J. Microbiol. Biotechnol. (2002), **12**(4), 603–609

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Molecular Characterization of the Levansucrase Gene from *Pseudomonas aurantiaca* S-4380 and Its Expression in *Escherichia coli*

JANG, EUN-KYUNG[1], KI-HYO JANG[2], ISAAC KOH[3], IN-HWAN KIM[1], SEUNG-HWAN KIM[1], SOON AH KANG[2], CHUL HO KIM[1,3]*, SANG-DO HA[4], AND SANG-KI RHEE[3]

[1]*RealBioTech Co. Ltd., Korea Research Institute of Bioscience and Biotechnology (KRIBB), Yusong, Taejon 305-333, Korea*
[2]*Department of Medical Nutrition, Graduate School of East-West Medical Science, Kyung Hee University, Suwon 449-701, Korea*
[3]*Biotechnology Research Division, KRIBB, Yusong, Taejon 305-333, Korea*
[4]*Department of Industry and Technology, Korea Health Industry Development Institute, Seoul 156-800, Korea*

Received: February 19, 2002
Accepted: May 31, 2002

Abstract DFA IV is di-D-fructose-2,6':6,2'-dianhydride, consisting of two fructose residues. It can be enzymatically synthesized from levan by levan fructotransferase, and can be used for mineral absorption. Understanding of the structure and composition of levan is important to obtain high-level production of DFA IV. A bacterial strain, *Pseudomonas aurantiaca* S-4380, was identified to produce low-branched levan, and the levansucrase gene (*lsc*A) from this bacterium was found to be composed of 1,275 bp coding for a protein of 424 amino acids, with an estimated molecular weight of 47 kDa. The bacterial levansucrase gene was expressed in *Escherichia coli* DH5α by its own promoter and *lac* promoter. The recombinant levansucrase was produced in soluble form with 170U of levansucrase activity from 1-ml *E. coli* culture broth. The expressed enzyme from the clone showed similar biochemical properties, such as size of active levansucrase, degree of branching, and optimum temperature, with *P. aurantiaca* S-4380 levansucrase.

Key words: Levansucrase, low-branched levan, *Pseudomonas aurantiaca*

DFA IV (di-D-fructose-2,6':6,2'-dianhydride IV) is a non-reducing and indigestible disaccharide consisting of two fructose residues, and expected to have novel physiological functions based on its unique structure [18, 20, 21, 25]. Baik *et al.* (1997) [2] demonstrated that DFA I and III were effective by promoting the iron uptake by the brush-border membrane of mucosal cells from the intestinal lumen of rats, when the mixture (iron:DFA=1:1, w/w) was used. In 1999, Saito *et al.* [19] reported that the apparent calcium absorption in rats fed with 3% (w/v) DFA IV was about 20% higher than the control rats. Therefore, DFA IV has been proposed as one of the candidates for mineral absorption. The Recommended Dietary Allowance (RDA) for calcium is 700 mg/day in Korea, requiring intake of large quantities (700 mg of DFA IV for daily intake) of pure and biologically active DFA IV.

During chemical synthesis, DFA IV is obtained as complex mixtures of isomers of DFAs [20]. Therefore, it is desirable to produce DFA IV by an enzymatic process for the production of a regio-specific DFA IV. The enzyme levan fructotransferase is capable of producing DFA IV from levan as a substrate [20]. In a previous study, we observed that the levan fructotransferase from *Arthrobacter ureafaciens* K2032 formed DFA IV from bacterial levan in a significant quantity [25, 29]. The drawback of this process, however, was its low product yield due to the structure of levan used.

Bacterial levansucrase (E.C. 2.4.1.10) is an extracellular protein found in Gram-negative and Gram-positive bacteria [6, 8, 10, 15, 24]. Levansucrase catalyzes synthesis of levan from sucrose. The catalysis is mediated by a coupled reaction of the enzyme through a ping-pong mechanism, including sucrose hydrolysis and polymerizing transfer of the fructose residues produced [5]. Levan is a homopolysaccharide composed of D-fructofuranosyl residues joined by β-(2,6) and β-(2,1) linkages. In some plants, levan is produced as a prevalent storage carbohydrate, and it was recently found that a recombinant levansucrase gene expressed in tobacco gave rise to increased tolerance to drought stress [16]. Plant levans, graminants, and phleins have shorter residues (ranging from 10 to ~200 fructose residues) than microbial levans, of which molecular weights are up to several

*Corresponding author
Phone: 82-42-863-4381; Fax: 82-42-863-4382;
E-mail: kim3641@mail.kribb.re.kr

million daltons with multiple branches [10]. Since the efficiency of final conversion of levan to DFA IV by levan fructotransferase is significantly affected by the degree of branching of the repeating unit, a low degree of branching levan is preferable for DFA IV production [18, 20, 21]. Saito *et al.* [18, 20, 21] proposed that levan from *Serratia levanicum* is the most suitable substrate for DFA IV production since the levan from this bacterium shows a 6% degree of branch. Recently, a levan-producing strain (*Pseudomonas aurantiaca* S-4380) was isolated from soil. The levan from this bacterium had a 6% degree of branch. Using various levans derived from *P. aurantiaca* S-4380, *Z. mobilis*, and *S. levanicum*, the highest conversion efficiency of 59% was obtained with *P. aurantiaca* S-4380 levan, whilst that from *S. levanicum* was 53% (unpublished data). In this paper, the cloning, sequencing, and expression of the *lscA* gene from *P. aurantiaca* S-4380 in *E. coli* are described.

