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POLYSACCHARIDE SYNTHESIS OF THE LEVANSUCRASE SACB FROM *BACILLUS MEGATERIUM* IS CONTROLLED BY DISTINCT SURFACE MOTIFS

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Running head: Polysaccharide synthesis process of the levansucrase SacB The atomic coordinates and structure factors (code 3OM2, 3OM4, 3OM5, 3OM6 and 3OM7) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/) Correspondence may be addressed to [3]: Helmholtz-Centre for Infection Research, Inhoffenstr. 7B, 38124 Braunschweig, Germany, phone +4953161817000, fax +4953161817098, email: dih@helmholtz-hzi.de. Correspondence may be addressed to [2]: University of Würzburg, Institute of Organic Chemistry, Am Hubland, D-97074 Würzburg, Germany, phone seibel@chemie.uni-wuerzburg.de.

Despite the widespread biological function of carbohydrates, the polysaccharide synthesis mechanisms of glycosyltransferases remain largely unexplored. Bacterial levansucrases (glycoside hydrolase (GH) family 68) synthesize high molecular weight, β-(2,6) linked levan from sucrose by transfer of fructosyl units. The kinetic and biochemical characterization of *Bacillus megaterium* levansucrase SacB variants Y247A, Y247W, N252A, D257A and K373A reveal novel surface motifs remote to the sucrose binding site with distinct influence on the polysaccharide product spectrum. The wild-type activity (k_{cat}) and substrate affinity (K_m) is maintained. The structures of the SacB variants reveal clearly distinguishable subsites for polysaccharide synthesis as well as an intact active site architecture. These results lead to a new understanding of polysaccharide synthesis mechanisms. The identified surface motifs are discussed in the context of related glycosyltransferases.

Carbohydrates represent one of the three major classes of biological macromolecules along with proteins and nucleic acids. They play a functional role in numerous biological recognition processes, including bacterial or viral infection, inflammation and innate/adaptive immunity (1-3). Thus, there is a great interest in glycans for developing new potential therapeutic agents, such as vaccines, glycoprotein

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therapeutics such as antibodies and glycosylated drugs (4). Their chemical synthesis is often expensive and laborious or not possible at all. Chemo-enzymatic glycoconjugate synthesis methods are on the rise to overcome these drawbacks (5). Poly-and oligosaccharides are synthesized by the linkage of activated monosaccharides and have remarkable structural variations. Their biological function depends on the degree of polymerization, the linkage type and the branching of the saccharide chain (6).

The biosynthesis of fructosyl polymers (fructan) is catalyzed by the action of enzymes called fructansucrases, also commonly referred to as fructosyltransferases. There are two types of fructansucrases known, levansucrases and inulosucrases. Levansucrases (EC 2.4.1.10) mainly form levan with β-(2,6)-linked fructosyl residues (7,8). Inulosucrases (EC 2.4.1.9) synthesize fructans containing primarily β-(2,1)

linked fructosyl units which are referred to as inulins (9,10). These enzymes cleave the glycosidic bond of their substrate sucrose and catalyze the transfer of a fructosyl unit from sucrose to a growing fructan chain (polysaccharide formation) or to water (hydrolysis) (11-13). Besides synthesizing high molecular weight polysaccharides, fructansucrases are also capable of forming short-chain fructo-oligosaccharides in the presence of suitable acceptors or by mutagenesis in the sucrose binding site (14-17). Each fructansucrase mainly forms one type of linkage in the synthesized fructo-oligosacharides. According to the database of Carbohydrate-Active enZYmes (CAZY) (18), bacterial fructansucrases are members of the family 68 of glycoside hydrolases (GH). Clan GH-J comprises bacterial fructansucrases of GH 68 and the enzymes of family GH 32, which occur mainly in plants and fungi. These members share a β-propeller fold consisting of four antiparallel β-strands and a central negatively charged cavity, first discovered in tachylectin-2 (19). Recently, it was discovered that the mutation of an amino acid not located in the active site of the fructosyltransferase SacB from *Bacillus megaterium* (Asn^{252}) eliminates its polysaccharide synthesis (20). Analysis of the crystal structure of the homologous *B. subtilis* levansucrase in complex with sucrose (pdb ID 1PT2) and raffinose (3BYN) provided insights into the functional role of Asn^{252} (8,21). Nevertheless, it remained unclear if structural elements on the enzyme`s surface outside the active site take part in the transfructosylation process.

