Biotechnological potential of novel glycoside hydrolase family 70 enzymes synthesizing α-glucans from starch and sucrose

Gangoiti, Joana; Pijning, Tjaard; Dijkhuizen, Lubbert

Published in:
Biotechnology Advances

DOI:
10.1016/j.biotechadv.2017.11.001

Biotechnology Advances 36 (2018) 196–207

Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Biotechnological potential of novel glycoside hydrolase family 70 enzymes synthesizing α-glucans from starch and sucrose

Joana Gangoiti[a,1], Tjaard Pijning[b], Lubbert Dijkhuizen[a,*,1]

[a] Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands
[b] Biophysical Chemistry, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands

ARTICLE INFO

Keywords:
GH70
Glucansucrase
Glucanotransferase
α-Glucan
Lactic acid bacteria
Structure-function

ABSTRACT

Transglucosidases belonging to the glycoside hydrolase (GH) family 70 are promising enzymatic tools for the synthesis of α-glucans with defined structures from renewable sucrose and starch substrates. Depending on the GH70 enzyme specificity, α-glucans with different structures and physicochemical properties are produced, which have found diverse (potential) commercial applications, e.g. in food, health and as biomaterials. Originally, the GH70 family was established only for glucansucrase enzymes of lactic acid bacteria that catalyze the synthesis of α-glucan polymers from sucrose. In recent years, we have identified 3 novel subfamilies of GH70 enzymes (designated GtfB, GtfC and GtfD), inactive on sucrose but converting starch/maltodextrin substrates into novel α-glucans. These novel starch-acting enzymes considerably enlarge the panel of α-glucans that can be produced. They also represent very interesting evolutionary intermediates between sucrose-acting GH70 glucansucrases and starch-acting GH13 α-amylases. Here we provide an overview of the repertoire of GH70 enzymes currently available with focus on these novel starch-acting GH70 enzymes and their biotechnological potential. Moreover, we discuss key developments in the understanding of structure-function relationships of GH70 enzymes in the light of available three-dimensional structures, and the protein engineering strategies that were recently applied to expand their natural product specificities.

1. Introduction

In recent years, there has been a rapidly growing demand for novel carbohydrate structures synthesized from renewable resources through environmentally friendly processes. Glycoside hydrolase family 70 (GH70) enzymes are promising tools for diversification of the commercially available α-glucan structures, which are difficult to produce via conventional chemical routes (Andre et al., 2010). GH70 enzymes use simple and low-cost sucrose or starch substrates to synthesize α-glucans, differing from Leloir glycosyltransferase enzymes, which require expensive nucleotide-activated sugars (e.g. UDP-glucose) (Desmet et al., 2012). Originally, the GH70 family was defined only for glucansucrases (GSs) exclusively found in lactic acid bacteria (LAB) catalyzing the synthesis of α-glucans from sucrose (Lombard et al., 2014). According to the CAZy classification system, the GH70 family belongs to clan GH-H, together with the GH13 and GH77 families, mainly comprising starch-modifying enzymes (http://www.cazy.org) (Stam et al., 2006). Despite differing in their overall activities, all GH-H enzymes are evolutionary related, sharing a catalytic $(\beta/\alpha)_8$-barrel with 4 catalytically important conserved amino acid sequence motifs and a similar α-retaining mechanism with Asp, Glu, and Asp as catalytic residues, acting as nucleophile, general acid/base and transition state stabilizer, respectively (Vujičić-Žagar et al., 2010; Meng et al., 2016c). This mechanism involves cleavage of the α-glycosidic bond of sucrose or starch/maltodextrins glucose donor substrates and the formation of a covalent enzyme-glycosyl intermediate.

Recently, we have identified 3 novel subfamilies of GH70 enzymes (designated as GtfB, GtfC and GtfD) which are inactive on sucrose, but display clear disproportionating activity with starch/maltodextrin substrates (Kralj et al., 2011; Gangoiti et al., 2015, 2016, 2017d). Compared to sucrose, starch is a cheaper and more abundant carbon source (Haydersah et al., 2012), as well as the largest source of carbohydrates in human food (Singh et al., 2010). These novel starch-acting GH70 enzymes considerably enlarge the repertoire of α-glucans that can be enzymatically synthesized, with (potential) commercial applications in food, health and as biomaterials. The discovery of the GtfB, GtfC and GtfD subfamilies of GH70 enzymes also is highly interesting from an evolutionary perspective, as they represent structurally

* Corresponding author.
E-mail address: L.Dijkhuizen@rug.nl (L. Dijkhuizen).
[1] Present address: CarbExplore Research BV, Zernikepark 12, 9747 AN Groningen, The Netherlands.

https://doi.org/10.1016/j.biotechadv.2017.11.001
Received 20 August 2017; Received in revised form 24 October 2017; Accepted 6 November 2017
Available online 10 November 2017
0734-9750/

and functionally evolutionary intermediates between sucrose-acting GH70 and starch-acting GH13 enzymes (Gangoiti et al., 2015, 2016; Bai et al., 2017).

In this review we focus on the variety of existing GH70 enzymes and α-glucan products with emphasis on the novel starch-converting GH70 enzymes discovered in recent years. Particular attention is given to the structural determinants involved in GH70 enzyme reaction/product specificity. A clear understanding of these structure-function relationships may allow their further optimization by enzyme engineering, and tailoring of their α-glucan products (linkage types, sizes, degree of branching) towards different functionalities.

2. Glucansucrases

Most of the enzymes classified within the GH70 family are GSs converting sucrose into α-glucooligo- and polysaccharides (http://www.cazy.org). > 60 GSs have been biochemically characterized; all of them are encoded by LAB of the genera *Leuconostoc*, *Streptococcus*, *Lactobacillus*, and *Weissella* (http://www.cazy.org). Some hypothetical GS genes are also found in other LAB, i.e. *Fructobacillus*, *Oenococcus*, and *Enterococcus*. In the oral cavity and gastrointestinal tract of mammals, α-glucans produced by GSs may be involved in biofilm formation, facilitating aggregation of bacteria and adherence to the tooth enamel or epithelium (Colby et al., 1999; Walter et al., 2008), and providing protection against oxygen (and other stresses) (Yan et al., 2016). Most LAB produce only a single GS enzyme, although some strains producing multiple GH70 enzymes have been described, i.e. *Leuconostoc mesenteroides* NRRL B1299 (Monchois et al., 1996, 1998; Passerini et al., 2015). At present the *in vivo* functional role of each of these multiple GSs has remained unclear. Some bacteria were found to produce more than one α-glucan product, and this ability was related to the expression of different GSs (Côté and Robyt, 1982). In such strains, the presence of two or more GSs may have a cooperative effect on α-glucan formation (Passerini et al., 2015). While the GSs from *Streptococcus* and *Lactobacillus* are constitutively expressed (van Geel-Schutten et al., 1998; Kralj et al., 2004a; Schwab et al., 2007), the expression of GS from *Leuconostoc* was found to be induced in the presence of sucrose (Kim and Robyt, 1994; Yan et al., 2016).

2.1. Synthesis of α-glucans by GSs

Depending on the GS and reaction conditions used, α-glucan polymers or oligomers can be produced that vary in terms of size, glycosidic linkage composition, and branching degree. This structural variability results in a wide range of physicochemical properties, which may be suitable for different applications. Based on their main glycosidic linkage type, these α-glucans are divided into four categories: dextran, mutan, reuteran and alternan (Fig. 1).

2.1.1. Dextran, with mainly (α1 → 6) linkages

Dextran is a water-soluble α-glucan mainly composed of (α1 → 6) glucan chain segments, connected by varying amounts and arrangements of (α1 → 2), (α1 → 3) and (α1 → 4) linkages. The GS enzyme responsible for the synthesis of dextran is designated as dextransucrase (EC 2.4.1.5) and was first reported in *Leuconostoc* (Hehre and Sugg, 1942). Subsequently, dextransucrases from various species of the genera *Lactobacillus*, *Streptococcus*, and *Weissella* were also identified and characterized (Hanada and Kuramitsu, 1989; van Leeuwen et al., 2008b; Kang et al., 2009) (Fig. 1). Most of the dextransucrases characterized so far produce dextran polymers with mainly (α1 → 6) linkages and minor amounts of (α1 → 3) linkages. The different techniques that are routinely used for the structural characterization of the α-glucan products synthesized by GH70 enzymes have been reviewed by Leemhuis et al., 2013b. Dextran for clinical and technical applications is marketed in most developed countries all over the world. Of industrial relevance is the dextran produced by *Leuconostoc mesenteroides* NRR B-512F DSR-S; the dextransucrase of this strain converts sucrose into a high molar mass (up to 1 MDa) polymer with 95% (α1 → 6) linkages in the main chains and 5% (α1 → 3) branching linkages (Monchois et al., 1997; Passerini et al., 2015; Zannini et al., 2016). This native or partially degraded dextran and its derivatives have found many industrial applications in medicine (e.g. blood plasma expander, anticoagulant and antithrombotic agents), pharmacy (e.g. lubricant and carrier), food (e.g. thickening, stabilizing, and gelling agent), and biotechnology (e.g. chromatography matrix) (Naessens et al., 2005a; Badel et al., 2011; Zannini et al., 2016). Another intensively studied dextransucrase is the *Lactobacillus reuteri* 180 Gtf180 GS producing an α-glucan with a high molecular weight of 30 MDa containing 69% of (α1 → 6) linkages plus single (α1 → 3) linkages in linear (21%) and branched (13%) orientations (van Leeuwen et al., 2008b; Meng et al., 2016c). Notably, different α-glucans with unique highly branched structures have been reported in *Leuconostoc* strains. The *Leuconostoc citreum* NRRL B-1299 was found to synthesize a dextran polymer with mostly (α1 → 6) linkages but also containing 27–35% of (α1 → 2) linkages, as well as a limited amount of (α1 → 3) branching linkages (Kobayashi and Matsuda, 1977; Seymour et al., 1979). This strain encodes six different GSs, namely DSR-A, DSR-B, DSR-E, DSR-M, DSR-P and BRS-A (Passerini et al., 2015). Biochemical studies revealed that DSR-A, DSR-B, DSR-DP and DSR-M produce (α1 → 6) rich dextrans individually (Monchois et al., 1996, 1998; Passerini et al., 2015). The (α1 → 2) branching linkages present in the native dextran produced by *L. citreum* NRRL B-1299 were proposed to result from the action of the DSR-E and BRS-A enzymes. DSR-E is an atypical enzyme which displays two fully active, highly regiospecific catalytic domains, connected by a glucan binding domain (Bozonnet et al., 2002). Biochemical characterization of truncated variants of DSR-E revealed that the first catalytic domain (CD1) synthesizes the main linear (α1 → 6) glucan chain, whereas the second catalytic domain (CD2) modifies dextran acceptors through the formation of (α1 → 2) branching linkages (Bozonnet et al., 2002; Fabre et al., 2005; Brison et al., 2012). The BSR-A enzyme was found to share significant sequence identity with the CD2 domain of DSR-E. In agreement with this observation, this enzyme shows (α1 → 2) branching specificity and introduces up to 37% (α1 → 2) linkages in dextran (Passerini et al., 2015). A different branching specificity was observed in *Leuconostoc citreum* NRRL B-742 which was found to produce a dextran with a comb-like structure consisting in a linear (α1 → 6) glucan main chain grafted with single (α1 → 3) linked glucosyl residues on every glucosyl unit (Seymour et al., 1979). Very recently, the genome analysis of this strain allowed the identification of an (α1 → 3) branching sucrase (named BSR-B) responsible for the high content of (α1 → 3) branching linkages present in the dextran produced by this strain (Vuillemin et al., 2016). Interestingly, GH70 enzymes with dextran branching specificity have also been identified in other strains (*Leuconostoc fallax* KCTC3537 and *Lactobacillus kunkei* EFB6) indicating that these types of enzymes occur more widespread in LAB (Vuillemin et al., 2016). The (α1 → 2)- and (α1 → 3)-branching sucrases constitute a novel subgroup in the GH70 family and display unique sequence variations in functionally important residues of the conserved GH70 family motifs II and IV (see section 2.2), allowing their rapid identification by using sequence alignments (Fig. 2A). Due to their dextran branching activity, these biocatalysts are useful for the production of dextrans with a controlled degree of branching (Brison et al., 2010, 2013, 2016; Vuillemin et al., 2016).

