Glycogen as Key Energy Storehouse and Possibly Responsible for Multidrug Resistance in *Mycobacterium tuberculosis*

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Abstract   Tuberculosis (TB) is a major public health problem with a high mortality rate worldwide due to *Mycobacterium tuberculosis* (*M. tuberculosis*) pathogen, claiming 9.6 million total cases were estimated in 2014 and more than 1.5 million people dead. *M. tuberculosis* and other pathogenic mycobacterial species produce a variety of glycogen or glycogen-associated molecules like lipoarabinomannan (LAM), trehalose monomycolate (TMM), phenolic glycolipids (PGLs), trehalose dimycolate (TDM), phosphatidylinositol-containing mannosides (PIMs), etc., that represent as major glycans present in the outermost layer of *M. tuberculosis*. The *M. tuberculosis* accumulate glycogen during harsh environmental condition, *i.e.* presence of reactive oxygen and nitrogen intermediates, limited nutrients availability and depletion of other essential elements required for their survival within the host. The glycosyltransferases (GTs) enzyme involves two families, glycogen transferase-3 (eukaryotes) and *GTs*-5 (eubacterial and archaeal), that play a major role in the regulation of glycogen metabolism. In bacteria, regulation of glycogen anabolism involves several glycogen synthase enzymes, *i.e.* α-D-glycogen synthase A (glgA), 1,4-α-D-glucan 6-glucosyltransferase (glgB) and glucose-1-phosphate adenylyltransferase (glgC), while catabolism involves glycogen phosphorylase (glgP) enzyme. In recent years, role of glycogen was investigated enormously in the pathogenesis of *M. tuberculosis*. Two major glycogen conjugates present in the cell wall of *M. tuberculosis* are TDM and TMM. These conjugates serve as precursors for the synthesis of mycolic acid that plays a key role in the invasion and pathogenesis of *M. tuberculosis*. This chapter summarizes the current updates of the presence of glycogen/glycoconjugates and their physiological role in the survival and pathogenesis mechanisms of *M. tuberculosis* during antagonistic conditions. Also, the chapter summarizes evidence of the putative GTs in the *Mycobacterium* spp.

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Abbreviations

ACP reductase	Enoyl-acyl carrier protein reductase
ADP	Adenosine diphosphate
AM	Arabinomannan
CR-3	Complement receptor 3
G1P	Glucose-1-phosphate
G6P	Glucose-6-phosphate
glgA	α-D-Glycogen synthase A
glgB	1,4-α-D-Glucan 6-glucosyltransferase
glgC	Glucose-1-phosphate adenylyltransferase
glgP	Glycogen phosphorylase
glgX	Glucan hydrolase
GS	Glycogen synthase
GTB	Glycosyltransferase B
GTs	Glycosyltransferases
KGD	α-Ketoglutarate decarboxylase
LAM	Lipoarabinomannan
LPS	Lipopolysaccharide
M. tuberculosis	*Mycobacterium tuberculosis*
MGLP	6-O-Methylglucosyl-containing lipopolysaccharides
NPP	Nucleotide pyrophosphate
ODHc	2-Oxoglutarate dehydrogenase complex
PAMP	Pathogen-associated molecular patterns
PGLs	Phenolic glycolipids
PGM	Phosphoglucomutase
PI	Phosphatidyl-myo-inositol
PIMs	Phosphatidylinositol-containing mannosides
STRE	*Cis*-element stress response element
TB	Tuberculosis
TLR2	Toll-like receptor
TMM	Trehalose monomycolate
UDP	Uridine diphosphate

1 Introduction

All living things store glucose (energy source) as glycogen (kingdom Monera, Protista, Fungi and Animalia) or starch (kingdom Plantae, some fungi and protists) (Roach et al. 1998; Gupta et al. 2014; Asención-Diez et al. 2015). Glycogen, a polysaccharide present in the cytosol of the cell, is principally used by bacteria, fungi or animals, while starch is synthesized and stored in plastids by plants, some protists or planktons (Calder 1991; François and Parrou 2001; Ball and Morell 2003; Preiss 2006). Glycogen has similar structure to amylopectin and is mostly

branched but more compact than starch. Glycogen is present in the cytosol/cytoplasm of cells in granular form and plays a significant role in the glucose metabolic cycle. Glycogen acts as energy reserve that can be quickly mobilized to overcome the quick need of glucose, because glycogen is less compact than other energy reserves such as triglycerides (lipids).

In humans, glycogen is produced and stored primarily in the liver and muscle cells in the hydrated form with three or four parts of water (Preiss 2006). The glycogen is stored as the chief energy store in the liver adipose tissues. In the liver cells (known as hepatocytes), glycogen can comprise up to 8 % of its dry weight (100–120 g in an adult) soon after a meal (Campbell et al. 2006), whereas only small amount (1–2 %) is present in the muscle cells. The common glycogen storage organs/tissues other than the liver and muscles are red blood cell (Moses et al. 1972; Ingermann and Virgin 1987). Nevertheless, in mammals, glucose uptake and its utilization is well regulated. Any error in the glucose regulation pathways results in the induction of different glycogen storage diseases (Buschiazzo et al. 2004).

The presence of glycogen molecules/granules in the cytoplasm has been described in more than 40 species of bacteria (Preiss and Romeo 1994). Both sugar molecules (glycogen and starch) are compact glucose polymers and act as reservoir of spontaneously accessible carbon and energy source in diverse organisms representing archaea, eubacteria, yeasts, plants and animals (Henrissat et al. 2002). In bacteria, it is usually synthesized when growth is limited by the nitrogen source, and the presence of excess amount of carbon source in in-vitro conditions. Hence, accumulation of glycogen supports the survival of bacteria under nutritional stresses. The inverse relation is observed between growth rate and the amount of the glycogen accumulation during the nitrogen restrictions. Its accumulation is quite rapid just prior to sporulation initiation in *Bacillus cereus* and exopolysaccharide production in all Corynebacteriaceae family members such as *Corynebacterium diphtheriae* and *M. tuberculosis* (Schwebach et al. 2002).

M. tuberculosis is an aerobic, gram positive and acid-fast bacillus that causes TB in humans. It accumulates glycogen during the unreceptive conditions—accumulation of reactive oxygen and intermediates of nitrogen, depletion of nutrients, low pH and during starvation for their survival within the host (Antoine and Tepper 1969). However, the accumulation of glycogen does not happen during the exponential growth of *M. tuberculosis* under laboratory conditions, but its presence may enhance the sustainability of *M. tuberculosis* during these hostile conditions. The role of glycogen in the pathogenic behaviour of *M. tuberculosis* has been reported by Pal et al. (2010). It was validated that if the bacteria are physically inactivated for long period of time, their stored sugar fulfils all energy needs of bacterium and becomes very important molecules for its survival. Various research groups have reported that glycogen or its intermediates may regulate biological pathways by binding glycogen molecules to their respective proteins/enzymes and that of lipids during the process of post-translational modifications. Quite a few uncharacterized glycosyltransferase (GTs) of *M. tuberculosis* are of major interest and will be discussed in detail in following prokaryotes.

