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Cloning and Sequence Analysis of a Levansucrase Gene from *Rahnella aquatilis* ATCC15552

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Abstract An intracellular levansucrase gene, *lscR* from *Rahnella aquatilis* ATCC15552, was cloned and its nucleotide sequence was determined. Nucleotide sequence analysis of this gene revealed a 1,248 bp open reading frame coding for a protein of 415 amino acids. The levansucrase was expressed by using a T7 promoter in *Escherichia coli* BL21 (DE3) and the enzyme activity was detected in the cytoplasmic fraction. The optimum pH and temperature of this enzyme for levan formation was pH 6 and 30°C, respectively. The deduced amino acid sequence of the *lscR* gene showed a high sequence similarity (59–89%) with Gram-negative levansucrases, while the level of similarity with Gram-positive enzymes was less than 42%. Multiple alignments of levansucrase sequences reported from Gram-negative and Gram-positive bacteria revealed seven conserved regions. A comparison of the catalytic properties and deduced amino acid sequence of *lscR* with those of other bacterial levansucrases strongly suggest that Gram-negative and Gram-positive levansucrases have an overall different structure, but they have a similar structure at the active site.

Key words: Levan, levansucrase, multiple sequence alignment, *Rahnella aquatilis*

Levan consists of β(2→6)-linked fructosyl units for the main chain and β(2→1)-linked fructosyl units for the branch chain. Levan offers a variety of industrial applications such as an emulsifier, a formulation aid, a stabilizing thickener, a surface-finishing agent, and a carrier for color or flavor in the fields of cosmetics, foods, and pharmaceutical goods [3, 8]. Levan produced as a prevalent storage carbohydrate in some plants is also known to serve as an osmoprotectant, and recently, the transgenic tobacco plant containing a levansucrase gene exhibited an increased tolerance to drought stress [17]. Levan is produced by several kinds of bacteria during their assimilation of sucrose through the action of levansucrase (Sucrose 2,6-β-fructan 6-β-D-fructosyl transferase; EC 2.4.1.10). Levansucrase hydrolyses sucrose, releasing fructose and glucose, and concomitantly polymerizes the fructose molecules to the acceptor molecules. All known levansucrases are recognized as extracellular proteins although different secretion routes can be used. The Gram-positive bacteria *Bacillus subtilis* [18] and *Streptococcus salivarius* [19] secrete levansucrases by a two-step mechanism involving signal peptide cleavage and protein folding. By contrast, the Gram-negative bacteria *Zymomonas mobilis* [11], *Erwinia amylovora* [7], *Pseudomonas syringae* [9], and *Rahnella aquatilis* [16] secrete levansucrases by a signal peptide-independent pathway.

Rahnella aquatilis is a sugar beets endophyte. This Gram-negative bacterium produces levansucrase, which shows a relatively high optimum temperature for the levan formation that has a broad acceptor specificity for transfructosylation reaction [16]. Interestingly, the levansucrase from *R. aquatilis* ATCC15552 is not released into an extracellular culture fluid unlike other plant pathogenic levansucrases. This phenomenon suggests that the sucrose metabolism of this bacterium in plant-microbe interactions might be somewhat different from other levan-producing bacteria. Therefore, such information about the biochemical and the genetic characteristics of this enzyme will be necessary for acquiring a detailed understanding of the sucrose metabolism of this strain. In the present work, we cloned and determined the amino acid sequence of the *lscR* gene and examined the structural feature of the enzyme by comparison with other reported levansucrases.

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MATERIALS AND METHODS

Materials

Sucrose, glucose, and fructose were obtained from Sigma. Levan was obtained from a laboratory-scale production by using *Microbacterium laevaniformans* levansucrase and was isolated by ethanol precipitation [23]. Restriction enzymes and modifying enzymes were purchased from Boehringer Mannheim (Mannheim, Germany) or Kosco. *Taq* DNA polymerase and dNTPs for PCR amplification were purchased from Takara (Shiga, Japan). All other compounds were of the reagent grade. All buffers were prepared in Millipore Milli-Q water.

