Bacillus anthracis tagO is required for vegetative growth and secondary cell wall polysaccharide synthesis

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Running title: Bacillus anthracis tagO and SCWP assembly

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ABSTRACT Bacillus anthracis elaborates a linear secondary cell wall polysaccharide (SCWP) that retains surface (S)-layer and associated proteins via their S-layer homology (SLH) domains. The SCWP is comprised of trisaccharide repeats \[\rightarrow 4\] -\(\beta\) -ManNAc-(1\(\rightarrow 4\)) -\(\beta\) -GlcNAc-(1\(\rightarrow 6\)) -\(\alpha\) -GlcNAc-(1\(\rightarrow \)) and tethered via acid-labile phosphodiester bonds to peptidoglycan.

Earlier work identified UDP-GlcNAc 2-epimerases, GneY (BAS5048) and GneZ (BAS5117), which act as catalysts of ManNAc synthesis, as well as a polysaccharide deacetylase (BAS5051) as factors contributing to SCWP synthesis. Here we show that tagO (BAS5050), which encodes a UDP-N-acetylglucosamine: undecaprenyl-P N-acetylglucosaminyl 1-P transferase, the enzyme that initiates synthesis of murein linkage units, is required for B. anthracis SCWP synthesis and S-layer assembly. Similar to gneY/gneZ mutants, B. anthracis strains lacking tagO cannot maintain cell shape or support vegetative growth. In contrast, mutations in BAS5051 do not affect B. anthracis cell shape, vegetative growth, SCWP synthesis or S-layer assembly. These data suggest that TagO-mediated murein linkage unit assembly supports SCWP synthesis and attachment to the peptidoglycan via acid-labile phosphodiester bonds. Further, B. anthracis variants unable to synthesize SCWP trisaccharide repeats cannot sustain cell shape and vegetative growth.
IMPORTANCE Bacillus anthracis elaborates a secondary cell wall polysaccharide (SCWP) to support vegetative growth and envelope assembly. Here we show that some, but not all SCWP synthesis is dependent on tagO-derived murein linkage units and subsequent attachment of SCWP to peptidoglycan. The data implicate secondary polymer modifications of peptidoglycan and subcellular distributions as a key feature of the cell cycle in Gram-positive bacteria, and establish foundations for work on the molecular functions of the SCWP and on inhibitors with antibiotic attributes.
The cell wall envelope of *Bacillus anthracis*, the causative agent of anthrax, is comprised of peptidoglycan and its attached secondary cell wall polysaccharide (SCWP)(1). The SCWP retains two S-layer proteins, surface array protein (Sap) and extractable antigen 1 (EA1)(2), as well as twenty-two S-layer associated proteins whose S-layer homology (SLH) domains associate with ketal-pyruvylated SCWP (3, 4). Unlike non-pathogenic *Bacillus* spp., for example *Bacillus subtilis* and *Bacillus cereus* AHU1030, *B. anthracis* does not synthesize wall teichoic acid (5, 6). Nevertheless, *B. anthracis* expresses functional *tagO* and *tagA* genes, whose products provide for the synthesis of murein linkage units [P-GlcNAc-(4→1)-β-ManNAc-R] that are phosphodiester linked to the C6-hydroxyl of N-acetylmuramic acid in the repeating disaccharide of peptidoglycan (MurNAc(P-GlcNAc-ManNAc-R)-GlcNAc)(4). In *B. subtilis* [-R] represents wall teichoic acid, *i.e.*, either polyglycerol-phosphate or polyribitol-phosphate (7). Here we test the hypothesis that in *B. anthracis* the SCWP is attached via the murine linkage unit and that the *tagO* gene is required for vegetative growth.

Earlier work characterized the structure of *B. anthracis* SCWP as repeating units of \([\rightarrow4]-\beta\text{-ManNAc-(1→4)}\beta\text{-GlcNAc-(1→6)}\alpha\text{-GlcNAc-(1→)}\]n, where \(\alpha\text{-GlcNAc is substituted with } \alpha\text{-Gal and } \beta\text{-Gal at O-3 and O-4, respectively, and } \beta\text{-GlcNAc with } \alpha\text{-Gal at O-3}(1). The terminal ManNAc of some SCWP molecules is ketal-pyruvylated by the product of *csaB*, a prerequisite for assembly of S-layer proteins (4, 8). Further, SCWP is acetylated by *patA1B1/patA2B2*, contributing to the assembly of proteins with SLH domains at discrete sites in the bacterial envelope (9). The enzymes that synthesize the trisaccharide repeats and galactosyl modifications of the SCWP are not yet known (10, 11). Comparative genome analysis identified the surface polysaccharide synthesis locus sps (BAS5116-BAS5127) (12). The *gneZ* gene
(BAS5117), a member of this locus, encodes a UDP-GlcNAc 2-epimerase and catalyzes the conversion of GlcNAc into ManNAc (13). gneZ is essential for B. anthracis growth (14) and its homologue, gneY (BAS5048), is not expressed under vegetative growth conditions (15). However, replacement of the endogenous gneY promoter with an IPTG-inducible spac promoter supports growth of the B. anthracis gneZ mutant (14). Removal of the gneY inducer causes defects in S-layer assembly, suggesting that a UDP-GlcNAc 2-epimerase indeed supplies ManNAc for SCWP synthesis (14). gneY (BAS5048) is located in the same gene cluster as tagO (BAS5050). In B. subtilis and Staphylococcus aureus, TagO (TarO) links UDP-GlcNAc and undecaprenyl-phosphate (C₅₅-P) generating C₅₅-PP-GlcNAc and initiating wall teichoic acid synthesis (WTA) (16-18). C₅₅-PP-GlcNAc is subsequently modified by TagA-catalyzed addition of ManNAc to generate C₅₅-PP-GlcNAc-ManNAc, the precursor of the murein linkage unit (7, 19). Glycerol moieties are polymerized onto this lipid precursor and LCP enzymes subsequently transfer the polymer onto peptidoglycan (20, 21). Although B. anthracis lacks WTA, expression of tagO (BAS5050) restores WTA synthesis in the S. aureus tagO mutant (4). Previous attempts to delete tagO in B. anthracis were unsuccessful (4). Earlier work suggested also that BAS5051 (BA5436), which encodes a putative polysaccharide deacetylase, may be required for SCWP synthesis (22), however a specific contribution of this enzyme has not yet been revealed.

