was assayed according to the method of Hoshino et al. (10), using NAD as an electron acceptor. One enzyme unit was defined as the amount of the enzyme catalyzing the formation of 1 μmol of reduced 2,6-DCIP or NADH per min.

Amino acid sequence analysis. The fraction containing SDH or SNDH was subjected to SDS-PAGE, and the proteins separated on the gels were electroblotted onto a polyvinylidenedifluoride membrane. After being visualized with ponceau S stain, the membrane was cut into pieces containing 58- or 50-kDa proteins, corresponding to SDH and SNDH, respectively. The membrane pieces were directly applied to a protein sequencer (model 470A; Applied Biosystems, Foster City, Calif.) for amino-terminal sequence analysis. To determine the internal amino acid sequences, in situ digestion with lysylendopeptidase (EC 3.4.21.50) (Wako Pure Chemical, Osaka, Japan) was carried out according to the method described by Iwamatsu (13). The peptide fragments were eluted from the membrane with 50 mM Tris-HCl (pH 9.0) containing 8% acetonitrile and separated by reversed-phase HPLC on a Cosmosil 5C4-300 column (i.d., 4.6 mm; length, 5 cm; Nacalai Tesque, Kyoto, Japan) and elution with a linear gradient from 8 to 83% acetonitrile in 0.05% trifluoroacetic acid over 75 min at a flow rate of 1.0 ml/min (detection wavelength, 214 nm).

Genetic engineering techniques. Oligodeoxyribonucleotides were prepared with a 381A DNA synthesizer (Applied Biosystems). All DNA manipulations were performed essentially according to Sambrook et al. (21). DNA sequencing was performed with a 373A DNA sequencer (Applied Biosystems).

Preparation of the probe for the SDH gene. PCR was carried out with 180 ng of genomic DNA from *G. oxydans* T-100 and 2.5 pmol of degenerate oligonucleotide primers designed from the elucidated partial sequences of the SDH: 5′ ACC (TA)(GC)C GGC TT(TC) GA(TC) TA(TC) AT(TCA) GT (forward primer for the amino-terminal sequence) and 5′ TC CCA (ATCG)GT (AG)TG (ATCG)GG (ATCG)CC (reverse primer for the internal sequence). The reaction was performed in a buffer consisting of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.2 mM deoxyribonucleotide 5′-phosphates, and 2.5 U of *Taq* DNA polymerase (Takara Shuzo), with an initial denaturation (95°C for 0.5 min), 50 cycles of amplification (95°C for 0.5 min, 42°C for 1.0 min, and 72°C for 2.0 min), and a final extension (72°C for 7 min) performed with a thermal reactor (model HB-TR1; Hybaid, Teddingstone, United Kingdom). The amplified DNA (180 bp) was isolated, treated with DNA polymerase (Klenow fragment), and cloned into pUC18 digested with *Sma*I to give pUC18SD180. After confirmation of the sequence of the insert DNA, the DNA (180 bp) was recovered and labelled with ^{32}P by nick translation (approximately 3.7×10^7 cpm/μg of DNA).

Cloning of genes for SDH and SNDH. The genomic DNA isolated from *G. oxydans* T-100 was partially digested with *Mbo*I. The 8- to 22-kb DNAs were separated by sucrose gradient ultracentrifugation and cloned into the *Bam*HI site of λ phage vector EMBL-3 (Promega, Madison, Wis.). The λ phage plaques on *E. coli* NM538 (Promega) were immobilized on nitrocellulose filters which had been incubated in a prehybridization buffer consisting of 50% formamide, 1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]), 0.1% SDS, and salmon sperm DNA (100 μg/ml) at 42°C for 4 h. Then, the filters were incubated in a prehybridization buffer containing the ^{32}P-labelled probe (approximately 10^7 cpm/ml) at 42°C for 18 h and successively in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.05% SDS at 42°C to remove the excess probe. The filters were exposed to X-ray film (HR-H; Fuji Film, Tokyo, Japan) at −80°C for 18 h. A λ phage DNA positive to the probe was digested with *Sal*I and *Eco*RI and analyzed by Southern blotting. An approximately 6-kb DNA fragment that hybridized to the probe was isolated and cloned into pUC19 between the *Sal*I and *Eco*RI sites to give pUC19SD5.

Preparation of shuttle vectors. A plasmid, pF4, was extracted from *G. oxydans* T-100 by the alkaline-SDS method and purified by ultracentrifugation with CsCl. pSDH155 was digested with *Nco*I and filled in with DNA polymerase (Klenow fragment) to give pSDH155-NK, in which the original *Nco*I site located at positions 4336 to 4341 (numbering correspond to GenBank sequence D86622) was destroyed. To introduce a new *Nco*I site in the 5′ region of the SNDH gene by PCR, oligodeoxyribonucleotides 5′ CGG TGC GTT ACG CGT CAG GAA G and 5′ TCA TGA GAA ATA TTC CTA CTG AC**C** ATG GTG CTG CC were synthesized (nucleotide positions correspond to 861 to 882 and 1100 to 1044, respectively, in GenBank sequence D86622 respectively) (restriction sites [*Mlu*I, *Ssp*I, and *Nco*I] are underlined, and the point-mutated position is shown in boldface type). PCR was carried out using a 4.6-kb *Eco*RI-*Pst*I DNA fragment from pSDH155 as a template. The PCR product (184 bp) was digested with *Mlu*I and *Ssp*I. The resulting 161-bp DNA was replaced with the corresponding region of pSDH155-NK to obtain plasmid pSDH155-NN, which carries a new and unique *Nco*I site in the upstream region of the SNDH gene (positions correspond to 1017 to 1022 in GenBank sequence D86622).

