Abstract

Polyfructans are synthesized from sucrose by plants (mostly inulin) and by both Gram-negative and Gram-positive bacteria (mostly levan). In the phylum Actinobacteria only levan synthesis by Actinomyces species has been reported. We have identified a putative fructansucrase gene (hugO) in Streptomyces viridochromogenes DSM40736 (Tü494). HugO was heterologously expressed and biochemically characterized. HPSEC-MALLS and 2D-1H-13C nuclear magnetic resonance (NMR) spectroscopy analysis showed that the fructan polymer produced in vitro has an Molar Weight of 2.5×10^7 Da and is an inulin that is mainly composed of (β2–1)-linked fructose units. This is the first report of a fructansucrase from Streptomyces and an inulosucrase from Actinobacteria. Database searches showed that fructansucrases clearly occur more widely in streptomycetes. Analysis of the active site of HugO and other actinobacterial Gram-positive fructansucrases revealed that their +1 substrate-binding sites are conserved, but are most similar to those in Gram-negative fructansucrases. HugO also resembles Gram-negative fructansucrases in not requiring calcium ions for activity. The origin and properties of HugO and other actinobacterial fructansucrases thus clearly differ from those of previously characterized Gram-positive fructansucrases.

INTRODUCTION

Streptomycetes are Gram-positive, G+C-rich soil bacteria with a complex life cycle that switches between mycelial growth and sporulation. They are an abundant source of anti-infective agents and other secondary metabolites, and consequently the genus has been researched extensively. In the mycelial growth phase streptomycetes are mainly saprophytic organisms and can utilize a multitude of different available substrates for growth by secreting extracellular enzymes [1]. Plant-derived polysaccharides such as cellulose [2], xylan [3], starch [4] or inulin are known to be degraded by Streptomyces species. Growth on inulin has been reported for Streptomyces turgidiscabies, and Streptomyces sp. MTCC-3119 has been used to create an inulinase enzyme production platform [5, 6].

Inulin is a fructan homopolymer consisting of d-fructose moieties connected by (β2–1) glycosidic linkages that can be produced by plants and a few Gram-positive bacteria, either as a polymer or as oligosaccharides (FOS) with various degrees of polymerization (DP <9) [7, 8]. Levan is a fructan that is mostly synthesized by both Gram-positive and Gram-negative bacteria and is composed of (β2–6) glycosidic residues [7, 9]. Bacterial fructans are produced from sucrose by fructansucrase or fructosyltransferase (Ftf) enzymes. Ftfs belong to the glycoside hydrolase family GH68 (www.CAZy.org) and produce either inulin (inulosucrases) or levan (levansucrases) [10]. In Gram-positive bacteria, GH68 enzymes generally consist of (i) a signal peptide for secretion, (ii) an N-terminal variable domain of unknown function, (iii) the core region containing all of the residues for catalysis and (iv) a C-terminal domain [10]. The structure of the core domain is reported to be a globular, five-bladed β-propeller enfolding a funnel-like cavity [11–13]. The catalytic triad consists of a catalytic nucleophile [11], a transition state stabilizer [14] and a general acid/base [15]. A retaining ping-pong mechanism for fructan synthesis was first proposed for SacB from Bacillus subtilis [16]. This mechanism was refined by Ozimek et al. concerning differences in processive and non-processive Ftfs of Lactobacillus reuteri [17]. In the active centre, sucrose is first bound in the substrate-binding subsites −1 and +1 with the fructose in subsite −1. Whereas subsite −1 is conserved, subsite +1 is reported to show variation between Gram-positive and Gram-negative bacteria [10]. Subsequently, the bound sucrose is cleaved and the glucose...
is released. The fructose moiety is considered to form a covalent bond with the nucleophile of the enzyme [11] and is then further transferred to either water (hydrolysis) or a growing fructan chain, or to another acceptor molecule (transglycosylation) [16, 17].

Bacterial inulosucrases have only been described for a few firmicutes, from lactic acid bacteria such as Lactobacillus reuteri, Streptococcus oralis and Leuconostoc citreum, and for a single enzyme from Bacillus sp. 217-C-11 [10, 18, 19]. Levansucrases, on the other hand, occur much more widely and have been described for both Gram-negative and Gram-positive bacteria, e.g. Pseudomonas, Zymomonas or Rahnella species, and Lactobacillus, Bacillus or Actinomyces species, respectively [9, 20].

When screening the CAZy database for novel Ftf enzymes we found a putative fructansucrase annotated in the genome of the plant pathogen Streptomyces scabiei 87.22 (CBG69007.1). Sucrose consumption is considered to be rare in streptomycetes [21], and because we were intrigued by this finding we searched the NCBI database using this protein sequence as a BLAST query [22]. We found various other putative ftf genes encoded in Streptomyces strains, including other S. scabiei strains, S. roseochromogenus subsp. oscitans DS12.976, S. lindensis and S. viridochromogenes DSM40736. Some (6 out of 21) of these streptomycetes are known as plant pathogens. We characterized the Ftf (HugO) enzyme from S. viridochromogenes DSM40736, the producer strain of the commercial herbicide phosphinotricin tripeptide (PTT) [23]. Our data show that HugO is in fact an inulosucrase that mostly produces fructooligosaccharides (FOS) from sucrose and smaller amounts of a high-molecular-mass polymer. Here we report the characteristics of the first non-firmicute inulosucrase enzyme and its products.

**METHODS**

**Strains, media and culture conditions**

The strains used in this study are listed in Table 1. S. viridochromogenes DSM40736 spores and genomic DNA were a kind gift from Professor Wolfgang Wohleben and Dr Yvonne Mast, Microbiology/Biotechnology, University of Tübingen, Germany. For spore collection the strain was streaked out on soy manitol agar (SFM) [21] and grown at 30 °C for approximately 5 days. Liquid cultures were grown in Difco nutrient broth (DNB) at 30 °C with shaking at 220 r.p.m.