Here we use phage transduction to demonstrate that a marked deletion of tagO can only be obtained in B. anthracis strains merodiploid for tagO and we generate a B. anthracis variant with conditional expression of tagO. Depletion of tagO leads to a loss of SCWP synthesis and S-layer assembly, as well as cell rounding and death, indicating that tagO is indeed essential.
for B. anthracis growth. We also show that BAS5051 (polysaccharide deacetylase) is not required for SCWP synthesis and S-layer assembly.

MATERIALS AND METHODS

**Bacterial growth and reagents.** B. anthracis wild-type strain Sterne 34F2 (23) and its variants were grown in brain heart infusion broth or agar (BHI) at 30-40°C. Escherichia coli was grown in Luria Bertani broth (LB) or agar at 37°C. Where necessary, 100 µg ampicillin /ml, 10 chloramphenicol µg/ml, 200 µg spectinomycin/ml or 20 µg kanamycin /ml were added to cultures. B. anthracis strains were sporulated in modified G medium (24). Spore preparations were heat-treated at 68°C for 2 hours to kill vegetative bacilli and then stored at 4°C. To monitor bacterial growth, spores were germinated at time 0 by inoculation into BHI at indicated temperatures and with appropriate antibiotics. Bacterial growth was monitored at timed intervals by recording the absorbance at 600 nm (A$_{600}$).

**B. anthracis strains and plasmids.** The temperature-sensitive vector pLM4 (25) was used to generate a clone where the tagO gene coding sequence was replaced with the aad9 gene. Briefly, 1 kbp flanking DNA sequences upstream and downstream of the tagO gene were amplified with primer pairs bearing sequences TTTCCCGGG CCTCCGTA AAAATTCA GATT, AACTCGAGAAATCACTTGTGAGTCCATTAATACC, AACTCGAGAAAAACGCGAAGCTTGAATT GTAG and TTTGAATTCCAAAAATAGGAAGGGTGGGAAG and ligated with the intervening spectinomycin resistance cassette aad9 as described (14). The resulting DNA fragment was ligated into pLM4 using K1077 (26). The recombinant plasmid was
electroporated into wild-type or several tagO merodiploid variants. Allelic replacement was
induced via changes in incubation temperature (25, 27) and verified by PCR using chromosomal
DNA as a template and PI and PII primers (TTAAAAAAATTACGGACTATTTAAAGG and
AATATTCATAATAGAAAGCTATAACTAC) flanking the cloning sites. Three complementing
plasmids ptagO\(^c\), ptagO\(^i\) and pts-tagO\(^i\) were used. Plasmid ptagO\(^c\) (c, constitutive expression)
carries the minimal coding sequence of tagO amplified with primer pairs bearing the sequences
AAACATATGGACTCACAAGTGATTTATGCC and AAAGGATCCTTAGTCTTCGCGTTTTTTCAC and
cloned under the constitutive hprK promoter of plasmid pWWW412 (28). Plasmid ptagO\(^i\)
carries the minimal coding sequence of tagO under the IPTG-inducible spac promoter of pJK4
(4, 29). Even in the absence of IPTG, expression from the spac promoter is leaky; the tagO gene
was therefore cloned into pLMS, a variant of pJK4 with a thermosensitive replicon (25) to
generate plasmid pts-tagO\(^i\). Primers with sequences TTTGGTACCTTCTCTCTCGGTCTTTTTACCT
and TTTTCAGATGGACTCACAAGTGATTTATGC were used to amplify tagO. The ftsZ gene,
BAS3757, was amplified by PCR without its stop codon from the genomic DNA of B. anthracis
using primer pairs AAAGGATCCTTCTAGGGGGATTTCGAC and
AAAGGTACCTCGTCTACGGCGATTAC. The fragment was digested with KpnI and ligated into the
pMutin-GFP plasmid (30). The inserts for pgfp and pftsZ-gfp were amplified using primer pairs
with sequences AAAACTAGGTGGATCCAGCTATCGTC, CGCAGTGTGGATCCATTATTTGAGAG
and AAACATATGACTAGTTAGTTTGAGTACTACTCAAGTC, respectively. The PCR products were cloned into
pWH1520 using the SpeI cloning site (31). The ΔBAS5051 mutant was obtained by allelic
replacement using plasmid pLM4 (25, 27). Here, 1 kbp flanking DNA sequences upstream and
downstream of BAS5051 were amplified with two primer pairs bearing sequences AAAGAATTCTCGGTATTTTAGATATGTACGAG, AAACCTCGAGTCGCTTGATCATAACATTCACC and AAACTCGAGACAAGATTGTAACAGCTTTAGTACC, and AAACCCGGGGACAGAGTCACGTTTCTTTGTC. The amplification products were ligated without intervening sequences into pLM4. The plasmid was transformed into B. anthracis for allelic replacement (25, 27). The BAS5051::bursa aurealis mutant allele was obtained by transposon mutagenesis with bursa aurealis (32-34). The mutant allele was transduced into wild-type B. anthracis with bacteriophage CP-51 (35, 36) and the position of transposon insertion was verified by DNA sequencing (34). B. anthracis strains with mutational lesions in sap, eag, bslO, csaB and bslR have been described (4, 6, 32, 37). Bacteriophage CP-51 was used to perform transduction experiments (35, 36).

**mCherry and mCherry hybrids.** Plasmids expressing mCherry or its hybrids from the T7 promoter of pET16b included pJK81 (mCherry alone), as well as pJK87 and pJK88, which express the mCherry coding sequence fused to the 3’ end of coding sequence for the Sap or EA1 SLH domain, i.e., residues 30–210 (pSapSLH-mCherry, pEA1SLH-mCherry)(4, 9). Plasmid pJK81 was digested with XhoI and ligated with the XhoI-restricted, PCR-amplified portion of plyG encoding the carbohydrate binding domain (amino acids 166 to 233 of PlyG; (38)) to create pPlyGCBD-mCherry. All cloning steps were performed in E. coli DH5α. E. coli BL21 (DE3) cultures harboring expression plasmids were induced for T7 polymerase expression with 0.1 mM IPTG overnight at 26°C. Cells were sedimented by centrifugation, lysed in a French Press and purified by affinity chromatography on Ni-NTA sepharose as described (4).