2.1.2. Mutan, with > 50% (α1 → 3) linkages

Mutan polymers are generally water-insoluble α-glucans containing > 50% (α1 → 3) glucosidic linkages in the linear backbone and minor amounts of (α1 → 6) linkages. Mutansucrase enzymes (EC 2.4.1.125) synthesizing mutan polymers are mainly found in *Streptococcus* strains. The ability of *Streptococcus* mutans and *Streptococcus sobrinus* to convert sucrose into water-insoluble glucans was found to be important in the etiology of dental caries by facilitating

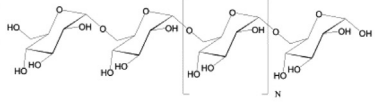
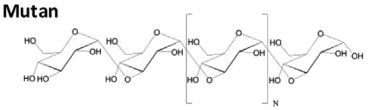
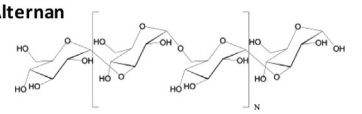
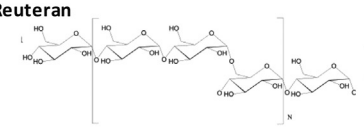
α-Glucan	Enzyme	Linkage composition of the product (%) (α1 → 2)	(α1 → 3)	(α1 → 4)	(α1 → 6)	References
Dextran 	*Leuconostoc mesenteroides* NRRL B-512F DSRS		5		95	(Monchois et al., 1997)
	Leuconostoc citreum B-1299 DSRE[a]	5	10	3	81	(Fabre et al., 2005)
	Leuconostoc citreum B-1299 BSR-A[b]	37			63	(Passerini et al., 2017)
	Leuconostoc citreum BSR-B[b]		50		50	(Vuillemin et al., 2016)
	Weissella cibaria DSRWC				100	(Kang et al., 2009)
	Lactobacillus reuteri 180 Gtf180		31		69	(van Leeuwen et al., 2008)
	Streptococcus mutans GS5 GtfD		30		70	(Hanada and Kuramitsu, 1989)
Mutan 	*Streptococcus mutans* GS5 GtfB		88		12	(Shiroza et al., 1987)
	Lactobacillus reuteri ML1		65		35	(Kralj et al., 2004)
	Leuconostoc mesenteroides NRRL B-1118 DSRI		50		50	(Cote and Skory, 2012)
Alternan 	*Leuconostoc mesenteroides* NRRL B-1355 ASR		43		57	(Côté and Robyt, 1982b)
Reuteran 	*Lactobacillus reuteri 121* GtfA			58	42	(van Leeuwen et al., 2008c)
	Lactobacillus reuteri ATCC 55730 GtfO			79	21	(Kralj et al., 2005)

Fig. 1. Examples of α-glucans made by characterized GH70 glucansucrases from sucrose. [a] DSRE displays two catalytic domains (GS and (α1 → 2) branching sucrase). [b] BSR-A and BSR-B are branching sucrases specialized in (α1 → 2) and (α1 → 3) transglucosylation from sucrose donor to dextran acceptor substrate.

the colonization of tooth surfaces (Tsumori and Kuramitsu, 1997). Consequently, GSs are regarded as potential targets for anticaries drugs (Zhang et al., 2011). Examples of mutansucrases have also been found in other *Lactobacillus* and *Leuconostoc* strains (Waldherr et al., 2010; Côte and Skory, 2012) (Fig. 1). Mutan is regarded as a potentially low-cost polymer, which may be used to develop new bio-based materials (Puanglek et al., 2016, 2017). In particular, chemical modification of mutans to ester derivatives has shown to improve the thermoplasticity of this polysaccharide (Puanglek et al., 2016, 2017). Moreover, chemically sulfated mutan showed fibrinolytic, anti-inflammatory and anti-microbial properties (Buddana et al., 2015). The use of mutan for a variety of applications as fibers, films and resins has been patented (Hiler, 1961; Nichols, 2002; O'Brien, 2015; Opper, 2013; Yokobayashi et al., 1978; Caimi and Hennessey, 2013).

2.1.3. Reuteran, with (α1 → 4) linear segments interconnected by single (α1 → 6) bridges

Reuteran is a water-soluble branched α-glucan mainly composed of (α1 → 4) linear segments interconnected by single (α1 → 6) bridges. Only two reuteransucrase enzymes (EC 2.4.1.-) have been characterized, both of them present in *Lactobacillus reuteri* strains and producing reuteran polymers differing in the amount of (α1 → 4) and (α1 → 6) linkages (Kralj et al., 2004b, 2005a) (Fig. 1). The most studied reuteransucrase is the GtfA from *L. reuteri* 121 synthesizing an α-glucan with a molecular weight of 40 MDa and 58% (α1 → 4) linkages and 42% single (α1 → 6) linkages in both branched (15%) and linear (27%) orientations (van Leeuwen et al., 2008c). Reuteran has been described as a potentially health-promoting food ingredient. When added in a white bread recipe, reuteran improves bread quality and texture (Plijter et al., 2009). At the same time, reuteran is regarded as a dietary fiber and was found to induce satiety in humans and animals (Ekhart et al., 2006).

2.1.4. Alternan, with alternating (α1 → 6) and (α1 → 3) linkages

L. mesenteroides NRRL B-1355 alternansucrase (ASR, EC 2.4.1.140) is a distinct glucansucrase which has the ability to synthesize an α-glucan with alternating (α1 → 6) and (α1 → 3) linkages in the main chain known as alternan (Côté and Robyt, 1982; Arguello-Morales et al., 2000). Due to its backbone structure of alternating (α1 → 6) and (α1 → 3) linkages, alternan is resistant to enzymatic digestion by most known mammalian and microbial hydrolytic enzymes and shows lower viscosity and higher solubility in water than dextran (Biely et al., 1994). These properties make alternan valuable as low-calorie food additive, and as texturizing agent in cosmetic preparations, to replace fat or oil (Frohberg and Pilling, 2009). So far the only enzymes reported to hydrolyze alternan are isomaltodextranases and alternanase (Biely et al., 1994; Côté and Ahlgren, 2001; Sawai et al., 1978, 1981).

2.2. The structure of Glucansucrase proteins and their mechanism of action

Although GSs have been extensively studied for > 60 years, the large size of these proteins (Mw ~ 120–200 kDa) for many years hampered the production of crystals suitable for X-diffraction studies. Only recently, the first high resolution three-dimensional structure of the N-terminally truncated GS Gtf180-ΔN from *L. reuteri* 180 has become available (Vujičić-Žagar et al., 2010) (Fig. 3A). Also the crystal structures of 3 other GSs with different product specificity subsequently were determined (Ito et al., 2011; Brison et al., 2012; Pijning et al., 2012). All of these crystal structures were obtained with truncated forms of GSs that lacked between ~240 and 840 N-terminal residues (745 amino acids in case of Gtf180-ΔN, Fig. 3A), but retained their full catalytic functionality. This N-terminal region varies significantly in length and composition between GSs, and has been proposed to act as a cell-wall attachment domain (Leemhuis et al., 2013b). Structurally, these GS proteins were found to be highly similar and also clearly related to GH13 α-amylases, confirming pioneering secondary-structure predictions and circular permutation predictions (MacGregor et al., 1996). In both GH13 and GH70 families, the catalytic core is formed by domains A, B and C that can be superimposed. Domain A comprises a TIM barrel or $(\beta/\alpha)_8$ barrel with the catalytic triad (the nucleophile Asp1025, the general acid/base Glu1063 and the transition state stabilizer Asp1136, Gtf180 numbering). The conserved I-IV motifs located in domain A, and first identified in GH13 enzymes, are also present in GSs (Fig. 2A). These motifs contain the catalytic residues as well as other substrate binding residues, and are regarded as sequence

Bacterial strain	Accession numbers	Specificity	Motif I		Motif II		Motif III		Motif IV	
A				1 2		3 4		5		67
Lactobacillus reuteri 180 (Gtf180)	AAU08001.1	Dextransucrase	1503	ADWVPDQ	1021	GIRVDAVDNVD	1058	HINILEDWGWD	1131	FVRAHDSNAQDQIRQ
Lactobacillus reuteri 121 (GtfA)	AAU08015.1	Reuteransucrase	1508	ADWVPDQ	1020	SVRVDAPDNID	1056	HINILEDWNHA	1128	FVRAHDNNSQDQIQN
Streptococcus mutans SI (GtfSI)	BAA26114.1	Mutansucrase	954	ADWVPDQ	473	SIRVDAVDNVD	510	HLSILEAWSYN	583	FIRAHDSEVQDLIRD
Leuconostoc mesenteroides NRRL-1355	CAB65910.2	Alternansucrase	1168	ADWVPDQ	631	GIRVDAVDNVD	668	HLSILEDWNGK	762	FVRAHDYDAQDPIRK
Leuconostoc citreum NRRL B-1299	CDX66820.1	(1→2) Branching sucrase	2688	ADVVDNQ	2206	SIRIDAVDFIH	2243	HISLVEAGLDA	2317	IIHAHDKGVQEKVGA
Leuconostoc citreum NRRL B-742	CDX65123.1	(1→3) Branching sucrase	1182	ADFVANQ	667	SMRIDAISFVD	704	HISIVEAPKGE	783	IVHAHDKDIQDTVIH
B										
Lactobacillus reuteri 121 (GtfB)	AAU08014.2	4,6-α-GTase	1478	EDIVMNQ	1011	GFRVDAADNID	1048	HLSYNEGYHSG	1120	FVTNHDQR-KNLINR
Lactobacillus reuteri ML1 (ML4)	AAU08003.2	4,6-α-GTase	1479	EDIVMNQ	1012	GFRVDAADNID	1049	HLSYNEGYHSG	1121	FVTNHDQR-KNLINR
Lactobacillus reuteri DSM 20016 (GtfW)	ABQ83597.1	4,6-α-GTase	1215	EDLVMNQ	748	GFRVDAADNID	785	HLVYNEGYHSG	858	FVTNHDQR-KNVINQ
Pediococcus pentosaceus IE-3	CCG90643.1	ND	841	EDIVMNQ	380	GFRIDAADNID	417	HLSYNEGYHSG	489	FVTNHDQR-KNLINS
Lactobacillus acidipiscis KCTC 13900	WP_035631372.1	ND	765	VDMVMNQ	296	GFRNDAADNID	333	HLVYNEGYHSG	406	FVTNHDQR-KNVINQ
Lactobacillus panis DSM 6035	KRM25865.1	ND	1455	EDLVMNQ	988	GFRVDAADNVD	1025	HLVYNEGYHSD	1097	FVTNHDQR-KNLINQ
C										
Lactobacillus fermentum NCC 2970	AOR73699	4,3-α-GTase	1446	EDIVMNQ	983	GFRIDAADDMD	1020	HLSYNEGYGPG	1092	YVTNHDIR-NNLING
D										
Lactobacillus reuteri NCC 2613	ASA47879.1	4,6-α-GTase	1134	VDIVMNQ	675	GFRIDAADHID	712	HLSYNEGYRSS	783	YVTNHDSR-ANLING
Lactobacillus reuteri NCC 2592	ASA47875.1	ND	1233	VDIVMNQ	774	GFRIDAADHID	811	HLSYNEGYRSS	882	YVTNHDSR-ANLING
Lactobacillus reuteri NCC 2603	ASA47878.1	ND	1097	VDIVMNQ	638	GFRIDAADHID	675	HLSYNEGYRSS	746	YVTNHDSR-ANLING
Lactobacillus reuteri NCC 3072	ASA47884.1	ND	749	VDIVMNQ	290	GFRIDAADHID	327	HLSYNEGYRSS	398	YVTNHDSR-ANLING
Lactobacillus delbrueckii NCC 828	ASA47848.1	ND	1129	VDIVMNQ	670	GFRIDAADHID	707	HLSYNEGYRSG	778	YVTNHDQR-ANLING
Streptococcus thermophilus NCC 2408	ASA47903.1	ND	1494	ADIVLNH	1031	GFRIDATDHID	1068	HLSYNEQYSRG	1140	FVTNHDQR-NNLING
Streptococcus thermophilus NCC 903	ASA47905.1	ND	1494	ADIVLNH	1031	GFRIDATDHID	1068	HLSYNEQYSRG	1140	FVTNHDQR-NNLING
E										
Exiguobacterium sibiricum 255-15	ACB62096.1	4,6-α-GTase	138	MDLVPNQ	403	GFRIDAASHYD	433	HLSYIESYKSE	504	FVNNHDQE-KNRVNQ
Exiguobacterium undae	WP_028105602.1	ND	138	MDLVPNQ	403	GFRIDAASHYD	433	HLSYIESYKSE	504	FVNNHDQE-KNRVNQ
Exiguobacterium antarcticum B7	AFS71545.1	ND	138	MDLVPNQ	403	GFRIDAASHYD	433	HLSYIESYKSE	504	FVNNHDQE-KNRVNQ
Bacillus kribbensis	WP_035322188.1	ND	130	EDLVPNQ	397	GFRIDAASHYD	429	HLSYIESYSNV	491	FVNNHDQE-KNRVNN
Bacillus coagulans DSM1	AJH79253.1	ND	128	EDLVPNQ	394	GFRIDAAGHYD	426	HLSYIESYQSA	497	FVNNHDQE-KNRVNK
Bacillus sporothermodurans	KYC94174.1	ND	140	EDLVPNQ	408	GFRVDAASHYD	440	HLSYIESYSSA	511	FVTNHDQE-KNRINN
Geobacillus sp. 12AMOR1	AKM18207.1	ND	140	LDLVPNQ	409	GFRIDAATHFD	441	HLSYIESYTSK	512	FVNNHDQE-KNRVNT
F										
Azotobacter chroococcum NCIMB 8003	AJE22990.1	4,6-α-GTase	202	VDVVPNQ	467	GFRIDAASHIN	500	HLSYIESYVTQ	567	FVNNHDQE-HNILVT
Dyella-like sp. DHo	WP_049623289.1	ND	212	VDLVPNQ	477	GFRIDAASHIN	510	HLSYIESYVTA	577	FVNNHDQE-HNLLAG
Burkholderia sp. NFACC38-1	2599741842	ND	103	ADIVPNQ	362	GFRFDAAGHYN	394	HLSVIESYVDP	465	FVTNHDQE-HNVIAK
Paenibacillus sp. Soil522	WP_056638435.1	ND	149	EDLVPNQ	403	GFRIDAASHLN	435	HLSFIESYTDN	505	FVNNHDQE-HNAIKP
Pseudomonadales bacterium GWC1 66 9	OHC12359.1	ND	153	VDVVPNQ	418	GFRIDAASHIN	451	HLSYIESYVAQ	518	FVNNHDQE-HNILVN
						NU		A/B		TS

