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Selected mutations in *Bacillus subtilis* levansucrase semi-conserved regions affecting its biochemical properties

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Levansucrases (LS) are fructosyltransferases (FTFs) belonging to family 68 of glycoside hydrolases (GH68) using sucrose as substrate to synthesize levan, a fructose polymer. From a multiple sequence analysis of GH68 family proteins, nine residues were selected and their role in acceptor and product specificity, as well as in biochemical *Bacillus subtilis* LS properties, was investigated. A product specificity modification was obtained with mutants Y429N and R433A that no longer produce levan but exclusively oligosaccharides. An effect of the mutation S164A was observed on enzyme stability and kinetic behavior; this mutation also induces a levan activation effect that enhances the reaction rate. We report the crystallographic structure of this mutant and found that S164 is an important residue to maintain the nucleophile position in the active site. We also found evidence of the important role of Y429 in acceptor specificity: this is a key residue coordinating the sucrose position in the catalytic domain-binding pocket. Some of these mutations resulted in LS with a broad range of specificities and new biochemical properties.

Keywords: *Bacillus subtilis*/fructosyltransferases/levan/levansucrase/site-directed mutagenesis

Introduction

Fructosyltransferases (FTFs) are enzymes synthesizing fructose polymers by direct transfer of the sucrose fructosyl moiety to a growing polymer chain (transfructosylation reaction); however, the transfer can also be made either to water (hydrolysis reaction) or toward an acceptor molecule different from sucrose or fructan, added to the reaction medium (acceptor reaction) (Chambert *et al*., 1974). FTFs are classified as inulosucrases (IS, EC 2.4.1.9), which synthesize β2-1 linked polymers (inulin), and levansucrases (LS, EC 2.4.1.10), which produce polymers with β2-6 linkages (levan) in the linear chain. Depending on the enzyme source, inulin and levan may be branched by β2-6 and β2-1 linkages, respectively. Both enzymes may also find applications in the synthesis of fructooligosaccharides or fructosides, depending on their transfer specificity (Ozimek *et al*., 2006a; Seibel *et al*., 2006).

The crystallographic structures of *Bacillus subtilis* and *Gluconacetobacter diazotrophicus* SRT4 LSs have been determined at 1.50 and 2.50 Å resolution, respectively (Meng and Futterer, 2003; Martinez-Fleites *et al*., 2005). The structures show a similar single-domain folded as a five-bladed β-propeller enclosing a funnel-like central cavity. LSs follow the pattern of five β-sheets, adopting the classical 'W' topology of four antiparallel β-strands. The molecular surface of *B. subtilis* LS has a negatively charged central pocket where sucrose binds. It has been reported that D86 and E342 are the catalytic residues (the nucleophile and the general acid/base residues, respectively), whereas D247, although required for catalysis, is not directly involved in the reaction mechanism (Meng and Futterer, 2003). Additionally, amino acids in contact with sucrose and constituting the subsites −1 and +1 have been identified. The subsite −1 is composed of W85, D86, W163, R246 and D247, whereas the subsite +1 is formed by R246, E340, E342 and R360 (Ozimek *et al*., 2006b). Recently, Homann *et al*. (2007) have reported that N242 is part of the subsite +2.

The role of amino acids in the catalytic domain on the reaction mechanism as well as on FTFs product specificity has also been investigated. Chambert and Petit-Glatron (1991) demonstrated that the mutation R360/K/S in *B. subtilis* LS drastically reduces polymerization activity. Later on, Batista *et al*. (1999) investigated the role of residue D309 in *Acetobacter diazotrophicus* LS, localized in the RDP motif, reporting that D309N did not alter the product profile, but increased the amount of hydrolyzed sucrose.

In spite of having the same active site architecture, it has been reported that *B. subtilis* LS synthesizes mainly high molecular weight fructans with a minor amount of oligosaccharides, whereas the homologous enzyme from *G. diazotrophicus* synthesizes only oligosaccharides. It has been found that region +2 could not be superimposed in these enzymes, suggesting that this structural feature could be responsible for turning the polymer synthesizing LS from *Bacillus megaterium* into an oligosaccharide-producing enzyme (Homann *et al*., 2007). It has also been reported that changes in the subsite −1 strongly influence the molecular weight of products synthesized by *L. reuteri* 121 IS (Ozimek *et al*., 2006b). A model to explain the polymerization mechanism in *L. reuteri* polymer and oligosaccharides producing FTFs has been recently proposed, suggesting a processive transglycosylation reaction for polymer synthesis and a non-processive mechanism for oligosaccharides production, which may be controlled by specific amino acids in the catalytic domain (Ozimek *et al*., 2006a).

