Genetics of Mycobacterial Trehalose Metabolism

RAINER KALSCHEUER and HENDRIK KOLIWER-BRANDL
Institute for Medical Microbiology and Hospital Hygiene, Heinrich-Heine-University Duesseldorf, Universitaetsstr. 1, 40225 Duesseldorf, Germany

ABSTRACT
Trehalose [alpha-\(\alpha\)-glucopyranosyl-(1\(\rightarrow\)1)-alpha-\(\alpha\)-glucopyranoside] is a highly abundant disaccharide in mycobacteria that fulfills many biological roles and has a plethora of possible metabolic fates. Trehalose is synthesized in mycobacteria de novo either from glycolytic intermediates or from alpha-glucans via two alternative routes, the OtsA-OtsB and the TreY-TreZ pathways, respectively. Intracellular trehalose can serve as an endogenous remobilizable carbon storage compound and as a biocompatible stress protectant. Furthermore, trehalose functions as the sugar core of many glycolipids with important structural or immunomodulatory functions such as the cord factor trehalose dimycolate, sulfolipids, and polyacyltrehalose. Moreover, trehalose plays a central role in the formation of the mycolic acid cell wall layer because it serves as a carrier molecule that shuttles mycolic acids in the form of the glycolipid trehalose monomycolate between the cytoplasm and the periplasm. In this process, a specific importer recycles the free trehalose that is extracellularly released as a by-product during mycolate processing via the antigen 85 complex, which might represent a specific adaptation to the intracellular lifestyle of Mycobacterium tuberculosis with limited carbohydrate availability. Finally, trehalose is converted to glycogen-like branched alpha-glucans by a four-step metabolic pathway involving the essential maltosyltransferase GlgE, which may be further processed to derivatives with intracellular or extracellular destinations such as polymethylated lipopolysaccharides or capsular alpha-glucans, respectively. In this article we summarize the current knowledge of the genetic basis of trehalose biosynthesis and metabolism in mycobacteria, the biological functions of trehalose-based molecules, and their roles in virulence of the human pathogen M. tuberculosis.

TREHALOSE: A UBIQUITOUS AND UNIVERSAL SUGAR
Trehalose is a natural nonreducing glucose disaccharide comprising an \(\alpha,\alpha\)-1,1-glycosidic linkage [\(\alpha\)-\(\alpha\)-glucopyranosyl-(1\(\rightarrow\)1)-\(\alpha\)-\(\alpha\)-glucopyranoside]. The discovery of trehalose is credited to H. A. L. Wiggers (1832), who isolated a nonreducing sugar-like substance from the ergot fungus (Claviceps purpurea) of rye. Later this sugar was chemically characterized by Mitscherlich (1857), who termed it “mycose.” About the same time (1858), the French chemist M. Berthelot isolated and characterized a sugar substance from “trehala manna,” the sweet-tasting cocoon from the lepidopterous beetle Larinus nidificans, and coined the term “trehalose.” In 1876, M. A. Müntz found that trehalose and mycose were identical (see reference 1 for an overview of the historical perspective). Since then, it has become clear that trehalose is a truly ubiquitous molecule that is synthesized by many different organisms including bacteria, yeasts, fungi, plants, and invertebrates. In the early days, trehalose was believed to exclusively serve as a carbon storage compound. Trehalose is nontoxic and, thus, accumulation to high intracellular concentrations is well tolerated. From this depot, glucose can rapidly be mobilized by hydrolysis mediated by the enzyme trehalase.

Today, however, it is evident that trehalose fulfills a plethora of potential biological functions that vary...
among the producing organisms (2, 3). Most notably, it can serve as a stress bioprotectant. Many organisms synthesize and accumulate high levels of trehalose in response to stimuli such as osmotic, heat, cold, dehydration, and other stresses in order to prevent denaturation and to maintain intracellular integrity. These characteristics are attributed to its peculiar physicochemical properties such as high hydrophilicity, chemical stability, formation of solid state polymorphs, and the absence of internal hydrogen bond formation. Due to these features, trehalose is capable of stabilizing macromolecular structures such as lipid membranes and proteins, partially by replacing hydration water (4). In fact, among other applications, trehalose is technically utilized as a preservative for enzyme preparations. In plants, where trehalose is typically found only at very low levels, trehalose-6-phosphate (a direct precursor in trehalose biosynthesis) is emerging as an important signaling molecule involved in the regulation of growth and development (5). An interesting notion is that in most insects the main “blood sugar” in the hemolymph is not glucose but trehalose, serving as the energy and carbon transport form but simultaneously also acting as an antifreeze cryoprotectant (6). Probably, trehalose has more than one biological function in most synthesizing organisms. This is particularly true for the many roles of trehalose in mycobacteria, as discussed below.

