

Assembly of α -Glucan by GlgE and GlgB in Mycobacteria and Streptomycetes

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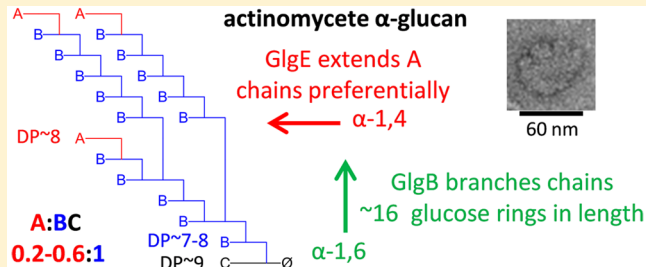
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Supporting Information

ABSTRACT: Actinomycetes, such as mycobacteria and streptomycetes, synthesize α -glucan with α -1,4 linkages and α -1,6 branching to help evade immune responses and to store carbon. α -Glucan is thought to resemble glycogen except for having shorter constituent linear chains. However, the fine structure of α -glucan and how it can be defined by the maltosyl transferase GlgE and branching enzyme GlgB were not known. Using a combination of enzymolysis and mass spectrometry, we compared the properties of α -glucan isolated from actinomycetes with polymer synthesized *in vitro* by GlgE and GlgB. We now propose the following assembly mechanism. Polymer synthesis starts with GlgE and its donor substrate, α -maltose 1-phosphate, yielding a linear oligomer with a degree of polymerization (~ 16) sufficient for GlgB to introduce a branch. Branching involves strictly intrachain transfer to generate a C chain (the only constituent chain to retain its reducing end), which now bears an A chain (a nonreducing end terminal branch that does not itself bear a branch). GlgE preferentially extends A chains allowing GlgB to act iteratively to generate new A chains emanating from B chains (nonterminal branches that themselves bear a branch). Although extension and branching occur primarily with A chains, the other chain types are sometimes extended and branched such that some B chains (and possibly C chains) bear more than one branch. This occurs less frequently in α -glucans than in classical glycogens. The very similar properties of cytosolic and capsular α -glucans from *Mycobacterium tuberculosis* imply GlgE and GlgB are sufficient to synthesize them both.



α -Glucans are widespread among bacteria, yeasts, plants, insects, and mammals, and in most cases they are thought to act as a store of carbon and energy. Arguably the best documented α -glucan in bacteria is glycogen, which typically accumulates under nitrogen-limiting growth conditions.¹ Glycogen comprises linear α -1,4-linked malto-oligosaccharides connected by α -1,6-linked branch points giving rise to an arboreal (tree-like) dendrimer and particles tens of nanometers in diameter. In the classical bacterial glycogen pathway, the α -1,4-links are synthesized by glycogen synthase GlgA (E.C. 2.4.1.21), using ADP-glucose as the sugar donor (Figure 1), and the α -1,6-linked branches are generated by GlgB branching enzyme (E.C. 2.4.1.18). About 32% of sequenced bacterial genomes contain the genes coding for the enzymes of the classical pathway.²

The alternative GlgE α -glucan pathway has been discovered recently,^{3,4} which is about half as widespread among bacteria as the classical pathway.² This involves trehalose synthase and maltose kinase that convert trehalose into α -maltose 1-

phosphate (Figure 1), which is the donor for the maltosyl transferase GlgE (E.C. 2.4.99.16) that creates α -1,4-linkages.⁵ Bioinformatic analysis suggests that even when organisms possess both the classical and GlgE pathways, they possess only one GlgB enzyme,² implying the branching enzyme is shared between both pathways. GlgB branching enzymes associated with these pathways are members of the glycoside hydrolase 13_9 CAZy family.^{6,7} Interestingly, the gene for GlgB is often clustered with the gene for GlgE rather than for GlgA in such cases. Some organisms possess only the GlgE pathway, and we have established using genetics that GlgE and GlgB are necessary and sufficient for the biosynthesis of α -glucan in one such organism, the actinomycete *Streptomyces venezuelae*.⁸

The GlgE pathway is widespread among other actinomycetes such as mycobacteria.² Indeed, both GlgE and GlgB have been

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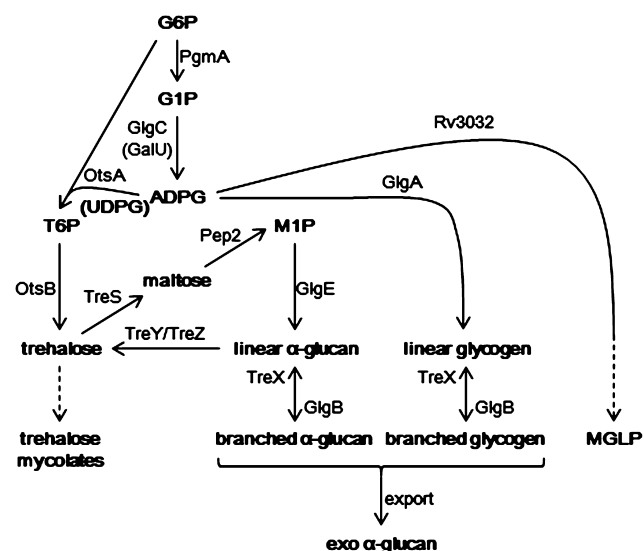


Figure 1. Metabolism of α -glucans thought to exist in *Mycobacterium tuberculosis*. G6P, glucose 6-phosphate; G1P, glucose 1-phosphate; M1P, α -maltose 1-phosphate; T6P, trehalose 6-phosphate; ADPG, ADP-glucose; UDPG, UDP-glucose; MGLP, methylglucose lipopolysaccharide; PgmA, phosphoglucomutase; GlgC, ADP-glucose pyrophosphorylase; GalU, UDP-glucose pyrophosphorylase; OtsA, trehalose 6-phosphate synthase; OtsB, trehalose 6-phosphate phosphatase; TreS, trehalose synthase; Pep2, maltose kinase; GlgE, maltosyl transferase; GlgB, branching enzyme; TreX, debranching enzyme; TreY, maltooligosyltrehalose synthase; TreZ, maltooligosyltrehalose trehalohydrolase; GlgA, glycogen synthase; Rv3032, putative glycosyltransferase.

genetically validated as potential drug targets for the treatment of tuberculosis.³ Some species, including *Mycobacterium tuberculosis*, possess not only the genes for the GlgE and classical pathways, but also genes for a methyl glucose lipopolysaccharide pathway (Figure 1). This pathway seems to involve Rv3032 in the formation of α -1,4 linkages in the highly modified malto-oligosaccharide product of this pathway.⁹ This specialized α -glucan is thought to chaperone fatty acid biosynthesis. Carbohydrate metabolism in mycobacteria is further complicated by the fact that the capsule coating the cells is mainly composed of a glycogen-like α -glucan.^{10,11} This polymer might contribute to virulence of *M. tuberculosis* by binding to DC-SIGN to evade the host immune response.^{12–15} The capsular and cytosolic α -glucan polymers appear to be very similar, with only subtle differences having been reported.^{15,16} Quite how each metabolic pathway contributes to each α -glucan product is not yet clear.

Mycobacterial α -glucans appear to resemble classical glycogen except for a higher degree of branching and correspondingly shorter linear chains.^{10,16,17} This implies that the branching enzyme in mycobacteria makes shorter branches than the corresponding enzymes in organisms that synthesize classical glycogen, such as in *Escherichia coli*.¹⁸ To address this issue, we first characterized GlgE-derived α -glucan from *S. venezuelae*, and α -glucans from mycobacteria. We then determined the chain length specificity of actinomycete GlgB branching enzymes and the chain specificity of *M. tuberculosis* GlgE. This enabled us to establish how the GlgE pathway-derived polymer is assembled, which has implications for how the cytosolic and capsular α -glucans of *M. tuberculosis* are synthesized.

MATERIALS AND METHODS

Materials. Dextrans (5, 12, 25, 50, 100, 150, 270, 410, and 1400 kDa), amylose from potatoes (average mass >150 kDa), amylopectin from potato, individual malto-oligosaccharides (degree of polymerization, DP2–7), rabbit liver glycogen and pullulanases were purchased from Sigma-Aldrich (Gillingham, United Kingdom). Malto-octaose (DP8) was purchased from Carbosynth (Compton, United Kingdom). Amylose 2800 (mean DP 12.6 ± 5.3 , median and mode DP11, DP6–16 contributing at least 5% of total malto-oligosaccharides according to capillary electrophoresis) was purchased from TCI Europe N.V. (Oxford, United Kingdom). α -Maltose 1-phosphate was synthesized as previously described.¹⁹ [^{13}C]- α -maltose 1-phosphate was a gift from John (Jack) Thompson.

Isolation of α -Glucans. *Streptomyces venezuelae*. ATCC 10712 was grown on sterile cellophane discs on MYM Tap agar plates at 30 °C for 30 h. The cells were removed from the cellophane, suspended in water (15 mL), and centrifuged at 4000g for 30 min. The cells were washed three more times, suspended in water (10 mL), and disrupted by sonication. The cell debris was pelleted by centrifugation at 30000g for 15 min, and the resultant supernatant was washed twice with a 1:1 (v/v) mixture of 0.2 M glycine, pH 10.5, and chloroform (10 mL). The aqueous layer was concentrated to ~8 mL using a rotary evaporator and then centrifuged at 108000g at 4 °C for 2 h. The gelatinous pellet was collected and dissolved in water (2 mL), and the α -glucan was precipitated with ethanol (3 vol) at 4 °C. The solid collected by centrifugation at 4000g for 10 min was redissolved in water (8 mL) and centrifuged at 12000g for 10 min to remove insoluble material. The ultracentrifugation and ethanol precipitation steps were repeated. Finally the pellet was dissolved in 2 mL of water and freeze-dried to yield the α -glucan as an amorphous white powder.

Mycobacterium smegmatis. Cells were grown on sterile cellophane discs for 72 h at 37 °C on Difco Middlebrook 7H10 agar solid medium supplemented with 10% ADS (5% w/v bovine serum albumin, 2% glucose, and 0.85% NaCl). Cells were resuspended in 10 mL of water and disrupted by sonication. α -Glucan was extracted as described above.

