L-Sorbose Reductase and Its Transcriptional Regulator Involved in L-Sorbose Utilization of Gluconobacter frateurii

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Upstream of the gene for flavin adenine dinucleotide (FAD)-dependent D-sorbitol dehydrogenase (SLDH), sldSLC, a putative transcriptional regulator was found in Gluconobacter frateurii THD32 (NBRC 101656). In this study, the whole sboR gene and the adjacent gene, sboA, were cloned and analyzed. sboR mutation did not affect FAD-SLDH activity in the membrane fractions. The SboA enzyme expressed and purified from an Escherichia coli transformant showed NADPH-dependent l-sorbose reductase (NADPH-SR) activity, and the enzyme was different from the NADPH-SR previously reported for Gluconobacter suboxydans IFO 3291 in molecular size and amino acid sequence. A mutant defective in sboA showed significantly reduced growth on L-sorbose, indicating that the SboA enzyme is required for efficient growth on L-sorbose. The sboR mutant grew on L-sorbose even better than the wild-type strain did, and higher NADPH-SR activity was detected in cytoplasm fractions. Reverse transcription-PCR experiments indicated that sboR comprises an operon. These data suggest that sboR is involved in the repression of sboA, but not in the induction of sldSLC, on D-sorbitol and that another activator is required for the induction of these genes by D-sorbitol or L-sorbose.

Gluconobacter strains are known to have the ability to oxidize various kinds of sugar-related compounds and alcohols to accumulate the corresponding oxidized products (3); however, in most cases, oxidation is not completed with water and carbon dioxide, since the strains possess an incomplete citrate cycle lacking the succinate dehydrogenase gene in the genome (17). Therefore, the growth efficiencies of these microorganisms are quite low compared to those of other aerobic bacteria. Their high oxidation ability and low biomass yield make them suitable for industrial applications for bioconversion to obtain a variety of valuable products, such as L-sorbose, keto-D-glucosones, L-erythrulose, and dihydroxyacetone (3). Such oxidation reactions are called oxidative fermentation, which is mostly performed using membrane-bound dehydrogenases (14).

NAD(P)-dependent dehydrogenases are proposed to work as reductases in vivo to assimilate the oxidized products produced by membrane-bound dehydrogenases (14). From D-sorbitol, Gluconobacter strains effectively produce L-sorbose, an important intermediate in industrial vitamin C production, with strong activities of two membrane-bound D-sorbitol dehydrogenases, FAD-SLDH and PQQ-SLDH (25). Both are categorized as EC 1.1.99.21. The latter enzyme is also known as a versatile polyol dehydrogenase, reacting with various sugar alcohols and D-glucuronic but strictly obeying the so-called Bertrand-Hudson rule (15), and we propose to call this enzyme PQQ-glycerol dehydrogenase (PQQ-GLDH) because it is its historical name and because glycerol is the most abundant substrate in nature. On the other hand, two cytosolic NAD(P)-dependent enzymes capable of oxidizing D-sorbitol have been reported for several Gluconobacter strains; they are D-sorbitol:NAD oxidoreductase (EC 1.1.1.14), producing D-fructose (1), and D-sorbitol:NADP oxidoreductase (EC 1.1.1.289), producing L-sorbose. The latter enzyme is also known as an NADPH-dependent L-sorbose reductase (NADPH-SR), and two different NADPH-SRs were purified and characterized, including one with two identical subunits of 30 kDa from Gluconobacter melanogenus IFO 3294 (2) and another with a subunit of 60 kDa from G. melanogenus N44-1 (22). In addition, a gene encoding an NADPH-SR with a 60-kDa subunit from Gluconobacter oxydans G624 was cloned and characterized, having one subunit with a molecular mass of 53,634 Da (19). A recent study revealed that a similar NADPH-SR with a calculated molecular mass of 53,541 Da plays the main role in L-sorbose assimilation in Gluconobacter suboxydans IFO 3291 (21).

