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Cloning and Nucleotide Sequencing of the Membrane-Bound L-Sorbosone Dehydrogenase Gene of *Acetobacter liquefaciens* IFO 12258 and Its Expression in *Gluconobacter oxydans*

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Cloning and expression of the gene encoding *Acetobacter liquefaciens* IFO 12258 membrane-bound L-sorbosone dehydrogenase (SNDH) were studied. A genomic library of *A. liquefaciens* IFO 12258 was constructed with the mobilizable cosmid vector pVK102 (*mob*$^+$) in *Escherichia coli* S17-1 (Tra$^+$). The library was transferred by conjugal mating into *Gluconobacter oxydans* OX4, a mutant of *G. oxydans* IFO 3293 that accumulates L-sorbosone in the presence of L-sorbose. The transconjugants were screened for SNDH activity by performing a direct expression assay. One clone harboring plasmid p7A6 converted L-sorbosone to 2-keto-L-gulonic acid (2KGA) more rapidly than its host did and also converted L-sorbose to 2KGA with no accumulation of L-sorbosone. The insert (25 kb) of p7A6 was shortened to a 3.1-kb fragment, in which one open reading frame (1,347 bp) was found and was shown to encode a polypeptide with a molecular weight of 48,222. The SNDH gene was introduced into the 2KGA-producing strain *G. oxydans* IFO 3293 and its derivatives, which contained membrane-bound L-sorbose dehydrogenase. The cloned SNDH was correctly located in the membrane of the host. The membrane fraction of the clone exhibited almost stoichiometric formation of 2KGA from L-sorbosone and L-sorbose. Resting cells of the clones produced 2KGA very efficiently from L-sorbosone and L-sorbose, but not from D-sorbitol; the conversion yield from L-sorbosone was improved from approximately 25 to 83%, whereas the yield from L-sorbose was increased from 68 to 81%. Under fermentation conditions, cloning did not obviously improve the yield of 2KGA from L-sorbose.

2-Keto-L-gulonic acid (2KGA) is an important intermediate in the production of L-ascorbic acid (vitamin C). Two approaches for improving microbial production of 2KGA have been described. One approach is to improve the production of 2KGA from L-sorbose or D-sorbitol. Tsukada and Perlman have reported that they obtained a low yield of 2KGA in the presence of L-sorbose with *Gluconobacter oxydans* (formerly *Gluconobacter melanogenus*) IFO 3293 (28). Zinsheng et al. have reported that a mixed-culture fermentation of *G. oxydans* and *Pseudomonas striata* produced 30 g of 2KGA per liter in the presence of 70 g of L-sorbose per liter (30). We have also studied production of 2KGA from L-sorbose or D-sorbitol with mutants of *G. oxydans* IFO 3293 which produced 60 g of 2KGA per liter in the presence of 100 g of L-sorbose or D-sorbitol per liter (24). The other approach involves improving the production of 2KGA from D-glucose. Sonoyama et al. have described a tandem fermentation process for 2KGA fermentation in which they used mutant *Erwinia* strains that converted D-glucose to 2,5-diketo-D-gluconic acid (2,5-DKGA) and *Corynebacterium* strains that converted 2,5-DKGA to 2KGA (23). Anderson et al. (1) and Grindley et al. (5) independently used recombinant DNA technology and constructed recombinant strains which converted D-glucose to 2KGA by introducing the 2,5-DKGA reductase gene of a *Corynebacterium* sp. into *Erwinia* cells. However, the single recombinant organism yielded only 0.6 g of 2KGA per liter in the presence of 20 g of D-glucose per liter (1) and 19.8 g of 2KGA per liter in the presence of 40 g of D-glucose per liter (5). Low yields were probably obtained because (i) 2,5-DKGA reductase in the soluble fraction requires an active NADPH regeneration system and transport of 2,5-DKGA across the inner cell membrane into the cytoplasm, (ii) 2KGA formed in the cytosol requires a system for efflux from the cell to the medium, and (iii) 2,5-DKGA and 2KGA are dissimilated by the host cells. The yield was improved mainly by producing mutant host cells; blocking the pathway that led toward the by-product L-idonate and toward dissimilation of 2,5-DKGA and 2KGA resulted in an increase in the yield. In these systems, two series of reactions are topologically separated: 2,5-DKGA-forming dehydrogenases are located in the membrane, and 2,5-DKGA reductase is located in the cytoplasm. In this context, these enzymes are not fully cooperative.

We also found that the two reactions (8) from L-sorbose to 2KGA via L-sorbosone in the 2KGA-producing strain *G. oxydans* IFO 3293 were catalyzed by topologically separated enzymes; one of these enzymes is L-sorbose dehydrogenase (SDH) bound to the membrane (25), and the other is L-sorbosone dehydrogenase (SNDH) in the cytoplasm (7). The metabolic pathway of L-sorbose is shown in Fig. 1. Our previous work revealed that one of the strains that produce high levels of 2KGA, *G. oxydans* UV10, wastefully consumes more than 50% of the initial substrate. Most of the carbon loss was found to be in the form of CO_2 gas that evolved mainly from the pentose phosphate pathway in the cytoplasm (cSNDH in Fig. 1) (21). In addition, we found that *Acetobacter liquefaciens* (formerly *G. melanogenus*) IFO 12258 converted L-sorbosone to 2KGA stoichiometrically (21a) and contained the membrane-bound SNDH.

In this paper we describe cloning of the gene coding for the membrane-bound SNDH of *A. liquefaciens* IFO 12258 and expression of this gene in the membrane of a 2KGA-producing

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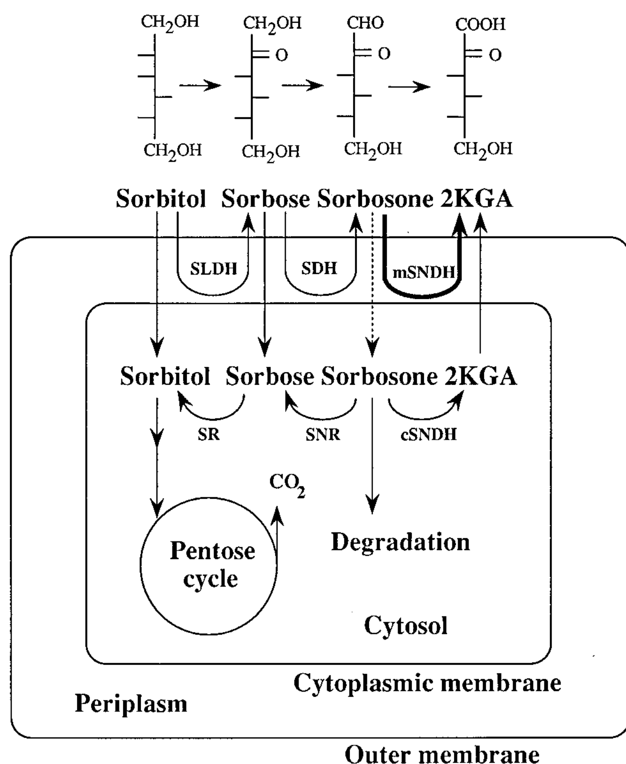