**Analysis of SCWP.** For the analysis of SCWP from the tagO variant, spores were derived from wild-type B. anthracis (pLM5) and the tagO::aad9 (pts-tagO*) mutant, each grown to lawns...
on six agar plates supplemented with 200 μg spectinomycin/ml, scraped off and suspended in modG. Spores were germinated at 40°C in 1 L BHI broth and grown with rotation for 5 hours. Bacilli were sedimented by centrifugation (10,000 ×g for 10 min) and washed in water. The remainder of the purification of SCWP from wild-type and tagO::aad9 (pts-tagO') bacilli as well as that of wild-type and BAS5051 mutant strains, which were propagated on BHI agar plates, was performed as described earlier (4, 37). Washed bacilli were boiled for 30 minutes in a 4% SDS solution, washed and suspended in water. The bacteria were mechanically lysed with 0.1 mm glass beads. The resulting murein sacculi were sedimented by centrifugation at 17,000 ×g for 15 min, suspended in 100 mM Tris-HCl (pH 7.5), and incubated for 4 hours at 37°C with 10 μg/mL DNase and 10 μg/mL RNase supplemented with 20 mM MgSO4. Samples were then incubated for 16 hours at 37°C with 10 μM trypsin supplemented with 10 mM CaCl2. Enzymes were inactivated by boiling for 30 minutes in a water bath in 1% SDS. The SDS was removed by repeated centrifugation and water-washing cycles. The murein sacculi were then washed with water, 100 mM Tris-HCl (pH 8.0), water, 0.1 M EDTA (pH 8.0), water, acetone, and finally twice with water. Murein sacculi were suspended in 5-7 mL water and 25 ml of 48% hydrofluoric acid (HF) was added. Samples were incubated overnight on ice with shaking. The acid-extracted murein sacculi were sedimented by centrifugation at 17,000 ×g for 15 min. SCWP-containing supernatant was mixed with ice-cold ethanol in a 1:5 ratio, causing SCWP to precipitate. The polysaccharide was sedimented by centrifugation at 17,000 ×g and 4°C for 15 min and the sediment was washed extensively with ice-cold ethanol. The SCWP was suspended in water and subjected to size exclusion high performance liquid chromatography (SEC-HPLC) on 300 mm × 7.8 mm BioBasic SEC300 (Thermos) column with a 3μm-particle-size guard column (Thermos).
equilibrated with 50 mM sodium phosphate buffer (pH 7.5) and a 0.4 ml/min flow rate. The absorbance at 206 nm was monitored to assess the retention time of SCWP material.

**Analysis of S-layer assembly.** For fractionation of S-layer proteins, spores derived from wild-type *B. anthracis* (pLM5) and the tagO::aad9 (pts-tagO') mutant were germinated at 40°C in BHI and grown for 2 or 5 hours. For fractionation studies involving the BAS5051 variants, overnight cultures of *B. anthracis* wild-type, ΔBAS5051 and BAS5051::bursa aurealis were diluted 1:100 into fresh BHI broth and incubated with rotation at 37°C to $A_{600}$ 2. *B. anthracis* cultures were then centrifuged at 16,000 × g to separate the medium (M) from the bacterial sediment. Bacteria were washed twice with PBS and boiled at 95°C for 10 min in 3 M urea. Cells were sedimented by centrifugation at 16,000 × g, and the supernatant containing S-layer proteins (S) was removed. Urea-extracted bacteria were washed twice with PBS and mechanically lysed by silica bead beating for 3 min (Fastprep-24; MP Biomedical) to generate the cell lysate (C). Proteins in all three fractions (M, S and C) were precipitated with 10% (vol/vol) trichloroacetic acid (TCA), separated on 10% SDS-PAGE gels and analyzed by Coomassie staining or electrotransferred onto a polyvinylidene difluoride (PVDF) membrane for immunoblotting (39). Proteins were detected with rabbit antisera raised against purified antigens.

**mCherry experiments.** For mCherry binding experiments, spores were germinated in BHI at 40°C for 5 hours and bacilli sedimented by centrifugation, suspended in PBS containing 3 M urea and heated at 95°C for 10 min. Bacteria were sedimented by centrifugation, washed three times with water, suspended in PBS, and normalized by optical density ($A_{600}$). Similar amounts of bacterial cell suspensions were incubated with equimolar quantities of purified
mCherry, SapSLH–mCherry, EA1SLH–mCherry or PlyGCBD-mCherry overnight at 4°C. Bacteria were sedimented by centrifugation, washed two times with PBS, and either imaged by fluorescence microscopy or fluorescence intensity measured in 96-well format with a Biotek Synergy HT microplate reader. The excitation and emission wavelengths were set at 590(±20) nm and 645(±40) nm, respectively. Fluorescence measurements were converted to % binding as compared to WT for which protein binding, i.e. fluorescence units, was set as 100%. P values were calculated using an unpaired, two-tailed Student’s t test.

Microscopy. To visualize bacterial growth, spores were germinated and at timed intervals, vegetative bacteria were fixed with buffered 4% formaldehyde. Fixed specimens were observed by light microscopy with a charge-coupled-device (CCD) camera on an Olympus IX81 microscope using a 100x objective. To visualize surface proteins, newly germinated bacilli were incubated with rabbit antisera raised against purified Sap (αSap), EA1 (αEA1), and BslR (αBslR) (14, 40), and bound antibodies were detected with DyLight594-conjugated goat anti-rabbit secondary antibody (Fisher). Cells were counterstained with boron dipyrromethene (bodipy)-vancomycin (Invitrogen). A Leica SP5 STED-CW Superresolution laser scanning confocal microscope with a 63× objective was used to acquire digital micrographs of bright field (BF) and fluorescence microscopy images which were then merged using ImageJ. B. anthracis cells, stripped of their S-layers via boiling with 3 M urea, and labeled with mCherry or mCherry hybrids were visualized in the same manner. For visualization of FtsZ, spores were germinated at 40°C in BHI broth and incubated for either four or six hours with shaking. Xylose was added to the culture medium at 0.02% (w/v) to induce *ftsZ* transcription during the final hour of incubation. Vegetative bacilli were sedimented by centrifugation, stained with 1:1000 Hoechst
33342 (Invitrogen), mounted and analyzed by microscopy. Images were obtained with the Olympus IX81 microscope using a 100× objective as described above.

