Polydiglycosylphosphate Transferase PdtA (SCO2578) of *Streptomyces coelicolor* A3(2) Is Crucial for Proper Sporulation and Apical Tip Extension under Stress Conditions

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**ABSTRACT**

Although anionic glycopolymers are crucial components of the Gram-positive cell envelope, the relevance of anionic glycopolymers for vegetative growth and morphological differentiation of *Streptomyces coelicolor* A3(2) is unknown. Here, we show that the LytR-CpsA-Psr (LCP) protein PdtA (SCO2578), a TagV-like glycopolymer transferase, has a dual function in the *S. coelicolor* A3(2) life cycle. Despite the presence of 10 additional LCP homologs, PdtA is crucial for proper sporulation. The integrity of the spore envelope was severely affected in a *pdtA* deletion mutant, resulting in 34% nonviable spores. *pdtA* deletion caused a significant reduction in the polydiglycosylphosphate content of the spore envelope. Beyond that, apical tip extension and normal branching of vegetative mycelium were severely impaired on high-salt medium. This growth defect coincided with the mislocalization of peptidoglycan synthesis. Thus, PdtA itself or the polydiglycosylphosphate attached to the peptidoglycan by the glycopolymer transferase PdtA also has a crucial function in apical tip extension of vegetative hyphae under stress conditions.

**IMPORTANCE**

Anionic glycopolymers are underappreciated components of the Gram-positive cell envelope. They provide rigidity to the cell wall and position extracellular enzymes involved in peptidoglycan remodeling. Although *Streptomyces coelicolor* A3(2), the model organism for bacterial antibiotic production, is known to produce two distinct cell wall-linked glycopolymers, teichuronic acid and polydiglycosylphosphate, the role of these glycopolymers in the A3(2) life cycle has not been addressed so far. This study reveals a crucial function of the anionic glycopolymer polydiglycosylphosphate for the growth and morphological differentiation of *S. coelicolor* A3(2). Polydiglycosylphosphate is attached to the spore wall by the LytR-CpsA-Psr protein PdtA (SCO2578), a component of the *Streptomyces* spore wall-synthesizing complex (SSSC), to ensure the integrity of the spore envelope. Surprisingly, PdtA also has a crucial role in vegetative growth under stress conditions and is required for proper peptidoglycan incorporation during apical tip extension.

The bacterial cell envelope forms a protective shield around bacteria, providing mechanical strength and conferring cell shape and size (1). The cell envelope is the interface for the interaction with the environment and one of the most important targets of antibiotics. The cell envelope of Gram-positive bacteria consists of a multilayered peptidoglycan (PG), a heteropolymer of peptide cross-linked glycan strands, membrane-anchored lipoteichoic acids, PG-bound wall teichoic acids (WTA), exopolysaccharides, and surface proteins (2, 3). WTAs, which can account for about 60% of the total mass of the cell envelope (4), are carbohydrate-based anionic glycopolymers often substituted with D-alanine ester residues, giving the molecule zwitterionic properties. They provide rigidity to the cell wall by attracting cations, such as magnesium and sodium, and play an essential role in growth, division, and morphogenesis, probably by acting as scaffolds for extracellular enzymes involved in PG remodeling (5).

WTA synthesis in *Bacillus subtilis* is initiated in the cytoplasm by TagO transferring N-acetylglycosamine 1-phosphate (GlcNAc-1-P) from UDP-GlcNAc to the membrane-anchored lipid carrier undecaprenylphosphate (6). Then, the glycosyltransferase TagA transfers N-acetylmannosamine (ManNAc) to make the linker unit ManNAc-beta-(1,4)-GlcNAc-pp-undecaprenyl (7). Subsequently, the primase TagB and the polymerase TagF add up to 60 glycerol-3-phosphate (GroP) units (8). The lipid carrier-linked teichoic acid is finally transported by the TagG/H ABC transporter (9) and covalently attached to the PG via phosphodiester linkage to the C6 hydroxyl of the N-acetylmuramic acid (MurNAc), carried out by the three redundant phosphotransferases TagT, TagU, and TagV (10). TagT, TagU, and TagV belong to the LytR-CpsA-Psr (LCP) family, originally characterized as cell envelope-associated transcriptional attenuator proteins (11). The LCP proteins are putative transmembrane proteins mainly found in Gram-positive bacteria and generally appear to be involved in cell envelope maintenance, although their mechanistic functions often remain unclear. They typically consist of a short intracellular N-terminal region, a transmembrane helix, and a large extracellular LytR-
CpsA-Psr domain (12). LytR was first described in B. subtilis as a transcriptional attenuator of both itself and the divergently transcribed lytABC autolysin operon (11). CpsA was characterized as a transcription activator of the capsular polysaccharide synthesis operon of Streptococcus pneumoniae (13). Psr was initially proposed to be a repressor of the synthesis of penicillin-binding protein 5 (PBP 5) in Enterococcus hirae, but its role in PBP 5 synthesis has not been confirmed (14). Moreover, a function of the LCP proteins in protein glycosylation was recently described (15).

