

Capsular glucan and intracellular glycogen of *Mycobacterium tuberculosis*: biosynthesis and impact on the persistence in mice

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Summary

Mycobacterium tuberculosis and other pathogenic mycobacterial species produce large amounts of a glycogen-like α -glucan that represents the major polysaccharide of their outermost capsular layer. To determine the role of the surface-exposed glucan in the physiology and virulence of these bacteria, orthologues of the *glg* genes involved in the biosynthesis of glycogen in *Escherichia coli* were identified in *M. tuberculosis* H37Rv and inactivated by allelic replacement. Biochemical analyses of the mutants and complemented strains indicated that the synthesis of glucan and glycogen involves the α -1,4-glucosyltransferases Rv3032 and GlgA (Rv1212c), the ADP-glucose pyrophosphorylase GlgC (Rv1213) and

the branching enzyme GlgB (Rv1326c). Disruption of *glgC* reduced by half the glucan and glycogen contents of *M. tuberculosis*, whereas the inactivation of *glgA* and *Rv3032* affected the production of capsular glucan and glycogen, respectively. Attempts to disrupt *Rv3032* in the *glgA* mutant were unsuccessful, suggesting that a functional copy of at least one of the two α -1,4-glucosyltransferases is required for growth. Importantly, the *glgA* mutant was impaired in its ability to persist in mice, suggesting a role for the capsular glucan in the persistence phase of infection. Unexpectedly, GlgB was found to be an essential enzyme.

Introduction

Tuberculosis remains a serious health problem worldwide, with an estimated one-third of the population infected by *Mycobacterium tuberculosis*, the causative agent of tuberculosis, and a death toll of more than 2 million people annually. Understanding the molecular mechanisms enabling the tubercle bacillus to multiply in a hostile environment such as the macrophage and to survive in the host is important for the design of novel strategies to combat the disease. The cell envelope of pathogenic mycobacteria plays important roles in host–pathogen interactions and in the resistance of these microorganisms to chemotherapeutic treatments (Jarlier and Nikaido, 1990; Daffé and Draper, 1998). The envelope consists of a typical plasma membrane, surrounded by a complex wall of carbohydrates and lipids that are organized into an outer membrane (Hoffmann *et al.*, 2008; Zuber *et al.*, 2008), which is in turn surrounded by an outer layer (Daffé and Draper, 1998). The unusual chemical nature of the envelope makes it difficult for the host to degrade.

The outermost compartment of the cell envelope of pathogenic mycobacterial species consists in a loosely bound structure called capsule (Chapman *et al.*, 1959; Hanks, 1961; Daffé and Draper, 1998). We have previously shown that the capsular material is composed of carbohydrates and proteins with only small amounts of species- or type-specific lipids (Ortalo-Magné *et al.*, 1995; Lemassu

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et al., 1996). Although mycobacteria shed some of this material in the culture medium during *in vitro* growth (Ortalo-Magné *et al.*, 1995), capsular components clearly coat *in vivo*-grown bacteria (Schwebach *et al.*, 2002), probably retained by the phagosomal membrane (Daffé and Draper, 1998). The major carbohydrate constituent of the capsule of *M. tuberculosis*, representing up to 80% of the extracellular polysaccharides, is a high-molecular-weight (> 100 000 Da) α -glucan composed of a $\rightarrow 4$ - α -D-Glc-1 \rightarrow core branched at position 6 every five or six residues by $\rightarrow 4$ - α -D-Glc-1 \rightarrow oligoglucosides (Lemassu and Daffé, 1994; Ortalo-Magné *et al.*, 1995; Dinadayala *et al.*, 2004). Because the carbohydrate nature of the main constituent of the surface of pathogenic mycobacterial species has been determined much later than the discovery of the mycobacterial capsule (Lemassu and Daffé, 1994; Ortalo-Magné *et al.*, 1995; Lemassu *et al.*, 1996; Dinadayala *et al.*, 2004), there is at present little information about the biological functions of these components. Using Complement Receptor 3 (CR3)-transfected Chinese hamster ovary cells, Cywes and collaborators showed that capsular polysaccharides, among which the α -glucan, mediated the non-opsonic binding of *M. tuberculosis* H37Rv to CR3 (Cywes *et al.*, 1997). Given that CR3 is one of the principal phagocytic receptors of monocytes and neutrophils and that CR3-mediated phagocytosis can result in the diminution or absence of oxidative burst and suppression of IL-12 secretion, it was proposed that this route of entry may be favourable to the intracellular survival of the tubercle bacillus (Ehlers and Daffé, 1998; Fenton *et al.*, 2005). In another study, capsular components of *M. tuberculosis* were shown to contain compounds that displayed antiphagocytic properties with certain types of macrophages (Stokes *et al.*, 2004). α -Glucan was also recently shown to induce monocytes to differentiate into altered dendritic cells that failed to present lipid antigens to CD1-restricted T cells, to upregulate CD80, and that produced IL-10 but not IL-12 (Gagliardi *et al.*, 2007). Finally, the cell wall α -glucan of *Pseudallescheria boydii*, which appears to be similar in structure to that of pathogenic mycobacteria, was involved in the internalization of this fungus by macrophages and the induction of the innate immune response by a mechanism dependent on toll-like receptor 2, CD14 and MyD88 (Bittencourt *et al.*, 2006).

Although these findings suggested an important role for capsular glucan in the regulation of phagocytosis and the modulation of the immune response, most of them were derived from *in vitro* studies using purified glucan and cellular models that may not accurately reflect its contribution during mycobacterial infections. The construction of mutants deficient in the synthesis of glucan was thus warranted to assess the relevance and individual contribution of this polysaccharide to the pathogenicity of *M. tuberculosis*.

Results

Identification of *M. tuberculosis* H37Rv genes potentially involved in α -D-glucan and glycogen biosynthesis

Mycobacterium tuberculosis and *M. bovis* BCG capsular glucans are composed of repeating units of $\rightarrow 4$ - α -D-Glc p -1 \rightarrow residues and some $\rightarrow 4$ - α -D-Glc p substituted at position 6 with one to six α -D-Glc p residues (Lemassu and Daffé, 1994; Ortalo-Magné *et al.*, 1995; Dinadayala *et al.*, 2004), indicating a glycogen-like highly branched structure. Given the structural similarities between α -D-glucan and glycogen, the synthesis of both polysaccharides is expected to involve similar catalytic reactions (Fig. 1). The two polysaccharides may even share, at least in part, a common biosynthetic pathway. In addition to the α -1,4-glucosyltransferase gene *Rv3032* which we recently showed to be involved in the synthesis of both 6-O-methylglucosyl lipopolysaccharides (MGLP) and glycogen (Stadthagen *et al.*, 2007), four genes were found to potentially participate in the synthesis of α -D-glucan and/or glycogen in the genome of *M. tuberculosis* H37Rv. *Rv1213* encodes a putative ADPGlc pyrophosphorylase. The product of this gene shares 35% amino-acid identity with the *Escherichia coli* ADPGlc pyrophosphorylase (GlgC) that is responsible for the synthesis of the glucosyl donor, ADP-Glc. *Rv1213* is immediately adjacent to the putative glycogen synthase gene, *glgA* (*Rv1212c*), whose orthologue in *Corynebacterium glutamicum* was recently implicated in glycogen synthesis (Tzvetkov *et al.*, 2003). *Rv1326c* encodes an α -1,4-glucan branching enzyme potentially responsible for the branching of the oligoglucosyl units on the glucosyl backbone (Garg *et al.*, 2007). *Rv1326c* shares 48% amino-acid identity (63% similarity) with the branching enzyme from *E. coli*. This *M. tuberculosis* *glgB*-like gene is arranged in an operon with *glgE* (*Rv1327c*), a gene encoding a glucanase whose orthologue in *Mycobacterium smegmatis* is required for the degradation and recycling of glycogen (Belanger and Hatfull, 1999). It is also adjacent to another catabolic gene, *glgP* (*Rv1328*), encoding a probable glycogen phosphorylase (Schneider *et al.*, 2000). Finally, *Rv1562c* (*treZ*) is the third gene of an operon that has been involved in the metabolism of trehalose (De Smet *et al.*, 2000; Woodruff *et al.*, 2004). The sequence similarity *Rv1562c* shares with bacterial α -1,4-glucan branching enzymes (26% identity with the *E. coli* GlgB protein on a 241-amino-acid overlap) suggested that it may also catalyse the branching of glycogen and/or α -D-glucan.

