Inorganic Phosphate Limitation Modulates Capsular Polysaccharide Composition in Mycobacteria*

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Mycobacterium tuberculosis is protected by an unusual and highly impermeable cell envelope that is critically important for the successful colonization of the host. The outermost surface of this cell envelope is formed by capsular polysaccharides that play an important role in modulating the initial interactions once the bacillus enters the body. Although the bioenzymatic steps involved in the production of the capsular polysaccharides are emerging, information regarding the ability of the bacterium to modulate the composition of the capsule is still unknown. Here, we study the mechanisms involved in regulation of mycobacterial capsule biosynthesis using a high throughput screen for gene products involved in capsular α-glucan production. Utilizing this approach we identified a group of mutants that all carried mutations in the ATP-binding cassette phosphate transport locus pst. These mutants collectively exhibited a strong overproduction of capsular polysaccharides, including α-glucan and arabinomannan, suggestive of a role for inorganic phosphate (Pi) metabolism in modulating capsular polysaccharide production. These findings were corroborated by the observation that growth under low Pi conditions as well as chemical activation of the stringent response induces capsule production in a number of mycobacterial species. This induction is, in part, dependent on σ factor E. Finally, we show that Mycobacterium marinum, a model organism for M. tuberculosis, encounters Pi stress during infection, which shows the relevance of our findings in vivo.

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‡ This article contains supplemental Tables S1 and S2.

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Despite enormous research efforts in global public health consortia, Mycobacterium tuberculosis, the causative agent of tuberculosis, continues to represent a major health problem that causes up to 1.5 million deaths annually. The chronic character of M. tuberculosis infection, covering one-third of the world’s population, coupled to the increasing insensitivity of the bacillus to many classes of antibiotics calls upon innovative strategies to combat this devastating disease. The unique and complex cell envelope of M. tuberculosis is not only a major virulence factor but also an attractive target for antituberculosis drug discovery. The mycobacterial cell envelope is composed of two membranes: a relatively normal plasma membrane and a membrane that resembles the outer membrane of Gram-negative bacteria. This outer membrane is composed of mycolic acids, partly linked to the arabinogalactan-peptidoglycan layer, in the inner leaflet and a variety of intercalating lipids and glycolipids in the outer leaflet (1–3). The outermost cell surface of mycobacteria is formed by a thick but loosely attached capsular layer that is mainly composed of polysaccharides and a minor amount of proteins and (glyco)lipids (4–8). Recent studies have indicated that capsular polysaccharide of M. tuberculosis is α-glucan, a polymer of linear α-D-1→4-linked glucose units with core substitutions at position 6 on every 5 or 6 residues by α-D-1→4-linked oligoglucosides (7, 9, 10). Recent studies have indicated that capsular α-glucan might play an important role in immune modulation upon entry into the host. For example, capsular α-glucan was shown to block CD1 expression in dendritic cells, suppress IL-12 production, and stimulate IL-10 production in a CD80-dependent manner (11). Additionally, capsular components have antiphagocytic properties that prevent uptake by macrophages (12) and abolish dendritic cell function, a process dependent on C-type lectin DC-SIGN (13). Furthermore, α-glucan interacts with complement receptor 3 (14), and inactivation of glycogen/α-glucan glycosyltransferase GlgA impairs virulence of M. tuberculosis in mice (10). Collectively, these findings suggest that M. tuberculosis capsular α-glucan has an important role in host-pathogen interaction and virulence. However, the exact function of capsular α-glucan in virulence as well as how the production of capsular polysaccharide is modified by the bacillus in response to stress signals is not known.
P₃ Limination Modulates Capsular Polysaccharide Composition

In our ongoing efforts to elucidate the capsular α-glucan biosynthesis routes and transport systems, we recently developed a monoclonal antibody-based screening and quantification method that allows for the comprehensive analysis of large libraries of mutants (n > 40,000) (15). Using this method, we identified several strongly induced capsular α-glucan mutants in the ATP-binding cassette phosphate transport pst locus of Mycobacterium smegmatis and M. tuberculosis (15). The Pst system is important for P₃ acquisition in mycobacteria (16, 17). Rifat et al. (16) showed up-regulation of the phosphate stress response via the pst system of M. tuberculosis. Several studies showed that the two-component system SenX3-RegX3 is involved in P₃ stress signaling (16) in which downstream signaling occurs via the MprAB-SigE-Rel₉₉ pathway (18). Recently, Chuang et al. (19) showed that P₃ stress increases the cell envelope thickness. Taken together, these reports and our own findings link Pi limitation in mycobacteria with capsular biosynthesis regulation.

In this study, we aimed to further characterize the panel of pst mutants that show strongly induced capsular α-glucan production. We demonstrate that P₃ starvation up-regulates the capsular α-glucan production in a SigE-dependent manner but not via the stringent response. Furthermore, we provide evidence that amino acid starvation also can induce the capsular α-glucan.

Experimental Procedures

Bacterial Strains and Growth Conditions—M. smegmatis mc²155, M. tuberculosis strains mc²6020, H37Rv, and CDC1551; and Mycobacterium marinum strains E11 and M–USA were grown at 37 or 30 °C, respectively, in Middlebrook 7H9 liquid medium supplemented with 10% albumin-dextrose-catalase (ADC); BD Biosciences) and 0.05% Tween 80 or in modified 7H9 medium supplemented with different concentrations of exogenous P₃ (0 μM–25 mM) and 10% ADC as described (16). For the serine hydroxamate (SHX) experiments, the bacterial strains were grown in modified 7H9 medium with or without ADC or supplemented with albumin or dextrose (both from Sigma) or a combination of these. When needed, the bacterial strains were grown on 7H10 solid medium supplemented with 10% ADC and Tween 80. For bacterial strains that required selective pressure, kanamycin, hygromycin, or streptomycin was added at a concentration of 50 μg/ml. Escherichia coli strain DH5α was grown at 37 °C on LB agar (Difco).

