Mycobacterium tuberculosis maltosyltransferase GlgE, a genetically validated anti-tuberculosis target, is negatively regulated by Ser/Thr phosphorylation

Jade Leiba¹, Karl Syson², Grégory Baronian¹, Isabelle Zanella-Cléon¹, Rainer Kalscheuer⁴, Laurent Kremer¹,⁵, Stephen Bornemann²,* and Virginie Molle¹,*

¹Laboratoire de Dynamique des Interactions Membranaires Normales et Pathologiques, Universités de Montpellier II et I, CNRS, UMR 5235, case 107, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France; ²Department of Biological Chemistry, John Innes Centre, Norwich Research Park, Norwich, NR4 7UH, United Kingdom; ³Institut de Biologie et Chimie des Protéines (IBCP UMR 5086), CNRS, Université Lyon1, IFR128 Biosciences, Lyon Gerland, 7 passage du Vercors, 69367 Lyon Cedex 07, France; ⁴Institute for Medical Microbiology and Hospital Hygiene, Heinrich-Heine-University Düsseldorf, Düsseldorf, Germany. ⁵INSERM, DIMNP, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France.

*Corresponding authors:
VM: Tel: (+33) 4 67 14 47 25, Fax: (+33) 4 67 14 42 86, E-mail: virginie.molle@univ-montp2.fr
SB: Tel: (+44) 1603 450741, Fax: (+33) 1603 450018, E-mail: stephen.bornemann@jic.ac.uk

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Background: GlgE is involved in the biosynthesis of α-glucans and is a genetically validated anti-tuberculosis target.

Results: Phosphorylation of GlgE by the Ser/Thr protein kinase PknB lowers enzyme activity in vitro and in vivo.

Conclusion: Phosphorylation of GlgE will negatively regulate flux through the GlgE pathway in actinomycetes.

Significance: This study provides new opportunities to target the GlgE pathway therapeutically.

GlgE is a maltosyltransferase involved in the biosynthesis of α-glucans that has been genetically validated as a potential therapeutic target against Mycobacterium tuberculosis. Despite also making a-glucan, the GlgC/GlgA glycogen pathway is distinct and allosterically regulated. We have used a combination of genetics and biochemistry to establish how the GlgE pathway is regulated. M. tuberculosis GlgE was phosphorylated specifically by the Ser/Thr protein kinase PknB in vitro on one serine and six threonine residues. Furthermore, GlgE was phosphorylated in vivo when expressed in Mycobacterium bovis BCG but not when all seven phosphorylation sites were replaced by Ala residues. The GlgE orthologues from Mycobacterium smegmatis and Streptomyces coelicolor were phosphorylated by the corresponding PknB orthologues in vitro, implying the phosphorylation of GlgE is widespread among actinomycetes. PknB-dependent phosphorylation of GlgE led to a two orders of magnitude reduction in catalytic efficiency in vitro. The activities of phosphoablative and phosphomimetic GlgE derivatives, where each phosphorylation site was substituted with either Ala or Asp residues, respectively, correlated with negative phosphoregulation. Complementation studies of a M. smegmatis glgE mutant strain with these GlgE derivatives, together with both classical and chemical forward genetics, were consistent with flux through the GlgE pathway being correlated with GlgE activity. We conclude that the GlgE pathway appears to be negatively regulated in actinomycetes through the phosphorylation of GlgE by PknB, a mechanism distinct from that known in the classical glycogen pathway. Thus, these findings open new opportunities to target the GlgE pathway therapeutically.

Tuberculosis (TB)¹ is caused by Mycobacterium tuberculosis and remains a major threat to global health, claiming the life of two million individuals annually (1). The ability to control the TB pandemic is limited by a lack of new therapeutic agents and the rapid emergence of multi- and extensively drug-resistant M. tuberculosis strains, which are essentially untreatable at this time. The development of new drugs targeting resistant strains is clearly now a priority.
In searching for new vulnerable processes in *M. tuberculosis* to enable the development of more efficient anti-TB chemotherapies, a novel antimiycobacterial drug target has recently been discovered. GlgE is an essential maltosyltransferase that elongates α-glucans. Kalscheuer et al. characterized this novel carbohydrate-active enzyme and defined its role within a new non-classical primary metabolic pathway for α-glucan biosynthesis (2). This four-step pathway comprises the TreS, Pep2, GlgE and GlgB enzymes for the conversion of trehalose into a branched α-glucan that resembles glycogen (3). A key enzyme in this pathway is the essential GlgE maltosyltransferase that uses maltose 1-phosphate (M1P) as a donor substrate to generate the linear glucan backbone that is further branched by the GlgB enzyme. Moreover, the GlgE pathway is of particular interest since it is feasible that it is associated with virulence and persistence of *M. tuberculosis* as it might participate in the formation of capsular α-glucan, an extracellular peri-cell wall component potentially involved in immune evasion (4-7). Surprisingly, the lethality associated with targeting GlgE is not due to the absence of product formation but is correlated with the hyper-accumulation to a toxic level of the GlgE donor substrate, M1P, which leads directly or indirectly to pleiotropic effects, toxicity, DNA damage and cell death (2). This novel mode of killing by self-poisoning, together with the lack of GlgE in humans, makes it an attractive candidate for future anti-tubercular drugs (8).

The primary metabolic GlgE pathway combines gene essentiality with a synthetic lethal interaction with another α-glucan pathway defined by the glycosyl transferase Rv3032 (2). In addition, there is a complex interplay between the GlgE and Rv3032 pathways with the classical GlgA-GlgC glycogen pathway in mycobacteria such that they appear collectively to produce three types of α-glucans; cytosolic glycogen, capsular α-glucan and methylglucose lipopolysaccharide (3,7,9). Therefore, each pathway is likely to be tightly regulated. To date, only the allosteric regulation of GlgC by metabolites has been reported (10).