The chapter presents the current status of the information about the various enzymatic processes leading to bacterial glycogen synthesis or degradation pathways that play critical role in the survival and possible drug resistance mechanisms in *M. tuberculosis*. Also, the chapter summarizes evidences of the putative GTs in the *Mycobacterium* spp.

2   Existence and Structure of Glycogen

The storage materials are usually accumulated as granular form in the cytosol of the bacteria. This accumulation conventionally takes place in response to depletion of nutrients or in the presence of excess of the relevant harmful substrates. These granules are superficially dispersed within the cytoplasm of bacteria. The size of granules is 20–100 nm in diameter and exists in the form of non-membrane-enclosed inclusions. However variations in the shape and size of the granules have been reported (Preiss 2006). In Cyanobacteria, glycogen stores may occur in the form of crystals, spherical or in rod forms. Glycogen stores can also be found in membrane-enclosed polyglucose inclusion bodies in several strains of Clostridia (Preiss 2006) with granule sizes ranging from 160 to 300 nm in diameter.

Glycogen accumulation is generally initiated in the stationary phase (Wilson et al. 2010). In a large number of bacteria, glycogen accumulates, while bacterial growth reaches to stationary phase due to the limitations of essential nutrients such as phosphorus, nitrogen or sulphur. Glycogen structure resembles that of amylopectin (a component of starch) with alpha glycosidic linkages. However, it is even more branched and has extra glucose units than amylopectin (Ball and Morell 2003). The complex molecules—glycogen and amylopectin—comprise of α-1-4-linked D-glucose unit with α-1-6-branching point. Branches are covalently linked to the main chain from which they are branching off by the glycosidic bonds. The glycosidic bond is formed between the first glucose (C-1) of the new branch and sixth glucose (C-6) on the stem chain. The branching takes place at every interval of 8–10 units of glucose, whereas in amylopectin 12–20 glucose units segregate the branches.

The variation in the size of branches (length and number) depends on the organism and size of the glycogen granules (Belanger and Hatfull 1999; Cid et al. 2002). In *Mycobacterium smegmatis* (*M. smegmatis*), the degree of branching is less and the rate of sedimentation coefficient is more due to bigger size of glycogen granules. *Mycobacterium phlei* (*M. phlei*) possesses a glycogen molecular weight 1.2×10^8 (Antoine and Tepper 1969) in comparison with 8.2×10^7 of *Escherichia coli* (*E. coli*) (Preiss and Romeo 1994) (Fig. 1). The branches of glycogen are extremely important to the quick response to metabolic needs, because the biosynthesis and degradation of glycogen molecules only happen from the non-reducing ends of amylose chain. Therefore, extremely branched glycogen has higher number of reducing ends per molecules of glycogen, which generate more glucose
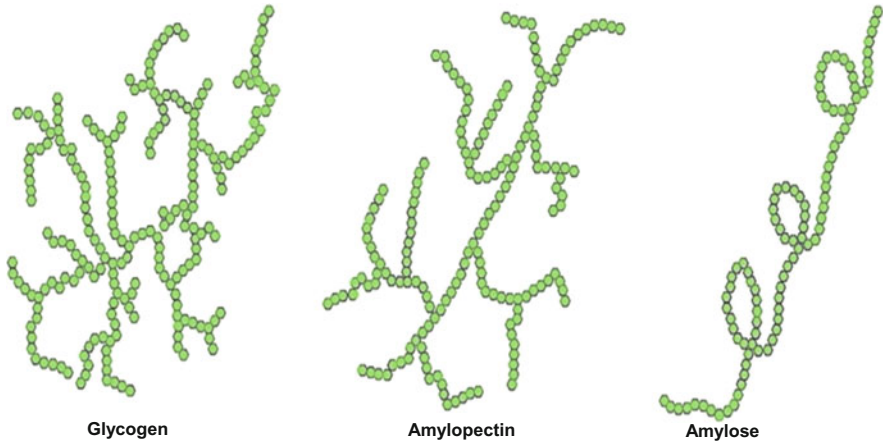
Fig. 1 Branched structure of glycogen and its component showing the helical structure known as amylose (α-1,4 linkage) and branched amylopectin (α-1,6-glucosyl linkages) structure

molecules at single time. Branches also increase solubility of glycogen in the water (Zmasek and Godzik 2014).

3 Glycogen: As Energy Storehouse

Many bacteria accumulate glucose in the form of glycogen, a polysaccharide comprising the glucose connected with α-1,4 glycosidic linkage and α-1,6 linked branched oligosaccharide chains with molecular weight of about 107–108 kDa. It acts as chief carbon source and provides energy to the organisms. Wilson et al. (2010) expressed that the compounds with energy-storage functions should fulfil three criteria:

1. Be accumulated intracellularly, in the presence of excess energy supplements.
2. Be consumed when the limited external carbon supplies are present for the growth maintenance or related processes necessary for the cell growth. The storage compound should be degradable to the energy for consumption by the cell for their survival or respond well to the environment.

Since glycogen meets all these criteria, it is one of the best storage materials in living things.

4   Biological Functions of Glycogen

It has been observed that glycogen-rich cells of *E. coli*, *Streptococcus mitis* (*S. mitis*) and *Enterobacter aerogenes* (*E. aerogenes*) present in media without exogenous or other carbon sources displays prolonged viability in comparison with glycogen-deficient strains during starvation. Under these conditions, glycogens containing *E. coli* and *E. aerogenes* do not degrade their protein components and nucleic acid to ammonia, while cells without glycogen rapidly degrade their proteins and their nucleic acid. Therefore, the presence of glycogen may protect cellular components in the stationary phase. However, survival rates of glycogen-rich and glycogen-deficient cells, in 0.5–1.0 mM magnesium chloride ($MgCl_2$) containing media, are similar. The possible reason behind this action could be that $MgCl_2$ is known to enhance the strength of the ribosomal constituents in the cell, resulting in the reduction of turnover rates of RNA and protein. There is a high probability that glycogen may prove effective in preserving and conserving the intracellular Mg^{2+} concentration. In Clostridia, up to 60 % of the cell dry mass may be accumulated as glycogen, prior to the beginning of sporulation (Shively 1974). This polysaccharide degrades rapidly during spore formation which suggests that glycogen serves as a solitary carbon and energy source for the development of spores and their maturation. Similarly, in sporulating hyphae of *Streptomyces viridochromeogenes* (*S. viridochromeogenes*), glycogen granules reach a maximal number in the beginning of maturation. In later stages of maturation, granules decrease in the fungal hyphae and accumulate in mature spores. Glycogen accumulation also occurs in *Bacillus cereus* (*B. cereus*) during early stage of sporulation and degrades during its maturation (Slock and Stahly 1974). Hence, it has been presumed in the sporulating microorganisms that glycogen acts as carbon and energy source. Several investigators suggested that glycogen helps bacteria to survive during starvation. However, some other observations are contrary to the concept of glycogen being an energy-storage house. As revealed earlier, the presence of higher amount of magnesium enhances survival rates of *E. aerogenes* and *E. coli* cells and is independent of the presence of glycogen (Shively 1974), while *Sarcina lutea* (*S. lutea*) dies at a faster rate in glycogen-rich conditions during starvation in the phosphate media (Rose and Tempest 1989). More efforts are needed to elucidate the role of glycogen in bacteria.

