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Published in:
Microbiology-Sgm

DOI:
10.1099/mic.0.27105-0

Publication date:
2004

Microbiology (2004), **150**, 2099–2112 **DOI** 10.1099/mic.0.27105-0

Biochemical and molecular characterization of *Lactobacillus reuteri* 121 reuteransucrase

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Received 18 February 2004
Revised 26 March 2004
Accepted 2 April 2004

Lactobacillus reuteri strain 121 uses sucrose for synthesis of a unique, soluble glucan ('reuteran') with mainly α-(1→4) glucosidic linkages. The gene (*gtfA*) encoding this glucansucrase enzyme had previously been characterized. Here, a detailed biochemical and molecular analysis of the GTFA enzyme is presented. This is believed to be the first report describing reuteransucrase enzyme kinetics and the oligosaccharides synthesized with various acceptors. Alignments of the GTFA sequence with glucansucrases from *Streptococcus* and *Leuconostoc* identified conserved amino-acid residues in the catalytic core critical for enzyme activity. Mutants Asp1024Asn, Glu1061Gln and Asp1133Asn displayed 300- to 1000-fold-reduced specific activities. To investigate the role of the relatively large N-terminal variable domain (702 amino acids) and the relatively short C-terminal putative glucan-binding domain (267 amino acids, with 11 YG repeats), various truncated derivatives of GTFA (1781 amino acids) were constructed and characterized. Deletion of the complete N-terminal variable domain of GTFA (GTFA-ΔN) had little effect on reuteran characteristics (size, distribution of glycosidic linkages), but the initial transferase activity of the mutant enzyme increased drastically. Sequential C-terminal deletions (up to six YG repeats) in GTFA-ΔN also had little effect on reuteran characteristics. However, enzyme kinetics drastically changed. Deletion of 7, 8 or 11 YG repeats resulted in dramatic loss of total enzyme activity (43-, 63- and 1000-fold-reduced specific activities, respectively). Characterization of sequential C-terminal deletion mutants of GTFA-ΔN revealed that the C-terminal domain of reuteransucrase has an important role in glucan binding.

INTRODUCTION

Reuteransucrase (GTFA) from *Lactobacillus reuteri* 121 is a 1781 amino acid glucosyltransferase (GTF) enzyme (EC 2.4.1.5; common name: glucansucrase), which synthesizes a unique soluble glucan polymer, reuteran, with mainly α-(1→4) glycosidic linkages and significant amounts of α-(1→6) and α-(1→4,6) glucosidic linkages (van Geel-Schutten *et al.*, 1999; Kralj *et al.*, 2002). Two different reactions are catalysed by glucansucrase enzymes, depending on the nature of the acceptor: (i) hydrolysis, in which water is used as acceptor, and (ii) glucosyl transfer (transferase). The latter reaction can be divided into: (a) polymerization, in which the growing glucan chain is used as acceptor, and (b) oligosaccharide synthesis, in which oligosaccharides (e.g. maltose, isomaltose) are used as acceptor. Where studied, the linkage specificity of glucansucrases is conserved in oligosaccharide synthesis (Dols *et al.*, 1997; Cote & Robyt, 1982; Robyt & Walseth, 1978), and oligosaccharides are elongated at their non-reducing end (Dols *et al.*, 1997; Argüello Morales *et al.*, 2001; Monchois *et al.*, 2000a; Mukasa *et al.*, 2000). However, a biochemical characterization of the reactions catalysed by GTFA of *Lb. reuteri* 121 remained to be carried out.

GTF proteins of lactic acid bacteria share a common structure and are composed of four distinct domains. Their N-terminal end starts with: (i) a signal peptide of 32–34 amino acids, followed by (ii) a highly variable stretch of 123–129 amino acids, (iii) a highly conserved catalytic

Abbreviations: DP, degree of polymerization; CGTase, cyclodextrin glycosyltransferase; GBA, glucan-binding activity; GBD, glucan-binding domain; GTF, glucosyltransferase.

or sucrose-binding domain of about 1000 amino acids, and (iv) a C-terminal domain of about 500 amino acids, composed of a series of tandem repeats (Monchois *et al.*, 1999c). We were interested in the precise roles of these various domains and repeats in the overall functioning of GTF enzymes.

Amino-acid sequence comparisons revealed that the catalytic core of GTF proteins is constituted of eight β-sheets alternated with eight α-helices [a $(\beta/\alpha)_8$ barrel structure], similar to glycoside hydrolases of family 13 (α-amylase family). This family includes, for instance, α-amylase and cyclodextrin glycosyltransferase (CGTase) (van der Veen *et al.*, 2000). In GTFs, however, this $(\beta/\alpha)_8$ barrel structure is circularly permuted (MacGregor *et al.*, 1996; Devulapalle *et al.*, 1997). Therefore GTFs are classified in family 70 of glycoside hydrolases (http://afmb.cnrs-mrs.fr/CAZY/).

Whereas the catalytic mechanism for the α-amylase family is known (McCarter & Withers, 1994; Uitdehaag *et al.*, 1999), the exact catalytic mechanism of GTF enzymes remains to be elucidated (Monchois *et al.*, 1999c). The amino-acid residues crucial for catalysis in glucansucrases of family 70 have been identified as Asp453 (putative catalytic nucleophile), Glu491 (putative acid/base catalyst) and Asp564 (putative transition-state stabilizer) in GTFI from *Streptococcus downei* Mfe28 (Fig. 1) (MacGregor *et al.*, 1996; Devulapalle *et al.*, 1997). The equivalent invariable residues in enzymes of α-amylase family 13 are Asp229, Glu257 and Asp328 (CGTase *Bacillus circulans* 251 numbering). In enzymes of both families, the first-mentioned Asp residue is involved in the formation of the covalent glucosyl–enzyme complexes (Mooser *et al.*, 1991; MacGregor *et al.*, 1996; Uitdehaag *et al.*, 1999). The importance of this Asp residue has also been shown for other GTFs by site-directed mutagenesis experiments (Kato *et al.*, 1992; Monchois *et al.*, 1997; Devulapalle *et al.*, 1997) (Fig. 1). Based on alignments with glucansucrases from lactic acid bacteria (Monchois *et al.*, 1999c), putative catalytic residues in *Lb. reuteri* strain 121 GTFA were identified and mutated (see Results and Fig. 1).

Different repeating units have been identified in the N-terminal variable domains of several glucansucrases: A-repeats in alternansucrase and dextransucrases from *Leuconostoc mesenteroides* (Janecek *et al.*, 2000), motif T in DSRT from *Ln. mesenteroides* NRRL B-512F (Funane *et al.*, 2000), and motif S in DSRE from *Ln. mesenteroides* NRRL B-1299 (Bozonnet *et al.*, 2002). The function of the N-terminal variable domain (and these repeats) has remained unclear. Deletion studies show that it does not play a significant role in glucansucrase activity (Abo *et al.*, 1991; Monchois *et al.*, 1999a).

The C-terminal domains of *Streptococcus* and *Leuconostoc* GTF enzymes consist of a series of different tandem repeats, which have been divided into four classes: A, B, C and D. In addition to A and C repeats, DSRS from *Ln. mesenteroides* NRRL-512F contains N repeats (Monchois *et al.*, 1998b), which have not been identified in other GTFs. Alternansucrase from *Ln. mesenteroides* NRRL-B 1355 contains a single A repeat and distinct short repeats $DG(X)_4APY$ (Janecek *et al.*, 2000). Within the A–D repeats, a repeating unit designated YG can be distinguished

Bacterial strain	Main α-linkages in glucan polymer			I		II		III
				†		‡		§
Lb. reuteri 121	1→4/1→6	GTFA	1016	ANFDSVRV***D***APDNIDADLMNI	1056	HINIL***E***DWNHADPEY	1126	YSFVRAH***D***NNSQDQI
S. mutans GS5	1→3	GTFB	443	ANFDSIRV**D**AVDNVDADLLQI	484	HLSILEAWSDNDTPY	555	YSFIRAHDSEVQDLI
S. mutans GS5	1→6	GTFD	457	ANFDGVRVDAVDNVNADLLQI	498	HLSILEAWSDNDPQY	577	YIFIRAHDSEVQTVI
S. downei Mfe28	1→3	GTFI	445	ANFDSIRV**D**AVDNVDADLLQI	486	HVSIV**E**AWSDNDTPY	557	YSFARAH**D**SEVQDLI
S. downei Mfe28	1→6	GTFS	388	ANFDGVRVDAVDNVNADLLQI	429	HLSILEAWSGNDNDY	470	YVFIRAHDSEVQTRI
S. salivarius ATCC 25975	1→3	GTFJ	463	ANFDGIRVDAVDNVDADMLQL	504	HISVLEAWSLNDNHY	605	YVFIRAHDNNVQDII
S. salivarius ATCC 25975	1→6	GTFK	453	AHFDGIRVDAVDNVSVDMLQL	494	NISILEAWSHNDPYY	575	YLFVRAHDSEVQTVI
Ln. mesenteroides NRRL B-1299	1→6	DSRB	525	ANFDGIRVDAVDNVDADLLQI	566	HLSILEDWSHNDPEY	637	YSFVRAHDSEVQTVI
Ln. mesenteroides NRRL B- 512F	1→6	DSRS	543	ANFDGIRV**D**AVDNVDADLLQI	584	HLSILEDWSHNDPLY	655	YSFVRAHDSEVQTVI
Ln. mesenteroides NRRL B-1355	1→6/1→3	ASR	626	ANFDGIRVDAVDNVDADLLKI	667	HLSILEDWNGKDPQY	759	YSFVRAHDYDAQDPI
				*:**.:**** **:.*::::		::.::* *. * *		* * **** : * *

Fig. 1. Amino-acid sequence alignment of highly conserved stretches (I, II, III) in catalytic domains of dextran-, mutan-, alternan- and reuteransucrases of lactic acid bacteria (see also Monchois *et al.*, 1999c). GTFA, *Lb. reuteri* 121 (Kralj *et al.*, 2002); GTFB, *S. mutans* GS5 (Shiroza *et al.*, 1987); GTFD, *S. mutans* GS5 (Honda *et al.*, 1990); GTFS, *S. downei* Mfe28 (Gilmore *et al.*, 1990); GTFI, *S. downei* Mfe28 (Ferretti *et al.*, 1987); GTFJ, *Streptococcus salivarius* ATCC 25975 (Giffard *et al.*, 1991); GTFK, *S. salivarius* ATCC 25975 (Giffard *et al.*, 1993); DSRB, *Ln. mesenteroides* NRRL B-1299 (Monchois *et al.*, 1998a); DSRS, *Ln. mesenteroides* NRRL B-512F (Monchois *et al.*, 1997); ASR, *Ln. mesenteroides* NRRL B-1355 (Argüello-Morales *et al.*, 2000). *, Identical residue; :, highly conserved residue; ., conserved residue. †, Putative nucleophile (MacGregor *et al.*, 1996; Devulapalle *et al.*, 1997); ‡, putative acid/base catalyst (MacGregor *et al.*, 1996; Devulapalle *et al.*, 1997); §, putative residue stabilizing the transition state (MacGregor *et al.*, 1996; Devulapalle *et al.*, 1997). Catalytic amino acids mutated in other studies (Monchois *et al.*, 1999c) are shown in bold underlined type; catalytic amino acids of *Lb. reuteri* 121 identified and characterized in this study are shown in bold italic type.