MATERIALS AND METHODS

Materials

DFA IV and levan fructotransferase were prepared using the method previously described [25]. A silica gel 60 F_{254} TLC plate purchased from Merck (Darmstadt, Germany) was used for sugar analysis. Unless otherwise specified, chemicals were purchased from Sigma. Restriction enzymes, calf intestinal alkaline phosphatase, Klenow fragment, T4 polynucleotide kinase, T4 DNA ligase, and dNTP mix were from Boehringer Mannheim or Takara.

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *P. aurantiaca* S-4380 (KCTC 0943BP) was grown aerobically in YPS broth (yeast extract 1%, peptone 2%, sucrose 5%) at 30°C. *E. coli* DH5α was used as a host in the cloning and heterologous levansucrase expression studies, and pET-21a(+) as a cloning vector. Recombinant *E. coli* cells were grown at 37°C in LB broth without the addition of carbohydrate. When necessary, ampicillin was added to a final concentration of 100 μg/ml.

DNA Manipulations and Reagents

Plasmid DNA from *E. coli* was obtained by alkaline lysis and purified on Qiagen (Hilden, Germany) columns. Chromosomal DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, ligation, transformation, restriction endonuclease mapping, and PCR were all performed as described elsewhere [17, 22]. After purification of the DNA product, direct sequencing was performed with an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer), as specified by the manufacturer [23]. The DNA fragments were analyzed on an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer). The homology search and construction of a phylogenetic tree were performed using the DNADIST program (Mac-molly, Mackintosh) and NEIGHBOR program of PHYLIP 3.5 package [7].

Cloning of *lscA*

Chromosomal DNA prepared from the isolated strain was partially digested with the restriction endonuclease *Sau*3AI, and DNA fragments of 2–4 kb were purified from agarose gel. The fragments were ligated to the *Bam*HI-cleaved, dephosphorylated vector pBluescript II KS+ and the plasmids were transformed into *E. coli* DH5α, which does not have T7 RNA polymerase. White colonies were transferred onto M9 minimal medium [22] supplemented

Table 1. Bacterial strains and plasmids used in this study.

Bacterial strain or plasmid	Relevant characteristics or genotype	Reference or source
P. aurantiaca S-4380	Wild-type, Lev^{+a}	Unpublished data
E. coli		
DH5α	*supE*44 Δ*lac*U169(p80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	BRL
Plasmids		
pBluescript II KS(+)	Ap^r	Stratagene
pBluescript II SK(+)	Ap^r	Stratagene
pET-21a(+)	Ap^r, a 5.4-kb vector containing an N-terminal T7 promoter sequence plus a C-terminal His-tag sequence.	Novagen
pLK1	Ap^r; 2.4-kb fragment containing the *lscA* gene from *P. aurantiaca* S-4380 cloned into pBluescript II KS(+). The gene in this plasmid has the same orientation as *lacZα*.	This study
pLKR1	Ap^r; 2.4-kb fragment containing the *lscA* gene from *P. aurantiaca* S-4380 cloned into pBluescript II KS(+). The gene is oriented opposite to the *lacZα*.	This study
pELK1	Ap^r; 2.5-kb *Bam*HI-*Eco*RI fragment from pLK1 inserted into pBluescript II SK(+). The *lscA* gene in this plasmid has the same orientation as *lacZα*.	This study

[a]Producing levansucrase.

with 5% sucrose and 100 μg/ml ampicillin. One colony, with a mucous morphology to indicate the presence of polymer formation activity in the cell, was obtained out of 4,000 colony libraries. This *lsc*A-inserted plasmid was named pLK1. Subsequently, the orientation of *lsc*A in pLK1 was reversed to produce pLKR1. Consequently, the *lsc*A in pLK1 had the same orientation as P_{lac}, while that in pLKR1 was oriented oppositely.

DNA Sequencing and Analysis

Sequencing was performed using pLK1 as the template. A series of nested deletions of the entire 2.4-kb DNA insert were constructed using an Exo Mung Bean Deletion Kit (Stratagene). DNA sequencing and analysis were performed as described above.

Enzyme Reaction

The levansucrase activity in the cell-free extracts and culture supernatant was assayed using 50-ml Falcon tubes in a temperature-controlled water bath. The reactions were initiated by adding levansucrase solution (1 or 3 U/ml) to 50 mM sodium acetate buffer (pH 5.6), containing 10% (w/v) sucrose. One hundred μl aliquot was taken at regular time intervals to check the levan formation. The enzyme reaction was terminated by the addition of 100 μl of 50 mM NaOH. Levansucrase activity represented sucrose-hydrolyzing activity. One unit of levansucrase activity was defined as the amount of the enzyme releasing one mmol of glucose per minute. The levan-degrading reaction was carried out using 50 ml sodium phosphate buffer (pH 5.8) containing 5% (w/vol) levan and 10 U of levan