FUNCTIONS OF TREHALOSE IN MYCOBACTERIA

Trehalose in mycobacteria is most widely recognized for being a structural component of cell wall glycolipids with important structural and/or immunomodulatory functions such as trehalose dimycolate (the cord factor). Less well known, free (i.e., nonconjugated) trehalose is also highly abundant intracellularly (7). In fact, it is probably the most prominent low-molecular-weight sugar compound present in mycobacteria during growth under regular laboratory culture conditions. Trehalose is an essential metabolite in Mycobacterium tuberculosis and Mycobacterium smegmatis and probably also in all other mycobacteria (8, 9). Mycobacteria can synthesize trehalose de novo from all kinds of unrelated carbon sources (such as glucose, glycerol, acetate, fatty acids, etc.), and they can also import and utilize exogenous trehalose. Trehalose auxotrophic mutants of M. smegmatis rapidly lose viability when they are starved for exogenous trehalose (9). Traditionally, free trehalose in microorganisms has been implicated in two main biological functions: as an intracellular carbon storage molecule and as a stress protectant. Likely, trehalose fulfills both of these functions in mycobacteria also. All mycobacteria tested so far can utilize trehalose as their sole source of carbon and energy, so it can serve as an intracellular storage compound that is mobilized during carbon source starvation. Furthermore, trehalose is probably the sole biocompatible stress protectant that M. tuberculosis is able to synthesize de novo. In line with its role in stress protection, trehalose auxotrophic mutants of M. smegmatis are sensitive to elevated temperatures when supplemented with suboptimal exogenous trehalose concentrations (9). The presence of an additional protective stress metabolite, ectoine, has been reported for M. smegmatis, but the corresponding biosynthetic genes are lacking in the M. tuberculosis genome (10).

However, beyond the two mentioned classical functions, trehalose is involved in many other biological processes in mycobacteria. Trehalose serves as the “sugar scaffold” for biosynthesis of numerous glycolipids, many of which play pivotal roles as structural cell wall components, carrier molecules in mycolic acid processing, and immune modulators influencing the interaction with infected host cells. Finally, it has recently been recognized that trehalose is also the substrate for a new metabolic four-step pathway in mycobacteria that converts it into glycogen-like branched alpha-glucans, which might be subject to further processing or export to yield molecules with different cellular destinations such as polymethylated alpha-glucan derivatives or extracellular capsular glucans. Our current knowledge of the genetic basis of the above-mentioned aspects of trehalose metabolism will be summarized below.

TREHALOSE DE NOVO BIOSYNTHESIS

To date, five natural metabolic routes for trehalose de novo biosynthesis have been described in the literature. Some organisms rely on only one pathway, whereas multiple pathways are present in others (5, 11). The most widespread route in prokaryotes and eukaryotes is the OtsA-OtsB pathway. This is the only biosynthetic route present in plants and has recently also been described in archaea (12). The trehalose-6-phosphate synthase OtsA catalyzes the transfer of nucleoside diphosphate-activated glucose (UDP-glucose) to glucose-6-phosphate to yield trehalose-6-phosphate with release of UDP. Subsequently, trehalose-6-phosphate phosphatase OtsB dephosphorylates trehalose-6-phosphate to trehalose (Fig. 1). The TreP pathway, which has been reported for fungi (13–16) and the protist Euglena (17),

Kalscheuer and Koliwer-Brandl
catalyzes the formation of trehalose from glucose-1-phosphate and glucose \textit{in vitro}. It is uncertain, however, whether this pathway operates \textit{in vivo} in the direction of trehalose production or rather in a reverse reaction in trehalose degradation. The other three pathways have been found exclusively in prokaryotes so far. In the TreY-TreZ pathway, first described in the thermophilic archaeon \textit{Sulfolobus acidocaldarius} \cite{18}, the maltoligosyltrehalose synthase TreY converts the terminal \(\alpha\)-1,4-glycosidic linkage at the reducing end of a linear \(\alpha\)-1,4-glucan into an \(\alpha\)-1,1-bond yielding maltooligosyltrehalose. Maltooligosyltrehalose trehalohydrolase, TreZ, then hydrolytically liberates trehalose (Fig. 1). Because this pathway requires linear glucans, branched alpha-glucans first need to be processed by glycogen phosphorylase GlgP, which reduces the branch length by releasing glucose-1-phosphate, followed by the debranching enzyme TreX, which hydrolyzes the \(\alpha\)-1,6-glycosidic branch linkages. In the TreS pathway, trehalose synthase, TreS (first cloned from \textit{Pimelobacter} \cite{19},

\begin{figure}
\centering
\includegraphics[width=\textwidth]{trehalose_biosynthesis}
\caption{Trehalose \textit{de novo} biosynthesis pathways in mycobacteria. Trehalose is synthesized either from glycolytic intermediates via the OtsA-OtsB pathway or from alpha-glucans via the TreY-TreZ pathway. UDP-glucose is formed from glucose-1-phosphate by the UTP-glucose-1-phosphate uridylyltransferase GalU (not depicted). This reaction, however, is not specific for trehalose biosynthesis. doi:10.1128/microbiolspec.MGM2-0002-2013.f1}
\end{figure}
(20), is a maltose α-d-glucosylmutase that reversibly interconverts maltose and trehalose by isomerizing the α-1,4- into an α-1,1-glycosidic linkage. Finally, in the TreT pathway, found in both bacteria and archaea and first described in the extremophilic archaea *Thermococcus litoralis* (21), trehalose glycosyltransferase, TreT, mediates the reversible formation of trehalose from ADP-glucose and glucose.