Disruption of the *glgA*, *glgB*, *glgC* and *treZ* orthologue genes by allelic exchange in *M. tuberculosis* H37Rv

glgA, *glgC* and *treZ* orthologues were disrupted by homologous recombination in *M. tuberculosis* H37Rv

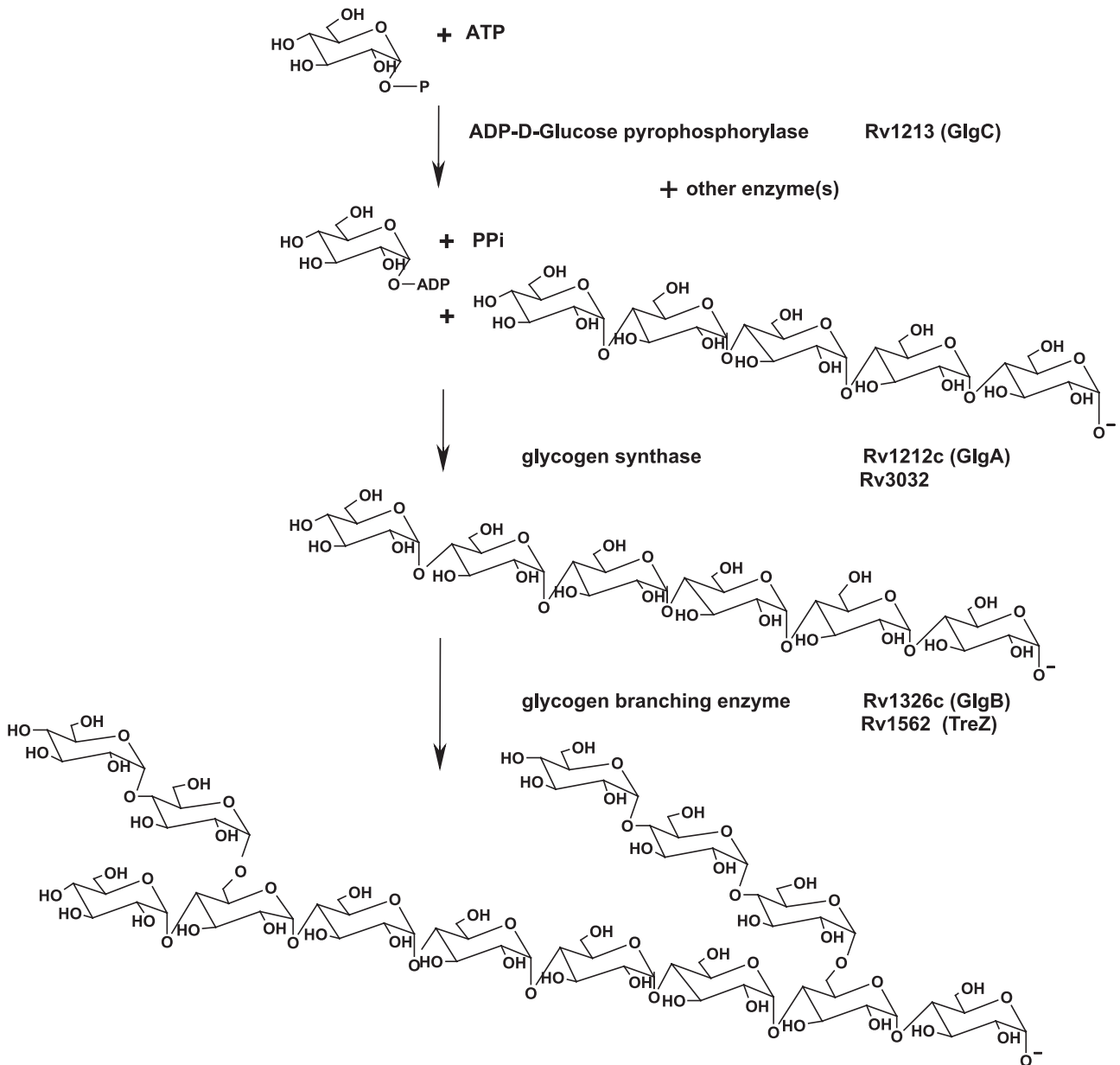


Fig. 1. Proposed pathway for the biosynthesis of glycogen and glucan in *M. tuberculosis*.

using standard protocols (Pelicic *et al.*, 1997; Jackson *et al.*, 2001). Allelic replacement at the *glgA*, *glgC* and *treZ* loci was confirmed in each mutant by Southern hybridization (Fig. S1). One *glgA* mutant, one *treZ* mutant and one *glgC* mutant were selected for further studies and named H37Rv Δ *glgA*, H37Rv Δ *treZ* and H37Rv Δ *glgC*, respectively. Unexpectedly, despite repeated attempts to knock out the *glgB* gene in *M. tuberculosis* H37Rv, no candidate allelic exchange mutants could be isolated. Single-cross-over was, however, achievable at the *glgB* locus (data not shown). To determine whether the failure to disrupt the *glgB* gene was due to its essentiality, we

then performed an allelic exchange experiment using an *M. tuberculosis* merodiploid strain. This strain was constructed by transforming the clone that had undergone a single cross-over at the *glgB* locus with pVV*glgBtb*. Gene replacement was then carried out following standard procedures (Jackson *et al.*, 2001). Analysis by PCR and Southern blotting of eight clones corresponding to putative double-cross-over recombinants showed that allelic exchange had occurred at the chromosomal *glgB* locus of five of them (Fig. S1). Therefore, the expression of *glgB* from pVV*glgBtb* was sufficient to rescue an *M. tuberculosis* knockout mutant, indicating that this gene

is essential for growth. Allelic replacement at the *glgB* locus of *M. tuberculosis* was also achievable in the presence of a wild-type copy of the *glgB* gene from *E. coli* expressed from pVV*glgBcoli*, suggesting that both *glgB* orthologues have a similar function. Finally, in spite of individual *glgA* and *Rv3032* knockouts being achievable (Stadthagen *et al.*, 2007), two independent attempts to disrupt the *Rv3032* gene in H37RvΔ*glgA* in order to create a mutant simultaneously deficient in both α -1,4-glucosyltransferases turned out to be unsuccessful. Thus, *M. tuberculosis* H37Rv requires a functional copy of at least one of these two genes for growth.