Escherichia coli. BL21(DE3) cells were transformed with a pUC19 vector conferring resistance to carbenicillin. Cells were grown at 37 °C for 22 h in nitrogen-limited M9 medium (1 L) comprising $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (6 g), KH_2PO_4 (3 g), NaCl (0.5 g), pH 7.4, glucose (3 g), $(\text{NH}_4)_2\text{SO}_4$ (0.22 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.25 g), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (15 mg), and thiamine (10 mg), supplemented with carbenicillin (100 mg). Cells were harvested by centrifugation and resuspended in 10 mL of water. Glycogen was isolated as described for α -glucan above.

M. tuberculosis. H37Rv cells were grown for 2 weeks in 100 mL cultures in a bovine serum albumin-free minimal medium containing 20 mM glucose, 0.5% glycerol, and 0.05% tyloxapol.²⁰ Because of the loose attachment of the *M. tuberculosis* capsule layer to the cell surface, treatment with detergent and physical agitation were sufficient to quantitatively strip off the capsule from cells and thus allow the satisfactory separation of capsular and cytosolic α -glucan polymers.²¹ The cells were harvested by centrifugation. The supernatant was sterilized by passing twice through a 0.22 μm sterile filter, heat inactivated at 65 °C for 12 h, and concentrated to 1.5 mL by centrifugation (4000g) through a 30 kDa cutoff membrane filtration device. The retentate was diluted with water (10 mL) and concentrated to 1.5 mL. The concentrate was treated with

absolute ethanol (5 vol), and the resulting precipitate was collected by centrifugation. The solid was dissolved in water (1 mL) and centrifuged, and the resulting supernatant was freeze-dried to yield capsular α -glucan as an amorphous solid. The cell pellet was washed twice with 20 mM sodium phosphate buffer, pH 7.4, containing 20 mM NaCl to remove all remaining capsular material, resuspended in water, and heated for 4 h at 95 °C. The cell extract was centrifuged to remove debris, and the supernatant was centrifuged at 108000g at 4 °C for 2 h. The gelatinous pellet was dissolved in water (0.5 mL), and the α -glucan was precipitated with ethanol (3 vol). The solid was collected by centrifugation, redissolved in water (0.5 mL), and freeze-dried to yield cytosolic α -glucan as an amorphous powder.

NMR Spectroscopy. Solution-state NMR spectra were recorded at 298 K on a Bruker Avance III 400 MHz spectrometer and analyzed using Bruker TopSpin 2.1 (Rheinstetten, Germany). Chemical shifts in D₂O are reported with reference to residual water at δ_{H} 4.79 or to the methyl resonance of internal acetone at δ_{C} 29.84 ppm. Assignments were made with the aid of COSY and HSQC experiments. The assignment of the peaks was based on our previous studies.^{3,22}

Pulse field gradient DOSY experiments²³ were carried out with up to 5 mg of polymer in 0.6 mL of D₂O at 313 K using TopSpin 2.1. The gradient strength was incremented longitudinally in 16 steps from 2% up to 95% of the maximum gradient strength. Diffusion times and gradient pulse durations were optimized for each experiment in order to achieve a 95% decrease in the resonance intensity at the largest gradient amplitude. Typically, diffusion time was set at 200 μ s and the diffusion gradient length at 3.5 ms. The 2D diffusion spectra were analyzed with T1/T2 processing to derive the corresponding diffusion coefficient. The relationship between diffusion coefficient and molecular mass was determined using a series of dextrans (Figure S2).

Molecular radii were calculated from the Stokes–Einstein equation, which relates the diffusion coefficient D to the macromolecular hydrodynamic radius r . The assumption that the macromolecules are spherical is reasonable given the morphology of particles according to electron microscopy (Figure 2).

$$D = \frac{k_{\text{B}}T}{6\pi\eta r}$$

where k_{B} = 1.38 $\times 10^{-23}$ J K⁻¹, T = 313 K, and dynamic viscosity of water η = 0.000653 N s m⁻²

(http://www.engineeringtoolbox.com/water-dynamic-kinematic-viscosity-d_596.html)

Solid-state NMR measurements were performed at 293 K using a Bruker 400 MHz Avance III spectrometer equipped with a triple resonance probe at frequencies 400.23 (¹H) and 100.64 MHz (¹³C). Amylose, amylopectin, and rabbit liver glycogen were each packed in 4 mm zirconia rotors, and α -glucan samples were packed into 12 μ L CRAMPS rotors 4 mm in diameter. Samples were rotated at a magic angle spinning rate of 10 kHz. ¹H–¹³C cross-polarization magic angle spinning (CP/MAS) spectra were acquired using the following parameters: ¹H $\pi/2$ pulse length 3.5 μ s, ¹³C $\pi/2$ pulse length 3.5 μ s, and ¹H–¹³C CP contact time 2 ms. SPINAL64 decoupling was used during signal acquisition with recycle delay of 20 s. The Hartmann–Hahn conditions for ¹H–¹³C CP/MAS NMR experiment were set with hexamethylbenzene. Typically, 256 scans were acquired for amylose, amylopectin,

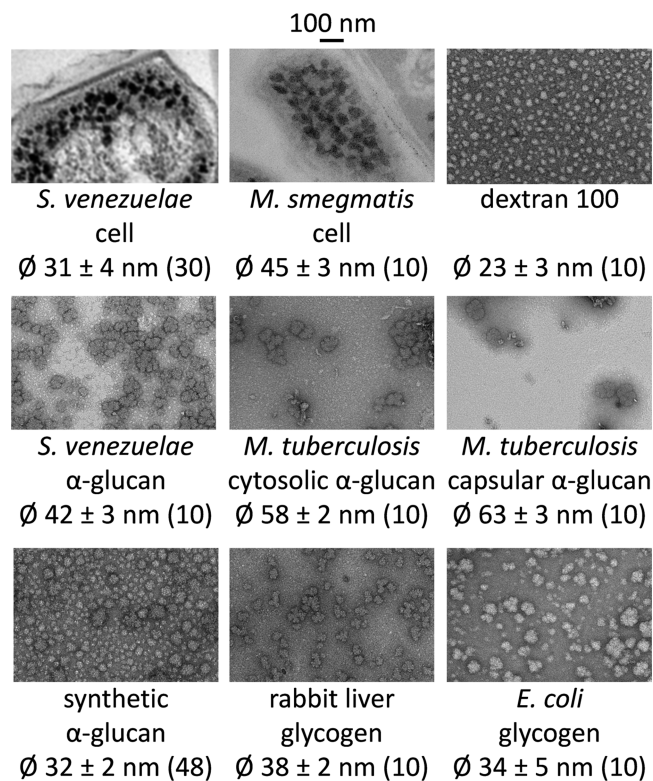


Figure 2. Transmission electron microscopy of α -glucans, glycogen, and dextran. Cells, isolated α -glucans, and dextran 100 were stained using periodic acid-thiocarbohydrazide-silver proteinate (PATAg), uranyl acetate, and phosphotungstic acid, respectively. Representative images are shown. The scale bar represents 100 nm. The diameters (\emptyset) of the particles (β particles in the case of α -glucans) are quoted as mean \pm SE (n). α -Glucan isolated from *M. smegmatis* had a diameter of 35 ± 3 (21). Synthetic α -glucan was produced by treating maltohexaose with *M. tuberculosis* GlgE and GlgB together with α -maltose 1-phosphate. See main text and Table 1 for other measured diameters.

and rabbit liver glycogen and 2048 scans for α -glucan. The ¹³C chemical shifts were recorded with respect to trimethylsilane.

Transmission Electron Microscopy. Aqueous polymer samples (typically 0.1 mg mL⁻¹) were allowed to settle briefly onto a 400 mesh copper grid with a carbon-coated pyroxylin support film before being negatively stained. Glycogen and α -glucan was stained with 2% aqueous uranyl acetate, pH 4.5, and dextran was stained with 1% phosphotungstic acid. The grids were viewed in a FEI Tecnai 20 transmission electron microscope (Eindhoven, Netherlands) at 200 kV, and digital TIFF images were taken using an AMT XR60B digital camera (Deben, Bury St Edmunds, UK).

S. venezuelae was grown for 5 days on MYM-TAP solid medium at 28 °C,²⁴ and *M. smegmatis* was grown on Difco Middlebrook 7H10 agar solid medium supplemented with 10% ADS (5% w/v bovine serum albumin, 2% glucose, and 0.85% NaCl) for 3 days at 37 °C. Single colonies of *S. venezuelae* and *M. smegmatis* were positively stained for α -glucan using periodic acid-thiocarbohydrazide-silver proteinate (PATAg) as previously described.⁸

Dynamic Light Scattering. Dynamic light scattering analysis was performed on a DynaPro Titan (Wyatt Technology Corporation, Santa Barbara CA, U.S.A.) equipped with DYNAMICS software (v6) using a 12 μ L sample cell at

293 K. The sample concentration was typically 1 mg mL^{-1} in water.

Iodine Complexes of α -Glucans. Lugol solution ($1 \text{ mg mL}^{-1} \text{ I}_2$ and $0.1 \text{ mg mL}^{-1} \text{ KI}$; typically 0.5 vol) was added to samples of isolated polysaccharides, with further dilution in water as appropriate, and absorbance was measured either using a PerkinElmer Lambda 25 spectrophotometer (Coventry, United Kingdom) or a BMG Labtech CLARIOstar microtiter plate spectrophotometer (Ortenberg, Germany).

Reduction and Debranching of α -Glucans. Saccharides were reduced by adding aqueous NaBH_4 (typically 1 mg in $5 \mu\text{L}$) to aqueous samples (in $25 \mu\text{L}$). The mixtures were incubated at $21 \text{ }^\circ\text{C}$ for 1 h, acidified with dilute acetic acid, and treated with absolute ethanol (5 vol). The resulting precipitated material was isolated by centrifugation and dried.