From bioinformatic analyses, it appeared that, of the five known trehalose biosynthesis pathways, three operate in *M. tuberculosis*: the OtsA-OtsB, TreY-TreZ, and TreS pathways (22). Enzymatic *in vitro* characterizations confirmed that all three pathways are in principle functional in both fast-growing (*M. smegmatis*) and slow-growing (*Mycobacterium bovis* BCG) mycobacteria (22). Of the three alternative routes, the OtsA-OtsB pathway is the dominant one for trehalose formation in *M. tuberculosis*. A Δ*otsA* (Rv3490) gene deletion mutant was significantly attenuated for *in vitro* growth in trehalose-free medium (8). Surprisingly, in contrast to the genetic dispensability of *otsA*, the *otsB2* gene (Rv3372) is obviously essential in *M. tuberculosis* and *M. bovis* BCG. The gene could not be inactivated even in the presence of exogenous trehalose to chemically complement for the biosynthetic defect (8). One explanation for this might be the potential toxic effect of the intermediate trehalose-6-phosphate that is expected to accumulate when OtsB is inactivated but OtsA is still active, similar to the toxicity of other phosphorylated sugar molecules, as will be discussed later for the intermediate trehalose-6-phosphate that is expected to accumulate when OtsB is inactivated but OtsA is still active, similar to the toxicity of other phosphorylated sugar molecules, as will be discussed later for the intermediate trehalose-6-phosphate that is expected to accumulate when OtsB is inactivated but OtsA is still active, similar to the toxicity of other phosphorylated sugar molecules, as will be discussed later for the intermediate trehalose-6-phosphate that is expected to accumulate when OtsB is inactivated but OtsA is still active, similar to the toxicity of other phosphorylated sugar molecules, as will be discussed later for the intermediate trehalose-6-phosphate that is expected to accumulate when OtsB is inactivated but OtsA is still active, similar to the toxicity of other phosphorylated sugar molecules, as will be discussed later for the intermediate trehalose-6-phosphate that is expected to accumulate when OtsB is inactivated but OtsA is still active, similar to the toxicity of other phosphorylated sugar molecules, as will be discussed later for the intermediate trehalose-6-phosphate that is expected to accumulate when OtsB is inactivated but OtsA is still active, similar to the toxicity of other phosphorylated sugar molecules, as will be discussed later for the intermediate trehalose-6-phosphate that is expected to accumulate when OtsB is inactivated but OtsA is still active, similar to the toxicity of other phosphorylated sugar molecules, as will be discussed later for the

In contrast to the dominance of the OtsA-OtsB pathway in *M. tuberculosis*, genetic experiments in the fast-growing mycobacterium *M. smegmatis* appeared to show that all three pathways were individually sufficient to synthesize trehalose *de novo* (9). This result was puzzling regarding the role of TreS in trehalose formation. Contribution of TreS to trehalose *de novo* biosynthesis from defined mineral salts media would require a substantial intracellular source of free maltose in *M. smegmatis*, which has never been reported in the literature so far. In order to unambiguously readdress the importance of TreS for trehalose biosynthesis in *M. smegmatis*, defined gene deletion mutants were generated with all possible combinatorial inactivations of the OtsA-OtsB, TreY-TreZ, and TreS pathways by targeting the genes *otsA*, *treY-Z*, and *treS*. These studies revealed that combined inactivation of the OtsA-OtsB and TreY-TreZ pathways was sufficient to result in trehalose auxotrophy in *M. smegmatis*, although in this genetic context the *treS* gene was still intact (24). This observation clearly defined that TreS plays no significant role in trehalose biosynthesis in *M. smegmatis* under the tested *in vitro* conditions. Furthermore, overexpression of TreS (Rv0126) in *M. tuberculosis* did not cause an elevation, but rather the opposite, a drastic reduction of the intracellular trehalose level, implying that TreS consumes rather than produces trehalose in mycobacteria (24). In fact, as described later in this article, TreS has recently been recognized as the first enzymatic step of a novel four-step pathway converting trehalose into branched alpha-glucans, which is widespread among prokaryotes (23, 25).

Although the equilibrium of purified TreS favors the formation of trehalose from maltose *in vitro*, flux through TreS *in vivo* is in the opposite direction (24), which is driven by the rapid and irreversible ATP-dependent phosphorylation of the formed maltose to maltose-1-phosphate by the maltose kinase Pep2 (Rv0127) (26, 27). The observed finding of the direction...
of flux through TreS for consumption of trehalose in *M. smegmatis* contradicts a previous study (9). The reason for this discrepancy is not obvious. However, in the previous study the authors did not employ a defined ΔotsAΔtreY double mutant to study the specific contribution of TreS in the *de novo* biosynthesis of trehalose, but rather, employed a surrogate strain (a ΔotsAΔtreSΔtreY triple mutant with a reconstituted treS gene constitutively expressed from an episomal multicopy plasmid) that likely exhibited a much higher treS expression level compared with the native gene. In this genetic context, TreS might suffice to form enough trehalose to support growth, given that a substantial source of maltose (e.g., from the medium) was present. The new findings in *M. smegmatis* are in agreement with the observed direction of flux in *M. tuberculosis* and with data from the closely related mycolic acid–producing actinomycete Corynebacterium glutamicum, in which the OtsA-OtsB and TreY-TreZ pathways, but not the TreS pathway, were demonstrated to be important for trehalose biosynthesis (28, 29). In conclusion, *M. tuberculosis*, *M. smegmatis*, and likely all other mycobacteria synthesize trehalose via two alternative routes, the OtsA-OtsB2 and the TreY-TreZ pathways (Fig. 1).

**TREHALOSE DEGRADATION**

All mycobacteria that have been tested for this metabolic capability so far (including *M. smegmatis*, *M. bovis*, *M. tuberculosis*, and *Mycobacterium marinum*) are able to grow on trehalose as the sole source of carbon and energy. Exogenous trehalose is imported by a highly specific and high-affinity ABC transporter (for ATP binding cassette), LpqY-SugABC (encoded by *Rv1235* to *Rv1238* in *M. tuberculosis*) (30), which will be discussed in detail below. It has been proposed that trehalose is hydrolyzed intracellularly by the enzyme trehalase (*α,α*-trehalose glucohydrolase; encoded by *Rv2402* in *M. tuberculosis*), which produces two glucose molecules that can then be further catabolized glycolytically (31). The enzyme has been biochemically characterized from *M. smegmatis*, but in an attempt to determine the role of trehalase for trehalose catabolism in this organism, the authors were unable to inactivate the respective gene, suggesting that it is essential (31). Our group, however, was able to delete the trehalase gene from *M. smegmatis* without noticing any negative effect on viability (32). According to its suggested role, the trehalase-deficient *M. smegmatis* mutant was unable to utilize trehalose as the sole carbon source, confirming its crucial role in intracellular trehalose breakdown (M. Alber and R. Kalscheuer, unpublished). However, the influence of trehalase on the regulation of the intracellular concentration of the endogenously *de novo* synthesized trehalose is currently not known.