5   Glycosyltransferases: An Enzyme Vital for Glycogen Synthesis

Glycogen biosynthesis is facilitated by the action of GT enzymes (Rini et al. 2009). A large number of enzymes grouped in GTs are involved in the biosynthesis of oligosaccharides, polysaccharides and other glycogen conjugates (Pederson et al. 2000; Pedersen et al. 2003). The GTs have immense diversity but mediate a

comprehensive variety of functions—structural and storage— important for molecular signalling. These enzymes are widely present in prokaryotic organisms and in eukaryotic organisms also, but illustrate enormous specificity for the glycosyl acceptor and donor molecules. In eukaryotes, many structural oligosaccharides are produced during the glycosylation process in Golgi apparatus (Breton et al. 2006; Possner et al. 2015). Prokaryotes produce different glycol conjugates and other polysaccharides, which vary in structures and complexity. In *E. coli*, glucose-1-phosphate adenylyltransferase (*glgC*), *alpha-D-glycogen synthase (glgA)* and 1,4-α-D-glucan 6-glucosyltransferase (*glgB*) genes encode for enzymes responsible for its synthesis, while glycogen phosphorylase (*glgP*) and glucan hydrolase (*glgX*) genes encode for its degradation. *glgC* gene is responsible for creation of charged glucose nucleotide pyrophosphate (NPP) and *glgA* gene for linear glucan chain. Glycogen branching enzyme (glgB), transfers 6–7 glucose units from the hydroxyl group of carbon number 6 of the non-reducing end, either on the similar or adjacent chains. GlgB in bacteria (Seibold et al. 2011) and fungi is responsible for glycogen branches. Additionally, bacterial glycans that include numerous unusual sugars such as Kdo, heptoses and modified hexoses (absent in vertebrates) take part in the pathogenesis of bacteria. Also, some other molecules, *i.e.* lipids coupled with glucose/mannose or a precursor of dolichol oligosaccharide, are responsible for the peptidoglycan, lipopolysaccharide (LPS) and capsules assembly (Rini et al. 2009).

6 Functional Classification of Glycosyltransferase Enzymes

According to nucleotide sequence and structural comparisons, glycogen synthase (GS) enzymes have been categorized as glycosyltransferase B (GTB). The structural characteristics of GS include two Rossmann fold domains among the catalytic and substrate-binding sites. Further, enzymes of GTB have been subclassified into GT3 and GT5 families (Fig. 2). The GT5 family consists of both eubacterial and archaeal GS enzymes, and GT3 family consists of eukaryotic enzymes and is regulated by the inhibitory phosphorylation and allosteric activator (G6P) (Unligil and Rini 2000).

The major difference between prokaryotic and eukaryotic enzymes is the use of adenosine diphosphate (ADP) glucose in bacteria and uridine diphosphate (UDP)-glucose in the eukaryotes. Apart from that, archaeal enzymes are proficient in utilizing ADP and UDP-glucose as substrates. Three-dimensional structures of the GT5 family members (three) have been determined in *E. coli* as monomeric, *Agrobacterium tumefaciens* (*A. tumefaciens*) as dimeric and in *Pyrococcus abyssi* (*P. abyssi)* as trimeric enzymes. This information is not proving very effective on elucidating the regulatory mechanisms in eukaryotes.
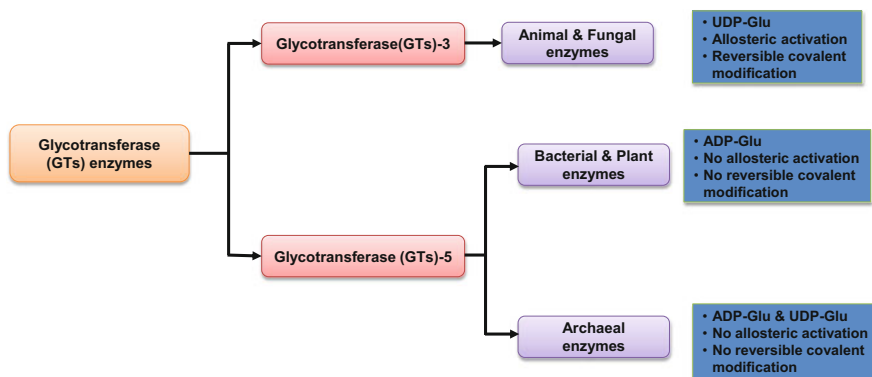
Fig. 2 Classification of prokaryotic and eukaryotic glycotransferase (GTs) enzymes, based on sugar donor molecule

7 Mechanisms of Action of Glycotransferase Enzyme

The mode of action of GT enzymes depends upon the activated donor, like NPP sugar, nucleoside monophosphosugar or lipid phosphor sugar and hydroxyl group of amino acid that acts as acceptor molecules. Monosaccharide component of activated nucleotide sugar is transferred to the glycosyl and forms glycosidic bonds. Inversion of GTs is supposed to resemble the inverting glycosyl hydrolase enzymes with an acidic amino acid that is responsible for the activation of the acceptor OH group by the deprotonation (Breton et al. 2006).

8 Synthesis and Regulation of Glycogen in Eukaryotes

In higher eukaryotic organisms, glycogen is synthesized when the nutrients are non-unlimiting. In eukaryotes, skeletal muscles, the liver, and red blood cells (RBCs) to some extent store glycogen. Several other organs like brain, adipose tissue, pancreas and kidney also synthesize minute amount of glycogen. In the skeletal muscles, glycogen is converted into glucose-6-phosphate (G6P) and enters into glycolysis cycle for the generation of ATP molecules, which are chiefly consumed as energy source for muscular contraction. Liver glycogen plays significant role in glucose homeostasis during fasting. Genetic or functional changes in the enzymes responsible for metabolizing glycogen result in the development of various glycogen storage diseases, affecting the liver, muscle, etc., and may be life-threatening (Gupta et al. 2014).