Despite the information available concerning the structure of LSs and the nature of the residues involved in substrate binding, structure–function studies determining reaction specificity (hydrolysis/transfructosylation, H/T), fructan molecular weight and linkage specificity, as well as acceptor

properties are still lacking. This information is essential not only to understand the basis of FTFs activity but also for a rational design of biocatalysts based on levansucrase specificities. Therefore, the principal aim of this work is to identify residues implicated in acceptor and product specificity, as well as in biochemical LS properties.

Materials and methods

Site-directed mutagenesis

The crystal structures of *B. subtilis* LS, including the sucrose-bound structure [Protein Data Bank (PDB) accession codes 1OYG and 1PT2, respectively], were used as templates in order to evaluate the spatial distribution of the selected amino acids (Meng and Futterer, 2003). The identification of conserved regions of FTF family members was carried out by structural alignment analysis of a set of sequences retrieved from the PDB (Berman *et al*., 2000a, 2000b) using *B. subtilis* LS sequences as probe in a Psi-BLAST search (Zhang *et al*., 1998). Final alignments were produced by clustal W (Chenna *et al*., 2003) and edited in JalView program V 2.3 (Clamp *et al*., 2004). The selected mutations were introduced in *B. subtilis* LS gene with the QuikChange site-directed mutagenesis system (Stratagene) according to the manufacturer's recommendations and were cloned into pET 22b+ vector under control of the promoter T7 (NOVAGEN). In order to obtain LS mutants with new biochemical properties, a site-directed mutagenesis strategy was designed based on the variability of amino acids found in semi-conserved regions of FTFs from family 68 of GH, as described in the Results section. The oligonucleotide sequences used for the mutagenesis reactions are summarized in Table I. Automatic DNA sequencing (3130XL-Applied Biosystems) was used to verify all constructions.

Escherichia coli BL21 [*E. coli* F^- ompT $hsdS_B$ ($r_B - m_B$) *gal dcm* (DE3)] transformants were grown in Luria-Bertani broth containing 10 μg/ml of ampicillin at 37°C, induced with 0.2 mM IPTG (Research Organics) after reaching an optical density of 0.5 and incubated at 22°C for another 4 h.

Table I. Oligonucleotides used for site-directed mutagenesis

Oligonucleotide	DNA sequence (5′ to 3′)
S164Afwd	aaacacaagaatgg gca ggttcagccacattta
S164Arvs	taaatgtggctgaacc tgc ccattcttgtgttt
S164Kfwd	aaacacaagaatgg aaa ggttcagccacattta
S164Krvs	taaatgtggctgaacc ttt ccattcttgtgttt
H243Wfwd	acagctcaggcgcacaac tgg acgctgagagatcct
H243Wrvs	aggatctctcagcgt cca gttgtcgcctgagctgt
I341Vfwd	cacagtaacagatgaa gtg gaacgcgcgaacgtc
I341Vrvs	gacgttcgcgcgttc cac ttcatctgttactgtg
A344Pfwd	agatgaaattgaacgc ccg aacgtctttaaaatg
A344Prvs	cattttaaagacgtt cgg gcgttcaatttcatct
R360Kfwd	cctgttcactgactcc aaa ggatcaaaaatgac
R360Krvs	gtcatttttgatcc ttt ggagtcagtgaacagg
R360Sfwd	cctgttcactgactcc tct ggatcaaaaatgac
R360Srvs	gtcatttttgatcc aga ggagtcagtgaacagg
G361Ffwd	gttcactgactcccgc ttc tcaaaaatgacgatt
G361Frvs	aatcgtcatttttga gaa gcgggagtcagtgaac
F414Wfwd	aacctttacttactcacac tgg gctgtacctcaagcg
F414Wrvs	cgcttgaggtacagc cca gtgtgagtaagtaaaggtt
Y429Nfwd	aatgtcgtgattacaagc aac atgacaaacagagga
Y429Nrvs	tcctctgtttgtcat gtt gcttgtaatcacgacatt
R433Afwd	agctatatgacaaac gcg ggattctacgcagac
R433Arvs	gtctgcgtagaatcc cgc gtttgtcatatagct

Cells were recovered by centrifugation (3214 g, 15 min) and the pellet resuspended in 8 ml of pH 6.0, 0.1 M potassium phosphate buffer and broken in a French pressure cell press (Thermo Spectronic). Wild type (WT) LS and mutants were purified by ion exchange chromatography as previously described (Meng and Futterer, 2003).

Levansucrase biochemical properties

All assays were conducted using purified WT and LS mutants.

