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The K296-D320 region of recombinant levansucrase BA-SacB can affect the sensitivity of *Escherichia coli* host to sucrose

Song Gao¹ · Bo Jiang² · Song Zhu² · Darren J. Hart³ · Xia Liu¹ · Hongling Wang^{1,4} · Yingfeng An¹

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Abstract

Purpose A mutant BA-SacB-Del encoding BA-SacB minus K296-D320 region was constructed to analyze its effect on catalytic characteristics of the enzyme as well as help deepen understanding of the catalytic mechanism of BA-SacB and even proteins of GH68 family.

Methods Based on the comparison of levansucrases from *Bacillus amyloliquefaciens* (BA-SacB) and *Spingopyxis macrogoltabida* (SM-Lev), a mutant BA-SacB-Del encoding BA-SacB minus K296-D320 region was constructed and its effect on catalytic characteristics of the enzyme was analyzed.

Results Deletion of this region would undoubtedly affect the conserved structure (i.e., central negatively charged cavity surrounded by five antiparallel β -strands) shared by the GH68 family. Therefore, *Escherichia coli*-expressing mutant BA-SacB-Del could more efficiently catalyze the production of levan in media containing high concentration of sucrose, which is unrealizable for BA-SacB.

Conclusions This result should be valuable for understanding this conditional lethal mechanism. Therefore, this study should be very valuable for understanding the catalytic mechanism of BA-SacB and even proteins of the GH68 family. More importantly, levan can be conveniently produced by direct fermentation of sucrose-containing media with *E. coli*-expressing BA-SacB-Del which is not sensitive to sucrose.

Keywords Bioinformatics analyses · Fructosyltransferase · Levansucrase · Molecular evolution · Recombinant protein expression

Introduction

Levansucrases (EC 2.4.1.10) are enzymes catalyzing sucrose hydrolysis by hydrolase activity and levan synthesis by fructosyltransferase activity. Levan has been widely used for

personal care, medical applications, prebiotics, aquaculture, films of packaging, foods, etc. (Öner et al. 2016). Therefore, increasing attention has recently been focused on biosynthesis of levan and Fructooligosaccharides (FOSs) by microbial levansucrase. Currently, levansucrases have been identified from a wide range of microorganisms belonging to the genera *Acetobacter*, *Bacillus*, *Erwinia*, *Geobacillus*, *Lactobacillus*, *Leuconostoc*, *Pseudomonas*, *Zymomonas*, etc. (Li et al. 2015). According to the database of carbohydrate-active enzymes (CAZY) (Henrissat and Davies 1997), levansucrases belong to family 68 of the glycoside hydrolases (GH68). All the GH68 enzymes use sucrose as their preferential donor substrate. The levansucrases could realize transfructosylation through a ping-pong mechanism involving the formation of a transient fructosyl-enzyme intermediate (Hernandez et al. 1995). At high sucrose concentrations levansucrase could transfer fructosyl units to various acceptors including fructan, glucose and sucrose, whereas at low sucrose concentrations it functions mainly as a hydrolase with water as acceptor. Commonly levansucrases from bacteria could catalyze all these reactions but with different efficiency (Öner et al.

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2016). The members of GH68 share a β -propeller fold consisting of a central negatively charged cavity and five antiparallel β -strands (Strube et al. 2011). Until now, structures of some levansucrases have been solved, such as the ones from *B. subtilis* (PDB ID: 1OYG) (Manival et al. 1997), *Gluconacetobacter diazotrophicus* SRT4 (PDB ID: 1W18) (Martínez-Fleites et al. 2005), *B. megaterium* (PDB ID: 3OM2) (Strube et al. 2011), *E. amylovora* (PDB ID: 4D47) (Wuerges et al. 2015), and *E. tasmaniensis* (PDB ID: 6FRW) (Polsinelli et al. 2019). All these structures show the basic features of enzymes from the GH68 family. In addition, the effect of some important amino residues on the structure and function of levansucrases have been studied (Chambert and Petit-Glatron 1991; Ozimek et al. 2004; Ozimek et al. 2006; Strube et al. 2011). However, deep understanding of the catalytic mechanisms of these enzymes is still necessary.