It has been reported that L-sorbose is incorporated into cells of Agrobacterium tumefaciens, Neurospora crassa, Aspergillus...
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TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant properties</th>
<th>Reference or source</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>G. frateurii strains</td>
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<tr>
<td>THD32</td>
<td>Wild-type strain; NBRC 101656</td>
<td>This study</td>
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<tr>
<td>shoR::Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>THD32 defective in shoR by insertion of Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>shoA::Tc&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>This study</td>
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<td>shoR::Tc&lt;pSA&lt;sub&gt;sboR&lt;/sub&gt;B&gt;</td>
<td>shoR disruptant harboring the plasmid pSA&lt;sub&gt;sboR&lt;/sub&gt;B</td>
<td>This study</td>
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<tr>
<td>shoA::Tc&lt;pSA&lt;sub&gt;E&lt;/sub&gt;asboA&gt;</td>
<td>shoA disruptant harboring the plasmid pSA&lt;sub&gt;E&lt;/sub&gt;asboA</td>
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<td>E. coli strains</td>
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<td>DH5α</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; endA1 hsdR17 recA1 supE44 thi-1 d800lacZΔM15</td>
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<td>BL21(DE3)</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; dcm ompT hsdS&lt;sub&gt;Δ&lt;/sub&gt;(rB&lt;sub&gt;−&lt;/sub&gt; m&lt;sub&gt;R&lt;/sub&gt;) gal</td>
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<td>E. coli cloning vector; Amp&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
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<td>Gluconobacter cloning vector; Amp&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pUC46</td>
<td>Carries 3.5-kb DNA fragment at SphI site of pUC119; Amp&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>pSA&lt;sub&gt;sboR&lt;/sub&gt;B</td>
<td>Carries 0.8 kb of shoA at Smal and Sall sites of pSA&lt;sub&gt;19&lt;/sub&gt;; Amp&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pUCSDR&lt;sub&gt;Tc&lt;/sub&gt;</td>
<td>Insertion of Tc&lt;sup&gt;+&lt;/sup&gt; cassette at HincII site of pUCSDR; Tc&lt;sup&gt;+&lt;/sup&gt; Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>pUC&lt;sub&gt;sboR&lt;/sub&gt;Tc</td>
<td>Insertion of Tc&lt;sup&gt;+&lt;/sup&gt; cassette at HincII site of pUC&lt;sub&gt;sboR&lt;/sub&gt;; Tc&lt;sup&gt;+&lt;/sup&gt; Amp&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pET-28a&lt;sup&gt;(+)&lt;/sup&gt; with the complete coding sequence of shoA</td>
<td>This study</td>
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<td>This study</td>
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<tr>
<td>pSA&lt;sub&gt;E&lt;/sub&gt;asboA</td>
<td>pSA&lt;sub&gt;19&lt;/sub&gt; with the complete coding sequence of shoA in the opposite direction from that of the lac promoter</td>
<td>This study</td>
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In contrast, in Escherichia coli, Lactobacillus casei, and Klebsiella pneumoniae, L-sorbose is phosphorylated to L-sorbos-1-phosphate at the cell surface during transport into the cell and reduced to D-sorbitol-6-phosphate by L-sorbose-1-phosphate reductase (26, 27, 30). In either case, an NAD(P)H-dependent

MATERIALS AND METHODS

Chromosomes. NAD, NADP, NADH, and yeast extract were a kind gift from the Oriental Yeast Co., Tokyo, Japan. The other chemicals used were of guaranteed grade from commercial sources. Plasmid pSA<sub>19</sub> (24) was kindly provided by Ajinomoto Co. Inc.

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1.

**Culture media and growth conditions.** Gluconobacter strains were maintained on potato-Ca<sub>3</sub>O<sub>4</sub> agar slants, which were prepared by adding 2% agar and 0.5% Ca<sub>3</sub>O<sub>4</sub> 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 performed in 5 ml of potato medium with shaking for 24 h at 30°C, and precultures were 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. Escherichia coli for plasmid construction and DNA sequencing was cultured in Luria-Bertani (LB) medium containing 50 μg/ml ampicillin, 25 μg/ml kanamycin, or 25 μg/ml tetracycline when required.