Initial reaction rates were measured by following the reducing power released from 120 g/l of sucrose in pH 6.0, 50 mM phosphate buffer at 37°C, unless otherwise specified, using the 3,5-dinitro-salicylic acid method. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of glucose equivalents per min. This corresponds to a global LS activity, as it includes released reducing sugars both by transfructosylation and hydrolysis activities. In all cases, assays were carried out adjusting protein concentration to an enzymatic activity of 0.5 U/ml.

Initial reaction rates were measured in a pH range between 4 and 8 in order to define the optimum pH for activity.

WT and LS mutants solutions (1 mg protein/ml) were incubated at 40°C and assayed for residual activity. The stability was reported either as the enzyme half-life or as the first-order deactivation rate constant at 40°C.

Global initial reaction rates and kinetic constants were measured in a sucrose concentration range of 0–1.2 M and also in the presence of levan (0–15 g/l). Kinetic constants were determined by linear regression analysis using the Lineweaver/Burk type plot.

Determination of H/T ratios

H/T ratios were determined by measuring the amount of glucose and fructose released from 120 g/l of sucrose in pH 6.0, 50 mM phosphate buffer. Fructose is the result of the hydrolytic activity, whereas the difference between glucose and fructose concentration is the result of the transfructosylation activity. H/T reaction ratio in the presence of acceptors was determined following the same procedure. Sugars involved in these experiments such as xylose, fructose, glucose, raffinose and sucrose were quantified by HPLC in a Waters 600E system controller (Waters Corp., Milford, MA, USA) equipped with a refractive index detector (Waters 410), and a Carbohydrate column (4.6×250 mm) kept at 35°C, using acetonitrile/water (75:25) as the mobile phase at 1.2 ml/min.

Levan molecular weight and oligosaccharide profile analysis

The effect of mutations on levan molecular weight and oligosaccharide synthesis was analyzed by HPLC in a Waters 600E HPLC system controller (Waters Corp.) equipped with a refractive index detector (Waters 410). Oligosaccharides were analyzed using a reversed-phase C-18 column at 30°C with water as the mobile phase at 0.7 ml/min, whereas levan molecular weight was analyzed by gel permeation chromatography, using a serial set of Ultrahydrogel (UG 500 and linear) columns at 35°C with water as the mobile phase at 0.8 ml/min.

Crystallization, data collection, structural determination and refinement

Crystals from S164A mutant were obtained after 12 days of micro-dialysis of the purified protein (8 g/l) against deionized water. The mutant S164A data set was collected using a

Rigaku RU200H rotating-anode copper Kα X-ray source fitted with a Yale optic and a Raxis-IV imaging-plate detector at 100 K using an Oxford Cryosystems 600 series cryostream cooler. Diffraction images were indexed and integrated using MOSFLM (Leslie, 1992) and data were scaled and truncated with SCALA and TRUNCATE respectively from the CCP4 suite (Collaborative Computational Project, 1994). The cryoprotectant solution used was an aqueous solution of glycerol 50% (v/v). The S164A mutant structure was determined by molecular replacement using MolRep (Vagin and Teplyakov, 1997) with PDB entry 1OYG (Meng and Futterer, 2003) as a search model. This was required due to the differences in the unit cell parameters between the native ($a = 51.1$ Å, $b = 67.0$ Å, $c = 123.7$ Å) and the S164A mutant ($a = 50.9$ Å, $b = 55.8$ Å, $c = 124.6$ Å) crystals. However, it was found that both crystals share the space group ($P2_12_12_1$) and the unit cell content (one monomer into the asymmetric unit).

The structure was refined using the program CNS (Brünger *et al*., 1998) following the protocol: rigid body minimization, restrained minimization, individual B-value refinement, $2F_o-F_c$ and F_o-F_c electron density map calculation and visual model fitting using the program Coot (Emsley and Cowtan, 2004). The refinement was concluded with REFMAC (Murshudov *et al*., 1997), but the same 5% R_{free} flagged reflections were used as for CNS (Supplementary Table I available at *PEDS* online).

The coordinates and the structure factors for the structure of LS S164A mutant have been deposited with the RCSB Protein Data Bank with accession code 2VDT.

Results

Design and selection of target residues

Basic site-directed mutagenesis design criteria were applied in an attempt to modify functionally important residues, which were likely involved in substrate and product binding sites and therefore in *B. subtilis* LS properties. Sequence and structural analyses of 40 family GH68 members performing transfructosylation, hydrolysis and acceptor reactions, were carried out. Six semiconserved regions were identified around the negatively charged central pocket of the LS catalytic domain, mainly located in β-strands and loops of the five-bladed β-propeller (Supplementary Figure 1 available at *PEDS* online). Amino acids that, due to their proximity to the active site (~15 Å from the sucrose glycosidic bond), were likely to be involved in substrate binding and have their side chains either directed toward sucrose or the catalytic surface were selected. In order to evaluate the role of these residues, the following mutants were constructed: S164K/A, H243L, R360K/S, I341V, A344P, G361F, F414W, Y429N and R433A. The substitutions of these residues were chosen following the variation already explored by nature in those positions, except for H243 and R433.