To overcome the stressful conditions imposed by a host, pathogens have evolved various protective and offensive responses generally achieved through cascades of phosphorylation reactions. Many of the stimuli encountered by *M. tuberculosis* are transduced via sensor kinases in the membrane, allowing the pathogen to adapt for survival in hostile environments. In addition to the classical two-component systems, *M. tuberculosis* contains 11 eukaryotic-like serine/threonine protein kinases (STPKs) (11,12). There is now an increasing body of evidence suggesting that many STPKs in *M. tuberculosis* are involved in regulating metabolic processes, transport of metabolites, cell division and virulence (13,14). Signalling through Ser/Thr phosphorylation has recently emerged as a key regulatory mechanism in pathogenic mycobacteria (13,14). This idea is supported by recent studies elucidating the network of post-translational modifications of complex metabolic pathway such as mycolic acids biosynthesis, where most enzymes are regulated by Ser/Thr phosphorylation (15-20).

This study was undertaken to establish whether GlgE is regulated by phosphorylation in *M. tuberculosis*, with the potential to provide new approaches to target it therapeutically. To this end, we have identified GlgE as a new substrate of *M. tuberculosis* STPKs and identified its phosphorylation sites. This allowed us to address the role and contribution of phosphorylation in regulating the maltosyltransferase activity of GlgE. Importantly, with both *in vitro* and *in vivo* approaches, we provide for the first time evidence that phosphorylation will negatively regulate flux through the GlgE pathway.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains and growth conditions.** Strains used for cloning and expression of recombinant proteins were *E. coli* 10G (Lucigen) and *E. coli* BL21(DE3)Star (Novagen) as detailed in Table S1. They were grown in LB medium at 37 °C. Media were supplemented with ampicillin (100 µg/ml), hygromycin (200 µg/ml), kanamycin (25 µg/ml) or spectinomycin (100 µg/ml) when required. Mycobacteria strains were grown aerobically on Middlebrook 7H10 agar plates with OADC enrichment (Difco) or in Middlebrook 7H9 medium supplemented with 10% (v/v) OADC enrichment, 0.5% (v/v) glycerol and 0.05% (v/v) Tyloxapol. Hygromycin (50 µg/ml) and kanamycin (25 µg/ml) were added for the selection of appropriate strains. Validamycin A (Research Products International Corp., Mount Prospect, IL) was used at 10 mM for inhibiting TreS activity when required.

**Cloning, expression and purification of *M. tuberculosis* GlgE and mutant proteins.** The glgE gene was amplified by PCR using *M. tuberculosis* H37Rv chromosomal DNA as the template and the forward and reverse primers listed in Table S2 containing *NdeI* and *BamHI*
Cloning, expression and purification of Mycobacterium smegmatis GlgE<sup>Mc</sup> and PknB<sup>Mc</sup>, and Streptomyces coelicolor GlgE<sup>Sc</sup> and AfsK.

The glgE gene from <i>M. smegmatis</i> was amplified by PCR using <i>M. smegmatis</i> mc<sup>2</sup>155 chromosomal DNA as the template and the forward and reverse primers containing NdeI and BamHI restriction sites (Table S2), respectively. The amplified product was digested with NdeI and BamHI, and ligated into the pETPhos plasmid that was previously digested with NdeI and BamHI, resulting in pETPhos <i>pknB<sup>Mc</sup></i>, which was used to prepare His-tagged PknB<sub>Ms</sub>. The glgE isoform I gene from <i>S. coelicolor</i> (Sco5443) was amplified using <i>S. coelicolor</i> M145 chromosomal DNA as the template with the forward and reverse primers containing NdeI and BamHI restriction sites (Table S2), respectively. The amplified product was digested with NdeI and BamHI, and ligated into pETPhos, thus generating pETPhos <i>glgE<sup>Sc</sup></i>, which was used to prepare Histagged GlgE<sub>Sc</sub>. The afsK kinase domain open reading frame from <i>S. coelicolor</i> was similarly amplified with the forward and reverse primers (Table S2) containing BamHI and HindIII restriction sites, respectively. The amplified product was digested with BamHI and HindIII, and ligated into the pGEX(M) vector, yielding pGEX <i>afsK</i>, which was used for the production of GST-tagged AfsK, as previously described (23).

**In vitro kinase assay.** In vitro phosphorylation was performed with 4 µg of wild-type GlgE or GlgE derivatives in 20 µl of buffer P (25 mM Tris-HCl, pH 7.0, containing 1 mM DTT, 5 mM MgCl<sub>2</sub> and 1 mM EDTA) with 200 µCi/ml [γ-<sup>33</sup>P]ATP (PerkinElmer, 3000 Ci/mmol), and 2 to 4 µg of kinase in order to obtain the optimal autophosphorylation activity for each mycobacterial kinase for 30 min at 37 °C. For in vitro phosphorylation with PknB<sub>Mc</sub> or AfsK, 3 µg of kinase were used. Each reaction mixture was stopped by addition of an equal volume of 5 × Laemmli buffer and the mixture was heated at 100 °C for 5 min. After electrophoresis, gels were soaked in 16% TCA for 10 min at 90 °C, and dried. Radioactive proteins were visualized by autoradiography using direct exposure to films.

**Mass spectrometry analysis.** Purified His-tagged hyper-phosphorylated GlgE (GlgE-P) from the <i>E. coli</i> strain carrying pDuet <i>glgE</i> and co-expressing the PknB kinase domain, was subjected to mass spectrometry without further treatment. Subsequent mass spectrometric analyses were performed as previously reported (16,20).

**Overexpression of GlgE and a derivative in <i>M. bovis</i> BCG and their purification.** <i>M. tuberculosis</i> glgE and glgE <i>Ala</i> genes were amplified from the corresponding pETPhos vector constructs and cloned into the shuttle vector pVV16 (24) using the primers listed in Table S2. The resulting constructs pVV16 <i>glgE</i> and pVV16 <i>glgE Ala</i> were electroporated into <i>M. bovis</i> BCG. Transformants were grown and used for the purification of the His-tagged GlgE...
proteins as described above. The purified recombinant proteins were used for immunoblotting using anti-phosphothreonine, anti-phosphoserine and anti-phosphotyrosine antibodies according to the manufacturer’s instructions (Invitrogen) and revealed with secondary antibodies labeled with IRDye infrared dyes (Odyssey Classic) to increase the detection sensitivity of phosphorylated proteins.

