Crystallization and preliminary X-ray diffraction analysis of levansucrase (LsdA) from 
Gluconacetobacter diazotrophicus SRT4

The endophytic bacterium *Gluconacetobacter diazotrophicus* SRT4 secretes a constitutively expressed levansucrase (LsdA; EC 2.4.1.10), which converts sucrose to fructo-oligosaccharides and levan. Fully active LsdA was purified to high homogeneity by non-denaturing reversed-phase HPLC and was crystallized at room temperature by the hanging-drop vapour-diffusion method using ammonium sulfate and ethanol as precipitants. The crystals are extremely sensitive, but native data have been collected to 2.5 Å under cryogenic conditions using synchrotron radiation. LsdA crystals belong to the orthorhombic space group *P2_12_12_2* with unit-cell parameters \( a = 53.80, b = 119.39, c = 215.10 \text{ Å} \).

1. Introduction
Levansucrase (EC 2.4.1.10; sucrose:6-β-D-fructan 6-β-D-fructosyltransferase) catalyses a transglycosylation reaction in which fructose, derived from sucrose, may be transferred to a variety of acceptors including water (hydrolysis), glucose (exchange), sucrose (oligosaccharide synthesis) and fructan (polymerization) (Cote & Ahlgran, 1993). These reactions all occur via a ‘ping-pong’ kinetic mechanism involving the formation of a fructosyl-enzyme intermediate and its subsequent interception by the acceptor (Chambert et al., 1974; Hernández et al., 1995; Song & Jacques, 1999).

Levansucrases are produced by a wide range of sucrose-utilizing bacteria. The endophytic species *Gluconacetobacter diazotrophicus* secretes a constitutively expressed levansucrase which yields high levels of β-2,1-linked oligosaccharides and levan from the natural substrate sucrose (Hernández et al., 2000). Levansucrase produced by the Cuban strain SRT4 has been extensively characterized (Arrieta et al., 1996; Hernández et al., 1999; Betancourt et al., 1999). The mature enzyme consists of a single polypeptide of molecular weight 60.4 kDa and isoelectric point 5.5 which contains an intramolecular disulfide bridge and an N-terminal blocking pyroglutamic acid residue.

Glycoside hydrolases have now been classified into over 90 families on the basis of amino-acid sequence similarities (CAZy database; http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). Bacterial fructosyltransferases are included in family GH32, comprise the β-fructosidase superfamily (reviewed in Pons et al., 2000; Naumoff, 2001). Here, we present the purification, crystallization and preliminary diffraction analysis of the *G. diazotrophicus* SRT4 levansucrase from family GH68.

2. Materials and methods
2.1. Purification of LsdA and protein assays
*G. diazotrophicus* SRT4 was cultured to stationary phase (96 h) in LGIT medium (2% glycerol, 0.2% tryptone, 0.1% KH₂PO₄, 0.1% K₂HPO₄, 0.02% MgSO₄.7H₂O, 0.002% CaCl₂.2H₂O, 0.0002% Na₂MoO₄, initial pH 7.0) at 303 K with shaking at 250 rev min⁻¹. Cells were removed by centrifugation, the culture supernatant was concentrated fivefold at 310 K using a rotatory evaporator and ethanol was added to 60% (final concentration) to precipitate high-molecular-mass exopolysaccharides. After centrifugation, LsdA remained in the supernatant fraction, from which ethanol was removed by rotary evaporation at 310 K. The enzyme solution was dialyzed against 10 mM Tris–HCl pH 7.0, filtered through 0.2 μm disposable filters and applied onto a semi-preparative butyl HPLC column (Vydac, USA). The column was washed with 0.1 M ammonium acetate pH 5.0 and proteins were eluted using an increasing gradient of 1-propanol (0–60%) in 0.1 M ammonium acetate pH 5.0. The peak fraction with LsdA activity was dialyzed against 10 mM Tris–HCl pH 7.0, concentrated to 10 mg ml⁻¹ by ultrafiltration using a 10 kDa molecular-weight cutoff membrane (Millipore) and used for crystallization experiments.

LsdA concentration was determined by absorbance at 280 nm considering the theoretical extinction coefficient of 1.4 AU
(1 mg ml\(^{-1}\)). LsdA activity was measured as the glucose released from sucrose hydrolysis using a glucose oxidase–peroxidase-coupled colorimetric kit (Sigma). SDS-PAGE was performed as described by Laemmli (1970). Proteins were revealed by Coomassie Blue staining.