The Escherichia coli strains XL1-Blue and NiCo21(DE3) were used for DNA manipulation and protein expression, respectively. Generally, E. coli was cultured in Luria Broth (LB) or on LB agar (Carl-Roth GmbH, Germany) supplemented with 100 µg ml⁻¹ ampicillin at 37 °C, unless otherwise indicated. Liquid cultures were incubated under shaking at 220 r.p.m.

**Bioinformatics analysis**

Sequences of characterized GH68 enzymes (http://www.cazy.org/GH68_characterized.html) and GH68 enzymes of interest were chosen from the CAZy database http://www.cazy.org/GH68_bacteria.html and downloaded from NCBI into one file in the FASTA format. Identical and therefore redundant sequences were removed from the selection. The sequences were used to construct a phylogenetic tree using Phylology ‘one click’ (http://www.phylology.fr/simple_phylogeny.cgi) [24, 25]. Conserved domains were identified using www.pfam.org and signal peptides were identified with the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) [26]

Alignment of GH68 enzymes for the comparison of conserved residues in the active centre was performed with T-Coffee (http://tcoffee.crg.cat/apps/tcoffee/index.html) [27] and analysed in Jalview [28].

**In vivo product formation**

*S. viridochromogenes* DSM40736 and *S. coelicolor* M145 cultures were inoculated with spores in 50 ml DNB medium with and without 300 mM sucrose to an initial OD₄₅₀ of 0.04. Cultures were grown for 14 days and 0.5 ml samples were collected and used for TLC and HPAEC-PAD analysis (see below).

**DNA isolation, gene amplification and construction of hugO-expression constructs**

General E. coli procedures, DNA electrophoresis, preparation of competent cells and transformation were performed as described in [29]. Phusion high-fidelity polymerase, fast-digest restriction enzymes and T4 ligase were purchased from Thermo Scientific.

The plasmid DNA for restriction analysis was isolated by isopropanol precipitation [30], while the plasmid DNA for sequencing was isolated using the Sigma-Aldrich GenElute HP plasmid miniprep kit following the manufacturer’s instructions. The elution of DNA from agarose gels was performed with GE Healthcare GFX PCR DNA and Gel Band purification kit.

Amplification of the putative fructansucrase gene *hugO* from the genomic DNA of *S. viridochromogenes* was performed with Phusion polymerase with the programme: 98 °C for 3 min (98 °C 10 s, 65 °C 15 s, 72 °C 45 s)×40 and 72 °C for 5 min with primers S.vir LS For and S.vir LS Rev (Table 2). The PCR products were eluted from agarose gels and ligated into vector pJET1.2 blunt (Table 2) (Thermo Scientific) according to the manufacturer’s instructions. The pJET1.2 clones were isolated from *E. coli*, tested by restriction with NdeI and BglIII, and sequenced at GATC (Konstanz, Germany). Inserts were excised from pJET1.2 with NdeI/BglII. The pET15b vector (Novagen) was linearized with NdeI and BamHI. The insert and vector were co-eluted from agarose gels and ligated at 4 °C overnight. The pET15b clones were tested for correct insertion by restriction analysis with NcoI/EcoRV.
Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomyces viridochromogenes</td>
<td>Wild-type, phosphinotricin tripeptide producer</td>
<td>[59]</td>
</tr>
<tr>
<td>DSM40736 (Tü494)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptomyces coelicolor M145</td>
<td>Plasmid-free wild-type derivative</td>
<td>[21]</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>endA1 gyrA96(malR) thi-1 recA1 relA1 lac glnV44 F' [:: Tn10 proAB lacI15Δ(lacZ)M15] hsdR17(rK mK) sBamH1o ΔEcoRI-B int [::lacI : PlacUV5 : T7 gene1] i21 Δnin5</td>
<td>[60] England Biolabs</td>
</tr>
<tr>
<td>NiCo 21(DE3)</td>
<td>can::CBD frucA2 [lon] ompT gal (λ DE3) [dem] arrA::CBD slyD::CBD glnSt66AAl ΔhsdS λ DE3 = λ lsd-1[:: Tn10 proAB lacI15Δ(lacZ)M15] hsdR17(rK mK) sBamH1o ΔEcoRI-B int [::lacI : PlacUV5 : T7 gene1] i21 Δnin5</td>
<td>[32] (New)</td>
</tr>
</tbody>
</table>

Expression and purification of fructansucrase HugO

The pET15b hugO construct was transferred to E. coli NiCo21(DE3) CaCl2-competent cells. Three randomly selected clones were each inoculated in 5 ml LB broth amp100 and grown for 5 h. The main expression cultures consisted of 400 ml LB broth amp100 (supplemented with 4 ml of 1 M MgSO4 and 3.5 ml of 60 % glycerol) inoculated with 1 % preculture and grown at 37 °C until OD600 = 0.5. Cultures were induced with 0.1 mM IPTG and cooled in a shaker to 14 °C for about 30 min. The induced cultures were grown at 14 °C and 220 r.p.m. for 48 h. Cells were harvested by centrifugation for 15 min at 4,200 g and 4 °C. Cell lysis was performed using B-PER solution (Thermo Scientific) following the manufacturer’s recommendations, including one freeze-thaw cycle. The purification (binding, washing and elution) of HugO protein was performed using an Ni-NTA matrix [31].

The elution fractions were combined into 15 ml tubes with 450 µl buffer-equilibrated chitin beads (NEB) per ml elution fraction and incubated overnight at 4 °C under continuous rolling. The chitin beads were removed by centrifugation at 4,400 g for 1 min at 4 °C and the supernatant transferred to a fresh tube [32], the HugO protein concentrations (Table S1, available in the online Supplementary Material) were determined by measuring the absorbance at 280 nm five times against the elution buffer and calculating the average.

SDS-PAGE

SDS-PAGE and gel staining was performed as previously described using Biorad Mini Protean equipment with homemade 10 % polyacrylamide gels [33]. Fairbanks A staining and de-staining was performed as described elsewhere, and documented with a Biorad Chemidoc MP [34].