**Preparation of membrane and soluble fractions.** Cells were harvested by centrifugation at 9,000 rpm for 10 min and washed once with distilled water. The washed cells were resuspended in 10 mM phosphate buffer (KP<sub>B</sub>; pH 6.0) at a concentration of 3 ml/g wet cells and passed twice through a French pressure cell (American Instruments Co., Silver Spring, MD) at 16,000 lb/in<sup>2</sup>. After centrifugation at 6,000 rpm for 10 min to remove the intact cells, the supernatants were ultra centrifuged at 40,000 rpm for 60 min. The resultant precipitate was resuspended in McIlvaine buffer (pH 5.0) and used as a membrane fraction, while the supernatant was used as the soluble fraction.

**Qualitative and quantitative analyses of ketohexose.** For qualitative measurement of the total amount of ketohexose, resorcinol was used as described previously (11). Enzyme activity was defined as the amount of enzyme which catalyzed 1 mol of substrate oxidation per min under the above conditions, which was equivalent to an absorbance of 4.0 at 660 nm. The enzyme activity of NAD(P)-dependent dehydrogenase was measured following the increase of NAD(P)<sub>H</sub> at 340 nm in a reaction mixture (1 ml) containing 100 μmol of substrate, 0.1 μmol of NAD(P)<sub>H</sub>, and the appropriate amount of enzymes in 50 mM KP<sub>B</sub> (pH 6.0). For the reduction reaction, enzyme activity was measured according to decreasing NAD(P)<sub>H</sub> at 340 nm in a reaction mixture (1 ml) containing 100 μmol of substrate, 0.1 μmol of NAD(P)<sub>H</sub>, and the appropriate amount of enzymes in 100
mM Tris-HCl (pH 9.0). One unit of enzyme activity was defined as the amount of enzyme catalyzing 1.0 μmol of substrate.

Determination of protein concentration. The protein concentration was measured by a modification of the Lowry method (6). Bovine serum albumin was used as the standard protein.

Measurement of molecular mass. The molecular mass of the native enzyme was determined using high-performance liquid chromatography with gel filtration column chromatography (Superdex 200; Amersham Biosciences). Glutamate dehydrogenase (290 kDa), lactate dehydrogenase (142 kDa), enolase (67 kDa), myokinase (32 kDa), and cytochrome c (12.4 kDa) were used as standard proteins.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12.5% (wt/vol) acrylamide slab gels by the method described by Laemmli (12). The following calibration proteins with the indicated molecular masses were used as references for the measurement of molecular mass: phosphorylase b (94 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and lysozyme (14.4 kDa). Proteins were stained with 0.1% Coomassie brilliant blue R-250.

Determination of N-terminal amino acid sequences. After SDS-PAGE, the proteins in the gel were transferred electrophoretically to a polyvinylidene difluoride membrane at 100 mA for 4 h. The proteins were stained with Coomassie brilliant blue R-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 PPSQ-21 protein sequencer (Shimadzu).

DNA manipulations. Restriction enzyme digestion, DNA ligation, and other DNA modifications were performed according to the vendor’s recommendations. The preparation of plasmid DNA from E. coli strains and other general molecular biological techniques were performed as described previously (18). Genomic DNAs of Glucobacter strains were isolated from cells grown to the mid-exponential phase in d-sorbitol medium by modification of the method of Marmur (13). PCR was performed using a Ready-To-Go PCR bead kit (Amersham Biosciences). DNA fragments obtained by PCR were isolated by agarose gel electrophoresis, purified with a QIAquick gel extraction kit (QIAGEN), and then cloned into the pGEM-T Easy vector (Promega). G. frutetii THD32 was transformed by transformation methods.