(Giffard & Jacques, 1994). The C-terminal repeats play an important role in glucan binding (Abo *et al.*, 1991; Shah & Russell, 2002; Lis *et al.*, 1995; Monchois *et al.*, 1998b).

The structure of *Lb. reuteri* 121 GTFA is unusual because the above-mentioned repeats are not present in its N- and C-termini. Instead, GTFA possesses a relatively large N-terminal variable domain (702 amino acids) with 5 RDV repeats. Its relatively short C-terminal domain (267 amino acids) contains 11 YG repeats only (Fig. 2A) (Kralj *et al.*, 2002). This raised questions about the precise role of the N- and C-terminal domains in GTFA.

Here we describe the first molecular (construction of site-directed and deletion mutants) and biochemical (analysis of the main reactions catalysed by wild-type and mutant enzymes) characterization of an α-(1→4) synthesizing glucansucrase. Furthermore, we provide evidence for involvement of the C-terminal YG repeats in glucan binding.

METHODS

Bacterial strains, plasmids, media and growth conditions. *Escherichia coli* DH5α (Phabagen, Utrecht, The Netherlands; Hanahan, 1983), *E. coli* TOP 10 (Invitrogen) and plasmid pBluescript II SK$^+$

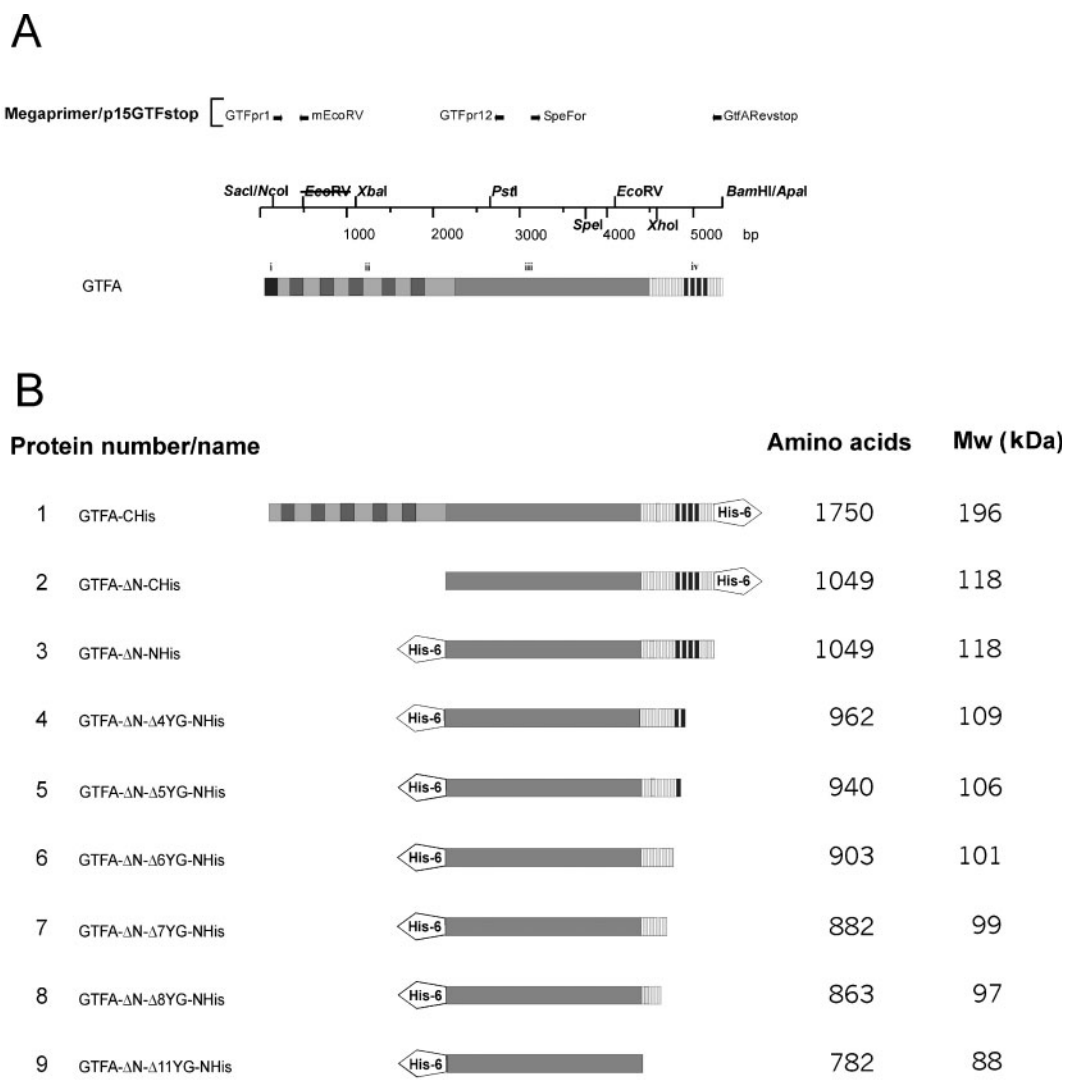

Fig. 2. Overview of primers and restriction sites used for cloning, expression and production of *Lb. reuteri* 121 GTFA protein and (deletion) mutant derivatives in *E. coli* together with domain organization of GTFA and (deletion) mutant derivatives. (A) Overview of primers used for the Megaprimer method and the construction of p15GTFstop. The different domains shown in GTFA are (i) N-terminal signal sequence (not present in mutants); (ii) variable region with five RDV repeats (dark-grey boxes); (iii) catalytic domain; (iv) C-terminal (putative) glucan-binding domain with five YG-repeating units (dark-grey boxes) according to the definition of (Giffard & Jacques, 1994) and seven less-conserved YG-repeating units (light-grey boxes). (B) Schematic representation of the domain structure of GTFA-CHis and the truncated derivatives constructed. Molecular sizes, number of amino acids and position of the His tag of the different protein constructs are indicated.

(Stratagene) were used for cloning. Plasmid pET15b (Novagen) was used for expression of the (mutant) *gtfA* gene(s) in *E. coli* BL21 Star (DE3) (Invitrogen). *E. coli* strains were grown aerobically at 37 °C in LB medium (Ausubel *et al.*, 1987). *E. coli* strains containing recombinant plasmids were cultivated in LB medium with the appropriate antibiotic (100 μg ampicillin ml^{-1}). Agar plates were made by adding 1·5 % agar to the LB medium.

Molecular techniques. General procedures for cloning, *E. coli* transformation, DNA manipulation, and agarose gel electrophoresis were as described (Sambrook *et al.*, 1989). Restriction endonuclease digestion and ligation with T4 DNA ligase were performed as recommended by the enzyme suppliers (New England Biolabs; Roche Biochemicals). Primers were obtained from Eurogentec, Seraing, Belgium. Cycle sequencing (Murray, 1989) was performed on double-stranded DNA, using the Thermo Sequence fluorescent primer cycle sequence kit (Amersham Pharmacia Biotech). Sequence reactions were performed on the Amersham ALF-Express sequencing machine at the BioMedical Technology Center, Groningen. DNA was amplified by PCR on a DNA Thermal Cycler PTC-200 (MJ Research), using *Pwo* DNA polymerase (Roche Biochemicals). Fragments were isolated from agarose gels using a Qiagen gel extraction kit, following the instructions of the supplier.

Plasmid construction. Plasmid pBGTF1, pBluescript carrying the *Lb. reuteri* 121 gene encoding GTFA (Kralj *et al.*, 2002), without signal sequence and fused at the C-terminus with six histidine residues (1750 amino acids), was used. To facilitate further mutagenesis and nucleotide sequencing, the first of two *Eco*RV restriction sites (492 bp, 4105 bp) was altered in pBGTF1, using the megaprimer method (Sarkar & Sommer, 1990) and the following primers: GTFpr1, 5′-GATGCAT*GAGCTC***CCATGG**ACCAACAAGTTCAGCAAGCTTCC-3′, containing *Sac*I (italics) and *Nco*I (bold) restriction sites, and m*Eco*RV, 5′-GGTTGTGAT**G**TCACGCACAATGATTTG-3′, containing a mutated *Eco*RV site (underlined, silent mutation by change of base shown in bold). In a subsequent PCR reaction, the amplified product (~500 bp) was used as forward primer, together with GTFpr12 (5′-GTGCATTAAAGTACGTAACCAATCAGTATTTCCGG-3′), 1600 bp downstream of an *Xba*I restriction site (Fig. 2A). The resulting product of 2600 bp was digested with *Sac*I and *Xba*I and ligated in the corresponding sites of pBGTF1, yielding pBGTF2.

Plasmid pBGTF2 now consisted (from 5′ to 3′) of four cassettes of 1000–1500 bp: i) *Sac*I&(*Nco*I)/*Xba*I (1001 bp), ii) *Xba*I/*Pst*I (1546 bp), iii) *Pst*I/*Eco*RV (1454 bp), and iv) *Eco*RV/(*Bam*HI)&*Apa*I (1269 bp) (Fig. 2A). This plasmid was digested with *Pst*I (2655 bp) and *Eco*RV (4105 bp), and the resulting fragment (1454 bp) was ligated in the corresponding sites of pBluescript II SK^+ (Stratagene), yielding pBPE1500. This small construct was used for site-directed mutagenesis, sequencing, and rapid exchange (using *Pst*I and *Eco*RV restriction sites) with pBGTF2 (see below). pBGTF2 was also digested with *Nco*I/*Bam*HI (5257 bp) and ligated in the corresponding sites of pET15b (Novagen), yielding p15GTF2 (encoding GTFA-CHis; Fig. 2B). This construct was used in subsequent cloning steps.

In order to create a construct without a C-terminal His tag, but with a stop codon, the following primers were used to exchange the C-terminal part of p15GTF2: SpeFor, 5′-GGGCTTTCAGATGCAACTAATCGTTGGGG-3′, lying 400 bp upstream of a *Spe*I restriction site, and GtfARevstop, 5′-TCGAT*GGGCCCC***GGATCC**TATTATAGTTTATTTTGATCAAGCATCTTACC-3′, containing *Bam*HI (bold) and *Apa*I (italic) restriction sites (Fig. 2A). The resulting PCR product (~2000 bp) was digested with *Spe*I and *Bam*HI (1592 bp) and ligated in the corresponding sites of p15GTF2 (see above), yielding p15GTFstop. In all cases, successful mutagenesis was confirmed by nucleotide sequencing.