Fig. 2. Alignment of conserved motifs I-IV of GH70 family enzymes with different reaction- and product specificities. (A) Sucrose-active enzymes, (B) (putative) IMMP-forming GtfB-like 4,6-α-GTase enzymes, (C) GtfB-like 4,3-α-GTase enzyme, (D) (putative) reuteran-forming GtfB-like 4,6-α-GTase enzymes, (E) (putative) GtfC-like 4,6-α-GTase enzymes, and (F) (putative) GtfD-like 4,6-α-GTase enzymes. Numbers 1 to 7 above the sequences indicate the seven highly conserved amino acid residues in GH70 enzymes. Amino acids that constitute the catalytic triad are highlighted in bold and lightly shaded. Residues forming acceptor subsites − 1, + 1 and + 2 in Gtf180 are indicated in green, red and blue, respectively. Abbreviations at the bottom: NU = nucleophile, A/B = general acid/base, TS = transition state stabilizer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fingerprints for the individual enzyme specificities (van Hijum et al., 2006; Janecek and Gabrisko, 2016). Interestingly, GSs also display unique structural features. Firstly, compared to GH13 and GH77 enzymes, their catalytic $(\beta/\alpha)_8$-barrel domain is circularly permuted. As a result, the conserved motif I is located at the C-terminal part of domain A (Fig. 2A). Secondly, in GSs the catalytic core is decorated with two extra remote domains IV and V (Fig. 3A) that are absent in GH13. Several experimental approaches including domain-truncation experiments and X-ray crystallography studies have shown that domain V is important for polymer elongation. Successive deletions of domain V in the *L. mesenteroides* NRRLB-1355 DSR-S GS resulted in less efficient polymer synthesis and favored a progressive decrease of the polymer molecular weight (Moulis et al., 2006). In contrast, the product size specificity of the Gtf180-ΔN GSs switched from polymer to oligomer synthesis by truncation of the remote domain V (Meng et al., 2015a). Domain V contains glucan binding pockets (Brison et al., 2016) and adopts different positions due to movements around a hinge located between domains IV and V (Pijning et al., 2014), suggesting that domains IV and V contribute to polysaccharide synthesis by moving bound intermediate α-glucan products away from or towards the active center. Most notably, GSs display an unusual "U-fold" domain structure in which 4 (domains A, B, IV and V) of the 5 domains (domains A, B, C, IV and V) are made up from two discontiguous segments of the polypeptide chain (Fig. 7). This unique GS domain organization was proposed to have emerged from a GH13 α-amylase ancestor through an evolutionary pathway based on gene duplication (Vujičić-Žagar et al., 2010).

The three-dimensional structure of GTF180-ΔN and its complexes with sucrose (donor substrate) and maltose (acceptor substrate) confirmed that GSs share the same catalytic machinery and α-retaining double displacement mechanism with GH13 and GH77 enzymes. According to this mechanism, first, the α-1,2-glycosidic linkage of sucrose is cleaved between enzyme subsites − 1 and + 1, and an α-glucosyl-enzyme intermediate is formed at subsite − 1. Second, the covalently bound glucosyl moiety is transferred to the accepting non-reducing end sugar of a growing glucan chain, with reformation of the α-glycosidic bond (transglycosylation or polymerization reaction). Alternatively, the glucosyl moiety may be transferred to a low molecular mass acceptor substrate such as maltose (acceptor reaction), or to a water molecule (hydrolysis reaction). Elucidation of crystal structures of GSs with bound substrates combined with mutagenesis studies have deepened our understanding of their structural features determining product specificity, thus providing clues for more rational approaches to design enzyme variants synthesizing (more of) the desired products (Meng et al., 2014, 2015a,b, 2016a,c, 2017). In particular, the linkage specificity is determined by the orientation in which the acceptor substrate binds to the enzyme. The crystal structure of Gtf180-ΔN in complex with maltose revealed that residues interacting with the acceptor substrate in subsites + 1 and + 2 are from conserved motifs II, III and IV, but also from other non-conserved regions of domain B and of the helix α4 (Fig. 3B) (Vujičić-Žagar et al., 2010). Mutagenesis studies have shown that the amino acid residues at the acceptor binding

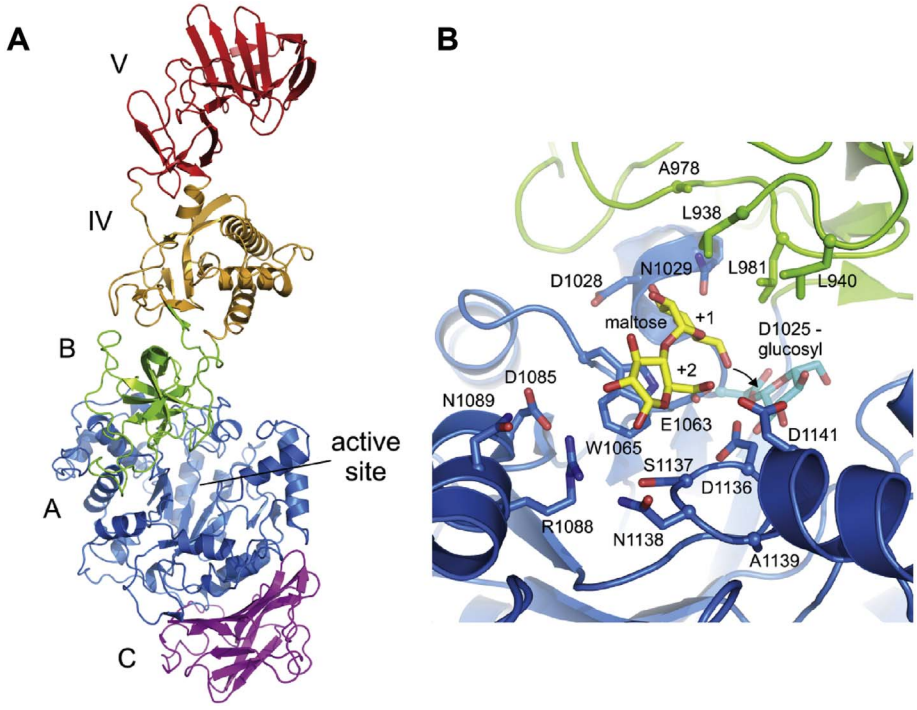


Fig. 3. Crystal structure of Gtf180-ΔN from *Lactobacillus reuteri* 180 (Vujičić-Žagar et al., 2010). (A) Overall structure with, from bottom to top, the three core domains C (magenta), A (blue) and B (green), followed by the auxiliary domains IV (yellow) and V (red). The N- and C-terminus of the construct both reside in domain V. (B) Close-up of the active site of Gtf180-ΔN with bound maltose (yellow carbon atoms), and with a modeled glucosyl-enzyme intermediate (cyan carbon atoms) linked to the catalytic nucleophile, D1025. The other two catalytic residues, the general acid/base residue E1063 and the transition state stabilizing residue D1136 are also indicated. Maltose is bound to subsites + 1 and + 2, oriented such that its non-reducing end O6 can attack the C1 of the covalent glucosyl intermediate to form a glycosidic α-1,6 linkage (see arrow). Other residues surrounding the acceptor binding subsites are indicated: D1028 and N1029 in motif II; W1065 in motif III; S1137, N1138, A1139 and D1141 in motif IV; D1085, R1088 and N1089 in helix α4; L938, L940, A978 and L981 in domain B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

subsites + 1 and + 2 together form a particular physicochemical micro-environment that determines the linkage specificity (Meng et al., 2014, 2015b). However, these mutagenesis experiments have also revealed that a limited number of amino acid substitutions are sufficient to provide GSs with distinct product specificity. Most notably, mutations in subsite + 2 residues immediately downstream the transition state stabilizer (motif IV) significantly altered the glycosidic linkage ratios of several GSs (Shimamura et al., 1994; Kralj et al., 2005b, 2006; Moulis et al., 2006; Hellmuth et al., 2008; van Leeuwen et al., 2008a; van Leeuwen et al., 2009). Interestingly, combining mutations in this region with mutations in motif II resulted in GS mutants able to introduce significant amounts of (α1 → 4) glucosidic linkages that were not present in the wild-type α-glucan products (van Leeuwen et al., 2008a; Kang et al., 2011). Mutagenesis approaches were also useful for tailoring the amount of branching points present in the α-glucans. For example, mutating the subsite + 1 residues L940, A978 and D1028 in *L. reuteri* Gtf180 GS into bulky residues, partially or completely blocked a groove above the + 1 subsite required for the formation of branches, resulting in a reduced amount of branching linkages in the products synthesized (Meng et al., 2014, 2015b). On the contrary, some mutations in residues of helix α4 near subsite + 2 yielded hyper-branched polymers (Meng et al., 2016a).

Mutations resulting in the production of polysaccharides with reduced or increased molecular masses also have been reported (van Leeuwen et al., 2009; Irague et al., 2012; Meng et al., 2014; Chen and Ganzle, 2016; Meng et al., 2015a), although the structural determinants of α-glucan product size are not fully understood. Mutagenesis of some of the + 1 and + 2 acceptor binding residues significantly affected the ratio of polysaccharide versus oligosaccharide synthesis by reducing or increasing the affinity of the enzyme for the growing acceptor substrate chain (Kralj et al., 2005b, 2006; Moulis et al., 2006; Hellmuth et al., 2008; Meng et al., 2014, 2016b). GSs can also shift their activity from polysaccharide synthesis to oligosaccharide synthesis when efficient acceptor substrates (e.g. maltose) are available. This strategy has been used to synthesize novel non-digestible oligosaccharides with prebiotic properties (Valette et al., 1993; Sanz et al., 2005; Monsan et al., 2010). Finally, in addition to structural features at the amino-acid level, large scale structures may also be of importance. Crystallographic and small-angle X-ray scattering (SAXS) studies indicated that *L. reuteri* Gtf180 has intrinsic flexibility (Pijning et al., 2014); during the polymerization reaction, the enzyme may undergo large conformational changes involving remote domains that bind (intermediate) α-glucan products (Ito et al., 2011). Recent studies with DSR-E (Brison et al., 2016) indeed identified carbohydrate binding sites in these remote domains.

3. Starch-converting family GH70 enzymes

In the last 6 years, 3 different GH70 subfamilies of enzymes designated as GtfB, GtfC and GtfD have emerged and biochemically characterized as α-glucanotransferase (α-GTase) enzymes. Instead of sucrose, these enzymes convert maltodextrins and starch substrates into a novel class of α-glucooligo- and polysaccharides, which are potentially of great interest for agro-food, health and biomaterial applications. The microbial origin, domain organization, reaction- and product specificity of α-GTase enzymes differ from those of GSs, as discussed below. The discovery of these 3 novel starch-converting GH70 subfamilies has significantly enlarged the diversity of enzymes in the GH70 family. The number of characterized starch-converting GH70 enzymes is still limited and is far exceeded by the number of GSs studied, but the rapidly increasing availability of bacterial genome sequences provides a rich source for identification of these novel GH70 enzymes. Remarkably, the existence of GtfB, GtfC and GtfD type of enzymes, active on starch and maltodextrins, but showing higher sequence relatedness with GH70 GSs further supports the evolutionary relationships between GH13 and GH70 families (Leemhuis et al., 2013b). The GH13-GH70 intermediate character of these GtfB, GtfC and GtfD type of enzymes is also reflected in their intermediate phylogenetic position, in between GSs and GH13 α-amylases (Fig. 4). In particular, phylogenetic studies have suggested that GH70 enzymes are closely related to and likely evolved from a malto-oligosaccharide-processing α-amylase belonging to GH13 subfamily 5 (Bai et al., 2017). In contrast, GH13 family members active on sucrose (e.g. amylosucrase of GH13 subfamily 4) or GH13 α-transglycosylases acting on starch (e.g. cyclodextrin glucanotransferases of GH13 subfamily 32 or branching enzymes of GH13 subfamily 9) are more distantly related. It is worth to note that GH77 family proteins, also active on (α1 → 4) glucan substrates and placed within the GH-H