RESULTS

Mutations affecting \textit{B. anthracis} expression of \textit{tagO}. Previous attempts to inactivate the \textit{tagO} gene of \textit{B. anthracis} were unsuccessful (4). We generated a merodiploid strain carrying endogenous \textit{tagO} on the chromosome in addition to the plasmid-encoded \textit{tagO}, placed under the constitutive \textit{hrpK} promoter (\textit{tagO}$/\textit{ptagO}^C$) (Fig. 1AB). For allelic replacement, we used pJML82, a vector temperature-sensitive for replication containing the spectinomycin resistance marker (\textit{aad9}) flanked by 1 kb DNA segments derived from sequences upstream or downstream of chromosomal \textit{tagO}. pJML82 co-integrants into \textit{B. anthracis} carrying an empty vector could not be resolved to yield colonies with viable bacilli. In contrast, pJML82 co-integrants into \textit{B. anthracis} (\textit{ptagO}^C) were readily resolved. Oligonucleotides PI and PII with sequences flanking the \textit{tagO} gene were designed to amplify \textit{tagO} from the chromosome (Fig. 1A). PCR amplification of \textit{tagO} from wild-type \textit{B. anthracis} yielded a 1.1 kb DNA fragment (predicted size 1,147 nucleotides) with a unique NheI restriction site (Fig. 1A and Fig. 1C, left image). Replacement of \textit{tagO} with \textit{aad9} in \textit{B. anthracis} JML360 (\textit{tagO}/\textit{aad9}, \textit{ptagO}^C) generated a PCR fragment of 1,282 nucleotides; as expected, this PCR fragment was cleaved with KpnI but not with NheI (Fig. 1AC). The chromosomal lesion of the \textit{tagO}/\textit{aad9} mutation was verified by DNA sequencing.
Bacteriophage CP-51 was used to generate lysates from the 

\textit{tagO::aad9 (ptagO\textsuperscript{C})}

merodiploid strain for transduction experiments with the 

\textit{tagO::aad9} allele into \textit{B. anthracis}

carrying pJK4 vector, \textit{ptagO} or \textit{pts-tagO} with a temperature-sensitive plasmid replicon. In the 

\textit{ptagO} and \textit{pts-tagO} plasmids, expression of \textit{tagO} is placed under control of the IPTG-inducible 

\textit{spac} promoter (4) (Fig. 1B). CP-51 transductants of 

\textit{tagO::aad9} were plated in the presence and 

absence of IPTG on BHI agar with spectinomycin. As a control, aliquots of the 

\textit{B. anthracis}

recipient strains were transduced with CP-51 phage lysate derived from the 

\textit{B. anthracis}

\textit{bslO::aad9} mutant, which carries a deletion of the non-essential BslO murein hydrolase 

coding the \textit{bslO} gene (32). Transduction of 

\textit{tagO::aad9} or \textit{bslO::aad9} CP-51 lysates generated 

spectinomycin-resistant colonies in \textit{B. anthracis ptagO} or \textit{B. anthracis pts-tagO} recipients (Fig. 

1D). In contrast, CP-51 transduction of \textit{bslO::aad9}, but not of \textit{tagO::aad9}, generated 

spectinomycin-resistant colonies in \textit{B. anthracis} (WT) or \textit{B. anthracis} (pJK4) (Fig. 1D). In twelve 

independent experiments, nine spectinomycin-resistant \textit{tagO::aad9} candidate colonies were 

isolated using the haploid (wild-type \textit{tagO}, pJK4) recipient. When examined for genomic 

content, these isolates appeared to be the result of spontaneous mutation conferring 

spectinomycin resistance as the \textit{aad9} resistance cassette could not be PCR amplified from their 

genomic content and they contained a wild-type \textit{tagO} gene (Fig. 1AC). Genomic DNA of 21 

spectinomycin-resistant transductants into the merodiploid \textit{tagO}\textsuperscript{+ (ptagO\textsuperscript{C})} recipient was 

analyzed by PCR with the PI and PII primer pair. All PCR products were restricted by KpnI but 

not by NheI. A representative sample of such a restriction digest is shown in Fig. 1C. These data 

demonstrate that the \textit{tagO::aad9} mutation can be effectively crossed into \textit{tagO} merodiploid
recipients but not into haploid strains, suggesting that tagO is essential for vegetative growth under laboratory conditions.

*B. anthracis tagO::aad9 (pts-tagO*) cannot form colonies under non-permissive plasmid replication conditions.* To analyze the role of tagO for *B. anthracis* vegetative growth, we took advantage of tagO::aad9 merodiploid strains carrying tagO on plasmids ptagOi and pts-tagOi (Fig. 1B). When the tagO gene is placed solely under the control of the IPTG-inducible spac promoter, leaky transcription occurs in *B. anthracis* growth media and strains do not require the addition of IPTG for vegetative growth (Fig. 1D). However, pts-tagOi carries the thermosensitive repF allele and cannot replicate at temperatures above 30°C, whereas ptagOi replicates under both low and high temperature conditions (4, 29). When propagated in liquid broth at 30°C, *B. anthracis* tagO::aad9 (ptagOi) and tagO::aad9 (pts-tagOi) grew at a similar rate and formed spores when inoculated into modified G medium (Fig. 2A, data not shown). Here too, addition of IPTG was not required for *B. anthracis* tagO::aad9 (ptagOi) and tagO::aad9 (pts-tagOi) growth.

Spore inoculation into rich broth and incubation at 40°C revealed that, in contrast to *B. anthracis* tagO::aad9 (ptagOi), the tagO::aad9 (pts-tagOi) variant was unable to grow beyond a small increase at A600 (Fig. 2A); further, the tagO::aad9 (pts-tagOi) variant could not propagate colonies on BHI agar with 200 µg spectinomycin/ml incubated at 40°C. Bright field (BF) microscopy of bacilli grown at 40°C revealed cell shape distortions in *B. anthracis* tagO::aad9 (pts-tagOi) but not in *B. anthracis* tagO::aad9 (ptagOi). The tagO::aad9 (pts-tagOi) mutant appeared for 3-4 hours as short chains of vegetative forms. Subsequently, however, the
tagO::aad9 (pts-tagOi) variant increased in cell diameter and assumed a coccoid morphology (Fig. 2B).