Assay of SDH and SNDH activities expressed in *E. coli*. *E. coli* strains were cultivated in 200 ml of a broth (pH 6.8) consisting of 1% Bacto Tryptone, 0.5% yeast extract, 0.5% sodium chloride, 1% glycerol, 0.3% KH_2PO_4, 0.8% $Na_2HPO_4 \cdot 12H_2O$, and 1% L-sorbose with (JM109/pUC19SD5) or without (JM109) ampicillin (100 μg/ml) at 25°C for 72 h. The harvested cells were suspended in 5 ml of saline and lysed by sonication. To determine the SDH activity, the lysate was incubated with 1 mM phenazine methosulfate, 0.1 M phosphate buffer (pH 8.0), and 1% L-sorbose at 30°C for 5 h with shaking. The reaction was stopped by adjusting the pH to 2 with 6 N H_2SO_4. A portion of the reaction mixture was directly analyzed by HPLC to determine the amount of L-sorbosone formed. To determine the SNDH activity, the lysate was incubated with 0.5 mM NAD, 0.1 M phosphate buffer (pH 8.0), and 1% L-sorbosone at 30°C for 5 h. After quenching the reaction by adjusting the pH to 2.0 with 6 N H_2SO_4, 2-KLGA formed was analyzed by HPLC.

Determination of L-sorbose, L-sorbosone, 2-KLGA, and L-idonate. HPLC conditions for the determination of the products were as follows: (i) for L-sorbose, an OA KC column (i.d., 7.8 mm; length, 300 mm; E. Merck) and elution with 0.05 N H_2SO_4 at a flow rate of 0.4 ml/min (detection, refractive index), (ii) for L-sorbosone, a model 3011N column (i.d., 46 mm; length, 30 cm; Hitachi, Tokyo, Japan), elution with 1 M borate buffer (pH 9.5) containing 0.02 M benzamidine hydrochloride and 0.25 M potassium sulfate at a flow rate of 0.8 ml/min, and a postcolumn labelling reaction at 80°C in a Teflon tube ("i.d., 0.5 mm; length, 10 m), with detection by monitoring of fluorescence (excitation wavelength, 315 nm; emission wavelength, 405 nm); and (iii) for 2-KLGA and L-idonate, a Capcellpak NH_2 column (i.d., 4.6 mm; length, 25 cm; Shiseido, Tokyo, Japan) and elution with 30% acetonitrile in 20 mM phosphate buffer (pH 3.0) at a flow rate of 1.2 ml/min (detection wavelength, 210 nm).

Transformation and chemical mutation of *Gluconobacter*. The cells of *G. oxydans* G624 cultivated in 100 ml of MB broth (2.5% D-mannitol, 0.3% polypeptone, 0.5% yeast extract [pH 6.0]) at 25°C for 20 h were washed with 10% glycerol and suspended in 1 ml of 10% glycerol to a concentration of approximately 10^{10} cells/ml. A portion of the suspension solution (160 μl) was used for electroporation of plasmids with a Gene-Pulsar (Bio-Rad, Richmond, Calif.) apparatus. Mutation of *G. oxydans* G624/pSDH155 by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) was performed essentially according to the method described by Sugisawa et al. (25, 26) and Tajima et al. (27). Each mutant was cultivated in 1 ml of a broth consisting of 5% D-sorbitol, 0.5% yeast extract, and 2% $CaCO_3$ at 30°C for 24 h. After centrifugation, the supernatant was acidified to pH 1.0 with 4 N HCl and heated at 80°C for 1 h to convert L-idonate to L-idono-γ-lactone. The chromogenic reaction was performed according to Herstrin's method (8), and absorbance at 540 nm was measured to determine the amount of L-idonate. From the mutants, strains with deficiency in L-idonate synthesis were selected. One of the selected strains, IA1069, was cultivated in MB broth containing novobiocin (0.5 μg/ml) at 30°C for 24 h to remove the plasmid. After five