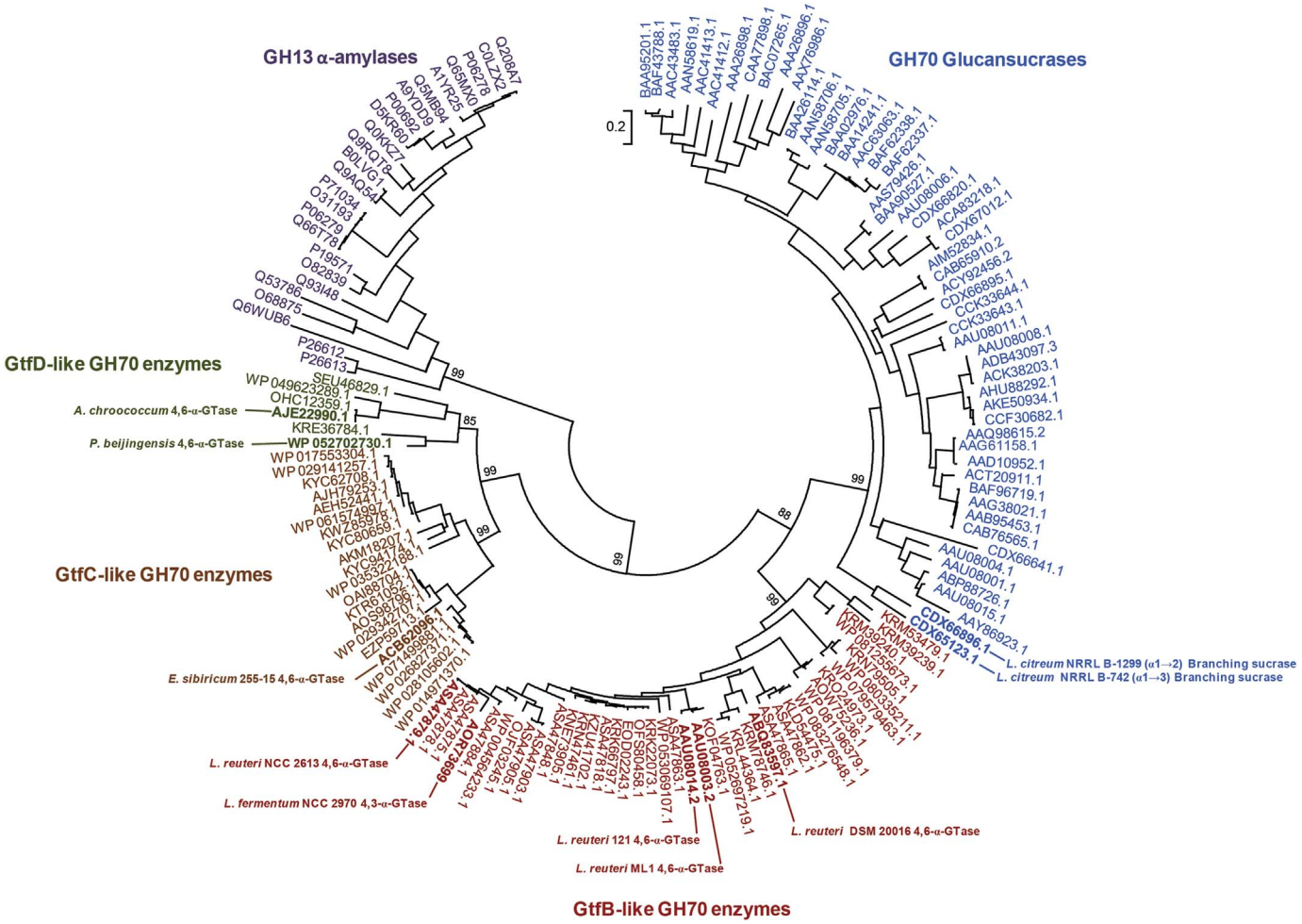


Fig. 4. Phylogenetic tree of representative family GH70 and GH13 protein sequences. The tree is based on the alignment of the complete amino acid sequences of some of the characterized GH70 proteins and GH13_5 α-amylases, and of some of the putative GH70 GtfB-, GtfC-, and GtfD-like proteins identified by BLAST searches using the *Lactobacillus reuteri* 121 GtfB, *Exiguobacterium sibiricum* 255-15 GtfC and *Azotobacter chroococcum* GtfD as query sequence, respectively. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The bar represents a genetic distance of 0.2 substitutions per position (20% amino acid sequence difference). The bootstrap values adjacent to the main nodes represent the probabilities based on 1000 replicates. The protein sequences are annotated by their Genbank accession numbers (also see Table S1). GH70 enzymes with new reaction/product specificities discovered in recent years are highlighted in bold.

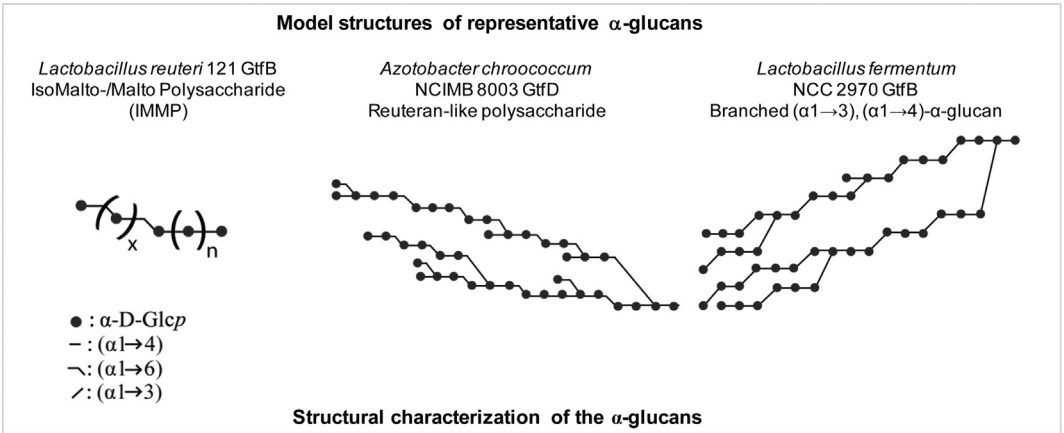
clan, are clearly less evolutionary related to GtfB, GtfC and GtfD type of enzymes with regard to its amino acid sequence and structure than the GH13 family proteins (e.g. GH77 enzymes lack domain C). Thus, it appears less likely that the novel GtfB, GtfC and GtfD subfamilies evolved from a GH77 enzyme intermediate.

3.1. GtfB-like GH70 subfamily of enzymes

The GtfB type of enzymes are exclusively found in lactic acid bacteria and constitute the first and most studied subfamily of starch-converting GH70 enzymes. According to current database searches (http://www.ncbi.nlm.nih.gov/, 5th July 2017), the GH70 GtfB subfamily contains 153 sequences. Most of these (hypothetical) enzymes are encoded by *Lactobacillus* strains with few exceptions (8, 2, 2, and 1 present in *Pediococcus*, *Streptococcus, Weissella* and *Leuconostoc* strains, respectively). The functional role of these GtfB enzymes remains unknown. In some strains the genes encoding GtfB-like proteins and GSs are arranged in tandem in the genome, which may reflect relatively recent gene duplications. *In vivo* experiments have revealed that not only GSs but also GtfB-like enzymes contribute to exopolysaccharide synthesis (Bai et al., 2016). Within this GH70 subfamily, 3 different reaction and product specificities have been described, represented by the *L. reuteri* 121 GtfB 4,6-α-GTase (Kralj et al., 2011; Leemhuis et al., 2013a; Bai et al., 2015), the *L. fermentum* 4,3-α-GTase (Gangoiti et al., 2017b), and the *L. reuteri* NCC 2613 GtfB 4,6-α-GTase (Gangoiti et al., 2017c).

3.1.1. Lactobacillus reuteri 121 GtfB 4,6-α-glucanotransferase

The *L. reuteri* 121 GtfB enzyme is the first reported starch-converting GH70 enzyme. The gene encoding this enzyme was found upstream of the gene encoding the GtfA GS converting sucrose into a reuteran polymer (Kralj et al., 2002, 2004b). Even though the *L. reuteri* GtfB shares high sequence identity with the GtfA GS, it turned out to be inactive on sucrose. Instead, the *L. reuteri* 121 GtfB displays clear hydrolase/transglycosylase activity on malto-oligosaccharides (MOS) and starch, cleaving (α1 → 4) linkages and synthesizing consecutive (α1 → 6) linkages. This 4,6-α-glucanotransferase (4,6-α-GTase) activity results in the synthesis of a soluble starch-derived product designated as Iso-Malto-Malto Polysaccharides (IMMPs) (Dijkhuizen et al., 2010; Kralj et al., 2011; Leemhuis et al., 2014). IMMPs consist of linear (α1 → 6) glucan segments attached to the non-reducing end of malto-oligosaccharides or starch fragments (Fig. 5). Later on, further homologs of this GtfB enzyme were characterized in other *Lactobacillus* strains, which showed the same reaction and product specificity (Leemhuis et al., 2013a) (Fig. 5). The 4,6-α-GTase activity displayed by these GtfB type of enzymes resembles the one reported for dextran dextrinases produced by some strains of *Gluconobacter oxydans* and placed within the GH15 family (Naessens et al., 2005b; Sadahiro et al., 2015).



Structural characterization of the α-glucans

Enzyme	Linkage composition of the product (%)					M_w (Da)	References
	Term	(α1 → 3)	(α1 → 4)	(α1 → 6)	Branch		
GtfB-like GH70 subfamily							
Lactobacillus reuteri 121 GtfB			9	91		15 x 10^3	(Leemhuis et al., 2014)
Lactobacillus fermentum NCC 2970 GtfB	6	28	58		8 (α1→4,3)	800 x 10^3	(Gangoiti et al., 2017a)
Lactobacillus reuteri NCC 2613 GtfB	15		59	10	16 (α1→4,6)	~7 x 10^3	(Gangoiti et al., 2017b)
GtfC-like GH70 subfamily							
Exiguobacterium sibiricum 255-15 GtfC			40	60			(Gangoiti et al., 2015)
GtfD-like GH70 subfamily							
Azotobacter chroococcum NCIMB 8003 GtfD	19		45	18	18 (α1→4,6)	13 x 10^6	(Gangoiti et al., 2016)
Paenibacillus beijingensis DSM 24997GtfD[a]	17		54	11	18 (α1→4,6)	27 x 10^6	(Gangoiti et al., 2017c)
	15		62	5	18 (α1→4,6)	~19 x 10^3	

Fig. 5. α-Glucan products generated by starch-converting GH70 enzymes from amylose. [a]Note that *Paenibacillus beijingensis* GtfD activity on amylose results in the synthesis of both low- and high molecular weight reuteran-like products (Gangoiti et al., 2017d). Amylose V with an average M_w of 200 kDa was used as substrate (AVEBE, Foxhol, The Netherlands).

However, the product synthesized by dextran dextrinases differ from the IMMP by the presence of a small proportion of (α1 → 4) branches intercalated within the (α1 → 6) glucosyl linear chains (Yamamoto et al., 1993; Wang et al., 2011). Both dextran and IMMP products represent a source of dietary fiber (Yamamoto et al., 1993; Tsusaki et al., 2006; Leemhuis et al., 2014). *In vitro* digestibility experiments simulating the passage through the upper gastrointestinal tract and the small intestine revealed that the IMMPs have a high dietary fiber content (Leemhuis et al., 2014). Various starches and maltodextrins have been evaluated as substrates for IMMP synthesis demonstrating that substrates with longer linear (α1 → 4) linked glucose segments and lower amounts of (α1 → 4,6) branching points are preferred substrates of the *L. reuteri* 121 GtfB and yield products with higher amounts of (α1 → 6) linkages (Leemhuis et al., 2014). In fact, amylose V from potato starch (average M_w = 200 kDa) and high amylose starches (amylose content > 52%) yielded products with 91 and 61% of (α1 → 6) linkages. In contrast, only 28% and 33% of (α1 → 6) linkages were present in the products derived from potato starch (amylose content = 36%) and Paselli MD10 maltodextrins (average DP = 12), respectively. Interestingly, pullulanase-debranched potato starch delivered IMMP products containing up to 91% of (α1 → 6) linkages. Sequence alignments showed that GtfBs with 4,6-α-GTase activity somewhat differ from GSs regarding the residues forming the + 1 and + 2 acceptor binding subsites (Kralj et al., 2011; Leemhuis et al., 2013b) (Fig. 2). In motif III of GSs the well-conserved Trp (W1065 *L. reuteri* 180 Gtf180 numbering) residue (Fig. 2A) forms a stacking interaction with the acceptor substrate. In GtfB proteins this Trp is replaced by a Tyr residue (Y1055 in GtfB) (Fig. 2B). In motif IV, amino acid residues downstream of the transition state stabilizer, known to determine the correct orientation of the sugar moiety at subsite + 2 in GSs, are completely different in GtfBs. Whereas GSs show variation in this region (Fig. 2A), the GtfB-type 4,6-α-GTases display an invariant motif QRK (note that the alignment depicted in Fig. 2B predicts an one amino acid gap). Mutagenesis studies revealed the importance of residues Y1055 and K1128 for the transglycosylation activity of *L. reuteri* 121 GtfB (Bai et al., 2017). In fact, mutations at these positions converted the *L. reuteri* 121 4,6-α-GTase into an α-amylase with only hydrolytic activity.