Determination of optimal reaction conditions

Optimal reaction conditions were determined by measuring the enzyme activity (initial rates) with sucrose by following glucose release with the D-glucose assay kit (GOPOD Format, Megazyme). Determination of the optimal pH was carried out at 37 °C for 40 min. The reactions contained 100 mM sucrose, 1 mM CaCl2 and 50 mM MMT buffer (PACT Buffer protocols, Qiagen) at pH 4.0–9.0 in 0.5 increments.

The optimal temperature was determined in increments of 5 °C with 100 mM sucrose, 1 mM CaCl2 and 50 mM MMT buffer (pH 6.0). The reaction mixtures were pre-warmed to the reaction temperature for >5 min and started by the addition of 20 µg of enzyme. To measure the inhibition of the enzyme by EDTA, the enzyme was pre-incubated with 1–3 mM CaCl2 and 50 mM MMT buffer (pH 6.0) with or without the addition of 2 mM EDTA at 45 °C for about 5 min, and then the reaction was started by the addition of sucrose to a final concentration of 100 mM.

After starting the reaction, 100 µl samples were taken every 5 min over a time course of 40 min and mixed with 20 µl 1 M NaOH to stop the enzyme reaction for 20 min at room temperature. Subsequently, 195 µl GOPOD solution (Megazyme) was mixed with 5 µl sample and incubated for 30 min at 37 °C. Absorbance was measured at 510 nm. The three HugO enzyme preparations from different clones were tested in parallel to give one triplicate. The total and hydrolytic activities were determined by hexokinase assay as previously described [32].

Table 2. Primers and plasmids used in this study

The bold sequence shows the NdeI restriction site. The underlined sequence corresponds to 6xHIS plus a stop codon.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.vir LS For</td>
<td>TGGCATATGTCAGCAGCAAGCAAGGTG</td>
<td>This study</td>
</tr>
<tr>
<td>S.vir LS Rev</td>
<td>TTAGTGGTATGTTGATCGAATGAAGCGTAGTGG</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pET12</td>
<td>Linear cloning vector, blunt-end, AmpR</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>pET15b</td>
<td>Expression vector for N-Terminal 6xHis-fusion, T7-promoter, AmpR</td>
<td>Novagen</td>
</tr>
<tr>
<td>pET12 HugO</td>
<td>Cloning vector containing hugO</td>
<td>This study</td>
</tr>
<tr>
<td>pET15b HugO</td>
<td>Expression vector containing hugO with N- and C-terminal His-tag</td>
<td>This study</td>
</tr>
</tbody>
</table>
described [35, 36]. Transfructosylation activity was calculated by subtracting the free fructose (hydrolysis activity) from the free glucose (total activity). One unit of total activity is defined as the synthesis of 1 µmol of glucose per min in a reaction containing 100 mM sucrose, pH 6.0, at 45 °C.

**Product analysis**

For product analysis, 50 ml reactions were performed with each of the three HugO enzyme preparations using 100 mM sucrose, 50 mM MMT buffer (pH 6.0), 1 mM CaCl₂ and 0.15 units/ml enzyme, incubated for 18 h at 45 °C. These reaction mixtures were used for TLC- and HPAEC-PAD-analysis, purification of oligosaccharides for H/C nuclear magnetic resonance (NMR) analysis and purification of polymer for 2D 13C and 1H NMR spectroscopy analysis.

**TLC**

TLC-analysis was performed as in Gangoiti et al. [37], with one exception: the plates were developed with a urea staining specific for fructose and fructans [38].

**High-pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)**

1:100 dilutions of the reaction mixtures were run on an ICS3000 (Thermo Scientific) equipped with an ICS3000 amperometric detection cell and a CarboPac PA-1 column (4×250 mm; Thermo Scientific) run at 1.0 ml min⁻¹. The gradient was 30–300 mM NaOAc in 100 mM NaOH over 30 min, followed by a wash phase and a re-equilibration phase. Solutions of 1% short-chain inulins (Fratafit CLR, Sensus, Roosendaal, The Netherlands), 1 mM glucose and a mixture of 1% sucrose and 1% fructose were used as standards in 1:50 dilutions.

**Purification of oligosaccharides for NMR spectroscopy analysis**

Samples of 3 ml reaction mixture were applied to GRACE extract clean carbo columns (150 mg/4 ml). Non-carbohydrates and monosaccharides were washed off with 3 ml Milli-Q water and 3 ml 4% acetonitrile. Oligosaccharides were eluted by 2×0.75 ml and 1×0.5 ml 40% acetonitrile. The samples were frozen at −80 °C and lyophilized.

**Polymer purification**

The polymer was isolated from the reaction mixtures by precipitation with 1 vol isopropanol and incubation at −20 °C overnight. The precipitated polymer was collected by centrifugation and re-dissolved in Milli Q water. Precipitation was repeated once more.

**NMR spectroscopy analysis**

Samples of −1 mg for 1D 1H NMR spectra were exchanged twice in 250 µl 99.9 atom% D₂O (Cambridge Isotope Laboratories Ltd, Andover, MA, USA) with intermediate lyophilization. Finally, the samples were dissolved in 650 µl D₂O containing internal acetone (δ 1H 2.225; δ 13C 31.08). Samples of −5 mg for 2D 13C-1H HSQC NMR were exchanged twice in 400 µl D₂O with heating to 60 °C to assist solubilization and finally dissolved in 650 µl D₂O containing internal acetone. All NMR spectra were recorded with a Varian Inova 600 spectrometer (NMR department, University of Groningen, The Netherlands). 1D 600-MHz 1H NMR spectra were recorded at 25 °C with a 4800 Hz spectral width, collecting 16k complex data points in 32 transients. A WETID pulse was applied to suppress the HOD signal. 2D 13C-1H HSQC spectra were recorded at 80 °C with a t2 spectral width of 4800 Hz and a t1 spectral width of 10 000 Hz; 200 increments of 64 transients were recorded. All of the spectra were processed using MestReNova 5.3 (Mestrelabs Research SL, Santiago de Compostela, Spain). Manual phase correction was applied, followed by Whittacker Smoother baseline correction.