Site-directed mutagenesis of putative catalytic residues of GTFA. Plasmid pBPE1500 (see above) was used as template for mutagenesis. The QuickChange site-directed mutagenesis kit (Stratagene) was used to construct mutants D1024N, E1061Q and D1133N, using different primer pairs (Table 1A). Successful mutagenesis resulted in the appearance or disappearance of restriction sites, allowing rapid screening of potential mutants. After successful mutagenesis (confirmed by nucleotide sequencing), pBPE1500 (mutation-containing) was digested with *Pst*I and *Eco*RV and ligated in the corresponding sites of pBGTF2. The resulting plasmid, pBGTF2 (mutation-containing), was digested with *Nco*I/*Bam*HI, and the resulting 5·3 kb fragment was ligated into the corresponding sites of the expression vector pET15b (Novagen), yielding mutation-containing p15GTF2.

Construction of truncated *gtfA* genes. Appropriate primer pairs and template DNA were used (Table 1B) to construct eight different deletion mutants of *gtfA* (Fig. 2B). Deletion mutants were constructed by exchange of restriction fragments, and checked by nucleotide sequencing.

Purification of GTFA (mutant) proteins. Cells of *E. coli* BL21star (DE3) harbouring the different pET15b derivatives were harvested by centrifugation (10 min, 4 °C, 10 000 ***g***) after 16 h of growth at 37 °C without induction. The pellet was washed with 50 mM phosphate buffer, pH 8·0. Pelleted cells were resuspended in 50 mM sodium phosphate buffer, pH 8·0, containing 250 mM NaCl, 5 mM β-mercaptoethanol and 10 mM imidazole. Cells were broken by sonication (7 × 15 s at 7 micron with 30 s intervals), and centrifuged (10 min, 4 °C, 10 000 ***g***). The clear lysate containing GTF activity was loaded on a Ni-NTA column (Qiagen). Binding was realized using 50 mM sodium phosphate buffer, pH 8·0, containing 250 mM NaCl, 5 mM β-mercaptoethanol and 10 mM imidazole, followed by washing with the same buffer. Elution of His-tagged protein(s) was performed using 50 mM sodium phosphate buffer, pH 8·0, with 250 mM NaCl, 1 mM β-mercaptoethanol and 200 mM imidazole. Eluted proteins were desalted with 20 mM Tris buffer, pH 8·0, using a 5 ml Hi-Trap desalting column (Amersham Pharmacia Biotech). Subsequently, the samples were purified on an ACTA prime FPLC system (Amersham Pharmacia Biotech), using a 1 ml Resource-Q column (Amersham Pharmacia Biotech) and a linear gradient of 30 ml with 1 M NaCl in 20 mM Tris buffer, pH 8·0, as eluent, at a flow rate of 1 ml min^{-1}. Proteins present in the elution peak were desalted with 25 mM sodium acetate buffer, pH 4·7, supplemented with 1 mM $CaCl_2$, using a 5 ml Hi-Trap desalting column (Amersham Pharmacia Biotech). At each stage of the purification, the GTF-transferase activity was quantified, as previously described (van Geel-Schutten *et al.*, 1999). The degree of purity of the different mutants was determined by SDS-PAGE (Laemmli, 1970).

Protein concentrations were determined by the Bradford method, using the Bio-Rad reagent and BSA as a standard (Bio-Rad). From 250 ml cultures of *E. coli* strains expressing full-length (wild-type or mutant) and N- or C-terminally truncated protein, approximately 0·25 and 1 mg of highly purified protein were obtained, respectively.

Glucan binding assay. The glucan-binding ability of GTFA (deletion mutants) was measured as described by Lis *et al.* (1995), and as improved by Shah & Russell (2002). Briefly, 30 pmol of GTFA (mutant) protein in 200 μl PBS-T (20 mM sodium phosphate, pH 7·3, 150 mM NaCl, 0·05 % Tween 20) was incubated overnight at 4 °C in Ni-NTA-coated HisSorb plates (Qiagen). Proteins containing a His tag bound to the Ni-NTA; non-binding protein was removed by washing with PBS-T (four times 1 min). After incubation for 20 min with 200 μl biotin-labelled dextran (100 μg μl^{-1}; Fluka) in PBS-B (20 mM sodium phosphate, pH 7·3, 150 mM NaCl, 0·2 % BSA), which may bind to the glucan-binding domain

Table 1. Oligonucleotides used for site-directed (A) and deletion mutagenesis (B) of *Lb. reuteri* 121 *gtfA*

Nucleotides in bold type/italics represent mismatches with the sequence of *gtfA*. Underlined nucleotides represent introduced or removed (mutation E1061Q) restriction sites. H, histidine residue; st, stop codon.

Mutation	Forward primer (5′–3′)	Reverse primer (5′–3′)	Restriction sites and plasmids used to exchange fragments
A			
D1024N	GATTCTGTACGGGT***TAAC***GCACCGG *Hpa*I	CCGGTGC***GTTA***ACCCGTACAGAATC *Hpa*I	*Pst*I/*Eco*RV(pBGTF2)
E1061Q	GCATAT***A***AATATTCTT***C***AAGACTGGAATC (*Ase*I)	GATTCCAGTCTT***G***AAGAATATT***T***ATATGC (*Ase*I)	*Pst*I/*Eco*RV (pBGTF2)
D1133N	CGTTCGGGGC***C***CAC***A***A***C***AATAATTCTC *Apa*I	GAGAATTATT***G***T***T***GTG***G***GCCCGAACG *Apa*I	*Pst*I/*Eco*RV (pBGTF2)
B			
GTFA-ΔN-CHis	GATGCATGAGCTCCC**ATG**GGCATTAACGGTCAACAA *Sac*I *Nco*I ACCCTATTATATTG	GAGCATCTTGGTTATCACTTGCTCCAACTGG	*Nco*I/*Spe*I (p15GTF2)
GTFA-ΔN-NHis	GATGCATGAGCTCCCATGGGCCATCACCATCACCATCAC ***Sac*I *Nco*I H H H H H H** ATTAACGGTCAACAATATTATATTGACCC	GAGCATCTTGGTTATCACTTGCTCCAACTGG	*Nco*I/*Spe*I (p15GTFstop)
GTFA-ΔNΔ4YG-NHis	CGTGCTAATGTTCGAATTGCCCAAAATGCTG	ATATCGATGGGCCCCGGATCCTATTAAACGAAA *Apa*I *Bam*HI **st st** TGTTTATTTTCAACCATCTTACC	*Xho*I/*Bam*HI (p15GTFA-ΔN-NHis)
GTFA-ΔNΔ5YG-NHis	CGTGCTAATGTTCGAATTGCCCAAAATGCTG	ATATCGATGGGCCCCGGATCCTATTATTCAATA *Apa*I *Bam*HI **st st** AAGGTATTCTTAACCATATTACC	*Xho*I/*Bam*HI (p15GTFA-ΔN-NHis)
GTFA-ΔNΔ6YG-NHis	CGTGCTAATGTTCGAATTGCCCAAAATGCTG	ATATCGATGGGCCCCGGATCCTATTAAATTGGC *Apa*I *Bam*HI **st st** AAGAATTGCTTTGTAGTAGTACC	*Xho*I/*Bam*HI (p15GTFA-ΔN-NHis)
GTFA-ΔNΔ7YG-NHis	CGTGCTAATGTTCGAATTGCCCAAAATGCTG	ATATCGATGGGCCCCGGATCCTATTATACATAA *Apa*I *Bam*HI **st st** CCAGAACCACGATGGAGAATGTTTG	*Xho*I/*Bam*HI (p15GTFA-ΔN-NHis)
GTFA-ΔNΔ8YG-NHis	CGTGCTAATGTTCGAATTGCCCAAAATGCTG	ATATCGATGGGCCCCGGATCCTATTATTCAGTT *Apa*I *Bam*HI **st st** ATCTTTTGTGAAGGATCAATAGC	*Xho*I/*Bam*HI (p15GTFA-ΔN-NHis)
GTFA-ΔNΔ11YG-NHis	GGGCTTTCAGATGCAACTAATCGTTGGGG	ATATCGATGGGCCCCGGATCCTATTATTGGTCAG *Apa*I *Bam*HI **st st** GAACCCAATCCGCCATTAC	*Spe*I/*Bam*HI (p15GTFA-ΔN-NHis)

(GBD) of the protein, washing was performed with PBS-T, as described above. Extravidin–alkaline phosphatase conjugate (1/10 000; Sigma) in PBS-B, which binds to the biotin part of the labelled dextran, was added and incubated for 1 h, followed by washing with PBS-T. Subsequently, the alkaline phosphatase substrate 4-nitrophenyl phosphate (100 µl; Roche Biochemicals) was added, yielding a yellow colour upon hydrolysis by the bound alkaline phosphatase. The colour change was monitored using a SpectraMax Plus 384 plate reader (Molecular Devices).

Enzyme activity assays. The various reuteransucrase activities were determined by measuring glucose and fructose release (enzymically) from sucrose conversion (van Geel-Schutten *et al.*, 1999). The amount of fructose released (V_F) corresponds to total enzyme activity (initial formation of leucrose or other sucrose isomers was negligible; see Results and data not shown). The amount of free glucose (V_G) represents the hydrolytic activity of the enzyme. The amount of fructose minus the amount of free glucose reflects the transferase activity ($V_F - V_G$). Unless indicated otherwise, reactions were performed at 50 °C in 25 mM sodium acetate buffer, pH 4·7, containing 1 mM $CaCl_2$ and 30 nM purified (mutant) reuteransucrase enzyme. One unit of enzyme activity was defined as the release of 1 µmol of monosaccharide per min. In the case of very low activity of mutant proteins, assay conditions were modified as follows: reactions were performed with 25 times more protein, and aliquots were removed at 30 min intervals, instead of 1 min.

(i) Kinetic parameters. Kinetic assays were performed using 24 different sucrose concentrations, ranging from 0·25 to 100 mM. Over a 6 min incubation period, samples of 25 µl were withdrawn every minute and inactivated with 2·5 µl 1 M NaOH. Curve fitting of the data was performed with the SigmaPlot program (version 8.0), using either the Michaelis–Menten formula [$y=(a\times x)/(b+x)$] or the same formula with a substrate-inhibition constant [$y=(a\times x)/(b+x+(x^2/c))$]. In these equations, y is the specific activity (U mg^{-1}), x is the substrate concentration (mM sucrose), a is the maximal reaction rate [V_{max} (U mg^{-1})], b is the affinity constant for the substrate [K_m (mM sucrose)], and c is the substrate inhibition constant [K_i (mM sucrose)].

(ii) Effect of maltose on initial GTFA activity. The initial rate of oligosaccharide synthesis was examined by measuring the effect of maltose on (mutant) GTFA enzyme activity, using 50 mM sucrose and 100 mM maltose. The activity was determined by measuring fructose release.

