Polysaccharide Synthesis of the Levansucrase SacB from Bacillus megaterium Is Controlled by Distinct Surface Motifs*§

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[6] The abbreviations used are: GH, glycoside hydrolase; HPAEC, high performance anion exchange chromatography.

Polysaccharide synthesis of the levansucrase SacB from Bacillus megaterium is controlled by distinct surface motifs. The kinetic and biochemical characterization of Bacillus megaterium levansucrase SacB variants Y247A, Y247W, N252A, D257A, and K373A reveal novel surface motifs remote from the active site. The identified surface motifs are discussed in the context of related glycosyltransferases.

Despite the widespread biological function of carbohydrates, the polysaccharide synthesis mechanisms of glycosyltransferases remain largely unexplored. Bacterial levansucrases (glycoside hydrolase 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 from the sucrose binding site with distinct influence on the polysaccharide product spectrum. The wild type activity (Km) and substrate affinity (Km) are 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 therapeutics, such as antibodies; and glycosylated drugs (4). Their chemical 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 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). Polysaccharides 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-oligosaccharides. According to the data base of carbohydrate-active enzymes (CAZY) (18), bacterial fructansucrases are members of family 68 of the glycoside hydrolases (GHs). 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 (Asn252) eliminates its polysaccharide synthesis (20). Analysis of the crystal structure of the homologous Bacillus subtilis levansucrase in complex with sucrose (Protein Data Bank code 1PT2) and raffinose (Protein Data Bank code 3BYN) provided insights into the functional role of Asn252 (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.

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**Polysaccharide Synthesis Process of the Levansucrase SacB**

**EXPERIMENTAL PROCEDURES**

*Vector Construction, Cloning, Expression, and Purification—* The *Escherichia coli* expression vector pRBEc1, harboring the *sacB* gene, was described previously (20). The plasmid was employed as a template to generate variants of *sacB* by site-directed mutagenesis (QuickChange®, Stratagene). All introduced mutations and the integrity of the residual *sacB* gene were confirmed by DNA sequencing. Expression and purification of each variant were 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 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 the hanging drop vapor diffusion method. Crystals grew under two different crystallization conditions; the first condition contained 0.1 mM sodium phosphate/citrate, pH 4.1, 0.1 mM magnesium sulfate, 30% polyethylene glycol (PEG) 400 (condition A). The second condition was composed of 0.1 mM sodium phosphate/citrate, pH 4.1, 0.2 mM lithium sulfate, 0.1 mM calcium chloride, and 20% PEG 1000 (condition B). Using purified protein at a final concentration of 8.5 mg/ml, small crystals grew at 20 °C over a period of 2 weeks. Crystals of SacB D257A were obtained from condition A, whereas variants Y247A, Y247W, N252A, and K373A showed the 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, 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 ESRF (European Synchrotron Radiation Facility, Grenoble, France). Data for variants K373A, N252A, Y247A, and Y247W were recorded at BESSY (Berlin Elektronenspeicherring-Gesellschaft für Synchrotronstrahlung, Berlin, Germany) either on beamline BL 14.1 or 14.2. Indexing and processing of the intensity data were carried out with the XDS program package (22). Scaling was performed using SCALA from the CCP4 package (23). The crystal structure of SacB D257A in space group P212121 was determined by the molecular replacement method using the program MolRep in the CCP4 suite. Coordinates of homologue SacB from *B. subtilis* (Protein Data Bank code 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 P2₁. The atomic coordinates of variant D257A were used as the starting model to solve the structures of SacB variants in space group P2₁ 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/liter 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 1, top) by high performance anion exchange chromatography (HPAEC) (Dionex; precolumn, CarboPac PA1, 4 × 50 mm; main column, CarboPac PA1, 4 × 250 mm Dionex; conductivity detector PAD-2, at 1 ml/min). The specific glucose formation activity (units/mg) and the substrate concentration (S) were plotted. The resulting *Kₘ* and *Vₘₐₓ* values were determined by regression according to the Michaelis-Menten equation.

**Oligo- and Polysaccharide Analysis—** The wild type and variant 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/liter enzyme concentration by thin layer chromatography (supplemental Fig. 1). 3 μl of the reaction mixture was separated by thin layer chromatography (TLC; silica-coated aluminum plate, isopropyl alcohol (60%), ethyl acetate (30%), water (10%)). TLC staining was performed by *N*-1-naphthyl-ethylenediamine 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 after 1, 6, 24, and 72 h and analyzed by HPAEC under conditions A (Table 1, top, and supplemental Fig. 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 Fig. 3). The ethanol-precipitated oligo- and polysaccharides of the 72 h sample of the wild type and the variants were further analyzed.
TABLE 2
Diffraction data collection and refinement statistics