Enzymatic assays. Before assaying for enzyme activity, proteins were further purified using size exclusion chromatography using an Superdex S200 16/60 column (Pharmacia Biotech, Amersham, United Kingdom) with 20 mM Tris buffer, pH 8.5, containing 100 mM NaCl at 1 mL/min. Fractions containing GlgE were pooled, dialysed against 20 mM Tris, pH 8.5, concentrated to ~2.3 mg/mL and stored in aliquots at -80 °C. GlgE specific activity was monitored at 21°C using an end-point assay involving inorganic phosphate release detection with malachite green (2). Reaction mixtures of 25 µL comprised 1 mM maltohexaose, 0.25 mM M1P, 100 mM Bis-Tris propane, pH 7.0, and 50 mM NaCl. Enzyme concentrations were such to allow reactions to progress linearly for 30 min with total donor consumption being < 40%. Reactions were quenched with 175 µL of malachite green and incubated for 20 min at 21 °C and the absorbance at 630 nm was measured on a SpectraMAX Plus microplate spectrophotometer using SOFTmax PRO 3.1.1 software. The affinity for maltohexaose was determined using this method with a 0-10 mM maltohexaose concentration range and 0.25 mM M1P. The affinity for M1P was determined at 37 °C with a 0.25-5 mM M1P concentration range and 1 mM maltohexaose using a modified method allowing the monitoring of initial rates as previously described (2).

Circular dichroism spectroscopy. Circular dichroism spectra were collected on a Jasco J-710 spectropolarimeter. Samples comprised 10 mM sodium phosphate buffer, pH 7.0, and 0.5 mg/mL protein. Far-UV (180-260 nm) spectra were collected using a 0.02 mm quartz cuvette. Spectra were recorded with a sensitivity of 100 mdeg, 0.2 nm data pitch, 4 s response time and a 1.0 nm bandwidth at a scan speed of 20 nm/min. Triplicate spectra were averaged followed by the subtraction of buffer only control spectra. Far-UV spectra were analysed with Dichroweb (25) using the CDSSTR algorithm and reference set 1 (26).

Complementation studies. The M. smegmatis mc²7124 (ΔglgE) strain (2) was transformed with either pVV16, pVV16_glgE, pVV16_glgE_Ala or pVV16_glgE_Asp (Table S1) that were constructed as follows. The glgE derivatives (lacking the stop codon) were amplified using the corresponding pETPhos constructs as the template with the primers listed in Table S2, and cloned into the pVV16 vector digested with Ndel/HindIII. The resulting constructs were electroporated into M. smegmatis mc²7124. Clones selected on kanamycin were grown in 7H9 medium at 37 °C to mid-log phase and plated at 37 °C for 3-5 days on Middlebrook 7H10 agar plates with OADC enrichment plus 1 mM of trehalose and/or 10 mM Validamycin A (Research Products International Corp., Mount Prospect, IL) when required. Moreover, transformants were grown in similar growth conditions and Histagged GlgE proteins were purified as described above.

RESULTS

GlgE is phosphorylated in vitro by the mycobacterial Ser/Thr kinase PknB. The M. tuberculosis genome encodes eleven Ser/Thr protein kinases (STPKs) (11,12). While these mycobacterial kinases appear to be involved in different key pathways such as cell wall metabolism, antibiotic susceptibility, and virulence (13,14,27,28), little is known about the nature of the substrates that are phosphorylated. Due to our interest in Ser/Thr kinase regulation and central metabolism in M. tuberculosis, we investigated whether the newly discovered GlgE metabolic pathway for non-classical glucan biosynthesis is regulated by Ser/Thr phosphorylation. This was first investigated in vitro in the presence of purified STPKs. The soluble kinase domains of several transmembrane kinases from M. tuberculosis were expressed as His-tagged fusion proteins and purified from E. coli as reported earlier (19). The kinase enzymes were incubated with M. tuberculosis GlgE and [γ-³³P]ATP, the proteins were resolved by SDS-PAGE and the protein phosphorylation status was analyzed by autoradiography. The presence of an intense radioactive signal indicated that GlgE was phosphorylated by PknB (Fig. 1A). No signal was observed in the presence of PknA, PknD, PknE or PknF, all of which displayed autokinase activities as reported earlier (19). As expected, no radioactive band was observed in the absence of kinase. These results clearly indicate that GlgE interacts with and is specifically phosphorylated by PknB, at least in vitro, suggesting that this key enzyme of glucan biosynthesis could be regulated by phosphorylation in mycobacteria.
GlGE is phosphorylated on serine and threonine residues. Mass spectrometry was used to identify the nature and location of the phosphorylation site(s) on *M. tuberculosis* GlGE. Such a method has been successfully used to elucidate the phosphorylation sites in a sequence-specific fashion for several *M. tuberculosis* STPK substrates (15-17,20,29,30). Phosphorylated GlGE-P was purified from *E. coli* co-expressing PknB and GlGE (pDuet_glgE) and subjected to mass spectrometric analysis after tryptic digestion. A sequence coverage of 95% that included all Ser and Thr residues was obtained. Spectra were analyzed with the paragon algorithm from the ProteinPilot® 2.0 database-searching software (Applied Biosystems) using the phosphorylation emphasis criterion against a homemade database that included the sequences of GlGE and its derivatives. The phosphopeptides identified by the software were then validated by manual examination of the corresponding MS/MS spectra. Manual validations were performed based on neutral loss of H$_3$PO$_4$ from the precursor ions and the assignment of major fragment ions to b- and y-ion series or to the corresponding neutral loss of H$_3$PO$_4$ from these fragment ions. The MS/MS spectra unambiguously identified the presence of seven phosphate groups on peptides (Table 1 and Fig. S1), thus indicating that GlGE is phosphorylated on six threonines, Thr10, Thr59, Thr148, Thr191, Thr193, Thr370, and on a single serine, Ser85.