In contrast to most other bacteria, which divide by binary fission, the Gram-positive soil bacterium Streptomyces coelicolor A3(2) develops a mycelial lifestyle by apical tip extension (16). PG incorporation at the hyphal tips and branching of the mycelium depend on DivIVA and the coiled-coil proteins FilP and Scy, which form a tip-organizing center (TIPOC), also called the polarisome (17–19). The multiply branched mycelium contains sporadic cross walls, which separate mycelial compartments containing multiple chromosomes (20). Upon partial nutrient depletion, Streptomyces starts to differentiate, regulated by a hierarchical cascade of whi and bld genes (21). Under partial lysis of the vegetative mycelium, sporogenic aerial hyphae are erected and transformed into spore chains by the coordinated formation of dozens of cross walls. Following segregation of chromosome copies and spore wall thickening, the ellipsoid uninucleoid spores are released into the environment (22). Proper sporulation and synthesis of the thickened spore wall were shown to depend on the Streptomyces spore wall-synthesizing complex (SSSC), which highly resembles the elongasome of rod-shaped bacteria (23, 24). The activity of the SSSC is probably controlled by protein phosphorylation involving multiple eukaryote-like serine/threonine protein kinases (eSTPK) (25). Besides MreBCD, RodZ, and various penicillin-binding proteins, the SSSC also contains proteins possibly involved in anionic glycopolymer synthesis, suggesting that the SSSC not only directs PG synthesis but also determines the glycopolymer content of spore envelopes (24).

Streptomyces coelicolor A3(2) encodes several homologues of Tag proteins (26) directing WTA synthesis in B. subtilis, although the major glycolipid of S. coelicolor has been recently characterized as teichulosonic acid. The S. coelicolor teichulosonic acid is a phosphate-free polymer of up to seven repeating units composed of galactose (Gal) and the neuraminic acid-related 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (Kdn), often substituted with GlcNAc or a methyl group. As a minor component, a polyglycosylphosphate (PDP) polymer consisting of -6)-α-Galp-(1→6)-α-GlcNpNAc-(1→P- subunits [poly(Gal-GlcNAc-P)] was found (27).

The TagV-like LCP protein SCO2578 was identified in a genomic library of S. coelicolor M145 as an interaction partner of the SSSC proteins MreC, MreD, and Sfr (RodA). It also interacted with RodZ and with the penicillin-binding proteins PBP 2, FtsI, and SCO3580 (23). To study the role of SCO2578 in S. coelicolor, we constructed a knockout mutant of SCO2578 and characterized the effects of SCO2578 inactivation on proper sporulation and vegetative growth. Despite the presence of 11 homologous genes in S. coelicolor, SCO2578 had a nonredundant function. SCO2578 inactivation affected the integrity of the spore envelope and growth of vegetative mycelium, in particular when grown under high-osmolarity conditions. The defects correlated with the mislocalization of PG synthesis and a reduced PDP content in the spore envelope, identifying SCO2578 (PdtA) as a PDP transferase.
plasmids

Staining of newly synthesized PG using Van-Fl. Strains were grown on coverslips as described above. After 48 h, 15 µl of the fluorescent vancomycin (Van-Fl) staining solution (5 µg/ml in 0.85% NaCl) was pipetted into the gap between agar and coverslip. Since vancomycin has a high affinity to the terminal D-Ala-D-Ala structure of non-cross-linked PG, it binds to sites of active PG biosynthesis. After 15 min of incubation in darkness at 30°C, the coverslips were transferred to microscope slides and inspected as described above.

Determination of a growth defect in liquid cultures. To investigate biomass accumulation in liquid cultures, 500-ml Erlenmeyer flasks containing either LB or LB supplemented with 6% NaCl were inoculated with biomass accumulation in liquid cultures, 500-ml Erlenmeyer flasks containing either LB or LB supplemented with 6% NaCl were inoculated with S. coelicolor A3(2) M145 and the dry weight was determined (31). The biomass was harvested by centrifugation. The pellets were lyophilized, and the dry weight was determined (31). The biomass was harvested by centrifugation. The pellets were lyophilized, and the dry weight was determined (31). The biomass was harvested by centrifugation. The pellets were lyophilized, and the dry weight was determined (31). The biomass was harvested by centrifugation. The pellets were lyophilized, and the dry weight was determined (31). The biomass was harvested by centrifugation. The pellets were lyophilized, and the dry weight was determined (31). The biomass was harvested by centrifugation. The pellets were lyophilized, and the dry weight was determined (31). The biomass was harvested by centrifugation. The pellets were lyophilized, and the dry weight was determined (31). The biomass was harvested by centrifugation. The pellets were lyophilized, and the dry weight was determined (31). The biomass was harvested by centrifugation. The pellets were lyophilized, and the dry weight was determined (31).

Spore viability assays. Strains were cultivated on MS agar, as described above. After 4 days of growth at 30°C and 120 rpm, the biomass of 3-day-old HA liquid cultures was harvested. For isolation of mycelial cell walls, 300-ml Erlenmeyer flasks containing either LB or LB supplemented with 6% NaCl were inoculated with approximately 5 × 10⁸ spores. After 4 days of growth at 30°C and 120 rpm, the biomass was harvested by centrifugation. The pellets were lyophilized, and the dry weight was determined (n = 5).