**BIOSYNTHESIS OF TREHALOSE-DERIVED GLYCOLIPIDS**

The mycobacterial cell wall is characterized by an often high abundance of a rich variety of lipophilic molecules, including glycolipids with important structural functions and/or immunomodulatory properties. A major role of trehalose in mycobacteria is to serve as a “scaffold” for the buildup of many of these glycolipids. The biosynthesis of some of these glycoconjugates might start from free trehalose, whereas others might utilize the precursor trehalose-6-phosphate. Some of these trehalose-based glycolipids contain unusual and complex fatty acid derivatives synthesized by polyketide synthases. The metabolic pathways for production of the major trehalose-based glycolipids will be described in the following.

**Trehalose Mycolates**

The “cord factor” trehalose-6,6′-dimycolate (TDM) (Fig. 2) is probably the most well-known glycolipid of *M. tuberculosis*. The ability of *M. tuberculosis* to form rope-like structures in culture was first described by Robert Koch in 1884. Much later the substance responsible for this “cord” formation, the cord factor, was extracted with petroleum ether from *M. tuberculosis* cells and chemically identified as TDM (33, 34). TDM is now recognized as a critical structural component of the mycomembrane, forming the outer leaflet of this lipid bilayer along with other noncovalently linked (so-called extractable) lipids (35, 36). The biosynthesis of TDM is initiated in the cytoplasm, whereas the final steps take place extracellularly. The precursor of TDM, trehalose-6-monomycolate (TMM), not only is a structural component of the cell wall, but it also fulfills a crucial role in the formation of the mycolic acid envelope layer.

Mature mycolates are first synthesized in the cytoplasm and remain coupled by a thioester linkage to a C-terminal domain of the polyketide synthase Pks13 (mycolyl-S-Pks13), as discussed in detail elsewhere in this book. The mycolyl group is then transferred from mycolyl-S-Pks13 to an isoprenoid carrier in the cytoplasm membrane (β-mannopyranosyl-1-phosphoheptaprenol) by an unknown cytoplasmic mycolyltransferase, yielding 6-O-mycolyllmannopyranosyl phosphoheptaprenol (Myc-PL) (37). The mycolyl group is subsequently transferred from Myc-PL to trehalose-6-phosphate by a
yet unidentified, probably membrane-associated mycolyltransferase, forming 6-mycolyl-trehalose-6′-phosphate, which is finally dephosphorylated to TMM by an unknown proposed membrane-associated phosphatase (38). TMM serves as the mycolate transport form which is exported outside the cell by the membrane transporter MmpL3, as has recently been discovered independently by several groups (39–41). Extracellularly, the periplasmic TMM is the substrate of the mycolyltransferases of the antigen 85 complex, which transfers the mycolate moiety to either the arabinogalactan layer to form cell-wall-bound mycolates or to another TMM molecule, resulting in formation of TDM (42, 43), whereas the concomitantly released trehalose is recycled and reimported into the cytoplasm, as will be discussed below (see Fig. 4). Trehalose thus functions as a carrier molecule to shuttle mycolates from the cytoplasm to the periplasm in the form of TMM. According to its transport function, pharmacological inhibition of MmpL3 in M. tuberculosis or conditional silencing of MmpL3 in M. smegmatis resulted in intracellular accumulation of TMM and abrogation of TDM formation (39–41).

The antigen 85 complex in M. tuberculosis is composed of Ag85A (FbpA; Rv3804c), Ag85B (FbpB; Rv1886c), and Ag85C (FbpC; Rv0129c) (42, 44). The mycolyltransferases of the antigen 85 complex are partially functionally redundant, implying overlapping substrate specificities. All three members are individually dispensable for viability of M. tuberculosis. An M. tuberculosis mutant lacking Ag85C exhibited a 40% reduction in the amount of cell-wall-linked mycolic acids (45). Inactivation of Ag85A or Ag85B had no significant effect on the cellular mycolic acid content, but their overexpression could compensate for the defect in mycolic acid content in the ΔfbpC M. tuberculosis mutant (43, 46).

