Isolation and enzymic properties of levansucrase secreted by *Acetobacter diazotrophicus* SRT4, a bacterium associated with sugar cane

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Acetobacter diazotrophicus, a nitrogen-fixing bacterium associated with sugar cane, secretes a levansucrase (sucrose–2,6-β-D-fructan 6-β-D-fructosyltransferase; EC 2.4.1.10). This enzyme is constitutively expressed and represents more than 70% of the total proteins secreted by strain SRT4. The purified protein consists of a single 58 kDa polypeptide with an isoelectric point of 5.5. Its activity is optimal at pH 5.0. It catalyses transfructosylation from sucrose to a variety of acceptors including water (sucrose hydrolysis), glucose (exchange reaction), fructan (polymerase reaction) and sucrose (oligofructoside synthesis). *In vivo* the polymerase activity leads to synthesis of a high-molecular-mass fructan of the levan type. *A. diazotrophicus* levansucrase catalyses transfructosylation via a Ping Pong mechanism involving the formation of a transient fructosyl-enzyme intermediate. The catalytic mechanism is very similar to that of *Bacillus subtilis* levansucrase. The kinetic parameters of the two enzymes are of the same order of magnitude. The main difference between the two enzyme specificities is the high yield of oligofructoside, particularly 1-kestotriose and kestotetraose, accumulated by *A. diazotrophicus* levansucrase during sucrose transformation. We discuss the hypothesis that these catalytic features may serve the different biological functions of each enzyme.

INTRODUCTION

Acetobacter diazotrophicus is a recently identified species of the genus *Acetobacter* [1]. It is a Gram-negative bacterium and was isolated from roots, stems and leaves of sugar cane [2]. It is an acid-tolerant nitrogen-fixing bacterium and produces an exopolysaccharide of high molecular mass when grown in the presence of sucrose. We show in this study that this polymer is a fructan of the levan type. The polymer is synthesized by levansucrase from sucrose alone, which acts both as fructosyl donor and fructosyl acceptor, thereby allowing polymerization.

A wide range of micro-organisms produces levansucrase and it has been postulated [3] that the enzyme activity is involved in a variety of processes: survival of bacteria in soil (*Bacillus subtilis*) [4], phytopathogenesis (*Erwinia* and *Pseudomonas* species) or symbiosis (*Bacillus polymixa*) for plant interactive bacteria [5]. The question arises whether these different functions can be correlated with differences in enzyme specificities. In this study we isolated the levansucrase produced and secreted by *A. diazotrophicus* strain SRT4 and we attempted to elucidate the enzyme mechanism for polysaccharide production.

MATERIALS AND METHODS

Bacterial strain and media

A. diazotrophicus strain SRT4 isolated from sugar cane [6] was used throughout this work. The bacterium was maintained and grown on LGI medium [2]. In liquid culture, LGI medium was supplemented with yeast extract (0.2 g/l) and tryptone (1 g/l). Solid medium was obtained by supplementing liquid medium with agar (1.5%, w/v).

Substrates

[U-^{14}C]Sucrose and [U-^{14}C]glucose were purchased from Amersham and purified by paper chromatography before use. The levan of low molecular mass (15 ± 3 kDa) used was prepared as described by Chambert and Petit-Glatron [7]. Inulin from dahlia tubers (5 kDa) and Jerusalem artichoke were from Sigma.

Isolation of extracellular polysaccharide

Bacteria were grown on solid LGI medium for 1 week at 30 °C. The extracellular polymer synthesized was collected from the medium surface with distilled water. Bacteria were removed from the suspension by centrifugation, and the polymer was precipitated with 2 vol. of ethanol and redissolved in distilled water. Precipitation was repeated three times. The polymer was then treated with saturated phenol, precipitated twice with ethanol, dialysed against distilled water and freeze-dried.

Analysis of exopolysaccharide composition and molecular mass

Total acid hydrolysis of purified polymer was performed with 0.5 M H_2SO_4 at 100 °C for 15 min and samples were neutralized with $Ba(OH)_2$. Paper chromatography was carried out on Whatman 3MM paper in pyridine/butan-1-ol/water (4:6:3, by vol.). Monosaccharides were detected by spraying 2.3% (w/v) aniline phthalate dissolved in water-saturated butan-1-ol on to the paper which was kept at 100 °C until the spots became visible.