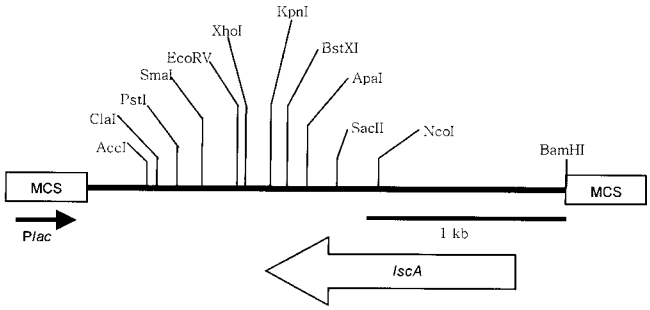

Fig. 1. Restriction map of pLKR1. pLKR1 has a 2,397 kb insert located in the *Bam*HI-restricted site of pBluescript II KS(+). The position and size of levansucrase gene, oriented in opposite direction to the *lacZα* gene, are indicated by the arrows below the map.

```
   1 GATCGCCTCCCTCCTTTCCCCTTCCTTGTCCCTCGAACCGACCAGCGGTTCCAAGCGCGCATTTTGCTGGCATCGTCGCTGATCAGGCCGAAAAACAAAC
                                                                  -35          -10
 101 TTCTGGAAACATAACTAGCTGAAACGTATCAGCTCCAGCAAAAACGGCAATTATGCATAAATGCAAATTTACCCTTGACCCGAAAAATCGCCTCGCTTAA
 201 GGTGAAATAACTGTATGCACATACAGTATTTTTGATAAACGAATCCATTCACGGACGAGGTATTACCTATGAAAAGCAACACTGAAAAATTCGGCAAGGC
                                                                 RBS          M  K  S  N  T  E  K  F  G  K  A   11
 301 ACCCCATCAACCCAGCCTCTGGACTCGGGCCGATGCGCTGAAAGTCCACGCGGACGATCCCACCACGACCCAGCCATTGGTCAGTGCGGACTTCCCGGTA
       P  H  Q  P  S  L  W  T  R  A  D  A  L  K  V  H  A  D  D  P  T  T  T  Q  P  L  V  S  A  D  F  P  V   44
 401 TTGAGCAACGAGGTGTTCATCTGGGACACCATGCCGCTGCGCGACCTGGACGGAAACATAACCTCCGTCGATGGTTGGTCGGTGATCTTCACCCTGACTG
       L  S  N  E  V  F  I  W  D  T  M  P  L  R  D  L  D  G  N  I  T  S  V  D  G  W  S  V  I  F  T  L  T   77
 501 CGGATCGTCATCCGAACGACCCGGAGTACATCGACGAAAACGGCAACTACGACATCACCCGCGACTGGAACGACCGTCACGGCCGGGCAAAGATGTACTA
       A  D  R  H  P  N  D  P  E  Y  I  D  E  N  G  N  Y  D  I  T  R  D  W  N  D  R  H  G  R  A  K  M  Y  Y  111
 601 CTGGTTTTCCCGCACCGGCAAGGACTGGAAGCTGGGTGGCCGGGTAATGGCCGAAGGCGTTTCGCCAACCGCTCGCGAATGGGCCGGCACGCCGATCCTG
       W  F  S  R  T  G  K  D  W  K  L  G  G  R  V  M  A  E  G  V  S  P  T  A  R  E  W  A  G  T  P  I  L  144
 701 TTGAACGAACAGGGTGACGTGGACCTGTATTACACCGCCGTCACTCCGGGCGCGACCATCGTCAAGGTGCGTGGCCGGGTGGTGACGACCGAGCATGGCG
       L  N  E  Q  G  D  V  D  L  Y  Y  T  A  V  T  P  G  A  T  I  V  K  V  R  G  R  V  V  T  T  E  H  G  177
 801 TCAGCATGGTCGGCTTCGAGAAGGTCAAGCCGCTGTTCGAGGCCGACGGCAAGATGTACCAGACCGAAGCGCAGAACCCCTTCTGGGGCTTCCGCGATCC
       V  S  M  V  G  F  E  K  V  K  P  L  F  E  A  D  G  K  M  Y  Q  T  E  A  Q  N  P  F  W  G  F  R  D  P  211
 901 ATGGCCGTTCCGCGACCCCAACGACGGCAAGCTGTACATGCTGTTCGAAGGCAACGTGGCCGGCGAACGCGGTTCGCACAAGGTCGGGAAAGCGGAAATC
       W  P  F  R  D  P  N  D  G  K  L  Y  M  L  F  E  G  N  V  A  G  E  R  G  S  H  K  V  G  K  A  E  I  244
1001 GGCGATGTGCCGCCAGGTTATGAAGACGTCGGCAACTCGCGCTTCCAGACTGCCTGTGTCGGTATCGCCGTGGCCCGCGACGAAGACGGCGACGACTGGG
       G  D  V  P  P  G  Y  E  D  V  G  N  S  R  F  Q  T  A  C  V  G  I  A  V  A  R  D  E  D  G  D  D  W  E  277
1101 AAATGCTGCCGCCGCTGCTGACCGCGGTCGGCGTCAACGACCAGACCGAACGCCCGCACTTCGTGTTCCAGGACGGCAAGTACTACCTGTTCACCATCAG
       M  L  P  P  L  L  T  A  V  G  V  N  D  Q  T  E  R  P  H  F  V  F  Q  D  G  K  Y  Y  L  F  T  I  S  311
1201 CCACACCTTCACCTATGCCGACGGTGTGACCGGGCCGGACGGGGTGTACGGTTTTGTCGCCGATTCGCTGTTCGGGCCCTACGTGCCGTTGAACGGTTCC
       H  T  F  T  Y  A  D  G  V  T  G  P  D  G  V  Y  G  F  V  A  D  S  L  F  G  P  Y  V  P  L  N  G  S  344
1301 GGCCTGGTGCTGGGCAACCCGTCTTCCCAGCCGTTCCAGACCTATTCGCACTGTGTGATGCCCAACGGCCTGGTGACCTCCTTCATCGACAGCGTACCGA
       G  L  V  L  G  N  P  S  S  Q  P  F  Q  T  Y  S  H  C  V  M  P  N  G  L  V  T  S  F  I  D  S  V  P  T  377
1401 CCGACGAAAGCGGCACGCAGATTCGCATCGGCGGTACCGAAGCCCCGACGGTGGAGATCAAGATCAAAGGGCAGCAGACATTCGTGGTGGCCGAGTACGA
       D  E  S  G  T  Q  I  R  I  G  G  T  E  A  P  T  V  E  I  K  I  K  G  Q  Q  T  F  V  V  A  E  Y  D  411
1501 CTACGGTTACATCCCGCCGATGCTCGATGTAACGCTCAAGTAATAGCGACACAGGGCGCTCAGGCAGCGGGCTCGAGTTCGATGAAGAACCCGTTGTGGA
       Y  G  Y  I  P  P  M  L  D  V  T  L  K  *                                                          425
1601 TATGATCGAGATATCCCGCCTGGCTCACTGCGACTTTGCTCAGGAGCTTCATTGAACAATGCGTAGTACCGACCCTTCGCAACTGGCTACACGGATGTAG
1701 CCAGTTCTGCTCCTGCTTGCGGAAATCGCAGCAGCACCCTCCTCTCTCCGGCCAGCAGTGGCCTTCCCAGGGTCAGCTCCCAGTCTTGACCTTGCTCCAC
1801 ACCCGGGTACGCACCCGTTCCACTTTCAGCGGCAGCGGCTCCAGCGGGAACAGCGTGGCCATCACTTCTGCAGACGGATAGACCCCAGGGTTGTCGCGGA
1901 TCTTCTGGGCCACAAGCGCCGTGGCATCGCGGTTGGCGTTGGGGTAGCCCAGGTAGTCGGAAGACTGGGCAATCACTTGCGGGCGCAGCAGGTAATCGAT
2001 GAACTTCAGGCCGTCCTGGGGATGCGGCGCATCCTTGAGCAGCACCAGGTTTTCCACCCAGACCGGCCCGCCTTCGCGGGGAATGCTGTAGACCAGATGG
2101 CGCCCATTATTGGCCTTCTGCGAGGCGACCTGGGCATCGAATACCCCTCCGCTCCAGCCCAGCACCACACAGATGTTGCCGTTGGCCAGGTCAGTGATGA
2201 ATTTCGACGAGTCGAAGTAGCGAATATGCGGCCGGACCTTGAGCAACAAGGCCTCGGCCTTCTTGTAGTCCTCGGGGTTCTGGCTGTTGTAGGGCAGGCC
2301 CAGGTAATGCAGGGCGATGGGGATGATTTCACTCGGAGCATCGAGCATGGCGACACCACACTGGCTGAGCTTGGCCAGGTTCTCTTCCTTGAAGATC
```