**HPSEC-MALLS analysis for the determination of Molecular Weight**

The Molecular Weight was determined as described in Meng et al. [39] using polymer concentrations of 0.5 mg ml⁻¹ in DMSO+ LiBr 5 g l⁻¹.

**Accession numbers**

The HugO protein is annotated under the accession number EFL36273.1.

**RESULTS**

**Bioinformatics analysis**

The phylogenetic position of streptomyctcal Ftfs was determined from a phylogenetic tree (Fig. 1) with the GH68 enzymes listed as characterized in the CAZy database. We curated the Ftf sequences selected by removing largely identical protein sequences and adding different Ftf proteins of interest. The final Ftf tree revealed a distribution into four major clades (Fig. 1). Clade 1 consists of the Ftfs in firmicutes, in particular bacillales and lactic acid bacteria (LAB) (Lactobacillus, Leuconostoc and Streptococcus). Clade 2 contains putative archaeal GH68 enzymes, but also Clostridium. Clade 3 contains most of the Gram-negative bacteria in the data set (Pseudomonas, Zymomonas, etc.) and Clade 4 contains all actinobacteria, including the putative fructansases from Streptomyces species, but also the Ftfs of a few Gram-negative bacteria, Zymomonas species, Azotobacter chroococcum and Gluconoacetobacter diazotrophicus. For comparison, the S. viridochromogones Ftf has 42, 45 and 27% identity with LsdA from G. diazotrophicus (Clade 4), Lsc1 from Pseudomonas syringae (Clade 3) and Inu from Lactobacillus reuteri 121 (Clade 1), respectively.

The S. viridochromogones HugO enzyme was selected for further characterization. The annotated hugO open reading frame is 1335 bp long and encodes a predicted protein of 444 amino acids. The deduced Ftf protein product has a theoretical MW of 49 kDa. We analysed the HugO amino acid sequence for conserved domains [40] and signal peptides [26]. A conserved GH68 domain was identified from the alignment coordinates aa47 to aa443, spanning almost the entire length of the protein. Also, a Gram-positive signal peptide for secretion (aa1 to aa30) [26] was identified. The signal peptide contains the amino acid motif R-R-V-V-L, which fits to the R-R-x-Φ-Φ-motif for secretion via the
twist-arginine translocation (TAT) pathway in Streptomyces [41]. The variable N-terminal domain appears to be relatively short, with about 17 amino acids between the proposed GH68 core domain and the signal peptide. The protein also lacks any C-terminal domain and therefore may not be bound to the cell wall of S. viridochromogenes.

Analysis of the active centre of HugO by sequence alignment with SacB (B. subtilis), Inul (Lb. johnsonii), Inu (Lb. reuteri 121), SacBSm (S. mutans), LsdA (G. diazotrophicus) and Lsc1 (Ps. syringae) showed that the –1 binding site with the catalytic triad D86, D247 and E342 (numbering from the SacB of B. subtilis) is largely conserved in all of the analysed fructansucrase enzymes (Table 3). It has been suggested that the sequence of the +1 site differs between the fructansucrases of Gram-positive and Gram-negative origin [10]. The three +1 site residues R360, K363 and N242 (SacB numbering) are indeed largely conserved in the Gram-positive SacB (B. subtilis), Inu, Inul and SacBSm proteins, as observed previously [36]. Analysis of HugO of S. viridochromogenes, however, showed that in its +1 site these conserved residues are more similar to those in Gram-negative fructansucrases, with the histidine H419 and threonine T422 (LsdA numbering) also conserved in S. viridochromogenes, with the histidine H419 and threonine T422 (LsdA numbering) also conserved in these enzymes (Table 3). As a result, HugO is classified as a Gram-negative fructansucrase.

Analysis of the active centre of HugO by sequence alignment with SacB (B. subtilis), Inul (Lb. johnsonii), Inu (Lb. reuteri 121), SacBSm (S. mutans), LsdA (G. diazotrophicus) and Lsc1 (Ps. syringae) showed that the –1 binding site with the catalytic triad D86, D247 and E342 (numbering from the SacB of B. subtilis) is largely conserved in all of the analysed fructansucrase enzymes (Table 3). It has been suggested that the sequence of the +1 site differs between the fructansucrases of Gram-positive and Gram-negative origin [10]. The three +1 site residues R360, K363 and N242 (SacB numbering) are indeed largely conserved in the Gram-positive SacB (B. subtilis), Inu, Inul and SacBSm proteins, as observed previously [36]. Analysis of HugO of S. viridochromogenes, however, showed that in its +1 site these conserved residues are more similar to those in Gram-negative fructansucrases, with the histidine H419 and threonine T422 (LsdA numbering) also conserved in HugO (Table 3), as well as in SucE2 and Lsc1 from Zymomonas mobilis and Ps. syringae, respectively. The third conserved residue of the +1 subsite is conserved in neither Gram-negative fructansucrases nor the HugO of S. viridochromogenes. According to [11], the corresponding residue in SacB (B. subtilis) binds weakly to the acceptor substrate. We also did not find the four residues forming the CaCl2-binding site in the Ftsfs of firmicutes [N310, D339, D241 and Q272 in SacB (B. subtilis) numbering] in HugO, suggesting that this protein does not require a metal co-factor, as is typical for Gram-negative fructansucrases [42] (Table 3). The same features were observed for other available actinobacterial Ftf sequences, displaying in these respects a clear similarity to Gram-negative Ffts, rather than the Ftsfs of firmicutes.