FIG. 1. Mechanism of production of 2KGA from D-sorbitol and L-sorbose in *G. oxydans*. Abbreviations: SLDH, D-sorbitol dehydrogenase; SDH, L-sorbose dehydrogenase; SR, L-sorbose reductase; SNR, L-sorbosone reductase; cSNDH, cytosolic SNDH; mSNDH, membrane-bound *A. liquefaciens* IFO 12258 SNDH. The thin lines show the metabolic pathway in *G. oxydans*; the thick line shows the catalytic reaction of the membrane-bound SNDH, which was the target enzyme which we studied to improve the 2KGA production yield in *G. oxydans.*

strain derived from *G. oxydans* IFO 3293 for the purpose of obtaining a recombinant strain that could carry out the two-step conversion by the two membrane-bound dehydrogenases (SDH and SNDH [mSNDH in Fig. 1]). We also describe the formation of 2KGA from L-sorbosone, L-sorbose, and D-sorbitol by resting and growing cells of transconjugants carrying the SNDH gene.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids which we used are listed in Table 1. *A. liquefaciens* IFO 12258 was used as the source of DNA in our gene cloning experiment. Strain OX4 (a strain which accumulates L-sorbosone in the presence of L-sorbose was derived from *G. oxydans* IFO 3293) and was used as the host strain for direct expression screening for the SNDH gene. *G. oxydans* IFO 3293 and its derivatives, strains UV10, N44-1, and U13, and *G. oxydans* IFO 3292 and IFO 3294 were used as the hosts in experiments to evaluate 2KGA formation by transconjugants carrying the SNDH gene. Mobilizable cosmid pVK102 was obtained from the American Type Culture Collection, Rockville, Md.

Media and culture conditions. *Escherichia coli* strains were grown at 37°C in Luria broth (LB) (16) medium. *A. liquefaciens* and *G. oxydans* were grown at 30°C in MB medium, which contained 2.5% mannitol, 0.5% yeast extract (Difco Laboratories, Detroit, Mich.), and 0.3% Bacto Peptone (Difco). FB agar medium, which contained 5.0% fructose, 1.0% yeast extract (Difco), 1.0% Polypepton (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 1.8% agar, was used for the conjugal mating experiments. No. 5 medium, which contained 10.0% L-sorbose, 1.5% yeast extract (Oriental Yeast Co., Osaka, Japan), 0.25% $MgSO_4 \cdot 7H_2O$, 0.05% glycerol, and 2% $CaCO_3$, was used to grow *G. oxydans* cells which were used to prepare SNDH and resting-cell reaction mixtures and to evaluate 2KGA production by growing cells. *Gluconobacter* strains carrying pVK102 or its derivatives were cultivated in medium containing kanamycin. Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were used in media at the following concentrations: streptomycin, 100 μg/ml; kanamycin, 50 μg/ml; tetracycline, 10 μg/ml; and polymyxin B, 10 μg/ml. LB medium containing streptomycin, LB medium containing kanamycin, LB medium containing streptomycin and kanamycin, and LB medium containing tetracycline were designated LS medium, LK medium, LSK medium, and LT medium, respectively. MB medium containing polymyxin B and kanamycin was designated MPK medium.

Chemicals. Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim GmbH, Mannheim, Germany, or Takara Shuzo Co., Ltd., Kyoto, Japan. L-Sorbose, L-sorbosone, and sodium 2KGA were supplied by Kilo Laboratories, F. Hoffmann-La Roche, Nutley, N.J.

Analytical methods. Analyses of 2KGA, L-sorbosone, L-sorbose, and D-sorbitol were performed by using thin-layer chromatography and high-performance liquid chromatography as described previously (24).

Preparation of DNA. Chromosomal DNA was prepared as follows. *A. liquefaciens* IFO 12258 was cultivated in 200 ml of MB medium at 30°C for 48 h. The cells were collected by centrifugation, washed with 100 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, and then resuspended in 50 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 20 mM EDTA. The cell suspension was treated with lysozyme at a final concentration of 400 μg/ml at 37°C for 30 min and then was treated with pronase (4,000 U) at 37°C for 30 min and with 1% sodium dodecyl sulfate (SDS) at 37°C for 1 h. Chromosomal DNA was extracted

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
G. oxydans IFO 3293	Produces low levels of 2KGA from L-sorbose (3 g/liter), PB^{r} [a]	24
G. oxydans OX4	Derived from IFO 3293, accumulates L-sorbosone in the presence of L-sorbose	
G. oxydans UV10	Derived from IFO 3293, produces 30 and 26 g of 2KGA per liter from L-sorbose and D-sorbitol, respectively	24
G. oxydans N44-1	Derived from IFO 3293, produces 55 and 50 g of 2KGA per liter from L-sorbose and D-sorbitol, respectively	24
G. oxydans U13	Derived from IFO 3293, produces 60 and 50 g of 2KGA per liter from L-sorbose and D-sorbitol, respectively	24
G. oxydans IFO 3292	Produces low levels of 2KGA from L-sorbose (less than 1 g/liter), PB^{r}	24
G. oxydans IFO 3294	Produces low levels of 2KGA from L-sorbose (less than 1 g/liter), PB^{r}	24
A. liquefaciens IFO 12258	Produces 2KGA from L-sorbosone, SNDH gene donor	Laboratory stock
E. coli ED8767	*supE supF58 hsdS3*($r_B^- m_B^-$) *recA56 galK2 galT22 metB1*	18
E. coli S17-1	*pro hsdR hsdM*$^+$ *thi recA* RP4-2-Tc::Mu Km::Tn7	22
Plasmids		
pVK102	*cos mob* Km^{r} Tc^{r}, 23 kb	11
p7A6	SNDH gene-carrying 25-kb insert in pVK102	This study
p7A6Δ4	SNDH gene-carrying 3.1-kb insert in pVK102 derivative (pVK102 with *Sal*I-*Sma*I-*Sma*I deletion)	This study

[a] PB^{r}, natural resistance to polymyxin B. All strains derived from *G. oxydans* IFO 3293 also express the PB^{r} phenotype.