According to the multiple alignments of deduced amino acid sequences, all bacterial levansucrases share seven conserved regions. However, many amino acid residues from Gram-positive and Gram-negative origin are obviously different (Arrieta et al. 1996; Seo et al. 2000). It is worth noting that the pairwise alignments between sequences of levansucrases from Gram-positive and Gram-negative bacteria show some alignment gaps. Whether the sequence of these alignment gaps could affect the characteristics of levansucrases deserves further study. Additionally, the sequence of these alignment gaps might be valuable for understanding molecular mechanism of levansucrases and provide evolution-related information of these enzymes. Molecular evolution of protein can provide an important means of studying the genetic relationship between protein molecules. It is a valuable tool for analyzing evolutionary relationships of protein molecules through comparing their sequences. According to the alignment of protein sequences, the regions with the same amino acids are commonly considered conservative regions which might share common ancestors. Whereas the gaps identified during pairwise alignments show that deletion, insertion, or replacement that might have taken place in these regions during the evolutionary process. Therefore, analysis of these alignment gaps would help us to understand the relationship between protein structures and functions, as well as the evolutionary process of the proteins (Chowdhury and Garai 2017). Moreover, the commonly used methods for modifying DNA molecules include random mutagenesis, site-directed mutagenesis, and DNA recombination, while introducing mutations through modifying alignment gaps has rarely been reported (Packer and Liu 2015). When protein sequence of levansucrase from *Bacillus amyloliquefaciens* (BA-SacB) (GenBank no AFJ64285.1) was searched using BLAST against all proteins in GenBank, the protein with minimum sequence identity to BA-SacB was levansucrase from *Sphingopyxis macrogoltabida* (SM-Lev). According to pairwise alignment of protein sequences of BA-SacB and

SM-Lev, the region of K296-D320 is missing in SM-LEV as compared with BA-SacB. This region should be very important because it contains one β -strand of the conserved “five antiparallel β -strands,” but it might be lost during evolution of levansucrase. Until now, how can this region affect the characteristics of levansucrases has not been reported. It is well known that the expression of SacB in the presence of sucrose is toxic to various Gram-negative bacteria including *E. coli* (Cai and Wolk 1990; Jäger et al. 1992; Jäger et al. 1995; Pelicic et al. 1996). Therefore, convenient production of valuable levan by direct fermentation of sucrose containing media with *E. coli*-expressing SacB is usually impractical.

In this study, a mutant BA-SacB-Del encoding BA-SacB minus the K296-D320 region was constructed and its effect on catalytic characteristics of enzyme was analyzed. In addition, sucrose sensitivity of BA-SacB-Del-expressing *E. coli* strain was detected to see the possibility of producing levan by direct fermentation of sucrose-containing media.

Materials and methods

Cloning of BA-SacB gene and BA-SacB-Del mutant encoding BA-SacB minus K296-D320 region

Genomic DNA was extracted from *B. amyloliquefaciens* and used as a template for PCRs. BA-SacB gene was amplified by PCR using primers: BA-SacB-For (5'-CTAGG ACGTC GATGA ACATC AAAAA ATTTG CAAAA CGAGC CAC-3', *Aat* II underlined) and BA-SacB-Rev (5'-TACCA CTAGT GTTGT TAACC GTAAG CTGTC CTTGT TCAAG G-3', *Spe* I underlined). The PCR product was cloned into the corresponding restriction enzyme sites of pETM11, to give plasmid pETM-BA-SacB containing BA-SacB gene. Then, BA-SacB-Del mutant encoding BA-SacB minus K296-D320 region was introduced by overlap extension PCR using BA-SacB-For and BA-SacB-Rev as external primers, and Inner-For (5'-CGAAG CCAAC ACTGG AACAG ATAAC AGTGC TAAAA AACGC GATGC TGAAT TAG-3') and Inner-Rev (5'-CTAAT TCAGC ATCGC GTTTT TTAGC ACTGT TATCT GTTCC AGTGT TGGCT TCG-3') as inner primers. The BA-SacB-Del mutant was cloned into pETM11 to give plasmid pETM-BA-SacB-Del.