Polymer samples (typically in $25 \mu\text{L}$) were debranched using *Pseudomonas* sp. isoamylase (with typically $2 \mu\text{L}$ of a 1000 U mL^{-1} stock solution) from Megazyme (Bray, Ireland) in 100 mM sodium acetate buffer, pH 4.0, at $37 \text{ }^\circ\text{C}$ for 3 h (as illustrated in Figure 3A). NMR spectroscopy confirmed the loss of α -1,6 linkages. Reactions were terminated by heating at $95 \text{ }^\circ\text{C}$ for 5 min, and the products were analyzed either directly or after precipitation with ethanol (5 vol). Precipitated samples were dissolved in water ($50 \mu\text{L}$) and freeze-dried to yield an amorphous powder.

Mass Spectrometry. Matrix-assisted laser desorption/ionization mass spectra were acquired as described previously³ on Bruker Ultraflex or Autoflex Speed MALDI-TOF/TOF mass spectrometers (Coventry, United Kingdom), equipped with a pulsed nitrogen laser emitting at 337 nm or Smartbeam-II 2 kHz laser, respectively. Samples were typically mixed 1:2 v/v with a 20 mg mL^{-1} solution of 2,5-dihydroxybenzoic acid in 50% aqueous acetonitrile and spotted on a target plate (Bruker MTP 384 Polished Steel TF Target). Data were processed using Bruker flexAnalysis software. The relative abundance of malto-oligosaccharides of $\text{DP} \geq 4$ was determined at least in duplicate by measuring the area under the peaks of all isotopes associated with each species of a given degree of polymerization using the Sophisticated Numerical Annotation Procedure with sugar-sodium adduct average composition. When experiments involved quantifying both reduced and nonreduced malto-oligosaccharides in a sample, the intensity of the main isotopologue was used to quantify each species, taking into account the contribution of any nonreduced isotopologues with $+2 \text{ Da}$ when quantifying reduced species of the same mass. The percentage contribution of such nonreduced isotopologues for each DP of malto-oligosaccharide was determined from experimental spectra of authentic compounds. Any difference between the ionization efficiencies of reduced and nonreduced malto-octaose was $<15\%$ in control spectra (data not shown), showing that quantitative comparison between such species is possible. Ion mobility mass spectrometry was carried out as described previously.²⁵ Samples were analyzed using a Synapt G2 HDMS mass spectrometer (Waters Corp., Manchester, UK) using electrospray ionization in traveling-wave mobility mode with nitrogen and helium gas flows in negative mode.

Capillary Electrophoresis. Reducing saccharides were labeled with 8-aminopyrene-1,3,6-trisulfonic acid to facilitate their separation on the basis of charge and their detection by fluorescence spectrophotometry. Samples (typically $5 \mu\text{L}$) were treated with 8-aminopyrene-1,3,6-trisulfonic acid ($1 \mu\text{L}$ of 50 mM in 15% aqueous glacial acetic acid) and freshly prepared NaCNBH_3 in tetrahydrofuran ($1 \mu\text{L}$ of 1 M). Reaction

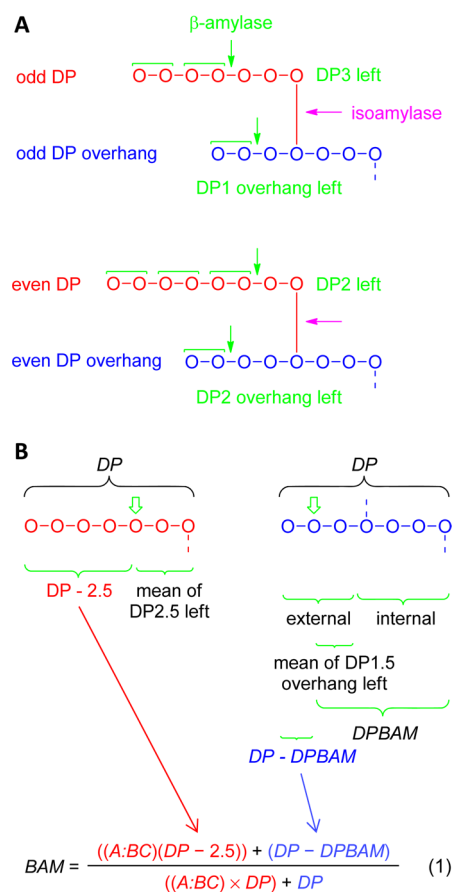


Figure 3. Cartoon showing the degradation of α -glucan by β -amylase and isoamylase. (A) Isoamylase hydrolyzes the α -1,6-linked branch points (indicated in purple). β -Amylase hydrolyzes maltose units iteratively from the nonreducing ends of linear chains (indicated in green). A chains (shown in red) are left with a DP of either 3 or 2, depending on whether the original DP was odd or even, respectively. B chains (shown in blue) are left with an overhang of either DP 1 or 2 depending on whether the original overhang was odd or even, respectively. C chains are degraded in the same way as for B chains (not shown). (B) A chains are therefore extensively hydrolyzed by β -amylase leaving a mean of 2.5 glucosyl residues (i.e., DP2.5). B and C chains are hydrolyzed leaving a mean overhang of 1.5 glucosyl residues (indicated by the broad green arrows). The contribution of the hydrolysis of A and B/C chains to the extent to which β -amylase degrades α -glucan (BAM) is indicated in the context of eq 1, which can be rearranged to give eq 2 that defines A:BC. Thus, the right-hand side of eq 1, in both the numerator and the denominator, relates to the extent of hydrolysis of B plus C chains. The left-hand side relates to the extent of hydrolysis of the A chains, where A:BC is the ratio of the numbers of A to B plus C chains normalized to the number of B plus C chains.

mixtures were incubated at $37 \text{ }^\circ\text{C}$ for 4 h, diluted 10-fold with water. Samples were subjected to capillary electrophoresis using a Beckman Coulter PA800 ProteomeLab Protein Characterization System (High Wycombe, United Kingdom) using laser-induced fluorescence detection with an excitation wavelength of 488 nm and an emission band-pass filter of $520 \text{ nm} \pm 10 \text{ nm}$. An SCIEX N-CHO capillary ($50 \mu\text{m}$ internal diameter \times 50.2 cm ; 40 cm effective length to detector) was used with a constant voltage of -30 kV , the anode at the detector, and a temperature of $25 \text{ }^\circ\text{C}$. The capillary was rinsed with SCIEX N-CHO buffer, and the samples were introduced at a pressure of 0.5 psi for 5 s.

Determination of the A:BC Chain Ratio. α -Glucan and glucogen were each exhaustively treated for at least 1 h at 37 °C with β -amylase from barley Megazyme (Bray, Ireland) in 10 mM MES pH 6.0. The only malto-oligosaccharide produced was maltose according to mass spectrometry as expected. The amount of maltose liberated by β -amylase was determined using the 3,5-dinitrosalicylic acid method. A solution of 3,5-dinitrosalicylic acid (43.8 mM 3,5-dinitrosalicylic acid containing 1.06 M potassium sodium L-(+)-tartrate and 0.4 M NaOH) was added to a sample (1:1 v/v) with water and incubated at 95 °C for 5 min. The mixtures were cooled on ice for 10 min, diluted 2-fold, and the absorbance of the mixtures was measured at 540 nm and compared with a glucose calibration curve. The amount of glucose residues liberated would have been 2 times the amount of maltose detected. In separate experiments, all glucosyl residues within a polymer were liberated with *Bacillus licheniformis* α -amylase (EC 3.2.1.1 in 50 mM MOPS buffer, pH 7.0, at 99 °C for 6 min to generate shorter dextrans) followed by *Aspergillus niger* amyloglucosidase (EC 3.2.1.3 in 200 mM sodium acetate buffer, pH 4.5, at 50 °C for 30 min to hydrolyze all remaining α -1,4 and α -1,6 linkages) from the Megazyme Total Starch Assay kit K-TSTA 05/2008 (Bray, Ireland),²⁶ allowing the total glucose content to be determined using 3,5-dinitrosalicylic acid. All measurements were carried out at least in duplicate. For example, three independent replicate experiments were carried out with *S. venezuelae* α -glucan giving 21.0 ± 4.7 (SD of replicate means).

The proportion of glucose residues liberated as maltose from A and B/C chains by β -amylase (BAM) is defined by eq 1 as illustrated in Figure 3.

$$\text{BAM} = \frac{((\text{A:BC})(\text{DP} - 2.5)) + (\text{DP} - \text{DPBAM})}{((\text{A:BC}) \times \text{DP}) + \text{DP}} \quad (1)$$

where A:BC is the ratio of the numbers of A to B plus C chains normalized to the number of B plus C chains (where, in this context, the sole C chain per molecule is equivalent to a B chain because it bears at least one branch also).

DP is the mean degree of polymerization of all chains (it is assumed that all types of chain have a similar mean DP; nevertheless, the lower the A:BC chain ratio, the less sensitive eq 2 below becomes to differences in the mean DP of A chains); e.g., five independent replicate experiments were carried out with *S. venezuelae* α -glucan giving 7.3 ± 0.4 (SD of replicate means).

DPBAM is the mean degree of polymerization of linear B chains after digestion with β -amylase determined by mass spectrometry (where maltose and malto-triose were ignored because they were derived from A chains); e.g., four independent replicate experiments were carried out with *S. venezuelae* α -glucan giving 6.5 ± 0.2 (SD of replicate means).