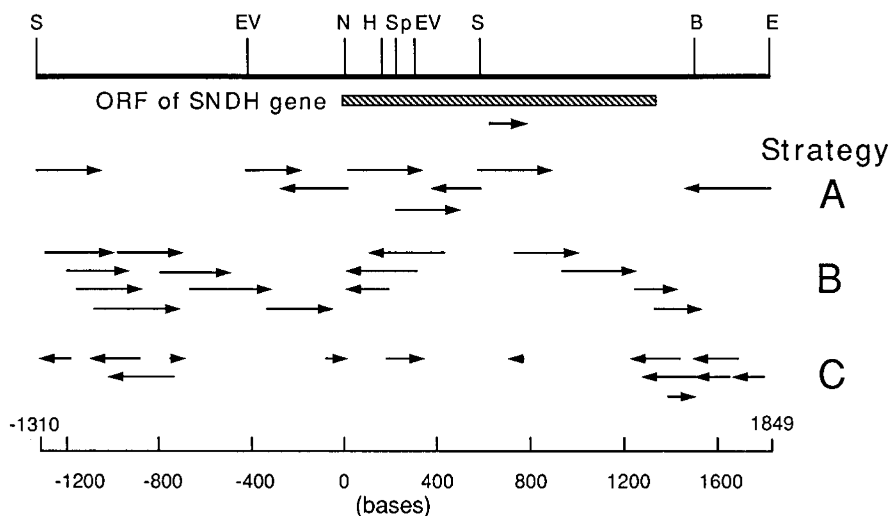

FIG. 2. Restriction map of the 3.1-kb *Sal*I-*Sal*I-*Eco*RI fragment and strategy for determining the sequence of the SNDH gene. The arrows beneath the map indicate the directions of sequencing and the amount of each sequence that was determined from the DNA fragment generated by the following three methods: restriction enzyme digestion (A); stepwise deletion with exonuclease III (B); and partial deletion with *Sau*3A (C). The numbers of the bases correspond to the numbers in Fig. 3. Abbreviations: B, *Bgl*II; E, *Eco*RI; EV, *Eco*RV; H, *Hin*cII; N, *Nru*I; S, *Sal*I; Sp, *Sph*I; ORF, open reading frame.

with phenol by the method of Maniatis et al. (16). DNAs of the vector pVK102 and its derivatives were prepared by a rapid alkaline lysis procedure (3).

Construction of the genomic library of *A. liquefaciens* IFO 12258 in *E. coli*. *A. liquefaciens* IFO 12258 total chromosomal DNA was partially digested with *Sal*I and electrophoresed in a 0.6% agarose gel. The 15- to 35-kb DNA fragments were recovered from the gel by an electroelution method and were ligated to cosmid pVK102, which was linearized with *Sal*I and dephosphorylated with calf intestine alkaline phosphatase. The ligation mixture was used for an in vitro packaging reaction, and the phage particles were used to infect *E. coli* ED 8767 cells at the log phase of growth. The cell suspension was plated onto LK agar plates and incubated at 37°C overnight. A total of 1,000 colonies having the Km^r Tc^s marker were collected in a single cell suspension. The cosmid DNAs were extracted from the cell suspension by a rapid alkaline lysis procedure and were used to transform *E. coli* S17-1 by the $CaCl_2$ method (16). A total of 1,400 of the resulting Km^r Sm^r transformant colonies were individually transferred to microtiter plates containing LSK liquid medium and incubated overnight; glycerol was then added to each well to a final concentration of 15% before the preparations were stored at −80°C.

Conjugal mating. The cosmid genomic library in *E. coli* S17-1 was transferred to *G. oxydans* OX4 by using the following biparental mating method. A 200-μl portion of a log-phase culture of recipient strain OX4 grown in MB medium was mixed with 100 μl of a log-phase culture of each transformant of the genomic library in *E. coli* S17-1, which we cultivated after we transferred the stock culture in a microtiter plate containing fresh LSK medium with a Nunc-TSP transplate cartridge (Nunc, Roskilde, Denmark). The mixtures were individually spotted onto nitrocellulose filters, each of which was placed on the surface of an FB agar plate (96 spots per filter). The plates were incubated at 30°C overnight. The organisms growing on the filters were streaked onto MPK agar plates, which were incubated at 30°C for 4 days. The resulting transconjugants were purified by restreaking them on MPK agar plates.

Resting-cell system. The resting-cell reaction experiment was performed as described below unless indicated otherwise. Each *Gluconobacter* strain was cultivated in 5 ml of No. 5 medium in a test tube at 30°C for 48 h. A 1-ml portion of the broth was transferred to 50 ml of the same medium, and the organism was cultivated again at 30°C for 48 h. The $CaCO_3$ remaining in the broth was removed by centrifugation at 500 rpm for 5 min. The cells were collected, washed twice with 25 ml of a sterile 3-g/liter NaCl solution, and suspended in 5 ml of the same solution. Each 5-ml resting-cell system reaction mixture contained 1 ml of cell suspension, 3 g of NaCl per liter, 10 g of $CaCO_3$ per liter, and a substrate, such as L-sorbosone, L-sorbose, or D-sorbitol, at the appropriate concentration. The reactions were carried out in test tubes at 30°C for 5 days with shaking.

Screening of the genomic library in *G. oxydans* by a direct expression method. Cells of each transconjugant of *G. oxydans* OX4 containing a recombinant cosmid were individually suspended in 50 μl of the reaction mixture used for resting-cell reactions containing 18 g of L-sorbosone per liter or 40 g of L-sorbose per liter and incubated in microtiter plates at 30°C for 1 to 5 days. The assays for 2KGA production and L-sorbosone accumulation were performed by using thin-layer chromatography (24). Clones that produced high levels of 2KGA in the presence of L-sorbosone and did not accumulate L-sorbosone in the presence of L-sorbose were screened; we selected one clone, 7A6, which contained plasmid p7A6, for further study.