Fig. 2. Nucleotide and deduced amino acid sequence of the *lsc*A gene from *P. aurantiaca* S-4380. Nucleotides and amino acid residues are numbered on the left and right, respectively. Putative promoter sequence and ribosome-binding site are marked. Two termination codons are indicated with a "★" mark.

fructotransferase at 37°C for 60 h with gentle shaking. One unit of enzyme activity was defined as the amount of enzyme releasing one mmol of sugar equivalent to glucose per min. The reaction products were analyzed by TLC, as described previously [31].

Analytical Methods

During batch cultivation, cell growth was monitored by measuring the OD_{600}. Protein samples were analyzed by electrophoresis on SDS-PAGE gels containing 10% (w/v) polyacrylamide, as described by Laemmli [14]. The gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad). The protein bands on the SDS-PAGE gels were densitometrically quantified (Bio-Rad Laboratories, U.S.A.). The amount of protein was determined with a protein assay kit (Bio-Rad), using bovine serum albumin as the standard [4].

Results and Discussion

Selection and Identification of Bacteria

About 250 isolates showed sucrose-hydrolyzing activity on sucrose-containing agar medium. Based on the migration rates and sugar compositions of the products on TLC analysis, 8 colonies from the above isolates were isolated as producers of levan from sucrose. Strain no. S-4380, which showed relatively high activity and produced the enzyme in a stable manner, was selected for further experiments. Phylogenic analysis with the 16S rRNA sequence showed that the microorganism shared a 99.7% sequence homology with *P. aurantiaca* ATCC 33663T (data not shown). This strain was named as *P. aurantiaca* S-4380 and registered in the Korean Collection for Type Cultures (KCTC) (reference number 0943BP).