Sulfolipids

The envelope of pathogenic mycobacteria contains complex trehalose-based glycolipids comprising up to five multiple methyl-branched long-chain fatty acids, including sulfolipids as well as di-, tri-, and polyacylated acyltrehaloses. Due to their high abundance in the cell wall, an important role of these abundant complex glycolipids for virulence of M. tuberculosis has been implicated. However, their definitive function has yet to be established. M. tuberculosis mutants lacking these cell envelope molecules typically exhibit no loss of virulence in the mouse infection model, but their role might be species specific and might reflect a specific adaptation to the human host. The most abundant sulfolipid in M. tuberculosis is SL-1, a tetraacyl-sulfotrehalose glycolipid. The biosynthesis of SL-1 is initiated by sulfation of trehalose at position 2 catalyzed by the sulfotransferase Stf0 (encoded by Rv0295c in M. tuberculosis), yielding trehalose-2-sulfate (Fig. 3A). Stf0 utilizes 3′-phospho-adenosine-5′-phosphosulfate (PAPS) as the sulfate donor. Deletion of stf0 abrogates trehalose-2-sulfate biosynthesis and subsequent formation of SL-1 in M. tuberculosis (47). The acyltransferase PapA2 then catalyzes the esterification of trehalose-2-sulfate at the 2′-position to generate the monoacylated intermediate SL659, which is further acylated by PapA1 at the 3′-position of SL659, forming diacylated SL1278. While PapA2 transfers a straight-chain fatty acid from acyl coenzyme A (CoA), PapA1 transfers a methyl-branched (hydroxy) phthioceranoyl chain likely directly from the acyl carrier protein domain of Pks2 (48). Pks2 synthesizes the required methyl-branched (hydroxy)phthioceranoyl chains using an activated fatty acid starter unit provided by the fatty acid AMP ligase FadD23 (Fig. 3A). Deletion of pks2 in M. tuberculosis resulted in abrogation of SL1278 and SL-1 biosynthesis (49).
sequential action in SL-1 synthesis, the *M. tuberculosis* Δ*papA1* mutant was deficient in SL\textsubscript{1278} synthesis but still produced trehalose-2-sulfate and SL\textsubscript{659}, whereas the Δ*papA2* mutant lacked SL\textsubscript{659} and SL\textsubscript{1278} while only retaining the ability to produce trehalose-2-sulfate (48). Additional acylations at the 6- and 6'-positions of SL\textsubscript{1278} finally lead to synthesis of mature SL-1.

While these acylation steps are biochemically similar to the reaction catalyzed by PapA1, this activity is mediated by a different acyltransferase, Chp1, which has been identified recently. Chp1 (cutinase-like hydrolase protein; Rv3822) is a transacylase that can utilize SL\textsubscript{1278} both as an acyl donor and acceptor and acylates SL\textsubscript{1278} twice to reveal SL-1, while the deacylated side products are recycled (Fig. 3A) (50). Chp1 probably needs association with the large membrane protein MmpL8, which is part of the SL-1 biosynthesis gene cluster (Fig. 3B), for full enzymatic activity. MmpL8 is required both for

---

**FIGURE 3** Biosynthesis of the major sulfolipid SL-1 and of polyacyltrehalose (PAT). (A) The synthesis of SL-1 and PAT is initiated by sulfation or acylation, respectively, at the 2-position of trehalose. The proposed reaction steps are depicted. (B) Molecular organization of the SL-1 and PAT biosynthesis gene clusters in *M. tuberculosis*. doi:10.1128/microbiolspec.MGM2-0002-2013.f3
proper SL-1 biosynthesis and for translocation across the cytoplasm membrane. Consistent with its transport function, inactivation of mmpL8 in M. tuberculosis correlated with accumulation of the intermediate SL-1278 retained in a deeper layer of the cell wall (51). A model has been proposed in which MmpL8 couples biosynthesis and transport by acting as a scaffold for the enzymatic machinery consisting of cytosolic PapA1, PapA2, Pks2, and membrane-associated Chp1 (50). By this macromolecular assembly, MmpL8 in a coordinated fashion might first facilitate biosynthesis and then translocate the final SL-1 synthesis product across the cytoplasm membrane. Another protein encoded within the SL-1 biosynthesis gene cluster, the integral membrane protein Sap (sulfolipid-1-addressing protein; Rv3821), probably associates with MmpL8 and modulates its transport function (50). While SL-1 deficiency per se does not impair virulence of M. tuberculosis in the mouse infection model as concluded from infection studies with the M. tuberculosis Δpks2 mutants (51, 52), deletion of mmpL8 leads to attenuation (51, 53). This may imply that MmpL8, in addition to SL-1, transports other unidentified lipid molecules important for virulence of M. tuberculosis. Alternatively, the phenotype of the ΔmmpL8 mutant might be explainable by the accumulation of a toxic intermediate that impairs M. tuberculosis viability in vivo.

**Polyacylated Trehalose**

Di-, tri-, and polyacylated acyltrehaloses are complex glycolipids containing methyl-branched fatty acids that are located in the outer layer of the mycobacterial cell envelope (54–58). They appear to be restricted to pathogenic mycobacteria. The major polyacyltrehalose (PAT) of M. tuberculosis, also referred to as pentaacyltrehalose, contains four mycolipenic acids and one saturated fatty acid (Fig. 3A). In contrast to the almost completely elucidated pathway for biosynthesis and export of SL-1, the available information on the molecular basis of biosynthesis of acytrehaloses is relatively scarce. However, the presumed PAT biosynthesis gene cluster of M. tuberculosis shows a molecular organization strongly resembling the SL-1 synthesis gene cluster (Fig. 3B). Hence, PAT formation likely follows a similar biochemical assembly series (Fig. 3A). PAT synthesis is initiated by the acyltransferase PapA3 (Rv1182), which transfers a straight-chain fatty acid moiety such as palmitate (C16), probably using acyl-CoA as the acyl donor to the 2-position of trehalose, yielding trehalose-2-palmitate (59). PapA3 then also catalyzes the subsequent acylation of the 3-position of trehalose-2-palmitate. Using palmitoyl-CoA as the donor substrate, purified PapA3 led to production of 2,3-dipalmitoyltrehalose in vitro (59). The mycolipenic acid moieties of PAT are produced by the polyketide synthase gene pks3/4 (also referred to as msl3) (60). In vivo, PapA3 thus probably associates with Pks3/4 and might transfer a mycolipenoyl moiety directly from the acyl carrier protein domain of Pks3/4 to trehalose-2-palmitate to form 2,3-diacyltrehalose (Fig. 3A). Deletion of the papA3 gene resulted in loss of PAT biosynthesis without affecting SL-1 formation in M. tuberculosis (59). Likewise, inactivation of pks3/4 also led to loss of PAT production with no effect on SL-1 production (60). Interestingly, the laboratory strain M. tuberculosis H37Rv harbors a frameshift mutation in the pks3/4 gene introducing a premature stop codon leading to a truncated nonfunctional polyketide synthase that abolishes PAT biosynthesis. Other M. tuberculosis strains such as CDC1551 and Erdman, however, contain a single continuous open reading frame for pks3/4 that encodes a functional enzyme (59, 61).