Subcloning of p7A6. Plasmid p7A6 was partially digested with *Sal*I, ligated with vector plasmid pVK102 linearized with *Sal*I and dephosphorylated. *E. coli* S17-1 was transformed with the ligation mixture. A total of 303 Km^r Tc^s transformant strains were individually transferred into strain OX4 by biparental conjugal mating. We examined the 303 transconjugants for the ability to efficiently convert L-sorbosone to 2KGA by a direct expression method as described above. The plasmid insert sizes of the organisms which produced high levels of 2KGA in the presence L-sorbosone were determined. The smallest plasmid with two *Sal*I fragments, one 7.3 kb long and the other 1.9 kb long, was selected and designated p7A6Δ2. Further subcloning was performed by deleting the internal *Eco*RI fragment from p7A6Δ2; this resulted in p7A6Δ3, which contained a 6.4-kb insert. Plasmid p7A6Δ3 was shortened by deleting the *Eco*RI-*Sma*I fragment. The smallest derivative (p7A6Δ4) contained 3.1 kb of insert DNA in pVK102 which had a small deletion of the *Sal*I-*Sma*I-*Sma*I fragment. A detailed restriction map of the 3.1-kb fragment is shown in Fig. 2.

DNA sequencing and analysis. DNA fragments obtained from the 3.1-kb fragment containing the SNDH gene with *Sal*I, *Nru*I, *Sph*I, and *Eco*RV were subcloned into vectors M13mp8 and M13mp9 (20) and then subjected to a sequence determination analysis in which we used the chain termination method with 7-deaza-dGTP in place of dGTP. Stepwise deletion with exonuclease III and partial deletion by *Sau*3A digestion followed by ligation were also used to obtain subclones. The nucleotide sequence was processed and analyzed by using the computer program of the Mitsui Information Service, Tokyo, Japan, and the Genetics Computer Group, University of Wisconsin, Madison. Other recombinant DNA techniques were performed essentially as described by Maniatis et al. (16).

Preparation of the membrane fraction and in vitro formation of 2KGA with the membrane fraction. *G. oxydans* N44-1 and its derivatives were cultivated in 500-ml portions of No. 5 medium at 30°C for 72 h. The cells were disrupted by treatment with a French pressure cell press (Ohtake Works Co., Ltd., Tokyo, Japan) at 1,500 kg/cm^2 in 10 mM potassium phosphate buffer (KPB) (pH 7.0) and were treated with DNase (Sigma) in the presence of 5 mM $MgCl_2$. The homogenate was centrifuged at 1,800 × *g* for 10 min to remove the cell debris. The resulting supernatant was centrifuged at 100,000 × *g* for 60 min. The precipitate was collected and designated the membrane fraction. Experiments to examine in vitro formation of 2KGA by the membrane fraction were performed at 30°C for 15 h with 3-ml portions of Tris-malate buffer (pH 7.0) containing 5 mg of membrane protein per ml and 18 g of L-sorbosone per liter or 20 g of L-sorbose per liter in 20-ml flasks containing glass beads.

Purification of SNDH from a *G. oxydans* N44-1 transconjugant and amino acid sequencing. All operations during purification were carried out at 4°C unless indicated otherwise. A membrane fraction prepared by using 10 g of wet strain N44-1(p7A6) cells as described above was suspended in 10 mM KPB (pH 7.0) containing 5 mM $MgCl_2$ and then collected by centrifugation at 100,000 × *g* for 60 min; this preparation was designated the washed membrane fraction. This fraction was suspended in 10 mM KPB (pH 6.0) to a final concentration of 10 mg/ml, and then 2% Triton X-100 was added. After the suspension was stirred