LS biochemical properties and kinetic parameters

WT LS and its mutants were expressed in *E. coli*, obtaining ~30 mg of purified enzyme per liter of culture medium. Activity, optimum pH and stability at 40°C of the constructed mutants were determined (Table II). It was found that S164K was inactive, whereas S164A, R360S and Y429N displayed a drastic decrease in activity. It was also found that mutants H243L and G361F were considerably less stable than the WT LS. The rest of the mutants had a reduced specific activity and stability, but not far from that measured for the WT LS, except for S164A, which resulted in a 12-fold higher $t_{\frac{1}{2}}$ at 40°C than WT LS, with a half-life of 628.0 (±51.0) min.

All mutants retained the optimal WT pH activity, except for Y429N and R433A, which showed an optimum at pH 5–6 and 6–7, respectively (Supplementary Figure 2 available at *PEDS* online).

WT LS and its mutant enzymes displayed Michaelis–Menten type kinetic behavior when global reaction rates were correlated with initial sucrose concentration (Table II). S164A was the only mutant showing a slightly higher affinity for sucrose, whereas the affinities of R360K, R360S, G361F, Y429N and R433A for sucrose were all reduced.

Effects of the LS mutations on H/T ratio and acceptor reactions

A shift of reaction specificity (H/T) was observed with H243L, R360K, R360S, G361F, Y429N and R433A mutants, which became more hydrolytic, reducing the percentage of sucrose used in polymer and oligosaccharide synthesis, whereas S164A resulted 15% less hydrolytic than WT LS (Fig. 1A).

In order to analyze the effect of each mutation on LS acceptor specificity, xylose and maltose were selected as model acceptor molecules and assayed in the presence of sucrose. It was found that the acceptor addition increased transfructosylation reactions, as a property of WT LS. In all cases, xylose is a better acceptor of the fructosyl residue than maltose, sucrose or levan, as deduced from results reported in Fig. 1B. On the other hand, only S164A, I341V, A344P and F414W had higher transfructosylation activity toward maltose than the WT LS (Fig. 1C).

Polymer molecular weight

WT LS synthesizes polymer with a bimodal distribution of ~3500 and 8.3 kDa average molecular weight (Fig. 2A). It was found that I341V, A344P and F414W mutants displayed the same product profile as WT LS (data not shown). Different specificities were found for some of the mutants:

Table II. Kinetic parameters, thermal stability and optimum pH of *B. subtilis* WT LS and its mutants

	Km (mM sucrose)	kcat (s^{-1})	kcat/Km (s^{-1} mM^{-1})	$t_{1/2}$ (min)	Optimum pH
WT LS	8	164.6	20.3	52.0 (±4.79)	6
S164A	2.5	6.4	2.5	628.0 (±51.0)	6
H243L	10.5	141.5	13.5	7.9 (±0.04)	6
I341V	7.4	166.3	22.6	31.4 (±0.54)	6
R360K	30	170.2	5.6	42.11 (±0.86)	6
R360S	154	13.5	0.09	17.21 (±1.16)	6
G361F	297.3	57.4	0.19	<1	6
Y429N	319.4	6.3	0.02	47.04 (±2.61)	5–6
R433A	29.3	87.5	2.98	16.0 (±1.61)	6–7

Activity assay reactions were carried out with 0.5 U/ml of enzyme activity at 37°C and pH 6.0. Stability was determined following the lost of activity after incubation of 1 mg/ml of protein at 40°C. Mutants A344P and F414W have the same behavior than WT LS and are therefore no longer included.