The sequential acylation steps to elaborate 2,3-diacyltrehalose with three further mycolipenoyl moieties to form PAT have not been identified yet. These may be mediated again by PapA3 and/or an unidentified acyltransferase. It is also not clear how PAT or its biosynthetic precursors are translocated across the cytoplasm membrane to reach their cell surface destination. However, by analogy to SL-1 biosynthesis, this transport likely involves the transmembrane protein MmpL11, which is encoded within the PAT gene cluster (Fig. 3B). Furthermore, the clustering of fadD21 within the PAT biosynthesis gene cluster suggests a role for this acyl-CoA synthetase/acyl-AMP ligase. In analogy to SL-1 biosynthesis, FadD21 may provide an activated fatty acid starter unit that is then elongated by Pks3/4 to mycolipenic acids. However, so far there has been no experimental evidence for this. Recently, a novel locus was implicated in acyltrehalose biosynthesis. M. tuberculosis transposon mutants with disrupted Rv1503c and Rv1506c genes were found to be impaired in the synthesis of diacyltrehalose (62). The homologues of these genes in M. marinum belong to a locus involved in the synthesis of lipoooligosaccharides (LOS) (63). However, as discussed below, M. tuberculosis strains do not synthesize LOS. The role of these genes and other genes of this cluster in acyltrehalose formation remains to be established.
Lipoooligosaccharides

A fourth class of trehalose-containing mycobacterial glycolipids is LOS, which are structurally highly diverse. They contain a trehalose-containing tetraglucosyl “core” \[\beta-D-glucose-(1\rightarrow3)\beta-D-glucose-(1\rightarrow4)\alpha-D-glucose-(1\rightarrow1)\alpha-D-glucose\], with the latter two \(\alpha,\alpha\)-1,1-linked glucose residues representing the trehalose moiety), which is then further glycosylated (64). Most LOS-producing mycobacteria include Mycobacterium kansasii (66) and M. marinum (67), but among members of the M. tuberculosis complex, LOS have only been found in Mycobacterium canetti (68), a rather distant relative of M. tuberculosis (69). Due to their absence from M. tuberculosis, relatively little research has been published regarding the biosynthesis of LOS. Most genetic information regarding LOS biosynthesis is available for M. marinum. Mutants of M. marinum impaired in various glycosylation steps have been isolated, e.g., in MMAR_2333, which has been identified as a glycosyltransferase involved in addition of a caryophylole moiety in LOS (70), or LosA, a glycosyltransferase involved in elongation of the tetraglucosyl core (67). Interestingly, losA is part of the supposed LOS biosynthesis gene cluster in M. marinum (63), which is partially conserved in M. tuberculosis and here encompasses the genes Rv1496 to Rv1505. However, the corresponding gene cluster in M. marinum is much larger and contains nine additional genes. Thus, it can be speculated that the loss of one or more of these genes is responsible for the loss of LOS biosynthesis in M. tuberculosis. However, as mentioned above, genes of this gene cluster have also been found to be involved in diacytrehalose formation, so that the precise function of the remnant LOS gene cluster in M. tuberculosis needs to be established (62).

Trehalose Uptake and Recycling

As mentioned above, M. tuberculosis and other mycobacteria can utilize trehalose as their sole source of carbon and energy. Recently, an ABC transporter was identified in M. tuberculosis that mediates the active transport of trehalose across the cytoplasm membrane (30). This transporter, which is highly conserved among mycobacteria, is composed of the two transmembrane proteins SugA and SugB, the ATP-hydrolyzing protein SugC, and the periplasmic solute-binding lipoprotein LpqY, which is tethered by a lipid anchor to the cytoplasm membrane. The overall structure of this transporter is LpqY-SugA-SugB-(SugC)_2. The genes coding for the four components of this transporter are organized as an operon in the genomes of M. tuberculosis (Rv1235-Rv1238), M. smegmatis, as well as all other mycobacteria. Biochemical studies revealed that this ABC transporter is a high-affinity highly trehalose-specific importer. The substrate specificity is so pronounced that this transporter can discriminate between trehalose \([\alpha-D-glucopyranosyl-(1\rightarrow1)\alpha-D-glucopyranoside]\) and maltose \([\alpha-D-glucopyranosyl-(1\rightarrow4)\alpha-D-glucopyranoside]\) and even between the \(\alpha,\alpha\)-trehalose and \(\alpha,\beta\)-trehalose stereoisomers. According to its transport function, gene deletion mutants with inactivated lpqY or sugC genes in M. tuberculosis and M. smegmatis were unable to grow in vitro with trehalose as the sole carbon source, whereas growth on other substrates was not impaired (30). Since the mycomembrane is supposed to constitute a relatively selective permeability barrier, the presence of some sort of transport mechanism in this cell wall layer facilitating trehalose uptake must be postulated, e.g., maybe a pore-forming membrane channel such as a porin specific for low-molecular-weight hydrophilic molecules (Fig. 4). However, so far no candidate for such a permease has been identified.