Bacterial Strains and Plasmids

Rahnella aquatilis ATCC15552 was used as a levansucrase gene source. *Escherichia coli* MC1061 [F^- *araD139 recA13 Δ(araABC-leu)7696 galU galK ΔlacX74 rpsL thi hsdR2 mcrB*] was used to propagate the plasmid and cloning. *E. coli* BL21 [F^- *ompT* rB^- mB^- λ(DE3)] [24] was used as a host for the pTRLSU to express the recombinant levansucrase. *E. coli* strains were grown in a Luria broth supplemented with ampicillin (50 μg/ml) as needed. *R. aquatilis* was grown in a LB medium with 3% sucrose. PCR II-TOPO plasmid (Invitrogen, Groningen, The Netherlands) was used for PCR cloning and expression of the levansucrase gene.

Genetic Manipulations and Sequence Analysis

A genomic DNA was prepared from *R. aquatilis* according to the method of Sambrook *et al.* [20]. *R. aquatilis* levansucrase gene (*lscR*) was obtained by the PCR method using two synthetic primers, LsuF (5'-ATGACAAATTTA-AATTATACA-3'), and LsuR (5'-TTAATTTAAAATAAT-GTTTTTC-3'), which were based on the nucleotide sequence of *lsrA* from *R. aquatilis* ATCC33071 [21]. Amplification of a genomic DNA was performed in a total volume of 50 μl: 40 μg of template DNA; 1 μM of each primer; dNTPs, 0.25 mM each; 1.5 mM of $MgCl_2$; 2 U of *Taq* DNA polymerase; and 1× *Taq* DNA polymerase buffer. PCR amplification was carried out on a thermal cycler (GeneAmp PCR Sytem 2400, Perkin Elmer) with a program of a predenaturation for 2 min at 95°C, followed by 30 cycles (denaturation 95°C, 1 min; annealing 50°C, 1 min; extension 72°C, 2 min), and a final incubation period for 7 min at 72°C. The amplified 1.25-kb PCR fragment was electroeluted from 1% agarose gel that was then ligated into the PCR II-TOPO for TA cloning, and the ligation mixture was tranformed into *E. coli* strain MC1061. The recombinant plasmid, pTRLSU, was isolated from *E. coli* transformants, and subjected to automatic sequencing with the ABI Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, U.S.A.). Resultant DNA sequence and the deduced amino acid sequence were analyzed with programs readily available on both the ExPasy Molecular Biology server (http://www.expasy.ch/tools/) and the National Center for Biotechnology Information Database. The alignment algorithm Clustal W program [25] was used to create a consensus multiple alignment of bacterial levansucrases.

Expression of Levansucrase

For the expression of cloned *R. aquatilis* levansucrase gene, *E. coli* BL21(DE3) containing a plasmid pTRLSU was grown in a LB medium supplemented with 50 μg/ml of ampicillin at 37°C for 12 h. The precultured cells were inoculated into the LB medium containing 50 μg/ml of ampicillin in a 250-ml flask. The cells were incubated with shaking at 37°C until OD_{600} reached approximately 0.8 to 1.0 and then a final concentration level of 1 mM IPTG was added to the culture and the incubation was continued for 3 to 4 h. The induced cells were harvested by centrifugation and the cell pellet was resuspended into the phosphate buffer, pH 7.0, and finally sonicated. The supernatant after centrifugation was saturated with 80% ammonium sulfate, then dissolved in phosphate buffer, pH 7.0, and dialyzed against the same buffer. The dialysate was loaded onto a 1 ml Resource-Q FPLC column (Pharmacia, Uppsala, Sweden) equilibrated with phosphate buffer, pH 7.0, and then eluted with a linear gradient of 1 M NaCl. The fractions containing the levansucrase activity were pooled, concentrated, and used as the enzyme solution for further analysis.