Analysis of the sucrose sensitivity of *E. coli* BL21(DE3) /pETM-BA-SacB-Del strain and its fermentation products

The plasmids pETM-BA-SacB-Del, pETM-BA-SacB, and pETM11 were transformed into *E. coli* BL21(DE3) to give strains *E. coli* BL21(DE3)/pETM-BA-SacB-Del, *E. coli* BL21(DE3)/pETM-BA-SacB, and *E. coli* BL21(DE3)/

pETM11, respectively. To assay sucrose sensitivity of *E. coli* BL21(DE3)/pETM-BA-SacB-Del strain, these strains were cultured on LB agar plate and then single colonies were spotted on LB agar plates supplemented with 0.1 mmol/L isopropyl- β -D-thiogalactopyranoside (IPTG), 50 mg/L kanamycin, and 5% sucrose. The plate was incubated at 25 °C for 72 h. Then, totally, 0.1 g cells were scraped from the colonies of *E. coli* BL21(DE3)/pETM-BA-SacB-Del and resuspended in 5 mL water. After centrifugation (13,000g, 10 min), the supernatant was recovered and the components of reducing sugars were detected using the method of 2,4-dinitrosalicylic acid (DNS) assay (Bailey 1988), and 5% sucrose was used as a control during the assay process. Then, these components were further analyzed by high-performance liquid chromatography (HPLC) on a Shodex Ionpack KS-802 column (Showa Denko, Tokyo). Fructofuranosyl-nystose (GF4), nystose (GF3), 1-kestose (GF2), sucrose (GF), glucose (G), and fructose (F) (Meiji Seika Kaisha Ltd.) were used as standards. The eluate was monitored using a RI detector, and the mobile phase was water at 0.5 mL/min. The temperature of the column and detector was 25 °C. *E. coli* BL21(DE3)/pETM-BA-SacB-Del and BL21(DE3)/pETM-BA-SacB were cultured in LB media and protein expression was induced by 0.1 mmol/L IPTG. The same amount of cells of the two strains were pelleted by centrifugation, resuspended in sodium phosphate buffer (50 mmol/L, pH 5.9), and lysed by 5 cycles of sonication (30 s, 50% cycle, 65% power) with an interval of 5 min between each cycle. The expressed BA-SacB-Del and BA-SacB proteins were purified using Ni-NTA agarose beads (Qiagen). Then, catalytic activities of BA-SacB-Del and BA-SacB were analyzed using crude enzyme or purified enzymes in presence of 5% sucrose. The reactions were incubated at 40 °C for 5 h and the production of glucose was estimated using Glucose Assay Kit (HUILI Biotech Co., China). Enzyme activity assays were performed in triplicate. *E. coli* BL21(DE3)/pETM-BA-SacB-Del was used to inoculate a TB medium supplemented with 5% sucrose and 100 μ g/mL kanamycin, and fermentations were performed under static conditions at 25 °C for 72 h to produce polysaccharide. The catalysis with purified BA-SacB was performed under the same conditions to compare with the direct fermentation of *E. coli* BL21(DE3)/pETM-BA-SacB-Del for time dependent yields of levan. After centrifugation (4,000g, 5 min), the supernatant of each reaction was recovered and mixed with four volumes of ethanol. After centrifugation at 13,000g for 10 min, the pellets were washed five times with 50% ethanol and dehydrated by lyophilization. Then the linkage type of the pellet of polysaccharide produced by direct fermentation of *E. coli* BL21(DE3)/pETM-BA-SacB-Del was analyzed using 13 C NMR spectrometry. 13 C NMR spectrometry was run at

500 MHz on AMX-500 (Bruker, Germany). In all the assays above, *E. coli* BL21(DE3)/pETM-BA-SacB and *E. coli* BL21(DE3)/pETM11 were used as a positive and negative control, respectively.

Results and discussion

In order to investigate the effect of the amino acids K296-D320 on the characteristics of BA-SacB, we deleted the DNA sequence encoding K296-D320 from BA-SacB to give BA-SacB-Del. The strains *E. coli* BL21(DE3)/pETM-BA-SacB-Del and *E. coli* BL21(DE3)/pETM-BA-SacB were cultured on LB plate supplemented with IPTG and 5% sucrose. As a result, *E. coli* BL21(DE3)/pETM-BA-SacB could not grow on the plate, while *E. coli* BL21(DE3)/pETM-BA-SacB-Del could form colonies (Fig. 1a). This result indicated that deletion of the region K296-D320 of BA-SacB made the *E. coli* host be less or even not sensitive to sucrose. Interestingly, the colonies formed by *E. coli* BL21(DE3)/pETM-BA-SacB-Del were mucoid, which are obviously different from that formed by *E. coli* BL21(DE3)/pETM11. Then, these mucoid colonies were scraped from the plate, resuspended in water, and DNS assay was performed DNS assay on the supernatant after centrifugation (Fig. 1b). The positive result indicated that BA-SacB-Del could introduce the production of reducing sugars on LB agar plate. Then the reducing sugars were identified as glucose and fructose