Yeast (*Saccharomyces cerevisiae*) is an unicellular eukaryotic microorganism, which is commonly present in the sugar-rich ingredients such as fruits, berries, plant exudates, etc. Yeast glycogen has structural similarity to other eukaryotic organisms (Wilson et al. 2010). Yeast glycogen is one of the chief reservoirs of

carbohydrate like several bacteria and it covers almost 20 % of the yeast cell mass. The quantity of glycogen accumulation increases exponentially in the stationary phase or in enervation of vital nutrients like nitrogen and phosphorus. Moreover, several studies supported that glycogen accumulation occurs in exponentially growing yeast when exposed to high temperature, salt, ethanol or oxidizing agents. Here, the yeast uses stored glycogen for survival (Silljé et al. 1999; Baskaran et al. 2010). The regulation of glycogen is done by various enzymes such as glycogen synthase (Farkas et al. 1991; Baskaran et al. 2010), glycogenin (Cheng et al. 1995), branching and de-branching enzymes (Wilson et al. 2010) and numerous other mechanisms including covalent modification (Lin et al. 1995), functioning of allosteric activators and translocation inside the cells. The regulation of GTs occurs via phosphorylation (Ramaswamy et al. 1998) and allosteric activation by G6P. However, the biological processes that inhibit these regulatory controls vary from tissue to tissue of the same or different organisms. Yeast has two isoforms of GS, which are designated as GSY-1 and GSY-2, from which the nutritionally regulated isoform-2 (GSY-2) is an utmost essential enzyme for glycogen accumulation in the cells. Predominantly, transcriptional and enzymatic mechanisms are involved in regulating glycogen metabolism. The transcription-mediated regulation is typically dependent on the promoter region of the genes, *i.e.* *cis*-element stress response element (STRE), while enzyme-mediated regulation of glycogen accumulation is completed by activation of the GS via G6P and inactivation of GPH via phosphorylation. The introduction of nutrients to the starved cells stimulates GPH, resulting in inhibition of GS and vice-versa.

9   Synthesis and Regulation of Glycogen in Prokaryotes

Biosynthesis and degradation processes of glycogen is highly conserved in prokaryotes (Ballicora et al. 2003; Preiss 2006). The enzymatic action of carbohydrate phosphotransferase system (PTS) takes up the extracellular glucose and converts it into G6P. Further G6P is then transformed into G1P via phosphoglucomutase (PGM) enzyme and in the end into ADP glucose (ADPG) in the presence of ATP molecules and Mg^{2+} (Ballicora et al. 2003). glgA utilizes ADPG as sugar donor nucleotide and produces linear glucose chain (amylose). Thereafter, development of branched oligosaccharide chain is initiated by the action of glgB by the formation of α-1,6-glucosidic linkages (Preiss 2006). Based on the genetic evidences of glycogen synthesis, it has been proposed that glgC is the solitary enzyme for generating ADPG (Leung et al. 1986; Ballicora et al. 2003).

   The glycogen metabolism regulation has been extensively studied in *E. coli*. The regulation of glycogen synthesis and degradation contain a complex set of factors, which adjust the biological and energy level of the cell (Alonso-Casajús et al. 2006; Montero et al. 2009), expression of analogous genes and communication between cells (Morán-Zorzano et al. 2008). At genomic level, numerous factors control glycogen accumulation in the bacteria such as allosteric regulation, etc. (Deutscher

et al. 2006; Preiss 2006). The higher level of glgC protein indicate the presence of higher amount of carbon and energy contents, while the existence of higher amount of inhibitors in growth medium may represent low metabolic energy levels inside the cells. The allosteric control of glgC has been broadly reviewed recently, which included structural and functional connections between glgA, glgB and glgC (Ballicora et al. 2003; Preiss 2006).

The glgP takes part in glycogen degradation pathway. The enzyme eliminates glucose monomers from the non-reducing ends (Dauvillée et al. 2005; Alonso-Casajús et al. 2006). These glucose-6-phosphate molecules enters in the glycolysis cycle. Through surface plasmon resonance (SPR) ligand fishing analysis (technique used for the detection and characterization of molecular interactions between interactive partners), it has been observed that glgP shows the specific interaction between glgP and HPr (Deutscher et al. 2006). Once HPr is wholly phosphorylated, it reduces the activity of glgP enzyme in log phase of bacteria and vice-versa. At this stage, binding of glgP to HPr is maximal. It has been projected that glgP activity is controlled by the phosphorylation level of HPr, which allows the glycogen to accumulate at the beginning of the stationary phase especially under situations where glucose is in excess (Seok et al. 2001; Deutscher et al. 2006).