Equation 1 can be rearranged to define the A:BC ratio to give eq 2

$$\text{A:BC} = \frac{\text{DP} - \text{DPBAM} - (\text{BAM} \times \text{DP})}{(\text{BAM} \times \text{DP}) - \text{DP} + 2.5} \quad (2)$$

Production of Recombinant Enzymes. The DNA sequences of the *glgB* genes from *M. tuberculosis* H37Rv, *Mycobacterium smegmatis* mc² 155, *Streptomyces coelicolor* A3(2) isoform 1, and *Streptomyces venezuelae* ATCC 10712 were synthesized with optimized codon usage for expression in *Escherichia coli* (Genscript Corporation, Piscataway, NJ, U.S.A.), allowing the production of each enzyme with an N-

terminal His₆ tag and TEV cleavage site. Each gene was flanked at the 5' end by an *NdeI* restriction site and a sequence encoding a His₆ tag and a TEV protease cleavage site, and at the 3' end by a *BamHI* restriction site. Each gene, with exceptions described below, was subcloned in to a pET-21a(+) (Novagen, Watford, United Kingdom) vector using *NdeI* and *BamHI* restriction sites and heterologously expressed in *E. coli* BL21(DE3). The *M. tuberculosis* H37Rv *glgB* gene sequence was mutated into that of *M. tuberculosis* CCDC5180 by changing the codon for Ser470 into one for Pro using QuikChange (Agilent Technologies, Santa Clara, CA, U.S.A.). Cells containing the resulting expression plasmid were cultured in Lysogeny Broth containing carbenicillin ($100 \mu\text{g mL}^{-1}$) at 37 °C. When the cells reached an OD_{600 nm} of 0.6, expression was induced by the addition of 0.5 mM isopropyl β -D-thiogalactopyranoside. After incubation at 30 °C for 2 h and then 16 °C for 17 h, the cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl, pH 8.0, containing 15 mM imidazole, 300 mM NaCl, DNase 1, and a Complete protease inhibitor cocktail tablet (Roche, Burgess Hill, United Kingdom). A similar strategy was used for the production of the other recombinant proteins. The cells were disrupted with a TS Series Benchtop 1.1 kW cell disruptor (Constant Systems Ltd.) at 25 kPSI. The resulting cell lysate was separated from the cell debris by centrifugation at 20000g for 15 min at 4 °C. Enzyme were purified from each cell lysate by application onto a 1 mL FF HisTrap column (GE Healthcare, Amersham, United Kingdom). Protein was eluted with 15–300 mM imidazole gradient. Enzymes were further purified to remove any traces of *E. coli* amylase activities using a HiLoad 26/60 Superdex 200 gel filtration column (GE healthcare) equilibrated with 20 mM sodium phosphate buffer, pH 7.4, containing 20 mM NaCl. The purified proteins were homogeneous according to SDS-PAGE with Coomassie staining.

The production of *S. venezuelae* GlgB was not successful with pET21a vector. The *glgB* gene was ligated into a lower copy-number pBAD43 vector²⁷ using *NcoI* and *HindIII* restriction sites, placing the gene under the more stringent control of an arabinose-inducible promoter. GlgB was produced in *E. coli* TOP10 cells (Novogen) grown at 37 °C to an OD at 600 nm of 0.6 in Lysogeny Broth before induction with 0.02% w/v L-arabinose followed by a further 5 h of incubation. The enzyme was purified using both Ni-affinity and size exclusion chromatographies as described above.

Enzyme-Catalyzed Reactions. To generate synthetic α -glucan and to determine the branch length specificity of GlgB enzymes, 1 mM malto-hexaose and 10.1 mM α -maltose-1-phosphate were incubated with 7 μg each of recombinant GlgE and GlgB (giving <2 μM final concentrations) in 100 mM bis-Tris propane, pH 7.0, containing 50 mM sodium chloride in a total volume of 50 μL . After 18 h at 22 °C, a second aliquot of α -maltose-1-phosphate was added. After a further 90 h, the sample was heated at 95 °C for 5 min, cooled, and washed 5 times using a 10 000 Da cutoff membrane with 100 mM sodium acetate buffer, pH 4.0, to remove small malto-oligosaccharides. The sample was analyzed by electron microscopy, mass spectrometry, or capillary electrophoresis with or without debranching as appropriate.

In other experiments, malto-oligosaccharides (typically 5 mg) were extended with GlgE (14 μg) and 6.6 mM α -maltose 1-phosphate in a total volume of 122 μL at 37 °C for 3 h. The enzyme was denatured by heating to 96 °C for 5 min. As appropriate, GlgB (70 μg) was then added, for example, and the

Table 1. Properties of α -Glucans and Glycogen

	<i>M. tuberculosis</i>		<i>S. venezuelae</i>	rabbit liver	<i>E. coli</i>
	capsular	cytosolic	cytosolic	glycogen	glycogen
	Size				
TEM β particle (nm) ^a	63 ± 7	58 ± 2	42 ± 3	38 ± 2	34 ± 5
after β -amylase ^{a,b}	nd ^c	nd ^c	42 ± 3	29 ± 2	nd ^c
DLS β particle (nm) ^d	64 ± 8	64 ± 5	51 ± 11	59 ± 10	nd ^c
after β -amylase ^{b,d}	nd ^c	nd ^c	54 ± 1	40 ± 1	nd ^c
NMR diffusion coefficient (m ² s ⁻¹) ^e	nd ^c	nd ^c	2.5 × 10 ⁻¹²	2.9 × 10 ⁻¹²	nd ^c
NMR α particle (nm) ^e	nd ^c	nd ^c	~280	~240	nd ^c
NMR α particle (Da) ^e	nd ^c	nd ^c	~5 × 10 ⁶	~4 × 10 ⁶	nd ^c
	Linear Chain DP ^f				
overall mean (DP)	7.0 ± 2.0	7.2 ± 1.9	7.3 ± 1.8	10.8 ± 4.6	11.0 ± 2.9
C mean ^g	nd ^c	nd ^c	9.1 ± 2.5	nd ^c	nd ^c
	Extent of Branching from				
overall mean DP ^h	0.14	0.14	0.14	0.09	0.09
NMR spectroscopy ⁱ	nd ^c	nd ^c	0.14	0.09	nd ^c
	β -Amylase Treatment				
mean DP (DPBAM) ^f	6.1 ± 1.4	6.2 ± 1.8	6.5 ± 1.6	8.6 ± 3.5	9.8 ± 3.3
residues liberated % (BAM)	31.1	33.0	21.0 ± 4.7	47.5	40.5
internal chain length ⁱ	4.6 ± 1.4	4.7 ± 1.8	5.0 ± 1.6	7.1 ± 3.5	8.3 ± 3.3
A:BC chain ratio ^k	0.6	0.6	0.2 ± 0.2	0.9	0.8
mean branches per B/C chain ^l	~1.6	~1.6	~1.2 ± 0.2	~1.9	~1.8

^aThe diameters of individual particles were determined by transmission electron microscopy (TEM) from at least 10 measurements (see Figure 2).

^bThe size of glycogen particles after treatment with β -amylase. ^cNot determined. ^dThe hydrodynamic diameter of the dominant species of <100 nm in each sample was determined using dynamic light scattering where at least five measurements were made. ^eThe diffusion coefficient, hydrodynamic diameter, and molecular mass of the dominant large species assigned as α particles were determined using pulse field gradient NMR spectroscopy (see Figure S2 for the calibration curve). ^fThe DP mean (mass average) was determined for products with a DP \geq 4 using mass spectrometry (see Figure 4 for representative spectra). Chain length distributions are expressed as SD. ^gThe DP of C chains was determined as described in Figure 5. ^hExtent of branching (α -1,6 linkages divided by the sum of α -1,6 and α -1,4 linkages) equates to the reciprocal mean branch length. ⁱExtent of branching was also determined by integrating the appropriate anomeric resonances in ¹H NMR spectra without solvent suppression of samples subjected to repeated freeze-drying and resolubilization in D₂O. ^jCalculated from DPBAM minus 1.5. ^kThe ratio of the numbers of A to B plus C chains normalized to the number of B plus C chains, from eq 2. ^lEssentially A:BC plus one.

mixture was incubated at 30 °C for 2 h. The enzyme was then denatured by heating and analyzed as described above.

The GlgE-dependent extension of acceptors with α -maltose 1-phosphate was monitored using malachite green to detect the production of inorganic phosphate as described previously.^{3,19,28}

RESULTS

Isolation of α -Glucan Particles from Actinomycetes and Classical Glycogen Particles from *E. coli*. Before characterizing GlgB branching enzymes from actinomycetes, we characterized the α -glucans produced by these bacteria together with classical glycogens to allow direct comparisons to be made. To this end, α -glucans/glycogen were first isolated from the cytosols of *S. venezuelae*, *M. tuberculosis* (the default strain in this study is H37Rv), and *E. coli*, from the capsule of *M. tuberculosis*, and from whole cells of *M. smegmatis*. We included *S. venezuelae* because we have previously shown that this organism generates α -glucan solely by the GlgE pathway.⁸ This contrasts with *E. coli*, which generates its glycogen only via the classical GlgA glycogen pathway.^{2,29} NMR spectra of the isolated materials gave characteristic resonances of α -1,4- and α -1,6-linked glucose polymers (examples of solution- and solid-state NMR spectra of *S. venezuelae* α -glucan are shown in Figure S1).^{17,30–32} Transmission electron microscopy showed that each of the isolated polymers comprised β particles that sometimes aggregated into larger α particles (Figure 2 and Table 1).³³ According to both transmission electron micros-

copy and dynamic light scattering, the β particles had diameters ranging from ~30 to ~60 nm, consistent with previous reports.^{15–17,30,34} Sectioned cells of *S. venezuelae* and *M. smegmatis* that were stained for α -glucan showed the presence of cytosolic β particles of a similar diameter (~30–45 nm) to that of isolated material (Figure 2). Only faint speckled staining was observed on the exterior of *M. smegmatis* cells, suggesting the presence of either little or diffuse capsular α -glucan in these growth conditions. Pulse field gradient NMR spectroscopy showed that isolated *S. venezuelae* α -glucan and rabbit liver glycogen contained slowly diffusing α particles with diameters of ~240–280 nm, equating to ~5 × 10⁶ Da assemblies comprising ~30 000 glucosyl residues (Table 1 and Figure S2). Similar values have been reported for material isolated from *M. smegmatis*.³⁵

Actinomycete α -Glucans Are Composed of Short Linear Chains of Length ~7–8. It has previously been reported that the α -glucans of mycobacteria contain shorter linear chains^{10,15–17,30,34,36} than those of classical glycogen.²⁹ In order to establish whether this was the case with *S. venezuelae* α -glucan, the isolated material was treated with isoamylase to hydrolyze the α -1,6 linkages (Figure 3A). Such a sample remained in solution and did not give strongly colored complexes with iodine according to absorbance spectroscopy (Figure S3), as would be expected with liberated linear chains having a degree of polymerization (DP) of <15.³⁷ Indeed the absorbance of the iodine complexes was only slightly greater than with malto-heptaose. By contrast, debranched rabbit liver glycogen yielded a little precipitate in the absence of iodine,