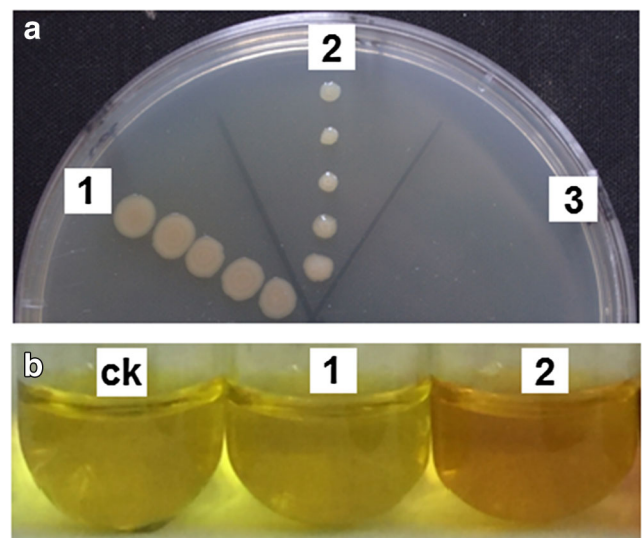


Fig. 1 The change of colony morphology of *E. coli* and the production of reducing sugars introduced by BA-SacB-Del in the presence of sucrose. **a** Shows the culture of *E. coli* BL21(DE3) harboring different plasmids on LB agar plate supplemented with IPTG and sucrose at 25 °C for 72 h. **b** Shows the DNS assays of reducing sugars produced by colonies scraped from agar plate. 1, 2, and 3 refer to colonies or DNS assays be associated with *E. coli* BL21(DE3) harboring plasmids pETM11, pETM-BA-SacB-Del, and pETM-BA-SacB, respectively. CK refers to DNS reaction only in the presence of 5% sucrose

according to the analysis of HPLC (Fig. 2), indicating that the mutant BA-SacB-Del has strong sucrose hydrolase activity. This result could account for formation of the mucoid colonies because fructose is very hygroscopic. In addition, nearly no fructooligosaccharides could be detected, indicating that BA-SacB-Del has high product specificity. Fermentation of sucrose-containing media with *E. coli* BL21(DE3)/pETM-BA-SacB-Del can produce turbid polysaccharide (Fig. 3a, b). Then structure of the polysaccharide was analyzed by ^{13}C NMR spectrometry. Assignment of the peaks was based on the report of Shimamura et al (1987). The results confirmed that the polysaccharide was levan of β -2,6-fructan (Fig. 4). It is worth noting that only linkage type of the insoluble polymer with high molecular mass has been analyzed by ^{13}C NMR spectrometry, because the soluble sucrose, glucose, and fructose in the reaction mixture have been eliminated by repeated washing with 50% ethanol. Levan can be conveniently produced by direct fermentation of sucrose containing media with *E. coli*-expressing BA-SacB-Del which is not sensitive to sucrose. By contrast, currently the commonly used strategy to produce levan with recombinant *E. coli* strains was in vitro catalysis using isolated levansucrase (Xu et al. 2017; Gao et al. 2017a; Porras-Domínguez et al. 2017; Xu et al. 2018; Núñez-López et al. 2019; Polsinelli et al. 2019; Szwengel and Wiesner 2019), which is less convenient than