3.1.1.1. Structure of Lactobacillus reuteri 121 GtfB and mechanism of action. Very recently, the 3D structure of *L. reuteri* 121 GtfB-ΔNΔV (N-terminally truncated and with a deleted domain V) has been determined in *apo* form as well as in complex with different oligosaccharides (Fig. 6), detailing the structural features that determine the unique reaction and product specificity of this enzyme (Bai et al., 2017), and confirming the evolutionary intermediate position of 4,6-α-GTases between GH13 α-amylases and GH70 GSs. As predicted, *L. reuteri* 121 GtfB displays the same U-fold domain organization as GSs (Fig. 6A, 7); however the general architecture of the active site region is different. Whereas GSs have only one donor subsite (− 1), with further donor subsites blocked by a loop (loop A2), a shorter corresponding loop in *L. reuteri* 121 GtfB results in the presence of multiple donor subsites, like in GH13 α-amylases (Fig. 6B and C). Consequently, *L. reuteri* 121 GtfB binds maltooligosaccharide donor substrates in a similar way as GH13 α-amylases, explaining their shared substrate specificity towards maltooligosaccharides. On the other hand, two long loops in *L. reuteri* 121 GtfB (A1 and B) create a unique tunnel-like architecture of the donor groove, likely related to the observation that, while most α-amylases transfer only one glucosyl unit, *L. reuteri* 121 GtfB-ΔNΔV is capable of transferring multiple glucosyl units at a time. The transglycosylation activity of GtfB is solely (α1 → 6) specific. Based on structural observations and kinetic characterization (Dobruchowska et al., 2012), a reaction scheme was proposed that fully explains the observed synthesis of a range of mixed IMMPs with (α1 → 6)-linked glucosyl units attached to the non-reducing end, from a series of MOSs and their corresponding alditols [degree of polymerization, DP2(-ol)-DP7(-ol)]. Finally, the 3D structure also

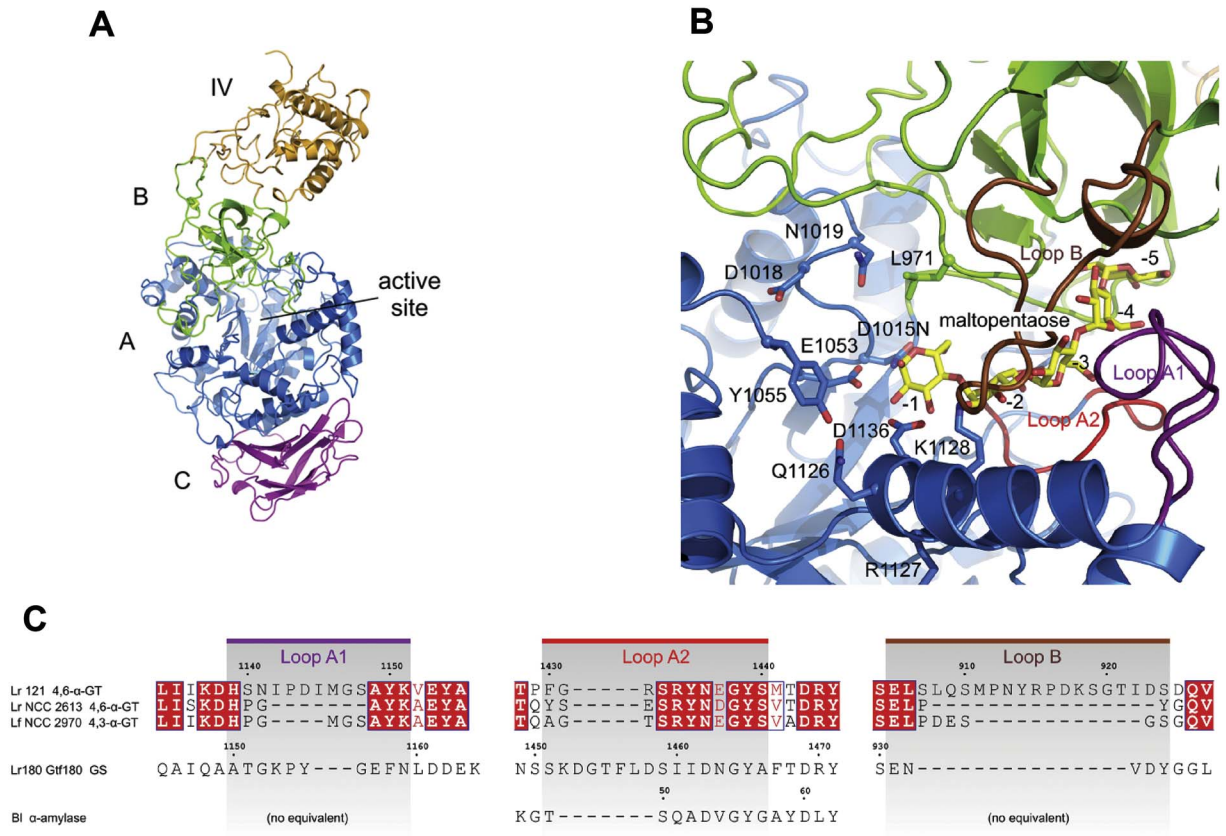


Fig. 6. Crystal structure of GtfB-ΔNΔV from *Lactobacillus reuteri* 121 (Bai et al., 2017). (A) Overall structure with the domains indicated, using the same colour scheme as in Fig. 3; note that domain V was not present in the crystallized (truncated) protein. (B) Crystal structure of the complex of an inactive mutant of GtfB-ΔNΔV with maltopentaose (yellow carbon atoms) bound to donor subsites − 1 to − 5. The catalytic residues D1015N (mutated), E1053 and D1136, as well as some other residues surrounding the active site, are indicated. Loop A1 and Loop B (purple and brown, resp.) partially cover the donor subsites, creating a tunnel in which the pentasaccharide binds. Loop A2 (red) is shorter than in glucansucrases and does not block donor subsites beyond subsite − 1. (C) Sequence alignment of loops A1, A2 and B near the active site, compared among enzymes of the GH70 GtfB subfamily, a GH70 glucansucrase and a GH13 α-amylase (Lr 121 4,6-α-GTase: *L. reuteri* 121 GtfB 4,6-α-glucanotransferase; Lr NCC 2613 4,6-α-GTase: *L. reuteri* NCC 2613 4,6-α-glucanotransferase; Lf NCC 2970 4,3-α-GTase: *L. fermentum* NCC 2970 4,3-α-glucanotransferase; Lr 180 GS: *L. reuteri* 180 Gtf180 glucansucrase Gtf180; Bl α-amylase: *B. licheniformis* α-amylase (has no equivalent to loops A1 and B)). Sequence alignment was performed with Clustal Omega; the alignment figures were prepared with ESPript 3 (Robert and Gouet, 2014). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

confirmed the importance of residues such as Y1055 and K1128 in the active site groove for the reaction specificity of GtfB.

3.1.2. *Lactobacillus fermentum* NCC 2970 GtfB 4,3-α-glucanotransferase

Recently we reported the identification and characterization of a GH70 glucanotransferase enzyme from *L. fermentum* NCC 2970 (Gangoiti et al., 2017b). Although this *L. fermentum* NCC 2970 GtfB shares 70% sequence identity with *L. reuteri* 121 GtfB, unique sequence variations of amino acid residues in motifs II (position 1029, Gtf180 numbering) and IV (positions 1137 and 1140) were found (Fig. 2C) that distinguishes L. *fermentum* NCC 2970 from most of the (putative) GtfB-type 4,6-α-GTase enzymes available in public databases. In GSs these positions are known to contribute to product specificity (Leemhuis et al., 2013b). In addition, loops A1 and B are 4 and 12 residues shorter, respectively, than in *L. reuteri* 121 GtfB (Fig. 6C). Homology modeling (not shown) indicated that this likely results in a completely open active site groove without a tunnel. These different features may have a large effect on the transglycosylation specificity. Indeed, we found that the *L. fermentum* NCC 2970 GtfB displays an entirely new product specificity: on amylose and maltoheptaose, it acts as a 4,3-α-glucanotransferase, providing the first evidence of this reaction and product specificity in the GH70 family and GH-H clan. The *L. fermentum* GtfB activity on amylose V from potato starch (average M_w = 200 kDa) results in the synthesis of a novel α-glucan consisting of maltooligosaccharide fragments interconnected via single (α1 → 3) linkages in linear or branching orientations (Fig. 5). The *L. fermentum* GtfB product is unique as none of the GSs characterized so far synthesize α-glucans consisting of (α1 → 4) and (α1 → 3) linkages. The structure of this α-glucan also differs from polysaccharides isolated from lichen and fungi, which contain the same linkage types but are mainly composed of (α1 → 3) linkages and do not present (α1 → 3,4) branching points. One may speculate that the formation of branched α-glucans by L. *fermentum* GtfB is related to its open active site architecture.

3.1.3. *Lactobacillus reuteri* NCC 2613 GtfB 4,6-α-glucanotransferase

An inventory of the GH70 enzymes present in the Nestlé Culture Collection genome database resulted in identification of 106 putative GtfB enzymes. Only seven of these GtfB-like protein sequences displayed further differences in motifs II and IV (Gangoiti et al., 2017c), namely at the same positions (1029, 1137 and 1140, Gtf180 numbering) as observed for L. *fermentum* GtfB 4,3-α-GTase (see Fig. 2C, D for more details). One of these GtfB proteins, encoded by *L. reuteri* NCC 2613, was biochemically characterized and its products from amylose were analyzed in detail. This revealed that, unlike most GtfB-type enzymes, which synthesize (or are predicted to synthesize) linear IMMPs, the *L. reuteri* NCC 2613 GtfB converts amylose V from potato starch (average M_w = 200 kDa) into a branched, low molecular mass (LMM) reuteran-like polymer built up of maltooligosaccharides (thus with (α1 → 4) linkages) from DP2 to 7, linked by (α1 → 6) linkages (Fig. 5). This polymer represents the first branched α-glucan product derived from amylose in LAB, and this reaction specificity is similar to the one previously described for GtfD GH70 enzymes found in non-LAB (see

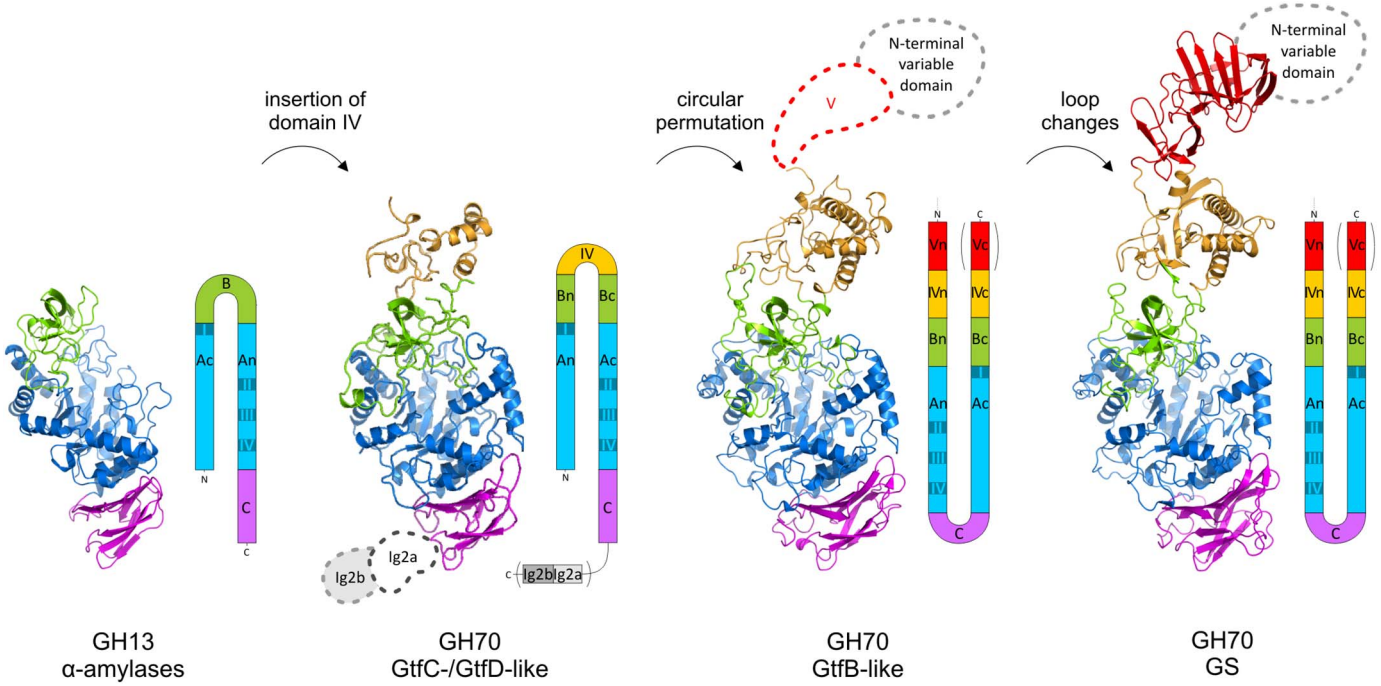


Fig. 7. Structures and modeled structures of representative members of the GH13 and GH70 families, with their domain organization. From left to right: crystal structure of α-amylase from *Bacillus licheniformis* (Accession number PDB ID: 1BLI; (Machius et al., 1998)); GtfC-type 4,6-α-glucanotransferase from *Exiguobacterium sibiricum* 255–15 (homology model); crystal structure of GtfB-ΔNΔV, the 4,6-α-glucanotransferase from *Lactobacillus reuteri* 121 (Accession number PDB ID: 5JBD; (Bai et al., 2017)); crystal structure of Gtf180-ΔN, the glucansucrase from *Lactobacillus reuteri* 180 (Accession number PDB ID: 3KLK; (Vujičić-Žagar et al., 2010)). Domains are colored as in Fig. 3; the positions of domains absent in the model or crystallized construct are indicated. The schematic domain organization depicted next to each model/structure highlights the circular permutation leading to a different order of the conserved motifs (II-III-IV-I instead of I-II-III-IV) in GH70 GtfB-like enzymes and GSs, but not in GH70 GtfC- and GtfD-like enzymes and GH13 α-amylases. Some of the evolutionary events related to domain organization and enzyme specificity are indicated. In α-amylase-like enzymes, insertion of domain IV (and sometimes attachment of Ig2 domains) may have led to a GtfC/GtfD-type domain organization (Gangoiti et al., 2015); gene duplication events resulted in circular permutation of the catalytic domain; changes in the loop arrangement around the active site then resulted in a shift of substrate specificity from starch to sucrose, as observed in glucanotransferases resp. glucansucrases (Bai et al., 2017).

below). Interestingly, 3D homology modeling (not shown) predicted that in *L. reuteri* NCC 2613 GtfB loops A1 and B, which are 7 and 16 residues shorter than in *L. reuteri* 121 GtfB (Fig. 6C), do not cover donor substrate binding subsites. As a result, the active site has a more open architecture, a feature that may explain its ability to form branched products.