*B. anthracis* tagO::aad9 (pts-tagOi) cannot assemble FtsZ rings under non-permissive plasmid replication conditions. To visualize the process of cell division, *B. anthracis* wild-type and tagO::aad9 (pts-tagOi) mutant strains were transformed with a plasmid expressing an *ftsZ-gfp* translational hybrid. Earlier work demonstrated that FtsZ and FtsZ-GFP assemble into a ring-structure at the cell division septum of bacteria (41). As expected, FtsZ-GFP Z rings were detected via microscopy in wild-type *B. anthracis* 4 and 6 hours following spore germination (white arrows, Fig. 2C). At 4 hours post germination, *B. anthracis* tagO::aad9 (pts-tagOi) formed misshapen cells, which appeared shorter and wider than wild-type and eventually assumed a coccoid cell shape by 6 hours of incubation (Fig. 2C). At 4 hours post germination, the *B. anthracis* tagO::aad9 (pts-tagOi) vegetative cells frequently displayed FtsZ-GFP rings, albeit that Z rings (white arrows) were positioned at irregularly intervals and some cells harbored diffuse FtsZ-GFP staining throughout the cytoplasm (green arrow, Fig. 2C). By 6 hours, tagO-depleted bacilli did not oligomerize FtsZ-GFP and some cells produced diffuse fluorescence staining (green arrow, Fig. 2C). Of note, FtsZ-GFP rings were no longer observed in *B. anthracis* tagO::aad9 (pts-tagOi) at 6 hours post germination. These data suggest that depletion of tagO does not immediately block cell division and initially affects expansions of the cell wall envelope impacting its physiological shape and function. Eventually, tagO depletion altogether blocks cell division in *B. anthracis*.

Depletion of tagO affects *B. anthracis* SCWP synthesis and S-layer assembly. The SCWP is tethered via acid-labile phosphodiester bonds to the cell wall peptidoglycan of *B. anthracis*.
We hypothesized that some or perhaps all SCWP molecules are anchored to the cell wall by TagO-initiated murein linkage units. Earlier work established a protocol for SEC-HPLC purification of SCWP that had been extracted with HF treatment from purified murein sacculi of B. anthracis (9). Spores of B. anthracis wild-type (WT) and merodiploid variant tagO::aad9 (pts-tagOi) were incubated at 40°C (the non-permissive temperature for pts-tagOi) in BHI broth for germination and vegetative expansion over 5 hours. Murein sacculi of vegetative bacilli were extracted with HF and the SCWP was analyzed by SEC-HPLC. The SCWP of wild-type B. anthracis eluted as a large absorbance peak at 206 nm, whereas tagO::aad9 mutant bacilli produced only small amounts of SCWP (Fig. 3A).

To determine whether tagO expression is essential for S-layer assembly, spores of B. anthracis wild-type and merodiploid variant tagO::aad9 (pts-tagOi) were incubated at 40°C in BHI broth for germination and vegetative expansion over 2 and 5 hours. Cultures were centrifuged to separate the extracellular medium (M fraction) from vegetative bacilli in the sediment. S-layer proteins associated with bacilli were solubilized with 3M urea extraction (S fraction). Sedimented bacteria were subsequently broken with glass beads to generate the cellular lysate (C fraction). Proteins in all three fractions (M, S and C) were analyzed by Coomassie-stained SDS-PAGE, which identified Sap S-layer protein assembly after 2 hours of incubation in wild-type but not in tagO::aad9 (pts-tagOi) mutant bacilli (Fig. 3B). After 5 hours of vegetative expansion, Sap was detected in the S-layer fraction of tagO::aad9 (pts-tagOi) mutant bacilli, albeit that S-layer abundance was diminished as compared to wild-type B. anthracis (Fig. 3B). Taken together, these data suggest that the tagO::aad9 mutation diminishes
SCWP synthesis and S-layer assembly in *B. anthracis* and that most, but not all, SCWP synthesis occurs in a TagO-dependent manner.

**Depletion of tagO affects Sap S-layers in *B. anthracis***. Spores of *B. anthracis* wild-type and merodiploid variants *tagO::aad9* (*ptagO*) or *tagO::aad9* (*pts-tagO*) were incubated at 40°C. At timed intervals of 2 or 5 hours, bacilli were stained with bodipy-vancomycin and with rabbit antibodies raised against purified Sap and secondary antibody conjugates (Fig. 4).

Fluorescence microscopy revealed bodipy-vancomycin deposition at cell division septa of wild-type *B. anthracis* and Sap staining throughout the bacterial envelope at 2 and 5 hours post-germination (Fig. 4AB). The S-layer protein EA1 and BslR, an S-layer associated protein, are assembled into the *B. anthracis* envelope during late stage logarithmic growth (33) and deposited near cell division septa of bacilli incubated for 8 hours in germination media (Fig. 4CD). After 2 hours of temperature-induced depletion of *tagO*, *B. anthracis tagO::aad9* (*pts-tagO*) did not yield immunofluorescence staining with Sap-antibodies. By 5 hours, *tagO::aad9* (*pts-tagO*) bacilli formed coccoid cells with weak but significant Sap-specific immunofluorescence and increased bodipy-vancomycin staining; compare with the fluorescence signals generated by the Δsap mutant (Fig. 4CD). We presume that *B. anthracis tagO::aad9* (*ptagO*), similar to *B. subtilis* ΔtagO and *S. aureus* ΔtagO, cannot properly localize factors involved in peptidoglycan synthesis and cytokinesis (PBP5 and MreB), causing the accumulation of lipid II precursors and uncrosslinked peptidoglycan with cell wall pentapeptides (D-Ala-D-Ala), which each bind bodipy-vancomycin (20, 42). After 8 hours of vegetative expansion, *tagO::aad9* (*pts-tagO*) bacilli displayed mislocalized EA1- and BslR-specific immunofluorescence (Fig. 4CD). Taken together, these data suggest that depletion of *tagO*
diminishes SCWP synthesis and Sap S-layer assembly, while the deposition of EA1 and BslR occurs in a tagO-independent and SCWP-dependent manner. These experimental results imply that the SCWP may be assembled in a tagO-dependent and tagO-independent manner; the purpose of the latter may be the deposition of S-layer and S-layer associated proteins at discrete sites in the bacterial envelope.