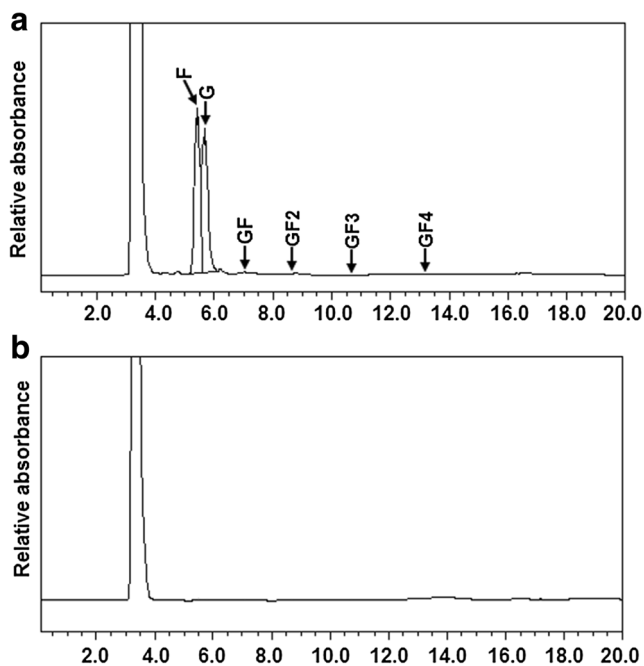


Fig. 2 HPLC chromatogram shows analysis of the substances introducing the formation of mucoid colonies by *E. coli* BL21(DE3)/pETM-BA-SacB-Del. **a** Shows the component of the solution after washing colonies formed by *E. coli* BL21(DE3)/pETM-BA-SacB-Del. **b** Shows the component of the solution after washing colonies formed by *E. coli* BL21(DE3)/pETM11. F, G, GF, GF2, GF3, and GF4 refer to predicted locations of fructose, glucose, sucrose, 1-kestose, nystose, and fructofuranosyl-nystose, respectively

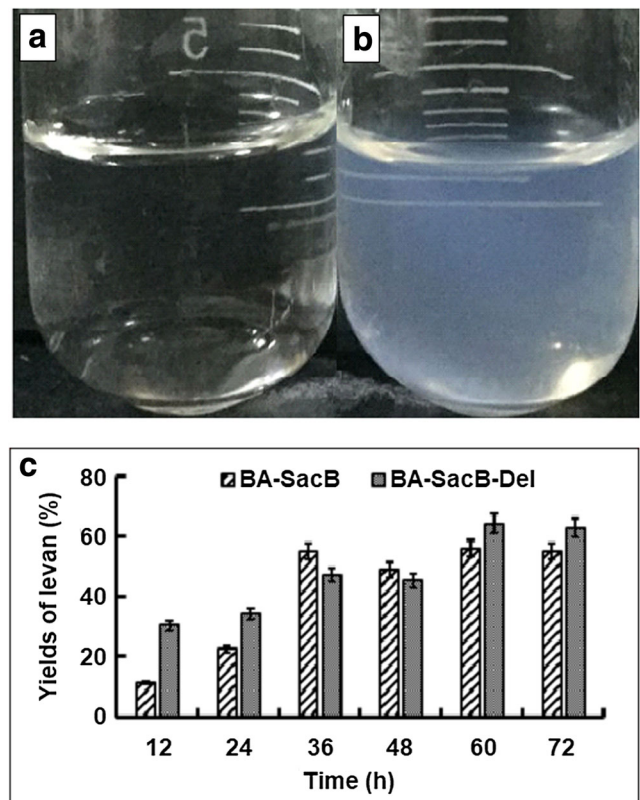


Fig. 3 Production of levan by direct fermentation with *E. coli*-expressing BA-SacB-Del or catalysis with purified BA-SacB. **a** and **b** refer to reactions associated with direct fermentation of sucrose-containing media with *E. coli* BL21(DE3)/pETM11 and *E. coli* BL21(DE3)/pETM-BA-SacB-Del, respectively. **c** shows comparison of catalysis with purified BA-SacB and direct fermentation with *E. coli* BL21(DE3)/pETM-BA-SacB-Del for time-dependent yields of levan

direct fermentation because the steps of protein purification are always required. In addition, during direct fermentation the by-product of glucose could provide nutrition for growing *E. coli*, thus making it possible to further reduce the cost of the culture medium. The direct fermentation of *E. coli*

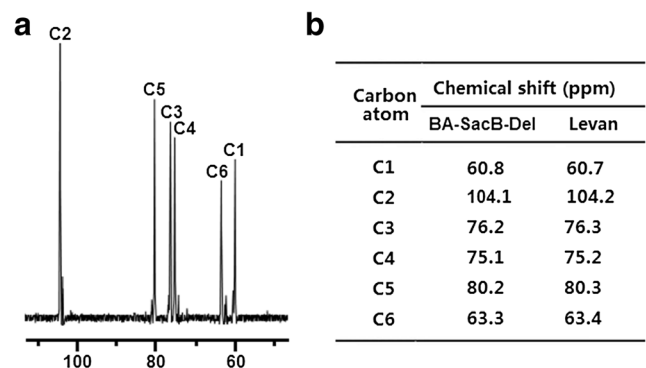


Fig. 4 ^{13}C -NMR spectra used for the analysis of polysaccharide produced by fermentation of sucrose-containing media with *E. coli* BL21(DE3)/pETM-BA-SacB-Del. **a** ^{13}C -NMR spectra shows polysaccharide catalyzed using BA-SacB-Del. **b** Shows chemical shifts for ^{13}C -NMR spectra of levan and polysaccharide synthesized using BA-SacB-Del