Enzyme Assay

Levan-forming activities were assayed by using cell-free extracts prepared by ultrasonic disruption. A reaction mixture consisting of 1 M sucrose, 0.1 M phosphate buffer, pH 7.0, and an appropriate amount of enzyme was incubated at 37°C for 2 h, and the levan that was produced was measured at 540 nm by the change of turbidity. The amount of levan was calculated based on the established standard curve of an absorbance rate against each concentration of levan ranging from 1 to 10 mg/ml. One unit of levan-forming activity was defined as the amount of enzyme that was required for the formation of 1 μg of levan per minute under the experimental conditions. Zymogram staining for levan-forming activities was performed as described [15]. *E. coli* transformants grown as described above were centrifuged and the pellet was resuspended in 100 mM phosphate buffer, pH 7.0. The cells were then disrupted by sonication with a Branson sonifier equipped with a microtip at setting 7. After removing cell debris, samples were applied to 8% non-denaturing PAGE gel and stained for levansucrase activity by immersing the gel into 100 mM sodium phosphate buffer (pH 6.0)-5% sucrose solution.

Analytical Methods

Protein concentration was quantified by the Bradford method [4] with bovine serum albumin as a standard. SDS-

PAGE was performed as described [12]. Reduced sugar released from the enzyme reaction was examined by applying the DNS method [13].

RESULTS AND DISCUSSION

Cloning and Expression of the Levansucrase Gene in *E. coli*

Isolation of the levansucrase gene from *R. aquatilis* ATCC15552 genomic DNA was conducted by following a direct PCR method using the amino and the carboxyl terminal regions of *lsrA* from *R. aquatilis* ATCC33071. Approximately 1.25-kb PCR product was cloned into the PCR II-TOPO vector and transformed into *E. coli* MC1061. Four recombinant colonies were isolated and sequenced. All of the purified plasmids contained 1.25-kb inserts. One of the recombinant plasmids with the *lscR* gene was named pTRLSU. The nucleotide sequence analysis revealed that the *lscR* gene from the cloned pTRLSU was composed of 1,248 bp encoding 415 amino acid residues and its molecular weight was estimated to be 45 kDa. The nucleotide sequence of *lscR* was deposited in the GenBank Databases under the accession number of AY027657. The *lscR* protein exhibited 96.9% amino acid sequence identity with the *R. aquatilis lsrA*. The content of the guanine and the cytosine was 48.5 mol% and that of the third base codon was 48.6 mol%. A plasmid pTRLSU carrying the T7 promoter was transformed into *E. coli* BL21(DE3) to express the recombinant levansucrase. When cells were induced by addition of IPTG, the activity of levansucrase was detected. Most of the levansucrase activity was found in the cytoplasmic fraction. An ability of *E. coli* transformant for levansucrase activity was also confirmed by active staining of the cell-free extract (Fig. 1). A white and turbid band indicating the formation of levan polymer was observed on the nondenaturing PAGE gel that was soaked in the sucrose solution. This band was located in the same position as that of the cell-free extract of *R.*

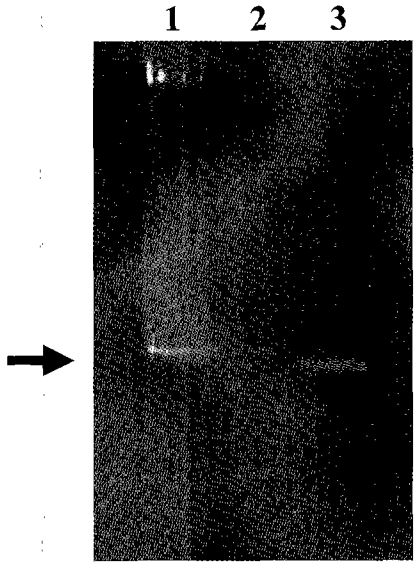

Fig. 1. Zymogram staining of levansucrase.
Lane 1, *R. aquatilis* ATCC15552 cell-free extract; lane 2, *E. coli* BL21(DE3) cell-free extract; lane 3, *E. coli* BL21(DE3)/pTRLSU cell-free extract. Each sample was applied to 8% nondenaturing PAGE gel. The gel was placed in 0.1 M sodium phosphate buffer (pH 6.0)-5% sucrose solution and incubated for 12–16 h at 37°C.