10 Glycogen Metabolism in *Mycobacterium tuberculosis*

The biosynthesis of glycogen is an endergonic reaction involving monomers of uridine diphosphate (UDP)-glucose. The biosynthesis and degradation of glycogen pathways in *M. tuberculosis* are similar to *E. coli* that is widely studied. Three genes reported as *glgA*, *glgB* and *glgC* encode glycogen biosynthesis enzymes, and other two genes *glgX* and *glgP* encode enzymes for glycogen degradation (Dauvillée et al. 2005; Bourassa and Camilli 2009). It is assumed that *M. tuberculosis* synthesizes glycogen through glgC-glgA pathway as shown in Fig. 3. The nucleotide diphosphoglucose pyrophosphorylase (glgC) utilizes G1P phosphate and generates activated glucose nucleotide diphosphate which is followed by generation of linear glucans by the action of glgA enzyme (Ball and Morell 2003; Chandra et al. 2011). After that, glgB enzyme transforms linear glucan into glycogen via addition of oligoglucan to the non-reducing end of the residual chain (at position 6) for the elongation of side chains (Palomo et al. 2009; Chandra et al. 2011). The regulation of gene expressions of *glgA* and *glgC* happens in bacteria via intracellular signals, which indicates the energy status of the cell (Fig. 4) (McMeechan et al. 2005). Any defect, or mutations in the *glgC* gene, prevents the synthesis of glycogen (Preiss and Romeo 1994). Few scientific reports are available in the database, which suggest that *glgC*-deleted mutant strains could be able to synthesize small amount of glycogen during the growing stage of bacteria under specific conditions (Leung et al. 1986; Bourassa and Camilli 2009). Another enzyme glgS is also involved in the glycogen synthesis pathway; however its role is still not clear. Recent studies suggest that it could play significant role in glycogen accumulation in *E. coli*
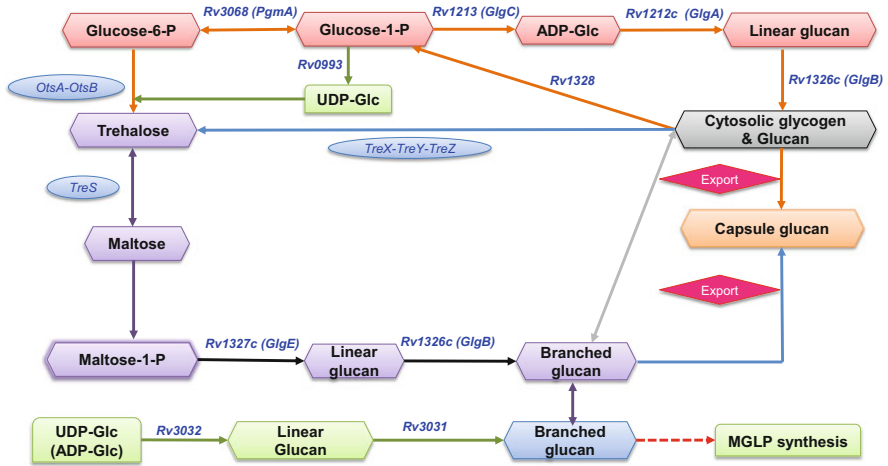
Fig. 3 Mycobacterial glucan pathways. In classical glycogen pathways *Rv1213* (*GlgC*) and *Rv1212c* (*GlgA*) are in central. The new Rv3032 pathway is connected to the MGLP. The recently identified Rv1327c (GlgE) pathway (*purple*) may contribute to capsular glucan, cytosolic glycogen and/or MGLP (Gupta et al. 2014)
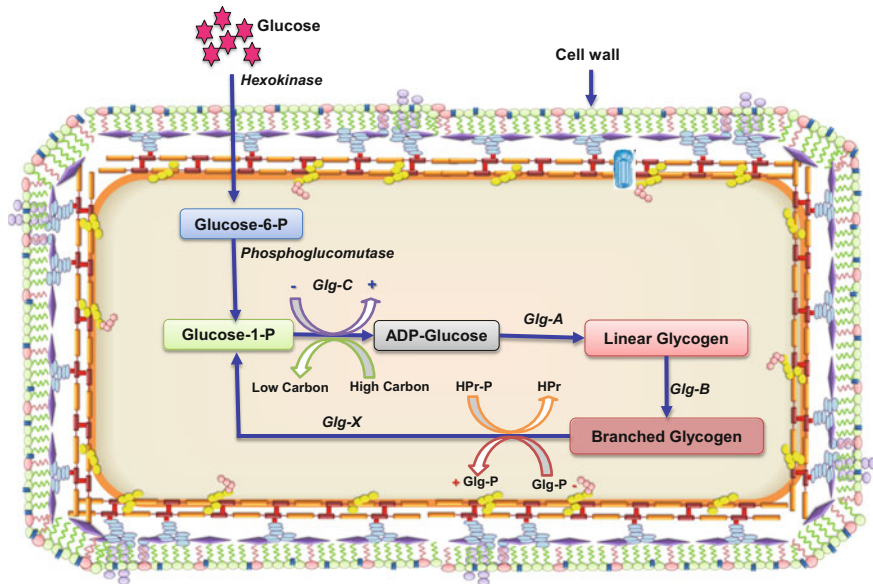

Fig. 4 Regulatory pathway of glycogen synthesis in *Mycobacterium tuberculosis*

(Hengge-Aronis and Fischer 1992; Morán-Zorzano et al. 2007; Bourassa and Camilli 2009).

The glycogen degradation in *M. tuberculosis* is mediated by the joint action of two enzymes, glgP and glgX, and this degradation produces G1P, which is consequently consumed by the bacteria (Chandra et al. 2011). The glgP removes glucose units serially and glgX eliminates α-1,6 linkages of glycogen through hydrolysis (Dauvillée et al. 2005). Both glycogen-degrading enzymes glgX and glgP regulate energy requirement of cells by glycogen degradation process (Fig. 4). Bourassa and Camilli (2009) reported that deletion of either glgX or glgP or both genes make the bacteria unable to degrade glycogen internally.

11 Possible Role of Glycogen in *Mycobacterium tuberculosis* Drug Resistance Development

The emergence of drug-resistant tuberculosis has increased significantly during the past decade in several countries. Generation of resistance to individual antitubercular drug occures after mutations in the concerned genes or chromosomal genes, resulting overproduction of particular gene product (protein), which alter the drug target against existing drugs. The multidrug-resistant (MDR) strains of *M. tuberculosis* display resistance to isoniazid and rifampicin anti-tuberculosis drugs. Rifampicin is a bactericidal antibiotic, which inhibits bacterial RNA synthesis by obstructing bacterial DNA-dependent RNA polymerase, hence blocking of RNA transcription. The mechanism of the generation of drug resistance against rifampicin has been determined and found mutation in the ß subunit of *rpoB* gene of RNA polymerase (Kumar and Jena 2014). The mutations have been detected in the 81-bp region (codons 507–533) of the *rpoB* gene, and codons 516, 526 and 531 are the most dominant mutations in the *rpoB* subunit (Koch et al. 2014; Singh et al. 2014).

Isoniazid is a prodrug activated by the action of catalase/peroxidase enzyme that is encoded by *katG* gene. The activated isoniazid inhibits mycolic acid synthesis through the action of *inhA*-encoded enzyme NADH-dependent enoyl-acyl carrier protein (ACP) reductase (Palomino and Martin 2014). Several mutations have been detected in the *katG*, *inhA*, *ahpC*, *oxyR* and *kasA* genes, which take part in the development of drug resistance against isoniazid. The detailed mechanism of isoniazid resistance is shown in Fig. 5.