Transposon Mutagenesis and Capsular α-Glucan Mutant Screen—Transposon mutagenesis and the capsular α-glucan screen were performed as described previously (15). Briefly, the obtained transposon mutant libraries of M. smegmatis and M. tuberculosis (mc²6020) were screened in a double filter assay for altered expression of capsular α-glucan using the colony-based immunoblot approach as described earlier (15). Then the transposon insertion sites of the mutant strains were identified by using ligation-mediated PCR as described previously (21). For the primer sequences used in the ligation-mediated PCR, see supplemental Table S2.

Genetic Constructs and Complementation of pstS::Tn—The genetic knock-out strains of M. tuberculosis H37Rv ΔsigE and ΔrelA and complemented strains were constructed as described previously (22, 23). M. smegmatis MSEG pstS::Tn strain was constructed as described previously (15) and complemented with M. smegmatis pstS. In short, primers were designed (see supplemental Table S2), and the gene was amplified from M. smegmatis genomic DNA by PCR. The PCR products were purified and digested with PstI and HindIII. pSTM3 E. coli-Mycobacterium shuttle vector was purified from E. coli strain DH5α and digested with PstI and HindIII. The digested plasmid and the insert (pstS) were ligated by T4 ligase and subsequently transformed into E. coli XL-10 by heat shock. Next, the transformants were selected on hygromycin-containing LB plates, and the pSTM3 complementation plasmid was confirmed by sequencing. Subsequently, the plasmid was electroporated into M. smegmatis pstS::Tn. The complemented strain was confirmed by PCR (supplemental Table S2). For the construction of the M. marinum phosphate marker strains, the putative promoters of genes pppk1, senX3, and phoS2 were cloned in front of the fluorescent reporter gene mCherry. The promoter regions of pppk1, senX3, and phoS2 were amplified by PCR from M. marinum E11 genomic DNA with forward and reverse primers containing an XbaI and BamHI site, respectively. The XbaI- and BamHI-digested insert and pSMT3 vector were ligated by T4 ligase (Fermentas) to generate the genetic constructs pSTM3::Prv2983-mCherry, pSTM3::Pppk1-mCherry, pSTM3::PsenX3-mCherry, and pSTM3::PphoS2-mCherry. The constructs were introduced into wild-type M. marinum M–USA by electroporation. To quantify the bacterial infection in the zebrabfish experiments, mEos3.1 was integrated in the genome using L5 integrase. Briefly, mEos3.1 was obtained from Zhang et al. (24) and amplified by PCR with flanking regions corresponding to the target vector. The primers were designed to amplify hsp60-mEos3.1 with 15 bp overlapping the target vector (supplemental Table S2). The vector pMV361 was digested with PmlI and NsiI. Subsequently, the insert was introduced into the digested vector by In-Fusion cloning (Clontech) to produce the integrative plasmid pMV-hsp60-mEos3.1 as described previously (25). The plasmid was subsequently transformed into M. marinum M–USA containing the genetic constructs as describe above. All primers that were used are listed in supplemental Table S2.

SHX Experiments—M. smegmatis mc²155 and M. tuberculosis mc²6020 and H37Rv were grown as described above, and at the early log phase (A₆₀₀ = ±0.5), 100–150 μg/ml or a concentration series of 0–250 μg/ml SHX (Sigma) was added for the induction of the stringent response. At different time points, capsular polysaccharides were extracted and analyzed as described below.

Capsule Polysaccharide Extraction—M. smegmatis, M. tuberculosis (H37Rv, CDC1551, and mc²6020), and M. marinum strains were grown in liquid or solid medium as described above, and capsular extraction was performed as described previously (15).

4 The abbreviations used are: ADC, albumin-dextrose-catalase; SHX, serine hydroxamate; AM, arabinomannan; PBS, phosphate-buffered saline; Tween 80; dpi, days postinfection.
Briefly, cells were diluted to equal amounts of \( A_{600} \) units in liquid medium, and 900 \( \mu l \) of cell suspension was collected and supplemented with 100 \( \mu l \) of 5% tyloxapol (Sigma) to a final concentration of 0.5%. Bacterial cells from agar plates were collected and subsequently suspended at a concentration of 20.0 mg/ml in PBS supplemented with 0.5% tyloxapol. The capsular polysaccharides were extracted using a Bead Beater (BioSpec Products) (without beads) by vigorous shaking (1 min) and subsequently centrifuged for 10 min at 2,000 \( \times g \). The capsular extracts (supernatants) were collected and filtered (0.45 \( \mu m \); Millipore) to remove remaining bacterial cells, and subsequently the capsular polysaccharides were analyzed by using an immunodot-blotting or sandwich ELISA assay.