After total acid hydrolysis, the polymer composition was also analysed by HPLC using a Nucleosil NH_2 column (25 cm × 0.8 cm) eluted with 80% (v/v) acetonitrile in water at a flow rate of 0.4 ml/min in an isocratic way. Monosaccharides were detected with a Knauer differential refractometer operating

Abbreviation used: DP, degree of polymerization.

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at 37 °C. Glucose and fructose standards were prepared at 10 mg/ml in the elution buffer.

The molecular mass of the polymer was estimated by gel filtration on a Sephacryl S-500 column (20 cm × 0.8 cm) eluted with 0.2 M NaCl at a flow rate of 12 ml/h. Dextran Blue and a mixture of dextran T-500 with sucrose were used as molecular-mass standards. The anthrone assay [7a] was used to monitor column effluents.

Initial-velocity measurements

Transfructosylation reaction from sucrose to water: sucrose hydrolysis

Reaction mixtures (45 μl) containing [^{14}C]sucrose at various concentrations (all lower than 50 mM) in 0.1 M sodium acetate, pH 5.8, were incubated at 30 °C. The reaction was initiated by the addition of 5 μl of the enzyme solution. Aliquots (5 μl) were removed at intervals and ^{14}C-labelled sugars were quantitatively analysed by paper chromatography [8]. v_F and v_G, the initial velocity of fructose and glucose release respectively, were calculated.

Transfructosylation from sucrose to fructose polymer: fructose chain elongation

Levan (or inulin) at various concentrations (1–20 mM) was added to the reaction mixture described above. Initiation of the reaction and analysis of the products were as described previously [8].

Transfructosylation from sucrose to glucose: exchange reaction

Glucose was added to concentrations up to 200 mM to the reaction mixture as described above. The conditions of the reaction initiation and product analysis were as described above. The initial velocity of the exchange reactions is $v_G - v_F$.

Isolation of extracellular levansucrase

A. diazotrophicus SRT4 was grown to stationary phase in LGI medium supplemented with 2% (v/v) glycerol as carbon source. After centrifugation, the culture supernatant was concentrated 5-fold using a rotatory evaporator and dialysed through an 8000 Da cut-off membrane against distilled water at 4 °C. Dialysed samples were submitted to preparative electrophoresis on a non-denaturating SDS/7.5% (w/v) polyacrylamide gel (15 mm × 10 mm × 6 mm) in Tris/glycine buffer, pH 8.3, using a constant current (40 mA) at 4 °C. Protein bands were visualized by the reverse staining method [9]. The levansucrase band was cut out of the gel, electroeluted and dialysed against distilled water at 4 °C. The enzyme was lyophilized and kept at −20 °C.

Oligofructan analysis by TLC

TLC was performed on silica-gel TLC foils (Schleicher and Schuell) as described by Cairns and Pollock [10]. The TLC foil was developed twice in butan-1-ol/propan-2-ol/water (3:12:4, by vol.). Fructans with a degree of polymerization (DP) of more than 15 do not migrate and stay at the application site.

Isoelectric focusing

Isoelectric focusing was performed using carrier ampholites (pH 3.5–10.5); samples were run for 72 h at constant voltage (700 V) and 4 °C.

RESULTS

Characterization of the exopolysaccharide produced by *A. diazotrophicus*

The exopolysaccharide produced by *A. diazotrophicus* grown in solid LGI medium supplemented with sucrose was isolated as described in the Materials and methods section. This polymer was readily soluble in water. It was completely excluded from a Sephacryl S-500 column (not shown) and thus had a molecular mass of more than 2×10^6 Da.

After total acid hydrolysis, the products released were analysed by paper chromatography or HPLC. The two methods gave the same result: more than 95% of the material released was identified as free fructose. These results suggest that this exopolysaccharide is a fructose homopolymer.