The mycobacterial cell wall accounts around 2–3 % of bacterial dry mass and are generally of polysaccharide and proteins (94–99 %). The structure of *M. tuberculosis* cell wall has been represented as pathogen-associated molecular patterns (PAMP), which includes glycolipids, LAM, lipopeptides, etc. The arrangement of triglycerides and glycolipids on the outer surface of the mycobacterial cell wall protects the bacilli against degradation by host enzymes, impenetrability to
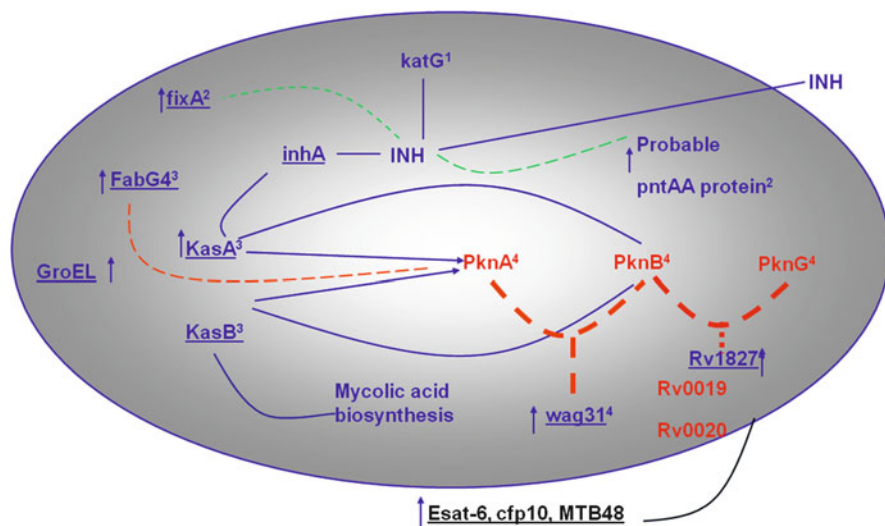
Fig. 5 Schematic illustration of proposed isoniazid drug resistance mechanism of *Mycobacterium tuberculosis*. Alternate mechanism of activation of INH (*dashed green lines*), normal mechanism of activation of INH (*blue lines*) and possible mechanism involved in drug resistance (*dashed red lines*). [1]KatG (encoding catalase and peroxidase) gene harbouring mutation resulting in compromised activation of prodrug molecule of INH into active form. The expression of superoxide dismutase may activate INH to overcome the oxidative stress caused by the INH action. [2]fixA gene is reported to be involved in INH activation in a cell-harbouring *KatG* gene mutation. Probable pntAA protein and PEP kinases are stress-related protein, and overexpression of pntAA protein may favour the INH resistance. [3]The kasA, kasB and fabG4 are primarily involved in fatty acid metabolism, which are known primary target of INH. The overexpression of these proteins supports that INH mechanism of action. [4]wag31 (cell division protein) and Rv1827 (FHA domain-containing protein associated with exponential phase growth and glycogen accumulation) were overexpressed while acquiring resistance to INH and RIF. Both these proteins regulate cell growth, size and morphology through signal transduction pathway primarily regulated by serine/threonine protein kinases. Only three (PknA, PknB and PknG) out of 11 STPKs in *M. tuberculosis* are essential for sustained growth. Rv1827 protein having a unique phosphorylation site for PknB and PknG and playing a regulatory role in glycogen metabolism (Unpublished data from Amit Singh, Krishnamoorthy Gopinath and Sarman Singh)

poisonous macromolecules (antibiotics), inactivation of reactive oxygen and nitrogen derivatives (Korf et al. 2005).

Trehalose, a disaccharide typically found in mycobacteria, acts as storage compound and is used as energy reservoir, and a stress protectant such as survival under desiccation, cold, osmotic and other traumatic situations (Argüelles 2000; Bolat 2008) consists of α-1-1 linkage of di-glucose. In *M. tuberculosis*, trehalose is synthesized from all three pathways; these are using TreY-TreZ, TreS, (De Smet et al. 2000) and GalU-OtsA-OtsB pathway (Pan et al. 2004). It serves as the precursor for the synthesis of mycolyl acetyl trehalose (also known as cord factor or mycolic acid) (Gibson et al. 2002; Chen and Haddad 2004; Takayama et al. 2005). The combined association of mycolic acid and peptidoglycan has

established the core of the cell wall (Crick et al. 2001). The peptidoglycan layer is interconnected by a variety of glycolipids, *i.e.* TMM, LAM, PGLs, PIMs and TDM, which increase the rigidity of *M. tuberculosis* cell wall. Hence, this complexity of the *M. tuberculosis* cell wall restricts the entry of drug within the cell and prompt phagocytosis (Elbein et al. 2003; Carroll et al. 2007).

Antoine and Tepper (1969) demonstrated that if the concentration of nitrogen/ sulphur contents is dropped in medium of *M. phlei* and *M. tuberculosis*, the accumulation of glycogen and lipids increased significantly. In the deficiency of exogenous carbon, these substrates or accumulated glycans are consumed by the bacteria and sustain their growth. Besides energy sources, glycogen also prevents *M. tuberculosis* phagocytosis by macrophages and also participates in host-pathogen interaction during the entry of the pathogen by altering the cell wall permeability, resulting in chronicity of the disease (Geurtsen et al. 2009). Glycogen or its intermediates (UDP-glucose) serve as precursor for production of 6-O-methylglucosyl-containing lipopolysaccharides (MGLP) and trehalose. The pathway of biosynthesis of MGLP is shown in Fig. 6. The MGLP is present in slow- and rapid-growing mycobacteria and plays a critical role in the control of fatty acid synthesis. A cluster of genes has been identified in *M. smegmatis* and *M. tuberculosis* and plays the main role in the MGLP biosynthesis. It has been observed that overexpression of the putative GT enzyme (encoded by *Rv3032* and *Rv3030*) in *M. tuberculosis* significantly increased MGLP production, and disruption of *Rv3032* gene dramatically reduces the amounts of MGLP vice-versa, which results in accumulation of UDP-glucose in the *M. tuberculosis* and ultimately reduction of glycogen contents in *M. tuberculosis* (Stadthagen et al. 2007). Significant changes were found in the glycogen contents between MDR-TB strains and drug-sensitive TB strains grown under drug pressure in initial stage (Singh et al. 2015). The mRNA level of corresponding gene (*GarA*) was confirmed by real-time PCR. The glycogen accumulation was relatively higher from the 7th day to 15th day only (Figs. 7 and 8), but no significant changes were observed after 15th day of growth (Singh et al. 2015). This observation confirmed the role of *GarA* gene in the multidrug-resistant tuberculosis at initial stage.