RNA isolation. Total RNA was isolated from the G. frutetii THD32 wild-type strain grown on d-sorbitol medium for 18 h (corresponding to the late exponential phase). RNAs were prepared quickly according to the hot phenol method. The preparation of plasmid DNA from G. frutetii THD32 in pUC119 were grown on an LB agar plate containing 50 μg/ml of ampicillin, transferred to a Hybrid N+ membrane, and lysed. Hybridization and detection were performed using the DIG system (Roche Diagnostics) according to the protocol provided by the supplier.

RESULTS

Construction of gene disruption mutants of G. frutetii THD32. To construct an sboA mutant, a 1.4-kb HindIII-SphI fragment was subcloned from a DNA fragment obtained by colony hybridization into pUC19, resulting in pUCSDR. The tetracycline resistance gene (Tc) obtained from pKRP12 (10) was ligated into pUCSDR digested with PstI, to generate pUCSDRt. Construction of an sboA-defective mutant was carried out using a 0.75-kb PCR fragment amplified using the primers SlDr-F1 (5′ AGCTAGAAGAGCGCCAGAGTCC 3′) and SlDr-R1 (5′ GTGCCGTCAGCTCCAGTAC 3′) under PCR conditions consisting of 30 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The amplified fragment was subcloned into pUC19, resulting in pUCsboB. The tetracycline resistance gene (Tc) obtained from pKRP12 was ligated into pUCsboB digested with HindIII, to generate pUCsboBt. Each plasmid harboring the disrupted gene was introduced into G. frutetii THD32 by electroporation. Transformants harboring the plasmid sequence integrated at the corresponding chromosome locus by homologous recombination were selected on d-sorbitol-glycerol agar containing 25 μg/ml of tetracycline. Disruption of sboA was confirmed by Southern hybridization, whereas disruption of the sboR gene was confirmed by PCR using the primers described above.

Complementation of sboR and sboA in disrupted mutant strains. The sboR gene was obtained from pUC86 digested with SalI and Sall and subcloned into pSA19 (24) to obtain plasmid pASboR. To construct a complementing sboA plasmid under control of the lac promoter, the Smal-EcoRI sboA gene fragment obtained from pBlueBoA was subcloned into pSA19, resulting in pSABsoBoA. The sboA gene transcribed in the opposite direction of the lac promoter of pSA19 was prepared from plasmid pETsboB digested with Sall and Sall and subcloned into pSA19, resulting in pSAETsboA. The resulting plasmids were incorporated into the gene-disrupted mutant by electroporation and selected on 1% d-sorbitol and glycylc plates containing 500 μg/ml of ampicillin and 25 μg/ml of tetracycline.

Southern hybridization. Genomic DNA was digested with suitable restriction enzymes, electrophoresed in an agarose gel, and then transferred to a Hybrid N+ membrane (Amersham Biosciences) by capillary blotting. DNA bands were then fixed to the membrane by exposure to UV light for 5 min. Hybridization and detection were carried out using the ECL direct nucleotide labeling system (Amersham Biosciences) according to the protocol provided by the supplier.

Nucleotide sequence analysis. Plasmids for sequencing were prepared with a QIAprep Spin miniprep kit (QIAGEN). Sequencing was performed using an ABI PRISM 310 instrument (Applied Biosystems). Sequence data were analyzed with Genedoc (Research Systems) and Align (Research Systems). Homology search analysis and alignment were performed with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

Expression of sboA in E. coli. Expression of sboA was assessed under the control of the T7 promoter. The 1.4-kb DNA fragment harboring sboA was subcloned from pUCSDR into pET28a (+) to generate pETsboA. The resulting plasmid was then transformed into the E. coli BL21(DE3) by electroporation. The transformant was cultured at 30°C in 100 mL of LB medium containing 50 μg/mL of kanamycin. After 6 h to 8 h, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the culture medium at a final concentration of 0.5 mM and further cultured for 5 h. The cells were then harvested by centrifugation at 6,000 rpm for 10 min, washed with 10 mM KPB (pH 6.0), and disrupted with a French press. The debris was removed by centrifugation at 6,000 rpm for 10 min.