In this work, an extensive mutagenesis study combined with biochemical analyses and structural information of the GH 68 levansucrase SacB from *B. megaterium* was performed. Novel variants of amino acid residues located on the enzyme`s surface remote from the active site were rationally chosen, characterized and crystallized. The five structures of SacB variants (Y247A, Y247W, N252A, D257A and K373A)obtained at resolutions between 2.0 and 1.75 Å support a surface-modulated transfructosylation mechanism.

Experimental Procedures

Vector construction, cloning, expression and purification -The *Escherichia coli* expression vector pRBEc1, harbouring the *sacB* gene, was described previously (20). The plasmid was employed as template to generate variants of *sacB* by site-directed mutagenesis (QuikChange®, Stratagene). All introduced mutations and the integrity of the residual *sacB* gene were confirmed by DNA sequencing. Expression and purification of each variant was performed as described previously (20).

Crystallization and structure refinement – For crystallization, SacB variants were additionally purified using a hydroxyapatite column, eluting with a linear gradient of phosphate buffer with increasing phosphate concentration. Eluted protein was applied to a Superdex 75 16/60 column (GE Healthcare) equilibrated with 50 mM sodium chloride and 10 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 6.0. The purified SacB variants were analyzed by SDS-PAGE and concentrated by centrifugal filtration in protein concentrators (VivaSpin). The protein concentrations of the fractions were measured photometrically at 280 nm (NanoDrop Spectrophotometer ND-100, peqLab Biotechnology). All crystallization experiments were performed by hanging-drop vapour-diffusion method. Crystals grew under two different crystallization conditions; the first condition contained 0.1 M sodium-phosphatecitrate pH 4.1, 0.1 M magnesium sulfate, 30 % polyethylene glycol (PEG) 400 (condition A). The second condition was composed of 0.1 M sodium-phosphate-citrate pH 4.1, 0.2 M lithium sulfate, 0.1 M calcium dichloride and 20 % PEG 1000 (condition B). Using purified protein at a final concentration of 8.5 mg ml^{-1} small crystals grew at 20 °C over a period of two weeks. Crystals of SacB D257A were obtained from condition A, whereas variants Y247A, Y247W, N252A and K373A showed best crystallization results under condition B. Initial crystals were used for microseeding with a decreased protein concentration between 5.0 and 6.0 mg ml^{-1} resulting in diffraction quality crystals. The cryoprotectant solution consists of reservoir solution supplemented with 20 % glycerol. Crystals grown under condition A were not supplied with additional cryoprotectant. An X-ray data set of SacB D257A was collected on beamline BM16 at the ESRF synchrotron (Grenoble, France). Data for variants K373A, N252A, Y247A and Y247W were recorded at the Bessy (Berlin, Germany) either on beamline BL 14.1 or 14.2. Indexing and processing of the intensity data was carried out with XDS program package (22). Scaling was performed using SCALA from the ccp4 package (23). The crystal structure of SacB D257A in space group $P2_12_12_1$ was determined by the molecular replacement method using the program MolRep in the ccp4suite. Coordinates of homologue SacB from *Bacillus subtilis* (PDB ID 1OYG) served as the search model. The results showed a clear solution for one molecule in the asymmetric unit and a solvent content of 43 %. Crystals of variants K373A, N252A, Y247A and Y247W belong to space group $P2_1$. The atomic coordinates of variant D257A were used as the starting model to solve the structures of SacB variants in space group $P2_1$ by molecular replacement. The asymmetric units of these variants contained four copies of the enzyme with a solvent content of about 43 %. The final structures were modeled after iterative cycles of manual model building and refinement using COOT (24) and REFMAC5 (25), respectively. All five final models include amino acid residues 34 – 481. The quality of the final structures was validated with the MOLPROBITY server (26) and SFCHECK from the ccp4 package (23).