3.2. GtfC-like GH70 subfamily of enzymes

The second GH70 subfamily of enzymes (designated as GtfC) was identified in different non-lactic acid Gram-positive bacteria of the genera *Exiguobacterium*, *Bacillus* and *Geobacillus* (Fig. 4) (Gangoiti et al., 2015; Meng et al., 2016c). The *Exiguobacterium*, *Bacillus* and *Geobacillus* genera are members of the low GC phylum of Firmicutes and belong to the class Bacilli. A total of 30 (putative) GtfC protein sequences are now available in public databases, but only the GtfC encoded by *Exiguobacterium sibiricum* 255–15 has been characterized so far. Bacteria encoding GtfC enzymes have been isolated from different sources, including extreme environments requiring low- and high-temperature adaptations. For example, *E. sibiricum* 255–15 is a psychrophilic non-spore forming bacterium that was isolated from ancient Siberian permafrost estimated to be 2–3 million years old (Rodrigues et al., 2008). In contrast, *Geobacillus* sp. 12AMOR1, also encoding a putative GtfC enzyme, is a thermophilic bacterium isolated from a 90 °C hot deep-sea sediment sample (Wissuwa et al., 2016). The amino acid sequence of *E. sibiricum* 255-15 GtfC clearly classifies it as a GH70 GtfB-type 4,6-α-GTase, in view of its high conservation of motifs I-IV, particularly in some of the residues forming acceptor binding subsites in GSs and conserved in most GtfB enzymes (Fig. 2B, E). Nevertheless, the overall sequence identity is low (e.g. 30% with *L. reuteri* 121 GtfB). Rather surprisingly, sequence comparisons revealed that GtfC-type enzymes lack the circular permutation of the $(\beta/\alpha)_8$ barrel characteristic for GSs and GtfB enzymes (compare sequence numbering of conserved motifs I–IV, Fig. 2A–E), and instead display a non-permuted GH13-like domain organization. GtfC-type enzymes share the core domains A, B and C with GH13 α-amylases, but differ in the presence of an extra continuous domain IV interrupting domain B, so far only identified in GH70 family proteins (Fig. 7). The N-terminal variable region and the remote domain V typical of GSs and GtfB-like proteins, however, are not found in GtfC enzymes. Moreover, several GtfC proteins have one or two extra Ig2-like motifs of unknown function at their C-terminus. The unique domain organization of the GtfC subfamily further clarifies the evolutionary pathway of GH13 and GH70 enzymes (Fig. 7), since it shows that a domain insertion event (namely of domain IV) took place in a GH13 α-amylase precursor as a first step. Later on, duplication and fusion of a GtfC ancestor gene, followed by its partial truncation, and the subsequent insertion of domain V may have led to the unusual U-fold domain organization with a circularly permuted $(\beta/\alpha)_8$ catalytic barrel found in the GtfB GH70 subfamily and in GSs (Fig. 7). Similar to the *L. reuteri* 121 GtfB, the *E. sibiricum* GtfC acts as a 4,6-α-GTase, cleaving the (α1 → 4) linkages of starch-like substrates (e.g. amylose V and MOS from DP 4 to 7) and synthesizing consecutive (α1 → 6) linkages (Fig. 5). However, *E. sibiricum* GtfC activity results in the synthesis of oligosaccharides (designated as IsoMalto-Malto/Oligosaccharides, IMMOs), instead of polymeric material. The percentage of (α1 → 6) linkages in the product mixtures was found to be positively correlated with the DP of the substrate (e.g. 33%, 44%, and 52% of (α1 → 6) linkages were present in the products derived from maltohexaose, maltoheptaose and amylose V, respectively). Isomaltooligosaccharides and oligodextrans have been described as promising prebiotics (Goffin et al., 2011). Commercial isomaltooligosaccharides are produced from starch by a 3-step enzymatic process, which includes starch

liquefaction, saccharification, and transglycosylation (Niu et al., 2017). The novel GtfC type of enzymes provide an alternative method for the production of oligosaccharides containing (α1 → 4) and (α1 → 6) linkages from starch-like substrates, involving use of a single enzyme (Kralj, 2017).

3.3. GtfD-like GH70 subfamily of enzymes

The GtfD-like proteins identified in taxonomically diverse plant-associated bacteria constitute the third GH70 subfamily of enzymes with 4,6-α-GTase activity on different starch-like substrates, such as potato starch, amylose and MOS from DP 4 to 7 (Gangoiti et al., 2016, 2017d). Members of the GtfD subfamily are phylogenetically very closely related to GtfC type of enzymes (Fig. 4) and display a similar non-permuted GH13 like-fold architecture (Fig. 7). However, the GtfD enzymes differ in their microbial origin as they are present not only in Gram-positive bacteria (i.e. *Paenibacillus* species), but also in diverse Gram-negative proteobacteria (i.e. *Dyella*-like sp. DHo, *Burkholderia* sp. NFACC38–1 and *Pseudomonadales* bacterium GWC1 66 9). So far only the GtfD enzymes encoded by the nitrogen-fixing bacteria *Azotobacter chroococcum* NCIMB 8003 (Gangoiti et al., 2016) and *Paenibacillus beijingensis* DSM 24997 (Gangoiti et al., 2017d) have been biochemically characterized and their products from amylose V from potato starch (average M_w = 200 kDa) characterized in detail. Both GtfD enzymes act as 4,6-α-GTases on (α1 → 4) glucans, but display a novel product specificity. Instead of catalyzing the formation of consecutive (α1 → 6) linkages, GtfD activity on amylose results in the synthesis of relatively highly branched polysaccharides consisting of maltooligosacharide units linked by single linear (α1 → 6) glycosidic linkages and (α1 → 4,6) branching points (Fig. 5). Structurally, these GtfD products resemble the reuteran polymer synthesized by the *L. reuteri* 121 GtfA GS from sucrose, and thus these polymers may have the same positive effects on satiety and bread quality as described for this reuteran (Ekhart et al., 2006; Plijter et al., 2009). The reuteran-like products synthesized by the *A. chroococcum* GtfD and *P. beijingensis* GtfD differ in size, with either a monomodal (13 MDa) or a bimodal (27 MDa and 19 kDa) distribution, respectively. The structural characteristics of the different reuteran-like polymers that can be synthesized from amylose are summarized in Fig. 5. These reuteran-like polymers differ in their size, amount of linear (α1 → 6) linkages and length of the linear (α1 → 4) segments. *In vitro* experiments have shown that these reuteran-like polymers are clearly less susceptible to hydrolysis by digestive enzymes than the amylose substrate. Moreover, *A. chroococcum* and *P. beijingensis* GtfD activity on wheat starch resulted in the production of both resistant and slowly digestible starch-derived products. The use of GtfD 4,6-α-GTases for the production of reuteran from starch, and their application for the reduction of the glycemic index of starchy food has been patented (Gangoiti et al., 2017a).

4. Conclusions and future directions

The discovery and characterization of GH70 enzymes with new reaction and product specificities has opened new directions for the synthesis of novel α-glucan products, not only from sucrose but also from starch. This has been facilitated by the availability of genomic data that can now easily be mined to identify putative enzymes of interest, in case of GH70 enzymes guided by analyzing conserved motifs I-IV (Passerini et al., 2015; Brison et al., 2016; Vuillemin et al., 2016). Such an approach can be expected to lead to the discovery of even more GH13-GH70 enzymes with interesting specificities and further detailing evolutionary pathways between these GH families.

For GSs, enzyme engineering studies and the availability of 3D structures demonstrated that their product specificity is determined by the interplay of various amino acid residues shaping the acceptor binding subsites (Meng et al., 2016c). This makes it difficult to predict the effect of single mutations, even more so because it may differ between GSs of different sources (Chen and Ganzle, 2016). Mutations may also lead to changes in more than one structural feature of the product, and/or affect the enzyme transglycosylation activity (Hellmuth et al., 2008; Meng et al., 2014, 2016a,c). While mutational and 3D structural studies have advanced our understanding of the structure-function relationship in GSs, little is still known about the later stages of α-glucan synthesis, when (intermediate) reaction products become large enough to extend beyond the catalytic domain and possibly bind to remote domains. Therefore, also given the observed intrinsic domain flexibility of GSs, approaches such as SAXS, electron microscopy and molecular dynamics may be needed to help clarify their structure-function relationship. At present, the rational, structure-based enzyme engineering to synthesize tailor-made α-glucans is still a challenge. An alternative powerful strategy to further enlarge the panel of α-glucans available is to combine directed evolution approaches with high-throughput NMR screening methods (Irague et al., 2011). Finally, directed evolution and structure-guided mutagenesis approaches can also be used to develop GS variants that are more thermostable and/or are catalytically more efficient (e.g., less hydrolytic) in synthesizing α-glucans for future industrial applications.

Compared to GSs, current understanding of the structure-function relationships and product specificity of starch-converting GH70 enzymes is still relatively limited, with only one experimental 3D structure known (*L. reuteri* 121 GtfB-ΔNΔV) and only donor subsites experimentally mapped. Complexes with acceptor substrates are needed, as well as the 3D structures of other GtfB, GtfC and GtfD enzymes. Such structures may help understand linkage specificity (e.g. the 4,3-specificity of *L. fermentum* GtfB 4,3-α-GTase GtfB, or the branching capability of *L. reuteri* NCC 2613 GtfB) or help identify the structural features in GtfC and GtfD enzymes that favor transglycosylation compared to the more hydrolytic GH13 α-amylases. New 3D structures will allow rational enzyme engineering to alter linkage specificity and to expand the repertoire of α-glucans that can be produced from starch. In the future this information may allow the development of engineering approaches to confer GH13 α-amylases, which are more common in nature, with transglycosylation activities.

Finally, GtfB, GtfC and GtfD 4,3-/4,6-α-GTases provide novel approaches for modification of starches into soluble slowly digestible carbohydrates or dietary fibers, thereby reducing the blood glucose levels after food consumption (Dijkhuizen et al., 2010; Gangoiti et al., 2017a; Kralj, 2017). Such low-glycemic index carbohydrates are generally believed to have a positive impact on health, and may be particularly important for individuals having diabetes or pre-diabetes. Investigation of the physicochemical (viscosity, solubility, etc.) and health promoting properties of these novel α-glucans is key for use of these enzymes as a tool to produce functional food ingredients.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biotechadv.2017.11.001.

Acknowledgments

The authors thankfully acknowledge financial support from the University of Groningen, the TKI Agri&Food program as coordinated by the Carbohydrate Competence Center (CCC-ABC; www.cccresearch.nl), AVEBE U.A. and Nestec Ltd.

References

Andre, I., Potocki-Veronese, G., Morel, S., Monsan, P., Remaud-Simeon, M., 2010. Sucrose-utilizing transglucosidases for biocatalysis. Top. Curr. Chem. 294, 25–48.
Arguello-Morales, M.A., Remaud-Simeon, M., Pizzut, S., Sarcabal, P., Willemot, R., 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.
Badel, S., Bernardi, T., Michaud, P., 2011. New perspectives for Lactobacilli exopolysaccharides. Biotechnol. Adv. 29, 54–66.
Bai, Y., van der Kaaij, R.M., Leemhuis, H., Pijning, T., van Leeuwen, S.S., Jin, Z., et al., 2015. Biochemical characterization of the *Lactobacillus reuteri* glycoside hydrolase