Product analysis

(i) Product spectrum from sucrose. After complete depletion of sucrose (100 mM, 60 h at 50 °C), the concentrations of fructose, glucose and leucrose in the reaction medium of GTFA (mutants) were determined using anion-exchange chromatography (see below). The amount of fructose released (97·9 %) and leucrose (2·1 %) synthesized from sucrose corresponds to 100 %. Subtracting the free glucose (23·1 %; due to hydrolysis) from the free fructose (97·9 %) concentration allowed calculation of the yield of reuteran synthesis (74·8 %) from sucrose (data of GTFA-CHis used here as an example, see Table 4).

(ii) Oligosaccharide synthesis with maltose and isomaltose as acceptors. Oligosaccharide synthesis was analysed using 100 mM sucrose together with maltose or isomaltose (100 mM each). After complete consumption of sucrose (60 h at 50 °C), samples were diluted 500–1000 times in 90 % DMSO solution. Maltose, isomaltose, maltotriose, panose (Sigma), isomaltotriose (TNO Nutrition and Food Research, Groningen), sucrose (Acros Organics, Geel, Belgium), fructose, glucose (Merck) and leucrose (Pfeiffer & Langen, Köln, Germany) were used as standards. The percentage of oligosaccharide synthesis from sucrose and acceptor was determined by subtracting the amount of unused acceptor from the initial acceptor concentration. Separation of oligosaccharides was achieved with a CarboPac PA1 anion exchange column (250 mm × 4 mm; Dionex) coupled to a CarboPac1 guard column (Dionex). The following gradient was used: eluent A at 100 % (0 min), 100 % (5 min), 92 % (50 min), 0 % (55–58 min), 100 % (60 min), 100 % (75 min). Eluent A was sodium hydroxide (0·1 M) and eluent B was sodium acetate (0·6 M) in sodium hydroxide (0·1 M). Detection was performed with an ED40 Electrochemical detector (Dionex), with an Au working electrode and an Ag/AgCl reference electrode with a sensitivity of 300 nC. The pulse program used was: +1·0 V (0–0·40 s); +0·7 V (0·41–0·60 s); −0·1 V (0·61–1·00 s). Data were integrated using a Turbochrom (Applied Biosystems) data integration system.

To determine the degree of polymerization (DP) of unknown oligosaccharides, a BC-200 Ca^{2+} column (at 85 °C; 300 mm × 7·8 mm; Benson Polymeric, Reno, USA) eluted with water (0·2 ml min^{-1}) was used. Detection was done by using a model 830-RI refractive index detector at 40 °C (Jasco, Tokyo, Japan).

In vitro glucan production by (mutant) GTFA enzymes and glucan structure analysis

(i) Polymer production. Purified (mutant) enzyme preparations were incubated overnight with 146 mM sucrose, using the conditions described above under enzyme activity assays. Glucans produced were isolated by precipitation with ethanol (van Geel-Schutten *et al.*, 1999).

(ii) Methylation analysis. Polysaccharides were permethylated using methyl iodide and Na-Dimsyl in DMSO at room temperature (Hakomori, 1964). After hydrolysis with 2 M trifluoric acetic acid (1 h, 125 °C), the partially methylated monosaccharides were reduced with $NaBD_4$ (Harris *et al.*, 1984). The mixtures of partially methylated alditol acetates obtained were analysed by GLC on a CP Sil 5 CB column (25 m × 0·53 mm; Chrompack) and by GLC-MS on an RTX Sil MS column (30 m × 0·25 mm; Restek) (Chaplin, 1982; Jansson *et al.*, 1976).

(iii) Molecular masses of the glucans. Molecular mass analysis was performed as described previously, using high-performance size-exclusion chromatography (HPSEC) coupled on-line with multi-angle laser light scattering (MALLS) and differential refractive index detection (Kralj *et al.*, 2002).

RESULTS

Purification of (mutant) GTFA enzymes

Lb. reuteri 121 GTFA-CHis enzyme expressed in *E. coli* was purified to homogenity. Also, three site-directed (D1024N, E1061Q and D1133N; Fig. 1) and eight deletion mutants (GTFA-ΔN-CHis, GTFA-ΔN-NHis, GTFA-ΔNΔ4YG-NHis, GTFA-ΔNΔ5YG-NHis, GTFA-ΔNΔ6YG-NHis, GTFA-ΔNΔ7YG-NHis, GTFA-ΔNΔ8YG-NHis and GTFA-ΔNΔ11YG-NHis; Fig. 2B) were expressed in *E. coli* and purified to homogeneity. The predicted M_r of the different deletion variants (Fig. 2B) was in agreement with the results obtained by SDS-PAGE analysis (data not shown).

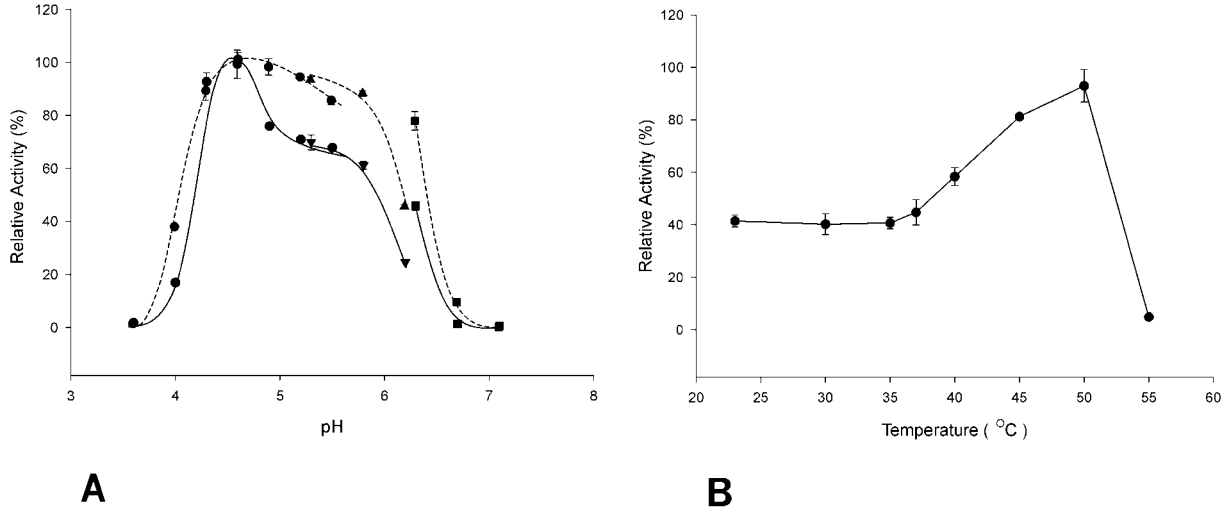

Fig. 3. (A) Effect of pH on *Lb. reuteri* 121 GTFA activity. Enzyme activity was determined at 50 °C in the presence of 1 mM $CaCl_2$ by measuring the amount of glucose and fructose released in 30 min from 50 mM sucrose by 30 nM GTFA-CHis (mean ± SEM; $n=3$). Solid line, transferase activity; dashed line, hydrolysis activity. (●) 25 mM Potassium acetate buffer; (▼) 25 mM MES buffer; (■) 25 mM MOPS buffer. (B) Effect of temperature on transferase activity of GTFA was evaluated as described for Fig. 3A (mean ± SEM; $n=3$).

Effects of pH, temperature and metal ions on GTFA activity

In order to define the best conditions for subsequent kinetic studies, the pH and temperature optima of GTFA activity were examined. The pH optimum for the hydrolysing activity was in the range pH 4·5–5·5 (Fig. 3A). For the transferase activity, a pH optimum at pH 4·7 was observed. The temperature optimum for both reactions was 50 °C. At lower temperatures, activity remained relatively high (Fig. 3B; only shown for transferase activity).

Different metal ions had strongly varying effects on the hydrolytic and transferase activities of GTFA (Table 2). Zn^{2+}, Hg^{2+}, Fe^{3+}, Fe^{2+} and Cu^{2+} ions significantly inhibited both enzyme activities, but especially the transferase activity, which was not detectable in the presence of these ions. EDTA also inhibited transferase activity completely, but hydrolysis remained almost unaffected. In contrast, Ca^{2+}, K^{+}, Na^{2+} and Mg^{2+} ions had stimulating effects on GTFA enzyme activities. Of these ions, Ca^{2+} had the most stimulating effect, with hydrolysis and transferase activities increased two and eightfold, respectively.

Kinetic studies of GTFA

(i) Kinetic parameters. In the presence of sucrose, GTFA-CHis displayed Michaelis–Menten type kinetics for the transferase reaction ($V_F - V_G$) and total enzyme activity (V_F). The hydrolysis reaction (V_G) displayed Michaelis–Menten kinetics with substrate inhibition (Fig. 4A and Table 3). The initial hydrolysis rate represents 95 % of the initial sucrose consumption rate in the presence of 0·5 mM sucrose, 75 % with 2·5 mM sucrose and only 65 % with 10 mM sucrose. These data reveal that GTFA-CHis favours hydrolysis at low sucrose concentrations and polymerization at high sucrose concentrations.

(ii) Effect of maltose on initial GTFA activity. Maltose stimulated the GTFA-CHis total activity (V_F) and transferase

Table 2. Effects of various compounds on *Lb. reuteri* 121 GTFA-CHis-catalysed activities

Activity measurements were done at 50 °C in 25 mM sodium acetate buffer, pH 4·7, with 50 mM sucrose. Glucose release (V_G; hydrolysis) and fructose minus glucose release ($V_F - V_G$; transferase) from sucrose by 30 nM GTFA-CHis after 30 min of incubation were taken as measures of enzymic activity. Results are given as mean ± SEM ($n=3$). ND, Transferase activity could not be detected.