Structural features of the glycogen of *M. tuberculosis*

The structural features of the α -glucans from *M. tuberculosis* and *M. bovis* were previously established (Lemassu and Daffé, 1994; Dinadayala *et al.*, 2004). The polysaccharide from both origins exhibited a glycogen-like structure, i.e. α -4- α -D-Glcp-1 \rightarrow core, substituted at some six positions with short α -4- α -D-Glcp-1 \rightarrow oligoglucoside chains. As these similarities were based on the structures of glycogens from non-mycobacterial sources, we first investigated the structure of *M. tuberculosis* glycogen. Knowing that the structural features of glycogen are age-dependent (Antoine and Tepper, 1969a), the mycobacterial α -glucan and intracellular glycogen analysed herein were purified from the same culture batches. The α -glucan was extracted from culture filtrates as described (Lemassu and Daffé, 1994). The corresponding cells were disrupted and the intracellular glycogen was recovered from the cytosolic fraction as described under *Experimental procedures*. The mycobacterial glycogen run in a Bio-Gel P-200 gel gave a single chromatographic peak at a position corresponding to an apparent molecular mass of 100 000 Da, as reported for the α -glucan of *M. tuberculosis* (Lemassu and Daffé, 1994; Ortalo-Magné *et al.*, 1995). Surprisingly, this molecular mass was 100-fold less than that estimated earlier by analytical ultracentrifugation (Antoine and Tepper, 1969b).

Gas chromatography (GC) analysis of the trimethylsilyl derivatives of the acid hydrolysis products of the purified glycogen showed that it was exclusively composed of Glc. GC-mass spectrometry (GC-MS) analysis of the partially *O*-methylated, partially *O*-acetylated alditols derived from the polysaccharide showed that it consisted of the three expected types of residues, i.e. (i) terminal Glc-(1 \rightarrow); (ii) \rightarrow 4)-Glcp-(1 \rightarrow or 5-linked-Glcf; and (iii) \rightarrow 4,6)-Glcp-(1 \rightarrow or 5,6-Glcf. The pyranosyl conformation of the glycosyl residues that composed the polysaccharide was established from the examination of the ^{13}C -NMR spectra (Fig. S2) that showed a signal at 100.5 p.p.m. corresponding to the resonances of anomeric carbons in α -glucopyranosyl units (Zang *et al.*, 1991; Dinadayala

et al., 2004). The ^1H -NMR spectrum of the *M. tuberculosis* glycogen (Fig. S2) was similar to that published for the α -glucan of *M. bovis* BCG (Dinadayala *et al.*, 2004) and eukaryotic glycogen (Zang *et al.*, 1991).

The lengths of the 6-branched chains in the glycogen from *M. tuberculosis* were determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS of per-*O*-acetylated fragments released after digestion with pullulanase, an enzyme that specifically hydrolyses Glc- α (1 \rightarrow 6)-Glc linkages (Dinadayala *et al.*, 2004). The efficiency of the enzymatic hydrolysis was monitored by the disappearance of the signal resonances at 5.00 p.p.m. in the ^1H -NMR spectra. The reaction was found to be complete after 72 h of digestion. The digestion of pullulan into triglucosides was used as the positive control whereas maltoheptaose, from which no degradation was observed, was the negative control. The digestion products of the glycogen from *M. tuberculosis* consisted mainly of two to six residues; minor oligosaccharides with seven to nine units were also present in the mixtures of per-*O*-acetylated oligosaccharides analysed (Fig. S3). No oligosaccharide derivatives containing 10 or more sugar residues were detected. These data indicated that the lengths of the branched chains in the glycogen of *M. tuberculosis* are similar to those of the glucan from the same species (Lemassu and Daffé, 1994).

^1H -NMR signals at 5.38 p.p.m., which correspond to the resonances of terminal, branched or linear α -D-Glcp-(1 \rightarrow 4) anomeric protons, as well as those at 5.00 p.p.m. assignable to the resonances of 4-linked or terminal α -D-Glcp-(1 \rightarrow 6) anomeric protons, were observed in the spectra of glycogen (Fig. S2) and α -glucan (Dinadayala *et al.*, 2004). The optical rotatory ($[\alpha]_D$) values measured for *M. tuberculosis* glycogen was $165^\circ \pm 5$, and thus significantly different from that determined for *M. bovis* BCG glycogen (75°), indicating a difference in the conformation of the two polysaccharides (Dinadayala *et al.*, 2008). Consistent with this, the dynamic radius of the glycogen of *M. tuberculosis* (25–26 nm) was different from that measured for the glycogen from *M. bovis* (35 nm, Dinadayala *et al.*, 2008).

Glycogen and α -D-glucan contents of the *M. tuberculosis* knockout mutants

To analyse the impact of the disruption of the various genes on the production of glucan, two complementary approaches were used. The relative percentages of Ara, Glc and Man, the sugar constituents of the extracellular arabinomannan glucan and mannan (Lemassu and Daffé, 1994) were determined by GC in the culture filtrates of three to four independent batches of the wild-type and mutant strains. Additionally, the amounts estimated by GC analysis of the three sugar constituents of the extracellu-

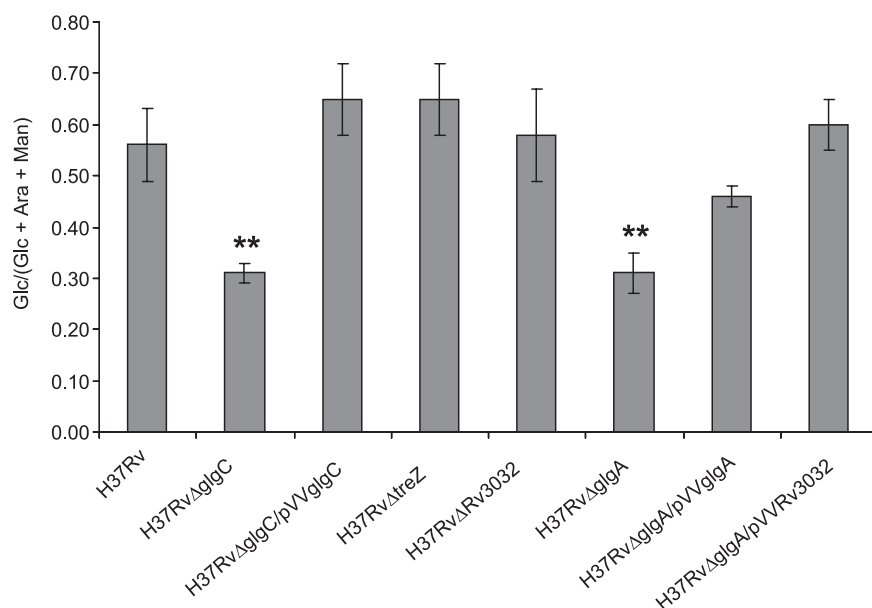


Fig. 2. Glucan contents of *M. tuberculosis* H37Rv, its isogenic H37RvΔglgA, H37RvΔglgC, H37RvΔRv3032 and H37RvΔtreZ mutants, and the complemented mutant strains. Exocellular polysaccharides were isolated from three to four different batches, hydrolysed by trifluoroacetic acid, trimethylsilylated and analysed by GC, using erythritol as an internal standard. The percentages of glucose in the monosaccharide mixture were determined. The values correspond to the mean of at least three different batches. Asterisks (**) denote *P*-values < 0.01 (Student's *t*-test).