family 70 GTFB type of 4,6-alpha-glucanotransferase enzymes that synthesize soluble dietary starch fibers. Appl. Environ. Microbiol. 81, 7223–7232.
Bai, Y., Boger, M., van der Kaaij, R.M., Woortman, A.J., Pijning, T., van Leeuwen, S.S., et al., 2016. *Lactobacillus reuteri* strains convert starch and maltodextrins into homoexopolysaccharides using an extracellular and cell-associated 4,6-alpha-glucanotransferase. J. Agric. Food Chem. 64, 2941–2952.
Bai, Y., Gangoiti, J., Dijkstra, B.W., Dijkhuizen, L., Pijning, T., 2017. Crystal structure of 4,6-α-glucanotransferase supports diet-driven evolution of GH70 enzymes from α-amylases in oral bacteria. Structure 25, 231–242.
Biely, P., Côté, G.L., Burgess-Cassler, A., 1994. Purification and properties of alternanase, a novel endo-α-1,3-α-1,6-d-glucanase. J. Biochem. 226, 633–639.
Bozonnet, S., Dols-Laffargue, M., Fabre, E., Pizzut, S., Remaud-Simeon, M., Monsan, P., et al., 2002. Molecular characterization of DSR-E, an alpha-1,2 linkage-synthesizing dextransucrase with two catalytic domains. J. Bacteriol. 184, 5753–5761.
Brison, Y., Fabre, E., Moulis, C., Portais, J.C., Monsan, P., Remaud-Simeon, M., 2010. Synthesis of dextrans with controlled amounts of alpha-1,2 linkages using the transglucosidase GBD-CD2. Appl. Microbiol. Biotechnol. 86, 545–554.
Brison, Y., Pijning, T., Malbert, Y., Fabre, E., Mourey, L., Morel, S., et al., 2012. Functional and structural characterization of alpha-(1- > 2) branching sucrase derived from DSR-E glucansucrase. J. Biol. Chem. 287, 7915–7924.
Brison, Y., Laguerre, S., Lefoulon, F., Morel, S., Monties, N., Potocki-Véronèse, G., et al., 2013. Branching pattern of gluco-oligosaccharides and 1.5 kDa dextran grafted by the α-1,2 branching sucrase GBD-CD2. Carbohydr. Polym. 94, 567–576.
Brison, Y., Malbert, Y., Czaplicki, G., Mourey, L., Remaud-Simeon, M., Tranier, S., 2016. Structural insights into the carbohydrate binding ability of an α-(1 → 2) branchingsucrase from Glycoside Hydrolase family 70. J. Biol. Chem. 291, 7527–7540.
Buddana, S., Varanasi, Y., Shetty, P., 2015. Fibrinolytic, anti-inflammatory and anti-microbial properties of alpha-(1-3)-glucans produced from *Streptococcus mutans* (MTCC 497). Carbohydr. Polym. 115, 152–159.
Caimi, PG, Hennessey SM. Increased poly (alpha - 1, 3 - glucan) yield using tetraborate. WO2013096511 A1. 2013.
Chen, X.Y., Ganzle, M.G., 2016. Site directed mutagenesis of dextransucrase DsrM from *Weissella cibaria*: transformation to a reuteransucrase. J. Agric. Food Chem. 64, 6848–6855.
Colby, S.M., McLaughlin, R.E., Ferretti, J.J., Russell, R.R., 1999. Effect of inactivation of gtf genes on adherence of *Streptococcus downei*. Oral Microbiol. Immunol. 14, 27–32.
Côté, G.L., Ahlgren, J.A., 2001. The hydrolytic and transferase action of alternanase on oligosaccharides. Carbohydr. Res. 332, 373–379.
Côté, 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 goes to 6), (1 goes to 3)-alpha-D-glucan. Carbohydr. Res. 101, 57–74.
Côte, G.L., Skory, C.D., 2012. Cloning, expression, and characterization of an insoluble glucan-producing glucansucrase from *Leuconostoc mesenteroides* NRRL B-1118. Appl. Microbiol. Biotechnol. 93, 2387–2394.
Desmet, T., Soetaert, W., Bojarova, P., Kren, V., Dijkhuizen, L., Eastwick-Field, V., et al., 2012. Enzymatic glycosylation of small molecules: challenging substrates require tailored catalysts. Chemistry 18, 10786–10801.
Dijkhuizen L, Van der Maarel MJEC, Kamerling JP, Leemhuis RJ, Kralj S, Dobruchowska JM. Glucooligosaccharides Comprising (alpha 1- > 4) and (alpha 1- > 6) glycosidic bonds, use thereof, and methods for producing them. CA 2761257 A1. 2010.
Dobruchowska, J.M., Gerwig, G.J., Kralj, S., Grijpstra, P., Leemhuis, H., Dijkhuizen, L., et al., 2012. Structural characterization of linear isomalto-/malto-oligomer products synthesized by the novel GTFB 4,6-alpha-glucanotransferase enzyme from *Lactobacillus reuteri* 121. Glycobiology 22, 517–528.
Ekhart P, van Geel-Schutten GH, Van Binsbergen M, Timmerman E. Branched alpha-glucans for weight management. US 20060100171 A1. 2006.
Fabre, E., Bozonnet, S., Arcache, A., Willemot, R.M., Vignon, M., Monsan, P., et al., 2005. Role of the two catalytic domains of DSR-E dextransucrase and their involvement in the formation of highly alpha-1,2 branched dextran. J. Bacteriol. 187, 296–303.
Frohberg C, Pilling J. Use of alternan as texturizing agent in foodstuffs and cosmetics. WO 2009109407 A3. 2009.
Gangoiti, J., Pijning, T., Dijkhuizen, L., 2015. The *Exiguobacterium sibiricum* 255-15 GtfC enzyme represents a novel glycoside hydrolase 70 subfamily of 4,6-alpha-glucanotransferase enzymes. Appl. Environ. Microbiol. 82, 756–766.
Gangoiti, J., van Leeuwen, S.S., Vafeiadi, C., Dijkhuizen, L., 2016. The gram-negative bacterium *Azotobacter chroococcum* NCIMB 8003 employs a new glycoside hydrolase family 70 4,6-alpha-glucanotransferase enzyme (GtfD) to synthesize a reuteran like polymer from maltodextrins and starch. Biochim. Biophys. Acta 1860, 1224–1236.
Gangoiti J, van Leeuwen SS, Dijkhuizen L, Vafeiadi C, Lamothe L. Branched α-glucans. WO2017046040 A1. 2017a.
Gangoiti, J., van Leeuwen, S.S., Gerwig, G.J., Duboux, S., Pijning, T., Dijkhuizen, L., 2017b. 4,3-α-Glucanotransferase, a novel reaction specificity in glycoside hydrolase family 70 and clan GH-H. Sci. Rep. 7, 39761.
Gangoiti, J., van Leeuwen, S., Meng, X., Duboux, S., Vafeiadi, C., Pijning, T., et al., 2017c. Mining novel starch-converting glycoside hydrolase 70 enzymes from the Nestlé culture collection genome database: the *Lactobacillus reuteri* NCC 2613 GtfB. Sci. Rep. 7, 9947.
Gangoiti, J., Lamothe, L., van Leeuwen, S., Vafeiadi, C., Dijkhuizen, L., 2017d. Characterization of the *Paenibacillus beijingensis* DSM 24997 GtfD and its glucan polymer products representing a new Glycoside Hydrolase 70 subfamily of 4,6-α-glucanotransferase enzymes. PLoS One 12, e0172622.
van Geel-Schutten, I.G.H., Flesch, F., ten Brink, B., Smith, M.R., Dijkhuizen, L., 1998. Screening and characterization of *Lactobacillus* strains producing large amounts of exopolysaccharides. Appl. Microbiol. Biotechnol. 50, 697–703.
Goffin, D., Delzenne, N., Blecker, C., Hanon, E., Deroanne, C., Paquot, M., 2011. Will isomalto-oligosaccharides, a well-established functional food in Asia, break through the European and American market? The status of knowledge on these prebiotics. Crit. Rev. Food Sci. Nutr. 51, 394–409.
Hanada, N., Kuramitsu, H.K., 1989. Isolation and characterization of the *Streptococcus mutans gtfD* gene, coding for primer-dependent soluble glucan synthesis. Infect. Immun. 57, 2079–2085.
Haydersah, J., Chevallier, I., Rochette, I., Mouquet-Rivier, C., Picq, C., Marianne-Pepin, T., et al., 2012. Fermentation by amylolytic lactic acid bacteria and consequences for starch digestibility of plantain, breadfruit, and sweet potato flours. J. Food Sci. 77, M466–72.
Hehre, E.J., Sugg, J.Y., 1942. Serologically reactive polysaccharides produced through the action of bacterial enzymes: I. Dextran of *Leuconostoc mesenteroides* from sucrose. J. Exp. Med. 75, 339–353.
Hellmuth, H., Wittrock, S., Kralj, S., Dijkhuizen, L., Hofer, B., Seibel, J., 2008. Engineering the glucansucrase GTFR enzyme reaction and glycosidic bond specificity: toward tailor-made polymer and oligosaccharide products. Biochemistry 47, 6678–6684.
van Hijum, S.A., Kralj, S., Ozimek, L.K., Dijkhuizen, L., van Geel-Schutten, I.G., 2006. Structure-function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. Microbiol. Mol. Biol. Rev. 70, 157–176.
Hiler, MJ. Fibers, yarns and filaments of water-insoluble dextran. US 2972178 A. 1961.
Irague, R., Massou, S., Moulis, C., Saurel, O., Milon, A., Monsan, P., et al., 2011. NMR-based structural glycomics for high-throughput screening of carbohydrate-active enzyme specificity. Anal. Chem. 83, 1202–1206.
Irague, R., Rolland-Sabaté, A., Tarquis, L., Doublier, J., Moulis, C., Monsan, P., et al., 2012. Structure and property engineering of a-D-glucans synthesized by dextransucrase mutants. Biomacromolecules 13, 187–195.
Ito, K., Ito, S., Shimamura, T., Weyand, S., Kawarasaki, Y., Misaka, T., et al., 2011. Crystal structure of glucansucrase from the dental caries pathogen *Streptococcus mutans*. J. Mol. Biol. 408, 177–186.
Janecek, S., Gabrisko, M., 2016. Remarkable evolutionary relatedness among the enzymes and proteins from the alpha-amylase family. Cell. Mol. Life Sci. 73, 2707–2725.
Kang, H.K., JS, Oh., Kim, D., 2009. Molecular characterization and expression analysis of the glucansucrase DSRWC from *Weissella cibaria* synthesizing a alpha(1– > 6) glucan. FEMS Microbiol. Lett. 292, 33–41.
Kang, H.K., Kimura, A., Kim, D., 2011. Bioengineering of *Leuconostoc mesenteroides* glucansucrases that gives selected bond formation for glucan synthesis and/or acceptor-product synthesis. J. Agric. Food Chem. 59, 4148–4155.
Kim, D., Robyt, J.F., 1994. Production and selection of mutants of *Leuconostoc mesenteroides* constitutive for glucansucrases. Enzym. Microb. Technol. 16, 659–664.
Kobayashi, M., Matsuda, K., 1977. Structural characteristics of dextran synthesised by dextransucrases from *Leuconostoc mesenteroides* NRRL B-1299. Agric. Biol. Chem. 41, 1931–1937.
Kralj S. Compositions and methods comprising the use of *Exiguobacterium acetylicum* and *Bacillus coagulans* α-glucanotransferase enzymes. WO 2017062687 A1. 2017.
Kralj, S., van Geel-Schutten, G.H., Rahaoui, H., Leer, R.J., Faber, E.J., van der Maarel, M.J., et al., 2002. Molecular characterization of a novel glucosyltransferase from *Lactobacillus reuteri* strain 121 synthesizing a unique, highly branched glucan with alpha-(1→4) and alpha-(1→6) glucosidic bonds. Appl. Environ. Microbiol. 68, 4283–4291.
Kralj, S., van Geel-Schutten, G.H., Dondorff, M.M., Kirsanovs, S., van der Maarel, M.J., Dijkhuizen, L., 2004a. Glucan synthesis in the genus *Lactobacillus*: isolation and characterization of glucansucrase genes, enzymes and glucan products from six different strains. Microbiology 150, 3681–3690.
Kralj, S., van Geel-Schutten, G.H., van der Maarel, M.J., Dijkhuizen, L., 2004b. Biochemical and molecular characterization of *Lactobacillus reuteri* 121 reuteransucrase. Microbiology 150, 2099–2112.
Kralj, S., Stripling, E., Sanders, P., van Geel-Schutten, G.H., Dijkhuizen, L., 2005a. Highly hydrolytic reuteransucrase from probiotic *Lactobacillus reuteri* strain ATCC 55730. Appl. Environ. Microbiol. 71, 3942–3950.
Kralj, S., van Geel-Schutten, I.G., Faber, E.J., van der Maarel, M.J., Dijkhuizen, L., 2005b. Rational transformation of *Lactobacillus reuteri* 121 reuteransucrase into a dextransucrase. Biochemistry 44, 9206–9216.
Kralj, S., Eeuwema, W., Eckhardt, T.H., Dijkhuizen, L., 2006. Role of asparagine 1134 in glucosidic bond and transglycosylation specificity of reuteransucrase from *Lactobacillus reuteri* 121. FEBS J. 273, 3735–3742.
Kralj, S., Grijpstra, P., van Leeuwen, S.S., Leemhuis, H., Dobruchowska, J.M., van der Kaaij, R.M., et al., 2011. 4,6- α -Glucanotransferase, a novel enzyme that structurally and functionally provides an evolutionary link between glycoside hydrolase enzyme families 13 and 70. Appl. Environ. Microbiol. 77, 8154–8163.
Leemhuis, H., Dijkman, W.P., Dobruchowska, J.M., Pijning, T., Grijpstra, P., Kralj, S., et al., 2013a. 4,6-alpha-Glucanotransferase activity occurs more widespread in *Lactobacillus* strains and constitutes a separate GH70 subfamily. Appl. Microbiol. Biotechnol. 97, 181–193.
Leemhuis, H., Pijning, T., Dobruchowska, J.M., van Leeuwen, S.S., Kralj, S., Dijkstra, B.W., et al., 2013b. Glucansucrases: three-dimensional structures, reactions, mechanism, alpha-glucan analysis and their implications in biotechnology and food applications. J. Biotechnol. 163, 250–272.
Leemhuis, H., Dobruchowska, J.M., Ebbelaar, M., Faber, F., Buwalda, P.L., van der Maarel, M.J., et al., 2014. Isomalto/malto-polysaccharide, a novel soluble dietary fiber made via enzymatic conversion of starch. J. Agric. Food Chem. 62, 12034–12044.
van Leeuwen, S.S., Kralj, S., Gerwig, G.J., Dijkhuizen, L., Kamerling, J.P., 2008a. Structural analysis of bioengineered alpha-D-glucan produced by a triple mutant of the Glucansucrase GTF180 enzyme from *Lactobacillus reuteri* strain 180: generation of (alpha1→4) linkages in a native (1→3)(1→6)-alpha-D-glucan. Biomacromolecules 9