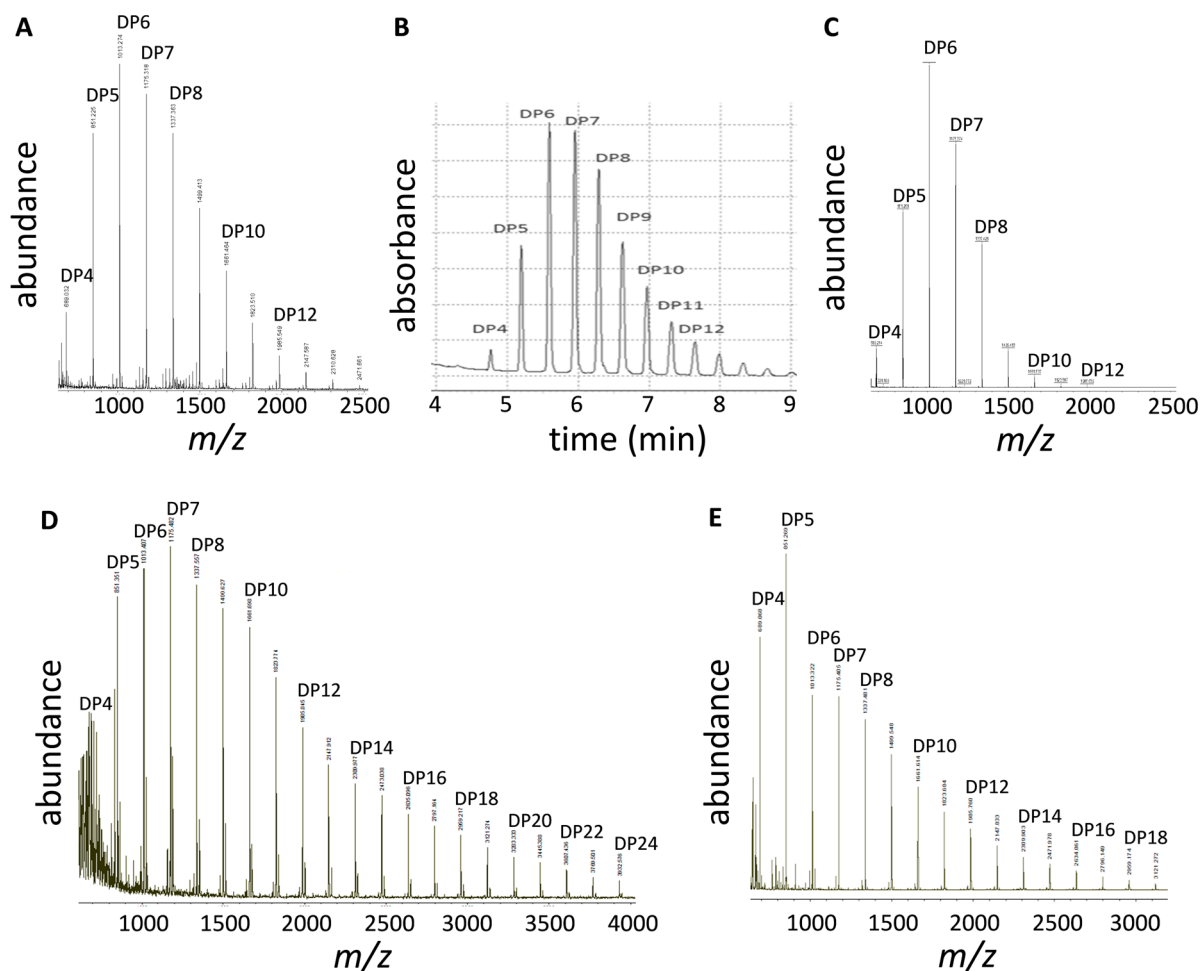


Figure 4. Branch length distribution of *S. venezuelae* α -glucan and rabbit liver glycogen. The α -1,6 linkages in the α -glucan were hydrolyzed using isoamylase to <1% according to ^1H NMR spectroscopy and subjected to either (A) MALDI MS or (B) capillary electrophoresis after treatment with 8-aminopyrene-1,3,6-trisulfonic acid and NaCNBH_3 , showing similar chain length distributions. Only after treatment with isoamylase were malto-oligosaccharides observed (data not shown). (C) A sample treated with β -amylase before debranching was analyzed by MALDI-MS and showed a loss of longer DPs and a relative gain of medium DPs of ~ 6 . Mass spectrometry of a sample that was treated solely with β -amylase showed the presence of only maltose as expected (data not shown). Debranched rabbit liver glycogen was subjected to MALDI MS either (D) without or (E) with pretreatment with β -amylase, leading to the substantial net reduction in DP. Only after treatment with isoamylase were malto-oligosaccharides observed, and a sample that was treated solely with β -amylase showed the presence of only maltose as expected (data not shown). Additional samples were analyzed as summarized in Table 1.

indicative of the liberation of linear malto-oligosaccharides that were too long to remain in solution ($> \text{DP}20$). In the presence of iodine, such a sample exhibited significant absorbance with a λ_{max} of ~ 537 nm, revealing the liberation of linear malto-oligosaccharides of DP up to ~ 30 .³⁷

Matrix assisted laser desorption/ionization mass spectrometry showed that that debranched *S. venezuelae* α -glucan and rabbit liver glycogen had mean DPs (mass average) of 7.3 and 10.8, respectively (Figure 4 and Table 1), the latter consistent with published values.²⁹ The narrower distribution of DPs in α -glucan compared with glycogen was also reflected in the standard deviations of their means of 1.8 and 4.6, respectively. The reliability of this analysis was independently verified using capillary electrophoresis (Figure 4). For example, the *S. venezuelae* material gave a value of 7.7 ± 2.2 , which was similar to the value of 7.3 ± 1.8 obtained with mass spectrometry. Analysis of the materials from *M. tuberculosis* capsule and cytosol showed that they comprised DPs of ~ 7 that were similar not only to that of *S. venezuelae*, but also each other (Table 1). A similar value was obtained with material isolated

from whole *M. smegmatis* colonies (7.1 ± 2.0). By contrast, the *E. coli* material had a longer mean DP of 11.0 ± 2.9 (Table 1), as expected for a classical glycogen.²⁹

Polymer samples treated with pullulanase from either *Bacillus acidopullulyticus* or *Klebsiella pneumoniae* also led to the cleavage of α -1,6 linkages. However, the hydrolysis of α -1,4 linkages also occurred such that glucose and maltose were the sole products after 24 h. This side reaction and/or contamination of pullulanase preparations might explain the very narrow distribution of very short chain lengths reported in one study.¹⁶

The degree of branching (the ratio between the number of α -1,6 linkages and the total number of linkages) can be calculated from the reciprocal of the mean chain DP. This gave 0.14 and 0.09 for actinomycete α -glucans and glycogens, respectively (Table 1). These values were independently verified by integrating the anomeric resonances observed using ^1H NMR spectroscopy (Table 1). Furthermore, the values were consistent with previous reports for materials isolated from mycobacteria based on either methylation analysis, which gave a degree of branching of 0.14,^{10,17,36} or enzymatic analyses.³⁴

The C Chain of *S. venezuelae* α -Glucan Has a DP of ~ 9 . Classical glycogen is an arboreal (tree-like) polymer. From the sole C chain with its reducing end stem one or more α -1,6-linked B chains that, in turn, bear further α -1,6 linked branches. The extremities of the nonreducing ends of the polymer comprise A chains that do not themselves bear branches. The C chain is uniquely addressable through reduction with sodium borohydride leading to an increase in mass by 2 Da. The debranching of reduced *S. venezuelae* α -glucan allowed the detection of small amounts of reduced linear malto-oligosaccharides using mass spectrometry. For example, chains with a DP of 8 in nonreduced control samples gave the natural isotopic distribution of isotopologues (Figure 5). However, a

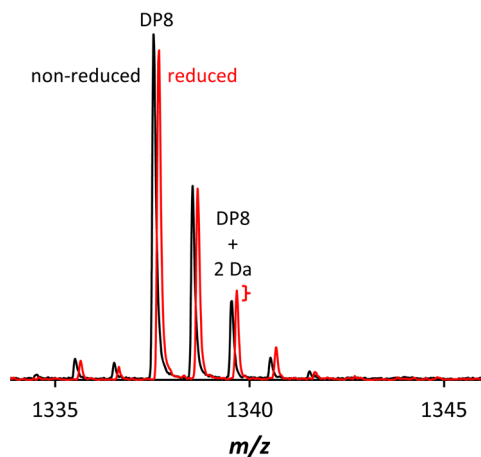


Figure 5. Detection of short C chains in *S. venezuelae* α -glucan. The mass spectra show the isotopic distributions of the DP8 product of debranched α -glucan with or without prior reduction. Without prior reduction (black), the natural abundance isotopic distribution of isotopologues was observable. With prior reduction with NaBH_4 (red; displaced slightly along the horizontal axis for clarity), a small but significant relative enhancement of the isotopologue with two additional mass units (1339.4 m/z for DP8) was observed (highlighted by the bracket), indicative of the presence of small quantities of C chains of DP8 that had been reduced at their reducing ends. Spectra of products of DP4 to 14 were similarly recorded, and the absolute differences in the signals with two additional mass units for each DP allowed the chain length distribution of C chains to be calculated.

reduced sample showed a relative increase in the abundance of the isotopologue with an additional two mass units. Such reduced species must have been liberated C chains because malto-oligosaccharides are detected by mass spectrometry in samples of α -glucan only when they are debranched. The reduced products had a mean DP \pm SD of 9.1 ± 2.5 (Table 1). Therefore, the C chains were slightly longer than the average of ~ 7 of all chains within the polymer.