lar polysaccharides were calculated relative to an external standard, erythritol (Ery). Very similar Glc contents (0.56 ± 0.07), determined as Glc versus Ara+Man+Glc ratios, were obtained for the different batches of the wild-type H37Rv strain (Fig. 2), indicating that the synthesis of capsular glucan was relatively constant and the preparation method reliable. Importantly, the ratios Glc/Ery varied according to the strains examined whereas those of Ara/Ery and Man/Ery were unchanged. Notably, twofold less glucan was recovered from the culture medium of H37RvΔglgC (Fig. 2). The molecular radius of the glucan synthesized by H37RvΔglgC, which reflects the conformation of the polysaccharide, was also slightly (22 versus

26 nm) but significantly affected by the mutation (Table 1). Complementation of the mutant with a wild-type copy of *glgC* restored glucan production to wild-type level as well as the native structure of the polysaccharide (Fig. 2; Table 1). The production of glucan was similarly affected in the H37RvΔglgA mutant but not in H37RvΔRv3032 and H37RvΔtreZ (Fig. 2). Interestingly, glucan production could be restored in H37RvΔglgA upon complementation with a wild-type copy of the *Rv3032* gene, indicating a partial redundancy of the two enzymes (Fig. 2). However, unlike the H37RvΔRv3032 mutant (Stadthagen *et al.*, 2007), H37RvΔglgA produced wild-type amounts of MGLP (data not shown).

Table 1. Optical rotatory values ($[\alpha]_D$ in degrees) and molecular radius (nm) of the glucans and glycogens purified from the wild-type, isogenic ΔglgC, ΔglgA and ΔRv3032 mutants, and complemented mutant strains of *M. tuberculosis* H37Rv.

Strain	Glucan		Glycogen	
	$[\alpha]_D$	Radius (nm)	$[\alpha]_D$	Radius (nm)
H37Rv	212 ± 2	25.7 ± 1.1	167 ± 6	26.6 ± 0.6
H37RvΔglgC	186 ± 1	22.7 ± 0.7	134 ± 6	24.0 ± 1.0
H37RvΔglgC/pVVglgC	209 ± 8	27.9 ± 2.5	158 ± 6	27.5 ± 1.0
H37RvΔglgA	206 ± 5	23.8 ± 0.8	161 ± 15	25.6 ± 0.2
H37RvΔglgA/pVVglgA	208 ± 2	25.3 ± 0.5	173 ± 22	25.1 ± 1.1
H37RvΔglgA/pVVRv3032	204 ± 2	25.9 ± 1.4	nd	nd
H37RvΔRv3032	215 ± 2	25.8 ± 0.7	166 ± 1	25.0 ± 0.1
H37RvΔRv3032/pVVRv3032	nr	nr	166 ± 1	26.2 ± 1.1
H37RvΔtreZ	nr	27.3 ± 0.7	nd	24.2 ± 0.6

nd, not determined; nr, not relevant.

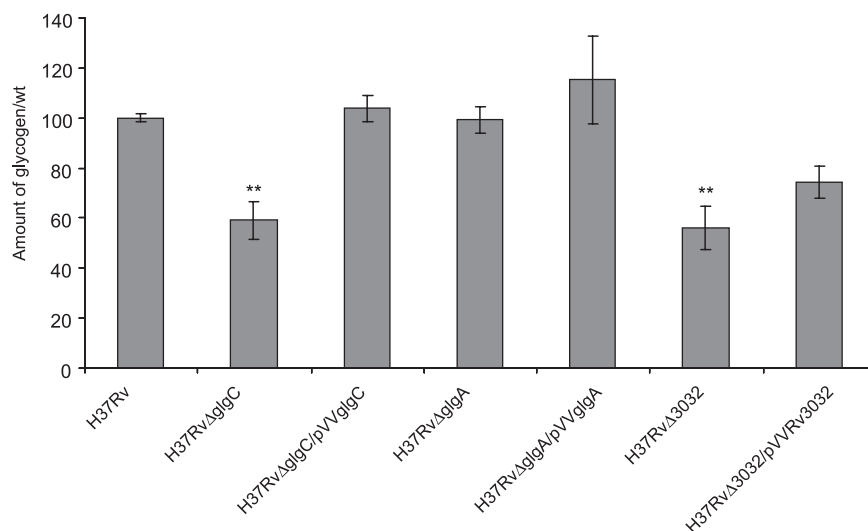


Fig. 3. Glycogen contents of *M. tuberculosis* H37Rv, its isogenic H37RvΔglgA, H37RvΔglgC and H37RvΔRv3032 mutants, and the complemented mutant strains. The intracellular glycogens were isolated from disrupted cells originating from three to four independent batches, purified as described under *Experimental procedures* and weighed. The quantity of glycogen per gram of bacterial dry mass was determined and compared with the value in the parental H37Rv strain arbitrary set to 100%. The values correspond to the mean of at least three different experiments. Asterisks (**) denote *P*-values < 0.01 (Student's *t*-test).

Determination of the glycogen content of the wild-type and mutant strains indicated that both the H37RvΔglgC and H37RvΔRv3032 strains were affected in the production of this polysaccharide. Complementation of the mutants with the wild-type copy of the corresponding disrupted gene restored, either fully or partially, glycogen production (Fig. 3). The global structural features of the glycogens produced by the mutants were otherwise similar to those of *M. tuberculosis* H37Rv as determined by ¹H-NMR analyses (data not shown) and dynamic light scattering (Table 1). Finally, no significant changes were observed in the glycogen content and structure of the H37RvΔglgA (Fig. 3) and H37RvΔtreZ mutants (data not shown).

Altogether, our results thus indicated that (i) the ADP-Glc pyrophosphorylase GlgC is involved in the synthesis of both glucan and glycogen in *M. tuberculosis* although the GlgC-dependent ADPGlc pathway is not the only route to the production of glycogen/glucan in *M. tuberculosis*, (ii) GlgA and Rv3032 have partially redundant α-1,4-glucosyltransferase activities although GlgA is preferentially utilized to synthesize glucan whereas Rv3032 is predominantly used in the synthesis of glycogen and MGLP (Stadthagen *et al.*, 2007), and (iii) TreZ is involved in the biosynthesis of trehalose (De Smet *et al.*, 2000; Wooldruff *et al.*, 2004) but not in that of glucan and glycogen.