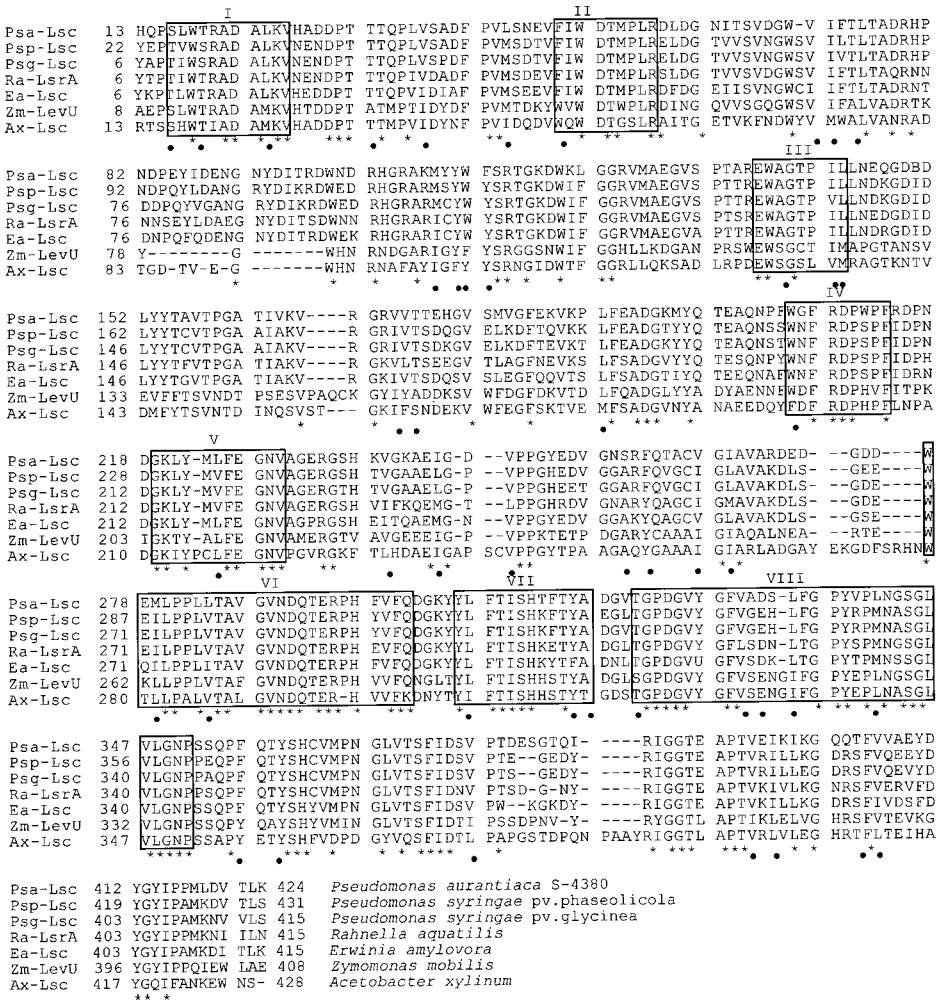

Fig. 3. Multiple alignment of the deduced amino acid sequences of Gram-negative bacterial levansucrases.
Origins of levansucrases are indicated at the ends of the sequences. Asterisks indicate identity and dots indicate conservative changes (S-T-A, L-V-I-M, K-R, D-E, Q-N, F-Y-W). Key amino acids for the enzyme activity are put in the box and numbered from I to VIII. Conserved acidic residues in bacterial fructosyltransferases are shown in bold letters.

Sequence Analysis of the *lscA* Gene

The entire nucleotide sequence of the inserted 2,397-bp *Bam*HI DNA of *P. aurantiaca* S-4830 on pLK1 was determined, using plasmids produced by the unidirectional deletion reactions on both strands. A large open reading frame (named as *lscA*: GenBank accession number AF306513) was found in the sequenced region (Figs. 1 and 2). The *lscA* gene was composed of 1,275 bp encoding a protein of 424 amino acid residues with an estimated molecular mass of 47 kDa. This was in agreement with the molecular mass measured by SDS-PAGE analysis. The *lscA* gene was preceded by a potential ribosomal binding site, GAGG, at 9 bp upstream from the start codon. In comparison with the consensus promoter sequences of *E. coli*, a potential promoter (-35,TTGACC and -10, TAAGGT) was identified upstream of the initiation codon. The coding region of the *lscA* gene ended with two adjacent termination codons, TAA and TAG.

The deduced amino acid sequence of *P. aurantiaca* S-4380 was aligned with those reported for other Gram-negative bacterial levansucrases (Fig. 3). The LscA protein was found to be highly identical with levansucrases from other Gram-negative bacteria, including *Pseudomonas syringae* pv. phaseolicola (73%), *P. syringae* pv. glycinea (72%) [11], *Rahnella aquatilis* (72%) [30], *Erwinia amylovora* (71%) [9], *Z. mobilis* [28], *Acetobacter xylinum* (39%) [32], and *Acetobacter diazotrophicus* (25%) [1]. On the other hand, the LscA protein was only 17–19% identical with levansucrases from Gram-positive bacteria such as *Bacillus amyloliquefaciens* (19%) [33], *Bacillus subtilis* (18%) [8], and *Bacillus stearothermophilus* (17%) [15]. Multiple alignment of the deduced amino acid sequences showed that there are eight conserved regions in the *lscA* from *P. aurantiaca* S-4380 [24, 28]. The conserved regions are thought to be important for their enzymatic activities, i.e. sucrose hydrolysis and subsequent transfer of fructose to the proper acceptor molecule. On the other hand, the cloned *lscA* showed two gaps in the alignment, which suggests a different property.