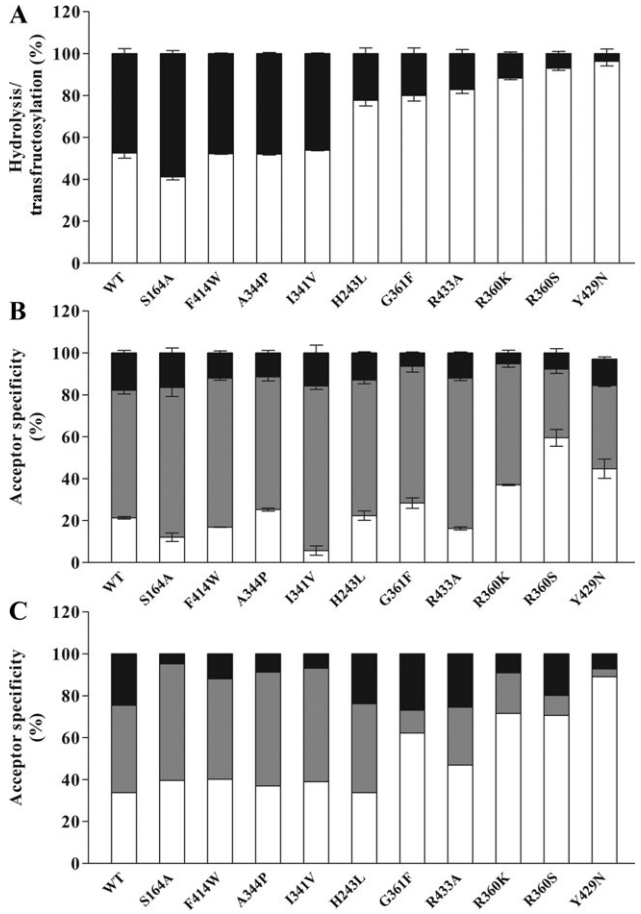

Fig. 1. Hydrolysis, transfructosylation and acceptor specificity of *B. subtilis* WT LS and its mutants. Reactions were carried out with: (**A**) sucrose; (**B**) sucrose/xylose and (**C**) sucrose/maltose, at a concentration of 120 g/l. The transfer of the fructosyl residue toward sucrose or levan (black), xylose or maltose (grey) and water (white) was analyzed after 80% sucrose conversion. All reactions contained 0.5 U/ml of enzymatic activity.

unlike WT LS, H243L and S164A synthesize either high or low levan molecular weight, respectively, whereas R360S, Y429N and R433A synthesize only oligosaccharides (Fig. 2B). Finally, although G361F and R360K were observed to synthesize mainly oligosaccharides, they still catalyze the synthesis of low amounts of polymer.

LS activation by levan

Although WT LS hydrolyzes sucrose early in the reaction, transfructosylation gradually increases reaching a maximum when sucrose conversion is ~90% (Fig. 3A). On the other hand, reaction rate decreases both as a consequence of lower sucrose concentrations and product inhibition. This common behavior was not observed with S164A, which maintained the initial sucrose consumption rate nearly constant throughout the reaction, reaching the final conversion faster than the WT LS. As shown in Fig. 3B, S164A seems to be activated after a few minutes of reaction. Levan activation in *B. subtilis* LS has already been reported at polymer concentrations lower than 5 mM, using a purified monomodal levan distribution (Chambert *et al.*, 1974). Additionally, it has been shown that *B. subtilis* FTF activity was dependent on levan at concentrations up to 5 g/l (Tanaka *et al.*, 1981). The effect of levan on WT LS, S164A and Y429N is shown in Fig. 4, where it may be

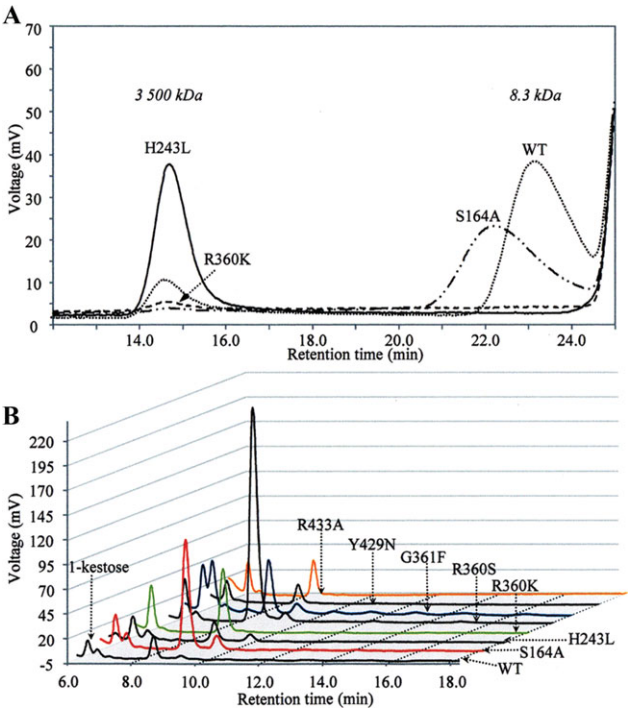

Fig. 2. Polymer and oligosaccharide profile obtained from sucrose by *B. subtilis* WT LS and its mutants. Levan molecular weight (**A**) was analyzed by HPLC using a serial set of Ultrahydrogel (UG 500 and Linear) columns, whereas oligosaccharides (**B**) were analyzed using a C-18 column. The same reaction media was analyzed for both type of products after 80% of sucrose conversion. Residual sugars and oligosaccharides are not shown in A.