Glycogen and α-D-glucan contents of the merodiploid *M. tuberculosis* glgB knockout mutants

GlgB appears to be an essential enzyme of *M. tuberculosis*. Allelic replacement at the *glgB* locus was, however,

achievable in the presence of wild-type copies of the *glgB* genes from *M. tuberculosis* or *E. coli* expressed from the multicopy plasmid pVV16 (see above). With the goal of establishing a link between GlgB and glucan and/or glycogen production, we thus set out to determine whether the deregulated expression of the *glgB* genes in the merodiploid strains and the expression of a heterologous *glgB* gene in H37RvΔglgB/pVVglgBcoli had any impact on the production and structural features of glucan and glycogen. Interestingly, the production of both the extracellular glucan and the intracellular glycogen was affected in H37RvΔglgB/pVVglgBtb. This strain synthesized only 57% and 36% of the glucan and glycogen normally produced by H37Rv respectively (Table 2). Moreover, while these polysaccharides were eluted from the permeation gel at a similar position as those isolated from the parental strain, contained only Glc and displayed NMR spectra superimposable to those of wild-type glucan and glycogen, their conformations were significantly different from those of wild-type H37Rv. The molecular radii measured for the glycogen and the glucan of H37RvΔglgB/pVVglgBtb were significantly lower than those of the native polysaccharides (Table 2). Furthermore, the rotatory power values of both polysaccharides were affected in the merodiploid strain (Table 2). Although the merodiploid H37RvΔglgB/pVVglgBcoli produced amounts of glycogen and glucan comparable to those of the wild-type strain, the conformation of glycogen was also significantly affected by the mutation (Table 2). Results thus indicated that, *in vivo*, the GlgB enzyme from *M. tuberculosis* (i) is involved in the biosynthesis of both glucan and glycogen and (ii) has the same function as its *E. coli* counterpart.

Table 2. Quantification and conformational analyses of glycogens and glucans from the wild-type and merodiploid $\Delta glgB$ mutants of *M. tuberculosis* H37Rv.

Strain	Glucan			Glycogen		
	Glc/(Glc + Ara + Man)	$[\alpha]_D$	Radius (nm)	Amount/wild-type	$[\alpha]_D$	Radius (nm)
H37Rv	0.56 ± 0.07	211 ± 2	25.7 ± 1.1	100 ± 2	167 ± 6	26.6 ± 0.6
H37Rv $\Delta glgB$ /pVVglgBtb	0.32 ± 0.02	127 ± 3	21.5 ± 0.4	36 ± 8	44 ± 1	22.5 ± 0.1
H37Rv $\Delta glgB$ /pVVglgBecoli	0.54 ± 0.05	nd	27.3 ± 0.5	97 ± 9	91 ± 3	30.0 ± 1.6

nd, not determined.

Multiplication and persistence of the glg mutants in cultured BALB/c mouse bone-marrow macrophages and in mice organs

To analyse the effects of disrupting *glg* genes on the virulence of *M. tuberculosis* H37Rv we used both BALB/c bone-marrow macrophages and mouse infection models. We first evaluated the possible influence of the α -glucan content on the uptake and intracellular growth of the H37Rv $\Delta glgA$ mutant and its parental strain in cultured mouse macrophages. Indeed, the α -glucan has been implicated in the CR3-mediated phagocytosis of *M. tuberculosis*, a route of entry that may be favourable to the intracellular survival of the tubercle bacillus (Ehlers and Daffé, 1998; Fenton *et al.*, 2005). However, the H37Rv $\Delta glgA$ mutant, which produces twofold less glucan than did the wild-type strain, did not show any defects in the examined parameters in both resting and activated mouse cells (Fig. S4). As α -glucan was also recently shown to impact on monocyte differentiation into dendritic cells, which then produce IL-10 but not IL-12 and fail to present lipid antigens to CD1-restricted T cells and to upregulate CD80 (Gagliardi *et al.*, 2007) it was important to determine the putative impact of the disruption of *glg* genes on the virulence of *M. tuberculosis* H37Rv in the context of the whole animal. Accordingly, the ability of the wild-type and H37Rv $\Delta glgA$, H37Rv $\Delta treZ$, H37Rv $\Delta glgC$ and H37Rv $\Delta Rv3032$ mutant strains to replicate and persist in the lungs and spleen of BALB/c mice was studied by colony-forming unit (cfu) analysis. As shown in Fig. 4, bacterial organ burdens in the mice infected with H37Rv $\Delta treZ$, H37Rv $\Delta glgC$ and H37Rv $\Delta Rv3032$ were comparable to those of mice infected with the parental H37Rv strain throughout a 90- to 100-day period. In contrast, the ability of H37Rv $\Delta glgA$ to persist in both organs during the late stage of infection was significantly reduced. At 42 and 90 days post infection, the lungs of mice infected with the H37Rv $\Delta glgA$ mutant carried about 10-fold less cfu than those of mice infected with the control *M. tuberculosis* H37Rv strain.

Discussion

The first purpose of the present study was to identify the

genes involved in the biosynthesis of the capsular glucan and intracellular glycogen of *M. tuberculosis*. We reasoned, based on the structures of the two polysaccharides, that their biosynthetic pathways were likely to be very similar to that established for bacterial glycogen in general. Accordingly, putative orthologues of the *glgA*, *glgB* and *glgC* genes of *E. coli* and *Bacillus subtilis* were identified in the genome of *M. tuberculosis* H37Rv. Inactivation of *glgC* (*Rv1213*) resulted in an important reduction in the production of both glucan and glycogen, whereas disruption of the putative α -1,4-glucosyltransferase genes *glgA* (*Rv1212c*) and *Rv3032* specifically affected glucan and glycogen synthesis, respectively. Given the very similar structures of glycogen and glucan, the molecular mechanisms underlying the preferred involvement of GlgA and Rv3032 in one or the other pathway are unknown. A possible mechanism may be related to the putative participation of the two enzymes in different macromolecular complexes, one being dedicated to the cytosolic biosynthesis of glycogen, the other – perhaps coupled to an export system – to that of glucan. Clearly, further studies will be required to verify this hypothesis. Our data indicate, however, that Rv3032 and GlgA can at least partially compensate for one another and that no other α -1,4-glucosyltransferase participates in the biosynthesis of glycogen and glucan in *M. tuberculosis*. This conclusion is based on the three observations that (i) both H37Rv $\Delta glgA$ and H37Rv $\Delta Rv3032$ still produced glycogen and glucan, (ii) the overexpression of *Rv3032* in H37Rv $\Delta glgA$ fully restored glucan synthesis, and (iii) at least one of the two α -1,4-glucosyltransferase genes needs to be functional for *M. tuberculosis* to sustain growth *in vitro*. A correlate of these results is that the production of glycogen, glucan and/or MGLP is an essential physiological requirement of the tubercle bacillus. In this context, it is interesting to note that while not all mycobacterial species display functional *glgA* and *Rv3032* genes, they all have retained at least one of the two enzymes. *Mycobacterium leprae*, for instance, which produces MGLP (Hunter *et al.*, 1986), carries an orthologue of *Rv3032* in its genome, but *glgA* is a pseudogene. Conversely, a frameshift mutation at the 5' end of the *Rv3032* gene of *M. smegmatis* causes this gene to be inactive (our unpublished results). *M. smegmatis* does, however, express a *glgA* gene that