Determination of the kinetic parameters

-The determination of the kinetic parameters was performed with purified enzyme under optimized reaction conditions (7.36 mg l^{-1} SacB at a pH of 6.6 and 37 °C). Substrate concentrations of 500 to 1 mM were investigated (500, 250, 100, 50, 25, 10, 5, 2.5 and 1 mM). After 1 h, the reaction was stopped by heating at 100 °C for 10 min. Spontaneous heat-mediated sucrose hydrolysis was excluded by the analysis of inactive SacB variants. Here, no glucose or fructose was detected using heat inactivation of the enzyme. The glucose content was determined using conditions A (table 2a) by high-performance anion exchange chromatography (HPAEC, Dionex, pre column, CarboPac PA1, 4 mm x 50 mm; main column, CarboPac PA1, 4 mm x 250 mm Dionex; conductivity detector PAD-2, at 1 ml min^{-1}). The specific glucose formation activity (U mg^{-1}) and the substrate concentration (S) were plotted. The resulting K_m and v_{max} values were determined by regression according to the Michaelis-Menten equation.

Oligo- and polysaccharide analysis -The wild-type and variant`s polysaccharide synthesis process was monitored in 50 mM Sorenson's phosphate buffer (pH 6.6) at 37 °C, 500 mM sucrose and 7.36 mg l^{-1} enzyme concentration by thin-layer chromatography

(Supplemental Figure 1). 3 µl of the reaction mixture was separated by thin layer chromatography (TLC, silica coated aluminium plate, isopropanol (60 %), ethylacetate (30 %), water (10 %). TLC staining was performed by N-(1-Naphthyl)ethylene diamine dihydrochloride (2.5 % w/v, in 5 % sulfuric acid and methanol) and subsequent heating (160 °C) for 5 min.

For the wild-type SacB, additional samples were taken after 10 and 30 min, and 1, 6, 24 and 72 h and analyzed by HPAEC under conditions A (table 2a, Supplemental Figure 2). To determine the polysaccharide synthesis kinetics of the variants, the 24 and 72 h samples were analyzed by HPAEC to determine the sucrose consumption (Supplemental Figure 3). The ethanol precipitated oligo-and polysaccharides of the 72 h sample of the wild-type and the variants was further analyzed using HPAEC without sodium acetate gradient (conditions B, table 2b, Figure 4 and Supplemental Figure 4). Thus, oligosaccharide peaks were separated as opposed to conditions A (table 2a).

Hydrolysis versus *transfer activity* -For the determination of the hydrolysis *versus* transfer activity of the wild-type SacB and its variants, the polysaccharide synthesis reaction was performed at pH 6.6, 37 °C, 7.36 mg l^{-1} SacB and 500 mM sucrose for 72 h. The glucose and fructose content of the reaction mixture was analyzed by HPAEC according to conditions A (table 2a).

RESULTS

Structures of SacB variants Y247A, Y247W, N252A, D257A and K373A maintain the wild type active site architecture - The structures of levansucrase SacB variants Y247A, Y247W, N252A, D257A and K373A have been refined at resolutions between 1.75 and 2.0 Å. Crystals of variants D257A and Y247A, respectively, were grown-together crystals resulting in multiple diffraction patterns. This may explain the relatively high values of R_{merge} (table 1). Furthermore, the analysis of data sets Y247A, Y247W and N252A revealed slightly pseudo-merohedral twinning (twinning fractions of 8.5 to 10 %). For each variant, amino acid residues 34 – 481 are well resolved in the electron density. The SacB variants contain a single globular domain with a five-fold β-propeller motif as described for all known structures of clan GH J enzymes (8,13,18). Each blade of the five-fold β-propeller consists of four antiparallel β-strands showing the classical `W` topology. The highly conserved sequence motifs