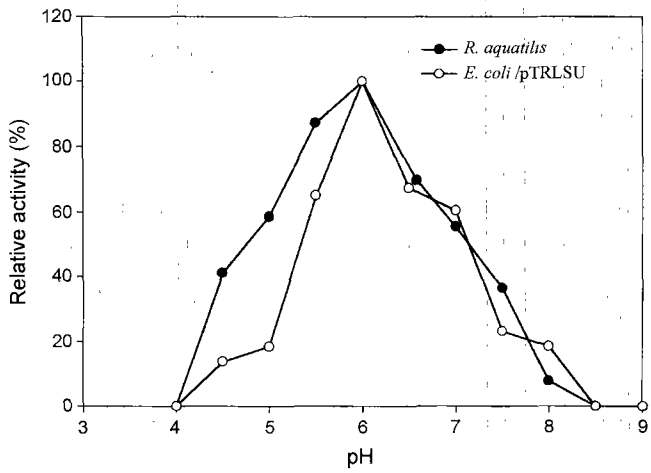

Fig. 2. Effect of pH on the activity of levansucrase.
The enzyme activity of *R. aquatilis* and *E. coli* BL21(DE3)/pTRLSU was examined by using 100 mM buffers in the pH range of 3.0–10.0 at 37°C. Buffers used were acetate buffer (pH 3.0–5.5), phosphate buffer (pH 6.0–7.5), Tris-HCl buffer (pH 8.0–9.0), Boric acid/NaOH buffer (pH 9.5–10.0). Enzyme activity was evaluated via the levan polymerization reaction as described in Materials and Methods.

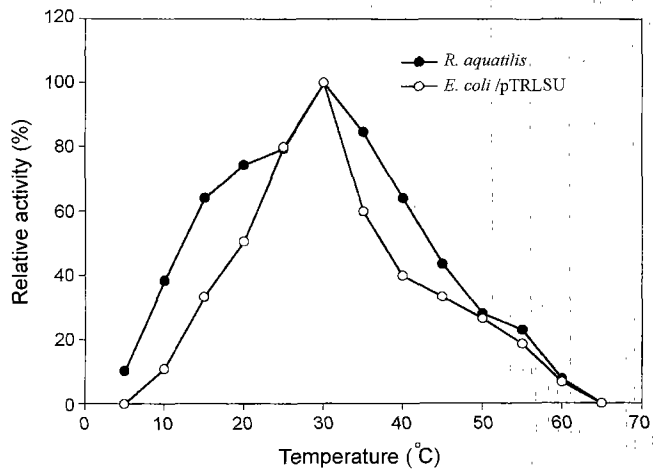

Fig. 3. Effect of temperature on the activity of levansucrase.
The temperature dependence of *R. aquatilis* and *E. coli* BL21(DE3)/pTRLSU for the levan formation was examined by using 100 mM phosphate buffer (pH 6.0) at a temperature ranging from 5–70°C. The enzyme activity was evaluated by using 1.0 M of sucrose solution as described in Materials and Methods.

aquatilis. A white band was not observed in *E. coli* cells without pTRLSU. What this result indicates is that the levansucrase activity found in *E. coli* indeed originated from *R. aquatilis*.

Enzymatic Properties of the Recombinant Levansucrase

Under the control of the T7 promoter, the levansucrase was produced in *E. coli*. After the cells were disrupted, the cell-free extract was partially purified with 80% ammonium sulfate and the Resource-Q FPLC ion-exchange column. The pH dependence of the recombinant enzyme for the formation of levan polymer was examined by using 1 M sucrose solution as a substrate. The levan-forming activity in both *R. aquatilis* and recombinant *E. coli* showed a broad pH optimum around 6.0. The enzyme activity greatly decreased below pH 5.0 or above pH 7.0 (Fig. 2). The pH-activity profile indicates that catalysis involves two ionic groups with pK_a values of 4.5–5.0 and 7.0–7.5. The lower pK_a is ascribed to the acidic residue based on its pK_a values of the solution. The acidic residue (aspartate or

Table 1. The comparison of amino acid homology among various bacterial levansucrases.