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-300                                                        -250
GGCGGAACCTATTCCTGCTATCTGGGACAGGGCCGAAAAATTTACGGAAACCTGACTTATAACTGGTGATATTTCCGGATTTTCAATAAAAACCCGGCTC
-200                                                        -150
CATCTCCCCCCGGCTCCTCATGCCCAGCCAACATTAAACGCAGCCATTCCCGACATATGGCACCGGGGGATGAACAGGCAAGATGCACACTATCAGGCGC
-100                                                        -50
CATCCAGACCATCCCTCCGCAACCACTTGGCGATCCGCGCTTTTCATTCCGACCGGCTCCGCTTACCGTTCACGGAGCCACAGAATGCAAGGCCGCCCTC
                                                            50                                     100
ATGACCCGTTCCCAGATCAGGCTTCTCGTCGCGACCACCGCCGTCACCGCGCTGCTGGTGGCAGCCGGCTACCGCGCGGTCGTCTCGCCCGAGGAAGCCC
M  T  R  S  Q  I  R  L  L  V  A  T  T  A  V  T  A  L  L  V  A  A  G  Y  R  A  V  V  S  P  E  E  A  R   34
                                                            150                                    200
GGCAGACGGTCGCGGCCGGAACCGGCCCCCACCCCGTCCTGCCGCCGCCCAACCCCACCTTCATGCCCACGGTCAACATCGCCACGCCCGTCGGCTGGCA
 Q  T  V  A  A  G  T  G  P  H  P  V  L  P  P  P  N  P  T  F  M  P  T  V  N  I  A  T  P  V  G  W  Q    67
                                                            250                                    300
GGGCACGCAGGCCCCGACCCCGGCGGCGGGGCTGGCGGTGCATGCCTTCGCCACCGGCCTGGACCACCCCCGCTGGCTGTACAAGCTGCCCAACGGCGAT
 G  T  Q  A  P  T  P  A  A  G  L  A  V  H  A  F  A  T  G  L  D  H  P  R  W  L  Y  K  L  P  N  G  D   100
                                                            350                                    400
ATCCTGGTGGCGGAATCCGAGTCCCCCGGCACCGACATCAAGACGGTGAAGAACCGCATCGCCGGCCTGGTCATGGGCCAGGTCGGCGCGGGCGGAAAAA
I  L  V  A  E  S  E  S  P  G  T  D  I  K  T  V  K  N  R  I  A  G  L  V  M  G  Q  V  G  A  G  G  K  S  134
                                                            450                                    500
GCCCCGACCGCATCATCCTGCTGCGCGATACCGACGGCGACGGCATCGCCGACCAGCGCAGCGTGTTCCTCGACCACCTCTACTCGCCCTTCGGCATGGC
 P  D  R  I  I  L  L  R  D  T  D  G  D  G  I  A  D  Q  R  S  V  F  L  D  H  L  Y  S  P  F  G  M  A   167
                                                            550                                    600
GCTGGTCGGCGACACGCTCTACGTGGCCAACGCCAACGCGCTGGTCCGCTTCCCCTATCACGAGGGCGAAACCCACATCGACGCACCGGGCGAGAAAGCC
 L  V  G  D  T  L  Y  V  A  N  A  N  A  L  V  R  F  P  Y  H  E  G  E  T  H  I  D  A  P  G  E  K  A   200
                                                            650                                    700
GTCGACCTCCCGGCCGGCTACAACCACCACTGGACCAAGAACATCCTGGCCAGCCCGGACGGCAGCACCCTCTACGTGACCGTCGGCTCCAACAGCAACG
V  D  L  P  A  G  Y  N  H  H  W  T  K  N  I  L  A  S  P  D  G  S  T  L  Y  V  T  V  G  S  N  S  N  V  234
                                                            750                                    800
TCGCCGACAACGGCATGGAGGTCGAGGAAGGCCGCGCCCGGATCGACCGGTTCGACATCGCCACCGGCAAGCTCACCCCCTACGCCACCGGCCTGCGCAA
 A  D  N  G  M  E  V  E  E  G  R  A  R  I  D  R  F  D  I  A  T  G  K  L  T  P  Y  A  T  G  L  R  N   267
                                                            850                                    900
CCCCAACGAGCTGGCGTGGGAGCCCAAGACCGGCGCCCTGTGGGTCGCAGTGAACGAACGCGACGAAATCGGCAGCGACCTGGTGCCCGACTACATCACG
 P  N  E  L  A  W  E  P  K  T  G  A  L  W  V  A  V  N  E  R  D  E  I  G  S  D  L  V  P  D  Y  I  T   300
                                                            950                                    1000
GCGGTGAAGGAGGGCGCGTTCTACGGCTGGCCCTACAGCTATTACGGCCAGCATGTCGATGTCCGCGTCAAGCCGCAGCGGCCCGACCTGGTGGCCAGCG
A  V  K  E  G  A  F  Y  G  W  P  Y  S  Y  Y  G  Q  H  V  D  V  R  V  K  P  Q  R  P  D  L  V  A  S  A  334
                                                            1050                                   1100
CCATCGCCCCCGACTACGCGCTCGGCCCGCACACCGCCTGGTTTGGCATCGCCTTCTCGCAGGACAGCAGCCTGCCCGCGGCCTGGCGCAATGGCCTGTT
 I  A  P  D  Y  A  L  G  P  H  T  A  W  F  G  I  A  F  S  Q  D  S  S  L  P  A  A  W  R  N  G  L  F   367
                                                            1150                                   1200
CGTCGCCCAGCACGGCTCATGGAACCGCAAGCCCAAGAGCGGCTACCGCGTCATCTACGTCCCCTTCACCGACGGCCACCCCGACGGCACCCCCCGCGAC
 V  A  Q  H  G  S  W  N  R  K  P  K  S  G  Y  R  V  I  Y  V  P  F  T  D  G  H  P  D  G  T  P  R  D   400
                                                            1250                                   1300
GTGCTGACCGGCTTCCTCACACAGGACGAAGACCACGCCCACGGCCGCCCGGTCGGCCTGGCGCTGGACAAATCCGGCGCCCTCCTGGTCGCCGACGATG
V  L  T  G  F  L  T  Q  D  E  D  H  A  H  G  R  P  V  G  L  A  L  D  K  S  G  A  L  L  V  A  D  D  V  434
                                                            1350                                   1400
TCGGCAACACCGTGTGGCGCGTCACCGGCACGGACCAGAAGACCGACTGACGCGCCCTTTCAGGCCCAGTACCACGACCGGATATTCCAGCAGTTCGACC
 G  N  T  V  W  R  V  T  G  T  D  Q  K  T  D  *                                                       449
                                                            1450                                   1500
GCACGTTGATATTGCTTTTATATCCGACCAGCGCGGATTTGACCCCTTCGATCGAACTGCGCTGGTAAAGCGGCAGAACCGGCAGGTCATGCCGGATGAC
                                                            1550                                   1600
GGCCTGGATCTTCCGGTAGATCTGCGCCGCTTCATCCGGTCCTGCTCATGCATCCCCGCAACCATTAGTTCGTCGGCGACGGTGGACGCGTACTGGAAGA
                                                  1640
CATTGCTGCCCACGCCCCCTCGCCGACCGACTGGTGGCTGTCGAAAT
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FIG. 3. Nucleotide sequence of the region encoding *A. liquefaciens* SNDH and deduced amino acid sequence. The amino acid sequences which are consistent with the N-terminal and internal peptide sequences are underlined. A possible ribosome binding site sequence is enclosed in a box. The transcription termination stem-and-loop structure is indicated by arrows.

for 15 h, it was centrifuged at 100,000 × *g* for 60 min to obtain the solubilized enzyme in the supernatant. The solubilized enzyme was precipitated by adding polyethylene glycol 6,000 to a concentration of 20%, was dissolved in 25 mM histidine-HCl buffer (pH 6.2) containing 0.5% Triton X-100, was placed on a PBE 94 chromatofocusing column (0.9 by 50 cm; Pharmacia, Uppsala, Sweden), and was eluted with a pH gradient solution (pH 6 to 4) made with buffer containing 0.5% Triton X-100. The active fraction that eluted at pH 4.9 was pooled and dialyzed against 5 mM KPB (pH 6.5) containing 0.5% Triton X-100. The dialysate was applied to a hydroxyapatite column (0.8 by 6 cm). The enzyme was eluted with a 30-ml 5 to 200 mM KPB (pH 6.5) gradient in the presence of 0.5% Triton X-100. The active fraction was further purified by preparative SDS-polyacrylamide gel electrophoresis (PAGE) (gel size, 3 by 120 by 140 mm). After electrophoresis, the band in the gel corresponding to a molecular weight of 47,500 was cut out, and the enzyme protein in the gel was electrophoretically

eluted and dialyzed against distilled water. About 300 μg of purified enzyme was obtained.