Spore viability assays. Strains were cultivated on MS agar, as described above. After 4 days of incubation, spore chains were transferred directly on agar plates by pipetting 15 µl of PBS containing 5 µM SYTO9 dye and 30 µM propidium iodide into the gap between agar and coverslip. After 10 min of staining at room temperature in darkness, the coverslips were transferred to microscope slides. Images were taken using a Leica DM550B microscope equipped with appropriate filter cubes (SYTO9, bandpass [BP] 470/40 nm [excitation] and BP 525/50 nm [emission]; propidium iodide, BP 535/50 nm [excitation] and BP 610/75 nm [emission]) and a Leica DFC360FX camera. Images for counting the living (green) and dead (red) spores were selected based on the phase-contrast pictures. Individual images were taken as one data point, and at least 16 images from at least 3 independent plates were counted.

To verify the results of LIVE/DEAD staining, CFU were determined. Spores were isolated after 5 days using 4 ml of 0.85% NaCl. The optical density (OD) of the spore solution was adjusted to 1.0, and the spore concentration was additionally controlled in a counting chamber. Dilution series were plated on LB agar and incubated at 30°C, followed by inspection for CFU after 48 h.

Sensitivity to salt stress was determined by spotting serial spore dilutions (10 µl) onto LB agar containing 6% NaCl. After 3 days of incubation at 30°C, the different spots were checked for growth. Wild-type and mutant spore suspensions (10⁷ in 100 µl of H₂O) were streaked out side by side on LB agar plates. Filter disks carrying either lysozyme (5 KU) or various antibiotics were applied immediately. Pictures were taken after 3 days of incubation at 30°C.

Phage assays. Streptomyces phages (ΦTU1 to ΦTU4) were isolated from soil sampled in Tübingen and propagated on Streptomyces collinus Tü365. About 10⁴ spores were poured in 3 ml of phage soft agar (10 g of glucose, 5 g of tryptone, 5 g of beef extract, 0.74 g of CaCl₂, 5 g of agar [all per liter]) onto phage agar (10 g of glucose, 5 g of tryptone, 5 g of beef extract, 0.74 g of CaCl₂, 15 g of agar [all per liter]) plates. Subsequently, 5 µl of phage lysates was spotted onto the plates and incubated for 24 h at 30°C.

Separation of cell/spore wall fractions and determination of peptidoglycan proportion in spore walls. For isolation of mycelial cell walls, the biomass of 3-day-old HA liquid cultures was harvested. For isolation of spore walls, spores were collected after 8 days of incubation from approximately 80 MS agar plates by wiping them from the surface with wet cotton pads. Cell/spore walls were isolated as described previously (31).

In order to detach cell/spore wall polymers from peptidoglycan, a defined amount (5 to 10 mg) of purified cell/spore walls was hydrolyzed.
under moderately acidic conditions (50 mM HCl, 90°C), according to Wolters et al. (32). This treatment leaves glycosidic and peptide bonds intact, while it is harsh enough to cleave the phosphodiester bonds by which cell wall polymers usually are attached to peptidoglycan. After 90 min of incubation, the samples were neutralized, and the soluble polymer fraction was separated from the insoluble peptidoglycan fraction by centrifugation (30 min, 21,000 × g, 4°C). The supernatant was freeze-dried, resolved in 100 μl of H2O, and stored for further chemical analysis. The pellet was washed twice and freeze-dried. The remaining weight in relation to the starting weight was interpreted as the peptidoglycan proportion of the spore wall.

**Determination of PG cross-linking level.** Spore wall isolation, PG purification, and PG digestion were performed as described previously (31). The resulting PG digestion products were separated by high-performance liquid chromatography (HPLC), and the muropeptides were identified using mass spectrometry. The PG cross-linking level was determined by calculating the amount of actual performed cross-links in relation to possible cross-links. In detail, the number of cross-links in dimers (1) and trimers (2) was divided by the number of muropeptides in monomers (1), dimers (2), and trimers (3). Higher-oligomer molecules were also detectable by the method used, but reliable quantification gets more difficult with increasing molecular weight. Therefore, tetramers and pentamers were not included in the cross-linking calculation.

**HPLC-MS analysis.** Samples were analyzed by HPLC-electrospray ionization-mass spectrometry (HPLC-ESI-MS) with an Agilent 1200 HPLC series equipped with an Agilent LC/MSD Ultra trap system XCT 6330 (Agilent Waldbronn, Germany) by linear gradient elution with eluent A at 0.1% formic acid in water and eluent B at 0.06% formic acid in methanol. The gradient was tA = tB = 5% B, t100 = t10 = 20.8% B (where t is time in minutes), at a flow rate of 0.5 ml/min. Subsequently, 80 μl of sample volume was injected on a Reprosil Gold 300 C18 column (250 by 4.6 mm inside diameter [i.d.], 5 μm; Dr. Maisch HPLC, Ammerbuch, Germany). The column temperature was 52°C. ESI-MS analysis (positive and negative ionization, alternating) was performed in ultrasmall mode with a capillary voltage of 3.5 kV and drying gas temperature of 350°C. Data evaluation was done with DataAnalysis for the 6300 series Ion Trap LC/MS software 6.1, version 3.4; Bruker Daltonik).