	Gram-negative bacteria					Gram-positive bacteria				
	Rahnella aquatilis	*Pseudomonas syringae*	*Erwinia amylovora*	*Zymomonas mobilis*	*Acetobacter xylinus*	*Bacillus subtilis*	*Bacillus stearothermophilus*	*Paenibacillus polymyxa*	*Streptococcus mutans*	*Streptococcus salivarius*
Rahnella aquatilis	**100**									
Pseudomonas syringae	**89**	**100**								
Erwinia amylovora	**87**	**88**	**100**							
Zymomonas mobilis	**68**	**67**	**66**	**100**						
Acetobacter xylinus	**59**	**60**	**61**	**63**	**100**					
Bacillus subtilis	41	40	44	43	41	**100**				
Bacillus stearothermophilus	40	40	42	42	42	**97**	**100**			
Paenibacillus polymyxa	42	42	42	44	43	**76**	**97**	**100**		
Streptococcus mutans	42	39	40	39	39	**56**	**57**	**56**	**100**	
Streptococcus salivarius	42	37	39	39	39	**56**	**58**	**56**	**71**	**100**

The homology scores are the percentage of similarity. The GenBank accession numbers of the bacterial levansucrases are described in Table 2.

Table 2. The conserved regions of the amino acid sequences of Gram-negative and Gram-positive bacteria levansucrases.

Source	Conserved regions						
	I	II	III	IV	V	VI	VII
RALSC	WDAMPL	EWAG	FRDP	VFEGN	VNDQTER	YLFTISH	GPYSPMNGSGLVL
RALSR	WDTMPL	EWAG	FRDP	VFEGN	VNDQTER	YLFTISH	GPYSPMNGSGLVL
PSLSC	WDTMPL	EWAG	FRDP	VFEGN	VNDQTER	YLFTISH	GPYRPMNASGLVL
EALSC	WDTMPL	EWAG	FRDP	LFEGN	VNDQTER	YLFTISH	GPYTPMNSSGLVL
ZMSAC	WDTWPL	EWSG	FRDP	LFEGN	VNDQTER	YLFTISH	GPYEPLNGSGLVL
ASLSX	WDTGSL	EWSG	FRDP	LFEGN	VNDQTER	YIFTISH	GPYEPLNASGLVL
BSSUR	WDSWPL	EWSG	LRDP	VFEAN	VTDEIER	YLSTDSR	GPYKPLNKTGLVL
BASAC	WDSWPL	EWSG	LRDP	VFEAN	VTDEIER	YLFTDSR	GPYKPLNKTGLVL
PPSAC	WDSWPL	EWSG	FRDP	IFEAN	VTDEIER	YLFTSTR	GPYKPLNGTGLVL
SMFTF	WDSWPL	EWSG	MRDP	VFEAS	VSDELER	YLFTASR	NGTKPLNNSGVVL
SSFTF	WDSWPV	QWSG	LRDP	IFESN	VTDEVER	YLFTASR	GKYRPLNGSGVVL
	* *	* *	* * *	* *	* * * *	* *	* * * * *

RALSC: lscR, *Rahnella aquatilis* (Accession number AY027657); RALSR: lsrA, *Rahnella aquatilis* (Accession number U91484); PSLSC: lsc, *Pseudomonas syringae pv. glycinea* (Accession number AF037443); EALSC: lsc, *Erwinia amylovora* (Accession number X75079); ZMSAC: sacB, *Zymomonas mobilis* (Accession number L33402); AXLSX: lsxA, *Acetobacter xylinus* (Accession number AB034152); BSSUR: surB, *Bacillus stearothermophilus* (Accession number U34874); BASAC: sacB, *Bacillus subtilis* (Accession number X02730); PPSAC: sacB, *Paenibacillus polymyxa* (Accession number AJ133737); SMFTF: ftf, *Streptococcus mutans* (Accession number M18954); SSFTF: ftf, *Streptococcus salivarius* (Accession number L08445).