The acidic residues, Asp and Glu, have been found to be responsible for catalysis in the majority of the fructotransferase and levanases studied to date, acting either as proton donors or as nucleophiles [3, 27]. Five well-conserved motifs containing acidic residues were found on aligning levansucrases from Gram-negative bacteria [3] (which are shown as bold letters in Fig. 3). Among these, it was considered that the Asp residue in the RDP motif in the fourth conserved regions was a putative catalytic residue and may be responsible for the formation of a covalent fructosyl-enzyme intermediate with sucrose [3]. The Glu residue (bold) in the sixth conserved region, which is invariant in levansucrases and glycosylhydrolases, was suggested to be a component of the active center in enzymes of the glycosylhydrolases families [12].

Expression of *lscA* in *E. coli*

The *lscA* expression in *E. coli* was monitored through enzyme assay and SDS-PAGE analysis over the time course (Fig. 4). Thus, the *E. coli* DH5α containing pLK1 was cultured as described in Materials and Methods and harvested at various growth phases. A dense protein band that was absent in the *E. coli* control (pBluescript KS or pBluescript SK) was detected in the position corresponding to about 47 kDa (data not shown). Although no signal sequence was found in the deduced amino acid sequence of *P. aurantiaca* levansucrase (Fig. 3), a large quantity of enzyme was found in both the periplasm and cytoplasmic space of the recombinant *E. coli* DH5α (pLK1), indicating that the levansucrase might possess another secretion system (data not shown). Since the bacterial levansucrases are known to be extracellular proteins, the possible secretion mechanism of *P. aurantiaca* levansucrase might be independent

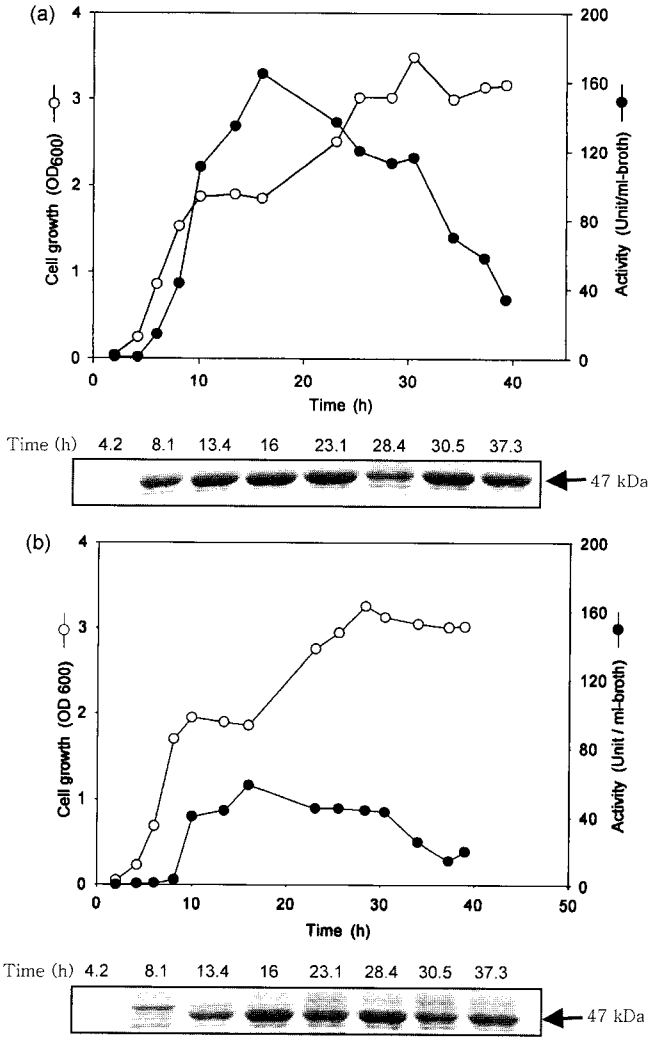

Fig. 4. SDS-PAGE analysis of levansucrase overexpressed in *E. coli* DH5α (pLK1) (a) and *E. coli* DH5α (pLKR1) (b).
Legends: (○) cell growth; (●) levansucrase activity.

of a signal peptide. However, further investigation is needed.

The gene on pLK1 has the same orientation as on the *lacZα* and was constitutively expressed in *E. coli* without any IPTG addition (Fig. 4A). The production of levansucrase in *E. coli* containing pLK1 progressively increased as the cultivation time increased, reached to 170 U/ml of culture broth after 16 h of cultivation, and began to decrease thereafter. By SDS-PAGE analysis, it was revealed that prolonged cultivation of *E. coli* containing pLK1 for more than 16 h increased the ratio of insoluble aggregate (inclusion body) over the soluble fracton, as described by Song and Rhee [29]. To determine whether the expression of the levansucrase-coding *lsc*A gene was led by its own promoter or not, the plasmid pLKR1, in which the *lsc*A gene was orientated in the opposite direction on the plasmid pBluescript II KS$^+$, was constructed as a counterpart of pLK1 (Fig. 4b). The gene on pLKR1 had the direction opposite to the *lacZα* and was expressed in a growth phase-dependent way (Fig. 4B). Interestingly, the *lsc*A expression was repressed during the early-phase of cell growth (0–8.1 h), initiated at the mid-log phase (8.1–10.0 h), and significantly increased during the late-phase, suggesting that its natural promoter exists in the upstream region and works on the recombinant plasmid in the *E. coli* strain (Fig. 4B). This growth-related expression pattern by its own promoter is different from the general constitutive expression of Gram-negative bacterial levansucrases [24].