Internal Chain Length of Actinomycete α -Glucan B Chains Is 4.6–5.0. To define the internal chain length of B chains (i.e., between the branch point of B chains and the furthest branch they bear as illustrated in Figure 3), α -glucan samples were treated with β -amylase. This exoacting enzyme hydrolyzes maltose units from the nonreducing ends of B chains to within one or two glucosyl residues of the first branch point it encounters, depending on whether the external chain has an odd or even number of sugar residues.^{29,38} Given that there is no detectable odd/even bias within the polymer chains, the average overhang beyond the outermost branch after digestion is 1.5 glucosyl residues in length. By contrast, A

chains are almost completely digested to give maltose and maltotriose (Figure 3). After treatment, the mean DPs of actinomycete α -glucans of DP ≥ 4 were 6.1–6.5 (Table 1 and Figure 4), showing that a typical B chain of seven residues bears a branch 4.6–5.0 residues from its own branch point (i.e., roughly half way along their length on their fourth or fifth residue). This contrasts with classical glycogens from rabbit liver and *E. coli* that have internal chain lengths of ~ 7 –8 (Table 1).

α -Glucan from *S. venezuelae* Has a Smaller A:BC Chain Ratio than Classical Glycogen. The ratio of the number of A vs B plus C chains (A:BC) of classical glycogen is typically close to 1.³⁹ The A:BC chain ratio can be determined from the mean DP before and after treatment with β -amylase together with the extent of hydrolysis by this enzyme (where eq 2 is derived from eq 1, which is also illustrated in Figure 3). The mean number of branches per B/C chain in a polymer of finite size is essentially the A:BC chain ratio plus one. For example, consider a 17 kDa polymer consisting of 105 glucosyl residues in 15 chains with a mean DP of 7 (Figure S4). If each B and C chain bore only one branch (Figure S4A), the polymer would comprise 1 A chain, 13 B chains one C chain, giving an A:BC chain ratio of close to zero (0.07). If the polymer comprised B and C chains that bore two chains each (Figure S4B), it would have eight A chains, six B chains, and 1 C chain, giving an A:BC chain ratio of close to 1 (1.14).

α -Glucans were conspicuously less prone to hydrolysis by β -amylase (21–33% glucose liberated) than *E. coli* classical glycogen (~ 40 –47%) (Table 1). This was also evident from electron microscopy and dynamic light scattering (Table 1) that showed that only glycogen particles were significantly diminished in size. The A:BC chain ratio with *S. venezuelae* α -glucan was 0.2 ± 0.2 , giving $\sim 1.2 \pm 0.2$ branches per B/C chain (Table 1). The values for both types of *M. tuberculosis* α -glucan were a little higher at 0.6 and ~ 1.6 , respectively. These values contrast with those of 0.8–0.9 and 1.8–1.9, respectively, for classical glycogens (Table 1).²⁹

GlgB Enzymes from Actinomycetes Generate Relatively Short Branches of DP ~ 7 –8. It might be expected that the branch length specificity of GlgB branching enzyme from actinomycetes reflects the shorter lengths present in the α -glucans from these bacteria. The treatment of α -1,4-linked amylose with a short or medium DP with *M. tuberculosis* GlgB branching enzyme led to a loss of absorbance in the presence of iodine (Figure S5), as previously described.³⁹ The chain length specificity of this enzyme was then determined by exposing α -maltose 1-phosphate to GlgE and GlgB to generate a branched α -glucan product that could be debranched and subjected to mass spectrometry (Figure 6). The chains generated by *M. tuberculosis* GlgB in the presence of *M. tuberculosis* GlgE had a mean DP of 8.6 ± 2.5 (Table 2). This value was verified using capillary electrophoresis, which gave 8.0 ± 3.1 (Table 2). No branched products were observed when malto-octaose was exposed to GlgB suggesting this malto-oligosaccharide was not long enough to be branched. Mean values of between 7.2 and 8.5 were obtained when using *S. coelicolor* GlgE with GlgB from either *M. tuberculosis* or a number of other *Mycobacterium* and *Streptomyces* species (Table 2). This contrasts with chain DPs of >10 observed with branching enzymes from organisms that produce classical glycogen, such as that from *E. coli*.^{18,40} The actinomycete GlgB branching enzymes therefore generate relatively short branches, as predicted.

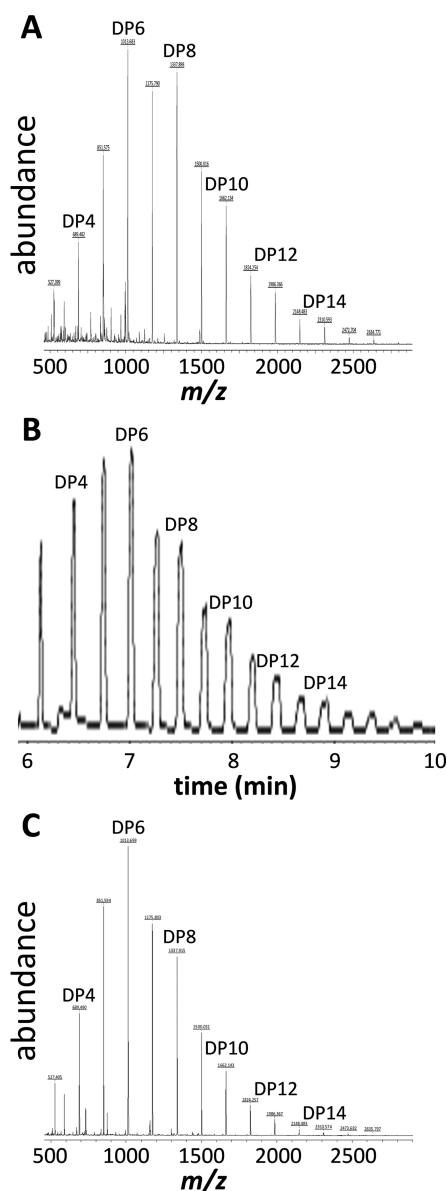


Figure 6. Branch length specificity of GlgB branching enzymes. The polymer, generated using *M. tuberculosis* GlgB, *S. coelicolor* GlgE, and α -maltose 1-phosphate, was filtered to remove small malto-oligosaccharides and then treated with isoamylase. The resulting debranched sample was then analyzed using either (A) MALDI mass spectrometry or (B) capillary electrophoresis. (C) A mass spectrum is also shown of a sample generated with GlgB enzyme from *S. venezuelae*. The mean branch lengths are shown in Table 1.

***M. tuberculosis* GlgB Transfers a Nascent A Chain of DP ~ 8 to a Recipient Nascent C Chain of DP ~ 9.** The mean chain DPs of the polymers generated in the presence of GlgB and GlgE would include all A, B, and C chains, where the B chains would dominate, and the C chains would be the least represented. We therefore devised an experiment to define the DP of nascent A chains that are transferred onto nascent C chains (Figure 7A). Malto-octaose (DP8) was first treated with GlgE and α -maltose 1-phosphate to extend it by maltose units to give a series of products with an even number of glucosyl residues. The sample was then boiled, to denature the GlgE, followed by treatment with GlgB to introduce a single branch. The sample was boiled again, to denature the GlgB, reduced

Table 2. Branch Length Distributions Generated by GlgB Branching Enzymes in the Presence of GlgE

source of GlgB	source of GlgE	branch length ^a	
		MS ^b	CE ^c
<i>M. tuberculosis</i>	<i>M. tuberculosis</i>	8.6 ± 2.5	8.0 ± 3.1
	<i>S. coelicolor</i>	8.1 ± 2.7	7.5 ± 3.5
A chains	na ^d	8.3 ± 1.9 ^e	nd ^f
	odd length C chains	na ^d	9.0 ± 2.9 ^e
<i>M. tuberculosis</i> CCDCS180	<i>S. coelicolor</i>	8.4 ± 2.5	nd ^f
<i>M. smegmatis</i> mc ² 155	<i>S. coelicolor</i>	8.5 ± 2.5	nd ^f
<i>S. venezuelae</i>	<i>S. coelicolor</i>	7.9 ± 2.4	nd ^f
<i>S. coelicolor</i> isoform 1	<i>S. coelicolor</i>	7.2 ± 2.2	7.1 ± 3.1

^aLength distributions shown are mean ± SD of products of DP ≥ 3 of samples of malto-hexaose treated with GlgB, GlgE, and α -maltose 1-phosphate followed by isoamylase. ^bDetermined using mass spectrometry. ^cDetermined using capillary electrophoresis. ^dNot applicable. ^eThe A and C chain length distributions were determined from a single branching event as shown in Figure 7. ^fNot determined.

with NaBH₄, and treated with isoamylase to cleave the α -1,6 linkages. This would liberate reduced C chains and nonreduced A chains. All nonreduced species would be A chains. By contrast, the reduced C chains would be significantly contaminated with starting materials with an even number of residues that would also be reduced. GlgB does not discriminate between odd and even chain transfers. Therefore, reduced species that had an odd DP would be liberated C chains, with only a low level of contamination from hydrolytic side-products, as described below.

Mass spectrometry was used to quantify the abundance of reduced C chains that had an odd DP, giving a mean DP of 9.0 ± 2.9 (Figure 7B and Table 2). For A chains, the distribution of nonreduced species with an even DP (mean DP 8.1 ± 2.7; data not shown) was similar to that of the odd species (mean DP 8.5 ± 2.6 shown in Figure 7B) giving an overall mean DP of 8.3 ± 2.7 (Table 2). This value was consistent with the DP of 8.1–8.6 generated in the presence of GlgE as described above (Table 2). The overall higher abundance of reduced over nonreduced chains was consistent with GlgB generating some hydrolytic products. From this, the hydrolysis to branching ratio was estimated to be ~0.2:1 for such single branching events with short-chain substrates.