| Parameter | D257A | N252A | K373A | Y247A | Y247W |
|-----------|-------|-------|-------|-------|-------|
| Data collection | ESRF BM16 | BESSY 14.1 | BESSY 14.2 | BESSY 14.2 | BESSY 14.2 |
| Space group | P2,2,1 | P2,1 | P2,1 | P2,1 | P2,1 |
| Unit cell dimensions | a (Å) 49.0 93.4 | b (Å) 55.3 100.2 | c (Å) 163.6 95.2 | β (degrees) 90.0 90.7 | No. of protein chains in asymmetric unit 1 4 |
| Wavelength (Å) | 0.97881 | 0.91841 | 0.91841 | 0.91841 | 0.91841 |
| Resolution (Å) | 24.66-1.90 47.59-2.0 | 24.66-1.90 47.59-2.0 | 24.66-1.90 47.59-2.0 | 24.66-1.90 47.59-2.0 | 24.66-1.90 47.59-2.0 |
| Unique reflections | 35,974 (5140) 117,898 (16,939) | 117,898 (16,939) 118,542 (16,927) | 118,542 (16,927) 135,669 (18,574) |
| Multiplicity | 4.5 (4.6) | 3.6 (3.0) | 4.0 (4.0) | 4.5 (3.2) | 3.4 (2.6) |
| Completeness (%) | 99.9 (100) | 99.7 (98.6) | 98.8 (97.9) | 99.6 (97.9) | 98.1 (92.4) |
| I/σ(I) | 7.5 (2.9) | 9.4 (2.9) | 10.50 (2.6) | 7.5 (2.4) | 8.3 (3.8) |
| Rmerge (%)* | 21.5 (7.8) | 10.0 (4.34) | 107.5 (52.2) | 22.5 (58.0) | 12.6 (42.4) |
| Wilson B-factor (Å²) | 17.6 | 33.0 | 22.7 | 26.4 | 26.4 |
| Solvent content (%) | 42.3 | 42.6 | 42.9 | 42.9 | 42.8 |

Refinement statistics

| Parameter | D257A | N252A | K373A | Y247A | Y247W |
|-----------|-------|-------|-------|-------|-------|
| Rmerge | 16.06 | 18.11 | 20.15 | 22.23 | 20.06 |
| Rfree | 21.75 | 20.84 | 21.33 | 22.98 | 21.57 |
| Rwork | 16.10 | 18.21 | 20.20 | 22.33 | 21.00 |
| Total number of protein atoms | 3601 | 14182 | 14260 | 14184 | 14235 |
| Total number of sulfate molecules | 5 | 7 | 5 | 8 |
| Citrate molecules | 2 | 1 | 1 | 1 |
| Calcium ions | 1 | 1 | 1 | 1 |
| Magnesium ions | 2 | 1 | 1 | 1 |
| Water molecules | 657 | 1156 | 1457 | 1277 | 1094 |
| Bond angle (degrees) | 0.015 | 0.007 | 0.006 | 0.006 | 0.006 |
| Bond length (Å) | 1.316 | 1.028 | 1.023 | 0.897 | 0.992 |
| Average B-factor | 7.08 | 24.93 | 12.33 | 13.36 | 16.03 |
| PEG (Å²) | 79.2 | 24.79 | 24.79 | 20.90 | 35.13 |
| Solvent (Å²) | 42.36 | 24.79 | 20.90 | 35.13 | 22.5 |
| Water molecules | 657 | 1156 | 1457 | 1277 | 1094 |

Values in parentheses refer to statistics in the highest resolution shell.

a Rmerge = Σ(Fobs) − Σ|Fcalc|/ΣFobs, where Fobs and Fcalc are the observed and calculated structure factor amplitudes, respectively.

b Rfree was computed using 5% of the data assigned randomly.

c r.m.s.d., root mean square deviation.

d Values in parentheses refer to statistics in the highest resolution shell.

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/liter 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 1, top).

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 Rmerge (Table 2). Furthermore, the analysis of data sets Y247A, Y247W, and N252A revealed slightly pseudomerohe-
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 Trp172, Leu118, Trp94, and Pro414. (Fig. 2). Except for proline, these amino acid residues were already part of a mutagenesis studied previously published (20). Their knock-out leads to inactivation of SacB.

**N252A Eliminates the Polysaccharide Synthesis after Tetrasaccharides**—In previous investigations, the crucial role of Asn252 in fructosyl transfer versus hydrolysis reactions was identified (20). SacB variant N252A does not form any polysaccharide but instead forms short-chain oligosaccharides of up to three fructosyl units (nystose) (20). In SacB from *B. megaterium*, Asn252 is not located in the sucrose binding site analogous to Asn242 of the levansucrase from *B. subtilis* co-crystallized with raffinose (Protein Data Bank code 3BYN (21)). The crucial question arises of whether the residue Asn252 is involved in interactions with the fructan chain or if alternative effects, perhaps toward 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 with D257A and K373A (Fig. 3). None of the catalytic amino acid residues changes its confor-
mation. Thus, conformational effects of N252A on the fructosyl transfer mechanism are excluded.