Fig. 1. Phylogenetic analysis of GH68 Ftf enzymes from the CAZy database. Red, actinobacteria; green, Gram-negative bacteria; violet, archaea; blue, lactic acid bacteria; orange, bacillales. Hp, hypothetical protein; LS, levansucrase; IS, inulosucrase; Bff, B-fructofuranosidase; *, uncharacterized protein of interest. Clade 1 consists of the Ftfs of firmicutes, clade 2 of archaea, clade 3 of Gram-negative bacteria and clade 4 of actinobacteria and some Gram-negative bacteria. Streptomyces viridochromogenes is listed here as a putative levansucrase based on its current annotation in the NCBI database.
During these studies, many more ftf genes were annotated in streptomycetal genomes (about 24 at the time of manuscript submission). With two exceptions, all are closely related to HugO (not shown). In view of the phylogenetic position of Ftf proteins of actinobacteria, the high identity to the putative Ftf from the plant pathogen *S. scabiei* (87 %), and the complete lack of data on the properties of streptomycetal fructansucrases in general, we decided to study the *in vivo* activity of *S. viridochromogenes* cultures with sucrose in more detail, and to biochemically characterize its Ftf enzyme and identify the products formed.

### Table 3. Amino acid residues in the active site (−1, +1 and +2 binding sites) and CaCl₂-binding sites of bacterial Ftf proteins and the HugO of *S. viridochromogenes* (adapted from [43]43)

| Subsite in SacB* | Inuf† | Inuf‡ | SacBsum§ | HugO|| | LsdA¶ | SucE2# | Lsc1** |
|------------------|-------|-------|-----------|-----------|-------|-------|-------|-------|
| −1               | D86   | D272  | D272      | D246      | D87   | D135  | D48   | D62   |
| −1               | D247  | E425  | D424      | D400      | D240  | D309  | D194  | D219  |
| −1               | E342  | E524  | E523      | E499      | E324  | E401  | E278  | E303  |
| −1               | L109  | M296  | M296      | M270      | L110  | L158  | L71   | L85   |
| −1               | R433  | R623  | R622      | R598      | V406  | H172/V501 | I361 | V385  |
| +1               | R360  | R542  | R541      | R517      | H342  | H419  | H358  | H321  |
| +1/+2            | K363  | R545  | R544      | H520      | T345  | T422  | T299  | T324  |
| +1/+2            | N242  | N420  | N419      | N395      | I235  | F304  | F189  | S214  |
| +2               | R246  | R424  | R423      | R399      | R239  | R308  | R193  | R218  |
| +2               | Y237  | N415  | N414      | F390      | –     | –     | –     | –     |
| +2               | A116  | V302  | Q302      | T276      | V116  | -/A164 | K77  | P91   |
| CaCl₂            | N310  | N489  | N488      | N464      | T291  | K368  | A245  | V270  |
| CaCl₂            | D339  | D521  | D520      | D496      | Q321  | D398  | D275  | D300  |
| CaCl₂            | D241  | D419  | D418      | D394      | I234  | E303  | N188  | S213  |
| CaCl₂            | Q272  | Q450  | Q449      | Q425      | –     | –     | –     | –     |

*B. subtilis CAA26513.1.
†Lb. johnsonii AAS08734.1.
‡Lb. reuteri AAN05755.1.
§S. mutans AAN59631.1.
||S. viridochromogenes DSM40736 (see M and M).
¶G. diazotrophicus AAW47883.1.
#Z. mobilis BAA04475.1.
**Ps. syringae AAO54974.1.

During these studies, many more ftf genes were annotated in streptomycetal genomes (about 24 at the time of manuscript submission). With two exceptions, all are closely related to HugO (not shown). In view of the phylogenetic position of Ftf proteins of actinobacteria, the high identity to the putative Ftf from the plant pathogen *S. scabiei* (87 %), and the complete lack of data on the properties of streptomycetal fructansucrases in general, we decided to study the *in vivo* activity of *S. viridochromogenes* cultures with sucrose in more detail, and to biochemically characterize its Ftf enzyme and identify the products formed.

### In vivo analysis of sucrose consumption by *S. viridochromogenes*

According to the manual, *Practical Streptomyces Genetics*, most *Streptomyces* species do not consume sucrose [21]. Consequently, we checked whether cultures of *S. viridochromogenes* and *S. coelicolor* M145, which does not have an Ftf gene, use sucrose *in vivo*. Both strains were grown in Difco nutrient broth (DNB) with and without 300 mM sucrose and sampled regularly; the supernatants were analysed for sucrose utilization and product formation by TLC and HPEAC-PAD. Despite the normal growth of *S. coelicolor* M145 in DNB medium, both TLC and HPEAC-PAD analysis of its culture supernatants failed to show any detectable changes in sucrose concentration from day 1 to day 14. TLC analysis of the *S. viridochromogenes* culture supernatants showed that during growth, sucrose was converted into a series of oligosaccharides of different sizes, represented by a ladder of distinct blue colour, indicating that synthesis of fructooligosaccharides (FOS) occurred (Fig. S1). This result was confirmed by the HPEAC-PAD-analysis (see Fig. 2). Production of FOS with DP >3 was first detected at day 2; the product size gradually developed until DP13 on day 14. Under these conditions the cells clearly also formed polymeric material. Over the course of time the sucrose concentration decreased and glucose accumulated. The fructansucrase reaction also may result in sucrose hydrolysis, and we indeed observed that about 60.8 % (SEM±17.3 n=2) of the converted sucrose was hydrolysed to fructose and glucose (Fig. 2). When grown in DNB medium, this strain apparently does not use glucose and fructose. As FOS synthesis and glucose release are characteristic of fructansucrase activity, we concluded that *S. viridochromogenes* expressed a functional Ftf enzyme. In subsequent steps we characterized the single Ftf enzyme encoded in the *S. viridochromogenes* genome and compared its products with those synthesized *in vivo* by *S. viridochromogenes* cultures.
Expression and purification of the *S. viridochromogenes* HugO fructansucrase

HugO was initially cloned with a C-terminal His-tag, introduced by PCR into pET3b and expressed in *E. coli* BL21 (DE3), but no expression was achieved. This may have been due to several rare codons being present at the beginning of the gene. With *E. coli* Rosetta2 (DE3) as host, low expression of HugO was observed (data not shown). To improve expression, the C-terminally His-tagged full-length gene was subcloned into pET15b, thus also incorporating an N-terminal His-tag. HugO expression in BL21(DE3) was now significantly improved, but it was still rather low and the Ni-NTA-purified HugO enzyme was still highly contaminated. Therefore, we switched to *E. coli* NiCo21(DE3). In this strain the major contaminants of Ni-NTA purification were tagged with a chitin-binding domain and could be removed with an additional chromatography step. This resulted in sufficiently clean HugO enzyme for further characterization (see SDS-PAGE analysis, Fig. S2). With this procedure we achieved protein yields of ~14 mg per litre of culture. It should be noted that our *E. coli* NiCo21(DE3) pET15 HugO glycerol stock lost expression over time, for unknown reasons. Therefore the expression experiments were started with fresh *E. coli* transformants.