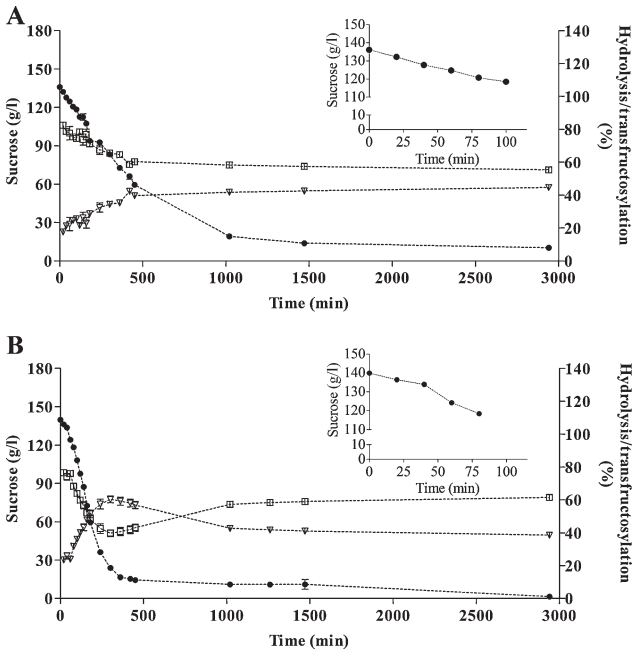

Fig. 3. Sucrose conversion kinetics of (**A**) WT LS and (**B**) S164A variant. Sucrose conversion (filled circle), hydrolysis (open square) and transfructosylation (open inverted triangle) were measured in reactions carried out with 0.5 U/ml of enzymatic activity. An inset is included in each figure to observe the data involved in the determination of initial reaction rates.

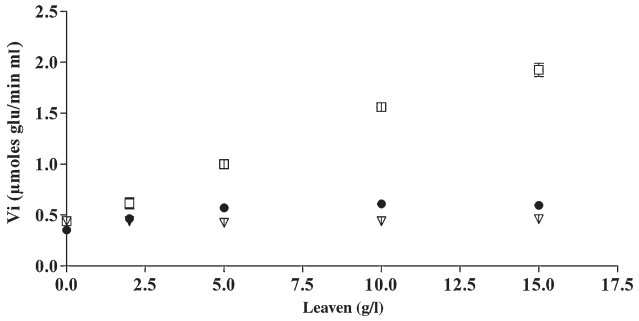

Fig. 4. LS activation by levan. Effect of levan concentration on the initial reaction rates for WT LS (filled circle); S164A (open square) and Y429 N (open inverted triangle) at 120 g/l of sucrose. Y429N was used as a negative control, as this mutant is unable to synthesize and elongate levan. All reactions contained 0.5 U/ml of enzymatic activity.

observed that up to 15 g/l, the activity of S164A is dependent on polymer concentration. Levan has no effect on Y429N, a mutant that has lost the fructan-synthesizing activity.

The kinetic behavior in the presence of levan is not described by any of the known activation models (essential and non-essential activation), since the activation becomes important only at high sucrose concentrations. Nevertheless, it is clear that levan exerts a greater influence on S164A than on the WT LS. A 3.7 and 1.4-fold increase in Vmax of S164A and WT LS, respectively, in the presence of 15 g/l of levan was observed.

Crystallographic structure of S164A

Mutant S164A was successfully crystallized and information extracted from the structure at 3.2 Å resolution in order to explain its acquired kinetics properties. It is important to emphasize that in spite of the resolution of the crystallographic data, the described structural modifications are supported by clear electron densities (Supplementary Figure 3 available at *PEDS* online). A comparison between S164A and both WT LS crystallographic structures (Meng and Futterer, 2003) is shown in Fig. 5.

Discussion

Effect of the exchanged amino acids on LS thermal stability

It has been proposed that buried residues are important determinants of protein stability, whereas surface residues are involved in protein function, suggesting the following destabilization gradient of substitutions: Asp>Glu>Lys>Arg>Ala (Bajaj *et al*., 2005). This is probably the case of substituting the hydrophilic serine, a buried residue, by the non-polar alanine in the variant S164A, which was found to be 12-fold more stable than WT LS.

Some of the consequences observed in the behavior of the constructed mutants can be readily deduced from the crystallographic structure of the WT LS (Meng and Futterer, 2003). The reduction in half-life when H243 is replaced by leucine could be explained by the elimination of polar interactions involving K189, D219, Q226 and V228 through two water molecules (Fig. 6A). We also found that G361 is an important position for stability, as a drastic decrease in half-life results from G361F, which could be explained by the destabilization of the β-hairpin formed between R360 and I374. Thermal stability was not considerably modified in the rest of the mutants.