Characterization and isolation of the exocellular levansucrase released in the culture supernatant by *A. diazotrophicus*

The culture supernatant was analysed by non-denaturating SDS/PAGE. The levan synthetase activity was detected directly in the gel [11] by soaking the gel in a solution of sucrose. Only one white opalescent spot due to the formation of levan was detected. This enzyme activity co-migrated with the major protein in the culture supernatant revealed by protein staining. The enzyme was mainly synthesized during the late phase of growth. The effects of various carbon sources on the production of levansucrase by *A. diazotrophicus* was investigated. It was not induced by sucrose (Figure 1).

The *A. diazotrophicus* levansucrase was isolated (see the Materials and methods section). SDS/PAGE analysis indicated that the protein consists of a single 58 kDa polypeptide and its isoelectric point, measured as described in the Materials and methods section, was estimated to be 5.5.

Examination of the enzymic properties of levansucrase and mechanism of transfructosylation reaction

The optimum pH for catalytic activity was determined so as to define the best conditions for the study of the enzyme mechanism; maximum activity was obtained around pH 5 (Figure 2). The effects of different metal ions, or of the metal ion chelator EDTA, on the enzyme activity were also tested. The results (Table 1) suggest that this enzyme does not need a metal cofactor.

Transformation of [U-^{14}C]sucrose catalysed by the enzyme under various initial conditions was studied as described in the Materials and methods section. With sucrose alone as the substrate, at low concentration (< 0.1 M), the enzyme acted mainly as a sucrose hydrolase. Large amounts of oligofructosides were synthesized at higher concentrations of sucrose. In the presence of fructan (of the levan type), the enzyme acted as a fructosyl polymerase: the amount of ^{14}C-labelled polymer in the spot remaining at the origin was much larger and the fructose release lower than in the absence of fructan. In the presence of unlabelled glucose, the enzyme had a low hydrolytic activity and catalysed an exchange reaction. The fructosyl moiety of uniformly labelled sucrose was not released but exchanged with unlabelled glucose to give sucrose labelled only on the fructosyl moiety.

This exchange reaction strongly suggests that the *A. diazotrophicus* levansucrase like that of *B. subtilis* [8] works via a Ping Pong mechanism involving the formation of a transient fructosyl-enzyme complex. The transfructosylation reaction can thus be described by a sequence of a minimum of elementary steps

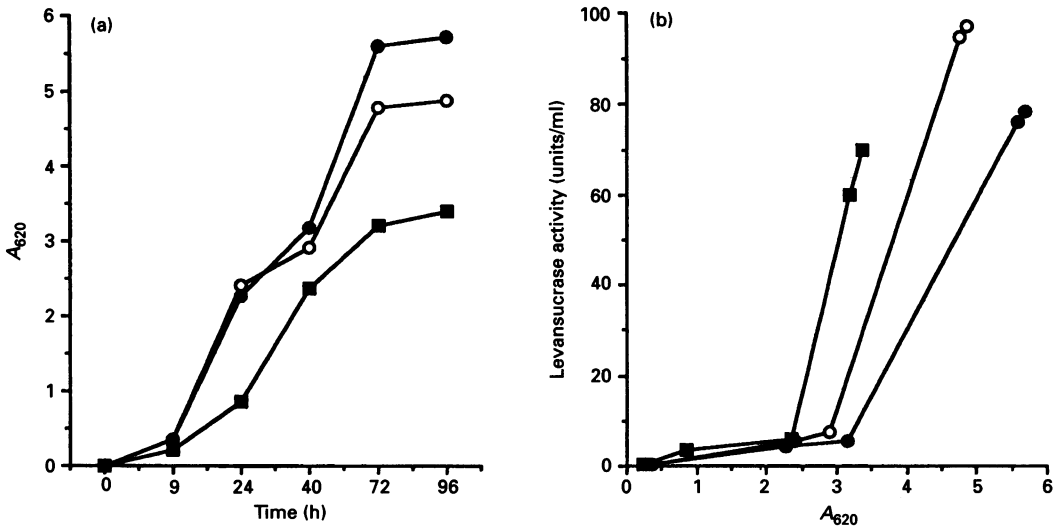


Figure 1 Production of exocellular levansucrase by *A. diazotrophicus* SRT4 in the presence of various carbon sources at 30 °C

During growth, samples of cell suspension were taken at intervals and centrifuged. The culture supernatants were dialysed against 0.1 M sodium acetate buffer, pH 5.2. Enzyme activity was measured as the glucose released from sucrose hydrolysis using a glucose oxidase/peroxidase reagent kit (Boehringer). One unit of enzyme is defined as the amount of enzyme releasing 1 μmol of glucose/min under the following conditions: 0.25 M sucrose in 0.1 M sodium acetate buffer, pH 5.2. Carbon sources in the culture medium were 1% sucrose (○), 1% glycerol (●) or 1% mannitol (■). Each assay was repeated three times. Cell density was measured in a 1 cm-path-length cuvette after appropriate dilution. (**a**) Growth curves; (**b**) production of enzyme versus growth.