To identify the recombinant levansucrase activity, a cell-free lysate of *E. coli* harboring pLKR1 was analyzed by enzymatic reaction, as described in Materials and Methods. The levan produced in the present work was used as a substrate for enzymatic production of DFA IV by levan fructotransferase (Fig. 5). A product corresponding to DFA IV (lane 4 in Fig. 5) was detected as a main product when the levan fructotransferase and levan derived from the cell-free lysate of *E. coli* harboring pLKR1 were reacted (lane 3 in Fig. 5). The sugar compositions of the products (lanes 3 and 4 in Fig. 5) were analyzed after acid hydrolysis for 1 h at 100°C with 1% oxalic acid; fructose was the only sugar detected by the reaction of the two products (lanes 3 and 4 in Fig. 5). The identity of DFA IV was confirmed by ^{13}C-NMR analysis, as described previously [25]. From these results, it could be concluded that the recombinant levansucrase obtained in this work had a potency equivalent to the potency of material produced by *P. aurantiaca* S-4380. According to Saito and Tomita [20], levan produced by *S. levanicum* was the most suitable substrate for DFA IV production [13]. In a previous report, it was found that the conversion yield from levan to DFA IV by levan fructotransferase was similar (53% in *S. levanicum* and 59% in *P. aurantiaca* S-4380) (unpublished data). Therefore, the recombinant levansucrase from this work can be used for large-scale DFA IV production. In addition, since *E. coli* DH5α containing pLK1 produced active levansucrase, it was concluded that the promoter and ribosome-binding site of the *P. aurantiaca* S-4380 levansucrase gene were recognized in *E. coli*. A study on the details of the upstream region of the *lsc*A gene is in progress. Also, a way to secrete the recombinant levansucrase by genetically modified *lsc*A or by modification of the cell surface structure of *E. coli* is currently being explored. This will be useful for industrial production of DFA IV.

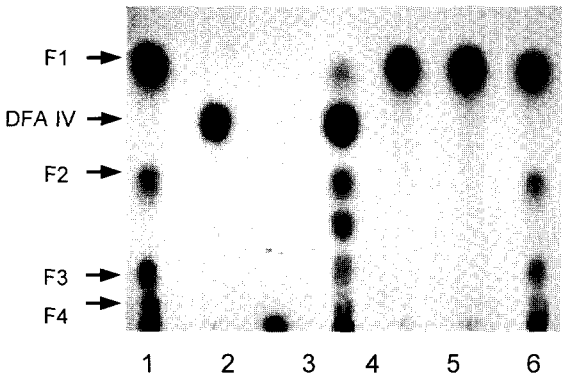

Fig. 5. TLC analysis of reaction products from levan produced from cell-free extracts of *E. coli* DH5α (pLK1) and levan fructotransferase.

Lanes 1 and 7, levan-oligosaccharides standard generated by partial acid hydrolysis of levan. The numbers on the left indicate the number of fructose; Lane 2, authentic DFA IV; Lane 3, the levan produced from *E. coli* DH5α (pLKR1); Lane 4, the reaction products of levan fructotransferase with levan (lane 3); Lanes 5 and 6, acid hydrolysis products from levan (lane 3) and DFA IV (lane 4).

Acknowledgments

This work was supported by the Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea. The authors deeply appreciate its financial assistance.

References

1. Arrieta, J., L. Hernandez, A. Coego, V. Suarez, E. Balmori, C. Menendez, M. F. Petit-Glatron, R. Chambert, and G. Selman-Housein. 1996. Molecular characterization of the levansucrase gene from the endophytic sugarcane bacterium *Acetobacter diazotrophicus* SRT4. *Microbiology* **142:** 1077–1085.
2. Baik, B. H., Y. W. Lee, and Y. B. Lee. 1997. Antianemic agent containing iron and difructose. *US Patent 5,700,832.*
3. Batista, F. R., L. Hernandez, J. R. Fernandez, J. Arrieta, C. Menendez, R. Gomez., Y. Tambara, and T. Pons. 1999. Substitution of Asp-309 by Asn in the Arg-Asp-Pro (RDP) motif of *Acetobacter diazotrophicus* levansucrase affects sucrose hydrolysis, but not enzyme specificity. *Biochem. J.* **337:** 503–506.