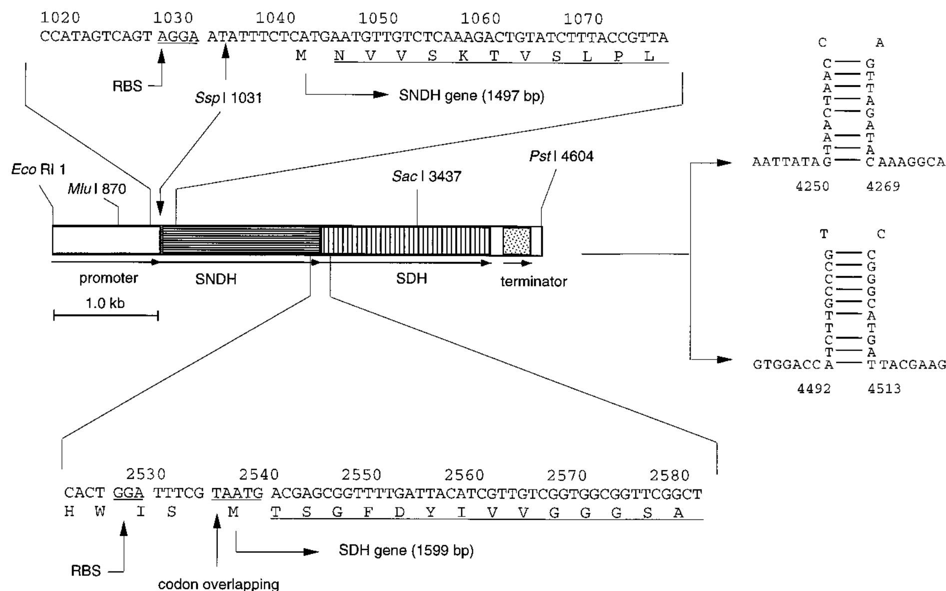

FIG. 1. Schematic structure of genes for SDH and SNDH. The amino-terminal sequences which are consistent with those from amino acid sequence analyses of the purified enzymes are underlined. RBS, putative ribosome-binding sequence; terminator, possible transcription termination sequence to form a stem-and-loop structure.

passages, a bacterium sensitive to kanamycin (50 μg/ml) was obtained and designated *G. oxydans* NB6939.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been deposited in the DNA Data Bank of Japan (DDBJ), EMBL, and GenBank databases as sequence D86622.

RESULTS

Purification of SDH and SNDH. *G. oxydans* T-100 was isolated from a Japanese persimmon (kaki) followed by mutation with MNNG. Fermentation of the bacteria in a broth containing 5% D-sorbitol yielded 7 mg of 2-KLGA per ml; however, further improvement of the conversion yield by classical mutations was unsuccessful (less than 13%). On the other hand, we had isolated *G. oxydans* G624 that was resistant to high concentrations of D-sorbitol and converted 20% D-sorbitol to L-sorbose almost quantitatively by fermentation at 30°C for 18 h. Therefore, we intended to clone the genes for SDH and SNDH from *G. oxydans* T-100 and introduce them to *G. oxydans* G624.

SDH and SNDH have been purified from *G. oxydans* UV10 by Sugisawa et al. (26) and Hoshino et al. (10), respectively; however, neither of the genes coding for these activities has been cloned. In preliminary studies, *G. oxydans* T-100 was revealed to have SDH activity in the membrane fraction and SNDH activity in the cytosolic fraction, results which are similar to the data from *G. oxydans* UV10 (10, 26). To clone the genes coding for SDH and SNDH, we isolated both SDH and SNDH from *G. oxydans* T-100. The SDH purified from the membrane fraction was revealed by SDS-PAGE analysis to be a 58-kDa protein. The amino-terminal sequences of the SDH protein (TSGFDYIVVGGGSA) (Fig. 1) and two peptide fragments (MTTGPHTWDLLTEPQK and LMMLSGVGP) generated from in situ digestion with lysylendopeptidase of the electroblotted enzyme were determined. The optimal pH (approximately 9.0) of the enzyme was higher than that of the SDH from *G. oxydans* UV10 (pH 7.0); however, other enzymatic properties were similar to those of the SDH from *Gluconobacter* as reported by Sugisawa et al. (26). SNDH was purified from the cytosolic fraction as a 50-kDa protein by SDS-PAGE analysis. The amino-terminal sequence was determined to be NVVSKTVXL (Fig. 1). It showed properties similar to those of the NAD(P)-dependent cytosolic SNDH from the *Gluconobacter* (10) (data not shown).

Cloning of the genes for SDH and SNDH. The gene for SDH was cloned from the genomic library prepared from *G. oxydans* T-100 with the 180-bp PCR product as a probe (see Materials and Methods). Thirty positive clones were obtained from 72,000 plaques of the λ phage. The λ DNA isolated from one of the positive phage was digested with *Eco*RI and *Sal*I. An approximately 6-kb DNA fragment was identified to hybridize the probe DNA and was cloned into pUC19 between the *Eco*RI and *Sal*I sites to give pUC19SD5.