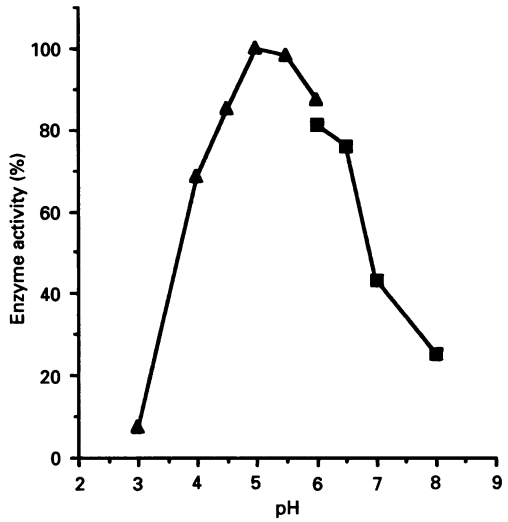


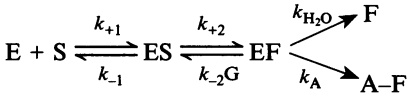
Figure 2 Effect of pH on enzyme activity

The pH effect was examined using 0.1 M sodium acetate buffer in the pH range 3.0–6.0 and 0.1 M sodium phosphate buffer in the pH range 6.0–8.0 at 42 °C. Enzyme activity was evaluated via the sucrose hydrolysis reaction as described in Figure 3. ▲, Acetate buffer; ■, phosphate buffer.

Table 1 Effects of metal ions on enzyme activity

Concentrations of metal ions and EDTA were 1 mM. Enzyme activities were evaluated from the rate of sucrose hydrolysis in the presence of the indicated concentration of the salt. Each enzyme activity assay was replicated three times.

Compound	Relative activity (%)
None	100
NaCl	99
$CaCl_2$	101
$CoCl_2$	127
$NiCl_2$	95
$ZnCl_2$	92
$HgCl_2$	4
$FeSO_4$	93
$Fe_2(SO_4)_3$	57
EDTA (disodium)	107



Scheme 1 Transfructosylation reaction of levansucrase

E, S, G, F and A represent enzyme, sucrose, glucose, fructose and fructosyl acceptor respectively.

(Scheme 1). The kinetic equations of the initial rate of each transfructosylation reaction can be derived from this mechanism [7].

Transfructosylation from sucrose to water: sucrose hydrolysis

The initial rate of sucrose hydrolysis was measured within a concentration range of 1–50 mM. The kinetic behaviour of the enzyme with respect to the sucrose concentration may be expressed by the Michaelis equation:

$$v_F = v_G = \frac{k_{cat.}[E_T][S]}{K_m + [S]}$$

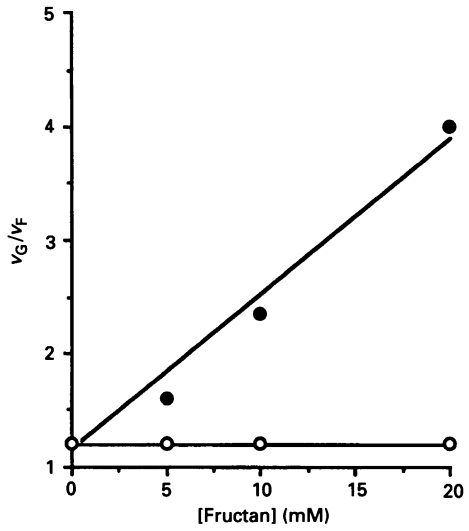


Figure 3 Plot of the v_G/v_F ratio against fructan concentrations

The initial concentration of labelled sucrose was 0.1 M. ●, Levan; ○, inulin from dahlia or Jerusalem artichoke. The enzyme concentration was 1 μM.

where v_F and v_G are the initial rate of fructose and glucose release respectively. K_m, $k_{cat.}$ and $k_{cat.}/K_m$ were estimated, according to the weighted regression procedure of Wilkinson [12], to be 11.8 ± 1.4 mM, $(3.6 \pm 0.4) \times 10^3$ min^{-1} and 3.1×10^5 M^{-1}·min^{-1} respectively [means $\pm$ S.D. ($n = 12$); enzyme concentration 1 μM, temperature 30 °C].