**Capsular Polysaccharide Quantification by Immunodot-blotting and Sandwich ELISA**—The capsular extracts of the diverse mycobacterial strains were obtained as described above, and immunodot-blotting and sandwich ELISA were performed as described earlier (15). In short, for the immunodot-blotting, 5.0 \( \mu l \) of the capsular polysaccharide extracts was spotted onto nitrocellulose sheets (Millipore) in a 3-fold dilution series, and the sheets were baked for 1 h at 70 °C and blocked in PBS supplemented with 4.0% skimmed milk (Nutricia) for 1 h at room temperature. Next, the sheets were immunolabeled with anti-\( \alpha \)-glucan mAb at 1.5 \( \mu g/ml \) (20) or anti-arabinomannan (AM) mAb (F30.5) (8) for 3 or 1.5 h, respectively, at room temperature. Subsequently, the sheets were washed three times with phosphate-buffered saline-Tween 80 (PBST) and incubated with a horseradish peroxidase (HRP)-labeled secondary antibody in PBST for 1.5 h. The capsular polysaccharides were visualized by coloring agent 3,3'-diaminobenzidine tetrahydrochloride/4-chloro-1-napthol. For the sandwich ELISA, ELISA plates, the preincubated secondary antibody solution was incubated with a horseradish peroxidase (HRP)-labeled secondary anti-\( \alpha \)-glucan mAb (F30.5) (8) for 3 or 1.5 h, respectively, at room temperature. Subsequently, the plates were washed six times with PBST, and subsequently blocked with 200 \( \mu l \) of PBST and subsequently blocked with 200 \( \mu l \) of 1% bovine serum albumin (BSA; Sigma) for 1 h at room temperature. Then 100 \( \mu l \) of capsular extracts or a glycogen reference (Sigma) dilution series (1.0 \( \mu g/ml \)–1.95 ng/ml) was incubated for 1.5 h at room temperature, the plates were washed four times with PBST, and 50 \( \mu l \) of the preincubated secondary antibody solution was incubated for 1.5 h at room temperature as previously described (15). Subsequently, the plates were washed six times with 200 \( \mu l \) of PBST and visualized by adding 100 \( \mu l \) of ortho-phenylenediamine dihydrochloride (Sigma) coloring solution (10 mg of ortho-phenylenediamine dihydrochloride, 10 ml of ortho-phenylenediamine dihydrochloride buffer (0.1 M citric acid; 0.2 M Na2HPO4, pH 5.5), and 5.0 \( \mu l \) of 30% \( H_2O_2 \)) for 4 min and subsequently stopped by adding 50 \( \mu l \) of 10% \( H_2SO_4 \). Next, the optical density was measured at 492 nm with an ELISA plate reader (Epoch, Biotek, SN253854) and analyzed by Gen 5, version 1.11, software.

**Electron Microscopy and Immunogold Visualization of Capsular \( \alpha \)-Glucan and AM**—The immunogold labeling and visualization of capsular polysaccharides \( \alpha \)-glucan and AM by electron microscopy were performed as earlier described (15). Briefly, the bacterial strains were grown as described above and fixed as described previously (15). The fixed bacteria (3.0 \( \mu l \), 0.55 OD/ml) were spotted onto carbon-coated EM grids (EMS, 15800) and dried for 15 min at 37 °C. Then EM grids were incubated two times for 2 min with 0.15% glycine (Sigma) in PBS and blocked for 10 min at room temperature in PBS supplemented with 1% BSA (Aurion). Next, the EM grids were immunolabeled for 3 h at room temperature using anti-\( \alpha \)-glucan mAb (IV58B6) or anti-AM mAb (F30.5), both at a concentration of 10 \( \mu g/ml \). The EM grids were washed three times for 3 min at room temperature in PBS supplemented with 0.1% BSA and incubated with secondary antibody biotinylated goat anti-mouse IgM (0.1 \( \mu g/ml \); Zymed Laboratories Inc., 62-6840) in 1.0% BSA, PBS for 45 min at 37 °C. The EM grids were washed three times for 3 min in 0.1% BSA, PBS at room temperature and immunogold-labeled with 10-nm gold particle-labeled tertiary antibody goat anti-biotin (1:20 dilution; Aurion, 810.088) in 1.0% BSA, PBS for 30 min at 37 °C. Subsequently, the grids were washed and incubated with 1% glutaraldehyde (Zymed Laboratories Inc.), PBS for 5 min at room temperature and washed six times for 1 min with \( H_2O_2 \), dried overnight, and subsequently stored in a grid box for EM analysis. The EM pictures were obtained by an electron microscope (CM100 BIOTRIM, Philips) and digital camera (Morada 9.2, Olympus) with ITEM imaging software (version 5.2).

**Flow Cytometry Analysis of Phosphate Stress Markers in M. marinum**—M. marinum strains were grown to an \( A_{600} \) of 0.6–0.9 in modified \( P_i \) limitation 7H9 medium with or without hygromycin for the controls as described above. Pellets of 1-ml cultures were collected by centrifugation. The pellets were washed with PBS and resuspended in 500 ml of PBS + 0.05% Tween 80. Flow cytometry analysis was subsequently performed on a BD Accuri C6 flow cytometer (BD Biosciences) equipped with a 488 nm laser and 585/40 nm filter. 20,000 gated events were collected per sample, and data were analyzed using BD CFlow software.

**Zebrafish Embryo Experiments and Bacterial Infection**—**M. marinum** M-USA wild type expressing the fluorescent marker mEos3.1 and **M. marinum** strains containing the genetically engineered promoter-fusion mCherry constructs (see above) expressing the fluorescent marker mEos3.1 were grown to the logarithmic phase and microinjected into Danio rerio zebrafish embryos. Subsequently, the bacterial infection was followed over time and analyzed as described previously (26). For the confocal microscope imaging, embryos were fixed overnight in 4% (v/v) paraformaldehyde (Electron Microscopy Sciences, 100122) dissolved in PBS and stored in 100% methanol at −20 °C. Subsequently, embryos were embedded in 1% low melting point agarose (Amresco, 0815) dissolved in egg water (60 \( \mu g/ml \) Instant Ocean sea salts (Sigma) in an 8-well microscope 

**mSla**-slide (ibidi). Next, the bacterial infection (mEos3.1) and the phosphate marker constructs (mCherry) were visualized with a confocal laser scanning microscope (Leica TCS SP8 X; microscope, Leica DMi6000) and analyzed with LAS (Leica Application Suite) AF 2.6.3 software. All zebrafish experiments were done according to the guidelines stated by the Dutch animal welfare laws.
Pi Limitation Modulates Capsular Polysaccharide Composition

**M. smegmatis pstS::Tn**

| α-glucan | AM |
|----------|----|
| WT       | WT |
| pstS::Tn | WT |
| C_pstS   | WT |

FIGURE 1. Immunodot-blotting analysis of capsular polysaccharides α-glucan and AM in *M. smegmatis* pstS::Tn and complemented C_pstS strains. Mycobacterial capsular extracts of liquid cultures (t = 48 h) were spotted in 3-fold serial dilution steps (Dil) onto nitrocellulose filters and immunostained with IgM anti-α-glucan (IV56B6) and anti-AM (F30.5) antibodies, respectively. Data shown are representative for three independent experiments.