**Binding of SLH domains to the envelope of tagO mutant B. anthracis.** To quantify the binding capacity of *B. anthracis* SCWP produced with or without tagO for S-layer proteins, spores of *B. anthracis* wild-type and merodiploid variants tagO::aad9 (ptagO) and tagO::aad9 (pts-tagO) were germinated and incubated at 40°C for 5 hours to induce tagO depletion. Vegetative cells were washed and stripped of S-layer proteins by extraction with urea (39).

Bacilli were then incubated with purified SapSLH-mCherry or EA1SLH-mCherry, hybrid polypeptides with the SLH domains of Sap or EA1 fused to the fluorescent reporter mCherry (4, 9). Following incubation, bacilli were washed and protein binding was quantified by fluorometry or visualized by fluorescent microscopy (Table 1; Fig. 5). As controls, mCherry alone did not bind to urea-extracted wild-type bacilli, whereas SapSLH-mCherry and EA1SLH-mCherry bound SCWP in the envelope of wild-type *B. anthracis*, but not in the ΔcsaB mutant; the csaB mutation blocks ketal-pyruvylation of the SCWP, a modification necessary for SCWP-SLH domain association (4). In *tagO*-depleted bacilli, the binding of SapSLH-mCherry and EA1SLH-mCherry to *tagO::aad9* (pts-tagO) vegetative bacilli was diminished (Fig. 5). In contrast, *tagO::aad9/ptagO* bacilli bound to the SLH domains of SapSLH-mCherry and EA1SLH-mCherry in a manner indistinguishable from wild-type bacilli (Table 1).
Previous work demonstrated that bacteriophage lysins of \textit{B. anthracis}, PlyG and PlyL, associate with the SCWP through their C-terminal cell wall binding domain (CBD) in a manner independent of \textit{csaB}-mediated ketal-pyruvlation (38, 43-45). We generated purified PlyG\textsubscript{CBD-mCherry}, where the C-terminal CBD of PlyG (38) is fused to the mCherry reporter, to assess the overall abundance and subcellular distribution of the SCWP (with or without ketal-pyruvate) in wild-type and \textit{tagO::aad9 (pts-\textit{tagO})} vegetative forms. PlyG\textsubscript{CBD-mCherry} associated with the SCWP of wild-type bacilli and appeared uniformly distributed throughout the bacterial envelope (Fig. 5). The abundance and uniform distribution pattern of PlyG\textsubscript{CBD-mCherry} deposition in the bacterial envelope was not affected in \textit{csaB} mutant \textit{B. anthracis} (Table 1; Fig. 5). As expected, the overall chain length of the \textit{B. anthracis} \textit{csaB} mutant is increased as compared to wild-type (2, 32). Depletion of \textit{tagO} in \textit{tagO::aad9 (pts-\textit{tagO})} mutant bacilli decreased the binding of PlyG\textsubscript{CBD-mCherry} to the bacterial envelope by 61%, in agreement with the hypothesis that TagO is required for the synthesis and cell wall attachment of some, but not all SCWP (Table 1).

Fluorescence microscopy experiments suggest that residual SCWP attachment in \textit{tagO} depleted bacilli occurs near the cell septa but not throughout the envelope of the coccoid bacilli that are formed in the absence of TagO (Fig. 5). Taken together, these data indicate that TagO activity is required for the synthesis and cell wall deposition of the SCWP in the cylindrical envelope of \textit{B. anthracis}, whereas TagO-independent SCWP synthesis and deposition appears to occur near cell septa. In agreement with this model, \textit{tagO}-depleted bacilli display a large defect in the assembly of the Sap S-layer protein along the cylindrical axis of the \textit{B. anthracis} envelope, whereas the deposition of EA1 and BslR near cell septa is only moderately affected (Fig. 4).
Contribution of BA5436 (BAS5051) deacetylase towards SCWP synthesis. Balomenou et al. reported that B. anthracis lacking the polysaccharide deacetylase BA5436 (ΔBA5436) produce long chains of vegetative bacilli and that HF extraction of peptidoglycan did not release SCWP in this mutant (22). Further, the ΔBA5436 mutant assembled reduced amounts of S-layer proteins (Sap and EA1) in the bacterial envelope, as determined by urea extraction and SDS-PAGE analysis of these polypeptides (22). Nevertheless, ΔBA5436 mutant bacilli did not display defects in growth, which was surprising, as studies of gneZ, gneY (12, 14) and tagO (this report) suggest that SCWP synthesis is essential for B. anthracis growth. Of note, the structural gene for BA5436 (BAS5051 in B. anthracis Sterne) is located immediately adjacent to tagO (BAS5050).

We isolated a bursa aurealis insertion at position 412 of the 737 base pair BAS5051 open reading frame (ORF) (32, 34) and transduced the mutation into wild-type B. anthracis (Fig. 1A). Further, a temperature-sensitive plasmid construct was generated to delete the entire ORF in B. anthracis, yielding the variant ΔBAS5051 (Fig. 1A). The ΔBAS5051 and BAS5051::bursa aurealis mutants did not display growth or sporulation defects (data not shown). HF-extracts of murein sacculi were examined by SEC-HPLC for SCWP production (Fig. 6A); wild-type, BAS5051::bursa aurealis and ΔBAS5051 mutant B. anthracis released SCWP with similar abundance. Cultures of B. anthracis wild-type or its BAS5051::bursa aurealis and ΔBAS5051 variants were grown to A600 2 and subjected to S-layer analysis on Coomassie stained SDS-PAGE and immunoblotting for S-layer proteins Sap and EA1. Similar total amounts and a similar distribution of Sap and EA1 in the medium and S-layer were detected by Coomassie-stained SDS-PAGE and immunoblotting (Fig. 6B). Taken together, these data suggest that mutations in BAS5051 do not affect B. anthracis growth, SCWP synthesis or S-layer assembly.
DISCUSSION