Purification of SboA. One gram of wet cells was resuspended in 4 mL of 10 mM KPB (pH 6.0) containing 1 mM EDTA and 10% (wt/vol) glycerol (buffer A). The cell extract was obtained by passage through a French press followed by ultracentrifugation at 40,000 rpm for 60 min at 4°C. The soluble fraction was put into DEAE-cellulose which had previously been equilibrated with buffer A. After being washed in the column with the same buffer, the enzyme was eluted using a linear gradient of 0 to 0.2 M KCl and eluted around 0.1 to 0.15 M KCl. The active fraction was pooled and dialyzed against 0.2 M KCl. The enzyme was then transferred to the chelating DE3 by electrophoresis. The enzyme was dialyzed and stored at –80°C.

Determination of protein concentration. The protein concentration was measured by a modification of the Lowry method (6). Bovine serum albumin was used as the standard protein.

Gel electrophoresis, purified with a QIAquick gel extraction kit (QIAGEN), and subcloned into pUCSDR into pET28a (+) to generate pETsboA. The resulting plasmid was then transformed into the E. coli BL21(DE3) by electroporation. The transformant was cultured at 30°C in 100 mL of LB medium containing 50 μg/mL of kanamycin. After 6 h to 8 h, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the culture medium at a final concentration of 0.5 mM and further cultured for 5 h. The cells were then harvested by centrifugation at 6,000 rpm for 10 min, washed with 10 mM KPB (pH 6.0), and disrupted with a French press. The debris was removed by centrifugation at 6,000 rpm for 10 min.

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Nucleotide sequence accession number. Sequence data obtained for this study are available under accession number AB192961 in the DNA Data Bank of Japan (DDBJ).

Nucleotide sequence upstream of sldSLC. In a previous study, part of a gene showing similarity to the araC transcriptional regulator was found upstream of sldSLC (25). Thus, a new 3.5-kb SphI gene fragment containing a further upstream region was cloned and showed a positive signal with a 0.75-kb PCR fragment containing part of the transcriptional regulator as a probe (Fig. 1). The total amino acid sequence of the transcriptional regulator also showed significant similarity to...
the AraC/XylE family of transcription activator proteins. We designated the gene sboR, encoding a L-sorbose oxidoreductase regulatory protein, according to several results, as described below. Downstream of sboR, another ORF was found, encoding 263 amino acid residues similar to those of many proteins in the short-chain dehydrogenase/reductase family (30 to 38% identity) in the protein database, especially two putative dehydrogenases predicted from the genome sequences of Rhizobium etli and Azotobacter vinelandii (71 and 69%, respectively). This ORF was designated sboA. The calculated molecular mass and pI of the encoded protein were 28,320 Da and 5.72, respectively. From the genome sequence of G. frateurii ATCC 621H (17), the genes for proteins showing the highest similarity were GOX0646 and GOX2040 (30% identity each). ATP-citrate lyase (EC 1.1.1.32) and acetoacetyl-CoA thiolase (EC 2.3.1.9) were not expressed because the genes were interrupted by a tetracycline resistance gene (sboA::Tc)

FIG. 1. Schematic representation of gene organization, including that of sboRA, in the DNA fragment obtained from G. frateurii THD32. ORFs are represented by arrows indicating their orientation. ORF1 is not complete at its N terminus.

To understand the biological function of sboA existing in this Gluconobacter strain, a mutant defective in sboA was obtained by homologous recombination, with the sboA gene fragment inserted by the tetracycline resistance gene (sboA::Tc). Growth of the mutant on L-sorbose was severely impaired, and that on D-sorbitol was also impaired to some extent (Fig. 3a). The growth yield of the mutant strain on D-sorbitol was much more dependent on the concentration of D-sorbitol in the culture medium, and at 5% (wt/vol) or higher, its growth was similar to that of the wild type (data not shown). Gluconobacter cells strongly oxidized D-sorbitol to L-sorbose by using two membrane-bound enzymes, PQQ-GLDH and FAD-SLDH (25); therefore, it seemed that to gain efficient growth, mutant cells required a higher concentration of D-sorbitol to utilize this growth substrate before complete conversion to L-sorbose occurred by membrane-bound enzymes. NADPH-SR activity in the cytoplasmic fraction was high for wild-type cells grown