The amino acid sequence of the purified enzyme was determined with an Applied Biosystems model 470A gas phase sequencer. Seven amino acid sequences were obtained by determining the NH_2-terminal ends of whole polypeptide and nine tryptic fragments of the purified enzyme protein (performed by Yu-Ching Pan, F. Hoffmann-La Roche). The sequences which we determined are the sequences which are underlined in Fig. 3.

PAGE. SDS-PAGE was performed by the method of Laemmli (13) by using vertical slab gels containing 10% acrylamide. Protein bands were stained with Coomassie brilliant blue R-250. Native PAGE was performed by using 7.5% polyacrylamide in glass rods (0.6 by 10 cm).

Enzyme assay. SNDH activity was determined in a 0.5-ml reaction mixture containing 110 μM 2,6-dichlorophenolindophenol (DCIP), 250 μM phenazine methosulfate, 30 mM KPB (pH 7.0), and 2.2 mM L-sorbosone at 25°C by measuring the decrease in the A_{600} of DCIP with a Kontron Uvikon 810 spectrophotometer. The reaction was started by adding 5 to 10 μl of enzyme solution to the mixture. One unit of enzyme activity was defined as the amount of enzyme which catalyzed L-sorbosone-dependent reduction of 1 μmol of DCIP per min. The extinction coefficient of DCIP at pH 7.0 was assumed to be 14.5 mM^{-1} cm^{-1}. SNDH activity in native disc polyacrylamide gels was detected by soaking the gels in a solution containing 0.25 mM phenazine methosulfate, 0.1 mM nitroblue tetrazolium, 100 mM KPB, and 2 mM L-sorbosone at 25°C for 30 min. The activity was visualized as a dark purple band. Protein concentrations were determined by the method of Lowry et al. (14), using bovine serum albumin as the standard.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been deposited in the Genome Sequence Data Base (GSDB), DNA Data Bank of Japan (DDBJ), European Molecular Biology Laboratory (EMBL), and National Center for Biotechnology Information (NCBI) nucleotide sequence databases under accession number D28511.

RESULTS

Cloning of the SNDH gene by a direct expression method. The *A. liquefaciens* gene library in *G. oxydans* OX4 consisting of 1,400 transconjugants was screened for SNDH activity by a direct expression method as described in Materials and Methods. Clone 7A6 produced more 2KGA in the presence of L-sorbosone than host strain OX4 and the same amount of 2KGA as the donor strain, *A. liquefaciens* IFO 12258. In the presence of L-sorbose, host strain OX4 accumulated a considerable amount of L-sorbosone and a small amount of 2KGA, while clone OX4(p7A6) accumulated no L-sorbosone and produced much more 2KGA than strain OX4 produced.

To confirm that SNDH activity was encoded on p7A6 quantitatively, we performed resting-cell experiments by using cells grown in both No. 5 medium and MB medium. When L-sorbosone (27 g/liter) was used as the substrate, clone OX4(p7A6) produced three times more 2KGA (18.7 g/liter) than strains OX4 and OX4(pVK102) produced; the 2KGA yield was close to the yield produced by *A. liquefaciens* IFO 12258 (23.0 g/liter). When L-sorbose (30 g/liter) was used as the substrate, strain OX4(p7A6) did not accumulate L-sorbosone and produced more 2KGA (19.6 g/liter) than did strains OX4 and OX4(pVK102) (7.1 and 15.0 g/liter, respectively), which accumulated L-sorbosone (9.4 and 5.2 g/liter, respectively). *A. liquefaciens* IFO 12258 did not produce 2KGA from L-sorbose because it does not possess SDH activity (data not shown). These results indicated that plasmid p7A6 contained the SNDH gene and that it was expressed in strain OX4.

Purification and amino acid sequence of SNDH from strain N44-1(p7A6). Plasmid p7A6 was introduced into strain N44-1 from *E. coli* S17-1(p7A6) by biparental conjugal mating. Cells of transconjugant N44-1(p7A6) were used to purify the cloned SNDH. More than 90% of the enzyme activity was recovered in the washed membrane fraction from the cell homogenate. Then, cloned enzyme was solubilized and purified about 45-fold (yield, 3%) from the washed membrane by chromatofocusing chromatography and hydroxyapatite column chromatography. The resulting partially purified enzyme preparation had a specific activity of 36.1 U/mg of protein and produced two protein bands on native disc PAGE gels. When activity staining was used, SNDH activity was detected as a band at an R_f value of 0.31. Finally, the enzyme protein was isolated from the partially purified enzyme preparation by using preparative SDS-PAGE. The purified enzyme produced a single protein band on SDS-PAGE gels at an M_r of 47,500; this value was identical to the M_r of SNDH isolated from *A. liquefaciens* IFO 12258 (data not shown).

Nucleotide sequence of the 3.1-kb fragment containing the SNDH gene. The DNA sequence of the 3.1-kb fragment was determined by using the sequencing strategy shown in Fig. 2, and the results are shown in Fig. 3. We found one open reading frame (1,347 bp) which encoded a polypeptide that had 449 amino acid residues and a molecular mass of 48,222 Da. This estimated molecular mass was very close to the molecular mass determined for the purified enzyme (47,500 Da). The NH_2-terminal sequence and six internal amino acid sequences of the purified enzyme were also found, indicating that there was no processing of polypeptide at the NH_2 terminus after translation. The calculated amino acid composition was also similar to the composition determined for the purified enzyme (data not shown). The G+C content of the gene was 69.4 mol%, and the G+C contents of the first, second, and third members of the codons were 67.5, 48.3, and 93.5 mol%, respectively. The G+C content of the SNDH gene (69.4 mol%) was a little higher than the G+C content of *A. liquefaciens* IFO 12258 total DNA (66.2 mol%) (29). Codon usage in the gene revealed a bias for G and C, especially C, as the third member of codons, as reported previously for *Pseudomonas* strains (19). The typical consensus sequences for the promoter region that have been reported for *E. coli* and *Bacillus subtilis* were not found, while a possible Shine-Dalgarno sequence was found (AAGG) 8 bp upstream from the initiation codon.

The overall hydropathy along the amino acid sequence deduced from the nucleotide sequence of the SNDH was evaluated by the method of Kyte and Doolittle (12). The hydropathy profile (data not shown) indicated that the NH_2-terminal sequence (amino acids 3 through 27) is hydrophobic and may be an anchor in the membrane; in contrast, the COOH-terminal region contains several hydrophilic sequences.