**K373A Eliminates the Polysaccharide Synthesis after Hexasaccharides**—Lys373 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, whereas 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/stacking mechanism in this location. The characterization of the oligo- and polysaccharide products point toward the role of Tyr247 and Lys373 in the transfructosylation mechanism. Tyr247 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), whereas the hydrolysis products of Y247W are only slightly increased compared with 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 Asn252, Tyr247, and Lys373 were chosen for mutagenesis studies. On the basis of structural alignments with the levansucrases SacB from B. subtilis and the related LsdA from Gluconacetobacter diazotrophicus (27), six additional amino acid residues with a 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 with the wild type. The \(K_m\) of the variants ranges between 2.1 (Y247W) and 11.4 mM (Y247I), which is less than a
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 with the wild type except for K315A (Fig. 5). According to its strongly increased transfructosylation activity of 22% (mol/mol), K315A forms significantly more oligosaccharides. The $K_m$ and $k_{cat}$ of the variants are similar to those of the wild type 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 B. megaterium*—Despite rigorous studies during the recent years with numerous solved structures of enzymes acting on sucrose, their polysaccharide synthesis mechanism remains elusive (13). Even successful co-crystallizations 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 an impact on $\beta$-(2,6)-linked levan or $\beta$-(2,1)-linked inulin formation were located in the sucrose binding site. They interact with sucrose, either catalyzing the cleavage of the glycosidic bond (Asp95, Asp257, and Glu352), generating the enzyme-fructosyl complex (Asp95), or stabilizing sucrose in the active site (Trp94, Trp173, Arg256, Glu264, and Arg370) (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 Asn252 have an impact on the active site conformation? 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 with

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**TABLE 3**

Kinetic parameters of the WT and variants of SacB from *B. megaterium*

| Variant | $K_m$ (µM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (µM$^{-1}$ 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                            |
| N252A   | 4.1 ± 1.7  | 1480                | 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).

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**TABLE 4**

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

| Organism       | Reaction conditions | $K_m$ (µM) | $k_{cat}$ (s$^{-1}$) |
|----------------|---------------------|------------|---------------------|
| *B. megaterium*| pH 6.6, 37 °C       | 6.6        | 2272                |
| *B. subtilis*  | pH 6.0, 30 or 37 °C | 14–40      | 0.6–165             |
| *G. diazotrophicus* | pH 5.0, 30 °C | 11.9       | 1                   |
| *Lactobacillus reuteri* | pH 5.4, 37 °C | 9.7        | 147                 |
| *A. awamori*   | pH 4.5, 37 °C       | 40         | 1150                |

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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 with the wild type SacB, except for two variants, K373A and N312A. These have a $k_{cat}$ 3-fold lower than the wild type. However, this observed activity is still in the high range compared with fructosyltransferases from other organisms (Table 4). The other variants do not differ more than 40% from the wild type SacB 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 with the amount of free fructose. The difference must 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

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**FIGURE 5.** Relative hydrolysis versus transfer activities of SacB variants. The amount of glucose released, indicating hydrolysis, is compared with 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 versus transfer activity similar to the wild type SacB. Black bars, crystallized variants.
wild type SacB (Table 3). Moreover, the structural data point toward a possible surface arrangement for the binding of an acceptor fructosyl chain. Residues Asn252, Lys373, and Tyr247 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 Asn252 is located close to the sucrose binding site, whereas Lys373 and Tyr247 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, leading to longer oligofructosyl 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 with the wild type. This leads to the synthesis of short oligosaccharides of 3–5 carbohydrate units.

These results may lead to the implication of surface-dependent polysaccharide synthesis modulation on other enzymes of the structurally related clan GH-J and beyond. Enzymes of clan GH-J include fungal and plant enzymes that act on sucrose or exhibit an exclusively hydrolysis mechanism like the exo-inulinase from Aspergillus awamori (EC 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 suggesting hydrolysis versus polysaccharide-synthesizing glycosyltransferase is the narrow and unpolar catalytic site architecture of the GH 68-related domain of the exo-inulinase from A. awamori, which does not bind any acceptor oligofructosides (28, 29). Besides other factors, such as substrate concentration and reaction kinetics, the surface architecture of a polysaccharide-synthesizing glycosyltransferase is clearly the key to its product spectrum.

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Polysaccharide Synthesis Process of the Levansucrase SacB

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