4. Bradford, M. M. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
5. Chambert, R., G. Treboul, and R. Dedonder. 1974. Kinetic studies of levansucrase of *Bacillus subtilis*. *Eur. J. Biochem.* **41:** 285–300.
6. Choi, S. H., C. Sung, and W. Y. Choi. 2001. Levan-producing *Bacillus subtilis* BS 62 and its phylogeny based on its 16s rDNA sequence. *J. Microbiol. Biotechnol.* **11:** 428–434.
7. Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Department of Genetics, University of Washington, Seattle, U.S.A.
8. Gay, P., D. Le Coq, M. Steinmetz, E. Ferrari, and J. A. Hoch. 1983. Cloning structural gene *sacB*, which codes for exoenzyme levansucrase of *Bacillus subtilis*: Expression of the gene in *Escherichia coli*. *J. Bacteriol.* **153:** 1424–1431.
9. Geier, G. and K. Geider. 1993. Characterization and influence on virulence of levansucrase gene from the fireblight pathogen *Erwinia amylovora*. *Physiol. Mol. Plant Pathol.* **42:** 387–404.
10. Han, Y. W. and M. A. Clarke. 1990. Production and characterization of microbial levan. *J. Agric. Food Chem.* **38:** 393–396.
11. Hettwer, U., F. R. Jaeckel, J. Boch, M. Meyer, K. Rudolph, and M. S. Ullrich. 1998. Cloning, nucleotide sequence, and expression in *Escherichia coli* of levansucrase genes from the plant pathogens *Pseudomonas syringae* pv. glycinea and *P. syringae* pv. phaseolicola. *Appl. Environ. Microbiol.* **64:** 3180–3187.
12. Kim, H. J., J. Y. Yang, H. G. Lee, and J. H. Cha. 2001. Cloning and sequence analysis of a levansucrase gene from *Rahnella aquatilis* ATCC15552. *J. Microbiol. Biotechnol.* **11:** 693–699.
13. Kojima, I., T. Saito, M. Iizuka, N. Minamiura, and A. Ono. 1993. Characterization of levan produced by *Serratia* sp. *J. Ferm. Bioeng.* **75:** 9–12.
14. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.
15. Li, Y., J. A. Triccas, and T. Ferenci. 1997. A novel levansucrase-levanase gene cluster in *Bacillus stearothermophilus* ATCC 12980. *Biochim. Biophys. Acta* **1353:** 203–208.
16. Park, J. M., S. Y. Kwon, K. B. Song, J. W. Kwak, S. B. Lee, Y. W. Nam, J. S. Shin, Y. I. Park, S. K. Rhee, and K. H. Paek. 1999. Transgenic tobacco plants expressing the bacterial levansucrase gene show enhanced tolerance to osmotic stress. *J. Microbiol. Biotechnol.* **9:** 213–218.
17. Rochelle, P. A., J. C. Fry, R. J. Parkes, and A. J. Weightman. 1992. DNA extraction for 16S rRNA gene analysis to determine genetic diversity in deep sediment communities. *FEMS Microbiol. Lett.* **100:** 59–66.
18. Saito, K., H. Goto, A. Yokota, and F. Tomita. 1997. Purification of levan fructotransferase gene from *Arthrobacter nicotinovorans* GS-9 and production of DFA IV from levan by the enzyme. *Biosci. Biotech. Biochem.* **61:** 1705–1709.
19. Saito, K., T. Hira, T. Suzuki, H. Hara, A. Yokota, and F. Tomita. 1999. Effects of DFA IV in rats: Calcium absorption and metabolism of DFA IV by intestinal microorganisms. *Biosci. Biotech. Biochem.* **63:** 655–661.
20. Saito, K. and F. Tomita. 2000. Difructose anhydrides: Their mass-production & physiological functions. *Biosci. Biotechnol. Biochem.* **64:** 1321–1327.
21. Saito, K., A. Yokota, and F. Tomita. 1997. Molecular cloning of levan fructotransferase gene from *Arthrobacter nicotinovorans* GS-9 and its expression in *Escherichia coli*. *Biosci. Biotech. Biochem.* **61:** 2076–2079.
22. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
23. Sanger, F., S. Nichlen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74:** 5463–5467.
24. Seo, J. W., K. B. Song, K. H. Jang, C. H. Kim, B. H. Jung, and S. K. Rhee. 2000. Molecular cloning of a gene encoding the thermoactive levansucrase from *Rahnella aquatilis* and its growth phase-dependent expression in *Escherchia coli*. *J. Biotechnol.* **81:** 63–72.
25. Song, K. B., K. S. Bae, Y. B. Lee, K. Y. Lee, and S. K. Rhee. 2000. Characteristics of levan fructotransferase from *Arthrobacter ureafaciens* K2032 and difructose anhydride IV formation from levan. *Enzyme. Microb. Technol.* **27:** 212–218.
26. Song, K. B., H. Belghith, and S. K. Rhee. 1996. Production of levan, a fructose polymer, using an overexpressed recombinant levansucrase. *Ann. N. Y. Acad. Sci.* **799:** 601–607.
27. Song, D. D. and N. A. Jacques. 1999. Mutation of aspartic acid residues in the fructosyltransferase of *Streptococcus salivarius* ATCC 25975. *Biochem. J.* **344:** 259–264.
28. Song, K. B., H. K. Joo, and S. K. Rhee. 1993. Nucleotide sequence of levansucrase gene (*levU*) of *Zymomonas mobilis* ZM1 (ATCC10988). *Biochim. Biophy. Acta* **1173:** 320–324.
29. Song, K. B. and S. K. Rhee. 1994. Enzymatic synthesis of levan by *Zymomonas mobilis* levansucrase overexpressed in *Escherichia coli*. *Biotechnol. Lett.* **16:** 1305–1310.
30. Song, K. B., J. W. Seo, M. G. Kim, and S. K. Rhee. 1998. Levansucrase of *Rahnella aquatilis* ATCC33071. Gene cloning, expression, and levan formation. *Ann. N. Y. Acad. Sci.* **864:** 506–511.
31. Stahl, E. 1969. *Thin-layer Chromatography: A Laboratory Handbook*. Springer-Verlag. Berlin, Germany.
32. Tajima, K., T. Tanio, Y. Kobayashi, H. Kohno, M. Fujiwara, T. Shiba, T. Erata, M. Munekata, and M. Takai. 2000. Cloning and sequencing of the levansucrase gene from *Acetobacter xylinum* NCI 1005. *DNA Res.* **7:** 237–242.
33. Tang, L. B., R. Lenstra, T. V. Borchert, and V. Nagarajan. Isolation and characterization of levansucrase-encoding gene from *Bacillus amyloliquefaciens*. *Gene* **96:** 89–93.