Apart from that, we have found that protein kinases and GarA of *M. tuberculosis* $H_{37}Rv$ play an important role in acquisition of drug resistance (Singh et al. 2015). Significant amount of glutamate production was also observed when *pknG* deletion mutants were retreated with ethambutol. The positive impact of deletion of *PknG* on glutamate formation may be a consequence of an increased level of unphosphorylated odhI and results in inhibiting 2-oxoglutarate dehydrogenase complex (ODHc) action and an elevated efflux of 2-oxoglutrate towards glutamate. pknG may also be involved probably with other kinases in the switch-on or switch-off mechanisms among active (unphosphorylated) and inactive (phosphorylated) forms of *GarA* gene. GarA also modulates the activities of α-ketoglutarate decarboxylase (KGD) and glutamate dehydrogenase, and the OdhI phosphorylation is grossly determined by PknG (Fig. 9). The proteomic analysis of *M. smegmatis* has revealed glutamate-1-semialdehyde-2 and 1-aminomutase (*hem*L) overexpressed in exposure to EMB which suggested its role in glutamate efflux. Interestingly, our
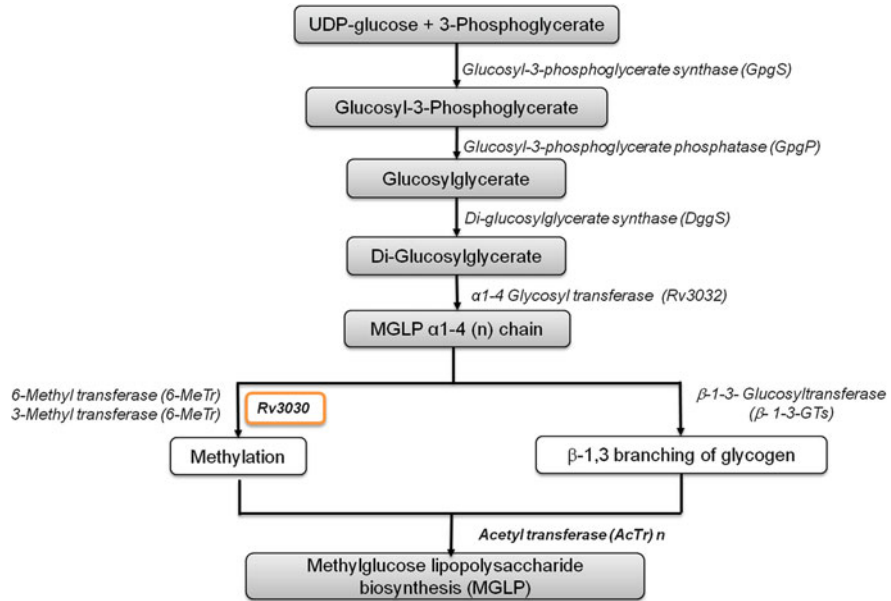
Fig. 6 MGLP synthesis pathway in *M. tuberculosis*. *Grey shades* represent confirmed activities, *white boxes* indicate putative/deduced enzyme activities. *Orange box* carries information on genes linked to the MGLP pathway: *GpgS* (*Rv1208*), *GpgP* (Rv2419), *DggS* and glucosyltransferases (Gupta et al. 2014)
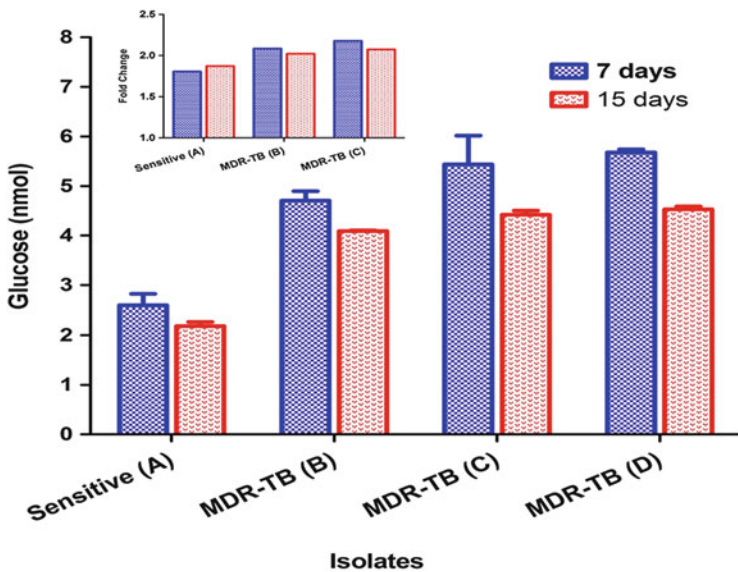

Fig. 7 Comparison of glycogen content in sensitive vs. MDR strain of *M. tuberculosis* (Singh et al. 2015)
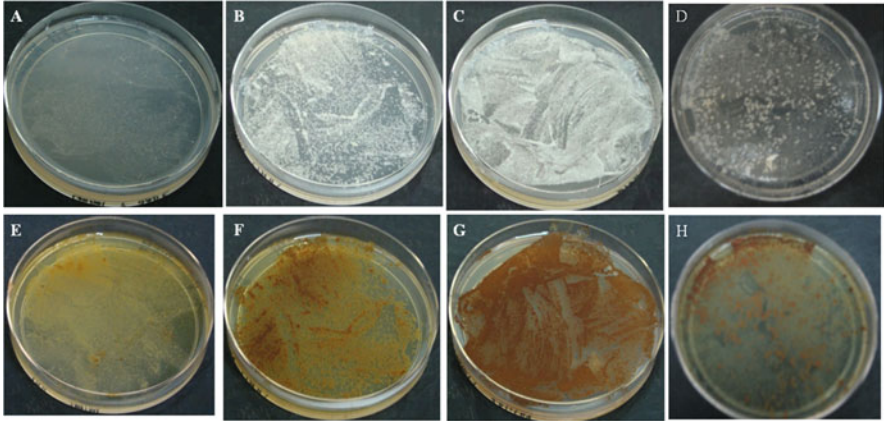
Fig. 8 Glycogen staining of sensitive and MDR isolates. (**a**) Sensitive isolate (Isolate A), (**b**) MDR isolate (Isolate B), (**c**) MDR isolate (Isolate C), (**d**) MDR isolate (Isolate D), (**e**) glycogen stained (Isolate A), (**f**) glycogen stained (Isolate B), (**g**) glycogen stained (Isolate C), (**h**) glycogen stained (Isolate D) (unpublished data from Amit Singh, Krishnamoorthy Gopinath and Sarman Singh)
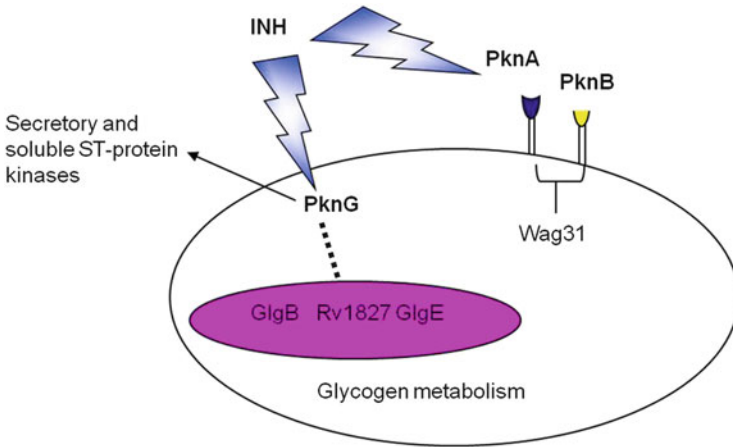

Fig. 9 PknG-Rv1827 signal cascade mechanism modulates the late growth effect. PknG—protein kinase G senses environment, involved in arresting the fusion of phagolysosome, regulates glutamine uptake and modulates the stationary phase growth of *M. tuberculosis*. Protein kinase A and protein kinase B (PknA, PknB) playing pivotal role in regulating cell morphology and size. Wag31—cell division protein, overexpressed when PknA-B acting together. GlgB, GlgE—genes regulating glycogen metabolism. Rv1827—(GarA—glycogen accumulation regulator)—FHA containing protein, phosphorylated by PknG and PknB. Regulates glycogen metabolism and expressed during exponential phase (Unpublished data from Amit Singh, Krishnamoorthy Gopinath and Sarman Singh)

study identified glutamyl-tRNA (gatA, Rv3011c, spot 11) overexpressed in MDR strains (Singh et al. 2015).