Nucleotide sequence of the insert DNA of pUC19SD5. From nucleotide sequence analysis of pUC19SD5, the sequenced DNA (4,624 bp) encoded two long open reading frames (ORFs) of 1,497 and 1,599 nucleotides (Fig. 1) (see also sequence D86622 in GenBank). The first ORF, from ATG to TAA (nucleotide positions 1041 to 2537; numbers correspond to sequence D86622), corresponds a protein of 498 amino acids with a molecular mass of 53.6 kDa. Interestingly, the amino-terminal sequence of SNDH (NVVSKTVXL) was identical to that of the putative amino acid sequence of the ORF and the molecular mass of the purified SNDH gave good agreement with that of the protein encoded by the first ORF. These data could indicate that the first ORF encodes the gene for the SNDH. The third nucleotide of the termination codon for the first ORF overlaps the initiation ATG (nucleotide 2537) of the second one, which encodes a protein of 531 amino acids with a molecular mass of 57.6 kDa. The putative amino acid sequence of the second ORF contained the amino-terminal sequence of the purified SDH, TSGFDYIVVGGGSA (Fig. 1), and the internal amino acid sequences derived from lysylendopeptidase digests, MTTGPHTWDLLTEPQK and LMMLSGVGPA (corresponding to nucleotides 2696 to 2734 and 3314 to 3340 in sequence D86622). The calculated molecular mass (57.6 kDa) gave good agreement with that obtained

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SDH (531) vs CDH (556); Total Identity: 37.7 %

SDH  1'   MTSGFDYIVVGGGSAGCVLAARLSENPSVRVCLIEAGRRDTH-PL-IHMPVGFA
              ****..*.**** ***.**.*.*...* *.*** .* .  . ..**...*
CDH  1"     MQFDYIIIGAGSAGNVLATRLTEDPNTSVLLLEAGGPDYRFDFRTQMPAALA
                    FAD binding site

SDH  469' HPTCTCKMGRDDMSVVDPRLKVHGLEGIRICDSSVMPSLLGSNTNAATIMISE
          **  ***** *.*****   .******.*.  *.*.** ....* **.****.*
CDH  473" HPCGTCKMGYDEMSVVDGEGRVHGLEGLRVVDASIMPQIITGNLNATTIMIGE
                active site


SNDH (498) vs BADH (489); Total Identity: 40.2 %

SNDH  226'  TEHQDIDMLSFTGSTGVGKSCIHAAADSNLKKLGLELGGKNPIVVFAD
            ***..*...****... **. . ..*.*.**....*****.*..**.*
BADH  217"  TEHPGIAKVSFTGGVASGKKVMANSAASSLKEVTMELGGKSPLIVFDD
                         NAD binding site