Then, to prevent *in vitro* phosphorylation, these residues were mutated to alanine. The corresponding phosphoablative GlGE_T10A/T59A/S85A/T148A/T191A/T193A/T370A mutant (GlgE_Ala) was expressed as a His-tagged protein in *E. coli* BL21(DE3)Star harboring pETPhos_glgE_Ala. The resulting GlGE_Ala mutant protein was purified, analyzed by circular dichroism to confirm its proper folding (see below and Table S3), and incubated with PknB in the presence of [γ-32P]ATP. Following separation by SDS-PAGE and analysis by autoradiography, total abrogation of the phosphorylation signal occurred when compared to the wild-type GlGE (Fig. 1B). As expected, GlGE_Ala was phosphorylated by neither PknA, PknD, PknE nor PknF (Fig. S2). These results suggest that specific phosphorylation of these seven residues by PknB may play a role in the regulation of *M. tuberculosis* GlGE activity.

GlGE is phosphorylated in mycobacteria. To address the relevance of *in vitro* phosphorylation, we next investigated the *in vivo* phosphorylation status of *M. tuberculosis* GlGE in overexpressing strains of *Mycobacterium bovis* BCG, a species frequently used as a non-virulent surrogate strain closely related to *M. tuberculosis*, by western blotting using either anti-phosphothreonine, anti-phosphoserine or anti-phosphotyrosine antibodies (20). First, the specificity of the antibodies was determined using the GlGE isoforms purified from either *E. coli* or *E. coli* co-expressing PknB, based on the strategy described by Molle *et al.* (2010) (22). Only the phosphorylated GlGE isoform derived from pDuet_glgE (GlgE-P) reacted with the anti-phosphothreonine and anti-phosphoserine antibodies as expected, while the unphosphorylated GlGE isoform derived from the pETPhos_glgE construct (GlGE) failed to generate a signal with these antibodies (Fig. 2). To address the phosphorylation state of GlGE in mycobacteria, the genes encoding GlGE or GlGE_Ala (harboring Ser/Thr to Ala substitutions at all seven positions) as C-terminal His-tag fusions were cloned into the shuttle vector pVV16, and the resulting constructs, designated pVV16_glgE and pVV16_glgE_Ala, respectively, were introduced into *M. bovis* BCG. The His-tag-purified proteins derived from these *M. bovis* BCG recombinant strains were subjected to western blotting using anti-phosphothreonine, anti-phosphoserine and anti-phosphotyrosine antibodies. As shown in Fig. 2, a clear signal for the wild-type GlGE was detected with anti-phosphothreonine and anti-phosphoserine antibodies, while no signal was observed with the GlGE_Ala (Fig. 2). No signal could be detected when probing the membrane with anti-phosphotyrosine antibodies (data not shown). Overall, these results not only confirm the presence of phosphorylated Ser and Thr residues only, but also demonstrate that GlGE is phosphorylated in mycobacteria.

Phosphorylation of GlGE is conserved in other actinomycetes. There is a high degree of sequence identity between the GlGE of *M. tuberculosis* and those of *M. smegmatis* (79%) and *S. coelicolor* (54%). However, not all of the phosphorylation sites identified for *M. tuberculosis* GlGE were fully conserved among GlGE orthologues (Fig. S3). We therefore wondered whether the GlGE proteins from these other actinomycetes are phosphorylated as well. Thus, the different GlGE orthologues were expressed in *E. coli*, purified and subjected to *in vitro* kinase assays in the presence of their corresponding kinase counterparts. Fig. 3 clearly shows that the GlGE isofrom I from *S. coelicolor* (GlGE$^{Sc}$) and *M. smegmatis* (GlGE$^{Ms}$) could be
Phosphorylation negatively regulates GlgE maltosyltransferase activity. Phosphorylation of metabolic enzymes can be associated with either positive or negative regulation. To test whether phosphorylation affects the maltosyltransferase activity of GlgE, we compared the activity of non-phosphorylated and phosphorylated GlgE. Recombinant His-tagged M. tuberculosis GlgE from E. coli carrying pETPhos GlgE was used as a source of non-phosphorylated GlgE (designated GlgE) as confirmed by western blot analysis (Fig. 2) and mass spectrometry (data not shown). By contrast, when co-expressed with PknB with the pDuet system, the purified protein was found to be substantially phosphorylated (designated GlgE-P; Fig. 2). Circular dichroism spectroscopy indicated that the net secondary structure of GlgE was not significantly affected by phosphorylation (Table S3) and was consistent with the known crystal structure of the S. coelicolor isoform I enzyme (31). It was then possible to determine the kinetics of the maltosyltransferase activity of GlgE and GlgE-P by monitoring the release of inorganic phosphate during the extension of maltohexaose by M1P (2). Phosphorylation of GlgE led to a 31-fold decrease in \( k_{cat}^{\text{app}}/K_m^{\text{app}} \) for maltohexaose by M1P (Table 2), clearly demonstrating that the enzyme was negatively regulated. The dependence of GlgE-P activity on M1P concentration did not give a normal saturation curve because activity decreased markedly above 1 mM M1P and this behavior did not adhere to normal substrate inhibition kinetics. Nevertheless, the maximum rate observed at 1 mM M1P was 63-fold lower than the corresponding \( k_{cat}^{\text{app}}/K_m^{\text{app}} \) observed with the wild-type enzyme, consistent with a reduction in enzyme activity by two orders of magnitude as a consequence of phosphorylation.

Phosphomimetic GlgE has reduced enzyme activity. In order to test the effect of GlgE activity in vivo, M. tuberculosis GlgE variants with constitutively altered activities were required. Earlier studies have shown that the acidic amino acids, Asp or Glu, mimic the functional effect of phosphorylation (18,20,32) while we have shown that Thr substitution by Ala prevents phosphorylation. Following a strategy successfully used to demonstrate the role of phosphorylation on Ser/Thr kinase substrates in other systems (33), we expressed and purified phosphomimetic GlgE_Asp and phosphoablative GlgE_Ala, where each phosphorylation site was mutated to mimic or ablate phosphorylation, respectively.