**Results**

**Inactivation of the Pst Phosphate Transporter System Induces Capsular α-Glucan and AM Production**—In an ongoing effort to gain insight into the molecular mechanisms underlying capsular α-glucan biogenesis in mycobacteria, we created transposon mutant libraries in *M. smegmatis* and screened for mutants with an altered capsular α-glucan profile using our double filter assay as described previously (15). In this species, we identified several mutants that carried transposon insertions in the ATP-binding cassette phosphate transport locus *pst* (supplemental Table S1). Using the same approach we also identified these P, transporter mutants in *M. tuberculosis*, which suggests a role for P, metabolism in capsular α-glucan regulation in both these mycobacteria (supplemental Table S1). As shown in supplemental Table S1, strains carrying mutations in genes coding for this high affinity P transport system expressed higher levels of capsular α-glucan.

Next, we complemented the *pstS::Tn* mutant of *M. smegmatis* with a plasmid containing an intact copy of *pstS* gene and analyzed the capsular α-glucan content by using an immunodot-blotting assay. As shown in Fig. 1, capsular α-glucan production was strongly up-regulated in the *pstS::Tn* mutant as compared with the wild-type strain, whereas complementation of this mutant restored wild-type levels of capsular α-glucan (Fig. 1, left panel). In addition to α-glucan, mycobacteria also produce capsular AM. To investigate whether inactivation of the Pst system also affected the production of this polysaccharide, we analyzed the expression of capsular AM in the *pstS::Tn* and complemented strains using an AM-directed immunodot-blotting experiment. We found that AM production, similar to α-glucan production, was strongly increased in the *pstS::Tn* mutant in comparison with the wild-type strain, whereas the complemented strain displayed normal AM production (Fig. 1, right panel). Taken together, these results demonstrate that inactivation of the Pst transport system in *M. smegmatis* and probably also in *M. tuberculosis* translates to elevated capsular α-glucan and AM production/secrection during growth in vitro.

**Exogenous Pi Levels Modulate Capsular Polysaccharide Production in Various Mycobacterial Species**—The results above suggest that capsular α-glucan levels are regulated by exogenous Pi levels. To further elucidate this, we first investigated the consequence of Pi, starvation on capsular α-glucan production in *M. smegmatis*. Wild-type *M. smegmatis* was grown in a modified 7H9 medium with Pi concentrations ranging from 25 mM to zero (Fig. 2). As expected, reducing the exogenous Pi concentration showed a stepwise reduction in mycobacterial growth. To investigate the effect of exogenous Pi levels on capsular polysaccharide production, capsular α-glucan levels were analyzed at the early stationary phase using immunodot-blotting (Fig. 2A, 49 h). As shown in Fig. 2B, an inverse correlation between Pi, concentration (25 mM to 0 μM) and capsular α-glucan production was observed. To examine whether other mycobacterial species behaved in a similar manner, capsular α-glucan levels produced by *M. marinum* (strain E11) and *M. tuberculosis* (both the auxotrophic strain mc² 6020 and strain CDC1551) when grown under Pi-rich (25 mM) or Pi-limiting (25 μM) growth conditions were compared. In both species, we detected strongly up-regulated capsular α-glucan production under Pi-limiting conditions (Figs. 2C and 3). To analyze capsular α-glucan production over time, produced capsular α-glucan was also quantified using a sandwich ELISA (Fig. 3B). Reducing the levels of exogenous Pi, correlated with a stepwise induction of capsular α-glucan, clearly visible at days 8, 11, and 16 (Fig. 3B). These results demonstrate that various mycobacterial strains and species, including the clinical isolate *M. tuberculosis* CDC1551, up-regulate capsular α-glucan production under Pi-limiting conditions.

**Chemical Activation of the Stringent Response Induces Capsular Polysaccharide Production**—To elucidate the pathway(s) involved in the Pi-dependent capsular polysaccharide production, we investigated the potential role of the stringent response in this process. Previously, Rifat et al. (16) showed up-regulation of *ppk1* and *relA* expression under Pi-limiting conditions, which is consistent with inorganic polyphosphate accumulation and stringent response activation. To investigate whether activation of the stringent response could play a role in Pi-dependent capsular polysaccharide production, we induced the stringent response by growing *M. smegmatis* in Pi, medium supplemented with SHX. As shown in Fig. 4A, growth of *M. smegmatis* was inhibited by the addition of SHX, presumably by activating the stringent response. Similar results were previously observed for *Pseudomonas aeruginosa* where supplementation of SHX induced a strong growth arrest (27). However, our results are in contrast with previous attempts to study an SHX-induced stringent response in *M. tuberculosis* (23). An explanation for the observed differences is the use of a different growth medium, i.e. 7H9 medium (Primm et al. (23)) versus Pi, medium (this study). To further sort out this difference, the...
The effect of SHX supplementation on \textit{M. smegmatis} growth in Pi versus 7H9 medium was tested (Fig. 5A). Consistent with what was observed by Primm et al. (23), addition of SHX to 7H9 medium did not result in a growth arrest of \textit{M. smegmatis}. One important difference between these two media is the supplementation of ADC.