SNDH  254'  SNLKKLGLELGGKNPIVVFADSNLEDAADAVAFGISFNTGQCCVSSSRLIVERS
            *.**....*****.*..**.*..*. ***  . ..  *..** *....*..*  .
BADH  243"  SSLKEVTMELGGKSPLIVFDDADLDLAADIAMMANFFSSGQVCTNGTRVFVPAK
                 active site                        active site
```

FIG. 2. Sequence homologies of SDH to CDH and SNDH to BADH. The sequences for SDH and SNDH were aligned with those for *E. coli* CDH and BADH as reported by Lamark et al. (15). Identical and homologous amino acids are indicated by asterisks and dots, respectively.

by SDS-PAGE analysis of the SDH. These results could indicate that the second ORF encodes SDH protein. In the 5′ region of the SNDH gene, there exists a putative ribosome-binding sequence, AGGA (nucleotides 1028 to 1031). In the 3′ region of the SDH gene, two stem-and-loop structures, which are characteristic for transcriptional termination in prokaryotes, were found at positions 4250 to 4269 and 4492 to 4513. These data suggest that the elucidated sequence (4624 bp) contains the whole operon of SNDH and SDH synthesis.

TABLE 1. SDH and SNDH activities of *E. coli* carrying pUC19SD5[a]

Strain	Treatment	SDH activity (U/ml)	SNDH activity (U/ml)
JM109/pUC19SD5	Sonication	43.2	97.7
JM109/pUC19SD5	Sonication and boiling	0.449	ND[b]
JM109	Sonication	0.393	37.3
Control[c]	No treatment	0.617	ND

[a] The cultivation of *E. coli* and the determination of L-sorbosone and 2-KLGA are described in Materials and Methods. One unit was defined as the activity capable of producing 1.0 μmol of L-sorbosone or 2-KLGA, formed from L-sorbose or L-sorbosone, respectively, per min.

[b] ND, not determined.

[c] Saline was used for the assay.

The primary sequences of SDH and SNDH showed high-level homology with *E. coli* choline dehydrogenase (CDH) and betaine aldehyde dehydrogenase (BADH) (15), respectively, on the basis of homology search analysis using the Swiss-Prot database (Fig. 2).

Expression of genes for SDH and SNDH in *E. coli*. The recombinant *E. coli* JM109 transformed with pUC19SD5 was cultivated in a phosphate-buffered L broth containing 1% glycerol, 1% L-sorbose and ampicillin (100 μg/ml). The cell lysates were used for assay of the amounts of L-sorbosone and 2-KLGA formed from L-sorbose and L-sorbosone, respectively (see Materials and Methods). As shown in Table 1, an activity to convert L-sorbose to L-sorbosone was found in the sonicated cells with the plasmid but not in the cells without the plasmid. The activity was diminished after treating the lysate at 100°C. The sonicated cell lysate also showed an ability greater than 2.5 times that of the host cells to convert L-sorbosone to 2-KLGA; however, *E. coli* JM109 itself expressed some SNDH activity

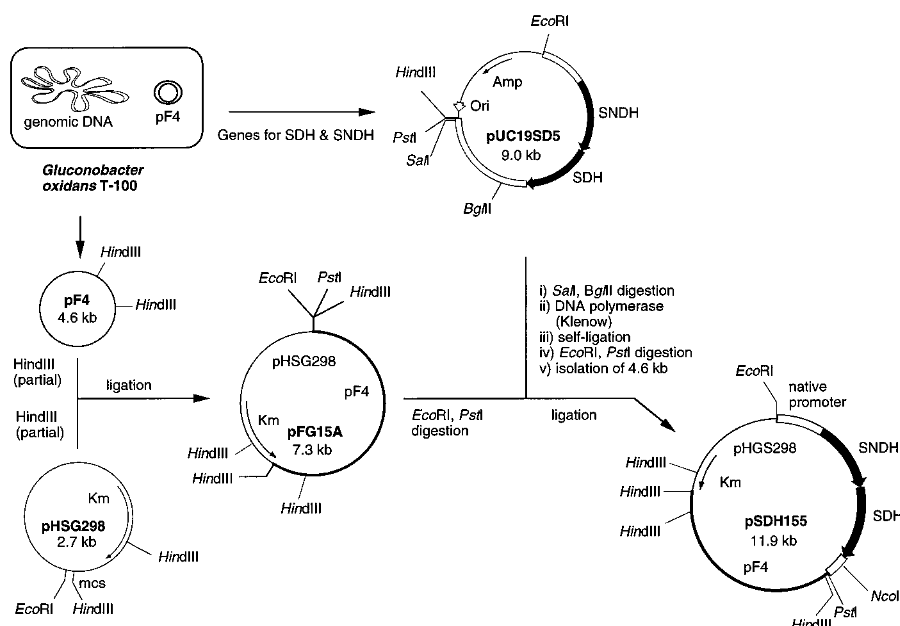

FIG. 3. Preparation of shuttle vector pSDH155. pUC19SD5 was digested with *Sal*I and *Bgl*II and filled in with DNA polymerase (Klenow fragment). The resulting 7-kb fragment was self-ligated and then digested with *Eco*RI and *Pst*I to give a 4.6-kb DNA carrying the promoter, genes for SNDH and SDH, and the terminator. A plasmid, pF4 (4.6 kb), isolated from *G. oxydans* T-100 was partially digested with *Hin*dIII and ligated to linearized pHGS298 (Takara Shuzo) which had been partially digested at the *Hin*dIII site in the multicloning site (mcs) to give pFG15A. pFG15A was digested with *Eco*RI and *Pst*I, and the resulting 7.3-kb DNA fragment was ligated to the 4.6-kb DNA fragment to give pSDH155, a vector for production of 2-KLGA in *G. oxydans.*

TABLE 2. Production of 2-KLGA in *G. oxydans*

G. oxydans strain	% D-Sorbitol in medium	Amt (mg/ml of broth)			
		L-Sorbose	L-Sorbosone	2-KLGA	L-Idonate
T-100	5.0[a]	36	1.0	7.0	5.0
G624/pSDH155	5.0[a]	23	4.0	16	13
IA1069	5.0[a]	29	4.0	22	0.0
NB6939/pSDH155	5.0[a]	28	4.0	31	0.0
NB6939/pSDH155	10.0[a]	55	1.0	57	3.0
NB6939/pSDHtac1	10.0[a]	47	1.0	71	2.0
NB6939/pSDH-tufB1	10.0[a]	42	1.0	88	3.0
NB6939/pSDH-PL1	10.0[a]	44	1.0	77	2.0