A search for proteins homologous to the SNDH protein was performed by using the TFASTA program of the Genetics Computer Group package and DNA databases (EMBL release 37.0, GenBank release 80.0); no significantly homologous protein was found.

In vitro 2KGA formation by the membrane fraction of strain N44-1 carrying plasmid p7A6. To evaluate the cooperation between cloned SNDH and the membrane-bound SDH of host strain N44-1, membrane fractions prepared from N44-1, N44-1 carrying pVK102, and N44-1 carrying p7A6 were used in in vitro reaction experiments (Table 2). The membrane fraction prepared from strain N44-1(p7A6) converted L-sorbosone to 2KGA seven- to eightfold faster than the membrane fractions prepared from strains N44-1 and N44-1(pVK102). Considering the physicochemical instability of L-sorbosone (data not shown), the conversion of L-sorbosone to 2KGA by the membrane of strain N44-1(p7A6) is probably carried out stoichiometrically. The membrane of strain N44-1(p7A6) converted L-sorbose to 2KGA 10-fold faster than the membranes of the other strains and accumulated no L-sorbosone; in contrast, the other strains accumulated considerable amounts of L-sorbosone. The rate of recovery of L-sorbose and 2KGA in the reaction mixture containing the membrane of strain N44-1(p7A6) was almost 100%, indicating that L-sorbosone formed by membrane-bound SDH of the host was converted by membrane-bound SNDH before nonenzymatic degradation of L-sorbosone.

TABLE 2. 2KGA formation by N44-1(p7A6) membranes

Strain	Substrate	Substrate concn (g/liter)	Concn (g/liter) of[a]:			% Recovery[b]
			L-Sorbose	L-Sorbosone	2KGA	
N44-1	L-Sorbosone	18.0		8.4 (46.4)	1.4 (6.9)	53.3
N44-1(pVK102)	L-Sorbosone	18.0		10.3 (57.3)	1.6 (7.8)	65.1
N44-1(p7A6)	L-Sorbosone	18.0		5.2 (28.7)	10.6 (53.5)	82.2
N44-1	L-Sorbose	20.0	12.2 (60.9)	1.4 (6.9)	0.6 (2.9)	70.7
N44-1(pVK102)	L-Sorbose	20.0	10.9 (54.5)	2.1 (10.4)	0.7 (3.3)	68.2
N44-1(p7A6)	L-Sorbose	20.0	13.8 (68.8)	0.0 (0.0)	6.7 (30.7)	99.5

[a] Concentrations of remaining substrates and products formed. The values in parentheses are the molar yields (expressed as percentages based on the initial concentration of the substrate).
[b] Total of molar yields of L-sorbose, L-sorbosone, and 2KGA.

Formation of 2KGA from L-sorbosone, L-sorbose, and D-sorbitol by SNDH cloned strains in resting-cell reactions. The effects of cooperation between two membrane-bound dehydrogenases, SDH from the host and cloned SNDH, on 2KGA formation in the presence of L-sorbose and D-sorbitol were studied by using five host strains (Table 3). 2KGA-producing strains UV10 and U13 carrying p7A6Δ4 converted L-sorbosone to 2KGA with a molar conversion yield of about 83%. Considering the physicochemical instability of L-sorbosone, the conversion seemed to be almost stoichiometric. We observed almost the same yield (about 80%) with three *G. oxydans* strains, IFO 3292, IFO 3293, and IFO 3294, indicating that strain IFO 3293 is not a specific host for expression of *A. liquefaciens* IFO 12258 SNDH.

The level of production of 2KGA from L-sorbose was high for strain U13(p7A6Δ4) (conversion yield, 81.2%) and low for strain UV10(p7A6Δ4) (39.2%). The difference in the yields might have been due to a difference in SDH activity; one of the major factors that improved the 2KGA productivity of *G. oxydans* IFO 3293 was the increase in SDH activity, as previously described (25). Strain U13 is a derivative of UV10 and exhibits higher SDH activity than UV10. In strains exhibiting weaker SDH activities, there is stronger carbon flow toward final assimilation of the substrate to CO_2, mainly via the pentose cycle, as reported previously (21).

L-Sorbosone was detected in the resting-cell reaction mixture when we used L-sorbose and UV10(pVK102) or U13 (data not shown). This characteristic, excretion of L-sorbosone in resting-cell reaction mixtures containing L-sorbose, possibly contributed to the increase in the 2KGA yield in the presence of L-sorbose when the excreted L-sorbosone was rapidly converted to 2KGA by cloned SNDH. L-Sorbosone could be accumulated more easily by resting cells than by growing cells. Because L-sorbosone is an aldehyde (aldoketohexose) that is toxic for cells, any unconvertible L-sorbosone in the cytosol of resting cells should be excreted. A poor supply of NAD and NADP might have been one of the reasons for reduced conversion of L-sorbosone to 2KGA in resting cells.

In the presence of D-sorbitol, U13(p7A6Δ4) produced more 2KGA than U13 or U13(pVK102) produced. The effect of cloning, however, was the smallest among the three substrates tested, because D-sorbitol is the most assimilable compound.

Production of 2KGA from L-sorbose in growing cells. Strain N44-1(p7A6Δ4) was evaluated in No. 5 medium containing L-sorbose under the conditions described in Materials and Methods. Table 4 shows that this clone produced more 2KGA (about 10 g/liter more) than strains N44-1 and N44-1(pVK102) produced. The selective pressure of kanamycin and the presence of the vector plasmid had little influence on the 2KGA yield. The conversion yield obtained with the clone (58%), however, was much lower than the yield obtained with resting cells (the yield obtained with U13 carrying p7A6Δ4 was 81% [Table 3]). As described above, the excretion of L-sorbosone observed in resting-cell reaction mixtures in the presence of L-sorbose resulted in a high 2KGA yield in the presence of L-sorbose and strains containing cloned SNDH.