Unlike *S. aureus* or *B. subtilis*, *B. anthracis* does not synthesize glycerol (GroP) or ribitol (RibP) phosphate WTA (5). Accordingly, homologs of *tarI*, *tarJ*, *tarL*, *tagB*, *tagD*, or *tagF*, genes whose products catalyze precursor activation and polymerization of wall teichoic acid, are absent from the genome of *B. anthracis* (46). Nevertheless, the genome of *B. anthracis* encodes homologs of *tagO* and *tagA* (*tagA1* and *tagA2*), which are involved in the synthesis of cell wall linkage units [-P-GlcNAc-ManNAc-R] that tether teichoic acid repeat units to peptidoglycan (47, 48). A genetic linkage between *tagA* and *csaB* genes and the presence of acid-labile SCWP led us to hypothesize that the *tagO* gene of *B. anthracis* may be involved in the synthesis of the SCWP (4). Here, this hypothesis was tested and validated. We used for the first time an experimental system of temperature-induced gene depletion in *B. anthracis* and demonstrated that the *tagO::aad9* mutant cannot grow without *tagO* expression. Shortly following spore germination and depletion of *tagO*, *tagO* mutants exhibit defective Z ring formation and cannot complete the cell division cycle. Instead, these cells form misshapen coccoid cells with reduced amounts of SCWP and are defective in their Sap S-layer protein assembly. After prolonged incubation, the *tagO* mutant eventually generates limited amounts of SCWP and supports assembly of S-layer protein in the vicinity of cell septa.

These experiments revealed also that not all SCWP synthesis and envelope attachment is dependent on *tagO* expression. Even though *tagO* mutant coccoid cells cannot grow, the mutant attaches SCWP at cell contact sites, which serve also as assembly sites for the EA1 S-
layer protein and the BslR S-layer associated protein. It is well established that the distribution
of S-layer proteins and S-layer associated proteins in the envelope of *B. anthracis* is not
homogeneous and occurs in an organized manner (2, 32, 33, 49). Two models have been
to explain this phenomenon. SLH domains of S-layer and S-layer associated proteins
are similar, but not identical in sequence, suggesting they have discrete differences in affinity
for SCWP molecules with or without modifications (50), for example PatA1-PatB1 and PatA2-
PatB2 acetylation (9). Do discrete differences in the affinity of SLH domains for specific SCWP
molecules function as determinants for their subcellular distribution? If this model, (here
designated *orchestrated assembly*), were correct, one would also predict that SCWP molecules
with discrete modifications were deposited at unique locations in the bacterial envelope. To the
best of our knowledge, experimental support for this conjecture was heretofore missing and is
now provided via the fluorescence microscopy experiments involving PlyG<sub>CBD</sub>-mCherry and
wild-type as well as *tagO* mutant bacilli (Fig. 4). On the other hand, Sap and EA1 are
transported across the bacterial envelope by a unique secretory pathway, defined by products
of the *secA2, slaP* and *slaQ* genes (39, 51). Also, mutations that affect Sap S-layer protein
expression impact the distribution of the other S-layer (EA1) and S-layer associated proteins
(BSLs) in the envelope; these observations imply that abundant secretion and assembly of Sap
blocks sites on the SCWP that then cannot be occupied by other SLH domain proteins (*preferred
seating model*) (33).

Our finding that *tagO* mutant bacilli synthesize small amounts of SCWP near cell septa,
thereby maintaining assembly sites for EA1 and BslR, but not Sap, provides new support for the
*orchestrated assembly* model. We would predict that specific LCP enzymes, catalysts of the
phosphodiester linkage tethering teichoic acids and polysaccharides to peptidoglycan (20, 52), also play a key role in the assembly of the SCWP at discrete sites. For example, \textit{tagO}-mediated SCWP attachment and subsequent assembly of Sap S-layers may require specific LCP enzymes from a cast of six different catalysts in \textit{B. anthracis} (LcpB1-4, LcpC and LcpD)(40), whereas \textit{tagO}-independent attachment of the SCWP would involve others. If so, some LCP enzymes may not utilize [-P-GlcNAc-ManNAc-SCWP] as a substrate and instead recognize SCWP assembled on undecaprenol-phosphate with or without amino sugars. In this regard, the conservation of two different \textit{tagA} alleles in the genome of \textit{B. anthracis} provides circumstantial evidence for the existence of another murein linkage unit.

\textit{TagO} is inhibited by tunicamycin, which at higher concentrations also inhibits MraY, an enzyme that links Park’s nucleotide (UDP-MurNAc-Ala-iGlu-Dpm-Ala-Ala) and undecaprenol-phosphate to initiate peptidoglycan synthesis (53). Even at very low concentrations, tunicamycin is an effective antibiotic for \textit{B. anthracis} (4). Tunicamycin toxicity in vertebrates, caused by inhibition of protein oligosaccharide-transferase (OST)(54), prohibits its clinical use. As supported by evidence in this report, SCWP synthesis and modification of peptidoglycan in Gram-positive bacteria with secondary polymers is essential for the replication of these microbes. Therefore, small molecules that specifically block TagO, TagA or LCP enzymes may be clinically useful for the therapy of infections with antibiotic-resistant Gram-positive bacteria such as \textit{Clostridium difficile}, \textit{Enterococcus faecium} and \textit{Staphylococcus aureus}.

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REFERENCES


FIGURE LEGENDS
FIG. 1. The tagO and BAS5051 genes of B. anthracis. (A) Organization of tagO and neighboring genes on the chromosome of B. anthracis Sterne 34F2. The arrowhead above gene BAS5051 denotes the bursa aurealis insertion site. Sites for restriction enzymes NheI and KpnI are shown as well as sites of hybridization of primers PI and PII. (B) Genetic layout of tagO complementing plasmids used in this study. P indicates promoters and rep indicates genes involved in plasmid replication. The bottom two plasmids are identical with the exception of pts-tagO containing a thermosensitive (ts) replication protein F. (C) Disruption of the tagO gene following allelic recombination or transduction was confirmed by PCR amplification of the deleted locus with flanking primers PI and PII followed by digestion with enzymes. Photographs of DNA gels are shown. (D) Transduction efficiency of the bslO or tagO alleles into wild type B. anthracis (WT) alone or carrying plasmid pJK4 (cloning plasmid), ptagO or thermo-sensitive pts-tagO plasmids. Spectinomycin-resistant colonies were enumerated after incubation of plates at 30°C for 24 hours with and without IPTG in the medium. Insertion of the correct alleles was verified as shown in panel C as well as by DNA sequencing. Phage lysate of bslO::aad9 was used as a control. Data from six independent experiments were averaged and plotted with their associated standard error.