Compound (1 mM)	Hydrolytic activity (%)	Transferase activity (%)
None	100 ± 10	100 ± 3
EDTA	89 ± 7	ND
$CaCl_2$	217 ± 18	846 ± 64
$MgCl_2$	109 ± 5	151 ± 13
$ZnCl_2$	54 ± 6	ND
KCl	122 ± 7	158 ± 3
$HgCl_2$	29 ± 2	ND
$FeCl_3$	26 ± 1	ND
$FeCl_2$	45 ± 1	ND
$CuCl_2$	23 ± 4	ND
NaCl	104 ± 3	121 ± 4

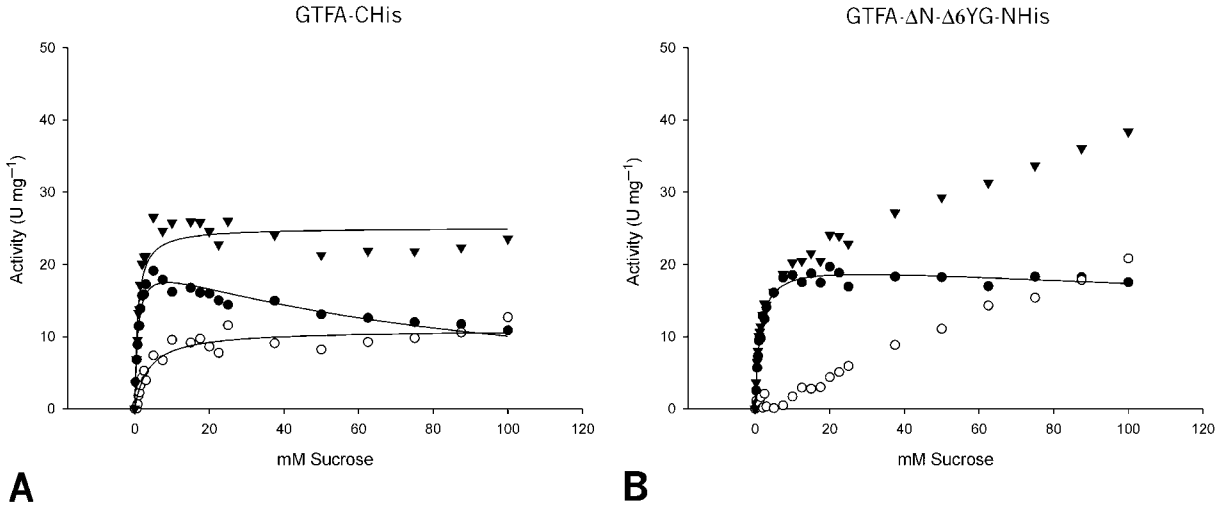

Fig. 4. (A) Effect of sucrose concentration on initial *Lb. reuteri* 121 GTFA-CHis enzyme activity at 50 °C in 25 mM sodium acetate buffer, pH 4·7, supplemented with 1 mM $CaCl_2$. (B) Effect of sucrose concentration on initial GTFA-ΔNΔ6YG-NHis enzyme activity. The mutants GTFA-ΔNΔ4YG-NHis and GTFA-ΔNΔ5YG-NHis displayed similar kinetics. Reactions were performed with 30 nM enzyme. (●) V_G (hydrolytic activity); (○) V_G-V_F (transferase activity); (▼) V_F (total activity).

activity (V_F-V_G) 3·5- and 7·5-fold, respectively. The hydrolysis (V_G) rate decreased 3·5-fold when maltose was present (Table 3B; data not shown).

Product analysis

(i) Product spectrum from sucrose. After complete depletion of sucrose, GTFA-CHis showed the following product distribution: reuteran synthesis, 74·8 %; hydrolysis, 23·1 %; and leucrose (α-D-glucopyranosyl-(1→5)-β-D-fructofuranoside) synthesis, 2·1 % (Table 4). Dionex analysis showed that a minor amount of isomaltose and significant amounts of an unknown product that eluted after 35 min were synthesized (data not shown).

(ii) Oligosaccharide synthesis with maltose and isomaltose as acceptor substrates. Dionex analysis showed that, in the presence of maltose, GTFA-CHis formed panose (α-D-Glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→4)-D-glucose) as the most abundant acceptor reaction product (from 100 mM sucrose and 100 mM maltose, approximately 44 mM panose was synthesized), indicating

Table 3. Kinetic parameters of wild-type and deletion mutants of *Lb. reuteri* 121 *gtfa*

(A) Kinetic parameters determined using 24 different concentrations of sucrose (0·25–100 mM); (B) initial total activity determined using 50 mM sucrose and 100 mM maltose. Kinetic parameters for GTFA-ΔN-Δ7YG-NHis, GTFA-ΔN-Δ8YG-NHis and GTFA-ΔN-Δ11YG-NHis were not determined due to their low activity (total activity 43, 63 and 1000 times reduced, respectively, compared to wild-type). *, Affinity for sucrose could not be determined when measuring transferase and total activity of GTFA-ΔN-Δ4YG-NHis, GTFA-ΔN-Δ5YG-NHis and GTFA-ΔN-Δ6YG-NHis, due to failure to reach saturation of the enzyme at the sucrose concentrations used, resulting in high standard errors with curve fits (see also Fig. 4B). Therefore, activities at a substrate concentration of 50 mM sucrose are depicted. Data obtained were fitted using the Michaelis–Menten equation, with or without (†) substrate inhibition.

A	k_{cat} (s^{-1})			K_m (mM)			K_i (mM), hydrolysis activity	B k_{cat} (s^{-1}), total activity
Enzyme	Total activity	Transferase activity	Hydrolysis activity	Total activity	Transferase activity	Hydrolysis activity		
GTFA-CHis	83·1±1·3	36·8±1·0	66·2±1·6	0·9±0·1	4·6±0·6	0·8±0·1	111±12	293
GTFA-ΔN-CHis	188·0±2·2	155·5±2·2	34·2±0·6	2·8±0·1	3·6±0·2	1·0±0·1	−†	354
GTFA-ΔN-NHis	152·0±2·4	112·9±3·7	45·6±0·6	7·9±1·0	3·9±0·3	0·9±0·1	−†	338
GTFA-ΔN-Δ4YG-NHis	139*	67*	81·0±1·8	−*	−*	1·7±0·1	297±48	630
GTFA-ΔN-Δ5YG-NHis	72*	33*	45·3±1·9	−*	−*	1·3±0·2	298±83	429
GTFA-ΔN-Δ6YG-NHis	49*	18*	33·5±0·8	−*	−*	1·3±0·1	635±195	363

Table 4. Effects of deletions in *Lb. reuteri* 121 GTFA on the product spectrum obtained with sucrose (100 mM), sucrose and maltose (100 mM each), or sucrose and isomaltose (100 mM each)

Oligosaccharide yield is expressed as a percentage of the total amount of acceptor used in the incubation.

Enzyme	Reuteran synthesis (%)	Leucrose synthesis (%)	Hydrolysis (%)	Acceptor reaction oligosaccharide yield (%) in the presence of:	
				Maltose	Isomaltose
GTFA-CHis	74·8±3·5	2·1±0·15	23·1±3·3	59·6±3·9	20·3±1·1
GTFA-ΔN-CHis	79·7±1·8	1·9±0·03	18·4±1·8	57·9±3·1	16·2±0·2
GTFA-ΔN-NHis	82·4±1·3	1·7±0·03	15·8±1·3	55·9±4·4	17·1±0·1
GTFA-ΔN-Δ4YG-NHis	86·0±2·5	1·8±0·08	12·2±2·5	59·1±9·1	22·1±2·6
GTFA-ΔN-Δ5YG-NHis	87·2±2·3	1·8±0·09	11·0±2·2	59·5±5·2	25·0±2·5
GTFA-ΔN-Δ6YG-NHis	85·7±0·6*	1·3±0·26*	13·0±0·4*	57·6±4·6	24·8±2·5

*Sucrose was not completely consumed.

that an α-1,6 linkage was formed at the non-reducing end of maltose (Fig. 5B). When isomaltose was used as acceptor, low amounts of isomaltotriose and isomaltotetraose (not shown in standard; elution between 35–36 min) were formed, together with two abundant unknown oligosaccharide products (Fig. 5C) with a DP of 3 (most likely isopanose, α-D-Glucopyranosyl-(1→4)-[α-D-glucopyranosyl-(1→6)]-D-glucose) and 4, respectively (DP determined by HPLC, data not shown).

Characteristics of mutants in the putative catalytic amino-acid residues

Based on alignments of GTFA with different GTF enzymes, three (putative) catalytic residues were identified in GTFA and mutated (Fig. 1). In the *E. coli* host strain, GTFA mutants D1024N, E1061Q and D1133N were expressed at levels similar to those of the wild-type enzyme (data not shown). Analysis of the purified proteins showed that they had almost completely lost their activity. Compared to wild-type, D1024N and E1061Q showed a 1000-fold reduction of total enzyme activity (V_F). Mutation D1133N resulted in an enzyme with a 300-fold reduced total activity (V_F).

Characteristics of GTFA deletion mutants

(i) Glucan binding. GTFA-ΔN-CHis displayed lower glucan-binding activity (GBA) than GTFA-ΔN-NHis (Figs 2B and 6). GTFA-CHis showed virtually no GBA. GTFA-ΔNΔ4YG-NHis completely lacked GBA. GTFA-ΔNΔ5YG-NHis, GTFA-ΔNΔ6YG-NHis and GTFA-ΔNΔ7YG-NHis were again active, but showed gradually lower GBA (Fig. 6). GTFA-ΔNΔ8YG-NHis and GTFA-ΔNΔ11YG-NHis were virtually inactive.

(ii) Kinetic analysis. GTFA-ΔN-NHis and GTFA-ΔN-CHis (Fig. 2B) displayed Michaelis–Menten type kinetics in all three reactions. Interestingly, both mutants had a three to fourfold increased transferase activity, whereas their hydrolytic activities decreased (Table 3). The Michaelis–Menten formula with a substrate inhibition constant was used to fit the hydrolytic activity of the mutants GTFA-ΔN-Δ4YG-NHis, GTFA-ΔN-Δ5YG-NHis and GTFA-ΔN-Δ6YG-NHis. The transferase and total activity of these mutants could not be fitted with the Michaelis–Menten formula. The affinity for sucrose had decreased drastically in both reactions, and no saturation was reached with the sucrose concentrations used (Fig. 4B). Sequential deletions of the YG repeats from the C-terminus resulted in initially lower hydrolytic activity compared to the wild-type enzyme (Table 3). However, the affinity for sucrose in the hydrolysis reaction was comparable to wild-type (Table 3). Kinetic parameters for mutants GTFA-ΔN-Δ7YG-NHis, GTFA-ΔN-Δ8YG-NHis and GTFA-ΔN-Δ11YG-NHis were not determined due to their low total activity (43, 63 and 1000 times reduced, respectively, compared to wild-type levels).

(iii) Effect of maltose on initial mutant GTFA activity. The stimulatory effect of maltose on GTFA-ΔN-NHis and GTFA-ΔN-CHis total activity was 1·5 to 2 times lower than on wild-type. In contrast, total activity of mutants GTFA-ΔN-Δ4YG-NHis, GTFA-ΔN-Δ5YG-NHis and GTFA-ΔN-Δ6YG-NHis was stimulated 5·5, 6 and 7·5 times, respectively, by maltose (Table 3B).

(iv) Product spectrum from sucrose. When incubated with sucrose, mutants with C-terminal deletions showed slightly increased reuteran synthesis and (slightly) decreased hydrolysis (Table 4).

(v) Oligosaccharide synthesis with maltose and isomaltose as acceptor substrates. In the acceptor reaction, all deletion mutants showed similar oligosaccharide yields to wild-type (Table 4). The deletion mutants produced the same oligosaccharides as wild-type (Fig. 5 and data not shown).