Structural insights into the kinetic parameters of S164A mutant

The effect of the mutated amino acids on the kinetic parameters of the variants was also analyzed considering the sucrose-bound crystallographic structure of *B. subtilis* LS (Meng and Futterer, 2003). S164 is localized as part of the βIIB, establishing contact mainly with D86 (2.69 Å). As the nucleophile, residue D86 has an essential role in catalysis and any modification around this position is expected to have a critical effect on LS activity. This is the case of S164K, which has no detectable activity, whereas for S164A, sucrose affinity was raised in spite of a 26-fold decrease in kcat. This is the only mutant in which a higher affinity for sucrose was observed. Homann *et al*. (2007) have recently reported similar results for the mutant S173A in *B. megaterium* LS (S164A in this work), confirming the critical role of this position. However, the authors did not report the consequences of this mutation on enzyme stability, product profile and levan activation.

In the LS native structure, S164 makes a hydrogen bond (2.7 Å) with the nucleophile D86. A shift in D86, W85 and R360 position was observed when comparing S164A with the WT LS structures previously reported (Meng and Futterer, 2003). As shown in Fig. 5, alanine is unable to maintain the contact with the nucleophile D86, which is therefore, shifted 1.20 Å in the opposite direction. Although the resolution of S164A crystallographic data is limited (3.2 Å resolution), the electron density countered at 1σ level, the estimated coordinated error based on sigma A statistics (0.44 Å) (Adams *et al*., 1997) and the comparison with the

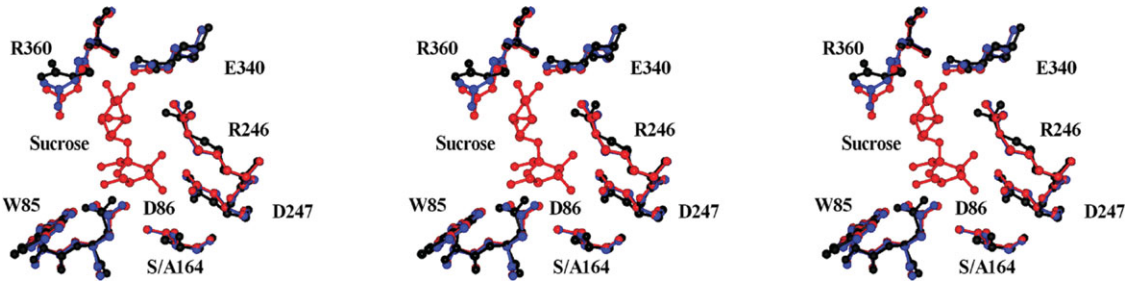

Fig. 5. Crystallographic structure of S164A LS. Stereo-view of the LS residues interacting with sucrose. The LSs are colored as follows: S164A mutant (black); WT LS (PDB 1OYG) crystal structure (blue) and sucrose-bound WT LS (PDB 1PT2) (red). The figure was prepared with CCP4 mg (Potterton *et al*., 2002).