glutamate) in the active site has been known to be responsible for the catalysis in most glycosylhydrolases [6]. The substitution of Asp-309 by Asn of *Acetobacter diazotrophicus* levansucrase dramatically decreased the sucrose hydrolysis activity, but not the transfructosylation activity [2]. This residue was conserved in the fructofuranosidase family including fructosyltransferases, invertases, levanases, inulinases, and sucrose-6-phosphate hydrolases. Therefore, the site-directed mutagenesis of the conserved aspartate residue will be necessary for determining the detailed mechanism of catalysis. The temperature dependence of recombinant *E. coli* for the levan formation was examined and compared with that of the *R. aquatilis*. The enzyme was incubated in 100 mM phosphate buffer, pH 6.0, with 1 M sucrose at various temperatures. Samples were taken after 2 h of incubation, and the levan-forming activity was measured by the turbidity at 540 nm. Both enzymes exhibited a similar temperature profile and the temperature optimum of 30°C (Fig. 3). The optimum temperatures of several levan-forming bacteria for the levan formation has been known to vary from 0 to 40°C. Levansucrases contain two substrate binding sites (levan and sucrose) and form more than one product by hydrolase and transferase activity. Comparative studies on the enzymatic and structural properties of various levansucrases will be quite interesting for obtaining a certain level of understanding of the catalytic mechanism of this enzyme.

Comparison with Other Bacterial Levansucrases

The deduced amino acid sequence of the *R. aquatilis lscR* gene was compared with those reported from other bacterial levansucrases. The comparison of the sequence homology revealed two distinct groups of levansucrases (Table 1). The deduced amino acid sequence of *lscR* showed a high degree of similarity with enzymes which were produced by the Gram-negative bacteria *Pseudomonas syringae* pv. glycinea (89%), *Erwinia amylovora* (87%), *Zymomonas mobilis* (68%), and *Acetobacter xylinus* (59%). However, the level of similarity decreased to below 42% in Gram-positive levansucrases. Within either Gram-negative or Gram-positive levansucrases, the similarities were at least above 58%. However, the similarity between two groups were 37–44%. This low similarity is probably related to a difference in the enzymatic properties of Gram-negative and Gram-positive levansucrases. To assess the structural features for further detail, the sequences of both groups were aligned by using the Clustal W program. Seven conserved regions (I-VII) are found by the alignment of the amino acid sequences of Gram-negative and Gram-positive levansucrases (Table 2). 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. Several acidic residues are highly conserved in these regions. Previously, the substitution of Asp-309 by Asn in region III (the RDP motif) of *Acetobacter diazotrophicus* levansucrase dramatically decreased the sucrose hydrolysis activity, but not the transfructosylation activity [2]. Therefore, the RDP motif conserved in fructosyltransferases, invertases, levanases, inulinases, and sucrose-6-phosphate hydrolases has been proposed to be quite important in catalysis of the fructofuranosidase family. Invariant glutamate and tyrosine residue in regions V and VI were also suggested as components