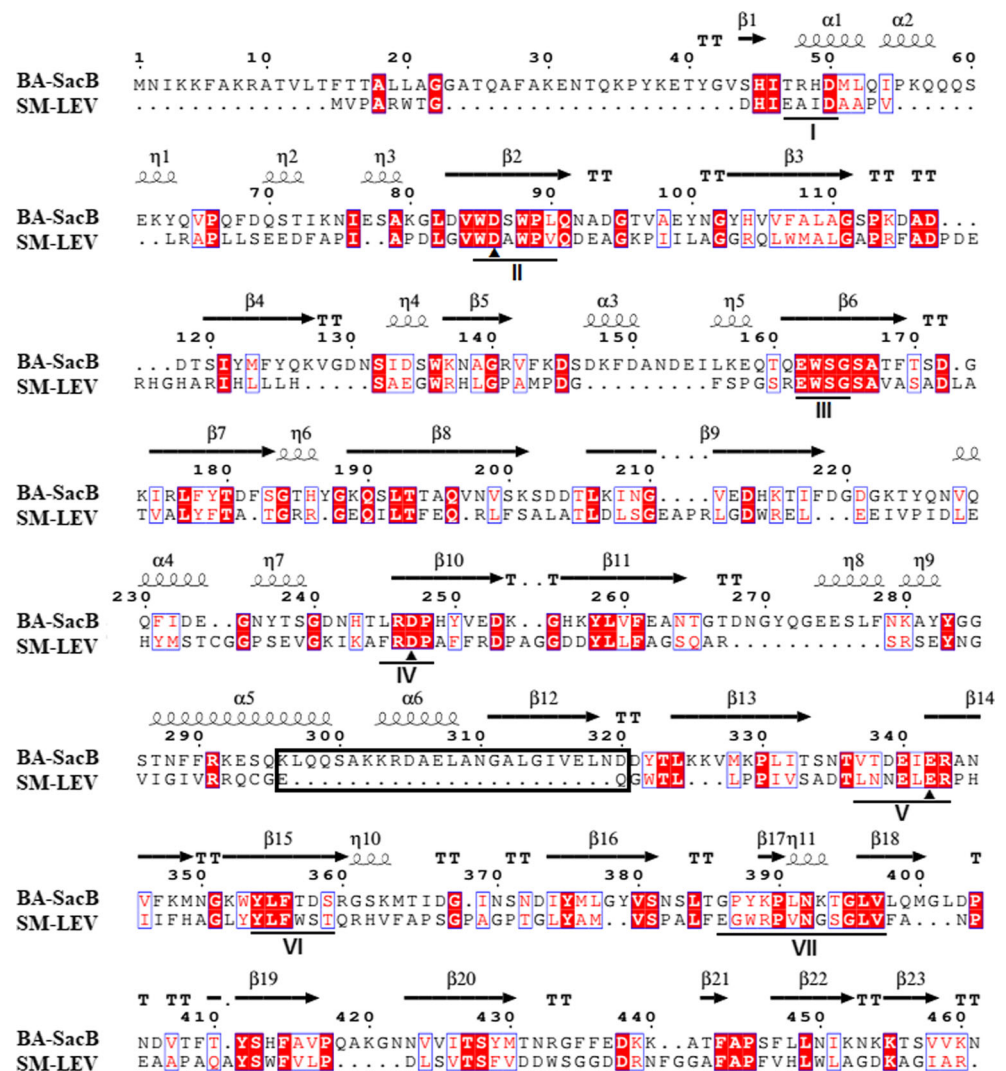
BL21(DE3)/pETM-BA-SacB-Del was compared with catalysis using purified BA-SacB for time-dependent yields of levan. As a result, although direct fermentation of *E. coli* BL21(DE3)/pETM-BA-SacB-Del could accumulate levan faster in the early stage, both methods could introduce about 60% yields of levan after 72 h of reaction (Fig. 3c). Therefore, direct fermentation of sucrose-containing media with *E. coli*-expressing BA-SacB-Del provided a valuable option for convenient and efficient production of levan.