The purified GlgE_Asp protein was atypical because size exclusion chromatography revealed that it was present not only as a dimer, the exclusive quaternary structure observed with all other GlgE derivatives, but also as a monomer in roughly equal proportions (Fig. S4). Circular dichroism spectroscopy showed that the distribution of protein secondary structures in both of the dimeric mutant proteins was very similar to that of the wild-type and phosphorylated proteins (Table S3). Monomeric GlgE_Asp was somewhat different because it possessed a little less \( \alpha \)-helix (12% rather than 18% for the wild-type), presumably as a result of the loss of subunit interfaces, but was otherwise similar to wild-type.

With all GlgE derivatives, the reduction in \( k_{cat}^{\text{app}}/K_m^{\text{app}} \) for maltohexaose by M1P with the GlgE_Asp dimer showed a 2 and 3-fold reduction, respectively, compared with GlgE_Ala (Table 2). The activity of monomeric GlgE_Asp was even lower with a 2.5 and 10-fold reduction, respectively, compared with GlgE_Ala. GlgE_Asp would most probably reflect intermediate values of ~2 to 7-fold. Although this reduction was not of the same magnitude as that between GlgE and GlgE-P, the strategy was nevertheless successful in terms of generating GlgE derivatives with a range of activities that could not be modified further by PknB.
for maltohexaose actually increased a little. No significant change in the affinity for the alternative acceptor, glycogen, was observed (data not shown), again consistent with a $k_{cat}^{app}$ effect. Although the reduction in $k_{cat}^{app}/K_m^{app}$ for the donor substrate, M1P, was also dominated by a lowering of $k_{cat}^{app}$, there was some contribution from an increase in the $K_m^{app}$ for M1P.

**GlgE_Asp does not complement a ΔglgE mutant strain.** To investigate the effect of GlgE activity in vivo, we took advantage of a ΔglgE *M. smegmatis* (m^2^7124) strain that had been generated previously (2). In contrast to GlgE essentiality in *M. tuberculosis*, null deletion of the *glgE* gene could be readily generated in *M. smegmatis* on minimal medium (2). Despite normal growth in minimal medium, the *M. smegmatis* ΔglgE mutant is unable to grow in complex media and is sensitive to the disaccharide trehalose (α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside) (2). Thus, the *M. smegmatis* ΔglgE mutant is not able to grow in minimal medium in the presence of exogenous trehalose. Trehalose-induced bacteriostasis in the *M. smegmatis* ΔglgE mutant correlates with hyper-accumulation of the GlgE substrate, M1P, which is formed from trehalose by the successive action of the trehalose synthase TreS and the maltokinase Pep2, and which becomes toxic at very high intra-cellular levels (2). *M. smegmatis* ΔglgE was therefore transformed with either the empty pVV16 vector or the pVV16 derivatives, allowing constitutive expression of the different *glgE* alleles in mycobacteria together with selection for kanamycin resistance. Cultures were then plated and grown at 37 °C in presence of 1 mM trehalose for 4-5 days. Fig. 4A shows that cells transformed with constructs carrying either the *glgE* or *glgE_Ala* alleles could grow on trehalose, indicating that functional complementation occurred, suggesting that the GlgE wild-type and GlgE_Ala isoforms were sufficiently active to metabolize M1P, preventing the trehalose-induced toxic effect. In sharp contrast, the *glgE_Asp* allele failed to restore growth on trehalose, showing that the reduction in activity of the GlgE_Asp isoform was severe enough to exhibit a phenotype in vivo (Fig. 4A). These phenotypes could not be attributed to alteration in the expression levels of the various GlgE variants, as demonstrated by SDS-PAGE analysis with Coomassie Blue staining following purification of the His-tagged proteins from the complemented *M. smegmatis* ΔglgE strains (Fig. 4B). We also noticed that the *M. smegmatis* GroEL1 protein was co-purified in each preparation, presumably due to the presence of a known His-rich region capable of binding to the Ni-bearing beads, as reported earlier (34). This inadvertently provided an internal standard supporting the conclusion that there were similar expression levels of the GlgE variants. Western blots with anti-His-tag antibody of crude extracts gave further support for the comparable expression levels of each GlgE variant (data not shown). The rate of proteolytic digestion of the GlgE variants was similar in each case (Fig. S5) with a small difference in the proteolytic cleavage pattern with the GlgE_Asp variant being rationalized as one of the Asp substitutions affecting one of the predicted cleavage sites. Therefore, the integrity of each GlgE variant appeared to be maintained in *M. smegmatis*. Attempts to measure GlgE activity in these strains were hampered by the deliberately low level of expression of each variant compounded by a significant background phosphatase activity interfering with the enzyme assay.

Finally, to correlate the trehalose-sensitive phenotype of the *M. smegmatis* ΔglgE mutant expressing GlgE_Asp with M1P hyper-accumulation, experiments were conducted under conditions where M1P-formation was suppressed by genetic or chemical inactivation of TreS trehalose synthase activity, as previously demonstrated with the ΔglgE mutant (2). First, we used an *M. smegmatis* ΔglgEΔtreS mutant strain that was transformed with the different pVV16 *glgE* derivatives. The *glgE_Asp* allele could be readily introduced into the double ΔglgEΔtreS mutant in the presence of trehalose (Fig. 5A), supporting the direct link between the GlgE_Asp lethal phenotype on trehalose (Fig. 4A) and the significantly reduced maltosyltransferase activity of this isoform (Fig. 6). Second, using an alternative to the genetic approach, TreS was chemically inhibited with Validamycin A to prevent M1P toxicity (Fig. 6), as previously demonstrated (2). As expected, it was possible to introduce the *glgE_Asp* allele into the ΔglgE mutant strain in the presence of trehalose and Validamycin A (Fig. 5B). As expected, an empty-vector control also allowed growth when TreS was blocked genetically or chemically, consistent with GlgE_Asp leading to lethality only through M1P toxicity (Fig. 4 and 5). Overall, these results show that introduction of the *glgE_Asp* GlgE allele into *M. smegmatis* ΔglgE cannot restore metabolic flux through the GlgE pathway in the presence of trehalose (Fig. 6), leading to mycobacterial death. Thus, both classical and chemical reverse genetic approaches are consistent with flux through the GlgE pathway.
correlating with GlgE activity.