The LpqY-SugABC transporter has previously been speculated to be involved in uptake and utilization of a carbohydrate compound from the host. However, trehalose is not present in mammals. Thus, it is unlikely to be involved in nutrient acquisition from the host. In contrast, it was demonstrated that this ABC transporter instead plays a role in recycling of trehalose that is extracellularly released from mycobacteria themselves as a by-product during cell wall biosynthesis (30). As outlined previously in this article, it has been established that trehalose functions as a carrier molecule in buildup of the mycolic acid cell wall layer (Fig. 4). Mycolic acids synthesized in the cytoplasm are first conjugated to trehalose to yield TMM. TMM subsequently serves as the mycolate transport form that is exported across the cytoplasm membrane by the membrane transporter MmpL3 (39–41). Extracellularly, the periplasmic TMM is the substrate of the mycolyltransferases of the antigen 85 complex, which transfers the mycolate moiety to either the arabinogalactan layer to yield cell-wall-bound mycolates or to another TMM molecule resulting in formation of TDM (42, 43). These enzymatic reactions lead to concomitant release of the trehalose moiety of TMM. The function of the LpqY-SugABC permease is the recycling of the released trehalose and its retrograde reimport into the
Cytoplasm, where it can be used as an acceptor for another mycolic acid moiety (Fig. 4). Inhibition of this recycling function in *M. tuberculosis* ΔlpqY or ΔsugC mutants led to secretion of substantial amounts of free trehalose into the culture medium *in vitro* (30). The importance of the LpqY-SugABC transporter for virulence of *M. tuberculosis* is highlighted by the strong attenuation of the ΔlpqY or ΔsugC mutants during the acute infection phase in the mouse model (30). The detailed molecular mechanisms causing attenuation during infection *in vivo* have not been elucidated yet. However, the primary niche for intracellular replication of *M. tuberculosis*, the arrested phagosome inside infected macrophages, is characterized as a nutrient-restricted microenvironment with very poor carbohydrate availability. In this regard, it is likely that continuous secretion of trehalose during intracellular growth of the ΔlpqY or ΔsugC mutants would gradually cause carbon starvation. Therefore, LpqY-SugABC-mediated trehalose recycling might represent a specific adaptation of *M. tuberculosis* to its nutrient-limited intracellular lifestyle.

**CONVERSION OF TRELHASE TO ALPHA-GLUCANS**

Previously, the trehalose synthase TreS was circumstantially implicated in glycogen formation in mycobacteria because it was observed that incubation of cells at high trehalose concentrations resulted in accumulation of large amounts of glycogen, and this effect was strictly TreS dependent. At this time, a direct conversion of trehalose to glycogen was speculated based on an alpha-amylase activity found to be associated with purified TreS (71). However, it has recently been established that TreS catalyzes the first step of a novel four-step biochemical pathway that converts trehalose into branched alpha-glucans (Fig. 5), which is now known as the GlgE pathway and which is widespread among prokaryotes (23, 25). TreS interconverts trehalose and maltose with formation of the α-anomer of maltose (24), which is subsequently rapidly and quantitatively phosphorylated in an ATP-dependent reaction to maltose-1-phosphate by the maltose kinase Pep2 (Rv0127) (27). Maltose-1-phosphate then acts as the activated donor substrate for the key enzyme of this pathway, the maltosyltransferase GlgE.

![Figure 4](https://www.asmscience.org/MicrobiolSpectrum/content/44312/44312_f4.png)

**FIGURE 4** Model of trehalose as a carrier molecule for mycolic acids in the formation of the mycobacterial cell wall. Mycolic acids synthesized in the cytoplasm are first conjugated to trehalose and then exported as TMM via the transporter MmpL3. Extracellularly, TMM serves as the substrate of the antigen 85 complex comprising the mycolyltransferases FbpA-C, which transfer the mycolate moiety either to the arabinogalactan layer or to another TMM molecule, yielding cell-wall-bound mycolates or TDM, respectively. The released trehalose moiety of TMM is recycled by the ABC transporter LpqY-SugABC. A putative porin might facilitate transport across the mycomembrane for uptake of exogenous trehalose. doi:10.1128/microbiolspec.MGM2-0002-2013.f4
(Rv1327c), which transfers the maltosyl moiety to the nonreducing 4′-hydroxyl group of an α-1,4-glucan acceptor molecule, yielding linear α-glucans. Finally, the branching enzyme GlgB (Rv1326c) introduces α-1,6-linked branches into the linear α-glucan, forming molecules structurally resembling glycogen (Fig. 5) (23).

By using a phosphosugar as the activated substrate, GlgE [systematic name (1→4)-α-D-glucan:phosphate α-D-maltosyltransferase], which is a member of the glycoside hydrolase subfamily GH13_3, represents an unusual glycosyltransferase, while most known members of this enzyme class rely on nucleotide-bound sugars as donor substrates. In addition to the maltose-1-phosphate-dependent maltosyltransferase activity, GlgE also exhibits disproportionating transglucosidase activity; i.e., it can remove a maltosyl moiety from one alpha-glucan molecule and transfer it to another (e.g., α-1,4-glucan$_x$ + α-1,4-glucan$_y$ → α-1,4-glucan$_{x+y}$, with $x,y =$ number of glucose units). Therefore, not only can GlgE synthesize linear alpha-glucans, but it is also capable of shaping the length of the branches of branched glucans (23).