FIG. 2. SDS-PAGE analysis of purified SboA. Fifteen micrograms of purified protein was applied to a 12.5% (wt/vol) polyacrylamide gel and stained with Coomassie brilliant blue R-250.
on either D-sorbitol or L-sorbose and low for cells grown on glycerol (Fig. 3b), indicating that this activity is inducible. Enzyme activity was not completely diminished in the mutant strain (sboA::Tcr), and residual activity seemed constant, irrespective of the growth substrate. This residual activity was similar to that in wild-type cells grown on glycerol, indicating that another enzyme has NADPH-SR activity but that it is constitutively expressed. For *Gluconobacter* cells, it is known that there is a NAD(H)-dependent dehydrogenase (NAD-SLDH) catalyzing the oxidation of D-sorbitol to D-fructose (1); therefore, NAD-SLDH activity in the cytosolic fraction was also measured. This enzyme activity changed very little with different carbon sources or with sboA disruption (Fig. 3b).

**Characterization of sboR disruptant.** It was speculated that sboR might be involved in the induction of *sldSLC* by D-sorbitol or L-sorbose (25). In order to investigate the physiological role of SboR, a mutant strain defective in sboR was constructed by insertion of the tetracycline resistance gene cassette. Unexpectedly, SLDH activity in membrane fractions from the mutant strain was similar to that of the wild-type strain under several conditions tested and was induced normally by D-sorbitol to D-fructose (1); therefore, NAD-SLDH activity in the cytosolic fraction was also measured. This enzyme activity changed very little with different carbon sources or with sboA disruption (Fig. 3b).

**SboR represses SboA activity involved in L-sorbose assimilation.** To confirm the repression of SboA by SboR, complementation of the mutant strain defective in the sboR gene was performed. Growth of the complemented strain on D-sorbitol was lower than that of either the sboR mutant or the wild-type strain (Fig. 4a), corresponding to a higher accumulation of L-sorbose (Fig. 4b) and to decreased NADPH-SR activity in cytoplasmic fractions (Fig. 4c). It seems that a higher copy number of sboR in the complemented strain prevented derepression by L-sorbose, even after L-sorbose accumulated in the culture medium. As a result, NADPH-SR activity remained low and L-sorbose assimilation did not occur, leading to its higher accumulation in the stationary phase. Furthermore, we also constructed an sboA-complemented strain. Growth of the complemented strain on D-sorbitol medium was restored to a level comparable to that of wild-type strains, as shown in Fig. 4a. NADPH-SR activity was much higher in the sboA-complemented strain than in other strains, resulting in assimilation of L-sorbose as well as that in the sboR disruptant strain (Fig. 4b). These results clearly indicated that sboR, encoding an NADPH-SR, is responsible for L-sorbose assimilation and is repressed by SboR.

**sboR and sboA are in the same transcriptional unit.** Between sboR and sboA, a gap of 132 bp exists, and a terminator-like sequence is found 32 bp downstream of sboA. To confirm whether sboR and sboA are in the same transcriptional unit, RT-PCR was performed, using the RNA fraction isolated from the *G. frateurii* THD32 wild-type strain as a template and three sets of primers, specific for a part within each gene and an intergenic region between the two genes, as shown in Fig. 5a. RT-PCR amplification products were obtained from all sets of primers (Fig. 5b), indicating that sboR and sboA are cotranscribed from the same upstream promoter. Moreover, comple-
menting plasmids harboring the complete sequence of sboA and the upstream intergenic region were constructed, in which the transcription direction was in either the same or opposite orientation (pSABsboA or pSAEsboA, respectively) to that of the lac promoter in the vector plasmid (Fig. 5a). The strain with plasmid pSABsboA exhibited high NADPH-SR activity, and the enzyme activity was not significantly different between cells grown on D-sorbitol and glycerol (Fig. 5c), indicating that expression occurred constitutively by the lac promoter, not by its own inducible promoter. Furthermore, complementation by plasmid pSAEsboA seemed not to induce sboA, since the enzyme activity of the complemented strain was similar to that of the disruptant (Fig. 5c). These results support the hypothesis that sboA and sboR are organized in an operon under the control of the same promoter.