For the Ping Pong mechanism expected, these kinetic parameters can be expressed as a function of individual rate constants, assuming that $k_{-1} \gg k_{+2}$.

$$k_{cat.} = \frac{k_{+2}k_{H_2O}}{k_{+2}+k_{H_2O}}; \quad K_m = \frac{k_{-1}}{k_{+1}}\left(\frac{k_{H_2O}}{k_{+2}+k_{H_2O}}\right)$$

The K_m for sucrose of this enzyme like those of the majority of bacterial levansucrases is low [3].

Transfructosylation from sucrose to fructose polymer: fructan chain elongation

The capacity of fructan (of the inulin or levan type) to act as a fructosyl acceptor, A, with this enzyme was evaluated from the measurements of v_G and v_F for the transformation of uniformly labelled sucrose in the presence of either levan of low molecular mass (15 ± 3 kDa) or inulin from dahlia or Jerusalem artichoke (5 kDa).

The following equation can be derived from a Ping Pong mechanism according to the King and Altman procedure [13]:

$$\frac{v_G}{v_F} = 1 + \frac{k_A}{k_{H_2O}}[A]$$

This ratio of reaction rates was determined at various fructan concentrations (Figure 3). The enzyme displays a chain elongation activity with levan but not with inulin, suggesting that fructan synthesized *in vivo* by this enzyme is of the levan type. The ratio of the individual rate constants, reflecting the competition between water and fructan to act as fructosyl acceptor, was determined from the straight line obtained.

$$\frac{k_{lev.}}{k_{H_2O}} = 190 \pm 10\,M$$

Transfructosylation from sucrose to glucose: exchange reaction

The activity of glucose as a fructosyl acceptor can be evaluated using the same method. In this case, the ratio of the initial rate of uniformly labelled glucose and labelled fructose release is:

$$\frac{v_G}{v_F} = 1 + \frac{k_{-2}}{k_{H_2O}}[G]$$

This ratio was measured in the presence of a fixed concentration of labelled sucrose and various concentrations of unlabelled glucose acceptor (Figure 4a). The slope of the straight line is

$$\frac{k_{-2}}{k_{H_2O}} = 65 \pm 3\,M$$

Thus, in the presence of 0.1 M glucose, more than 85% of the fructosyl moiety transferred from sucrose is exchanged with glucose.

The exchange activity was experimentally separated from the sucrose hydrolysis reaction by using labelled glucose rather than uniformly labelled sucrose. The rate of exchange, r_G, was thus evaluated from the formation of labelled sucrose.

As demonstrated by Chambert et al. [14], the simple rate equation for the initial velocity of exchange can be derived from the Ping Pong mechanism as follows:

$$\frac{E_0}{r_G} = \frac{1}{k_{+2}} + \frac{k_{-1}}{k_{+1}k_{+2}[S]} + \frac{1}{k_{-2}[G]}$$

r_G was measured in the presence of various concentrations of glucose and sucrose. The data fit this equation very well (Figure 4b).