^{94}WD^{95}

of GH 68, including the motifs ,^{256}RDP258

^{172}WSGS^{175}, , ^{349}DXXER^{353} and ^{421}YSH^{423}, which form the central framework of the β-propeller, were identified. The active site is located at the bottom of the central cavity of the propeller. A funnel-like opening provides access to the deep negatively charged pocket towards the molecular surface (Fig. 1). The active site contains the catalytic amino acid residues Asp^{95}/Asp^{257}/Glu^{352}. Glu^{352} acts as acid/base catalyst and Asp^{95} is the catalytic nucleophile supported by previous mutagenesis studies of SacB from *B. megaterium* (20). Asp^{257} has been shown to interact with the 3- and 4-OH of the fructosyl moiety in the active site (9-10) and stabilizes the transition state. Arginine in position 370 is analogous to the previously described Arg^{360} of SacB from *B. subtilis*, which influences the polysaccharide synthesis in subsite +1 (8,21). Structure refinement reveals a continuous stretch of electron density within the active site cavity of all variants, except for D257A. Fragments of PEG molecules sized between 100 and 300 Da and used as precipitants during crystallization best fit in the elongated density. The PEG molecules are located between residues Trp^{172}, Leu^{118} Trp^{94} and Pro^{414}. (Fig. 2). Except for proline, these amino acid residues were already part of a mutagenesis studied previously published (20). Their knockout leads to inactivation of SacB.

N252A eliminates the polysaccharide synthesis after tetrasaccharides - In previous investigations the crucial role of Asn^{252} in fructosyl transfer *versus* hydrolysis reactions was identified (20). SacB variant N252A does not form any polysaccharide but instead short-chain oligosaccharides of up to three fructosyl units (nystose) (20). In SacB from *B. megaterium*, Asn^{252} is not located in the sucrose binding site analogous to Asn^{242} of the levansucrase from *B. subtilis* co-crystallized with raffinose (pdb code 3BYN (21)). The crucial question arises if the residue Asn^{252} is involved in interactions with the fructan chain or if alternative effects, perhaps towards amino acid residues located in the catalytic site, are responsible for the observed termination of polymer formation. In order to answer this question, SacB variant N252A was crystallized and the structure of this variant was solved at a resolution of 2.0 Å. The structure of SacB variant N252A shows an intact active site compared to D257A and K373A (Fig. 3). None of the catalytic amino acid residues changes its conformation. Thus, conformational effects of N252A on the fructosyl transfer mechanism are excluded.

K373A eliminates the polysaccharide synthesis after hexasaccharides -Lys^{373} located outside the sucrose binding site was exchanged to alanine in order to examine its role in the polysaccharide synthesis process. Regarding variant K373A, the formation of tri- and tetrasaccharides is lowered while the synthesis of penta-and hexasaccharides (containing four or five fructosyl units, respectively) is slightly enhanced (Fig. 4). Oligosaccharides exceeding six units are not observed. Consistent with the eliminated polysaccharide synthesis, K373A has an increased hydrolytic activity of almost 33 % (Fig. 5). Superposition of SacB variant K373A
(1.75 Å) with the structures of variants D257A and N252A shows an intact active site (Fig. 3).

Y247A eliminates the polysaccharide synthesis after decasaccharides – We further investigated the role of tyrosine in position 247. We anticipated a potential protein-carbohydrate π-π stacking mechanism in this location. The characterization of the oligo- and polysaccharide products point towards the role of Tyr^{247} and Lys^{373} concerning the transfructoslyation mechanism. Tyr^{247} is not located in the active site of SacB, but has a strong influence on its polymer formation activity. The exchange of tyrosine in position 247 to alanine leads to the formation of short oligosaccharides (Fig. 4). The amount of octa- and nonasaccharides is slightly enhanced and the transfructosylation is totally eliminated after nine fructosyl units (decasaccharides, fig. 4). In contrast, the exchange of tyrosine to tryptophan results in the same oligosaccharide pattern as the wild-type SacB (Fig. 4). Consistent with the kinetic parameters (K_m 2.1 mM, k_{cat} 2653 s^{-1}, table 3), variant Y247W maintains the wild-type activity and substrate affinity. The hydrolysis activity of variant Y247A is enhanced by 10 % (mol mol^{-1}), while the hydrolysis products of Y247W are only slightly increased compared to the wild-type SacB (5 %, Fig. 4). Again, the structures of the SacB variants Y247A and Y247W (2.0 and

1.9 Å, respectively) point out that the wild type active site architecture is maintained (Fig. 3). K_m and k_{cat} of Y247A, which are similar to the wild type SacB, support this structural observation (Table 3).