FIG. 2. Limited expression of tagO causes growth and morphological defects. (A) Spores (1x10⁶/ml) derived from tagO::aad9 (ptagO) and tagO::aad9 (pts-tagO) strains were inoculated into BHI broth at 30 or 40°C and vegetative growth was monitored as increased optical density at 600 nm (A₆₀₀). Growth curves are representative graphs of three experimental replicates. (B) Bacilli were fixed at the specified time points following germination at 40°C and
observed by bright field (BF) microscopy. Scale bar: 10 µm. (C) Spores (1x10^6/ml) derived from wild-type and merodiploid tagO::aad9 (pts-tagO) strains carrying a plasmid expressing FtsZ-GFP were inoculated into BHI broth at 40°C. Bacilli were stained with Hoechst (DNA) at 4 and 6 hours following germination at 40°C and observed by phase and fluorescence microscopy. White arrows identify Z rings, whereas green arrows identify cells with aberrant FtsZ-Gfp distribution. Scale bar: 5 µm.

FIG. 3. Depletion of tagO causes SCWP synthesis and S-layer assembly defects in B. anthracis. (A) Spores derived from B. anthracis wild-type (WT) and tagO::aad9 (pts-tagO) were germinated at 40°C and vegetative forms expanded for 5 hours. Murein sacculi were isolated and treated with hydrofluoric acid (HF). Released SCWP was separated by SEC-HPLC and absorbance recorded at 206 nm (mAu). The SEC-HPLC data are representative of three experimental replicates. (B) Spores derived from B. anthracis wild-type (WT) and tagO::aad9 (pts-tagO) were germinated at 40°C and vegetative forms expanded for 2 and 5 hours. B. anthracis cultures were centrifuged to separate the extracellular medium (M) from the bacterial sediment. The S-layer (S) of bacilli was extracted by boiling in 3 M urea. Extracted cells (C) were broken in a bead beater. Proteins in all fractions were precipitated with TCA, washed in acetone, separated on 10% SDS-PAGE and stained with Coomassie.

FIG. 4. Limited expression of tagO causes decreased binding of the Sap S-layer. Spores from B. anthracis wild-type (WT), sap, eag and bslR mutants or tagO::aad9/ptagO variants were germinated in BHI broth at 40°C and incubated for 2, 5 or 8 h, fixed in
formalin, and stained with αSap antibodies (A-B), αEA1 antibodies (C), or αBslR antibodies (D).

Antibody binding and bacterial architecture were revealed with DyLight594 conjugated anti-rabbit secondary antibody and bodipy-vancomycin (bodipy-van), respectively. Bright field and fluorescent microscopy images were acquired. Data sets were merged to reveal the location of the proteins in relation to the cell septa (bodipy-van). The sap, eag and bslR mutants were stained as controls for specificity of antibody binding. Scale bar: 10 µm.

FIG. 5. Capacity of S-layer stripped bacilli to retain SCWP-binding proteins with or without tagO expression. Spores from B. anthracis Sterne (WT), csab mutant or tagO::aad9 (ptagO) and tagO::aad9 (pts-tagO) variants were germinated in BHI broth at 40˚C for 5 hours. Bacilli were then treated with 3 M urea to remove S-layer proteins attached to the bacterial envelope. Stripped bacilli were incubated with purified reporter hybrids, SapSLH-mCherry, EA1SLH-mCherry or PlyGCBD-mCherry, washed and bright field and fluorescent microscopy images were acquired. The csab mutant served as a control for loss of S-layer binding. Scale bar: 10 µm.

FIG. 6. Requirement of BAS5051 deacetylase for SCWP synthesis and S-layer assembly. (A) Equal amounts of purified murein sacculi from B. anthracis wild-type (WT) or isogenic variants ΔBAS5051 and BAS5051::bursa were treated with hydrofluoric acid. Released SCWP was separated by SEC-HPLC and absorbance recorded at 206 nm (mAu). (B) Cultures of B. anthracis Sterne (WT) or isogenic variants ΔBAS5051 and BAS5051::bursa were grown to the mid-log phase and centrifuged to separate the extracellular medium (M) from the bacterial sediment. The S-layer (S) of bacilli was extracted by boiling in 3 M urea. Extracted cells (C) were broken in
a bead beater. Proteins in all fractions were precipitated with TCA, washed in acetone, and separated over 10% SDS-PAGE gels for Coomassie staining or immunoblotting with rabbit antisera raised against purified Sap (αSap) or EA1 (αEA1).
Table 1. Relative binding of protein hybrids to stripped mutant bacilli as compared to wild-type bacilli.

<table>
<thead>
<tr>
<th>Protein hybrid</th>
<th>csaB</th>
<th>P value (pts-tagO&lt;sup&gt;i&lt;/sup&gt;)</th>
<th>tagO::aad9</th>
<th>P value (ptagO&lt;sup&gt;i&lt;/sup&gt;)</th>
<th>tagO::aad9</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SapSLH&lt;sup&gt;-&lt;/sup&gt;mCherry</td>
<td>6% (±16%)</td>
<td>1x10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>58% (±9%)</td>
<td>8.3x10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>119% (±9%)</td>
<td>0.07</td>
</tr>
<tr>
<td>EA1SLH&lt;sup&gt;-&lt;/sup&gt;mCherry</td>
<td>5% (±10%)</td>
<td>6.5x10&lt;sup&gt;-11&lt;/sup&gt;</td>
<td>36% (±4%)</td>
<td>3.5x10&lt;sup&gt;-9&lt;/sup&gt;</td>
<td>103% (±4%)</td>
<td>0.57</td>
</tr>
<tr>
<td>PlyGCBD&lt;sup&gt;-&lt;/sup&gt;mCherry</td>
<td>94% (±3%)</td>
<td>0.07</td>
<td>39% (±2%)</td>
<td>2.8x10&lt;sup&gt;-12&lt;/sup&gt;</td>
<td>93% (±4%)</td>
<td>0.06</td>
</tr>
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</table>

<sup>1</sup>Binding of fluorescent proteins was assessed by fluorescence measurement; means and associated standard errors were derived from six independent experimental determinations. Arbitrary units of fluorescence were converted to % binding. Binding to wild type bacilli was set as 100% for a given hybrid. The number of bacilli was normalized between experiments. In all cases, binding of mCherry alone was <5%.