**Characterization of the cell/spore wall polymers.** The amount of phosphatase in the polymer fraction was measured according to Chen et al. (33), with some modifications. An aliquot (10 μl) of supernatant was dried in ultrapure HPLC glasses. Complete hydrolyzation was achieved by adding 75 μl of 70% perchoric acid and incubation at 100°C for 2 h. After the samples had cooled down to room temperature, 250 μl of H2O, 100 μl of 1.25% ammonium molybdate, and 100 μl of 5% l-ascorbic acid were added. After an additional 10 min of incubation at 100°C, the absorbance at 750 nm was determined in a photometer and compared to a KH2PO4 standard curve.

The amount of hexosamine in the polymer fraction was measured as described previously (34).

The amount of 2-keto-3-deoxy-o-glycero-β-galacto-nononic acid (Kdn) in the polymer fraction was determined according to Matsumo and Suzuki (35). For the measurement, 10 μl of supernatant was mixed with 90 μl of ice-cold H2O. Kdn was oxidized by adding 10 μl of 10 mM sodium periodate, followed by 45 min of incubation on ice. The reaction was stopped using 50 μl of 50 mM sodium thiosulfate. After adding 250 μl of 4 M ammonium acetate (pH 7.5) and 200 μl of an ethanolic solution of hexanucleotide sequence GGATCC. The large intergenic regions of 99 to 582 bp in size indicate independent transcription of each gene, consistent with published microarray data (40). pdtA is probably cotranscribed with the nontetinate-nucleotide adenyllytransferase gene SCO2579 (Fig. 1A) and lies close to other putative anionic glycopolymer-related genes (26).

The ΔpdtA mutant forms irregular spore chains. To study the proposed function of PdtA in sporulation, we generated a nonpo- lar in-frame deletion mutant of pdtA (ΔpdtA). Upstream and downstream fragments, including the start and stop codons of pdtA, respectively, of 1.54 kb of size were amplified from S. coelicolor DNA and cloned into the nonreplicative pGus21 vector. After conjugative transfer to S. coelicolor, apramycin-resistant transconjugants were selected, which contained the knockout plasmid integrated into the chromosome via a single crossover. Following an additional round of sporulation on nonselective medium, colonies were isolated on distinct plates that had lost GusA activity, indicated by white color on X-Gluc plates. PCR analyses and Southern blotting (data not shown) confirmed ΔpdtA mutants, where the complete pdtA coding region had been replaced by the hexanucleotide sequence GGATCC.

Although the interaction of PdtA with the SSCS proteins RodZ, FtsI, MreC, MreD, PBP 2, and Sfr (23) suggested an involvement of PdtA in proper sporulation, the ΔpdtA mutant was able to sporulate and showed normal colony morphology (data not shown). The average spore (n = ~1,000) length (Fig. 1A), the number of spores within the sporogenic hyphae (n = 50), as well as the length of the sporogenic hyphae (n = 50) did not significantly differ from those of M145 (see Fig. S1 in the supplemental material). This indicated that pdtA inactivation did not substantially interfere with the development of the sporogenic compartment prior to its septation. However, a careful inspection of spore chains revealed a less-homogeneous composition of the spore chains, a phenotype also observed with the majority of other mutants affected in one of the SSCS genes. When analyzing the heterogeneity of the spore sizes within individual spore chains, as indicated by the higher standard deviations, statistically significant differences were ob-
TABLE 2 TagT, TagU, and TagV-like LCP family proteins of S. coelicolor A3(2)

<table>
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<tr>
<th>Protein</th>
<th>Size (aa)</th>
<th>Domain (aa)</th>
<th>TM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LytR&lt;sub&gt;cpsA_psa&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
<th>LytR&lt;sub&gt;C&lt;/sub&gt;&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Similarity (E value) to&lt;sup&gt;b&lt;/sup&gt;:</th>
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<tr>
<td>SCO2578</td>
<td>592</td>
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<td>273–421</td>
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<td>349–442</td>
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<td>085–247</td>
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<td>146–302</td>
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<sup>a</sup> BLASTP (TagT, TagU, and TagV of B. subtilis 168 were compared by BLASTP against all S. coelicolor proteins).

<sup>b</sup> Predicted by TMpred. TM, transmembrane.

<sup>c</sup> Predicted by SMART (HMMER3).

The mean standard deviation of the mean spore length within individual spore chains of M145 was ±0.16 μm, that of ΔpdtA mutant spores was ±0.23 μm (Fig. 1B). Genetic complementation by the integration of plasmid pRMpdtAc into the FC31 att site reduced the mean standard deviation of spore length to ±0.15 (Fig. 1B). The heterogeneity in the spore sizes suggests that pdtA inactivation subtly interfered with the precise placement of sporulation septa, affecting the uniformity of spore lengths within the spore chains.

Integrity of the ΔpdtA mutant spore envelope is impaired.