DISCUSSION

We have used a combination of genetic and biochemical approaches to provide the first reported evidence that the M1P-dependent maltosyltransferase GlgE, a recently identified and genetically validated anti-tuberculosis target (8), is negatively regulated by phosphorylation. M. tuberculosi s GlgE was shown to be phosphorylated both in vitro and in vivo, showing that GlgE was missed in a recent phosphoproteomic study performed on M. tuberculosis (35). One serine (Ser85) and six threonine (Thr10, Thr59, Thr148, Thr191, Thr193 and Thr370) residues were phosphorylated specifically by the Ser/Thr kinase PknB. This resulted in a reduction in enzyme activity in vitro that correlated with lethality in vivo with a GlgE phosphomimetic implying a toxic build-up of GlgE’s substrate, M1P, as previously observed for a GlgE null mutant (8). Therefore, the GlgE α-glucan pathway appears to be regulated by the STPK-dependent phosphorylation of GlgE, consolidating the emerging theme of Ser/Thr phosphorylation playing a critical role in the regulation of anabolic pathways in mycobacteria (Fig. 6) (13,14). We have also shown that the phosphorylation of GlgE occurs not only in M. tuberculosis but also in other actinomycetes such as Streptomyces. Importantly, phosphoregulation of the GlgE pathway is distinct from the regulation of the classical GlgC/GlgA-dependent glycogen biosynthetic pathway that involves allosteric regulation by metabolic intermediates (10).

The regulation of the GlgE pathway through inhibition of the GlgE-catalysed step raises an important question because blocking this step has been shown to be lethal in M. tuberculosis (8). The implication is that there is at least one other upstream step within the glucan pathway that is co-regulated to prevent the buildup of M1P to toxic levels in M. tuberculosis. Alternatively, the extent to which GlgE is down-regulated is limited to fine-tuning perhaps in coordination with the regulation of the GlgA-GlgC and Rv3032 pathways. Interestingly, a temperature-sensitive GlgE mutant in M. smegmatis has been reported to affect glycogen/α-glucan recycling and growth rate (36). Particularly noteworthy was the observation that the temperature-sensitive mutation was suppressed by the over-expression of GarA (36), a forkhead-associated domain protein that is known not only to regulate both carbon and nitrogen metabolism but also to be a substrate for PknB (37). Perhaps the over-expressed GarA acted as a decoy, suppressing the phosphorylation and down-regulation of an already compromised temperature-sensitive GlgE by PknB. Alternatively, GarA interacts directly with GlgE in some way.

There is no known structure of M. tuberculosis GlgE but there is one for S. coelicolor GlgE isoform I (31). In order to establish where the phosphorylation sites are, it was possible to map their locations on the known structure (Fig. S6) with reference to a sequence alignment (Fig. S3). All sites were solvent exposed, as expected. Three were within the S-domain, for which there is currently no confirmed role, three within the N-domain, a domain making subunit interfaces, and one within the B-domain, involved in defining the active site. Although the sites within the N-domain could have been responsible for the dissociation of the normally dimeric GlgE Asp, dissociation was not observed with GlgE-P, suggesting the dissociation of GlgE-Asp was due to an artefact. None of the phosphorylation sites are located within known substrate binding sites. Indeed, studies on the activity of single-site phosphomimetic GlgE derivatives suggest that no single site is solely responsible for the down-regulation of activity, suggesting that a cooperative inactivation involving several sites is required (K. Syson and S. Bornemann, unpublished observations). The phosphorylation-dependent reduction in GlgE activity appears to be primarily a result of the lowering of $k_{cat}^{app}$ (Table 2). This could therefore be a result of long range electrostatic effects on catalytic intermediates. Any contribution of a lowering of the affinity for M1P could also be a result of long range electrostatic effects on the binding of this negatively charged substrate. Why GlgE-P is inhibited at high M1P concentrations is currently unknown. More structure/function work on GlgE-P and GlgE phosphomimetic is therefore required to establish the physical and structural basis for phosphoregulation.

Our work suggests that a shift in the unphosphorylated/phosphorylated GlgE balance in favor of the phosphorylated form rapidly leads to the accumulation of toxic M1P levels, leading to mycobacterial death. Therefore, an elegant hypothesis arising from the present work is that by increasing the activity of the PknB kinase it may be possible to alter mycobacterial growth, opening new opportunities for future anti-tuberculosis drug development. Indeed, small molecules that modulate the activity of STPKs in general may be of great therapeutic value in inhibiting M.
tuberculosis growth. Bryostatin, a natural product synthesized by a marine bacterium, which activates eukaryotic intracellular STPKs (38), is one such molecule. Interestingly, bryostatin acts directly on the Bacillus subtilis Ser/Thr kinase PrkC which contains an extracellular domain able to bind to peptidoglycan fragments, and this signals the bacteria to exit dormancy by stimulating germination (39). PrkC, like M. tuberculosis PknB, possesses the PASTA (penicillin and Ser/Thr kinase associated) domains, which are found in the extracellular portion of membrane-associated STPKs and which have been proposed to bind to peptidoglycan and act as signaling molecules. In this context, bryostatin or other STPK-activating molecules, along with the recent structural determination of the M. tuberculosis PknB PASTA domains (40), may provide new therapeutic strategies to be developed against tuberculosis. Alternatively, if and when a phosphatase has been identified which dephosphorylates GlgE, it could also be a potential target.

In conclusion, the present study provides a foundation for further investigation of an important functional linkage between STPKs and the GlgE α-glucan biosynthetic pathway, which may contribute to the generation of the mycobacterial capsule. An abundant surface-exposed M. tuberculosis capsular α-glucan has been identified as a novel ligand for the C-type lectin DC-SIGN (41). Binding of α-glucan to DC-SIGN stimulated the production of immunosuppressive IL-10 by LPS-activated monocyte-derived dendritic cells. This observation suggests that the α-glucan capsule may fulfill an important role in pathogenesis. Recent work has demonstrated that the surface-exposed glucan plays an important role in the virulence of these bacteria and particularly in the persistence of infection in mice (7). While there is still much to understand about the interplay between the three α-glucan pathways and three α-glucan products in mycobacteria, the present work provides a conceptual advance in our understanding of the metabolic adaptation and regulatory mechanisms associated with the recently identified GlgE α-glucan pathway. Although challenging, future studies will help identify extracellular cues sensed by kinases leading to the phosphorylation of GlgE and other glucan pathway enzymes. This will not only allow us to understand how M. tuberculosis senses its environment and mediates its response in a coordinated manner to regulate α-glucan biosynthesis, but also establish whether there is a link between GlgE phosphorylation and the establishment of the non-replicating persistent state.