NB6939/pSDH-tufB1	11.0[b]	0.0	0.7	98	0.0
NB6939/pSDH-tufB1	13.0[b]	0.0	1.3	110	0.0
NB6939/pSDH-tufB1	15.0[b]	0.0	0.0	130	0.0

[a] Cultivation in 10 ml of a medium in a 100-ml flask at 30°C for 72 h. 5%, 5% D-sorbitol, 0.5% yeast extract, and 2.0% $CaCO_3$; 10%, 10% D-sorbitol, 1.5% corn steep liquor (Nihon Shokuhin, Osaka, Japan), and 2.0% $CaCO_3$. After centrifugation of the broth, the supernatant was directly analyzed by HPLC (Materials and Methods).

[b] Cultivation in 19 liters of a medium (11, 13, or 15% D-sorbitol, 2.0% corn steep liquor, 0.5% $CaCO_3$, and 6% glycerol in a 30-liter jar fermentor at 30°C for 72 h. After centrifugation of the broth, the supernatant was directly analyzed by HPLC (Materials and Methods).

that had not been reported in *E. coli*. We think that some kinds of aldehyde dehydrogenases (ALDHs) show SNDH-like activity in this assay. Although no clear homology with prokaryote promoters in the 5′ region of the SNDH gene was found, the DNA (nucleotides 1 to 1027) was active for the expression of the tetracycline resistance gene in *E. coli* as well as in *G. oxydans* (data not shown). Therefore, it is obvious that the region contains the promoter for the SNDH and SDH genes. These results indicate that pUC19SD5 contains the whole DNA for the SDH and SNDH synthesis system, in accordance with the expectation from the sequence analysis.

Preparation of shuttle vectors. A plasmid of 4.4 kb, designated pF4, was isolated from *G. oxydans* T-100 and ligated with pHSG298 (Takara Shuzo) at the *Hin*dIII site to prepare a shuttle vector, designated pFG15A (Fig. 3). pFG15A was introduced to *G. oxydans* G624, which accumulates L-sorbose in the presence of D-sorbitol. It was confirmed that pFG15A was stable across several passages in a broth without kanamycin and gave no effects to L-sorbose production in the host cells (data not shown). Then, an expression vector for SNDH and SDH, designated pSDH155, was prepared from pUC19SD5 and pFG15A (Fig. 3). *G. oxydans* G624 was transformed with pSDH155 and cultivated in the presence of D-sorbitol. The formulation of products as determined by HPLC are listed in Table 2. The recombinant *Gluconobacter*, *G. oxydans* G624/pSDH155, was 2.3-fold more active in the production of 2-KLGA than was *G. oxydans* T-100; however, *G. oxydans* G624 itself showed no ability to produce 2-KLGA. No unreacted D-sorbitol was detected in the broth. These data show that D-sorbitol was completely converted to L-sorbose and that active SDH and SNDH were expressed by pSDH155 in the recombinant *Gluconobacter*. Although considerable amounts of L-sorbose remained unconverted (23 mg/ml) and L-idonate was also a by-product (13 mg/ml), the stoichiometry of the reaction revealed that no catabolites were produced in the fermentation pathway.

MNNG mutation of host strain. To obtain a mutant whose enzymatic pathway from 2-KLGA to L-idonate was blocked, *G. oxydans* G624/pSDH155 was treated with MNNG according to the conventional method (25, 27). From one of the mutated strains, a mutant (IA1069) whose productivity of L-idonate was greatly reduced was isolated (Table 2). After treating the mutant with novobiocin to remove the plasmid, a mutant strain, designated NB6939, was obtained. The resultant *G. oxydans* NB6939 showed no effect on the metabolism of L-sorbose and L-sorbosone (data not shown). It was retransformed with pSDH155. The productivity of 2-KLGA by *G. oxydans* NB6939/pSDH155 increased by 90% over that by *G. oxydans* G624/pSDH155, indicating that MNNG mutated the genomic DNA of *G. oxydans* G624 participating in L-idonate synthesis.