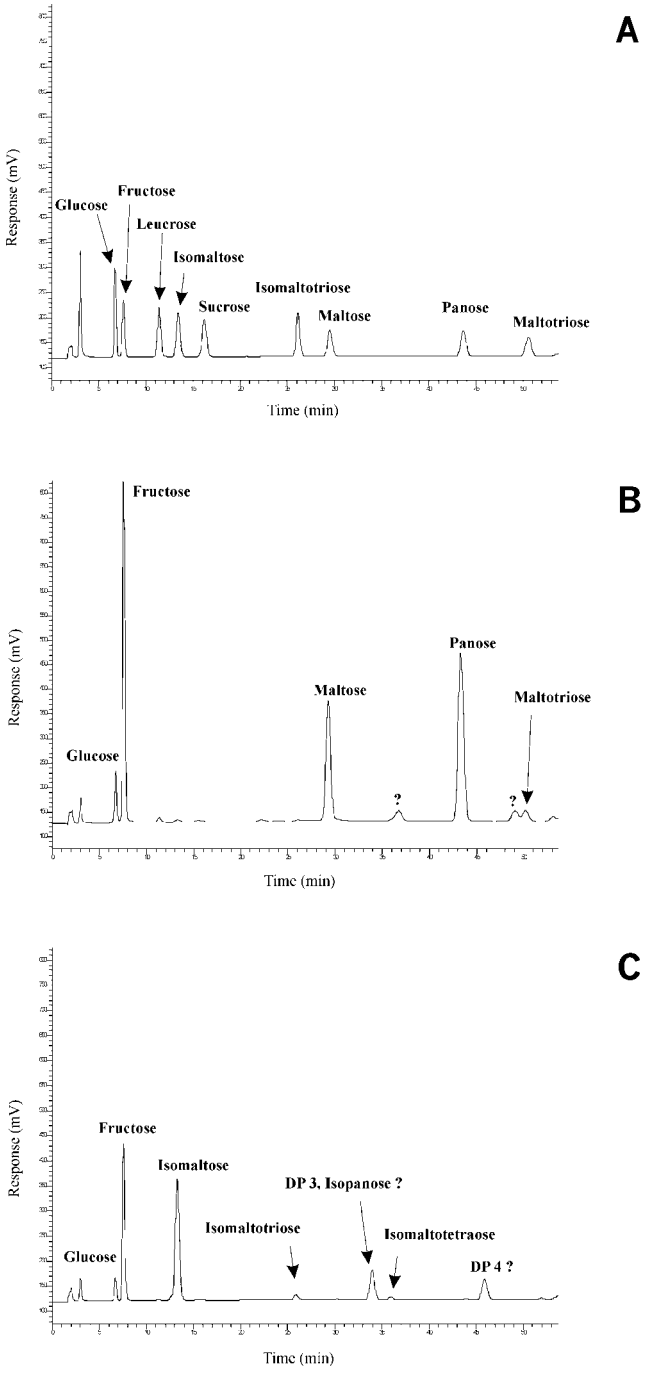

Fig. 5. Dionex analysis of *Lb. reuteri* 121 GTFA acceptor reaction products (as described in Methods). (A) Elution profile of a standard mixture; (B) products formed on incubation of 30 nM GTFA-CHis enzyme with 100 mM sucrose and 100 mM maltose for 60 h; (C) products formed on incubation of 30 nM GTFA-CHis enzyme with 100 mM sucrose and 100 mM isomaltose for 60 h.

(vi) Molecular sizes and linkage-type analysis of glucans produced. Compared to GTFA-CHis, the deletion mutants showed no drastic changes in the sizes and linkage types of the glucans produced (Table 5). However, the glucan products of mutants GTFA-ΔNΔ5YG-NHis and GTFA-ΔNΔ6YG-NHis showed less branching (lower percentage of both terminal and branched α-(1→4,6) glucosyl units), a higher percentage of α-(1→6) linkages, and slightly decreased molecular masses compared to wild-type enzyme and other deletion mutants (Table 5).

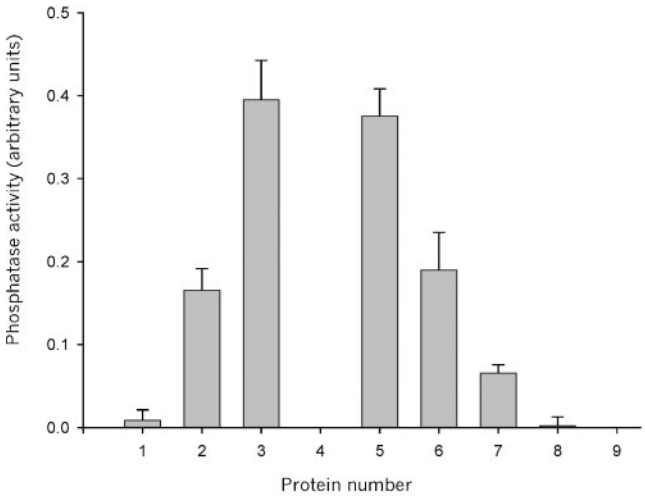

Fig. 6. Glucan-binding activity of the different *Lb. reuteri* 121 GTFA deletion mutants, measured 5·5 h after addition of substrate (mean ± SEM; $n = 3$). Protein numbers refer to the (deletion) mutants shown in Fig. 2B.

DISCUSSION

Characterization of GTFA

This paper is believed to report the first detailed biochemical and molecular characterization of the recombinant glucansucrase GTFA of a *Lactobacillus* strain, synthesizing a polymer with mainly α-(1→4) glucosidic linkages (a reuteransucrase).

The GTFA pH optimum was comparable to that of other glucansucrases. Interestingly, transferase activity showed a sharp optimum around pH 4·7, whereas the hydrolysis reaction had a broader optimal pH range (Fig. 3A). This is the first demonstration of different pH profiles for these GTF-catalysed reactions. A striking feature of the recombinant GTFA-CHis protein is its high optimal temperature of 50 °C (Fig. 3B). This high temperature optimum has also been observed for the inulosucrase and levansucrase enzymes of the same strain (van Hijum *et al.*, 2002, 2003, 2004).

Of all cations tested, Ca^{2+} had the most stimulating effect on enzyme activity (Table 2): hydrolysis and transferase activity were increased two and eightfold, respectively. Zn^{2+}, Hg^{2+}, Fe^{3+}, Fe^{2+} and Cu^{2+} ions significantly inhibited enzyme activity. These ions (except for Hg^{2+}, which was not tested) also inhibit the *gtfB*-, *gtfC*- and

Table 5. Methylation analysis and molecular masses of the glucans produced by purified wild-type *Lb. reuteri* 121 GTFA enzyme and derived deletion mutants

Type of glucosyl unit	Methylation (%)					
	GTFA-CHis	GTFA-ΔN-CHis	GTFA-ΔN-NHis	GTFA-ΔN-Δ4YG-NHis	GTFA-ΔN-Δ5YG-NHis	GTFA-ΔN-Δ6YG-NHis
Glc*p*-(1→	9	9	8	9	5	5
→4)-Glc*p*-(1→	49	46	49	48	46	47
→6)-Glc*p*-(1→	26	34	28	29	40	40
→4,6)-Glc*p*-(1→	15	12	15	14	9	8
	Molecular mass (1×10^6 Da)					
	45	50	48	47	38	39

gtfD-encoded enzymes of *Streptococcus mutans* (Wunder & Bowen, 1999).

Kinetics of GTFA

Kinetic analysis of GTFA-CHis revealed interesting features (Fig. 4). At high sucrose concentrations, substrate inhibition was observed for the GTFA-CHis hydrolysis reaction. GTFA-CHis clearly favours hydrolysis at low sucrose concentrations and transferase at high sucrose concentrations. The same phenomenon has otherwise been observed only for the family 13 enzyme amylosucrase from *Neisseria polysaccharea* (Potocki de Montalk *et al.*, 2000).

The GTFA affinity for sucrose in the total reaction was much higher (K_m 0·9 mM) than that observed for GTFI of *S. downei* Mfe28 and DSRS of *Ln. mesenteroides* NRRL B512F (K_m 49 and 26 mM, respectively; Monchois *et al.*, 1997, 2000a). The stimulating effect of maltose on initial GTFA velocity (three to fourfold activation of total activity) was also observed for DSRS of *Ln. mesenteroides* NRRL B-512F and GTFI of *S. downei* Mfe28 (two and fourfold stimulation of total activity, respectively; Monchois *et al.*, 1997, 2000a).

Product spectrum from sucrose

Upon depletion of sucrose, GTFA formed only low levels of leucrose (2·1 % of total sucrose consumed), even when incubated together with a high (100 mM) concentration of fructose (about 5 % leucrose synthesized) (Table 4 and data not shown). GTFI of *S. downei*, on the other hand, produced significant amounts (24·0 %) of leucrose (Monchois *et al.*, 2000a, b). GTFA synthesized more glucan (74·8 %), and was more effective in hydrolysis (23·1 %) than GTFI of *S. downei* Mfe28 (60·0 % and 16·2 %, respectively; Monchois *et al.*, 2000a). Fructose is thus a better acceptor for GTFI of *S. downei* Mfe28 than for GTFA of *Lb. reuteri* 121.

Oligosaccharide synthesis with maltose and isomaltose as acceptors

Analysis of the acceptor reaction of GTFA revealed that, with glucose and sucrose, only a small amount of isomaltose was formed (data not shown). Previous studies showed that the production of isomaltose by DSRS from *Ln. mesenteroides* NRRL B-512F could be improved by using high amounts of sucrose and glucose (Buchholz & Seibel, 2003).

Maltose is a good acceptor reaction substrate for DSRS of *Ln. mesenteroides* B-512F (Robyt & Walseth, 1978; Monchois *et al.*, 1997), for GTFI of *S. downei* Mfe28 (Monchois *et al.*, 1999b) and for GTFA of *Lb. reuteri* 121 (this study). GTFA produced panose as major product with maltose as acceptor (formation of an α-(1→6) linkage to the non-reducing end of maltose), together with low amounts of maltotriose and two unknown products (Fig. 5B). The formation of panose has also been observed for other GTF enzymes (Monchois *et al.*, 1996, 1997, 1998a, 2000b; Robyt, 1996). Compared to GTFI of *S. downei* Mfe28 (91·3 %) and DSRS of *Ln. mesenteroides* NRRL-B512F (93 %; Monchois *et al.*, 1998b, 2000a), the yield of oligosaccharides synthesized by GTFA-CHis with maltose as acceptor substrate (59·6 %) is lower. Maltose is thus a better acceptor reaction substrate for GTFI and DSRS than for GTFA.