Functional modifications of SacB surface motifs maintain wild-type kinetic parameters -To further investigate structural elements on the surface of SacB, amino acid residues in the vicinity of Asn^{252}, Tyr^{247} and Lys^{373} were chosen for mutagenesis studies. On the basis of structural alignments with the levansucrases SacB from *B. subtilis* and the related LsdA from *G. diazotrophicus* (27), six additional amino acid residues with potential impact on the polysaccharide synthesis mechanism of SacB are identified. The amino acid functionality of the new SacB variants (Y247A, Y247I, Y247W, N312A, K315A, K315R, S372A, K373A, K373R and Q381A) is eliminated by an exchange to alanine or partially maintained by the exchange to functionally similar amino acid residues (Table 3). In order to elucidate the conformation and substrate affinity of the active site of the new SacB variants, their kinetic parameters and product spectra were determined and analyzed by HPAEC. All SacB variants have a Michaelis-Menten constant comparable to the wild-type. The K_m of the variants ranges between 2.1 (Y247W) and 11.4 mM (Y247I) which is less than 50 % difference from the K_m of the wild-type. The intact active-site architecture of the variants is indicated by their substrate affinity in the wild-type range. Moreover, the turnover numbers of all variants are comparable to the wild-type SacB, except for two variants, K373A and N312A. These have a k_{cat} three-fold lower than the wild-type. However, this observed activity is still in the high range compared to fructosyltransferases from other organisms (Table 4). The other variants do not differ more than 40 % from the wild type SacB`s turnover number. This indicates the integrity of the polysaccharide synthesis machinery.

SacB variants show deviating hydrolysis versus *transfer activities* -The analysis of the hydrolysis *versus* transfer reaction elucidates the different product spectra of the SacB variants. The amount of glucose released, indicating hydrolysis, is compared to the amount of free fructose. The difference has to correspond to transfer products. The variant with significantly enhanced transfer activity is K315A (22 %, fig. 5). Variants K315R and N312A show a hydrolysis *versus* transfer activity similar to the wild-type. All other variants have an increased hydrolysis rate of up to 33 % (K373A) (Fig. 5). In order to elucidate the composition of the oligosaccharides formed by SacB, the precipitated reaction products were analyzed by HPAEC. On the basis of a carbohydrate standard, the HPAEC peaks were assigned to the corresponding number of fructosyl units. The hydrolysis activity of the variants is enhanced compared to the wild type except for K315A (Fig. 5). According to its strongly increased transfructosylation activity of 22 % [mol mol^{-1}], K315A forms significantly more oligosaccharides. The K_m and k_{cat} of the variants are similar to the wildtype SacB. The exchange of lysine in position 315 to arginine retains the wild type hydrolysis and transfructosylation activity and does not have any kinetic effect that differs significantly from any wild-type parameter.

DISCUSSION

Polysaccharide synthesis is modulated by structural elements on the surface of SacB from Bacillus megaterium - Despite of rigorous studies during the recent years with numerous solved structures of enzymes acting on sucrose, their polysaccharide synthesis mechanism remains elusive (13). Even successful cocrystallizations with di- and trisaccharides did not lead to a detailed explanation of the transfructosylation process regarding oligo- and polysaccharide synthesis (8,21). The previously described amino acids with impact on β-(2,6) linked levan-or β-(2,1) linked inulin formation were located in the sucrose binding site. They interact with sucrose either catalyzing the cleavage of the glycosidic bond (Asp^{95}, Asp^{257} , Glu^{352}), generating the enzyme-fructosyl complex (Asp^{95}) or stabilizing sucrose in the active site (Trp^{94}, Trp^{173}, Arg^{256}, Glu^{350}, Arg^{370}) (13). Recently, an asparagine residue in position 252 outside the sucrose binding site was described as crucial for polysaccharide synthesis (20). Hence, the following questions arise: First, does a mutation of Asn^{252} have an impact on the active site conformation? And second, are there other surface motifs influencing the polysaccharide synthesis and does their mutation influence the active site architecture?