Replacement of the promoter sequence in the shuttle vector. To investigate the effect of the promoter on the productivity of 2-KLGA, several types of *E. coli* promoter DNA containing −35 and −10 regions were synthesized based on the reported sequences (1, 7) and were inserted in place of the original promoter DNA (Fig. 4). Among the promoters, *tufB*, *tac*, and PL showed excellent activity for the production of 2-KLGA (Table 2). In particular, the production of 2-KLGA by *G. oxydans* NB6939/pSDH-tufB1 was 88 mg/ml with 72 h of fermentation in a broth containing 10% D-sorbitol.

DISCUSSION

Genes for SDH and SNDH. In the fermentation of acetic acid, oxidation of ethanol to acetic acid by *Acetobacter* and

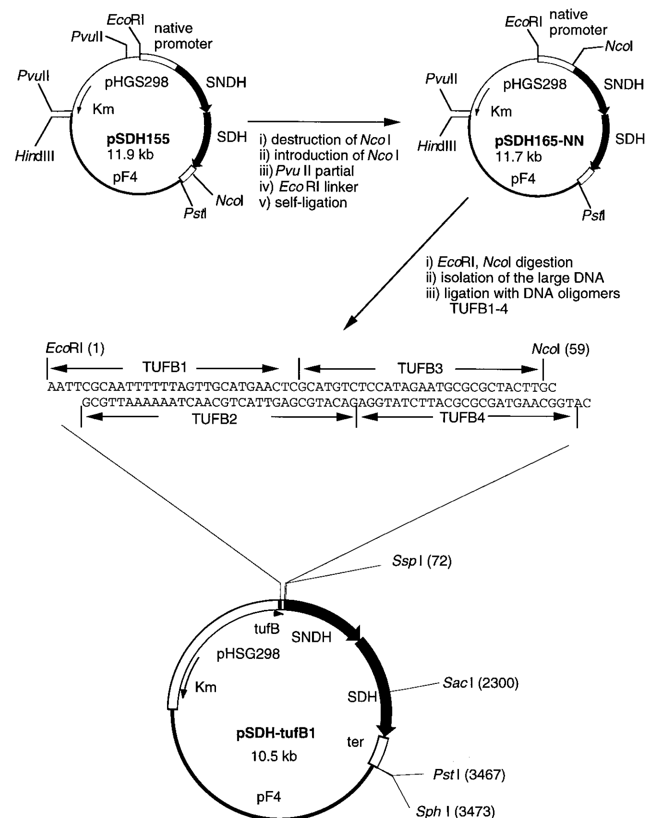

FIG. 4. Preparation of shuttle vector pSDH-tufB1. An *Nco*I site in the downstream region of the SDH gene of pSDH155 was destroyed and a new *Nco*I site was introduced in the upstream region of the SNDH gene by PCR mutation to give pSDH155-NN (11.9 kb) (see Materials and Methods). To remove the *lac* promoter, originating from pHSG298, pSDH155-NN was partially digested with *Pvu*II. The resulting DNA fragment (11.9 kb) was ligated with an *Eco*RI linker (Pharmacia), digested with *Eco*RI, and self-ligated to give pSDH165-NN. The *Eco*RI-*Nco*I region of pSDH165-NN was replaced with synthetic oligodeoxyribonucleotides (TUFB1, -2, -3, and -4) for the *E. coli tufB* promoter (1) to obtain the desired pSDH-tufB1. ter, terminator sequence.

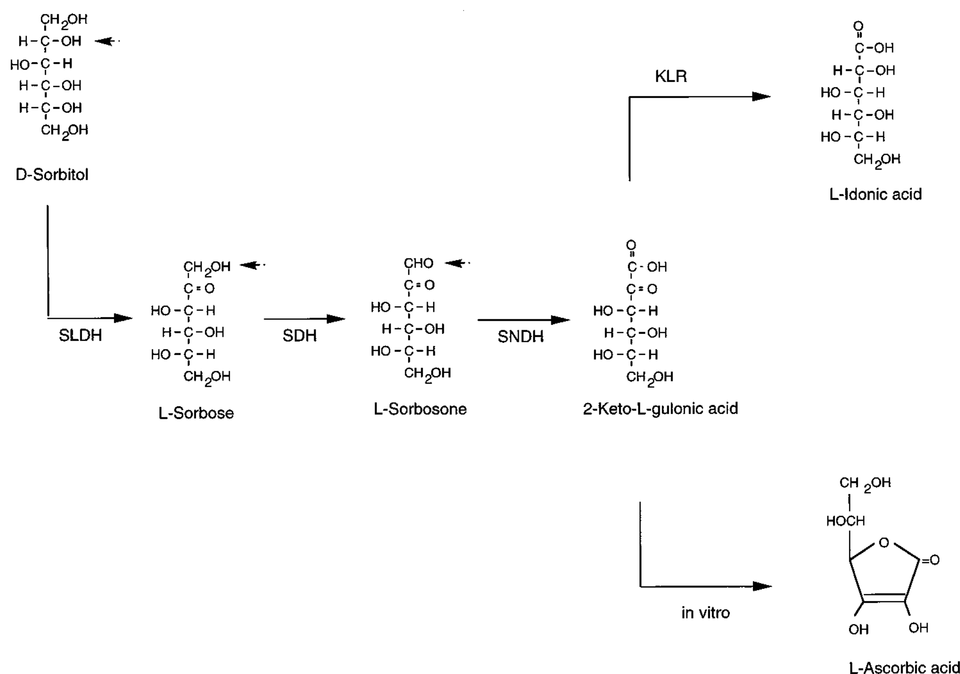

FIG. 5. Biosynthetic pathway of 2-KLGA in recombinant *G. oxydans*. SLDH, D-sorbitol dehydrogenase; KLR, 2-KLGA reductase.

Gluconobacter is catalyzed by alcohol dehydrogenase (ADH) and ALDH. Although the enzymatic processes of ADH and ALDH show some similarities to those of SDH and SNDH, respectively, in *G. oxydans* T-100, the cloned genes for SDH and SNDH showed no similarity to those for either ADH or ALDH in reports by several groups (11, 28, 29). The SDH from *G. oxydans* T-100 is a protein consisting of 531 amino acid residues. From a homology search using the Swiss-Prot database (Fig. 2), the primary sequence of the SDH was revealed to show high homology (37.7% identical) with that of a membrane protein, *E. coli* CDH, which catalyzes the oxidation of choline to betaine aldehyde by using FAD as a cofactor (2, 5, 15, 16). SDH and CDH resembled each other closely in hydrophobicity plot analyses. The sequence near the putative active site (C at position 478) of CDH is highly conserved in SDH. Furthermore, the first 35 amino acids of the SDH display 58 and 53% identity with the corresponding regions of the CDH and the flavoprotein methanol oxidase of *Hansenula polymorpha* (17), respectively. These data suggest that the SDH is also a flavoprotein, because the amino acid sequence of this region contains a so-called glycine box (GXGXXG), at (11)GGGSAG, which is typical for the binding site of the ADP moiety of FAD as reported by Wierenga et al. (32) and Lamark et al. (15). Interestingly, the gene for the SNDH consisting of 498 amino acids was found to be located in the upstream portion of the SDH gene whose translational initiation codon ATG overlapped with the termination TAA of the SNDH (Fig. 1). The primary sequence of the SNDH protein shows high similarity (40.2% identical) to that of a cytosolic protein, *E. coli* BADH, which converts betaine aldehyde to betaine by use of NAD as a cofactor (15, 16) (Fig. 2). In particular, the sequences near the putative NAD(P) binding site [(228)GGVA SG] and active sites (E and C at positions 251 and 285, respectively) of BADH are highly conserved in the SNDH. These data suggest that the SNDH is an NAD(P) dependent enzyme. These results are in good agreement with the enzymatic properties of SNDH, whose activity can be assayed in the presence of NAD (see Materials and Methods).