When isomaltose was used as acceptor reaction substrate, two unknown products (identified by HPLC as DP 3 and DP 4, data not shown) were produced. Maltotriose or panose eluted at different time points than the DP 3 oligosaccharide; its most likely identity is isopanose. The coupling of a glucose moiety, using one of the two linkage types synthesized by GTFA, α-(1→4) and α-(1→6), to the reducing end of isomaltose would have resulted in the formation of isomaltotriose (of which small amounts are indeed synthesized) or panose (not formed) (Fig. 5C). However, various studies show that glucansucrases elongate oligosaccharides at their non-reducing end (Mukasa *et al.*, 2000; Dols *et al.*, 1997; Argüello Morales *et al.*, 2001; Monchois *et al.*, 2000a). The small amount of isomaltotriose is thus probably formed by coupling of glucose with an α-(1→6) linkage to the non-reducing end of isomaltose. Linkage specificity (inside glucans) of several glucansucrases is conserved in oligosaccharide synthesis (Dols *et al.*, 1997; Cote & Robyt, 1982; Robyt & Walseth, 1978). This suggests that the DP 3 oligosaccharide is isopanose (coupling of a glucose unit by

an α-(1→4) glucosidic bond onto the non-reducing end of isomaltose). Besides the DP 3 oligosaccharide, a DP 4 oligosaccharide was also synthesized. The exact nature of both oligosaccharides will be analysed in future. However, it appears likely that reuteransucrase is capable of forming both α-(1→4) and α-(1→6) glycosidic bonds with acceptor reaction substrates (Fig. 5).

Characteristics of catalytic mutants

Mutagenesis of the *Lb. reuteri* strain 121 GTFA putative catalytic residues (mutants D1024N, E1061Q and D1133N) resulted in drastically reduced total enzymic activities. D1024 of GTFA is homologous to: (1) the Asp residues identified by Mooser *et al.* (1991) as part of the active site of *Streptococcus sobrinus* GTFI and GTFS, (2) Asp451 of GTFB from *S. mutans* GS5, (3) Asp453 of GTFI from *S. downei* Mfe28 and (4) Asp551 of DSRS of *Ln. mesenteroides* NRRL-B512F, which were also shown to be essential for activity (Kato *et al.*, 1992; Monchois *et al.*, 1997; Devulapalle *et al.*, 1997; Fig. 1). GTFA residues E1061 and D1133 correspond to residues E491 and D564 in GTFI of *S. downei* Mfe28. These residues are essential for activity in both GTFI and GTFA (Devulapalle *et al.*, 1997; this study).

Characteristics of deletion mutants

GTFA possesses a relatively large N-terminal variable domain (702 amino acids) containing 5 RDV repeats (Kralj *et al.*, 2002). Its complete deletion had a drastic effect on enzyme kinetics. The initial transferase activity of GTFA-ΔN-CHis and GTFA-ΔN-NHis increased three to fourfold, whereas the hydrolytic activity decreased (Table 3). Conceivably, the large N-terminal domain in the wild-type protein causes steric hindrance to the growing glucan chain, its deletion resulting in a strongly increased transferase activity. The affinities for the substrate sucrose remained similar, in both the transferase and the hydrolysis reaction (Table 3). The data show that the large N-terminal variable domain is important for initial activity with sucrose (Table 3), but has only small effects on the product spectrum with sucrose alone, or sucrose plus acceptor substrates (Table 4), and on glucan characteristics (Table 5).

The GTFA mutants with N-terminal and additional C-terminal deletions showed drastically decreased affinity for sucrose in the total (V_F) and transferase ($V_F - V_G$) reactions (Fig. 4B and Table 3). In contrast, C-terminal deletions in DSRS of *Ln. mesenteroides* NRRL-B512F have virtually no effects on K_m values with sucrose (Monchois *et al.*, 1998b). Sequential deletions of the YG repeats from the C-terminus onwards resulted in gradually lower activity of the mutant GTFA enzymes in all reactions (Table 3A). The stimulatory effect of maltose increased upon deletion of C-terminal YG repeats (Table 3B). Both phenomena have also been observed for DSRS from *Ln. mesenteroides* NRRL-B512F (Monchois *et al.*, 1998b). The increased stimulatory effect of maltose on C-terminal deletions in DSRS was explained by a change in a rate-limiting reaction step. C-terminal deletions in GTFI of *S. downei* Mfe28 have no effect on the distribution of glucosyl residues during mutan synthesis (Monchois *et al.*, 1999a). The reuterans produced by wild-type GTFA and C-terminal deletion mutants had comparable sizes and linkage-type distribution, except for GTFA-ΔNΔ5YG-NHis and GTFA-ΔNΔ6YG-NHis. The latter mutants showed less branching (lower amount of both terminal and branched α-(1→4,6) glucosyl units), a higher percentage of α-(1→6) linkages, and a slightly decreased molecular mass compared to the wild-type and other mutants (Table 5). C-terminal deletions in GTFI of *S. downei* Mfe28 do not affect glucan structure (Monchois *et al.*, 1999a). However, deletion of six internal units (A–C) of GTFG of *Streptococcus gordonii* also affects glucan structure (Vickerman *et al.*, 1996). GTFA enzyme activity could still be detected after removal of more than half (152 amino acids, 6 YG repeats) of the GBD (Table 3). When more than six YG repeats were removed, enzyme activity was reduced drastically (Table 3). C-terminal deletions in other glucansucrases also affect enzyme activity (Monchois *et al.*, 1998b; Kato & Kuramitsu, 1990). Only in the case of GTFI of *S. downei* Mfe28 could the C-terminal domain be removed completely without large loss of activity (Monchois *et al.*, 1999a).

The C-terminal domains of glucansucrases from leuconostocs and streptococci are involved in glucan binding (Abo *et al.*, 1991; Shah & Russell, 2002; Lis *et al.*, 1995; Monchois *et al.*, 1998b). The A repeats found in the C-terminal domains of these GTF enzymes are not present in *Lb. reuteri* 121 GTFA. Instead, 11 YG repeats have been identified (Kralj *et al.*, 2002). Their deletion revealed that the GTFA C-terminal domain and these YG repeats are involved in glucan binding (Fig. 6). GTFA-ΔNΔ4YG-NHis was not able to bind biotin–dextran, which may be due to conformational changes in this mutant protein. GTFA-CHis showed virtually no GBA. The location of the His tag (GTFA-ΔN-CHis also showed lower GBA than GTFA-ΔN-NHis), together with the presence of the large N-terminal variable domain, may block the access of the GBD to the biotin–dextran. We had to incubate the GTFA protein/biotin–dextran/extravidin–alkaline phosphatase complex for several hours with the 4-nitrophenyl phosphate substrate to be able to measure GBA, whereas in a previous report with GFTI of *S. downei* Mfe28, incubation for a few minutes was enough to measure comparable amounts of GBA (Shah & Russell, 2002). Reuteran, rather than biotin–dextran, may be the preferred ligand for the C-terminal repeats of GTFA. Nevertheless, we show for the first time that, in reuteransucrase also, an α-(1→4) synthesizing glucansucrase from a *Lactobacillus* strain, the C-terminal domain is involved in glucan binding. Furthermore, positions at which truncation prevented glucan binding (deletion of eight YG repeats, 100 amino acids after the end of catalytic core) were identified (Fig. 6).

The GTFA hydrolytic activity remained upon deletion of

YG repeats, whereas glucan binding and affinity for sucrose in the transferase reaction decreased. This may imply that the glucan-binding domain is involved in polymer chain growth, as previously postulated for the GTFI from *S. mutans* GS5 and GTFI from *S. sobrinus* 6715 (Kato & Kuramitsu, 1990; Abo *et al.*, 1991).

Conclusion

The first thorough molecular and biochemical investigation of GTFA, a GTF enzyme synthesizing linkages of the α-(1→4) glucosidic type, has been carried out. This includes analysis of the kinetics of wild-type enzyme and derived N- and C-terminally truncated mutants, analysis of the acceptor reaction, and analysis of glucan binding by the C-terminal domain. Finally, three catalytically important residues have been identified and characterized.

ACKNOWLEDGEMENTS

We thank Peter Sanders for help with the Dionex and GC analysis and the kind gift of isomaltotriose, Wim Soetaert for the kind gift of leucrose, Albert Woortman and Peter Steeneken for help with the methylation analysis, Gert-Jan Euverink for help with enzyme purification and assays, Elly Faber for GLC-MS analysis and Kees de Ruijter for the HPSEC-MALLS analysis.

REFERENCES

Abo, H., Matsumura, T., Kodama, T., Ohta, H., Fukui, K., Kato, K. & Kagawa, H. (1991). Peptide sequences for sucrose splitting and glucan binding within *Streptococcus sobrinus* glucosyltransferase (water-insoluble glucan synthetase). *J Bacteriol* **173**, 989–996.

Argüello-Morales, M. A., Remaud-Simeon, M., Pizzut, S., Sarçabal, P., Willemot R.-M & Monsan, P. (2000). Sequence analysis of the gene encoding alternansucrase, a sucrose glucosyltransferase from *Leuconostoc mesenteroides* NRRL B-1355. *FEMS Microbiol Lett* **182**, 81–85.

Argüello Morales, M. A., Remaud-Simeon, M., Willemot, R.-M., Vignon, M. R. & Monsan, P. (2001). Novel oligosaccharides synthesized from sucrose donor and cellobiose acceptor by alternansucrase. *Carbohydr Res* **331**, 403–411.

Ausubel, F. M., Brent, R. E., Kingston, D. D., Moore, J. G., Seidman, J. G., Smith, J. A. & Struhl, K. (1987). *Current Protocols in Molecular Biology*. New York: Wiley.

Bozonnet, S., Dols-Laffargue, M., Fabre, E., Pizzut, S., Remaud-Simeon, M., Monsan, P. & Willemot, R.-M. (2002). Molecular characterization of DSR-E, an α-1,2 linkage-synthesizing dextransucrase with two catalytic domains. *J Bacteriol* **184**, 5753–5761.

Buchholz, K. & Seibel, J. (2003). Isomaltooligosaccharides. In *Oligosaccharides in Food and Agriculture*, pp. 63–75. Edited by G. Eggleston & G. L. Côté. Washington: American Chemical Society.

Chaplin, M. F. (1982). A rapid and sensitive method for the analysis of carbohydrate components in glycoproteins using gas-liquid chromatography. *Anal Biochem* **123**, 336–341.

Cote, G. L. & Robyt, J. F. (1982). Isolation and partial characterization of an extracellular glucansucrase from *Leuconostoc mesenteroides* NRRL B-1355 that synthesizes an alternating (1→6), (1→3)-α-D-glucan. *Carbohydr Res* **101**, 57–74.

Devulapalle, K. S., Goodman, S. D., Gao, Q., Hemsley, A. & Mooser, G. (1997). Knowledge-based model of a glucosyltransferase from the oral bacterial group of mutans streptococci. *Protein Sci* **6**, 2489–2493.

Dols, M., Simeon, M. R., Willemot, R. M., Vignon, M. R. & Monsan, P. F. (1997). Structural characterization of the maltose acceptor-products synthesized by *Leuconostoc mesenteroides* NRRL B-1299 dextransucrase. *Carbohydr Res* **305**, 549–559.