(8), 2251.
van Leeuwen, S.S., Kralj, S., van Geel-Schutten, I.H., Gerwig, G.J., Dijkhuizen, L., Kamerling, J.P., 2008b. Structural analysis of the alpha-D-glucan (EPS180) produced by the *Lactobacillus reuteri* strain 180 glucansucrase GTF180 enzyme. Carbohydr. Res. 343, 1237–1250.
van Leeuwen, S.S., Kralj, S., van Geel-Schutten, I.H., Gerwig, G.J., Dijkhuizen, L., Kamerling, J.P., 2008c. Structural analysis of the alpha-D-glucan (EPS35-5) produced by the *Lactobacillus reuteri* strain 35-5 glucansucrase GTFA enzyme. Carbohydr. Res. 343, 1251–1265.
van Leeuwen, S.S., Kralj, S., Eeuwema, W., Gerwig, G.J., Dijkhuizen, L., Kamerling, J.P., 2009. Structural characterization of bioengineered alpha-D-glucans produced by mutant glucansucrase GTF180 enzymes of *Lactobacillus reuteri* strain 180. Biomacromolecules 10, 580–588.
Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P.M., Henrissat, B., 2014. The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res. 42, D490–5.
MacGregor, E.A., Jespersen, H.M., Svensson, B., 1996. A circularly permuted alpha-amylase-type alpha/beta-barrel structure in glucan-synthesizing glucosyltransferases. FEBS Lett. 378, 263–266.
Machius, M., Declerck, N., Huber, R., Wiegand, G., 1998. Activation of *Bacillus licheniformis* alpha-amylase through a disorder– > order transition of the substrate-binding site mediated by a calcium-sodium-calcium metal triad. Structure 6, 281–292.
Meng, X., Dobruchowska, J.M., Pijning, T., Lopez, C.A., Kamerling, J.P., Dijkhuizen, L., 2014. Residue Leu940 has a crucial role in the linkage and reaction specificity of the glucansucrase Gtf180 of the probiotic bacterium *Lactobacillus reuteri* 180. J. Biol. Chem. 289, 32773–32782.
Meng, X., Dobruchowska, J.M., Pijning, T., Gerwig, G.J., Kamerling, J.P., Dijkhuizen, L., 2015a. Truncation of domain V of the multidomain glucansucrase Gtf180 of *Lactobacillus reuteri* 180 heavily impairs its polysaccharide-synthesizing ability. Appl. Microbiol. Biotechnol. 99, 5885–5894.
Meng, X., Pijning, T., Dobruchowska, J.M., Gerwig, G.J., Dijkhuizen, L., 2015b. Characterization of the functional roles of amino acid residues in acceptor-binding subsite +1 in the active site of the glucansucrase Gtf180 from *Lactobacillus reuteri* 180. J. Biol. Chem. 290, 30131–30141.
Meng, X., Dobruchowska, J.M., Pijning, T., Gerwig, G.J., Dijkhuizen, L., 2016a. Synthesis of new hyperbranched alpha-glucans from sucrose by *Lactobacillus reuteri* 180 glucansucrase mutants. J. Agric. Food Chem. 64, 433–442.
Meng, X., Pijning, T., Dobruchowska, J.M., Yin, H., Gerwig, G.J., Dijkhuizen, L., 2016b. Structural determinants of alternating (α1→4) and (α1→6) linkage specificity in reuteransucrase of *Lactobacillus reuteri*. Sci. Rep. 6, 35261.
Meng, X., Gangoiti, J., Bai, Y., Pijning, T., Van Leeuwen, S.S., Dijkhuizen, L., 2016c. Structure-function relationships of family GH70 glucansucrase and 4,6-alpha-glucanotransferase enzymes, and their evolutionary relationships with family GH13 enzymes. Cell. Mol. Life Sci. 73, 2681–2706.
Meng, X., Pijning, T., Tietema, M., Dobruchowska, J.M., Yin, H., Gerwig, G.J., Kralj, S., Dijkhuizen, L., 2017. Characterization of the glucansucrase Gtf180 W1065 mutant enzymes producing polysaccharides and oligosaccharides with altered linkage position. Food Chem. 217, 81–90.
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 alpha (1-6) and alpha (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., 1998. Cloning and sequencing of a gene coding for an extracellular dextransucrase (DSRB) from *Leuconostoc mesenteroides* NRRL B-1299 synthesizing only a alpha (1-6) glucan. FEMS Microbiol. Lett. 159, 307–315.
Monsan, P., Remaud-Simeon, M., Andre, I., 2010. Transglucosidases as efficient tools for oligosaccharide and glucoconjugate synthesis. Curr. Opin. Microbiol. 13, 293–300.
Moulis, C., Joucla, G., Harrison, D., Fabre, E., Potocki-Veronese, G., Monsan, P., et al., 2006. Understanding the polymerization mechanism of glycoside-hydrolase family 70 glucansucrases. J. Biol. Chem. 281, 31254–31267.
Naessens, M., Cerdobbel, A., Soetaert, W., Vandamme, E.J., 2005a. *Leuconostoc* dextransucrase and dextran: production, properties and applications. J. Chem. Technol. Biotechnol. 80, 845–860.
Naessens, M., Cerdobbel, A., Soetaert, W., Vandamme, E.J., 2005b. Dextran dextrinase and dextran of *Gluconobacter oxydans*. J. Ind. Microbiol. Biotechnol. 32, 323–324.
Nichols, SE. Glucan-containing compositions and paper. US6465203 B2. 2002.
Niu, D., Qiao, J., Li, P., Tian, K., Liu, X., Singh, S., Lu, F., 2017. Highly efficient enzymatic preparation of isomalto-oligosaccharides from starch using an enzyme cocktail. Electron. J. Biotechnol. 26, 46–51.
O'Brien, JP. Novel composition for preparing polysaccharide fibers. US9540747 B2. 2015.
Opper, K. Fiber composition comprising 1,3-glucan and a method of preparing same. US9365955 B2. 2013.
Passerini, D., Vuillemin, M., Ufarté, L., Morel, S., Loux, V., Fontagné-Faucher, C., et al., 2015. Inventory of the GH70 enzymes encoded by *Leuconostoc citreum* NRRL B-1299 - identification of three novel α-transglucosylases. FEBS J. 282, 2115–2130.
Pijning, T., Vujicic-Zagar, A., Kralj, S., Dijkhuizen, L., Dijkstra, B.W., 2012. Structure of the alpha-1,6/alpha-1,4-specific glucansucrase GTFA from *Lactobacillus reuteri* 121. Acta Crystallogr. F Struct. Biol. Cryst. Commun. 68, 1448–1454.
Pijning, T., Vujicic-Zagar, A., Kralj, S., Dijkhuizen, L., Dijkstra, B.W., 2014. Flexibility of truncated and full-length glucansucrase GTF180 enzymes from *Lactobacillus reuteri* 180. FEBS J. 281, 2159–2171.
Plijter J, Jurgens A, Kats MP, Noort MWJ, Heddes CEA, Van Geel Schutten GH. Bread improver. US 20090297663 A1. 2009.
Puanglek, S., Kimura, S., Enomoto-Rogers, Y., Kabe, T., Yoshida, M., Wada, M., et al., 2016. *In vitro* synthesis of linear a-1,3-glucan and chemical modification to ester derivatives exhibiting outstanding thermal properties. Sci. Rep. 169, 245–254.
Puanglek, S., Kimura, S., Iwata, T., 2017. Thermal and mechanical properties of tailor-made unbranched a-1,3-glucan esters with various carboxylic acid chain length. Carbohydr. Polym. 169, 245–254.
Robert, X., Gouet, P., 2014. Deciphering key features in protein structures with the new ENDscript server. Nucleic Acids Res. 42, W320–4.
Rodrigues, D.F., Ivanova, N., He, Z., Huebner, M., Zhou, J., Tiedje, J.M., 2008. Architecture of thermal adaptation in an *Exiguobacterium sibiricum* strain isolated from 3 million year old permafrost: a genome and transcriptome approach. BMC Genom. 9 (547,2164-9-547).
Sadahiro, J., Mori, H., Saburi, W., Okuyama, M., Kimura, A., 2015. Extracellular and cell-associated forms of Gluconobacter oxydans dextran dextrinase change their localization depending on the cell growth. Biochem. Biophys. Res. Commun. 456, 500–505.
Sanz, M., Côté, G., Gibson, G., Rastall, R., 2005. Prebiotic properties of alternansucrase maltose-acceptor oligosaccharides. J. Agric. Food Chem. 53, 5911–5916.
Sawai, T., Tohyama, T., Natsume, T., 1978. Hydrolysis of fourteen native dextrans by *Arthrobacter* isomaltodextranase and correlation with dextran structure. Carbohydr. Res. 66, 195–205.
Sawai, T., Ohara, S., Ichimi, Y., Okaji, S., Hisaha, K., Fukuya, N., 1981. Purification and some properties of the isomaltodextranases *Actinornadura* strain R10 and comparison with that of *Arthrobacter globifomis* T6. Carbohydr. Res. 89, 289–299.
Schwab, C., Walter, J., Tannock, G.W., Vogel, R.F., Ganzle, M.G., 2007. Sucrose utilization and impact of sucrose on glycosyltransferase expression in *Lactobacillus reuteri*. Syst. Appl. Microbiol. 30, 433–443.
Seymour, F.R., Chen, E.C.M., Bishop, S.H., 1979. Methylation structural analysis of unusual dextrans by combined gas-liquid chromatography and mass spectrometry. Carbohydr. Res. 68, 113–121.
Shimamura, A., Nakano, Y.J., Mukasa, H., Kuramitsu, H.K., 1994. Identification of amino acid residues in *Streptococcus mutans* glucosyltransferases influencing the structure of the glucan product. J. Bacteriol. 176, 4845–4850.
Singh, J., Dartois, A., Kaur, L., 2010. Starch digestibility in food matrix: a review. Trends Food Sci. Technol. 21, 168–180.
Stam, M.R., Danchin, E.G., Rancurel, C., Coutinho, P.M., Henrissat, B., 2006. Dividing the large glycoside hydrolase family 13 into subfamilies: towards improved functional annotations of alpha-amylase-related proteins. Protein Eng. Des. Sel. 19, 555–562.
Tsumori, H., Kuramitsu, H., 1997. The role of the *Streptococcus mutans* glucosyltransferases in the sucrose-dependent attachment to smooth surfaces: essential role of the GtfC enzyme. Oral Microbiol. Immunol. 12, 274–280.
Tsusaki K, Kubota M, Fukuda S, Miyake T. Dextrin dextranase, method of producing the same and use of the same. WO 2006054474 A1. 2006.
Valette, P., Pelenc, V., Djouzi, Z., Andrieux, C., Paul, F., Monsan, P., et al., 1993. Bioavailability of new synthesized glucooligosaccharides in the intestinal tract of gnotobiotic rats. J. Sci. Food Agric. 62, 121–127.
Vuillemin, M., Claverie, M., Brison, Y., Severac, E., Bondy, P., Morel, S., et al., 2016. Characterization of the first alpha-(1– > 3) branching sucrases of the GH70 family. J. Biol. Chem. 291, 7687–7702.
Vujičić-Žagar, A., Pijning, T., Kralj, S., Lopez, C.A., Eeuwema, W., Dijkhuizen, L., et al., 2010. Crystal structure of a 117 kDa glucansucrase fragment provides insight into evolution and product specificity of GH70 enzymes. Proc. Natl. Acad. Sci. U. S. A. 107, 21406–21411.
Waldherr, F.W., Doll, V.M., Meissner, D., Vogel, R.F., 2010. Identification and characterization of a glucan-producing enzyme from *Lactobacillus hilgardii* TMW 1.828 involved in granule formation of water kefir. Food Microbiol. 27, 672–678.
Walter, J., Schwab, C., Loach, D.M., Ganzle, M.G., Tannock, G.W., 2008. Glucosyltransferase A (GtfA) and inulosucrase (Inu) of *Lactobacillus reuteri* TMW1.106 contribute to cell aggregation, *in vitro* biofilm formation, and colonization of the mouse gastrointestinal tract. Microbiology 154, 72–80.
Wang, S., Mao, X., Wang, H., Lin, J., Li, F., Wei, D., 2011. Characterization of a novel dextran produced by *Gluconobacter oxydans* DSM 2003. Appl. Microbiol. Biotechnol. 91, 287–294.
Wissuwa, J., Stokke, R., Fedoy, A.E., Lian, K., Smalas, A.O., Steen, I.H., 2016. Isolation and complete genome sequence of the thermophilic *Geobacillus* sp. 12AMOR1 from an Arctic deep-sea hydrothermal vent site. Stand. Genomic Sci. 11 (16,016-0137-y. eCollection 2016).
Yamamoto, K., Yoshikawa, K., Okada, S., 1993. Structure of dextran synthesized by dextrin dextranase from *Acetobacter capsulatus* ATCC 11894. Biosci. Biotechnol. Biochem. 57, 1450–1453.
Yan, M., Han, J., Xu, X., Liu, L., Gao, C., Zheng, H., et al., 2016. Gsy, a novel glucansucrase from *Leuconostoc mesenteroides*, mediates the formation of cell aggregates in response to oxidative stress. Sci. Rep. 6, 38122.
Yokobayashi, K, Ikeda, T, Misaki, A. Compound water-insoluble glucan and process for the production thereof. US4072567 A. 1978.
Zannini, E., Waters, D.M., Coffey, A., Arendt, E.K., 2016. Production, properties, and industrial food application of lactic acid bacteria-derived exopolysaccharides. Appl. Microbiol. Biotechnol. 100, 1121–1135.
Zhang, R., Zhang, W., Hu, T., 2011. Dextran glucosidase: a potential target of iminosugars in caries prevention. Med. Hypotheses 76, 574–575.