REFERENCES


**FOOTNOTES**

1 Abbreviations used in the text: *M. tuberculosis*, *Mycobacterium tuberculosis*; TB, tuberculosis; STPK, Ser/Thr protein kinase; M1P, maltose 1-phosphate.

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**FIGURE LEGENDS**

**Figure 1.** (A) *In vitro* phosphorylation of *M. tuberculosis* GlgE by PknB. The soluble domains of five recombinant *M. tuberculosis* STPKs (PknA to PknF) were expressed and purified as His-tagged fusions and incubated with purified His-tagged GlgE and [γ-32P]ATP. The amount of the STPKs used varied from 2 to 4 µg in order to obtain the optimal autophosphorylation activity for each kinase. Samples were separated by SDS-PAGE, stained with Coomassie Blue (upper panel) and visualized by autoradiography after overnight exposure to a film (lower panel). Upper bands reflect the phosphorylation signal of GlgE and the lower bands correspond to the autophosphorylation activity of each kinase. (B) *In vitro* phosphorylation of the GlgE_Ala mutant. Purified GlgE and phosphoaiblative GlgE (GlgE_Ala) were incubated with PknB and [γ-32P]ATP. Samples were separated by SDS-PAGE, stained with Coomassie Blue (upper panel) and visualized by autoradiography (lower panel) after overnight exposure to a film.

**Figure 2.** Phosphorylation of GlgE in mycobacteria. *E. coli* harboring pETPhos_glgE was used as a source of non-phosphorylated GlgE (GlgE), and the strain harboring pDuet_glgE coexpressing PknB and GlgE provided the phosphorylated GlgE isoform (GlgE-P). GlgE and GlgE_Ala were produced in *M. bovis* BCG strains harboring pVV16_glgE or pVV16_glgE_Ala, respectively. Three µg of purified His-tagged GlgE derivatives were analyzed by SDS-PAGE after staining with Coomassie Blue (upper panel), and detected on independent SDS-PAGE gels by immunoblotting using anti-phosphothreonine (middle panel) or anti-phosphoserine (lower panel) antibodies according to the manufacturer (Invitrogen) and revealed with secondary antibodies labeled with IRDye infrared dyes (Odyssey Classic).

**Figure 3.** *In vitro* phosphorylation of the GlgE orthologues from *M. smegmatis* and *S. coelicolor*. Recombinant *M. smegmatis* PknB kinase (PknB Ms) and *S. coelicolor* AfsK kinase (AfsK) were expressed as His-tagged fusion proteins in *E. coli*, purified and incubated with purified His-tagged *M. smegmatis* GlgE (GlgE Sm) and *S. coelicolor* GlgE (GlgE Sc), respectively, in the presence of [γ-32P]ATP. GlgE and PknB proteins from *M. tuberculosis* were included as a positive control. Samples were separated by SDS-PAGE and stained with Coomassie Blue (upper panel) and visualized by autoradiography (lower panel).

**Figure 4.** (A) Functional complementation of *M. smegmatis* ΔglgE with GlgE or GlgE_Ala but not with phosphomimetic GlgE_Asp. The ΔglgE mutant of *M. smegmatis*, which is sensitive to trehalose, was transformed with pVV16, pVV16_glgE, pVV16_glgE_Ala, or pVV16_glgE_Asp and grown with or without trehalose (1 mM). Plates were incubated at 37 °C for 4-5 days. (B) Expression of the different GlgE variants in the complemented *M. smegmatis* ΔglgE strains. GlgE proteins from *M. smegmatis* ΔglgE cultures complemented with the wild-type pVV16_glgE, pVV16_glgE_Ala or pVV16_glgE_Asp constructs were obtained by purification by Ni-affinity chromatography. Equal quantities of purified His-tagged GlgE proteins per unit volume of culture were separated by SDS-PAGE, stained with Coomassie Blue and identified using mass spectrometry. The mycobacterial GroEL1 protein was co-purified due to it harbouring a region rich in histidine residues, thus providing an internal standard.

**Figure 5.** Complementation of the *M. smegmatis* ΔglgE single and ΔglgEΔtreS double mutants with GlgE variants. Both genetic (A) and chemical (B) inactivation of TreS prevent M1P hyper-accumulation in the presence of exogenous trehalose. (A) *Genetic inactivation of treS*. The ΔglgEΔtreS mutant, which is insensitive to trehalose, was transformed with pVV16, pVV16_glgE, pVV16_glgE_Ala, or pVV16_glgE_Asp and grown with 10 mM trehalose. Plates were incubated at 37 °C for 4-5 days. (B) *Chemical inactivation of TreS in presence of Validamycin A*. The ΔglgE mutant was transformed with pVV16, pVV16_glgE, pVV16_glgE_Ala, or pVV16_glgE_Asp and grown in the presence of both Validamycin A (10 mM) and trehalose (1 mM). Plates were incubated at 37 °C for 4-5 days.

**Figure 6.** Proposed regulatory role of the phosphorylated isofom of GlgE as a molecular switch in α-glucan synthesis in *M. tuberculosis*. Distinct from the classic glycogen pathway, the newly identified GlgE pathway comprises four successive enzymatic steps from trehalose to an α-glucan polymer that may contribute to biosynthesis of cytosolic glycogen, capsular glucan and/or methylglucose lipopolysaccharide-related α-glucan derivatives. In response to external stimuli, the mycobacterial Ser/Thr kinase PknB is autophosphorylated. This induces GlgE phosphorylation on seven Ser/Thr residues. As a result, the maltosyltransferase activity of the Ser/Thr phosphorylated GlgE is inhibited.
Table 1. Phosphoacceptors identified after purification of *M. tuberculosis* GlgE from the *E. coli* strain co-expressing *M. tuberculosis* PknB. Sequences of the phosphorylated peptides identified in GlgE as determined by mass spectrometry following tryptic digestion are indicated, and phosphorylated residues (pT or pS) are shown in bold.