DISCUSSION

In a previous paper, we found a transcriptional regulator next to sldSLC and assumed that SboR might be an activator of the expression of sldSLC, whose activity was induced according to the increased concentration of D-sorbitol added to the culture medium (25). In this paper, however, we demonstrated that sboR is not involved in the induction of sldSLC because FAD-SLDH activity was unchanged and still induced in the sboR mutant as well as in the wild-type strain (data not shown), and instead, it was shown that sboR is involved in the repression of its own expression and of sboA, which is shown to be in the same transcriptional unit. The deduced amino acid sequence of SboR showed high similarity to proteins in the AraC-XylS family, having two helix-turn-helix DNA binding motifs in the C-terminal domain and a ligand binding site in the N-terminal domain (7). Most proteins in this family are transcriptional activators, whereas SboR is a repressor. L-Sorbose seems to be involved in the derepression of SboR and allows the expression of sboA, since higher NADPH-SR activity was found in cells grown on D-sorbitol or L-sorbose than in those grown on glycerol (Fig. 3). On the other hand, it was also shown that the induction of SboA by D-sorbitol or L-sorbose occurred even in sboR mutant cells, indicating that another transcriptional activator is involved in the activation of sboRA transcription, which might be the same as the activator for sldSLC transcription. We tried to obtain the recombinant SboR protein to examine its binding ability with DNA fragments; however, it was obtained as an inclusion body (data not shown), and thus, DNA binding experiments remain for future work.

Shinjoh et al. reported that an NADPH-SR of 60 kDa from...
G. *suboxydans* IFO 3291 was encoded by a gene belonging to the D-mannitol dehydrogenase superfamily and that the enzyme was required for L-sorbose assimilation (21). Similarly, SboA found in *G. frateurii* THD32 seemed to be essential for efficient L-sorbose assimilation, although SboA has 263 amino acid residues and its molecular size is 28,320 Da, similar to many proteins in the short-chain dehydrogenase/reductase family in the genome database and different from the enzyme of *G. suboxydans* IFO 3291. The physicochemical and catalytic properties of SboA were compared to those of other NADPH-SRs or D-sorbitol:NADP dehydrogenases reported from *Gluconobacter* strains, as shown in Table 2. Basically, NADPH-SRs are divided into two types, those of 30 kDa and those of 60 kDa. NADP-SLDH obtained from *G. oxydans* G624 (19) is similar to the enzymes from *G. suboxydans* IFO 3291 and *G. melanogenus* N44-1 (23), and the NADPH-SR from *G. melanogenus* 3294 (2) shows strong similarity to SboA in its molecular size and catalytic properties; however, there has been no molecular biological study of this enzyme. Since the disruption of *sboA* did not diminish NADPH-SR activity completely in *G. frateurii* THD32 and the remaining activity seemed to be constitutive, whereas SboA was induced by L-sorbose, it is supposed that another NADPH-SR exists in this strain, which might be similar to the 60-kDa NADPH-SR. In fact, in the genome sequence of *G. oxydans* ATCC 621H, two 60-kDa NADPH-SR-like genes, GOX1432 and GOX0849, showing 85 and 44% identities, respectively, to the NADPH-SR gene from *G. suboxydans* IFO 3291, were found, while homologs of SboA showing only 30% identity or less also existed (see Results). Recently, an NADPH-SR with a molecular mass of 31 kDa was reported for *Candida albicans* (8); however, its amino acid sequence showed only 27% identity to that of SboA, and it reacted with D-fructose, which was not utilized by SboA at all.