To analyze whether the sporulation defect causing increased heterogeneity of the ΔpdtA mutant spores also has consequences for the integrity of the spore envelope, the sensitivity of ΔpdtA mutant to salt stress and cell wall-targeting vancomycin and lysozyme was analyzed. When serial dilutions of ΔpdtA mutant spores were spotted on LB agar containing 6% NaCl, survival was reduced compared to the wild-type M145 (Fig. 2A). Whereas resistance of plated spores to vancomycin (Fig. 2B) and several other antibiotics (data not shown) was not affected at all, the ΔpdtA mutant showed a slightly increased sensitivity to lysozyme (Fig. 2B). Surprisingly, the ΔpdtA mutant was also highly sensitive to rifampin (Fig. 2B). Since rifampin does not target the bacterial cell wall but rather the RNA polymerase in the cytoplasm, the surface properties of the ΔpdtA mutant spores might be altered, facilitating the entry of rifampin.

To directly assess the integrity of the spore envelope, the viability of ΔpdtA mutant spores was analyzed by LIVE/DEAD staining, S. coelicolor and the ΔpdtA mutant were grown on coverslips inserted into MS agar for 72 h, and spore chains attached to the coverslips were double stained with the membrane-permeable DNA stain SYTO9, indicating viable spores, and propidium iodide, indicating dead spores (Fig. 3A). Whereas less than 2% of M145 spores (n = ~3,000) were nonviable under the tested conditions, spore chains of the ΔpdtA mutant contained 34% dead spores. In the complemented mutant ΔpdtA::pRMpdtAc, 13% of the spores stained with propidium iodide (Fig. 3A).

To confirm the validity of LIVE/DEAD staining, we also determined spore viability. Spores of M145, the ΔpdtA mutant, and the complemented mutant ΔpdtA::pRMpdtAc were grown for 5 days on MS agar, collected, and adjusted to an OD of 1. Then, the CFU were determined by plating to LB agar. Whereas 100% of the M145 spores were viable, only 67% of the ΔpdtA mutant spores were viable.
and 93.9% of the complemented mutant ΔpdtA::pRMpdtAc spores formed colonies on LB agar (Fig. 3B).

The hydrophobicity of the spore surface, determined by measuring the adherence of spores to chloroform drops in a biphasic water-chloroform mixture (41), was not impaired (data not shown). Cryo-scanning electron microscopy and atomic force microscopy did not reveal visible defects in the spore envelope. Also, the rodlet layer characteristic for Streptomyces spores did not seem to be affected in the ΔpdtA mutant (data not shown).

![FIG 2](image-url) **Effect of pdtA inactivation on sensitivity to osmotic stress, lysozyme, antibiotics, and phage infection.** Different dilutions of M145 and ΔpdtA mutant spores were spotted on LB agar and LB containing 6% NaCl. After 3 days of incubation at 30°C, the individual spots were checked for growth (A). The ΔpdtA deletion mutant showed a higher sensitivity to osmotic stress than the wild type. (B) The resistance to cell wall biosynthesis targeting vancomycin (Van), cell wall-degrading lysozyme (Lys), and rifampin (Rif) targeting RNA polymerase was tested by applying filter disks on freshly plated spores (10⁵) on LB agar. After 3 days of incubation at 30°C, the sizes of the inhibition zones were compared. The ΔpdtA mutant (top row) is more sensitive to lysozyme and rifampin but as resistant to vancomycin as the wild-type M145 (bottom row). (C) Phage resistance of the ΔpdtA mutant was analyzed by spotting 5-μl lysates of several Streptomyces phages (isolated from soil) onto ~10⁶ spores of M145 or ΔpdtA mutant embedded in soft agar. Lysis zones were imaged after 24 h of incubation at 30°C. Two of four tested S. coelicolor phages were not able to lyse the ΔpdtA mutant strain.

![FIG 3](image-url) **Effect of pdtA inactivation on spore viability.** (A) Spore viability of the S. coelicolor M145, ΔpdtA mutant, and the complemented mutant ΔpdtA::pRMpdtAc was determined by SYTO9-propidium iodide double staining. SYTO9 is a green membrane-permeable DNA stain, indicating viable spores. Propidium iodide marks dead spores (red), since it can only enter cells after membrane damage, displacing SYTO9, due to its use in higher concentrations. Cultures were grown on coverslips inserted into MS agar at 29°C. For statistical analysis, at least 1,500 spores of randomly selected images from at least three independent plates were counted. Compared to the wild type and the complemented mutant, the ΔpdtA mutant showed a strong decrease (Mann-Whitney test, ***, P ≤ 0.01; ****, P ≤ 0.0001) in average spore viability. (B) To confirm viability determination by LIVE/DEAD staining, spore viability was measured by determining the number of CFU. Equal amounts (calculated by OD and counting chamber) of spores (4 replicates) of M145, ΔpdtA mutant, and the complemented mutant ΔpdtA::pRMpdtAc were plated onto LB agar and incubated for 2 days. One hundred percent of M145 spores, only 67% of the ΔpdtA mutant spores, and 95.9% of the complemented mutant spores formed colonies. The data are presented as median with interquartile range.
The phosphate content of the ΔpdtA mutant spore envelopes was reduced to 32% of that of M145 (Fig. 4C). Also, the hexosamine content (48%) was significantly decreased (Fig. 4D). In contrast, the concentration of the Kdn sugar was not affected by the inactivation of pdtA (Fig. 4E). This indicated that only the production of the minor polymer PDP was impaired in the ΔpdtA mutant, while synthesis of the major polymer teichulosonic acid was not affected.