DISCUSSION

The membrane-bound SNDH gene of *A. liquefaciens* IFO 12258 was cloned in L-sorbosone-accumulating strain OX4 derived from *G. oxydans* IFO 3293 by direct expression screening. The cloned *Acetobacter* SNDH gene was transcribed and translated in members of the genus *Gluconobacter*, and the gene product, SNDH, was localized in the membrane. The cloned

TABLE 3. Production of 2KGA from L-sorbosone, L-sorbose, or D-sorbitol by resting cells of strains containing cloned SNDH[a]

Substrate	Substrate concn (g/liter)	Host	Plasmid	Concn of 2KGA (g/liter)	2KGA yield (mol%)[b]
L-Sorbosone	36	UV10[c]	None	11.3	28.8
			pVK102	11.2	28.6
			p7A6Δ4	32.6	83.1
		U13[c]	None	8.9	22.7
			pVK102	10.3	26.3
			p7A6Δ4	32.7	83.4
		IFO 3292[d]	p7A6Δ4	32.1	81.9
		IFO 3293[d]	p7A6Δ4	30.5	77.8
		IFO 3294[d]	p7A6Δ4	33.9	86.4
L-Sorbose	40	UV10	None	14.5	33.6
			pVK102	13.3	30.9
			p7A6Δ4	16.9	39.2
		U13	None	19.0	44.1
			pVK102	29.3	68.0
			p7A6Δ4	35.0	81.2
D-Sorbitol	40	UV10	None	14.1	33.1
			pVK102	12.4	29.1
			p7A6Δ4	14.3	33.5
		U13	None	9.9	23.2
			pVK102	15.9	37.3
			p7A6Δ4	21.6	50.4

[a] Resting-cell reaction experiments were performed as described in Materials and Methods.
[b] Molar yield of 2KGA calculated from the initial substrate concentration.
[c] Cells of strains UV10 and U13 were cultivated in No. 5 medium containing 100 g of L-sorbose per liter as described in Materials and Methods.
[d] Cells of strains IFO 3292, IFO 3293, and IFO 3294 were cultivated in MB medium.

TABLE 4. Production of 2KGA from L-sorbose in growing cells[a]

Strain	Kanamycin	Concn of 2KGA (g/liter)
G. oxydans N44-1(pVK102)	+	55.7
	−	53.8
G. oxydans N44-1(p7A6Δ4)	+	63.0
	−	64.3
G. oxydans N44-1	−	56.5

[a] The organisms were cultivated in 5 ml of No. 5 medium containing 100 g of L-sorbose per liter at 30°C for 5 days. Two of the plasmids were stably maintained (ca. 95% of the cells carried the plasmids after fermentation) even without kanamycin.

SNDH allowed oxidation of L-sorbosone to 2KGA without the addition of any artificial dye as an electron acceptor, indicating that the SNDH was able to couple with the electron transport chain in members of the genus *Gluconobacter.*

Our determination of the nucleotide sequence of SNDH revealed that SNDH is a new enzyme. We found no significant homologous regions when we compared SNDH with proteins whose sequences have been deposited in DNA databases, including membrane-bound dehydrogenases of acetic acid bacteria such as *Acetobacter aceti* alcohol dehydrogenase (9), *Acetobacter polyoxogenes* alcohol dehydrogenase (26) and aldehyde dehydrogenase (27), and *G. oxydans* glucose dehydrogenase (4). All of the other enzymes have signal sequences in the N-terminal region; in contrast, SNDH has no signal sequence. The alcohol dehydrogenases, aldehyde dehydrogenase, and glucose dehydrogenase have a common region reported to be related to pyrroloquinoline quinone binding (2, 10), but SNDH does not contain such a region, indicating that SNDH is not a quinoprotein. The addition of pyrroloquinoline quinone to purified SNDH did not stimulate 2KGA formation in the presence of L-sorbosone. SNDH and *A. polyoxogenes* aldehyde dehydrogenase exhibited no significant homology; these enzymes are different, even though they both belong to a group containing membrane-bound aldehyde dehydrogenases. SNDH is the first example of a membrane-bound dehydrogenase without a signal sequence in acetic acid bacteria. In addition, SNDH is the first membrane-bound aldehyde dehydrogenase that consists of a single subunit (molecular weight, 48,222); in contrast, *A. polyoxogenes* aldehyde dehydrogenase (27) consists of two subunits (molecular weights, 75,000 and 19,000). Actually, our data confirmed that introduction of only the SNDH gene into host *Gluconobacter* cells resulted in expression of L-sorbosone dehydrogenation activity. The amino acid sequence of SNDH also did not exhibit any homology with the sequences of the membrane-bound methanol dehydrogenases of *Methylobacterium organophilum* (15) and *Paracoccus denitrificans* (6), which possess alcohol and aldehyde dehydrogenase activities.

The hydropathy profile of SNDH deduced from the nucleotide sequence showed that the NH_2-terminal sequence consisting of 24 amino acid residues was hydrophobic and might play a role as an anchor in an inner membrane, whereas the region near the COOH terminus was hydrophilic. In general, membrane-bound dehydrogenases of acetic acid bacteria are believed to localize and act in the periplasmic space. Using a reverse-phase membrane, Matsushita et al. (17) confirmed that the membrane-bound D-glucose dehydrogenase of *E. coli*, which is a quinoprotein and exhibits homology with the *Gluconobacter* membrane-bound glucose dehydrogenase (4), is exposed in the periplasm. Since SNDH has no signal sequence, this enzyme must be partially trapped in the inner membrane at the NH_2-terminus and partially extend into the periplasmic space.

The effect of cloning of the SNDH gene on 2KGA formation was clearly recognized in the membrane fraction reaction and resting-cell reactions. In both reaction mixtures, in which we used N44-1 and U13, respectively, two strains which produce high levels of 2KGA, as the hosts for SNDH cloning, the conversion yields from L-sorbosone seemed to be stoichiometric. In the presence of L-sorbose, the conversion yield per consumed L-sorbose molecule in the membrane fraction reaction was almost 100% (Table 2), while the yield in the resting-cell reaction was slightly less (81%) (Table 4). This is because the membrane fraction did not contain cytosolic enzymes responsible for the assimilation of L-sorbose. In contrast, SNDH cloning had a minimal effect on 2KGA production in growing cells. The reasons for the low yield may be as follows: (i) L-sorbosone might be no sooner generated from L-sorbose by SDH than it is transported into the cytosol, and the active metabolic pathways in growing cells can assimilate the toxic compound L-sorbosone as soon as possible; and (ii) L-sorbose itself is assimilated faster by growing cells than by resting cells.

Considering the fact that SNDH gene cloning has only a slight effect on 2KGA production by growing cells, a bioreactor system in which the membrane fraction of the clone is used to produce 2KGA would be attractive. Immobilization of the membrane is one of the methods that should be used.

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