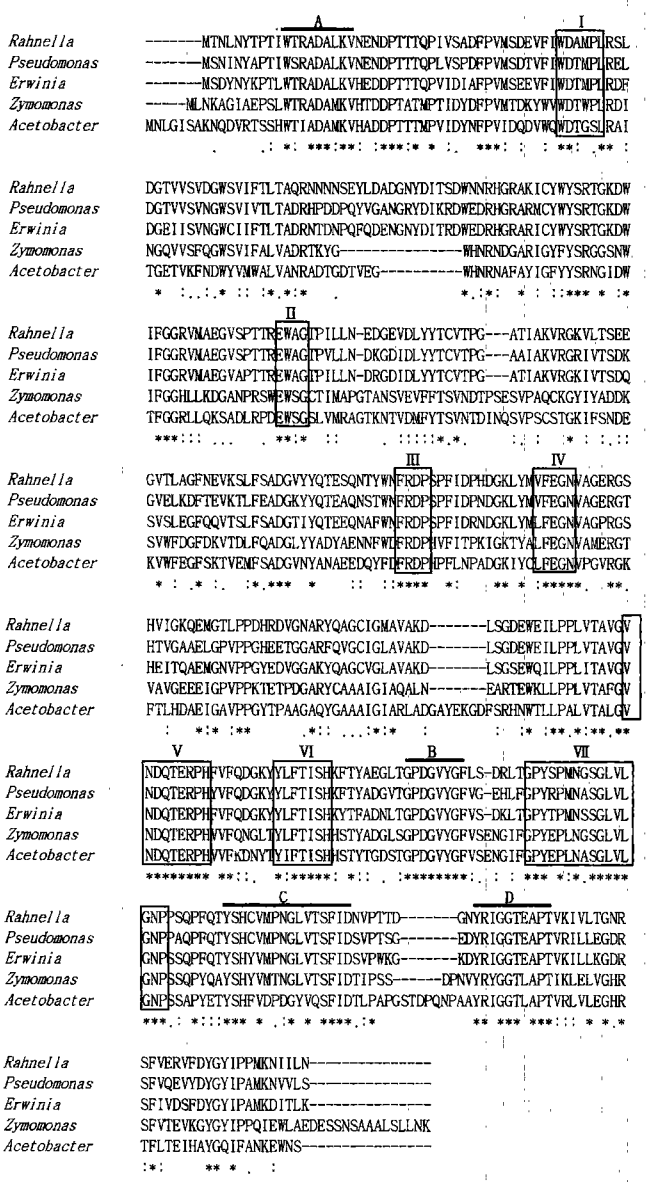

Fig. 4. Multiple alignment of deduced amino acid sequences of *lscR* with other reported Gram-negative levansucrases.
Asterisks and dots indicate both identity and similarity match across all sequences, respectively. Regions conserved in all levansucrases, including those from Gram-positive bacteria, are boxed and numbered from I to VII. Regions that are only conserved in Gram-negative levansucrases are overlined and labeled in A, B, C, and D.

of the active centers of the levansucrase and β-xylosidase families [14]. The arginine residue within the region VI has also been proposed to be important for the transfructosylation reaction of the enzyme [5]. In the *Bacillus subtilis* enzyme, substitution of Arg-331 by His or Lys affected the pattern of the products synthesized. His-mutant had the catalytic property similar to those of the Gram-negative enzymes. Both enzymes synthesized small amounts of high-molecular mass levan but accumulated the fructooligosaccharides, while the levansucrases from Gram-positive bacteria showed a tendency to make the high-molecular mass levan at high levels. These results support that Gram-negative and Gram-positive levansucrases have different enzymatic properties.

Extensive sequence similarities were found among the levansucrases from Gram-negative bacteria (Fig. 4). Four additional conserved regions (A, B, C, and D) besides the seven conserved regions found in Gram-negative and Gram-positive bacterial levansucrases were observed. Region A, W(T/S)RADA(L/M)KV, was located in the N-terminus close to the initiation codon of Gram-negative levansucrases. An extra N-terminal region was found in all Gram-positive levansucrases. Gram-positive bacteria share a common mechanism for levansucrase secretion involving the proteolytic processing of precursor proteins. The signal peptide cleavage motif, Ala-Xaa-Ala, was found just right in front of the conserved region A of the Gram-negative enzymes. Consequently, it might be a characteristic of Gram-positive levansucrases. This observation supports the idea that *Z. mobilis* and *E. amylovora* enzymes are not cleaved during the transport process, and they are subjected to a signal-peptide independent export mechanism [7, 22]. In the case of the dextran-forming enzyme, dextransucrase contains an N-terminal catalytic domain, responsible for the cleavage of sucrose, and a C-terminal glucan-binding domain with a series of tandem Gly-rich repeats [1, 10]. Although such a repeated structure could not be found in the primary structure of levansucrase, an abnormal Gly-rich region existed similarly in the C-terminal part (B, C, and D regions) of Gram-negative levansucrases. These highly conserved regions contribute very well to the production and accumulation of fructooligosaccharides observed in Gram-negative levansucrases. Therefore, it may be concluded that the Gram-negative and Gram-positive levansucrases have a different overall three-dimensional structure, but on the other hand, they have a similar structure at the active site.

Acknowledgments

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