The genes for SDH and SNDH in *G. oxydans* T-100 constitute an operon similar to those of CDH and BADH, which cooperatively play an important role to confer a high level of osmotic tolerance by controlling the choline-glycine betaine pathway in *E. coli* (2, 5, 15, 16, 31). SDH and SNDH show close similarity to CDH and BADH, respectively, in the amino acid sequences as well as the cellular locations. These data could indicate that SDH and SNDH are also involved in the regulation of stress from outside the cells. The overlapping of the termination codon of the SNDH gene with the initiation codon of the SDH gene by one nucleotide suggests that these gene products function by associating with each other, because the codon overlappings are often found in pairs of genes whose products are associated in a multisubunit enzyme complex (20, 33). Therefore, the SDH in the membrane might be located near the SNDH in the cytosol to interact cooperatively in *G. oxydans* T-100.

Optimization of the expression system. A schematic outline of the biosynthetic pathway from D-sorbitol to 2-KLGA in the recombinant *G. oxydans* is shown in Fig. 5. Although the productivity of 2-KLGA was improved by the introduction of pSDH155 to *G. oxydans* G624, it was still unsatisfactory for large-scale production of 2-KLGA by simple fermentation, because there still remained problems to be solved: (i) a considerable amount of L-idonate was made as a by-product and (ii) the original promoter did not have enough activity for high-level production of 2-KLGA.

First, we tried to obtain a mutant *Gluconobacter* that lacks the activity to produce L-idonate. From the metabolic analysis of MNNG mutants of *G. oxydans*, Hoshino et al. (9) and Sugisawa et al. (25) proposed that L-idonate was produced by reduction of 2-KLGA with NAD(P)-dependent L-idonate reductase. Since the original host, *G. oxydans* G624, showed no activity to produce 2-KLGA, *G. oxydans* G624/pSDH155 was

used as a starting strain for the MNNG mutation to block the L-idonate pathway. This newly obtained strain, designated *G. oxydans* NB6939, showed superior activity in the production of 2-KLGA when pSDH155 was reintroduced (Table 2), indicating that blocking the pathway to L-idonate by MNNG mutation resulted in reducing the formation of L-idonate as well as in improving the productivity of 2-KLGA. Therefore, we suggest that the DNA for the regulation of the 2-KLGA reductase and/or the gene for the enzyme itself had mutated in *G. oxydans* NB6939 and that the synthetic pathway to L-idonate is independent of L-sorbosone and 2-KLGA in the turnover of the coenzyme system. However, further investigation of a method for precise analysis is still necessary.

Next, we assumed that the productivity could be improved by introducing a more suitable promoter in place of the original one from *G. oxydans* T-100. Since there are few reports of analyses on the promoter sequences of *Gluconobacter*, we replaced the SNDH promoter with a synthetic promoter of ADH from *Acetobacter* (11), which is the strain closest to *Gluconobacter*. However, no improvement was observed (18a). Then, several types of *E. coli* promoters (PL, *tac*, and *tufB*) were introduced to pSDH165-NN (Fig. 4). The data in Table 2 show that the productivity of 2-KLGA was improved by replacement of the original promoter with *tac*, PL, and *tufB* promoters. These data indicate that strong *E. coli* promoters are also active in *G. oxydans* and that the productivity of 2-KLGA can be improved by optimization of the promoter system. Among them, the *tufB* promoter (pSDH-tufB1) showed excellent activity for the production of 2-KLGA (88 mg/ml) by simple fermentation in 10% D-sorbitol. Although unconverted 2-KLGA remained after flask fermentation, further improvement of the productivity of 2-KLGA was attained by optimization of expression system and cultivation conditions in a 30-liter fermentation jar. The production of 2-KLGA reached 130 mg/ml in 15% D-sorbitol medium, and L-sorbose was completely consumed (Table 2). Therefore, we propose that the method described in this report is a new promising way for mass production of vitamin C.

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