The three kinetic parameters were determined by a weighted least-squares polynomial regression from the rate-exchange measurements. The mean values $\pm$ S.D. ($n = 20$) were:

$$\frac{1}{k_{+2}} = (3.0 \pm 0.3) \times 10^{-5}\,min; \quad \frac{k_{-1}}{k_{+1}k_{+2}} = (3.2 \pm 0.2) \times 10^{-6}\,M \cdot min$$

$$\frac{1}{k_{-2}} = (4.9 \pm 0.3) \times 10^{-6}\,M \cdot min$$

Transfructosylation from sucrose to sucrose: kestose synthesis activity

In the presence of sucrose alone at high concentration, the enzyme activity released free glucose and fructose and mainly kestose as oligofructoside, during the initial phase. HPLC analysis on Dionex [15] of the kestose eluted from paper chromatography indicated that it is only 1-kestose. Thus sucrose acts both as a fructosyl donor and as a fructosyl acceptor competing with water. From the King and Altman procedure [13], the ratio of initial rates of kestose, v_i^K, and fructose release v_i^F is:

$$\frac{v_i^K}{v_i^F} = \frac{k_s}{k_{H_2O}}[S]$$

The slope of the straight line obtained (Figure 5) indicates that $k_s/k_{H_2O} = 0.5$. Thus, in 1 M sucrose, one-third of the fructosyl is transferred by the enzyme to sucrose acting as acceptor and the rest is released as free fructose.

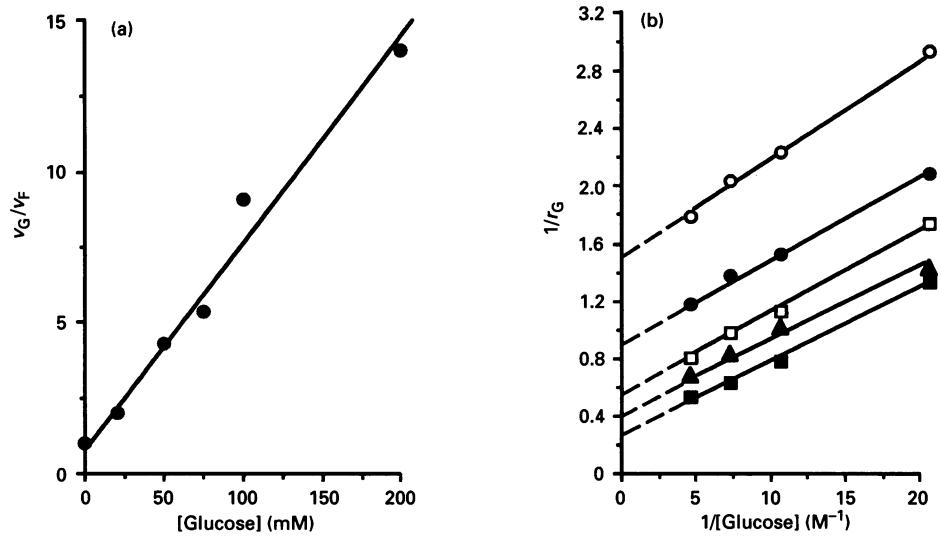


Figure 4 Exchange reaction

(**a**) Plot of the v_G/v_F ratio versus glucose concentration. The initial concentration of sucrose was 0.1 M; the enzyme concentration 0.1 μM. (**b**) Double-reciprocal plot of r_G, the initial rate of exchange of the free [^{14}C]glucose with unlabelled glucosyl moiety of sucrose ($1/r_G$ is expressed in $mM^{-1} \cdot min$) as a function of glucose concentration. The sucrose concentration was held constant at 20 mM (○), 40 mM (●), 80 mM (□), 125 mM (▲) or 250 mM (■). The enzyme concentration was 0.1 μM.

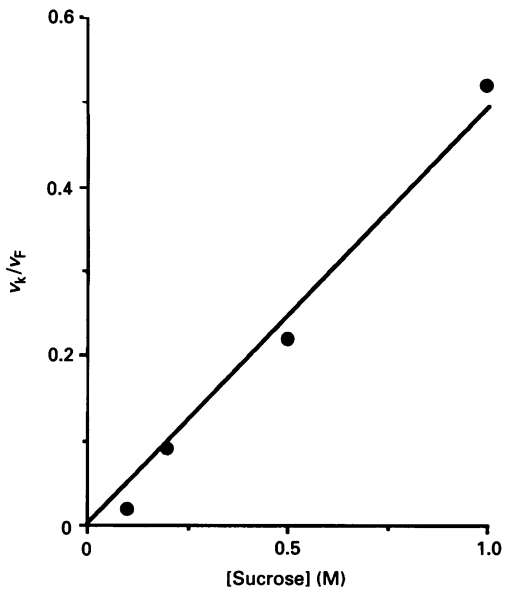


Figure 5 Kestose synthesis

Plot of the ratio v_k/v_F against sucrose concentration. The enzyme concentration was 0.1 μM.