In this work, it is shown for the first time that amino acids outside the active site of a polysaccharide-forming enzyme have a well-defined and rationally explainable effect on the polymer formation activity. Indirect effects on the position of other amino acids can be excluded due to the extensive structural data of SacB variants Y247A, Y247W, N252A and K373A. The structural data are consistent with the kinetic and biochemical analyses. Conformational analyses of variants Y247A, Y247W, N252A and K373A reveal retained active site architecture (Fig. 3). Supporting the crystallographic data, the kinetic parameters of these variants are not significantly different compared to wild-type SacB (Table 3). Moreover, the structural data point towards a possible surface arrangement for the binding of an acceptor fructosyl chain. Residues Asn^{252}, Lys^{373} and Tyr^{247} form a platform for a possible stabilization of the acceptor fructan chain. Clear subsites can be assigned to every exchanged amino acid (Fig. 4). The biochemical data along with the structural data show that Asn^{252} is located close to the sucrose binding site, whereas Lys^{373} and Tyr^{247} are clearly apart from the sucrose binding cavity of SacB. All exchanged amino acid residues are located on the surface of SacB. HPAEC analyses of the variant`s oligofructoside synthesis patterns show definite terminations of the polymerization process depending on the location of the mutated amino acid residue. Variants K373A, N252A and Y247A synthesize unique mixtures of oligosaccharides with clearly distinguishable chain lengths, correlating to their location on the surface of SacB. We examined further the exchange of lysine in position 373 to arginine leads to longer oligofructosides (9 fructosyl units) than its exchange to alanine (4-5 fructosyl units) (Fig. 4). Interactions between the functional amino groups of arginine in position 373 and the amino acid network as well as carbohydrate units are still possible although the interactions are reduced compared to the wild-type. This leads to an eliminated polymer formation. We further investigated the role of Tyr^{247}. SacB variants Y247A and Y247I form oligosaccharides consisting of up to nine fructosyl units while variant Y247W forms the whole range of oligosaccharides compared to the wild-type spectrum. These results indicate that an unpolar favoured π-π-stacking mechanism is possible with tyrosine as well as tryptophan in this location but not with alanine or isoleucine. Superposition of Y247W with D257A confirms the key supporting role of Tyr^{247} in the oligo- and polysaccharide-forming “assembly line”.

Structural comparison of SacB from Bacillus megaterium *with other members of clan GH-J points towards a general mechanism of polysaccharide synthesis* A structural comparison of the levansucrases SacB from *B. megaterium* and LsdA from *G. diazotrophicus* (27) enables insights into the functional role of Tyr^{247}. LsdA forms mainly the β-(2,1)-linked trisaccharide 1-kestose whereas the wild-type SacB forms mainly polysaccharides. In LsdA, the conformation of Phe^{304} might block the interaction of an oligofructosyl acceptor chain (Fig. 6). Thus, one structural element on the surface of both levansucrases might influence this difference in oligo- *versus* polysaccharide synthesis. These results may lead to the implication of surface-dependent polysaccharide synthesis modulation on other enzymes of the structural related clan GH-J and beyond. Enzymes of clan GH-J include fungal and plant enzymes which act on sucrose or exhibit an exclusively hydrolysis mechanism like the exo-inulinase from *Aspergillus awamori* (E.C. 3.2.1.80) (28,29) or the invertase from *Thermotoga maritima* (30,31). It remains to be investigated if similar surface structures influencing polysaccharide synthesis exist in these enzymes. One further indication towards hydrolysis *versus* polysaccharide surface-dependent synthesis mechanisms is the narrow and unpolar catalytic **ACKNOWLEDGEMENTS** site architecture of the GH 68-related domain of the exo-inulinase from *A. awamori*, which does The authors acknowledge financial not bind any acceptor oligofructosides (28,29). support by the German Research Foundation Besides other factors such as substrate (DFG) through the Collaborative Research concentration and reaction kinetics, the surface Centre (SFB) 578. We thank Christopher Warner architecture of a polysaccharide-synthesizing for critical reading of the manuscript. D.W.H. glycosyltransferase is clearly the key to its acknowledges support by the Fonds der product spectrum. Chemischen Industrie. We are grateful to the

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FIGURE LEGENDS

Figure 1. Stereopicture of *Bacillus megaterium* SacB variant D257A.
Exposed surface motifs of the levansucrase SacB from *B. megaterium* with distinct impact on the polysaccharide synthesis are indicated.