<table>
<thead>
<tr>
<th>Phosphorylated tryptic peptide sequence of GlgE purified from pCDFDuet co-expressing PknB</th>
<th>Number of detected phosphate groups</th>
<th>Phosphorylated residue(s)</th>
</tr>
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<tbody>
<tr>
<td>[5-17] AIGTEpTEWWVPGR</td>
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<td>[51-63] EGHEAVAAPTLVVR</td>
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<td>[185-192] TPGDPVpTR</td>
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<td>T191</td>
</tr>
<tr>
<td>[185-211] TPGDPVTRpTALALTPEIEELLADYPLR</td>
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<td>T193</td>
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<td>[361-379] QWFTELPDPpTIAYAENPK</td>
<td>1</td>
<td>T370</td>
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Table 2: Michaelis-Menten kinetic analysis of *M. tuberculosis* GlgE derivatives

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<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>$K_m^{app}$</th>
<th>$k_{cat}^{app}$</th>
<th>$k_{cat}^{app}/K_m^{app}$</th>
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<td></td>
<td></td>
<td>mM</td>
<td>s$^{-1}$</td>
<td>M$^{-1}$ s$^{-1}$</td>
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<td>maltohexaose$^b$</td>
<td>GlgE</td>
<td>5.5 ± 0.5</td>
<td>4.3 ± 0.2</td>
<td>780 ± 80</td>
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<tr>
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<td>GlgE_Ala</td>
<td>2.1 ± 0.2</td>
<td>0.72 ± 0.3</td>
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<td>GlgE_Asp (dimer)</td>
<td>1.6 ± 0.1</td>
<td>0.44 ± 0.01</td>
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<tr>
<td></td>
<td>GlgE_Asp (monomer)</td>
<td>1.64 ± 0.05</td>
<td>0.216 ± 0.002</td>
<td>132 ± 4</td>
</tr>
<tr>
<td></td>
<td>GlgE-P</td>
<td>2.2 ± 0.2</td>
<td>0.053 ± 0.002</td>
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<tr>
<td>M1P$^c$</td>
<td>GlgE</td>
<td>0.25 ± 0.03</td>
<td>1.25 ± 0.06</td>
<td>5,000 ± 600</td>
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<tr>
<td></td>
<td>GlgE_Ala</td>
<td>0.33 ± 0.07</td>
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<td>GlgE_Asp (dimer)</td>
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<td>GlgE_Asp (monomer)</td>
<td>0.9 ± 0.2</td>
<td>0.15 ± 0.01</td>
<td>170 ± 40</td>
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<tr>
<td></td>
<td>GlgE-P</td>
<td>~0.24$^d$</td>
<td>0.020 ± 0.001$^e$</td>
<td>~80$^f$</td>
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</table>

$^a$Enzyme activity was monitored by detecting Pi release in triplicate and values are expressed as the mean and standard error. Quoted constants are apparent because the enzyme obeys ping-pong (substituted enzyme) kinetics (2).

$^b$In the presence of 0.25 mM M1P.

$^c$Maltose 1-phosphate in the presence of 1 mM maltohexaose.

$^d$The GlgE-P enzyme was severely inhibited at concentrations of M1P > 1 mM with kinetics that did not adhere to simple substrate inhibition. Therefore this value reflects the concentration of M1P that gave half the maximum observed rate.

$^e$This value is the maximum observed rate that was with 1 mM M1P (see footnote$^e$ above).

$^f$This value reflects the ratio between the maximum observed rate$^e$ and the concentration of M1P that gave half the maximum observed rate$^f$. 
Figure 1

A

Autoradiogram

Coomassie

B

 Autoradiogram

Coomassie
Figure 2

An#-PhosphoThreonine

GlgE-P

pVV16_glgE_Ala

pVV16_glgE

M (kDa)

150 —

75 —

50 —

37 —

Coomassie

GlgE

Anti-PhosphoThreonine

GlgE

Anti-PhosphoSerine
Figure 3

Autoradiogram

Coomassie

Marker (kDa)

GlgE

PknB

GlgE + PknB

GlgE<sub>Ms</sub>

PknB<sub>Ms</sub>

GlgE<sub>Ms</sub> + PknB<sub>Ms</sub>

GlgE<sub>sc</sub>

GlgE<sub>sc</sub> + AfsK

AfsK

GlgE<sub>Ms</sub> + PknB<sub>Ms</sub>

GlgE

kinase*
**Figure 4**

**A**

*M. smegmatis* mc²155 Δ*glgE*

![Image of bacterial colonies with different plasmids and trehalose conditions]

**B**

<table>
<thead>
<tr>
<th>Marker (kDa)</th>
<th>pVV16</th>
<th>pVV16_ glgE</th>
<th>pVV16_ glgE_Ala</th>
<th>pVV16_ glgE_Asp</th>
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<tr>
<td>130</td>
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<td>🐧</td>
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<td>🐧</td>
<td>🐧</td>
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</tr>
</tbody>
</table>

iciencies
**Figure 5**

**A**

*M. smegmatis* mc²155 Δ*glgEΔtreS*

- pVV16
- pVV16* _glgE*
- pVV16* _glgE_Ala*
- pVV16* _glgE_Asp*

+ trehalose

**B**

*M. smegmatis* mc²155 Δ*glgE*

- pVV16
- pVV16* _glgE*
- pVV16* _glgE_Ala*

+ validamycin A
+ trehalose
Figure 6

Trehalose

TreS

Maltose

Pep2

Maltose 1P

GlgE

GlgE

PstP phosphatase?

Lineral glucan

GlgB

Branched glucan

Cytosolic glycogen and/or capsular glucan and/or methylglucose lipopolysaccharide

eexternal signal

cytosol

Validamycin A