Although production of alpha-glucan molecules through the GlgE pathway is not required for viability of *M. tuberculosis* in vitro as indicated by the genetic dispensability of treS and pep2, both the *glgE* and *glgB* genes are strictly essential in *M. tuberculosis*. Inactivation of GlgE leads to an intracellular accumulation of the phosphorylated intermediate maltose-1-phosphate, which is toxic to the cells by eliciting pleiotropic stress responses. These maltose-1-phosphate-induced stress responses include DNA damage as indicated by global induction of the DNA damage-responsive SOS regulon, causing rapid cell death of *M. tuberculosis* in vitro as well as in lung and spleens of infected mice (23). Since linear alpha-glucans rapidly become insoluble with increasing chain length, inactivation of GlgB indirectly retards GlgE activity by acceptor substrate depletion, eventually also causing maltose-1-phosphate accumulation. In agreement with the toxicity of maltose-1-phosphate as the basis of essentiality of GlgE and GlgB, prevention of maltose-1-phosphate formation through genetic or pharmacological inactivation of TreS allows deletion of both *glgE* and *glgB* in *M. tuberculosis* (23).

FIGURE 5 Conversion of trehalose to alpha-glucans. Trehalose is reversibly interconverted by the trehalose synthase TreS to α-maltose, which is subsequently phosphorylated to α-maltose-1-phosphate by the maltokinase Pep2. Maltose-1-phosphate serves as the activated donor substrate for the maltosyltransferase GlgE producing linear α-1,4-glucans by elongating the nonreducing end of an α-glucan acceptor molecule. Finally, the branching enzyme GlgB introduces α-1,6-linked branches into the linear molecule. doi:10.1128/microbiolspec.MGM2-0002-2013.f5
Recently, it was demonstrated that GlgE activity can be negatively fine-tuned via phosphorylation by the serine/threonine protein kinase PknB (72), although it is unclear if this regulation is important for M. tuberculosis virulence. Given the high toxicity of maltose-1-phosphate, its synthesis rate also needs to be subject to one or more regulatory mechanisms in order to balance production and consumption. However, how this is mediated is currently unknown.

The final cellular destination(s) and function(s) of the branched alpha-glucans synthesized by the GlgE pathway are not fully clear. It is likely that the GlgE pathway products, like glycogen, are deposited in the cytosol and serve as an intracellular carbon storage compound that is hydrolytically remobilized during starvation. Furthermore, alpha-glucans are also found extracellularly in mycobacteria, where they constitute the major polysaccharide component of the capsule representing the outermost cell envelope layer, which has been implicated in M. tuberculosis persistence (73–75). Currently, however, a contribution of the GlgE pathway to production of capsular alpha-glucans has not been established. Finally, a synthetic lethal interaction of the GlgE pathway with an alternative pathway leading to production of methyl-branched alpha-glucan derivatives, methylglucose lipopolysaccharides (MGLPs), has been revealed (23). MGLPs are alpha-glucan oligomers consisting of 19 to 20 glucose residues, which are extensively methylated and acylated (76). MGLP biosynthesis involves the glucosyltransferase Rv3032, which can utilize UDP-glucose as well as ADP-glucose as activated donor substrates (76). The synthetic lethal interaction was demonstrated by the inability to delete the genes treS and Rv3032 simultaneously in M. tuberculosis, while both genes individually are dispensable. This synthetic lethal interaction was further corroborated by demonstration that treatment of the M. tuberculosis ΔRv3032 mutant with the TreS inhibitor validamycin A resulted in rapid killing while having no effect on wild-type cells (23). Thus, it is likely that the branched alpha-glucan GlgE pathway products are at least partially subject to further chemical modifications, yielding derivatives that are structurally and/or functionally redundant to MGLP. However, these further modifications have yet to be identified. The combination of essentiality of GlgE within a synthetic lethal pathway makes GlgE an attractive drug target because it possibly enables two independent and synergistic killing mechanisms (77).

The question of the importance of alpha-glucans synthesized via the GlgE pathway for M. tuberculosis virulence has not conclusively been resolved yet. While the M. tuberculosis ΔtreS mutant was shown to be not attenuated in BALB/c mice following infection via the high-dose intravenous route (23), studies from a different laboratory of C57BL/6 mice following aerosol challenge suggested a role of TreS in late-stage pathogenesis (8). However, since genetic complementation was not able to restore virulence of this M. tuberculosis ΔtreS mutant, inadvertent secondary mutations likely contributed to the observed in vivo phenotype in C57BL/6 mice (unpublished data available at http://webhost.nts.jhu.edu/target/pdf/TARGET%20Report%20treS%20otsA.pdf).

CONCLUDING REMARKS

As outlined above, trehalose fulfills many roles in mycobacteria and has many metabolic fates. In this respect, the amount of the de novo synthesized trehalose is probably tightly regulated according to the intracellular demand. Likewise, the flux of trehalose into the many different metabolizing pathways probably requires tight regulation at multiple levels. However, while we now begin to understand the biochemical and genetic basis of trehalose-based metabolic pathways, the regulatory mechanisms underlying the balance of intracellular production and consumption of trehalose are completely unknown. Given the central importance of trehalose for M. tuberculosis pathogenesis, this aspect deserves increased attention in the future.

ACKNOWLEDGMENTS

Research related to this chapter in the laboratory of R. Kalscheuer is supported by the Juergen Manchot Foundation, the Strategic Research Fund of the Heinrich-Heine-University Duesseldorf, and the German Research Foundation.

REFERENCES

8. Murphy HN, Stewart GR, Mischenko VV, Apt AS, Harris R, McAlister MS, Driscoll PC, Young DB, Robertson DB. 2005. The OtsAB pathway is
Genetics of Mycobacterial Trehalose Metabolism