To test the effects of ADC supplementation on SHX activity, bacteria were grown in 7H9 medium supplemented with or without ADC and SHX. Indeed, in the absence of ADC, bacteria grown in 7H9 displayed an SHX-dependent growth arrest (Fig. 5B). Reversely, in an experiment where \textit{M. smegmatis} was grown in Pi medium supplemented with ADC, addition of SHX no longer demonstrated an inhibition of growth (Fig. 5C). These results clearly demonstrate that ADC has an inhibiting effect on the SHX-induced growth arrest. To further sort out which components of ADC are responsible for this effect, we tested Pi medium supplemented with or without albumin. As shown in Fig. 5C, albumin had no effect on SHX-induced growth arrest. Because dextrose is added to both media, these results indicate that the catalase component in ADC is probably responsible for the inhibiting effect.

Next, we investigated the effect of chemically induced stringent response activation on the production of capsular polysaccharides. For this, capsular extracts obtained at different time points during growth were analyzed for capsular \(\alpha\)-glucan levels and AM by using immunodot-blotting (Fig. 4). Three hours after addition of SHX, \textit{M. smegmatis} produced elevated levels of both \(\alpha\)-glucan (Fig. 4B) and AM (Fig. 4C). These levels further increased after 17 h as both polysaccharides were strongly overexpressed. To confirm these results, we also visualized and subsequently quantified capsular polysaccharides using immunogold labeling and electron microscopy. These experiments
showed that, upon addition of SHX, both capsular α-glucan (Fig. 4, D and E) and AM (Fig. 4, D and F) were highly up-regulated. Finally, to investigate whether *M. tuberculosis* exhibits a similar phenotype, the stringent response was induced in *M. tuberculosis* auxotrophic strain mc26020 by addition of SHX to a log phase culture for 4 days. As shown in Fig. 4G, 48 h after SHX supplementation, *M. tuberculosis* demonstrated a dose-dependent increase in capsular α-glucan production. Taken together, these results strongly suggest that SHX-mediated activation of the stringent response is linked to an induction of capsular polysaccharide production in both *M. smegmatis* and *M. tuberculosis*.

**Pi-mediated Capsular α-glucan Production Is Dependent on Factor SigE**—Previous studies reported the involvement of alternative σ factor SigE in downstream nutrient stress signaling, including P, stress, via polyphosphate-MprAB-SigE (16, 18) and induced expression of RelA (18, 28). Therefore, we investigated the consequence of SigE deficiency on the P, mediated induction of capsular polysaccharides. Wild-type *M. tuberculosis* H37Rv, ΔsigE, and ΔsigE complemented with an integrated wild-type sigE were grown under P,–rich (25 mM (+)) or P,–limiting (25 μM (−)) conditions, and capsular polysaccharide expression was analyzed over time by immunodot-blotting. As shown in Fig. 6A, wild-type *M. tuberculosis* showed a P,–dependent up-regulation of capsular α-glucan production at day 15 postinoculation. In contrast, the ΔsigE strain failed to induce α-glucan production during the course of the experiment (26 days), a phenotype that was partially restored by complementation with an intact sigE copy.

Next, wild-type *M. tuberculosis*, ΔsigE, and the complemented strains were incubated with SHX to directly activate the stringent response downstream of SigE. As expected, addi-
**FIGURE 4.** Chemical activation of the stringent response by SHX up-regulates the capsule polysaccharides α-glucan and AM in *M. smegmatis* and α-glucan in *M. tuberculosis*. *M. smegmatis* (mc²155) and *M. tuberculosis* (mc²6020) strains were grown in 7H9-based liquid P, medium without ADC, and SHX was added at 6.5 h and 4 days, respectively. Bacterial cells were collected at different time points and shaved with a detergent. The obtained capsular extracts were subsequently assayed by immunodot-blotting to analyze the capsular polysaccharides α-glucan or AM using specific anti-glucan (IV56B6) or anti-AM (F30.5) mAb. A, *M. smegmatis* challenged with 100 μg/ml SHX showed strong reduction in growth in comparison with bacteria grown without SHX. Error bars represent S.D. The cells grown in A were used to determine α-glucan (B) and AM (C) levels. A strong induction of α-glucan and AM is observed after 3 and 17 h of SHX addition. D, direct visualization of capsular polysaccharides α-glucan (upper panel) and AM (lower panel) in *M. smegmatis* WT by immunogold labeling. Cells were cultured in liquid, fixed 2 days after SHX addition, and labeled with IV56B6 (upper panel) or F30.5 (lower panel) mAb. Quantitative evaluation of the EM analysis of α-glucan (E) and AM (F) is shown. Values represent means of three experiments ± S.E. G, *M. tuberculosis* (mc²6020) strain treated with SHX (50, 100, 150, and 200 μg/ml) showed dose-dependent up-regulation of capsular α-glucan after 48 h. Data are representative for three independent experiments. Dil, diluted/dilutes or dilutions.
Pi Limitation Modulates Capsular Polysaccharide Composition