12   Glycogen in the Pathogenesis of *Tuberculosis*

Glycogen not only offers major nutrition to organisms but also plays significant role during host-pathogen interaction (Pal et al. 2010). Glycogen plays a minor role in colonization *of M. tuberculosis* within the host, but plays significant role in the intracellular survival of *M. tuberculosis* (McMeechan et al. 2005; Pal et al. 2010; Chauhan et al. 2012). The arabinomannan (AM) and α-glucans, which are the major composition of mycobacterial capsule and are located on the outer side of the mycolic acid layers in the cell wall and serve as receptor during host-pathogen interactions (Berg et al. 2007). The capsule mediates the adhesion to the host cell receptors and penetration of bacilli into the host cells (Daffé and Etienne 1999). Thereafter, mycobacterium modulates the environment conditions inside the host cell for their survival (Korf et al. 2005).

13   Immunology of *Mycobacterium tuberculosis* Glycans in the Host-Pathogen Interaction

Capsule of *M. tuberculosis* contains glycans, which can be up to 80 % of the extracellular polysaccharides, and is composed of α-4-D-Glc-1 core, which is branched at position 6 of each 5/6 residues by 4-α-D-Glc-1 oligoglucosides (Lemassu and Daffé 1994; Ortalo-Magné et al. 1995; Dinadayala et al. 2008). Since the discovery of capsular carbohydrates, with their role in bacterial pathogenesis, the focus has been on the macrophage receptors, which take part in the binding and phagocytosis of this microbe. The utility of carbohydrates in pathogenic mycobacterial species has followed the findings of the mycobacterial capsule (Dinadayala et al. 2008; Mendes et al. 2011). The AG, a disaccharide, contains 2–3 branched sugar chains and helps in the pathogenic role played by mycolic acid. These branched chains are connected at position 5 to Galf residue of the galactan's chain towards the reducing ends, consisting 22 Araf residues in D-arabinan chain (Besra et al. 1995). The D-arabinan contains backbone of α-1,5-linked Araf with numerous α-1,3-linkages and non-reducing ends are always terminated by β-1,2-Araf, resulting in hexa-arabinoside (Ara6) motifs shifted to the AG, and the dimers [β-D-Araf-1,2-α-D-Araf] form connecting site of mycolic acid. Both peptidoglycan and arabinoglycans form together covalently linked between mycolic acid layer and plasma membrane. Due to this, the cell wall of mycobacteria is enormously robust and difficult to penetrate by the drugs (Berg et al. 2007).

Unlike AG, cell envelope components are non-covalently bound to the LAM and might be linked to the mycolic acid layer or plasma membrane or both via phosphatidyl-myo-inositol (PI) unit. The reducing ends of the LAM show structural resemblances to the PIMs. The inositol residues of the phosphatidylinositol are mannosylated at the positions 2 and 6 of the LAM (Berg et al. 2007). The mycobacterial cell wall moieties (LAM) bind to the macrophage and prevent the attachment of mycobacteria to the complement receptors (Stokes et al. 2004). The capsular polysaccharides of *M. tuberculosis* facilitate non-opsonic binding of the bacteria to the complement receptor (CR-3) (Cywes et al. 1997; Dinadayala et al. 2008). Additionally, these glycans are able to generate innate immune responses through the attachment to the toll-like receptor (TLR2), CD14 and myeloid differentiation primary response gene-88 (MyD88-TLR) receptors (Bittencourt et al. 2006). Similarly, capsular components of the *M. tuberculosis* showed anti-phagocytic characteristics like that of macrophages (Stokes et al. 2004) and induced monocytes for proliferation and transformed into altered dendritic cells. These modified dendritic cells could not present lipid antigens to the CD1-restricted T cells (Gagliardi et al. 2007).

14 Glycans: Novel Drug Targets for MDR-TB

The emergence of MDR strains of *M. tuberculosis* (Koch et al. 2014) emphasizes the urgent need to find out the novel drug targets for *M. tuberculosis* (De Smet et al. 2000; Shriver et al. 2004). To find out the effective drugs, it is essential that appropriate drug targets are discussed before. The enzymes that take part in glycogen metabolism or those that mediate in the biosynthesis of vital components of *M. tuberculosis* cell envelope, cell wall or its survival machinery could be promising drug targets against mycobacteria. It has been validated that glgB auxotrophic strain of *M. tuberculosis* accumulates toxic polymers within the cells and induces cell death in *M. tuberculosis*. The deficiency of glycogen did not disturb macrophage infection by mycobacterium mutants; however, its presence showed the defending role during hostile stage of mycobacteria infections (Kalscheuer et al. 2010). Moreover, glgE-dependent pathway of glycogen synthesis was identified in mycobacteria. The product of glgE gene transfers activated glucose molecule to the maltose-1-phosphate through α-1-4 glycosidic linkage. Another enzyme Pep2 (encoded by Rv0127) is known to phosphorylate maltose and activate glycogen polymerization. Hence, the glgE enzyme induces cell death by two processes, the one known as glgE-dependent (induce self-poisoning via accumulation of maltose-1-phosphate followed by feedback inhibition of glgE) and the other glgE-independent mechanisms. Therefore, obstructing glgE activation could be an exciting drug target (Kalscheuer et al. 2010; Leiba et al. 2013).

Apart from that, biosynthesis of trehalose from glycogen is extensively studied in mycobacteria, and involved enzymes could also be possible drug targets, because of its significant role in bacterial cytosol and appearance in toxic glycolipids

(De Smet et al. 2000). It has been observed that any disruption in the enzyme—trehalose mycolyltransferase through 6-azido-6-deoxy-*a*,*a*-trehalose—inhibits growth of *M. tuberculosis* in-vitro (Belisle et al. 1997). In summary, the glycotransferase enzymes that are responsible for the synthesis of essential elements of the cell envelope in *M. tuberculosis* need to be explored as potential novel drug targets against mycobacterial pathogen.

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