**HugO optimal reaction conditions and biochemical properties**

The optimal reaction conditions for the HugO of *S. viridochromogenes* were determined as described in the Methods section by measuring glucose release, representing total activity. The optimal pH for HugO spans a relatively broad range, from pH 5.0 to 7.0 (Fig. S4a). A relatively substantial amount of activity continues at the pH extremes of pH 4.0 (79.6%) and pH 9.0 (49.2%). We chose to continue our experiments at pH 6.0, as this was in the middle of the activity plateau (Fig. S4a). The reaction conditions were further optimized by testing at different temperatures from 30 to 65 °C at pH 6.0. The total activity increased until a plateau was reached from 50–55 °C (Fig. S4b). Beyond 55 °C the enzyme lost activity rapidly. To avoid problems with protein inactivation at elevated temperatures, we performed the following experiments at 45 °C.

In contrast to the fructansucrases from *Lactobacillus* and *Bacillus* species, HugO does not appear to be dependent on CaCl$_2$. No significant change in activity could be observed after the pre-incubation of HugO with EDTA, CaCl$_2$ and a mixture thereof, and the reaction was started by the addition of sucrose (Fig. S4c). Under these conditions the $K_m$ and $K_{cat}$ values were determined to be 7.3±0.8 mM and 3.5 s$^{-1}$, respectively.

*S. viridochromogenes* grows optimally at 28 °C, and the maximal Pf activity was observed at 50–55 °C. We performed most of the other experiments at 45 °C, and determined the specific activity of the purified HugO enzyme preparations at all three temperatures and at pH 6.0 in 100 mM sucrose. The specific total activities varied from 2.6±0.26 units/mg (30 °C), to 6.3±0.11 units/mg (45 °C) to approximately 8.9
±0.8 units/mg protein (55 °C). The percentage of transglycosylation decreased with temperature from 50.6±7.4 % (30 °C), to 47.0±2.0 % (45 °C) to 38.0±0.6 % (55 °C).

**Characterization of HugO products from sucrose**

The initial characterization of the HugO products after a 18 h reaction at 45 °C with 100 mM sucrose was performed by TLC using fructose- and fructan-specific urea staining [38]. We observed an oligosaccharide ladder of distinct blue colour (Fig. 3a) that was very similar to the oligosaccharide pattern observed with supernatant samples of *S. viridochromogenes* cultures grown in sucrose (Fig. S1). This indicates that HugO is indeed responsible for FOS synthesis. TLC analysis did not show much polymer being produced. HPAEC-PAD analysis of the in vitro reaction products and the FOS from the *S. viridochromogenes* culture supernatants yielded highly similar product profiles, confirming that HugO is the enzyme responsible for FOS production in vivo (Fig. 3b).

To analyse the fructan polymer produced, the residual reaction (about 40 ml) was precipitated with 2-propanol and the polymeric material was collected. The precipitated polymer was used for structural analysis by NMR spectroscopy and molecular weight determination by HPSEC-MALLS. The polymer size was determined to be ~2.5*10^7 Da.

The polysaccharide and oligosaccharide fractions were isolated by precipitation with 2-propanol. The polysaccharide was analysed by 1D ^1^H NMR (Fig. 4), as well as by 2D ^13^C-^1^H NMR spectroscopy (Fig. S4), in comparison with inulin and levan references. From the 2D ^13^C-^1^H NMR spectra, all of the ^1^H and ^13^C chemical shifts could be assigned for the main structural element of the inulin, levan and polymer products (Table 4). The main difference in 1D ^1^H NMR was the H-3 signal at δ 4.26 that occurred in inulin, but was absent in levan, while the H-3 signal at δ 4.18 fit a 6-substituted β-D-Fruf residue and was a minor peak in inulin, indicating the level of (β2–6) linkages.

Further comparison to a short-chain inulin standard showed that the major product peaks of HugO had the same retention times as those from the standard, indicating the production of inulin oligosaccharides by HugO (Fig. 3B).

**DISCUSSION**

In this paper we identified and characterized the HugO enzyme of *Streptomyces viridochromogenes* as a true fructansucrase. HugO product analysis revealed that it is an inulosucrase. The oligosaccharide fraction showed a 1D ^1^H NMR spectrum fitting to inulooligosaccharides, with a relatively intense set of terminal α-D-Glcp-related peaks (δ 5.41, 3.55, 3.47 ppm), indicating a relatively low average DP. The NMR spectra of the HugO polymer product also showed a
A bioinformatics analysis showed that HugO and other streptomycetal GH68 enzymes form a clade of closely related proteins. To our initial surprise, the actinobacterial Ftfs displayed a close relationship with some Gram-negative Ftfs. Previously we [43] concluded that Ftf proteins can be divided into those of Gram-positive and Gram-negative origin. However, the study by Anwar et al. [44] focused strongly on the Ftfs of LAB, and no attention was paid to the specific phylogenetic position of the Ftfs from actinobacteria. The phylogenetic position of the HugO protein is in good agreement with the data of Velázquez-Hernández [7]. In this review, 51 microbial fructosyltransferases of diverse origin (including Actinomyces and Arthrobacter Ftfs,

---

Table 4. $^1$H and $^{13}$C chemical shifts of levan, inulin and HugO polysaccharide products, determined from 2D $^{13}$C-$^1$H HSQC NMR spectroscopy