To distinguish whether the anionic PDP is more rarely incorporated into the spore wall or whether pdtA inactivation affects...
the structure of PDP, the spore wall glycopyolmers of M145 and the \( \Delta pdtA \) mutant were partially hydrolyzed under mildly acidic conditions to obtain fragments of different lengths. The anionic glycopyomer fragments were separated on polyacrylamide gels and visualized by combined alcian blue and silver staining. The fragment pattern derived from \( \Delta pdtA \) mutant spore envelopes indicated the presence of more than 20 repeating Gal-GlcNAc-P units and was indistinguishable from that of the wild type (Fig. 4F). Consistent with the predicted role of PdtA as a possible TagV-like glycopyomer transferase, only the amount of PDP in the spore wall was affected but not its structure.

\( \Delta pdtA \) mutant is widely resistant to the Streptomyces phages. Phages of Gram-positive bacteria are known to use surface-exposed wall teichoic acids as phage receptors. To analyze whether the reduction in PDP interferes with phage adsorption, we tested the resistance of the \( \Delta pdtA \) mutant against phages (\( \Phi TU1 \) to \( \Phi TU4 \)) isolated from soil (Fig. 2C). About 10\(^3\) spores of M145 and the \( \Delta pdtA \) mutant were mixed with soft agar and poured on phage agar plates. After spotting about 10\(^4\) phages, the plates were incubated overnight at 30°C. Whereas M145 was completely lysed by all phages, the \( \Delta pdtA \) mutant was widely resistant to \( \Phi TU2 \) and \( \Phi TU3 \). This suggested that the reduced PDP content in the spore wall interfered with phage infection of the \( \Delta pdtA \) mutant.

A PdtA-mCherry fusion protein preferentially localizes to the tips of vegetative hyphae and to potentially new branching points. To localize PdtA in the mycelium, we fused the autofluorescent protein mCherry to the C terminus of PdtA. The fusion protein was functional, as demonstrated by the complementation of the growth defect on high-salt medium of the \( \Delta pdtA \) mutant (see below; see also Fig. 5S in the supplemental material). The fusion gene was placed under the control of the constitutive \( \Phi C31 \) attachment sites of M145 and the \( \Delta pdtA \) mutant. In both strains, but more clearly visible in the \( \Delta pdtA \) mutant background (Fig. 5, arrows), red fluorescent foci were observed preferentially at the tips. Moreover, red foci were also present at specific positions in the mycelium, maybe reflecting septa and new branching points. In contrast, uniform red fluorescence of the whole mycelium was observed when \( mCherry \) alone was expressed under the control of the \( ermE^* \) promoter (data not shown).

At a later stage in the life cycle, PdtA-mCherry showed ring-like structures underneath the spore wall (see Fig. S3 in the supplemental material), similar to those described for MreB-eGFP or Mbl-mCherry (42, 43). However, similar structures were also seen when plasmid pRM43-mCherry encoding the soluble mCherry protein was integrated into the M145 chromosome (Fig. S3). Perhaps, the ring-like fluorescence is caused by the distribution of mCherry surrounding the condensed chromosome in spores. Therefore, no valid conclusion on the PdtA localization in spores could be made.

\( \Delta pdtA \) mutant is also affected in apical tip extension and branching of vegetative hyphae. When cultures grown on coverslips inserted into MS agar were inspected by microscopy, occasionally (\( \sim 0.1 \) to 1\%), some aberrant hyphal tips were observed in the vegetative mycelium of the \( \Delta pdtA \) mutant, which were never seen in M145. Whereas M145 forms long vegetative hyphae, which start to branch at a distance to the hyphal tip of more than 20 \( \mu \)m (Fig. 5 and 6A), no regular branches and only staggered extensions with a bulbous morphology were formed in the aberrant \( \Delta pdtA \) mutant hyphae (Fig. 6B and C). The morphology of the aberrant hyphae suggested a defect in apical tip extension. As a consequence of the blocked tip extension, new tips are established close by, which also fail to elongate into normal hyphae. However, it is noteworthy to mention that the majority of the vegetative hyphae showed a normal morphology, explaining why cultivation of the mutant was not affected.

Growth defect of the \( \Delta pdtA \) mutant is potentiated under osmotic stress. When grown under osmotic stress, the defect of the \( \Delta pdtA \) mutant was more dramatic. While M145 hyphae were able to grow on LB agar supplemented with 6% NaCl, showing only slightly increased curling (Fig. 7A), apical tip extension of the \( \Delta pdtA \) mutant was dramatically impaired. All hyphal tips of the \( \Delta pdtA \) mutant showed hyperbranching directly at the tips, resulting in a bulky and dented mycelium (Fig. 7B, arrows). Complementation of the mutant by the integration of plasmid pRMpdtAc widely restored the wild-type growth pattern with normal branches (Fig. 7C).

The growth defect of the \( \Delta pdtA \) mutant on high-salt medium became even more evident when strains were incubated in liquid medium on a rotary shaker. Whereas the \( \Delta pdtA \) mutant yielded
95% of the wild-type biomass in LB, only 4% of the biomass was formed in LB containing 6% NaCl (Fig. 7D). Salt stress also affected the growth of wild-type M145, which in LB with 6% NaCl still accumulated 35% of the biomass obtained from LB medium. The complemented mutant ΔpdtA::pRMpdtAc in LB with 6% NaCl formed 21% of the biomass obtained from LB medium.