It is well known that insertional inactivation of a plasmid-borne SacB expression cassette can be used for selection of bacterial clones containing recombinant DNA inserts. Therefore, SacB genes have been widely used for marker exchange procedures to trap insertion sequences (Thulin et al. 2015; Sawisit et al. 2015; Rubio et al. 2015; Weissman 2016). There are two hypotheses to explain the lethality of SacB in the presence of sucrose: (i) production of toxic metabolites by transfer of fructose residues to inappropriate acceptor molecules (Pelicic et al. 1996; Dziewit and Bartosik

2015; Khetrpal et al. 2015), and (ii) accumulation of levan in the periplasm might disrupt periplasmic functions (Jäger et al. 1992; Pelicic et al. 1996). Recently, in our laboratory, we have proven that signal peptide-dependent protein translocation pathway is crucial for the sucrose sensitivity of SacB-expressing *E. coli*, indicating that accumulation of levan in the periplasm should account for this phenomenon (Gao et al. 2017b). However, in this study, although BA-SacB-Del contained signal peptide and had levansucrase activity, *E. coli* BL21 (DE3)-expressing BA-SacB-Del lost sensitivity towards sucrose. This result indicated that the reason for sucrose sensitivity of SacB-expressing *E. coli* should be complicated, which might be related to the specific protein structure of SacB. The reason for the loss of sucrose sensitivity of BA-SacB after deletion of the K296-D320 region deserves further investigation.

BA-SacB and SM-LEV sequence alignment based on structural superimposition was generated by ESPrnt 3.0 (Fig. 5). Secondary-structural elements were labeled using

Fig. 5 BA-SacB and SM-LEV sequence alignment based on structural superimposition. Secondary-structural elements are indicated by squiggles (α -helices) and arrows (β -strands). α -helices (labeled α) and β -strands (labeled β) are numbered consecutively. Regions considered important for the activity are underlined and numbered from I to VII. The catalytic triad at the center of the active site is marked with small triangles. The K296-D320 region deleted in this study is boxed