<table>
<thead>
<tr>
<th></th>
<th>Inulin</th>
<th></th>
<th>Levan</th>
<th></th>
<th>HugO</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^1$H</td>
<td>$^{13}$C</td>
<td>$^1$H</td>
<td>$^{13}$C</td>
<td>$^1$H</td>
<td>$^{13}$C</td>
</tr>
<tr>
<td>1a</td>
<td>3.90</td>
<td>62.2</td>
<td>3.75</td>
<td>61.6</td>
<td>3.91</td>
</tr>
<tr>
<td>1b</td>
<td>3.73</td>
<td>62.2</td>
<td>3.70</td>
<td>61.6</td>
<td>3.74</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>ND</td>
<td>–</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>4.24</td>
<td>78.4</td>
<td>4.18</td>
<td>77.9</td>
<td>4.24</td>
</tr>
<tr>
<td>4</td>
<td>4.10</td>
<td>75.7</td>
<td>4.09</td>
<td>76.5</td>
<td>4.11</td>
</tr>
<tr>
<td>5</td>
<td>3.87</td>
<td>82.2</td>
<td>3.94</td>
<td>81.2</td>
<td>3.88</td>
</tr>
<tr>
<td>6a</td>
<td>3.85</td>
<td>63.3</td>
<td>3.90</td>
<td>64.3</td>
<td>3.85</td>
</tr>
<tr>
<td>6b</td>
<td>3.76</td>
<td>63.3</td>
<td>3.63</td>
<td>64.3</td>
<td>3.78</td>
</tr>
</tbody>
</table>

---

Fig. 4. 1D $^1$H NMR spectra of (a) levan standard, (b) inulin standard, (c) HugO oligosaccharide fraction, (d) HugO polysaccharide fraction. Relevant peaks for structural determination are marked, $\beta$1 and $\beta$6 signify ($\beta$2–1)- and ($\beta$2–6)-linked fructose residues, respectively.

---

profile that matched that of inulin, indicating a ($\beta$2–1)-linked product, with small numbers of ($\beta$2–6)-linked branches.
but not *Streptomyces* Ftfs) were analysed and distributed into seven different clades (clades A–G) in a phylogenetic tree [7]. In fact the four major clades in the phylogenetic tree in Fig. 1 can be further differentiated to match clades A–F in [7] (not shown); we did not include the fungal Ftfs of clade G [7]. Similar observations were made in a phylogenetic study by Alm *et al.* [7] and by Bergeron *et al.* and Ito *et al.* when characterizing the levansucrases from *Actinobacillus naeslundii* and *Arthrobacter* sp. K-1, respectively [45–47]. To the best of our knowledge, our work provides the first phylogenetic analysis of Ftfs to focus specifically on the genus *Streptomyces* as a source.

The evolution of Ftfs proteins was discussed in detail by [7], with the conclusion that all Ftfs have a common ancestor that evolved further by gene duplication and horizontal gene transfer. This hypothesis was further supported by the fact that the Pf of *Leuconostoc mesenteroides* (AAAT81165.1) is closely related to the Pf of *Pseudomonas chlorophis* [7] (Fig. 1). Also, the phylogenetic analysis in the present study makes it seem likely that horizontal gene transfer of ftf genes has occurred, from Gram-negative bacteria to *Actinobacteria* and *Streptomyces* in particular (Fig. 1).

The overall architecture of HugO fits well within the framework of the described GH68 enzymes. The size and domain distribution of fructansucrases is rather diverse, but HugO is a good representative of smaller fructansucrases such as SacB from *B. subtilis*. With 473 aa, SacB has a similar size to HugO, and with 11 aa its N-terminal domain also has a similar size. In comparison, LslA, an inulosucrase from *Lc. citreum* CW28, has an overall size of 1490 aa, a 40 aa signal peptide and a 259 aa-long N-terminal domain, together with 9 C-terminal SH3-like domains [18].

Our biochemical characterization of HugO showed a broad optimal pH range of pH 5 to 7, and an optimal temperature of around 50–55°C. This pattern is similar to that for the levansucrase from *A. viscosus*, which has an optimal pH in the range 5.5 to 6.5, and shows approximately 40% continuing activity at pH 4.0 and pH 9.0 [20]. BffA from *Arthrobacter* sp. K-1 has a pH optimum at 6.5 [47]. Inul1 from *Lb. johnsonii* has a pH optimum at 7.0 and 85% continuing activity from pH 4.0 to 6.5, while it loses activity drastically above pH 7.5 [36]. Lsc1 from *Ps. syringae* has an optimal pH range from 5.6 to 6.6, and is inactive at pH 4.0 or pH 8.0 [48]. A temperature curve similar to that in Fig. S3 was also reported for Inul [36]. BffA from *Arthrobacter* sp. K-1 also has a temperature optimum of 55°C [47], and Lsc1 has an optimum around 55–60°C and also loses activity rapidly above 60°C [48]. The HugO enzyme thus has similar pH and temperature profiles to the other Ftfs enzymes characterized.

The activity of HugO is relatively low in comparison to that of the other fructansucrases characterized [44]. This may reflect the different physiological roles of these enzymes, as discussed below. Velázquez-Hernández [7] compiled a list of $K_m$ values for different fructansucrases. These values ranged from 4 mM for SacB [49–51] to 160 mM for Lsc1 [48]. The closest phylogenetic relative to HugO is the levansucrase of *A. viscosus*, with a $K_m$ value of 12 mM [20]. The HugO $K_m$ value of 7.3 mM therefore matches the published literature. The low $K_{cat}$ of HugO is due to its low total activity.

HugO does not require a metal co-factor, in agreement with our analysis of conserved residues, which found very little conservation of amino acids in the CaCl$_2$-binding site (Table 3) when compared to the Ftfs from firmicutes like SacB (*B. subtilis*) or Inul1 (*Lb. johnsonii*). The same has been reported for the levansucrase of *A. viscosus* [20], the BffA of *Arthrobacter* sp. K-1 [47] and the Lsc1 of *Ps. syringae* [48].