SboA was shown to be an NADPH-SR, whereas SboR is a repressor of the expression of *sboRA*, and the induction of *sboRA* occurred by another, unidentified activator with D-sorbitol or L-sorbose. Why is the repressor *sboR* required to comprise an operon with *sboA*? It might be a “break” for L-sorbose accumulation by preventing its assimilation or against the expression of *sboA* after L-sorbose is exhausted. When the strain starts to grow on D-sorbitol, the cells directly incorporate and utilize it in a small part as a carbon source by the pentose phosphate pathway through D-fructose, which is derived from...
NAD-SLDH and then phosphorylated to D-fructose-6-phosphate. A major part of the substrate is oxidized by membrane-bound PQO-GLDH and FAD-SLDH, and then L-sorbose accumulates in the culture medium. After L-sorbose accumulates to some extent and D-sorbitol is exhausted, the cells start to utilize L-sorbose, probably similar to the case in C. albicans (8), through fructose 6-phosphate. During the accumulation of L-sorbose, SboR is expressed together with SboA and may compete with the unknown activator by binding to the same operator site for \textit{sboRA} transcription; however, it probably does not repress its expression because of the presence of a derepressing molecule. In fact, when SboR was expressed under the control of the \textit{lac} promoter, which works constitutively in \textit{Gluconobacter}, no consumption of L-sorbose was observed (Fig. 4), indicating that L-sorbose is an inducer that derepresses SboR from the operator site. When L-sorbose is exhausted, the activator without the coactivator no longer works, and then complete repression of \textit{sboRA} by SboR is accomplished. SboR also works positively for the accumulation of L-sorbose at the beginning of growth on D-sorbitol by preventing the expression of SboA. \textit{Gluconobacter} strains may quickly oxidize D-sorbitol to an unfavorable substance, L-sorbose, creating acidic conditions to prevent the growth of other bacteria, and after expelling enemies, slowly utilize L-sorbose, similar to the hypothesis for acetic acid use by \textit{Acetobacter} (16). Actually, compared to L-sorbose, D-sorbitol is a good carbon source for many microorganisms, such as \textit{E. coli}, which utilizes it via the phosphotransferase system, which transports and phosphorylates the substrate to D-sorbitol-6-phosphate (28).

It has been shown that an NADPH-SR is responsible for the assimilation of L-sorbose in this strain and is required for efficient growth on L-sorbose; however, even though its activity was increased more than five times by introducing the gene via a plasmid, the assimilation rate was not much affected (Fig. 4). In NADPH-SR-overexpressing cells, something other than NADPH-SR activity limits the assimilation of L-sorbose, for example, the transport system of L-sorbose into the cytoplasm or coenzyme NADP regeneration in cells. There is no information about the transport system for L-sorbose. When cells grow on D-sorbitol, a small part of L-sorbose formed during the oxidation reaction can be transported and utilized by coupling with the hydrolysis of ATP (ABC transporter) or with proton motive force; however, it is more plausible that a small part of the substrate, D-sorbitol, is utilized for growth without oxidation and that the oxidized product is accumulated almost quantitatively until the substrate is exhausted (Fig. 4b). L-Sorbose is then gradually consumed with slower growth when the energy source for L-sorbose transport is already lacking, implying that the transporter is a permease requiring no energy source such as ATP or proton motive force. This also suggests that the transport system for L-sorbose is inducible and works only after a high concentration of L-sorbose is accumulated in the culture medium.

This is the first report about the regulation of L-sorbose assimilation in a \textit{Gluconobacter} strain, although the regulation mechanism is still not clear. To show direct evidence of the actual inducer of SboR, the expression of SboR in \textit{E. coli} and its use in a gel shift assay are desirable; however, we obtained the recombinant protein as an inclusion body (data not shown).
Further analysis to determine the repression mechanism of SboR is required.

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