Figure 2. PEG binding in the active site pocket of SacB N252A.
Amino acid residues in close proximity are shown as sticks. The σ_A-weightet $2F_o$-F_c map (blue mesh) is contoured at 1.0 σ; the F_o-F_c map (green mesh) is contoured at 3.0 showing the PEG electron density.

Figure 3. Superposition of A) SacB variants D257A (yellow) / Y247A (rosé) / Y247W (green) and B) D257A (yellow) / N252A (cyan) / K373A (purple).
The structures of the indicated SacB variants show a maintained active site architecture. The catalytic amino acid residues Asp^{95}/Asp^{257}/Glu^{352} do not change their conformation. Furthermore, also the amino acid residues in subsite +1 (Glu^{350}, Arg^{370}) and the polysaccharide interaction motifs (Asn^{252}, Lys^{373} and Tyr^{247} maintain their conformation.

Figure 4. The surface localization of the polysaccharide interaction motifs of the levansucrase SacB from *Bacillus megaterium* and their oligosaccharide product spectra.
The amino acid interactions with the polysaccharide chain indicated in A) are assigned according to the HPAEC analyses B). Black indicates wild-type SacB, red the indicated variant. The peaks are assigned according to previous carbohydrate standard measurements. A shift in retention time of some peaks may be explained by the formation of short branched oligosaccharides as described (20).

Figure 5. Relative hydrolysis *versus* transfer activities of SacB variants.
The amount of free glucose is divided by the amount of free fructose in ([mol], determined by HPAEC). The difference is considered as transfructosylation products. The wild-type percentage was subtracted. The hydrolysis activity is enhanced except for K315A. Y247W, N312A, K315R and S372A have a hydrolysis *vs*. transfer activity similar to the wild-type SacB. Black bars indicate crystallized variants.

Figure 6. Superposition of the levansucrases SacB from *Bacillus megaterium* and LsdA from *Gluconacetobacter diazotrophicus*.
The differential surface motif Tyr^{247} of the levansucrase SacB from *B. megaterium* and LsdA from *G. diazotrophicus* leads to the synthesis of polysaccharide and oligosaccharides, respectively. The thin-layer chromatography analyses of the two levansucrases show polysaccharide synthesis for SacB (A) and oligosaccharide synthesis for LsdA (B). The structural alignment of the levansucrases SacB and LsdA
(29) shows a conformational difference in the surface motif Tyr^{247} essential for polysaccharide synthesis in SacB (C). In LsdA, the orientation of this motif might block the polysaccharide chain, thus leading to the synthesis of short oligosaccharides of 3-5 carbohydrate units.

Variant

D257A
N252A
K373A
Y247A

Y247W

Beamline

ESRF BM16

Bessy 14.2
Bessy 14.2
Bessy 14.2

Spacegroup *Unit cell dimensions* No. of protein chains in AU
a (?) *b* (?)*c* (?) *?* (?)

Ramachandran plot regions
Favoured (%)
97.3
$P2_12_12_1$ 49.0 55.3 163 6 90.0 1
96.5
$P2_1$ 93.4 100.2 95.2 90.7 4
96.9
$P2_1$ 93.6 100.1 95.5 90.7 4
96.5
$P2_1$ 93.8 100.0 95.5 90.6 4
96.6
Allowed (%)
$P2_1$ 93.5 100.1 95.5 90.5 4

Wavelength (?) Resolution (?)a Unique reflectionsa Multiplicitya Completeness (%)a I/?1a R_{merge} (%)a,b Wilson B-factor (?2) Solvent content (%) *Refinement statistics*

2.5
3.4
0.97881 24.66?1.90 (2.0-1.9) 35,974 (5140) 4.5 (4.6) 99.9 (100) 7.5 (2.9) 21.1 (58.7) 17.6 42.3
3.1
0.91841 47.89-2.10 (2.22-2.0) 117,888 (16,939) 3.6 (3.0) 99.7 (98.6) 9.4 (2.9) 10.6 (43.4) 33.0 42.6
3.4
0.91841 47.77-1.75 (1.84-1.75) 174,958 (25,307) 4.0 (4.0) 98.8 (97.9) 10.50(2.6) 10.7 (52.2) 22.7 42.9
Outlier (%)
0.91841 47.77-2.0 (2.11-2.0) 118,542 (16,927) 4.5 (3.2) 99.6 (97.9) 7.5 (2.4) 22.5 (58.0) 26.4 42.9
2.2
0.91841 47.72-1.90 (2.0-1.9) 135,669 (18,574) 3.4 (2.6) 98.1 (92.4) 8.3 (3.8) 12.6 (42.4) 26.4 42.8
0.1
R_{cryst} c

Table 1. Diffraction data collection and refinement statistics.