We calculated the individual rate constants for each step of the Ping Pong mechanism proposed (Table 2). The kinetic constants are of the same order of magnitude as those published for *B. subtilis* levansucrase [7] except $k_{lev.}$ and k_{inu}. Apparently, the levansucrase of *B. subtilis* is a better fructosyl polymerase. Comparison of the rate constants with those of levansucrase from other origins has not been possible until now, as their transfructosylation catalytic pathways were not characterized [3].

Differences in enzyme specificity between the *A. diazotrophicus* and *B. subtilis* levansucrase

The *A. diazotrophicus* levansucrase released a large amount of kestose from sucrose in the initial phase. The oligofructans synthesized during the course of complete sucrose transformation were analysed and compared with those synthesized by *B. subtilis* levansucrase. The two enzymes gave different patterns of products (Figure 6). The *B. subtilis* levansucrase synthesized fructan with a DP higher than 15, as soon as the reaction was initiated. Oligofructans with a lower DP were not accumulated. In contrast, *A. diazotrophicus* levansucrase released large amounts of 1-kestose and kestotetraose which accumulated in the reaction mixture. Fructans with higher DP were synthesized during later stages of the reaction. The fructosyl acceptor

Table 2 Values of apparent kinetic constants and partial equilibria for the reaction in Scheme 1 catalysed by *A. diazotrophicus* levansucrase

Origin of levansucrase	k_{+2} (s^{-1})	k_{-2} ($M^{-1} \cdot s^{-1}$)	k_{H_2O} (s^{-1})	$k_{lev.}$ ($M^{-1} \cdot s^{-1}$)	$k_{inu.}$ ($M^{-1} \cdot s^{-1}$)	k_S ($M^{-1} \cdot s^{-1}$)	k_{+1}/k_{-1} (M^{-1})
A. diazotrophicus	550±60	3300±200	51±5	(9.7±1)×10^3	0	25±5	12±1
*B. subtilis**	260±20	4600±300	35±4	(3.9±0.4)×10^4	(7.0±0.7)×10^3	–	18±2

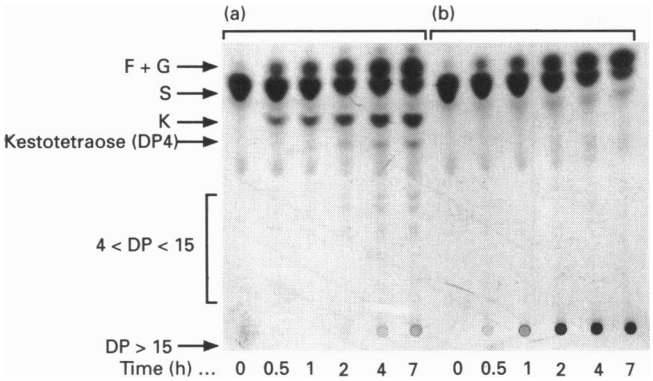


Figure 6 Time course of oligofructan synthesis from sucrose by *A. diazotrophicus* levansucrase (a) and *B. subtilis* levansucrase (b) at 30 °C

The reaction mixture (40 μl) contained 0.8 M [U-^{14}C]sucrose (initial concentration) in 0.1 M potassium acetate, pH 5.8. Reactions were initiated by addition of the enzyme (to a final concentration of 4 μM). At the indicated intervals, samples (5 μl) were removed, mixed with 20 μl of water and incubated for 5 min at 100 °C. Aliquots (2 μl) of each fraction were analysed by TLC. Kestose and kestotetraose were identified by their migration with respect to fructose migration according to the values published by Cairns and Pollock [10]. F, G, S and K refer respectively to fructose, glucose, sucrose and 1-kestose. DP is the degree of polymerization of the oligofructan.