Branching by *M. tuberculosis* GlgB Is Strictly Intra-chain. Branching enzymes act by transferring a segment from the nonreducing end of a malto-oligosaccharide to a hydroxyl group at the six position of a glucosyl residue along a malto-oligosaccharide, via cleavage of an α -1,4 linkage and a glycosyl-enzyme intermediate.⁴¹ Both intra- and interchain branching has been reported with GlgB enzymes.^{18,42,43} In order to establish whether *M. tuberculosis* GlgB carries out intra- and/or interchain transfers, we first generated ¹³C-labeled and unlabeled populations of malto-oligosaccharides (Figure 8 and Figure 9). This was possible by exposing unlabeled malto-tetraose (DP4) to GlgE in the presence of labeled or unlabeled α -maltose 1-phosphate. These two populations of molecules were then boiled to denature the GlgE, mixed, boiled again, and then cooled, to allow any intermalto-oligosaccharide associations that might potentially occur to be formed at random. On exposure to GlgB, there would be three possible outcomes (Figure 7). If the branching reaction were intrachain, the masses of the products would be identical to those of the substrates. If it were interchain, the label would be redistributed

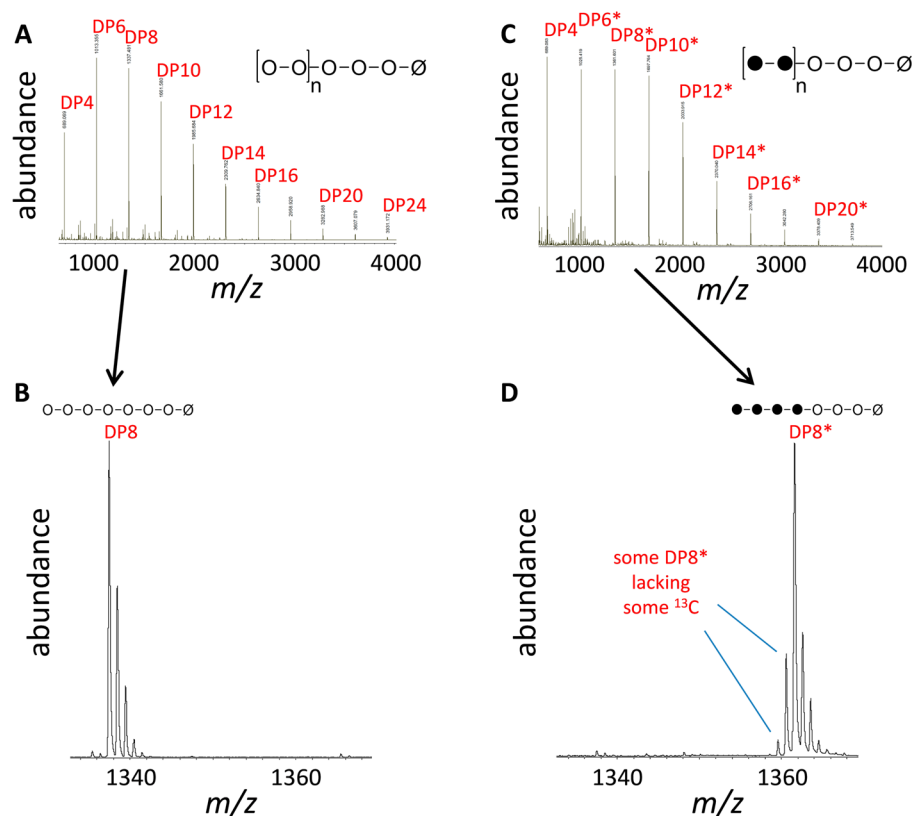


Figure 9. Enzymatic synthesis of labeled and unlabeled malto-oligosaccharides. Malto-tetraose (DP4) was extended by *M. tuberculosis* GlgE with either [U- ^{12}C] or [U- ^{13}C] α -maltose 1-phosphate as depicted in Figure 8. The filled circles and \emptyset in the structures refer to labeled and reducing end glucosyl residues, respectively. To stop the reaction, the samples were boiled to denature the enzyme. (A) A mass spectrum of unlabeled compounds showed the presence of a range of products. (B) The ions associated with each unlabeled product gave the expected natural abundance isotopic distribution, as illustrated by the DP8 product shown. (C) A mass spectrum of the labeled compounds also showed the presence of a range of products. (D) The ions associated with each labeled product showed the presence of the label, as illustrated by the DP8* product. The asterisk refers to the presence of ^{13}C -labeled glucosyl residues observed in all but the original DP4 acceptor.

with strictly intrachain branching because interchain branching would have given additional species with intermediate amounts of label.

In order to confirm that branching had indeed occurred, the sample was reduced and then debranched (Figure 8). It was immediately apparent that the overall distribution of species was shifted to shorter DPs (Figure 10C) consistent with the cleavage of branch points. Closer inspection of the products confirmed that this was indeed the case. For example, the reduced, labeled DP8 species had the identical isotopic distribution (Figure 10D) as the starting material (Figure 9D), except for the addition of 2 Da to all ions. This is consistent with starting material and liberated C chains (and likely some hydrolytic products). Furthermore, this shows that reduction was complete because no unreduced labeled species were detected. By contrast, the unlabeled DP8 product contained not only species with the same isotopic distribution as starting material plus 2 Da, but also nonreduced species amounting to over a quarter of the total unlabeled DP8 species (Figure 10D). The unreduced species can only have been derived from A chains formed by the branching reaction of GlgB. Such nonreduced species, with and without labels, were detected with a DP of between 5 and 10 giving a mean DP of 8.0 ± 1.4 for all liberated A chains, again consistent with the branching specificity experiments described above. These observations are therefore strongly supportive of a strictly intrachain mechanism.

Re-Evaluation of the Ability To Detect Malto-Oligosaccharides Containing an α -1,6 Branch Using Ion Mobility Mass Spectrometry. We previously interpreted the presence of two isomers of a malto-oligosaccharide with a DP of 11 in samples of amylose treated with *M. tuberculosis* GlgB as evidence for the ability to resolve linear and branched materials using ion-mobility mass spectrometry.²⁵ Careful re-evaluation of reaction mixtures with and without enzyme revealed that the relative abundance of the two observable isomers was in fact somewhat variable and, more critically, independent of GlgB (Figure S6). Reduction of a sample of linear malto-oligosaccharide (amylose 2800) with NaBH_4 clearly showed the collapse of the two isomers of DP11 into one. This phenomenon resembles what we reported with malto-hexaose (DP6), which was interpreted as the presence of two conformers, where one is extended and the other forms the first complete turn of a left-handed helix. Each turn of the helix involves the formation of O(6)–O(2) hydrogen bonds between glucosyl residues along the helical axis. Reduction of the reducing end would open up the terminal glucopyranose ring, disrupting the potential for such hydrogen bonding involving this residue. We therefore now interpret the presence of two isomers in the material with a DP of 11 in a similar way; it could adopt either a conformation with two complete turns of the helix or another more extended conformation. The variability in the relative abundance of the two isomers likely reflects very small differences in the stabilities of the two

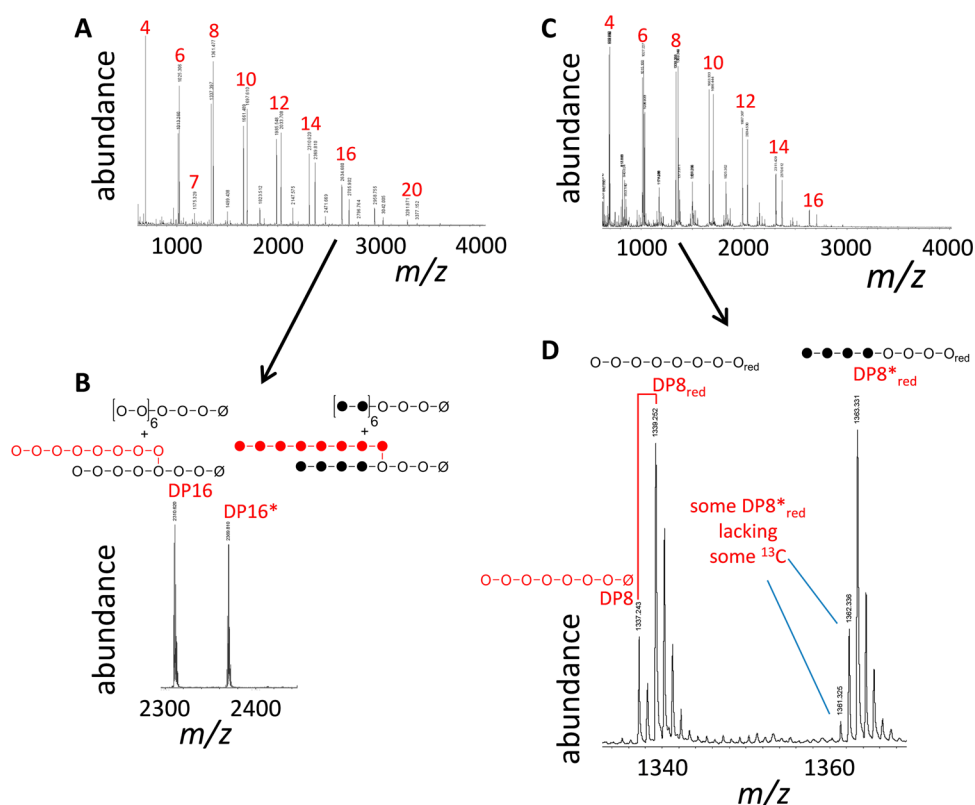


Figure 10. *M. tuberculosis* GlgB catalyzes intrachain branching. Labeled and nonlabeled compounds (see Figure 9) were mixed in equal proportions, boiled, cooled and treated with GlgB (see Figure 8 for experimental scheme). (A) A mass spectrum showed only minor changes in the profile of DP compared with starting materials (see Figure 9). (B) None of the products with an even DP showed any redistribution of ¹³C labels, as illustrated with the DP16 product. The filled circles, \emptyset , and O_{red} in the structures refer to labeled, reducing end, and reduced glucosyl residues, respectively. Structures in black were derived from linear molecules and C chains, while those in the color red were derived from A chains. (C) The sample was reduced with $NaBH_4$ and treated with isomylase to hydrolyze all of the α -1,6 linkages in order to confirm the presence of branches. The mass spectrum showed the loss of compounds with the highest DP, consistent with the cleavage of branches. (D) The reduced products illustrate how the reduced products ($DP8_{red}$ and $DP8^*_{red}$), derived from C chains and linear molecules, had the same isotopic distribution as the starting material, while the presence of nonlabeled, nonreduced product ($DP8$) showed that sample must have contained A chains generated by GlgB (see Figure 8 for the experimental scheme and the main text for further details).

conformations in the gas phase, and reduction would disrupt the formation of two complete helical turns.