M. marinum Experiences Phosphate-liming Conditions in Vivo—Phosphate limitation is believed to be a relevant physiological condition encountered by M. tuberculosis in vivo. In a previous study, Rifat et al. (16) showed that expression levels of gene clusters involved in the phosphate stress response, including the phosphate-specific transport operon pstS3-pstC2-pstA1, were induced in mouse lungs during infection. Furthermore, several reports show that the two-component regulator SensX3-RegX3 is directly involved in the mycobacterial phosphate stress response (16, 29, 30) and that ppk1 expression is RegX3-dependent (16, 31). To investigate whether Pi limitation is a relevant condition encountered by mycobacteria in vivo, three reporter strains were constructed. These reporter strains are transcriptional fusions of a promoter region of interest in front of an mCherry gene so that gene activation could be monitored by a fluorescence readout. Promoter regions of known phosphate stress-related genes ppk1, senX3, and phoS2 (ortholog to pstS3 in M. tuberculosis) were selected. First, these phosphate stress-sensing reporter strains were grown under Pi-rich (25 mM) or Pi-limiting (25 μM) conditions and subsequently analyzed by flow cytometry to see whether phosphate stress could be detected in vitro. Comparison of M. marinum strains containing the stress marker constructs revealed that the senX3-mCherry fusion was only 1.6-fold induced after 24 h under Pi-limiting condition (Fig. 7, A and B). Over time, this induction was slightly increased on day 2 and peaked on day 3, indicating that the Pi stress occurs within 24 h and stably increases (Fig. 7C). Surprisingly, the ppk1-mCherry reporter construct showed no significant difference between high or low Pi growth conditions over a 4-day period of time (Fig. 7, D and E, respectively). The phoS2-mCherry fusion showed constitutive activity, and no difference was observed between high and low Pi (Fig. 7, F and G). Therefore, the phoS2-mCherry fusion was excluded as a phosphate stress marker and was used as a positive control.

The in vitro induction of Pi stress might be different from the in vivo situation, therefore, all three constructed reporter strains were also analyzed in zebrafish embryos. First, an additional (integrative) plasmid harboring the hsp60 promoter and fluorescent protein-encoding gene mEos3.1 (GFP) was introduced in the reporter strains to allow the detection of the bacteria. Subsequently, zebrafish embryos were infected with these M. marinum promoter-fusion mCherry/mEos3.1 strains and a control strain that only contained the hsp60-mEos3.1 construct. The bacterial infection in the zebrafish embryos was

![Graph](image)

**FIGURE 5.** Mycobacterial growth medium 7H9 supplement ADC inactivates activity of stringent response activator SHX in M. smegmatis. M. smegmatis WT strain was grown in liquid 7H9 or in 7H9-based Pi medium. The 7H9 and Pi media were supplemented with or without ADC, and Pi medium was supplemented with albumin. The bacterial growth of M. smegmatis was monitored by A600 over time; at 4 h after inoculation, the bacterial growth is arrested in Pi medium upon addition of SHX in comparison with 7H9. The bacterial growth of M. smegmatis was slightly increased on day 2 and peaked on day 3, indicating that the Pi stress occurs within 24 h and stably increases (Fig. 7C). Surprisingly, the ppk1-mCherry reporter construct showed no significant difference between high or low Pi growth conditions over a 4-day period of time (Fig. 7, D and E, respectively). The phoS2-mCherry fusion showed constitutive activity, and no difference was observed between high and low Pi (Fig. 7, F and G). Therefore, the phoS2-mCherry fusion was excluded as a phosphate stress marker and was used as a positive control.

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To further elucidate the pathway of Pi-mediated up-regulation of capsular α-glucan, we investigated stringent response initiator RelA. Importantly, RelA in M. tuberculosis is a special enzyme variant and synthesizes and hydrolyzes (p)ppGpp. To highlight these two enzyme activities, M. tuberculosis RelA is designated as RelMb. Wild-type M. tuberculosis, ΔrelA strain, and complemented strains were grown under Pi-rich (25 mM (+)) or Pi-limiting (25 μM (−)) conditions and analyzed for capsular α-glucan by immunodot-blotting assay. As shown in Fig. 6C, both the wild-type and complemented strains showed normal Pi-mediated induction of capsular α-glucan. However, strikingly, the ΔrelA strain demonstrated the same α-glucan induction under Pi-starved conditions, indicating that RelMb activity is not needed for the increase in capsular α-glucan production. These data suggest that Pi stress signaling in M. tuberculosis induces capsular α-glucan in a SigE-dependent manner but independently of RelMb.

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analyzed from 1 to 4 days postinfection (dpi). At 3 dpi, the phosphate stress marker *senX3* is gradually induced, increasing even further at 4 dpi (Fig. 8A). This clearly indicates that Pi stress is sensed by *M. marinum in vivo*. This result is consistent with our *in vitro* data (Fig. 7, A and B) and with the previous findings that the expression of *senX3* is elevated under Pi stress in *M. tuberculosis in vivo* (16). In addition, the *ppk1* Pi stress marker strain now also showed induction of *ppk1* at 4 dpi, an indication that the *ppk1* promoter-fusion construct is expressed *in vivo* as reported previously by Rifat et al. (16) (Fig. 8A).

To examine possible differences in induction between bacterial populations, we analyzed these reporter strains by confocal microscopy. In all reporter strains, we detected similar severity of bacterial infection from 3 dpi onward (Fig. 8C). The *senX3* promoter-fusion construct is induced at 3 dpi, and the merged fluorescence pictures indicate induction of the *senX3* reporter, a clear sign that Pi limitation is sensed at 3 dpi *in vivo* (Fig. 8C). Furthermore, the *ppk1* Pi stress indicator is clearly detectable at 3 dpi and suggests *ppk1* induction *in vivo* (Fig. 8C). As observed *in vitro*, the *phoS2* promoter-fusion was constitutively induced (Fig. 8C) *in vivo*. In conclusion, these data show that Pi limitation is encountered by *M. marinum in vivo*.

**Discussion**

*M. tuberculosis* is a well adapted human pathogen that encounters multiple stress conditions during infection. To survive these harsh environments, the bacillus deploys a variety of strategies to adapt its complex cell envelope composition. The mycobacterial capsule, the outermost cell envelope layer, is expected to be important. The capsular polysaccharides have previously been associated with virulence and immune modulation (10, 13), which might be key for bacterial adaptation within the host. The purpose of this study was to investigate mechanisms involved in mycobacterial capsule polysaccharides α-glucan biogenesis under exogenous Pi stress and to pinpoint the biological relevance of Pi stress *in vivo*.