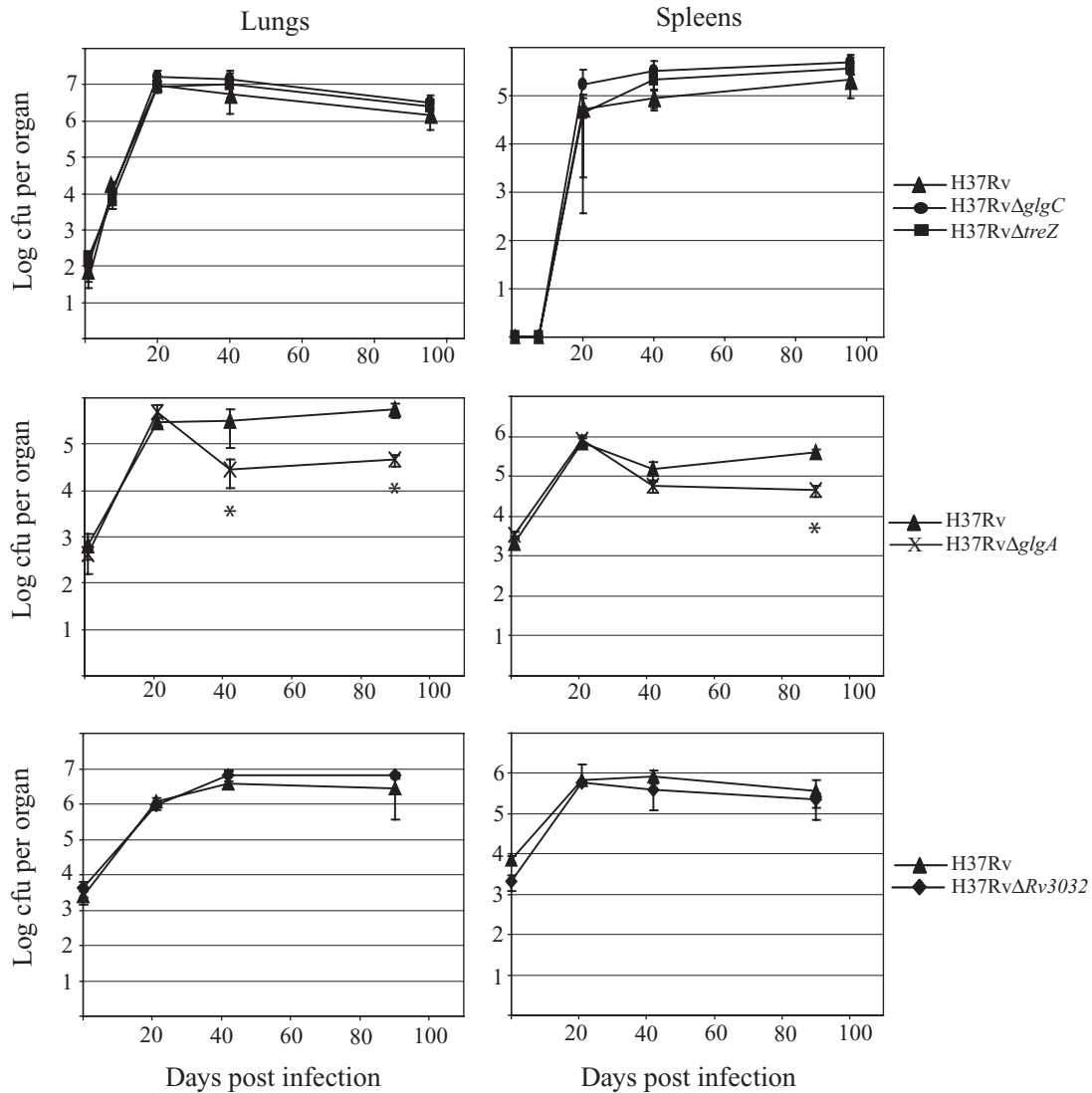


Fig. 4. Multiplication and persistence of wild-type H37Rv, H37RvΔtreZ, H37RvΔglgC, H37RvΔRv3032 and H37RvΔglgA in the lungs and spleen of BALB/c mice. Results are expressed as means ± standard deviations (error bars) of cfu counts for five infected mice. Mice were infected either intravenously (H37Rv, H37RvΔRv3032, H37RvΔglgA) or via the aerosol route (H37Rv, H37RvΔtreZ, H37RvΔglgC). Asterisks denote *P*-values < 0.05 (Student's *t*-test) for a comparison of H37Rv versus H37RvΔglgA.

most likely accounts for the production of MGLP in this species.

The fact that significant amounts of both glucan and glycogen were still produced in the ADPGlc pyrophosphorylase *glgC* mutant indicates that, unlike the situation in *E. coli* (Preiss and Romeo, 1989), the GlgC-dependent ADPGlc pathway is not the only route to glycogen/glucan synthesis in *M. tuberculosis* H37Rv. Other as yet unidentified enzymes with low sequence similarity to the usual prokaryotic ADPGlc pyrophosphorylases may exist in *M. tuberculosis* that compensate, at least partially, for the loss of GlgC in the mutant. Alternatively, one of the two α-1,4-glucosyltransferases, Rv3032 or GlgA, might utilize UDPGlc instead of ADPGlc as the sugar donor in the

elongation reactions. Providing support for this assumption, our preliminary studies on the substrate specificity of Rv3032 indicate that this enzyme has the ability to transfer Glc from UDPGlc onto short-chain gluco-oligosaccharides *in vitro* (G. Stadthagen and M. Jackson, unpubl. results). Finally, the presence of a putative amyloamylase gene (*Rv 1781c*, *malQ*) in the *M. tuberculosis* genome suggests that this bacterium might have the ability to synthesize α-1,4-glucans when grown on maltose or maltodextrin (Preiss and Romeo, 1989).

An important result of this study is the demonstrated essentiality of the branching enzyme GlgB. This result was unexpected as GlgB from *M. tuberculosis* is, to our knowledge, the first glycogen-branching enzyme reported

to be essential in a prokaryotic organism. The successful complementation of H37Rv Δ *glgB* with the *glgB* gene of *E. coli* proves that the GlgB proteins from both organisms have, *in vivo*, similar catalytic functions. This result is consistent with the recently demonstrated branching activity of GlgB from *M. tuberculosis* in an *in vitro* assay (Garg *et al.*, 2007). The reason(s) of the essentiality of this enzyme in the tubercle bacillus is (are) unknown. As GlgB seems to be the only branching enzyme committed in the synthesis of glucan and glycogen, its essentiality may be related to the physiological requirement of *M. tuberculosis* to produce at least one of these two polysaccharides as suggested above. By comparison with *glgB* mutants of *E. coli* (Lares *et al.*, 1974), one may also speculate that a deficiency in GlgB could lead to the accumulation of linear poorly water-soluble polymers of 4-linked α -glucosyl residues either in the cytosol or in the periplasmic space of the cell envelope (in the case of a putative precursor of the capsular α -glucan being exported) causing the death of the *Mycobacterium*. The latter mechanism would be similar to the one thought to account for the lethal character of the counterselectable marker *sacB* in Gram-negative bacteria, mycobacteria and corynebacteria, when sucrose is present in the culture medium (Reyrat *et al.*, 1998). The levansucrase SacB catalyses the formation of polymers of fructose from sucrose, whose accumulation in the periplasm is believed to lead to cell death.

The Rv3032 and *treZ* mutants were not affected in their multiplication or persistence in the BALB/c mouse model. The virulent phenotype of the latter mutant is consistent with the virulence data reported by Murphy and collaborators on a *treY* mutant of *M. tuberculosis* H37Rv affected in the same trehalose pathway (Murphy *et al.*, 2005). In contrast, the *glgA* mutant was significantly affected in its ability to persist in both the spleen and the lungs of mice. In view of the biochemical phenotype of this mutant and of the existence of two GlgA-independent pathways for the synthesis of trehalose in mycobacteria (de Smet *et al.*, 2000; Murphy *et al.*, 2005), we postulate that the virulence attenuation of the *glgA* mutant is due to a decrease in the production of capsular glucan, not in that of glycogen or trehalose. Two hypotheses may then account for the absence of an attenuation phenotype associated to H37Rv Δ *glgC* which displays *in vitro* a similar reduction in glucan content as H37Rv Δ *glgA*. First, an increase in UDPGlc production and UDPGlc-dependent activity of Rv3032 may compensate for the absence of (or decrease in) ADPGlc in the bacterial cell during host infection. Second, α -1,4-glucan synthesis from maltose or maltodextrin may be stimulated during the persistence stage of infection. Efforts are now ongoing in our laboratories to generate an *M. tuberculosis* mutant specifically deficient in the synthesis of the capsular glucan through the targeted inac-

tivation of the transport system(s) of this extracellular polysaccharide.