2.2. Crystallization, data collection and processing

The crystallization experiments were performed using the hanging-drop vapour-diffusion method in 24-well tissue-culture plates at room temperature. LsdA concentrated to 10 mg ml\(^{-1}\) in 10 mM Tris–HCl pH 7.0 was initially screened for crystallization conditions, varying the ammonium sulfate concentration (0.2–1.5 M) in the pH range 5.0–7.0. In each trial, 2 μl of purified enzyme and 2 μl of mother liquor were mixed over wells that contained 1 ml of the screening solution. Long needles grew over two weeks using ammonium sulfate in the concentration range 0.35–0.6 M in 0.1 M sodium citrate pH 5.6. Ethanol (30–60%) and the detergent SDS (1.5–5.0 mM) were used to optimize the crystallization conditions. Crystal formation was accelerated twofold by adding 50 mM guanidinium chloride and 50 mM MgCl\(_2\) to the crystallization solution. Crystals with improved morphology and size were transferred with difficulty to the cryoprotection solution (15% glycerol, 30% ethanol, 50 mM ammonium sulfate, 0.1 M sodium citrate pH 5.6). A single crystal was mounted in a rayon-fibre loop and stored in liquid nitrogen. For data collection, the crystal was placed in a stream of N\(_2\) gas at 100 K and 105° of data were collected with an oscillation angle of 0.5° per image at the ESRF (Grenoble, France) on beamline ID14-EH1 (λ = 0.934 Å) with a MOSFILM (Leslie, 1992) detector. Data were processed with SCALA from the CCP4 suite of programs (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The yield of LsdA was 58 mg per litre of culture with an initial purity of 70% from the culture supernatant of *G. diazotrophicus* SRT4 grown under shaking batch conditions. Conventional reversed-phase HPLC allowed LsdA purification to homogeneity, but the enzyme was irreversibly inactivated under the extremely acidic conditions (pH ≈ 2.0) of the solvent system water/acetonitrile/TFA. A second purification protocol using as the main step a non-denaturing reversed-phase HPLC with the solvent system water/1-propanol/ammonium acetate pH 5.0 rendered a fully active enzyme with high homogeneity and purity (Fig. 1).

Initial attempts to crystallize LsdA using ammonium sulfate as the sole precipitant agent in 0.1 M sodium citrate pH 5.6 resulted in long thin needles that were not suitable for X-ray analysis. The addition of ethanol or the detergents SDS and β-octylglucoside to the crystallization solution allowed a significant improvement in crystal morphology and size. More highly reproducible crystals with prismatic shape reaching up to 180 μm in their largest dimension were achieved in the presence of 30% ethanol as a second precipitant agent (Fig. 2), although the high ethanol concentrations make subsequent handling extremely challenging.

LsdA crystal diffraction statistics are presented in Table 1. The crystals belong to the primitive orthorhombic crystal system. Because of the elongated shape of the crystals and the difficulties in handling the crystals, the reflections along the [001] axis (corresponding to the long axis of the crystal) were not measured during data collection. The exact space group could thus be either *P2\(_1\)2\(_1\)2\(_1\)* or *P2\(_2\)2\(_2\)2\(_2\)*. Preliminary molecular-replacement attempts using *Bacillus subtilis* levansucrase (kindly provided prior to publication by Klaus Fütterer, University of Birmingham, England) gives better statistics for a two-molecule solution in space group *P2\(_1\)2\(_1\)2\(_1\)*. Levansucrases have been the target of structural studies since the preliminary work of LeBrun & van Rappenbusch (1980). We hope that a number of levansucrase crystal structures will soon be available to shed light on this fascinating family of enzymes.

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### References


### Table 1

<table>
<thead>
<tr>
<th>Wavelength (Å)</th>
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<tr>
<td>Crystal system</td>
<td>Primitive orthorhombic</td>
</tr>
<tr>
<td>Space group</td>
<td><em>P2(_1)2(_1)2(_1)</em></td>
</tr>
<tr>
<td>Unit-cell parameters (Å, °)</td>
<td>(a = 53.8, b = 119.4, c = 215.1), (\alpha = \beta = \gamma = 90)</td>
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<tr>
<td>Unique reflections collected</td>
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<tr>
<td>Resolution range (Å)</td>
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<td>Completeness (observed) (%)</td>
<td>99.2 (98.5)</td>
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<tr>
<td>Matthews coefficient</td>
<td>3.0 (2 molecules) or 2.0 (3 molecules)</td>
</tr>
</tbody>
</table>

### Figure 2

Typical crystals of LsdA obtained by equilibration against a solution of 0.5 M ammonium sulfate, 30% ethanol, 50 mM guanidinium chloride and 50 mM MgCl\(_2\) in 0.1 M sodium citrate buffer pH 5.6. The maximum dimension of the crystals is about 0.18 mm.

### Figure 1

Analysis by rp-HPLC (a) and SDS-PAGE (b) of LsdA purified from the culture supernatant of *G. diazotrophicus* SRT4 and used for crystallization experiments. LsdA eluted from the HPLC column at 40% 1-propanol.
crystallization papers