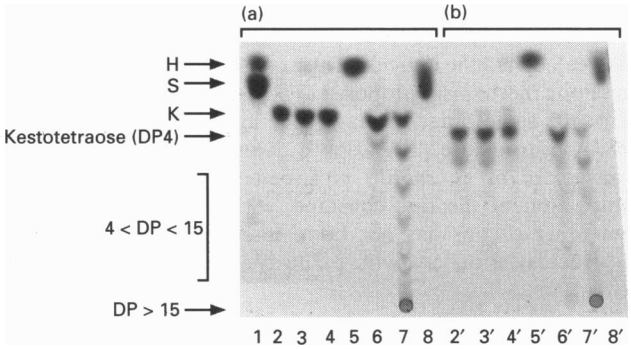


Figure 7 Transformation of 1-kestose (a) and kestotetraose (b) by *A. diazotrophicus* levansucrase and *B. subtilis* levansucrase and by yeast invertase

The reaction mixtures (10 μl) contained initially 0.02 M uniformly labelled 1-kestose (lanes 2–5), 0.02 M uniformly labelled kestose plus 0.2 M unlabelled sucrose (lanes 6–8), 0.01 M uniformly labelled kestotetraose (lanes 2′–5′) or 0.01 M uniformly labelled kestotetraose plus 0.2 M unlabelled sucrose (lanes 6′–8′). Reactions were initiated by addition of the enzyme (to 2 μM). After 5 h of incubation at 30 °C, aliquots (2 μl) of each reaction mixture were analysed by TLC. Lane 1, mixture of labelled sucrose and hexose as markers; lanes 2 and 2′, kestose and kestotetraose incubated without enzyme as controls; lane 3, 6, 3′, 6′, *A. diazotrophicus* levansucrase; lanes 4, 7, 4′, 7′, *B. subtilis* levansucrase; lanes 5, 8, 5′, 8′, yeast invertase (from Sigma). ^{14}C-labelled kestose and kestotetraose were obtained from the action of *A. diazotrophicus* levansucrase on ^{14}C-labelled sucrose followed by chromatographic purification.

efficiencies of 1-kestose and kestotetraose for each enzyme were tested (Figure 7). In a reaction mixture containing each of these labelled oligofructans in the presence of unlabelled sucrose, both enzymes synthesized labelled oligofructans with higher DP. 1-Kestose was a better fructosyl acceptor for *B. subtilis* levansucrase than for the *A. diazotrophicus* enzyme. The two labelled oligofructans incubated alone in the presence of each levansucrase remained unmodified (Figure 7). This means that the two levansucrases cannot use 1-kestose and kestotetraose as fructosyl donor in the experimental conditions.

DISCUSSION

Bacterial levansucrases [3] catalyse the transfructosylation reaction from sucrose to a variety of acceptors including water, glucose, fructan and sucrose. All the enzymes, whatever their origin, catalyse all these reactions but with different efficiency. Such differences were clearly shown in this study. *B. subtilis* levansucrase catalysed the formation of high-molecular-mass levan without accumulation of transient oligofructan with a low degree of polymerization. In contrast, the action of *A. diazotrophicus* levansucrase led to the accumulation of a large amount of tri- and tetra-saccharides. The rate of formation of the fructosyl-enzyme, expected to be a key intermediate for the transfructosylation process, is approximately the same for both enzymes. The molecular mechanism of the first step of the catalytic pathway may be conserved for these two levansucrases but the affinity of the fructose-enzymes for the various fructosyl acceptors, modulating the product specificity, could have diverged. A single amino acid substitution in the *B. subtilis* levansucrase [7] was demonstrated to modify the enzyme activity in such a way that polymerase reaction was completely inhibited.

On the other hand, levan produced by *B. subtilis* may constitute a defence mechanism against environmental stress or be a food reservoir. Exopolysaccharides synthesized by *A. diazotrophicus* may be expected to be involved in its interaction with sugar cane. Kestose is known to play a key role in plant fructan biosynthesis [16]. Thus the kestose accumulated by *A. diazotrophicus* levansucrase may be used by sugar cane for its own fructan metabolism. Another hypothesis is that exopolysaccharides prevent the growth of sugar cane pathogen fungi as shown for *Bacillus polymixa* levansucrase activity which is found in the rhizosphere of wheat [4]. It may therefore be assumed that the differences in the ecological niches of the bacterial species producing levansucrase may explain the differences in the catalytic specificities of these enzymes which may have a common ancestor.

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