Experimental procedures

Bacterial strains and growth conditions

Escherichia coli XL1-blue, the strain used for cloning experiments, was propagated in Luria–Bertani (LB) broth (pH 7.5) (Becton Dickinson, Sparks, MD). *M. tuberculosis* H37Rv was grown at 37°C in Middlebrook 7H9 broth supplemented with 10% ADC enrichment (Difco) and 0.05% Tween 80, in minimal Sauton's medium as surface pellicles or on agar Middlebrook 7H11 medium supplemented with OADC (Difco). When required, antibiotics were added at the following concentrations: kanamycin, 20 μ g ml⁻¹; hygromycin B, 50 μ g ml⁻¹. For selection of allelic exchange mutants, 2% sucrose was added to the solid medium.

Construction of the *treZ*, *glgB*, *glgA*, *glgC* and *glgA*-Rv3032 mutants

The *ts/sacB* methodology (Pelicic *et al.*, 1997) was used to achieve allelic replacement at the *treZ* (Rv1562c), *glgC* (Rv1213) and Rv3032 loci of *M. tuberculosis* H37Rv. The *glgA* (Rv1212c) gene was disrupted using a two-step homologous recombination procedure (Jackson *et al.*, 2001). The essentiality of *glgB* (Rv1326c) was investigated following a standard strategy based on the construction of a merodiploid strain (Jackson *et al.*, 2001). Standard PCR strategies were used to amplify the *M. tuberculosis* H37Rv *treZ*, *glgB*, *glgA*, Rv3032 and *glgC* genes. *glgA* and its flanking regions were amplified using primers *glgA*.1 (5'-gcggaattccgctgcgattttcacgtgg-3') and *glgA*.2 (5'-gcggaattccggtggtcagaccatattcc-3'), and a disrupted allele of the *glgA* gene was constructed by cloning the kanamycin (Km) resistance cassette from pUC4K (Amersham Pharmacia Biotech) into the NcoI sites of *glgA*. Digestion by NcoI resulted in the deletion of 253 bp of the coding sequence of *glgA*. *glgA::Km* was then cloned into pJQ200-*xylE*, yielding pJQ*glgAKX*, the plasmid used for allelic replacement. The *glgB* gene and flanking regions were amplified using primers B130.2 (5'-gcatttgctctgaagaagctacg-3') and B130.4 (5'-ggggatattccacagcgcgaagtggcg-3'), and a disrupted allele of the *glgB* gene was constructed by cloning the Km cassette into the Sall sites of *glgB*. Sall-cut resulted in the deletion of 740 bp of the coding sequence of *glgB*. *glgB::Km* was then cloned with the *xylE* coloured marker (Pelicic *et al.*, 1997) into the BamHI site of pJQ200 to yield pJQ*glgBKX*, the construct used for allelic replacement at the *glgB* locus. The *glgC* gene and flanking regions were amplified using primers C364.1 (5'-cccgaattcggtgggatagaccgcaac-3') and C364.2 (5'-cccgaattcggtttcatcagcagttcg-3') and inserted into pGEM®-T easy (Promega), yielding pGEMT*glgC*. A disrupted allele of the *glgC* gene was then constructed by cloning the Km cassette into the BamHI site of *glgC*. *glgC::Km* was finally cloned with the *xylE* gene into the BamHI site of pPR27 (Pelicic *et al.*, 1997), to yield p27*glgCKX*. *treZ* was amplified using the couple of primers B48.2 (5'-gcttctggcgccgcataccatc-3') and B48.3 (5'-cccgaattcgccgctccgagcccgag-3') and a disrupted allele of the *treZ* gene was constructed by cloning the

Km cassette into the *XhoI* site of *treZ*. *treZ::Km* was then cloned with the *xylE* gene into the *BamHI* site of pPR27, yielding p27*treZKX*. The *Rv3032* gene and flanking regions were PCR-amplified from *M. tuberculosis* H37Rv genomic DNA using primers *Rv3032.1* (5'-gggctgcagatgcggcgcgctggcc-3') and *Rv3032.2* (5'-tgagccatgctgcctccctgg-3') and a disrupted allele, *Rv3032::Str*, was obtained by inserting a streptomycin resistance cassette into the *SmaI* restriction site of *Rv3032*. *Rv3032::Str* was then cloned into the *NotI*-cut and blunt-ended pPR27-*xylE* to obtain pPR27*Rv3032SX*, the construct used for allelic replacement in H37RvΔ*glgA*.

Complementation of the *glgA*, *glgB*, *glgC* and *treZ* mutants

Wild-type copies of the *M. tuberculosis* H37Rv *glgA*, *glgB*, *glgC* and *treZ* genes carried by the mycobacterial expression plasmid pVV16 (Jackson *et al.*, 2000), in pVV*glgA*, pVV*glgB*, pVV*glgC* and pVV*treZ*, respectively, were used for complementation studies. The primers used to amplify *glgC* were *glgC.1* (5'-gggctgcagccatgagagaagtgccgcac-3') and *glgC.2* (5'-cccaagcttgatccaaacaccttgcgccacg-3'). *glgZ.2* (5'-tggtgggcatatgcctgaattccagatgaggc3') and *glgZ.3* (5'-gggaagcttggcggctccgcagcccgcag-3') were used to amplify *treZ*. *glgA.5* (5'-ccgcgcatatgctgggtggcgatgtgactc-3') and *glgA.6* (5'-cccaagcttcgcgccacaccttccggtagatg-3') were used to amplify *glgA*. *glgB.1* (5'-ccccccccatagatgcgtagagaaactacc-3') and *glgB.2* (5'-gggaagcttggcggcgctcagccacagcg-3') were used to amplify *glgB*. In addition, the *glgB* gene from *E. coli* was PCR-amplified using the primers *glgBc.5* (5'-ggcgggcatatgtccgatcgtatcgatagagac-3') and *glgBc.6* (5'-cccaagcttttgcctcccgaaccagccag-3') and cloned into pVV16, yielding pVV*glgBcoli*. The primers were designed to generate PCR products corresponding to the entire genes devoid of their stop codon and harbouring *NdeI* or *PstI* and *HindIII* restriction sites (underlined in the primers' sequences), enabling direct cloning into pVV16. In this vector, genes are constitutively expressed under control of the *hsp60* transcription and translation signals and the recombinant proteins produced carry a six-histidine tag at their carboxyl-terminus. The production of recombinant proteins in the complemented mutants was analysed by immunoblotting with the monoclonal Penta-His antibody from Qiagen as described previously (Jackson *et al.*, 2000).