In this study, we show that the Pst system is involved in the regulation of the capsular polysaccharides α-glucan in both *M. smegmatis* and *M. tuberculosis*. The Pst system is a high affinity inorganic Pi ATP-binding cassette transporter important for the acquisition of Pi (17). This is in line with previous data, which showed that expression of *pstS3* is significantly up-regulated under Pi depletion, suggesting an important role for the Pst system in bacterial survival at low Pi conditions (32). Consistently, the Pst system is linked via SenX3-RegX3 to the ESX-5 system in which Pi limitation controls the overexpression of ESX-5 genes and hypersecretion of its substrates EsxN.

**FIGURE 6.** Capsular α-glucan up-regulation under Pi starvation is SigE-dependent. *M. tuberculosis* H37Rv wild-type, targeted knock-out (sigE and relMtb), and complemented (C_sigE and C_relMtb) strains were collected from liquid 7H9-based Pi medium supplemented with normal Pi (+; 25 mM) or low Pi (−; 25 μM) concentrations. To activate the stringent response, SHX was added at a final concentration of 250 μg/ml. Cell suspensions were normalized to *A*$_{600}$ values at days 6, 11, 15, 19, and 26 and subsequently shaved with detergent to liberate the capsular polysaccharides. The capsular extracts were spotted in 3-fold serial dilutions (Dil) onto nitrocellulose sheets and subsequently analyzed for the amount of capsular polysaccharides α-glucan by immunodot-blotting with antibody directed against α-glucan (IV56B6). A, immunodot-blotting analysis of Pi stress-related capsular α-glucan regulation by SigE in *M. tuberculosis*. Upon Pi stress, capsular α-glucan is up-regulated in WT strain over time, whereas in the sigE mutant no Pi stress-mediated up-regulation is observed. The up-regulation of capsular α-glucan is restored by complementation with the sigE gene. B, immunodot-blotting analysis of capsular α-glucan regulation by SigE where the stringent response, downstream of SigE, is activated by SHX. WT, mutant sigE, and the complemented sigE strains showed induced capsular α-glucan levels by activation of the stringent response at day 15. C, in WT, mutant relMtb, and the complemented relMtb strains, normal Pi stress-related induction of α-glucan levels is observed. Experiments are representative for two, and all other data are representative for three independent experiments.
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FIGURE 7. Up-regulation of senX3 promoter fusion in *M. marinum* under Pi starvation in vitro. A, a plot of FACS data for the senX3-mCherry construct under high phosphate conditions. A representative WT fluorescence curve (black line) is plotted with senX3-mCherry after 1 (in red), 2 (in blue), 3 (in green), or 4 days (in orange) in high phosphate-containing medium. B, a plot of FACS data for the senX3-mCherry construct under low phosphate conditions. A representative WT fluorescence curve (black line) is plotted with senX3-mCherry after 1 (in red), 2 (in blue), 3 (in green), or 4 days (in orange) in low phosphate-containing medium. C, quantification of the -fold induction per day compared with WT *M. marinum*. Mean fluorescence intensity of the low phosphate cultures was divided by the mean fluorescence intensity of the high phosphate cultures, leading to a -fold induction.

FIGURE 8. In vivo activation of *M. marinum* low Pi reporter strains. *M. marinum* strains containing the mCherry promoter-fusion constructs (phoS2, ppk1, and senX3) and constitutive mEos3.1 expression were injected into zebrafish embryos. As a control, we used a strain expressing only mEos3.1. A, bright field (left panel) and fluorescence images of 3- and 4-dpi embryos with the P, stress promoter-fusion constructs in red and total infection in green. The bacterial infection inocula for strains phoS2, control, ppk1, and senX3 were 57, 243, 32, and 139, respectively. B, bright field image of zebrafish embryo indicating the region that was analyzed by confocal microscopy imaging. C, confocal microscopy imaging showing mEos3.1 (bacterial infection; left panel) and mCherry (Pi stress indicator; middle panel) expression. Data are representative for three individual experiments.

We hypothesized that blocking the Pst system by transposon inactivation would lead to Pi starvation in *M. tuberculosis*, and it is also suggested to have an important role in the Pst system in virulence (33). The ESX-5 system is essential for virulence in *M. tuberculosis*, and it is also suggested to have an important role in the Pst system in virulence (33).
Pi limitation 

Stress condition

Nutrient stress (stationary phase)

Other stresses

SHX (amino acid stress)

SenX3-RegX3 (TCS)

Poly-P

MprAB (TCS)

Pho regulon

Homeostasis

Capasular α-glucan

(p)ppGpp

Strongent reponse stress regulon

Capsular α-glucan

SigE

RelMtb

Other stresses

FigURE 9. Model of capsular α-glucan regulation routes in M. tuberculosis. The model is based on our finding of capsular induction under Pi starvation and SHX-mediated amino acid stress as well as the current literature on stress signaling pathways in M. tuberculosis. Low Pi is sensed by the Pst system and downstream signaling via the SenX3-RegX3-MprAB-SigE pathway. SigE activates the SigE-dependent stress regulon, which ultimately induces the capsular α-glucan (green arrows). SigE signaling to RelMtb occurs in Pi starvation, but the capsular α-glucan is not elevated via a RelMtb-dependent manner (red arrow with cross). Mimicking amino acid starvation by SHX activates the stringent response via RelMtb-mediated induction of (p)ppGpp and up-regulates the capsule polysaccharide α-glucan (green arrows). Nutrient stress as seen in the stationary phase cultured M. tuberculosis leads to induced capsular α-glucan levels mediated by RelMtb and/or SigE (black arrows). SigE and RelMtb communicate via feedforward and feedback loops and therefore might influence each other in the capsular α-glucan production (black arrows with question mark). Furthermore, M. tuberculosis encounters in vivo various stress conditions, like carbon, iron, hypoxia, or other stresses (marked Other stresses) that might up-regulate capsular α-glucan in a similar way via SigE and/or RelMtb. TCS, two-component system; Poly-P, polyphosphate.