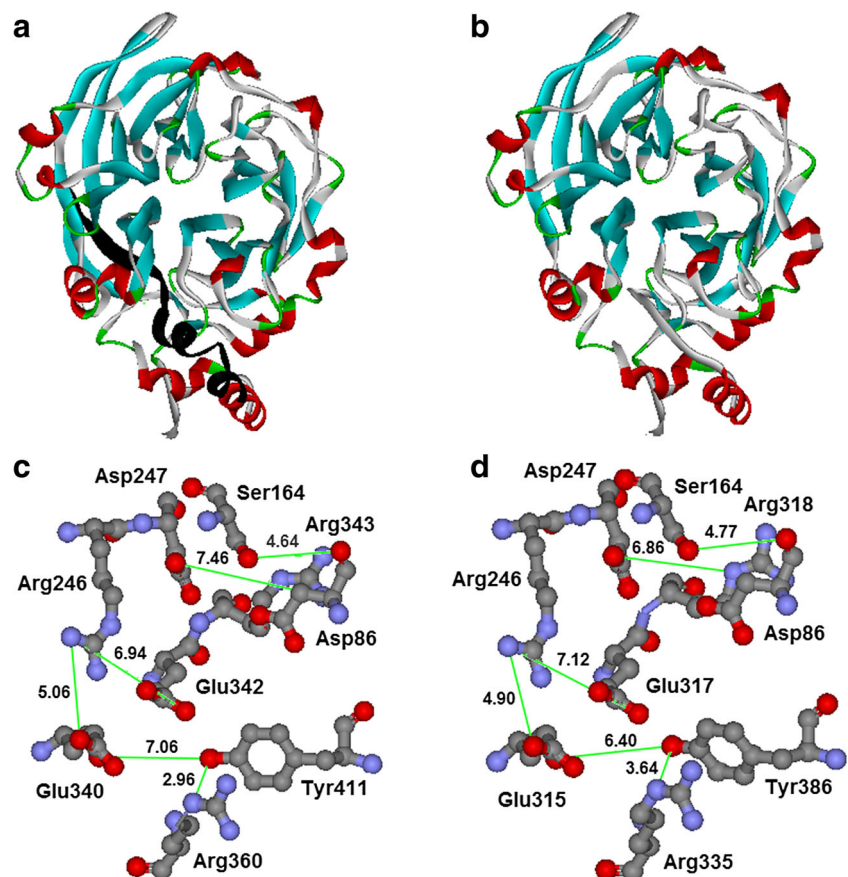


the structure of *B. subtilis* levansucrase as template. BA-sacB presents an overall sequence identity with SM-LEV of 23%. There are totally seven conserved region (Arrieta et al. 1996) and three crucial amino acid residues that function together at the center of the active site (i.e., catalytic triad) in reported levansucrases (Verhaest et al. 2005), but none of them are in the region of K296-D320. To further understand the effect of deleting the K296-D320 region on protein structure and functions, the protein models of BA-SacB and BA-SacB-Del were constructed using SWISS-MODEL (Fig. 6a, b). According to the models, both BA-SacB and BA-SacB-Del have the typical structure of the GH68 family (i.e., β -propeller fold consisting of five blades with antiparallel β -strands, and the β -propeller of each structure forms a central cavity, which is essential for activities). However, all the five blades in BA-SacB comprise four antiparallel β -strands, while one of the blades in BA-SacB-Del (Blade III) remains only two antiparallel β -strands after deletion of the K296-D320 region. According to the structural framework of *B. subtilis* levansucrase BS-SacB, some important functional motifs in the GH68 family locate in the five blades and form central cavity of the propeller (Meng and Fütterer 2003). The Asp86 and Glu342 form the pair of essential catalytic side chains, whereas Asp247 forms strong hydrogen bonding interactions with hydroxyls

of the fructosyl unit of the substrate. Enzyme-substrate hydrogen bonding interactions can promote catalysis by stabilizing the oxocarbenium ion-like transition state of the anomeric carbon. Glu342 is part of a complex network of interactions that includes Arg 246, Arg360, and Tyr411 as well as Glu340 and Glu262. Some of these amino acid residues may also have multiple hydrogen bonding contacts with the substrate (Meng and Fütterer 2003; Martínez-Fleites et al. 2005; Strube et al. 2011). The detailed space distribution and interactions of these amino acid chains were analyzed (Fig. 6c, d). For both BA-SacB and BA-SacB-Del, these amino acid chains are spaced 3.0–7.5 Å from each other. After, pairwise comparison of BA-SacB and BA-SacB-Del, only less than 0.6 Å differences are identified between these amino acids. Therefore, deletion of the K296-D320 region might have not remarkably affected the spatial arrangement of the critical amino acids, which may partly account for the fact that BA-SacB-Del maintains both sucrose hydrolase and fructosyltransferase activities.

Similarities in functions and protein sequences of BA-SacB and SM-Lev suggest that these proteins may originate from same ancestral protein. An interesting question is that: was the K296-D320 region removed from the ancestral protein, or obtained by the ancestral protein during molecular evolution? To assess evolutionary characteristics of levansucrases, pro-

Fig. 6 Protein models of BA-SacB and BA-SacB-Del. **a** and **b** show models of BA-SacB and BA-SacB-Del, respectively. The region of K296-D320 is marked in black. **c** and **d** Show interatomic distances between critical residues in the active sites of BA-SacB and BA-SacB-Del, respectively. Hydrogen bonds are shown by solid lines in green; distances are given in Å



tein sequences of levansucrases having similarity with that of BA-SacB were searched using BLAST against all proteins in GenBank. These levansucrases were found to exist in microorganisms of up to 77 genera. All the characterized levansucrases from each genera (including *B. amyloliquefaciens* and *S. macrogoltabida*) were performed phylogenetic analysis, and a dendrogram was generated using the MEGA software (Supplementary Fig. 1). According to this dendrogram, levansucrases from nearly all the Gram-positive bacteria from different genera (22/24) contain the K296-D320 region. On the other hand, this region is lost or incomplete in all the levansucrases from archaea (i.e., *Halogeometricum pallidum*, *Halogramum salarium*, and *Halalkalicoccus paucihalophilus*) according to this dendrogram. Analysis of evolutionary principle revealed that archaea are more closely related to eukaryotes than to eubacteria (Iwabe et al. 1989; Zaremba-Niedzwiedzka et al. 2017). Therefore, the K296-D320 region might originally exist in the ancestral levansucrase, which might be lost during the evolution of the levansucrases from Gram-negative eubacteria into archaea.

Conclusions

To sum up, in this study, after comparison and analysis of BA-SacB and SM-LEV, a mutant BA-SacB-Del encoding BA-SacB minus K296-D320 region was constructed. The effect of this deleted region on characteristics of the BA-SacB was investigated. This result should be very valuable for understanding sucrose sensitivity of SacB-expressing *E. coli*. In addition, levan can be conveniently produced by direct fermentation of sucrose-containing media with *E. coli*-expressing BA-SacB-Del which is not sensitive to sucrose. Therefore, this study should be valuable for understanding the mechanisms of BA-SacB and even enzymes of GH68, and lay the foundation for more convenient and efficient production of levan.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

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