Analysis of glycogen and glucan

For the analysis of glycogen and α -D-glucan, *M. tuberculosis* strains were grown in Sauton's medium as surface pellicles. High-molecular-weight carbohydrates and proteins were precipitated from 10-fold concentrated culture filtrates with six volumes of cold ethanol overnight at 4°C. Precipitates were then recovered by centrifugation at 14 000 *g* for 1 h, dissolved in distilled water, dialysed for 1 day against water to eliminate traces of salts and glycerol, lyophilized and weighed. The extracellular α -D-glucan content was determined by GC of sugar derivatives resulting from the acid hydrolysis of the lyophilized macromolecules. To this end, 100 μ g of polysaccharides and 20 μ g of erythritol (used as an internal standard) were hydrolysed in 200 μ l of 2 M trifluoro-

acetic acid for 1 h at 110°C and then dried under nitrogen. The resulting free hydroxyl groups of liberated sugars were trimethylsilylated as described (Sweeley *et al.*, 1963), solubilized in petroleum ether and analysed by GC. Further purification of α -D-glucan was carried out by chromatography on a (7 × 1 cm) DEAE-trisacryl gel (IBF, Villeneuve-La-Garenne, France). The neutral fractions, eluted with 0.01 M NH₄Cl (pH = 8.3), were re-chromatographed on a Bio-Gel P-60 column (Bio-Rad) (90 × 1.8 cm) using 0.5% acetic acid as the eluent and refractive index detection. The purity of the excluded fractions was checked by ¹H-NMR analysis as described previously (Dinadayala *et al.*, 2004).

For the purification of glycogen, heat-killed cells were weighed, suspended in water and centrifuged twice at 1100 *g* for 15 min to remove loosely attached compounds among which the extracellular α -D-glucan. Washed cells were then broken in a French press at 140 bars to liberate the intracellular glycogen. The material was centrifuged at 1100 *g* for 30 min to eliminate non-broken cells and the supernatant was re-centrifuged at 27 000 *g* for 15 min to remove cell walls. Glycogen was extracted as described by Bueding and Orrell (1964) with only minor modifications. Briefly, one volume of supernatant was mixed with three volumes of 0.2 M glycine buffer (pH 10.5) and two volumes of chloroform at 4°C. The upper phase was removed and the lower phase was re-extracted three times with two volumes of glycine buffer. The aqueous phases were pooled, concentrated and centrifuged at 100 000 *g* for 4 h at 4°C. The gelatinous pellet was homogenized in 15 ml of water, shaken for 15 min with 5 ml of chloroform/1-octanol (3:1, v/v), and decanted. The upper glycogen phase was recovered and the treatment with chloroform/1-octanol was repeated for longer periods, i.e. 30 and 150 min, until the proteinaceous interphase disappeared. The aqueous phase was then precipitated with six volumes of cold ethanol overnight at 4°C, centrifuged for 1 h at 1500 *g*, lyophilized and weighed. The purification process was monitored by determining the carbohydrate content of the fractions and by ¹H-NMR analysis. The glycogen content was defined by the mass of glycogen versus mass of wet cells.

Samples of polysaccharides purified from the wild-type and mutant strains were comparatively *O*-methylated (Blakeney and Stone, 1985); portions of the per-*O*-methylated products were hydrolysed with 2 M CF₃COOH at 110°C for 2 h, reduced with NaBH₄ and acetylated. The different partially *O*-methylated and partially *O*-acetylated glucitols were identified by GC and GC-MS.

Samples of polysaccharides purified from the wild-type and mutant strains were comparatively hydrolysed with pullulanase and the resulting fragments were *O*-acetylated and analysed by MALDI-TOF mass spectrometry as described (Dinadayala *et al.*, 2004; 2008)

MALDI-TOF mass spectra (in the positive mode) were acquired on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a pulsed nitrogen laser emitting at 337 nm. Samples were analysed in the Reflector mode using an extraction delay time set at 100 ns and an accelerating voltage operating in positive ion mode of 20 kV. To improve the signal-to-noise ratio, 150 single shots were averaged for each mass spectrum and typically, four individual spectra were accumulated to gener-

ate a summed spectrum. Mass spectrum calibration was performed using the calibration mixture 1 of Sequazyme Peptide Mass Standards Kit (Perseptive Biosystems), including known peptide standards in a mass range from 900 to 1600 Da. Internal mass calibration was performed as previously described (Laval *et al.*, 2001). Stock solutions of per-acetylated oligosides were prepared in chloroform, at a concentration of 1 mM and were directly applied on the sample plate as 1 μ l of droplets, followed by the addition of 0.5 μ l of matrix solution [2,5-dihydroxybenzoic acid (10 mg ml⁻¹) in CHCl₃/CH₃OH (1:1, v/v)]. Samples were allowed to crystallize at room temperature.

The ¹H- and ¹³C-NMR analyses were performed on a Bruker AMX-500 spectrometer at 500.13 MHz and 125.77 MHz, respectively, using a 5 mm BBI probe, at 343°K in D₂O. A basic pulse programme was used for ¹H-NMR spectra, without pre-saturation of HOD signal, in order to preserve the integration of the signal at 5.00 p.p.m. A pulse of 45° was applied before a 2 s recycle delay. Free induction decay (FID) was recorded on 16 k points, and processed by a sine-bell apodization function before Fourier transformation and integration. Bruker DEPT (distortionless enhancement by polarization transfer) sequence was applied for ¹³C-NMR analyses.

Gas chromatography of monosaccharide derivatives was performed on a Hewlett-Packard 5890 series II apparatus equipped with an OV1 capillary column (0.30 mm \times 25 m) using helium gas. The temperature separation programme involved an increase from 100°C to 300°C at the rate of 5°C min⁻¹, followed by 10 min at 300°C.

GC-MS analyses were performed on a HP 5889X mass spectrometer (electron energy, 70 eV) coupled to a HP 5890 series II gas chromatograph fitted with a column identical to that used for GC. GC-MS analyses were performed in the electron impact mode.

Conformational studies by dynamic light scattering

The diffusion parameters of the purified polysaccharide solutions (1 mg ml⁻¹ in distilled water) were determined by dynamic light scattering (DLS). The apparatus consisted of a DynaPro-MS/X Dynamic light scattering instrument (Protein-Solutions, Charlottesville, VA), providing an 830 nm light beam. Samples were analysed in a 12 μ l quartz sample cell at 20°C. Using the Dynamic V6 software, DLS autocorrelation function were collected at a scattering angle of 90° with a data acquisition time of 200 s.

M. tuberculosis growth and persistence in mice

Female BALB/c mice (6–8 weeks old) purchased from CER-Janvier (Le Genest St Isle, France) were infected through the aerosol route with approximately 100 cfu of wild-type *M. tuberculosis* H37Rv or the *treZ* and *glgC* mutant strains. *M. tuberculosis* aerosols were generated from bacterial suspensions consisting of 5 \times 10⁶ cfu ml⁻¹ in phosphate-buffered saline. Mice were exposed to the aerosols for 15 min. Mice were infected intravenously with 10⁵ cfu of wild-type *M. tuberculosis* or the *glgA* and *Rv3032* mutants as described (Jackson *et al.*, 1999). Five mice were used per experimental point and per strain.

Infection of BALB/c bone marrow-derived macrophages with *M. tuberculosis*

BALB/c bone-marrow macrophages were prepared, activated or not, and infected at a multiplicity of infection of one bacillus per macrophage as described (Rousseau *et al.*, 2004).

Statistics

Statistical significance was determined using paired Student's *t*-tests.

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