**pdtA is involved in positioning PG synthesis.** The preferential localization of PdtA-mCherry to the tips is consistent with the defect of the ΔpdtA mutant in proper tip elongation. To address whether PdtA affects PG synthesis, we visualized sites of active PG synthesis by staining living mycelium with Van-Fl and compared the staining patterns of the M145 wild type and the ΔpdtA mutant. Since the effect of pdtA inactivation was more dramatic on high-salt medium, we grew the strains on coverslips inserted into LB agar supplemented with 6% NaCl. Whereas Van-Fl staining detected PG incorporation mainly at the hyphal tips and at cross walls in M145, the staining pattern of the ΔpdtA mutant looked completely different. Here, the short branches emerging from the bulbous hyphae often did not bind Van-Fl. In contrast, many sites of the lateral wall, distributed in a nonordered manner, were stained (Fig. 8). This staining pattern is consistent with the permanent establishment of new branching points that after a short period of PG synthesis collapse and fail to elongate.

**DISCUSSION**

Anionic glycopolymers are crucial components of the cell envelope of Gram-positive bacteria. Their structures are highly varied, and they are often synthesized in a species- or growth condition-specific manner (4, 44). The vegetative mycelium of *S. coelicolor* M145 was shown to contain two distinct glycopolymers. While PDP [poly(Gal-GlcNAc-P)] represents a minor component, teichulosonic acid, a phosphate-free polymer of galactose, and 2-ke-to-3-deoxy-D-glycero-D-galacto-nononic acid (Kdn), in part substituted by α-GlcNAc, is produced as the major component (27). In this study, we demonstrated that the spore walls of *S. coelicolor* M145 contain the same two glycopolymers, teichulosonic acid and PDP, as the vegetative cell walls in a quantity of around one-quarter of the spore wall dry weight.

Due to their important role in the bacterial physiology, it is not astonishing that the final step in glycopolymer synthesis, the transfer of the glycopolymer chain to PG and its covalent linkage via phosphoester bonds, is carried out by several redundant LCP

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**FIG 6** Aberrant morphology of vegetative hyphae of *S. coelicolor* ΔpdtA. Spores of *S. coelicolor* M145 (A) and the ΔpdtA mutant (B) were grown on coverslips inserted into MS agar at 30°C. After 48 h of incubation, the hyphae were inspected by phase-contrast microscopy. Beside normal hyphae (white arrows), aberrant branching hyphae (black arrows) were formed in the ΔpdtA mutant. The dashed rectangle indicates the area enlarged in image C. Scale bar = 5 μm.

**FIG 7** Aberrant growth of ΔpdtA mutant under osmotic stress. Cultures of M145 (A), ΔpdtA (B), and the complemented mutant ΔpdtA::pRMpdtAc (C) were grown on coverslips inserted into LB agar supplemented with 6% NaCl. After 48 h of incubation at 30°C, the hyphal morphologies were inspected by phase-contrast microscopy. Under osmotic stress, tip elongation of the ΔpdtA mutant was impaired, resulting in hyperbranching bulbous hyphae (white arrows). The wild-type M145, ΔpdtA mutant, and the complemented mutant ΔpdtA::pRMpdtAc grow to equal densities in the absence of osmotic stress (LB) in liquid culture. Under osmotic stress conditions (LB plus 6% NaCl), the ΔpdtA mutant accumulated significantly (Mann-Whitney test, **, P ≤ 0.01) smaller amounts of biomass (D) than the wild-type M145 and the complemented mutant. The data are presented as means with SD. Scale bar = 5 μm.
enzymes (10, 12). In *B. subtilis*, single-deletion mutants had no obvious defect, and only a triple disruption of *tagT*, *tagU*, and *tagV* was lethal (10). Whereas *B. subtilis* contains three LCP transferases (10), *S. coelicolor* M145 encodes 11 LCPs, which fall into two groups defined by the presence of the C-terminal LytR_C domain of unknown catalytic function (Table 2). The TagV-like PdtA (SCO2578) is encoded as part of a putative gene cluster involved in anionic glycopolymer synthesis (26) and was previously identified in a screen of a genomic library as an interaction partner of several SSSC proteins, suggesting a role in sporulation (23). Despite the presence of 10 further homologs, the deletion of *pdtA* caused a severe phenotype. Apparently, none of the other TagT-, TagU-, or TagV-like enzymes could substitute the loss of PdtA, as was the case with TagV in *B. subtilis* (10). The deletion of *pdtA* led to a 48% decrease in spore wall glycopolymer content. Quantification of Kdn, hexosamine sugars, and phosphate allowed the discrimination between the two glycopolymers of *S. coelicolor* M145, teichulosonic acid and PDP. Unchanged Kdn amounts but the reduction in the phosphate content to 32% of the wild-type amount indicates that the production of only PDP was impaired.