Ferretti, J. J., Gilpin, M. L. & Russell, R. R. (1987). Nucleotide sequence of a glucosyltransferase gene from *Streptococcus sobrinus* MFe28. *J Bacteriol* **169**, 4271–4278.

Funane, K., Mizuno, K., Takahara, H. & Kobayashi, M. (2000). Gene encoding a dextransucrase-like protein in *Leuconostoc mesenteroides* NRRL B-512F. *Biosci Biotechnol Biochem* **64**, 29–38.

Giffard, P. M. & Jacques, N. A. (1994). Definition of a fundamental repeating unit in streptococcal glucosyltransferase glucan-binding regions and related sequences. *J Dent Res* **73**, 1133–1141.

Giffard, P. M., Simpson, C. L., Milward, C. P. & Jacques, N. A. (1991). Molecular characterization of a cluster of at least two glucosyltransferase genes in *Streptococcus salivarius* ATCC 25975. *J Gen Microbiol* **137**, 2577–2593.

Giffard, P. M., Allen, D. M., Milward, C. P., Simpson, C. L. & Jacques, N. A. (1993). Sequence of the *gtfK* gene of *Streptococcus salivarius* ATCC 25975 and evolution of the *gtf* genes of oral streptococci. *J Gen Microbiol* **139**, 1511–1522.

Gilmore, K. S., Russell, R. R. & Ferretti, J. J. (1990). Analysis of the *Streptococcus downei gtfS* gene, which specifies a glucosyltransferase that synthesizes soluble glucans. *Infect Immun* **58**, 2452–2458.

Hakomori, S. (1964). A rapid permethylation of glycolipid and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. *J Biochem* **55**, 205–208.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.

Harris, P. J., Henry, R. J., Blakeney, A. B. & Stone, B. A. (1984). An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. *Carbohydr Res* **127**, 59–73.

Honda, O., Kato, C. & Kuramitsu, H. K. (1990). Nucleotide sequence of the *Streptococcus mutans gtfD* gene encoding the glucosyltransferase-S enzyme. *J Gen Microbiol* **136**, 2099–2105.

Janecek, S., Svensson, B. & Russell, R. R. (2000). Location of repeat elements in glucansucrases of *Leuconostoc* and *Streptococcus* species. *FEMS Microbiol Lett* **192**, 53–57.

Jansson, P.-E., Kenne, L., Liedgren, H., Lindberg, B. & Lönngren, J. (1976). A practical guide to the methylation analysis of carbohydrates. *Chem Commun Univ Stockholm* **8**, 1–74.

Kato, C. & Kuramitsu, H. K. (1990). Carboxyl-terminal deletion analysis of the *Streptococcus mutans* glucosyltransferase-I enzyme. *FEMS Microbiol Lett* **72**, 299–302.

Kato, C., Nakano, Y., Lis, M. & Kuramitsu, H. K. (1992). Molecular genetic analysis of the catalytic site of *Streptococcus mutans* glucosyltransferases. *Biochem Biophys Res Commun* **189**, 1184–1188.

Kralj, S., van Geel-Schutten, G. H., Rahaoui, H., Leer, R. J., Faber, E. J., van der Maarel, M. J. & Dijkhuizen, L. (2002). Molecular characterization of a novel glucosyltransferase from *Lactobacillus reuteri* strain 121 synthesizing a unique, highly branched glucan with α-(1→4) and α-(1→6) glucosidic bonds. *Appl Environ Microbiol* **68**, 4283–4291.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lis, M., Shiroza, T. & Kuramitsu, H. K. (1995). Role of C-terminal direct repeating units of the *Streptococcus mutans* glucosyltransferase-S in glucan binding. *Appl Environ Microbiol* **61**, 2040–2042.

MacGregor, E. A., Jespersen, H. M. & Svensson, B. (1996). A circularly permuted α-amylase-type α/β-barrel structure in glucan-synthesizing glucosyltransferases. *FEBS Lett* **378**, 263–266.

McCarter, J. D. & Withers, S. G. (1994). Mechanisms of enzymatic glycoside hydrolysis. *Curr Opin Struct Biol* **4**, 885–892.

Monchois, V., Willemot, R. M., Remaud-Simeon, M., Croux, C. & Monsan, P. (1996). Cloning and sequencing of a gene coding for a novel dextransucrase from *Leuconostoc mesenteroides* NRRL B-1299 synthesizing only α(1-6) and α(1-3) linkages. *Gene* **182**, 23–32.

Monchois, V., Remaud-Simeon, M., Russell, R. R., Monsan, P. & Willemot, R. M. (1997). Characterization of *Leuconostoc mesenteroides* NRRL B-512F dextransucrase (DSRS) and identification of amino-acid residues playing a key role in enzyme activity. *Appl Microbiol Biotechnol* **48**, 465–472.

Monchois, V., Remaud-Simeon, M., Monsan, P. & Willemot, R. M. (1998a). Cloning and sequencing of a gene coding for an extracellular dextransucrase (DSRB) from *Leuconostoc mesenteroides* NRRL B-1299 synthesizing only a α(1-6) glucan. *FEMS Microbiol Lett* **159**, 307–315.

Monchois, V., Reverte, A., Remaud-Simeon, M., Monsan, P. & Willemot, R. M. (1998b). Effect of *Leuconostoc mesenteroides* NRRL B-512F dextransucrase carboxy-terminal deletions on dextran and oligosaccharide synthesis. *Appl Environ Microbiol* **64**, 1644–1649.

Monchois, V., Arguello-Morales, M. & Russell, R. R. B. (1999a). Isolation of an active catalytic core of *Streptococcus downei* MFe28 GTF-I glucosyltransferase. *J Bacteriol* **181**, 2290–2292.

Monchois, V., Vignon, M. & Russell, R. R. (1999b). Isolation of key amino acid residues at the N-terminal end of the core region *Streptococcus downei* glucansucrase, GTF-I. *Appl Microbiol Biotechnol* **52**, 660–665.

Monchois, V., Willemot, R. M. & Monsan, P. (1999c). Glucansucrases: mechanism of action and structure-function relationships. *FEMS Microbiol Rev* **23**, 131–151.

Monchois, V., Vignon, M., Escalier, P. C., Svensson, B. & Russell, R. R. (2000a). Involvement of Gln937 of *Streptococcus downei* GTF-I glucan-sucrase in transition-state stabilization. *Eur J Biochem* **267**, 4127–4136.

Monchois, V., Vignon, M. & Russell, R. R. (2000b). Mutagenesis of Asp-569 of glucosyltransferase I glucansucrase modulates glucan and oligosaccharide synthesis. *Appl Environ Microbiol* **66**, 1923–1927.

Mooser, G., Hefta, S. A., Paxton, R. J., Shively, J. E. & Lee, T. D. (1991). Isolation and sequence of an active-site peptide containing a catalytic aspartic acid from two *Streptococcus sobrinus* α-glucosyl-transferases. *J Biol Chem* **266**, 8916–8922.

Mukasa, H., Shimamura, A. & Tsumori, H. (2000). Nigero-oligosaccharide acceptor reaction of *Streptococcus sobrinus* glucosyl-transferase GTF-I. *Carbohydr Res* **326**, 98–103.

Murray, V. (1989). Improved double-stranded DNA sequencing using the linear polymerase chain reaction. *Nucleic Acids Res* **17**, 8889.

Potocki de Montalk, G., Remaud-Simeon, M., Willemot, R. M., Sarcabal, P., Planchot, V. & Monsan, P. (2000). Amylosucrase from *Neisseria polysaccharea*: novel catalytic properties. *FEBS Lett* **471**, 219–223.

Robyt, J. F. (1996). Mechanism and action of glucansucrase. In *Enzymes for Carbohydrate Engineering*, pp. 1–22. Edited by K. H. Park, J. F. Robyt & Y. D. Choi. Amsterdam: Elsevier Science.

Robyt, J. F. & Walseth, T. F. (1978). The mechanism of acceptor reactions of *Leuconostoc mesenteroides* B-512F dextransucrase. *Carbohydr Res* **61**, 433–445.

Sambrook, J., Fritsch, E. F. & Maniates, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sarkar, G. & Sommer, S. S. (1990). The 'megaprimer' method of site-directed mutagenesis. *Biotechniques* **8**, 404–407.

Shah, D. S. H. & Russell, R. R. (2002). Glucan binding domain of streptococcal glucosyltransferases. *Biologia* **57**, 129–136.

Shiroza, T., Ueda, S. & Kuramitsu, H. K. (1987). Sequence analysis of the *gtfB* gene from *Streptococcus mutans*. *J Bacteriol* **169**, 4263–4270.

Uitdehaag, J. C., Mosi, R., Kalk, K. H., van der Veen, B. A., Dijkhuizen, L., Withers, S. G. & Dijkstra, B. W. (1999). X-ray structures along the reaction pathway of cyclodextrin glycosyltrans-ferase elucidate catalysis in the alpha-amylase family. *Nat Struct Biol* **6**, 432–436.

van der Veen, B. A., Uitdehaag, J. C., Dijkstra, B. W. & Dijkhuizen, L. (2000). Engineering of cyclodextrin glycosyltransferase reaction and product specificity. *Biochim Biophys Acta* **1543**, 336–360.

van Geel-Schutten, G. H., Faber, E. J., Smit, E., Bonting, K., Smith, M. R., Ten Brink, B., Kamerling, J. P., Vliegenthart, J. F. & Dijkhuizen, L. (1999). Biochemical and structural characterization of the glucan and fructan exopolysaccharides synthesized by the *Lactobacillus reuteri* wild-type strain and by mutant strains. *Appl Environ Microbiol* **65**, 3008–3014.

van Hijum, S. A., van Geel-Schutten, G. H., Rahaoui, H., van der Maarel, M. J. & Dijkhuizen, L. (2002). Characterization of a novel fructosyltransferase from *Lactobacillus reuteri* that synthesizes high-molecular-weight inulin and inulin oligosaccharides. *Appl Environ Microbiol* **68**, 4390–4398.

van Hijum, S. A., van der Maarel, M. J. & Dijkhuizen, L. (2003). Kinetic properties of an inulosucrase from *Lactobacillus reuteri* 121. *FEBS Lett* **534**, 207–210.

van Hijum, S. A. F. T., Szalowska, E., van der Maarel, M. J. E. C. & Dijkhuizen, L. (2004). Biochemical and molecular characterization of a levansucrase from *Lactobacillus reuteri*. *Microbiology* **150**, 621–630.

Vickerman, M. M., Sulavik, M. C., Minick, P. E. & Clewell, D. B. (1996). Changes in the carboxyl-terminal repeat region affect extracellular activity and glucan products of *Streptococcus gordonii* glucosyltransferase. *Infect Immun* **64**, 5117–5128.

Wunder, D. & Bowen, W. H. (1999). Action of agents on glucosyltransferases from *Streptococcus mutans* in solution and adsorbed to experimental pellicle. *Arch Oral Biol* **44**, 203–214.