levels of Pi in M. tuberculosis. These results strongly indicate that Pi stress modulates the immunomodulatory capsular α-glucan. In contrast to α-glucan levels, the AM levels were not significantly induced upon Pi stress in M. tuberculosis (data not shown). This result is inconsistent with our findings in M. smegmatis where an MS_pst::Tn mutant showed elevated levels of AM (Fig. 1). This suggests a different regulation of AM production under Pi starvation in M. smegmatis in comparison with M. tuberculosis. Interestingly, the capsular α-glucan production is detectable from the early stationary phase onward, suggesting that some form of stress (other than low phosphate) can initiate the production of capsular α-glucan (Fig. 3). Interestingly, the stringent response is activated as the metabolic state of mycobacteria is changed due to various nutrient starvation. Consistently, Primm et al. (23) reported a mild activation of the stringent response and (p)ppGpp production in the early stationary phase (23). Here, we have used chemical activation of the stringent response by SHX, which elevated the α-capsular glucan production. So these findings suggest a strong link between the stringent response and capsular α-glucan up-regulation in M. tuberculosis. Interestingly, inactivation of the Pst system is associated with immune modulation and reduced virulence of M. tuberculosis in mice (34, 35). This effect might be due to high capsular α-glucan levels, which have also been associated with immunomodulation, in these strains (11, 13, 14, 33). Because we identified up-regulation of capsular α-glucan production in three different mycobacterial species, M. smegmatis, M. marinum, and M. tuberculosis, upon Pi starvation, this phenotype seems to be a conserved feature. In this study, we also show that SHX-mediated activation of the stringent response in M. smegmatis causes bacterial growth arrest and up-regulation of α-glucan and AM. Therefore, our study clearly demonstrates that SHX is a useful tool to investigate the stringent response in mycobacteria. Previous attempts by Primm et al. (23) were unsuccessful, and we show here that this is probably due to the presence of catalase, which may inactivate SHX, in the medium.

A full understanding of the stringent response pathway is difficult due to a highly dynamic interplay of σ and anti-σ factors, RelA activity (levels of (p)ppGpp), and different nutrient stresses (36–43), and the situation in M. tuberculosis is no different. To further complicate matters, several of the σ factors involved in this process, like SigE, are also capable of activating genes not related to the stringent response (22, 44). In the context of Pi starvation, SigE is of particular interest as previous reports link SigE to the signal transduction pathway activated under Pi stress (16, 18). In this study, we show that the phosphate stress-mediated up-regulation of capsular glucan is SigE-dependent. To our surprise, inactivation of relMtb had no influence on the Pi stress-mediated induction of capsular α-glucan (Fig. 6). This strongly suggests that the pathway for the up-regulation under Pi starvation is initiated by SigE and is primarily not stringent response-dependent. In contrast, downstream SHX-mediated activation of relMtb clearly induces the capsular α-glucan levels. These results highlight the complexity of capsular α-glucan production and regulation mediated by different stress conditions and divergent activated pathways at least for amino acid versus Pi starvation. Based on our findings and the current literature, we suggest a model of the capsular α-glucan production under stress conditions in M. tuberculosis (Fig. 9). This model shows that different stresses can activate the SigE stress regulon and/or the stringent response via RelMtb to mod-
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ulate capsular α-glucan levels. The Pst system senses low $P_i$ and activates the SigE regulon via downstream signaling via the SenX3-RegX3-MprAB-SigE pathway to elevate capsular α-glucan production. In addition, amino acid stress via chemical activation of RelMtb by SHX induces (p)ppGpp production, activates the stringent response stress regulon, and therefore leads to higher levels of capsular α-glucan (Fig. 9, green arrows). Furthermore, SigE and RelMtb can be activated by various stresses encountered by $M. tuberculosis$ in vivo that could therefore also up-regulate capsular α-glucan (Fig. 9, black arrows).

Finally, we have revealed that $P_i$ limitation is encountered by $M. marinum$ in vivo in zebrafish embryos. These findings are consistent with several reports suggesting that $P_i$ limitation is encountered by $M. tuberculosis$ in vivo (16, 18, 33). However, in our hands, the ppk1 promoter-fusion construct was not induced in vitro; apparently additional stimuli that only occur in vivo are necessary for induction of this gene. A full comprehension of both capsular polysaccharide and lipid changes, especially in multiple stresses that mimic the in vivo condition, might be the key to a better understanding of the pathogenesis of $M. tuberculosis$. In this context, our work provides a first glimpse that the outermost capsule layer, i.e. capsular α-glucan, is modified under stress conditions. The identification of possible routes in stress-mediated biosynthesis regulation of capsular α-glucan might lead to novel ways to intervene with the capsular polysaccharide biosynthesis in $M. tuberculosis$. Finally, the discovery how to activate the stringent response in mycobacteria with SHX is of great practical value and might therefore boost our understanding of this essential in vivo stress pathway in $M. tuberculosis$.

Author Contributions—R. v. d. W., J. G., and B. J. A. conceived and designed the experiments. R. v. d. W., M. B., J. M., M. S., T. V., L. M. v. L., M. J. B., N. J. P., E. D., and R. M. performed the experiments. R. v. d. W., M. B., and B. J. A. analyzed the data. R. v. d. W., M. B., W. B., J. G., and B. J. A. wrote the paper.

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