As expected, the reduction in the amount of hexosamine sugars was less pronounced (48%), since GlcNAc is also a substituent in teichulosonic acid. Moreover, hexosamine sugars might also be present in the yet-unidentified linker unit of both glycopolymers. The negative charge of poly(Gal-GlcNAc-P) enabled the electrophoretic separation of PDP fragments of different length, generated by partial hydrolysis. Alcian blue/silver staining revealed a polymer length consisting of more than 20 repeating units. No difference between M145 and Δ*pdtA* mutant fragments could be observed, suggesting that only the amount of PDP was reduced in the spore envelope of Δ*pdtA* mutant but not its structure.

The reduction in PDP had a severe phenotype. It interfered with the precise regular placement of sporulation septa, as indicated by the heterogeneity in spore length within spore chains. Moreover, more than one-third of the produced spores were nonviable, and the remaining spores (or the developing mycelium) showed an increased sensitivity to lysozyme and osmotic stress. A similar phenotype was also reported for SSSC mutants defective in the mre genes *mreb, mrec, mred, pbp2, sfr*, and *mbl* (23, 42) or the Ser/Thr kinase gene *pkaI*, which directs the phosphorylation of MreC and PBP 2 (25). Surprisingly, the Δ*pdtA* mutant was highly sensitive to rifampin, which targets the RNA polymerase and does not interfere with envelope synthesis. Rifampin crosses the bacterial cell wall, presumably by diffusion, and for mycobacteria, it was shown that the arabinogalactan content of the cell wall influences rifampin uptake (45). Increased sensitivity of the Δ*pdtA* mutant to rifampin indicates that PDP constitutes an important barrier preventing the entrance of larger-sized antibiotics, like rifampin (823 Da). The PDP layer itself seems to be responsible for the barrier function, since besides the reduction in the PDP glycopolymer, changes in neither teichulosonic acid, PG cross-linking, spore hydrophobicity, or the arrangement of the rodlet layer on the spore surface were affected.

Interestingly, the deletion of *pdtA* affected not only proper sporulation, as was suggested from its interaction with other SSSC proteins (23), but vegetative tip growth was also impaired. Under normal growth conditions, the morphological defect was mild, and aberrant hyphae or spores were formed only quite rarely. Such aberrant hyphae did not show normal branching, but the whole hyphae were composed of staggered bulges. The hyphal morphology could be interpreted as a defect in apical tip elongation. As a consequence, new tips were established next to the stalled tip, which again could not develop into a growing tip, and so forth. *Streptomyces* growth by apical tip extension is mainly determined by the TIPOC, or polarisome, composed of the cytoskeletal FilP, Scy, and DivIVA proteins (17, 18, 46). The aberrant branching of the Δ*pdtA* mutant highly resembled the phenotype of *scy* mutants (18). *scy* mutants had pleiotropic phenotypes and frequently showed tip splitting and apical branching. This correlated with alterations in tip geometry. Functional Scy fusion proteins co-localized with DivIVA to established and new tips but also to sites in the lateral hyphal wall, possibly marking future branching points. The Scy localization pattern matched the sites of new cell wall synthesis, identified by Van-Fl staining (18). Like for *pdtA*, the deletion of *scy* not only affected vegetative tip growth and branching but also caused irregularly spaced and often tilted sporulation septation (18). The localization pattern of PdtA-mCherry, which preferentially localized to the mycelial tips, suggests that PdtA could also have a role in the polarisome. PdtA might anchor PDP to the hyphal tip. PDP at the tip might serve as a scaffold for PG

FIG 8 Mislocalization of PG synthesis under salt stress in Δ*pdtA* mutant. Strains were grown for 48 h on coverslips inserted into LB agar supplemented with 6% NaCl. Following staining with a fluorescent vancomycin derivative (Van-Fl) to visualize sites of PG synthesis, the mycelium was observed by phase-contrast (pc) and fluorescence microscopy (Van-Fl). The wild-type M145 incorporates new PG (arrows) only at septal cross walls and at the tips of growing hyphae (A). In contrast, many of the tips of the aberrant branches of Δ*pdtA* mutant did not bind Van-Fl, and PG incorporation occurred at many places at the lateral walls (B). Scale bar = 5 μm
synthesis during apical tip growth, in particular under stress conditions. It will be interesting to see whether PdtA is also part of the TIPOC and interacts with one of its other components, Scy, FilP, or DivIVA. One can speculate that PDP attached by PdtA is essential for the functionality of the TIPOC under (osmotic) stress conditions. Also, the rarely formed aberrant hyphae on MS agar might reflect stress. The insertion of coverslips into agar plates for cultivation for microscopy does not produce uniform growth conditions over the whole area of the coverslip. During insertion of the coverslip, the agar cracks, resulting in local changes in oxygen, hydration, and nutrient supply. Therefore, different hyphae of the mycelium can experience different kinds of stress, explaining why not all hyphae show the same phenotype. In agreement, the crucial role of \( \text{pdtA} \) became more apparent under salt stress. Osmotic change is one of the most frequent types of stress for a soil organism. In case of osmotic upshift, bacteria need to increase the internal osmolyte concentration to avoid efflux of water leading to desiccation. In case of osmotic upshift, bacteria need to increase the internal osmolyte concentration to avoid efflux of water leading to desiccation. In case of osmotic upshift, bacteria need to increase the internal osmolyte concentration to avoid efflux of water leading to desiccation.

REFERENCES