GlgE Preferentially Extends A Chains over B and C Chains. GlgE must extend chains to attain the requisite length for GlgB to act. The B and C chains of an α -glucan cannot be frequently extended by GlgE because the majority of B and C chains bear only one branch, and their lengths clearly remain unchanged after being formed by GlgB. To test this hypothesis, the activity of *M. tuberculosis* GlgE was determined with rabbit liver glycogen and *S. venezuelae* α -glucan, which have A:BC chain ratios of 0.9 and 0.2, respectively (Table 1). This was done by monitoring inorganic phosphate release from the donor substrate of GlgE, α -maltose 1-phosphate. The k_{cat}/K_m of the enzyme was 6.4-fold higher with rabbit liver glycogen (Figure S7), consistent with GlgE exhibiting a preference for A chains. We then treated an equal mass of either oligosaccharides that contained a single branch, glycogen or α -glucan with GlgE and ¹³C-labeled α -maltose 1-phosphate (for example, see Figure S8). Mass spectrometry of debranched samples showed the chains were extended by 94, 38, and 18%, respectively, again showing a correlation between the number of available A chains and the degree of extension.

Particles of α -Glucan Generated by GlgE and GlgB Resemble Those Isolated from Cells. Polymers generated by *M. tuberculosis* GlgE and GlgB from α -maltose 1-phosphate

and malto-hexaose (DP6) resembled those isolated from cells according to electron microscopy (Figure 2). The mean diameter \pm SE of the synthetic material was about half (32 ± 2 nm) that of the biological polymer isolated from *M. tuberculosis*, but their morphologies were similar. Similar sized particles were generated when the reaction was carried out with GlgE from *S. coelicolor* (30 ± 2 nm, $n = 40$). Comparable diameters were also obtained when both *S. coelicolor* enzymes were used either with malto-hexaose (29 ± 5 nm, $n = 10$) or without (25 ± 4 nm, $n = 10$), showing that polymer initiation is possible without a primer.

DISCUSSION

GlgB enzymes from actinomycetes generate polymers in the presence of GlgE with DPs of typically 8.1 ± 2.5 , which is reasonably similar to the typical DP of 7.2 ± 1.9 observed in polymer material isolated from cells. These are, to the best of our knowledge, among the shortest branch lengths reported. The shortest reported thus far for GH13 branching enzymes appear to be 7.9 ± 3.4 from *Vibrio vulnificus*⁴⁴ and 8.0 ± 2.6 from *Deinococcus geothermalis*.⁴⁵ We and others¹⁸ have attempted to correlate the specificity of branching enzymes with their amino acid sequence but have failed to identify any obvious motif or domain responsible for any particular property. Nevertheless, the two N-terminal domains of

actinomycete GlgB enzymes, which include a family 48 carbohydrate binding module, do appear to be important because chimeric enzymes have altered chain length specificities.^{44,45}

Interestingly, branching by *M. tuberculosis* GlgB was shown to be strictly intrachain. This implies that the nascent recipient chain, and by definition the remainder of the polymer, must bind very tightly and noncovalently to the enzyme until the nascent A chain is fully transferred to the recipient chain via a glycosyl-enzyme intermediate. We and others⁴¹ have observed that branching enzymes can generate some hydrolytic by-products. This was more prevalent with short substrates, implying fewer potential contacts with the protein lead to weaker binding. With the *M. tuberculosis* enzyme at least, it would appear that water, rather than another polymer chain, acts as the acceptor after premature dissociation of the original recipient chain.

We have shown that the internal chain length of α -glucan is shorter than for classical glycogen. Many previous studies on glycogens have estimated internal chain lengths based solely on the degree of hydrolysis by β -amylase. This is inappropriate given that glycogen contains almost as many A chains and B chains, and A chains are almost completely digested.³⁸ This artificially decreases the calculated internal chain lengths to such an extent that they would often not be long enough to support the degree of branching that is known to occur. In order to avoid this problem, we directly determined the length of B chains with mass spectrometry after treatment with β -amylase.

It is now possible to conceive of how α -glucans are generated in organisms such as *S. venezuelae* (Figure 11), which possesses the GlgE pathway and no other α -glucan pathway.⁸ It is known that GlgE can initiate polymer synthesis without any primer, such as glycogenin or a malto-oligosaccharide. It does this by slowly hydrolyzing α -maltose 1-phosphate to give maltose, which in turn is slowly extended to give malto-tetraose, one of its preferred acceptor substrates.^{3,19} As soon as the malto-oligosaccharide attains a length of ~ 16 glucosyl residues, GlgB will generate the first branch giving an A and a C chain of DP ~ 8 –9. GlgE will then preferentially extend the A chain until it is long enough to undergo branching to generate a new A chain and the first B chain. The minimum length that seems to be transferred can occasionally be three but is more typically four or more *in vivo* and *in vitro* (Figure 4 and Figure 6). Each new A chain forms a branch as close as three residues from the branch point of the recipient chain, but more often on the fourth or fifth. By contrast, the *E. coli* enzyme has been reported to prefer the third residue for the first branch to be formed.¹⁸ GlgE and GlgB will continue to extend and create new A chains, respectively. Normally, a newly formed B chain will not be long enough to allow a second branch to be introduced by GlgB. However, GlgE appears to occasionally extend a B chain and, when it attains sufficient length, GlgB introduces a second branch into this chain. These additional events ultimately generate the arboreal structure of the α -glucan. However, the introduction of a second branch in a B chain is not a frequent event because the mean number of branches per B chain is 1.2–1.6, considerably less than the value of 1.8–1.9 routinely observed in classical glycogens. Indeed the A:BC chain ratios for α -glucan are the smallest that have, to our knowledge, been reported.²⁹

Importantly, the majority of chains in an α -glucan are B chains. Therefore, the overall mean linear chain DP of an α -

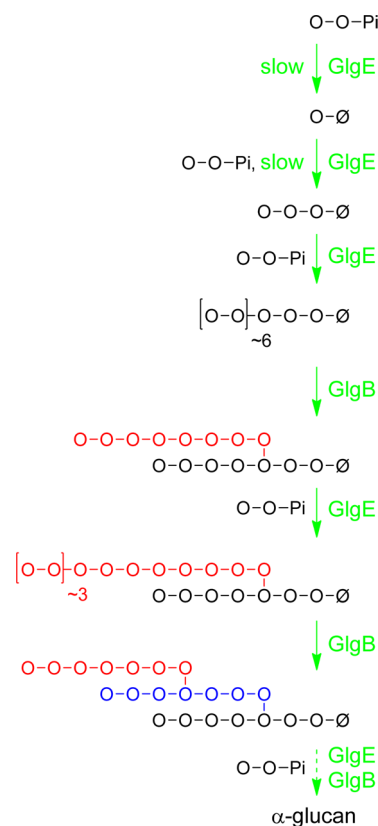


Figure 11. Scheme of α -glucan assembly by GlgE and GlgB. Linear molecules and C chains are shown in black, A chains in red, and B chains in blue. The \emptyset and Pi in the structures refer to the reducing end glucose residue and phosphate, respectively.

glucan must be principally defined by GlgB. This is because the length of each B chain is initially defined by the recipient chain length specificity of GlgB. We have established that *M. tuberculosis* GlgB creates C chains of DP ~ 9 (Table 1 and Figure 5), which matches that observed in the polymer isolated from cells. It is reasonable to assume that it also generates B chains of a length that matches those observed in the polymer of ~ 7 –8. If GlgE readily extended B chains, one would expect that the mean linear chain DP in the polymer would be much greater, but this is clearly not the case. Rather, it would appear that the occasional extension of individual B chains by GlgE simply leads to GlgB acting again, significantly restricting any potential to extend B chains within the polymer. Consistent with this, the branch lengths generated by GlgB *in vitro* are not obviously influenced by the presence or absence of GlgE in our *in vitro* assays.

GlgE is capable of not only extending malto-oligosaccharides with α -maltose 1-phosphate but also moving maltosyl units between them in a disproportionation reaction.^{3,19} Perhaps this second activity is important to prevent any chains from being excessively extended because linear malto-oligosaccharides with a DP of >20 have limited solubility in aqueous conditions.

Having previously used a genetic approach to show that the GlgE pathway is both necessary and sufficient for the biosynthesis of α -glucan in *S. venezuelae*,⁸ we have now shown that GlgE and GlgB are sufficient to generate the polymer from solely α -maltose 1-phosphate *in vitro*. Importantly, the α -glucans isolated from the cytosol and capsule of *M. tuberculosis* were very similar not only to each other, but also to that from *S. venezuelae*, which is derived exclusively from the

GlgE pathway. Although others have reported differences between the two mycobacterial materials,^{15,16} such as in optical rotation, particle size, and hydrolytic susceptibility, these differences are subtle and could reflect differences in the way in which the materials are isolated. The similarity in the structures of the cytosolic and capsular materials implies that they could be synthesized solely by the GlgE pathway. It therefore remains to be established what role GlgA and Rv3032 have in the biosynthesis of α -glucan polymers in mycobacteria, despite genetic evidence suggesting they could have a direct role.¹⁵ Furthermore, how a kDa-MDa dendrimer with a diameter of tens of nanometer is exported from a mycobacterial cell remains unclear, since secretion via vesicles appears to have been ruled out.⁴⁶

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.biochem.6b00209](https://doi.org/10.1021/acs.biochem.6b00209).

NMR spectra of α -glucan, calibration curve for pulse field gradient NMR spectroscopy, UV/visible absorbance spectra of iodine complexes, cartoons of theoretical structures, ion mobility mass spectra, and kinetics and mass spectrometric analysis of polymer extension by GlgE (PDF)

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Notes

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■ ABBREVIATIONS

DP, degree of polymerization of linear α -1,4-linked chains

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