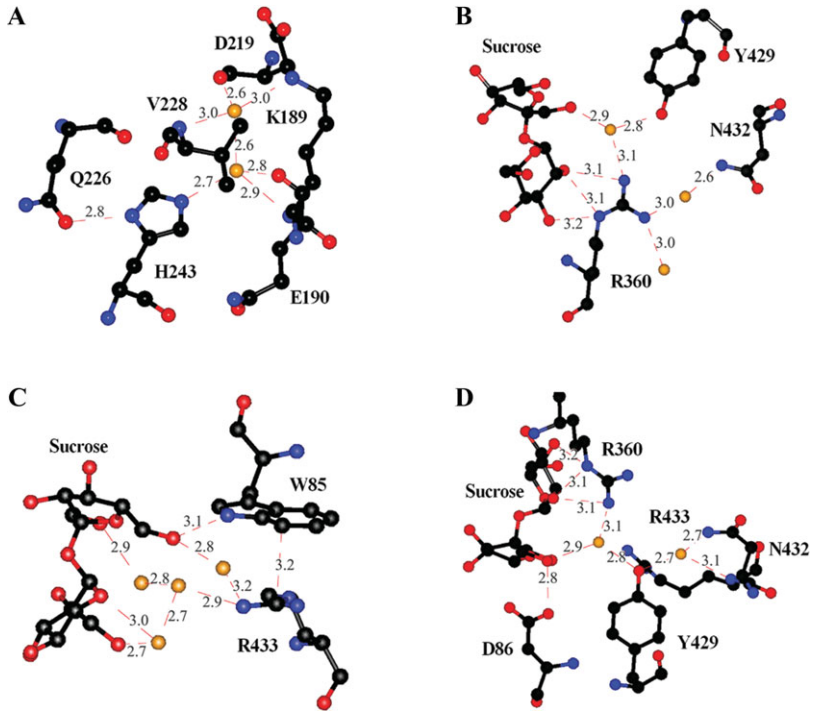

Fig. 6. Interatomic distances between selected amino acids for site-directed mutagenesis and critical residues in *B. subtilis* WT LS. Interactions involving (**A**) H243, (**B**) R360, (**C**) R433 and (**D**) Y429 with sucrose, water molecules (yellow) and/or other near residues may explain the changes observed in the kinetic parameters when these amino acids are substituted in the mutants H243L, R360K/S, R433A and Y429N, respectively. Hydrogen bonds are shown as red dashed lines and distances are given in Å.

LS sucrose-bound structure (PDB entry 1PT2, 2.1 Å resolution) support the existence of a different conformer at residue D86 in mutant S164A. It is likely that the shift observed in D86 position contributes to the low activity shown by this variant. S164A displayed the same WT LS pH profile, suggesting that no important changes had taken place on the catalytic site ionization state (Supplementary figure 2 available at *PEDS* online).

As there is either a direct or an indirect interaction of the mutated residues with sucrose (Fig. 6), the elimination of such contacts in mutants R360/K/S, R433A and Y429N may be the origin of their poor catalytic behavior. On the other hand, the reduction in the catalytic efficiency of G361F could be explained by the destabilization of the β-hairpin found between R360 and I374. However, we have no structural bases for a rational explanation of the observed changes in the kinetic properties.

Influence of the mutations on LS properties

Reaction specificity (H/T), molecular weight and acceptor specificity of LS were analyzed (Figs 1 and 2). H243L, R360/K/S, G361F, R433A and Y429N became more hydrolytic than WT LS. It was found that R360 interacts with a water molecule and glucose 2-OH and 3-OH (Fig. 6B). Owing to their localization, R360/K/S and G361F mutations may explain the decrease in the acceptor affinity (sucrose and/or levan) at subsite +1, whereas H243L (Fig. 6A) could decrease the affinity in subsite +2, as N242 has been reported to be involved in this subsite (Homann *et al*., 2007). As shown in Fig. 6C, it is possible that the high hydrolytic activity displayed by mutant R433A could be explained by the substitution of a bulk residue (R433) by alanine, which may increase water availability. The highest hydrolytic activity observed among these mutants was displayed by Y429N, reducing its transfructosylation activity to only 4% of the converted sucrose. This residue coordinates a water network in the active site indirectly involving subsites +1 and −1 (Fig. 6D). S164A was the only mutant reducing the hydrolytic activity of the WT LS, probably through an increase in the hydrophobicity of the nucleophile environment.

Differences on molecular weight were observed among fructans produced by some of the LS mutants. Ozimek *et al*. (2006a) proposed that polymerization is a processive reaction requiring a high enzyme affinity for the growing polymer chain at subsites +2, +3 and further. Product specificity of R360S, Y429N and R433A may result from reduction of polymer affinity for the subsites, as these mutants exclusively synthesize oligosaccharides. On the other hand, H243L synthesized high molecular weight levan exclusively, whereas S164A produced a low molecular weight polymer.

The modification in substrate and product binding sites already discussed could also explain the changes observed in acceptor specificity. In the particular case of Y429N, it is possible that the effect of the Y429-coordinated water network disruption could affect the affinity in subsite +2. This is supported by the fact that Y429N was the only mutant displaying only hydrolytic activity when raffinose was used as substrate (results not shown). Experimental data obtained with xylose and maltose seems to support this assumption, given that the transfructosylation reaction increases in the presence of the monosaccharide and decreases with maltose (Fig. 2). Owing to its monomeric structure, xylose is proposed to occupy subsite +1 once glucose is released, and therefore, when xylose acts as

acceptor, both, hydrolysis and polymer synthesis are considerably reduced.

Conclusions

The strategy to design site-directed LS variants based on subsite interactions with sucrose and/or acceptors was found successful to obtain mutants with new properties. It was found that Y429 plays an indirect but important role in catalysis and acceptor specificity, as this is a key residue coordinating the sucrose position in the LS binding pocket through a complex water network. We found that S164 is catalytically important, as it maintains the nucleophile in an appropriate position regarding the sucrose molecule. S164A results in a 12-fold more stable and less hydrolytic enzyme than the WT LS, and susceptible to product activation, an effect that renders the enzyme more efficient for transfructosylation.

The WT LS product profile was modified by Y429A, R433A and R360S variants, which no longer synthesize levan. The combination of mutants bearing different properties such as pH, product profile or increased stability may open possibilities to obtain tailor-made LS biocatalysts in the future.

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