The MW of the isolated HugO polymer is also within the range of previously reported Ftfs-derived inulins and other polysaccharides. The inulin polymer produced by Inul was reported to be $4*10^7$Da [36], and the levan produced by Lsc1 was reported to be between $10^8$ and $10^9$Da [48]. Furthermore, the levan produced by the more closely related Ftfs from *A. viscosus* ($10^8$Da) falls within this range [20]. The glucans produced from sucrose by glucansucrases show similar values, e.g. $4.5*10^7$Da and $3.1*10^7$Da for reuteran and EPS180, respectively [52, 53]. Generally, microbial inulins (DP 20–10 000) are also much larger than inulins derived from plants (DP 30–150) [10].

The in vivo function of HugO has so far remained elusive. Many bacteria use Ftfs to produce an exopolysaccharide (EPS) for biofilm formation to acquire resistance against environmental stresses. However, streptomycetes usually escape from such stresses through the formation of exospores, and slimy material on an agar plate inoculated with streptomycetes cells is generally regarded as a sign of contamination. In view of the sessile life cycle of streptomycetes and the presence of putative GH32 enzymes (degrading fructans) (data not shown), we speculate that the inulin produced is a storage compound that is synthesized in the presence of sucrose and hydrolysed in the absence of other carbon sources. A similar dynamic equilibrium of fructan synthesis and hydrolysis has also been suggested for *Actinomyces* species during dental plaque/cavity formation [20, 54]. However, over a 14-day cultivation period of *S. viridochromogenes* with sucrose we only observed FOS and more, the levan produced by the more closely related Ftfs from *A. viscosus* ($10^8$Da) falls within this range [20]. The glucans produced from sucrose by glucansucrases show similar values, e.g. $4.5*10^7$Da and $3.1*10^7$Da for reuteran and EPS180, respectively [52, 53]. Generally, microbial inulins (DP 20–10 000) are also much larger than inulins derived from plants (DP 30–150) [10].

The in vivo function of HugO has so far remained elusive. Many bacteria use Ftfs to produce an exopolysaccharide (EPS) for biofilm formation to acquire resistance against environmental stresses. However, streptomycetes usually escape from such stresses through the formation of exospores, and slimy material on an agar plate inoculated with streptomycetes cells is generally regarded as a sign of contamination. In view of the sessile life cycle of streptomycetes and the presence of putative GH32 enzymes (degrading fructans) (data not shown), we speculate that the inulin produced is a storage compound that is synthesized in the presence of sucrose and hydrolysed in the absence of other carbon sources. A similar dynamic equilibrium of fructan synthesis and hydrolysis has also been suggested for *Actinomyces* species during dental plaque/cavity formation [20, 54]. However, over a 14-day cultivation period of *S. viridochromogenes* with sucrose we only observed FOS and polymer accumulation, making degradation less likely. In addition, the relatively low $K_{cat}$ value of HugO suggests a rather high specificity for sucrose. Taken together with the low turnover rate ($K_{cat}$), this could mean that the HugO in vivo function is to produce FOS or polymer under low-sucrose conditions; it is a slow enzyme to prevent hydrolysis until a suitable acceptor has been encountered. Streptomycetes are also regular inhabitants of plant rhizospheres, where they show growth-enhancing effects [55] or prevent root infection by pathogens [56]. The exact mechanism by which streptomycetes establish such interactions is currently unclear, but fructans (levans) are supposed to play a role in host colonization for *Rhizobacterium* and *Erwinia* species [57, 58]. It should also be considered that a large
number of Streptomyces strains are known to interact with plants, but ftf genes are not widespread in the genus Streptomyces (see above). More experimental work on the in vivo function of HugO remains to be performed in future work.

Conclusion
We have cloned and characterized HugO as the first GH68 enzyme from the genus Streptomyces. The predicted architecture of HugO follows the domain structure outlined by van Hijum et al. [10], but the Fffs of the genus Streptomyces form a distinct group within the phylogenetic tree of known Fffs. HugO produces a ladder of FOS and a polymer with predominantly (β2–1) linkages, fitting inulo-oligosaccharides. The polymeric material was structurally characterized for their critical reading of this manuscript. We would like to thank Navickas from BASF SE for their valuable input during the project and the University of Groningen.

Acknowledgements
We thank Dr Kai-Uwe Baidenius, Dr Michael Breuer and Dr Vaidotas Navickas from BASF SE for their valuable input during the project and for their critical reading of this manuscript. We would like to thank Dr Mirjan Petrusma and Dr Joana Gangoiti Munecas for advice on pro-

References
1. McCarthy AJ, Williams ST. Actinomycetes as agents of biodegra-

2. Thomas L, Crawford DL. Cloning of clustered Streptomyces viridi-

4. Martínez-Fleites C, Ortiz-Lombardía M, Pons T, Tarbouriech N,
5. Taylor EJ et al. Crystal structure of levansucrase from the Gram-

7. Yanase H, Maeda M, Hagiwara E, Yagi H, Taniguchi K et al. Identifi-
cation of functionally important amino acid residues in Zymomo-

8. Chambert R, Treboul G, Dedonder R. Kinetic studies of levansu-

9. Ozimek LK, Kralj S, Forsberg K, Dijkhuizen L. The levan-
sucrase and inulosucrase enzymes of Lactobacillus reuteri 121 catalyse processive and non-processive transglycosylation reac-
10. Olivares-Ilíana V, López-Munguia A, Olvera C. Molecular charac-
terization of inulosucrase from Leuconostoc citreum: a fructosyl-
15. Schinko E, Schad K, Eys S, Keller U, Wohlleben W. PhosphOTHIN-
cin-tripeptide biosynthesis: an original version of bacterial sec-
16. Dereeper A, Guignon V, Blanc G, Audic S, Buffet S et al. Phylo-

18. Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: dis-

20. Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ. Jalview Version 2—a multiple sequence alignment editor and analy-