16.06 0.1
18.11
20.15
21.1
22.23

[a] Values in parentheses refer to statistics in the highest resolution shell. [b] $R_{merge} = \Sigma|Iobs - \langle I \rangle| / \Sigma Iobs$. [c] $R_{crys} = (\Sigma|Fo - Fc|/ \Sigma Fo) \times 100$, where *Fo* and *Fc* are the observed and calculated structure-factor amplitudes, respectively. [d] R_{free} was computed using 5% of the data assigned randomly. *e* r.m.s.d., root mean square deviation.

20.06
R_{free} d

21.75
20.84
21.33
21.98

Table 2. HPAEC protocol for carbohydrate analysis. Eluent is 0.1 M sodium hydroxide / 1 M sodium acetate in water.

21.57
Total number of

a) Mono- and disaccharides

t [min] % eluent 0-5 0 5-30 0-50 30-35 50 35-40 to 0 40-60 0

b) Oligosaccharides

t [min] % eluent 0-5 0 5-10 0-30 10-40 30 40-45 30-0 45-60 0

Protein atoms
3601
14182
14260
14184
14235

PEG molecules
6
7
1
6

Sulfate molecules
5
7
5
8

Citrate molecules
2

Calcium ions
1
1
1
1
1

Magnesium ions Water molecules
2 657
1156
1457
1277
1094

0.015
0.007
0.006
0.006

0.006
Average *B*-factor
Bond angle (?) Protein (?2) PEG (?2) Solvent (?2)

1.316 7.08 20.79
1.028 24.93 42.36 34.44
1.023 12.33 24.79 21.32
0.897 13.36 20.90 24.67

0.992 16.03 35.1323.87

Table 3. Kinetic parameters of the wild-type (WT) and variants of SacB from *B. megaterium*. Given errors are calculated based on the Michaelis-Menten curve regression of SacB activity at different substrate concentrations as described in the experimental procedures.

Variant K_m k_{cat} k_{cat} K_m^{-1}

[mM] [s^{-1}] [mM^{-1} s^{-1}]

WT* 6.57 ± 1.1 2272 ± 134 254 Y247A 8.98 ± 1.6 1505 ± 57 168 Y247I 11.4 ± 3.0 2030 ± 121 178 Y247W 2.13 ± 1.3 2653 ± 284 1243

1480

N252A* 4.1 ± 1.7 361 N312A 10.8 ± 3.0 776 ± 48 72 K315A 5.28 ± 2.0 2415 ± 175 457 K315R 9.46 ± 3.6 2122 ± 142 224 R370A* 29.2 ± 11.6 179 6 S372A 7.54 ± 2.4 2617 ± 175 347 K373A 3.50 ± 1.4 699 ± 48 200 K373R 11.0 ± 4.2 1708 ± 145 155 Q381A 9.46 ± 1.9 3295 ± 142 348 Y421A* 51.9 ± 16.3 335 7

* data previously published (20)

Table 4. Kinetic data of Clan J sucrose-active enzymes from GH 32 (*Aspergillus awamori*) and GH 68 (all other entries).

Bacillus megaterium (20) pH 6.6, 37° C 6.6 2272 *Bacillus subtilis* (15,17,32) pH 6.0, 30 or 37 °C 14 - 40 0.6 - 165 *Gluconacetobacter* pH 5.0, 30° C 11.9 1

diazotrophicus (27) *Lactobacillus reuteri* (33) pH 5.4, 37° C 9.7 147 *Aspergillus awamori* (28) pH 4.5, 37° C 40 1150

Figure 1.

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73704d534654000000049454320735247420000000000000000000000000000f6d6000100 **22.**
000000d32d48502020000000000000000000000000000000000000000000000000000000 **33.**
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Figure 6

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