Another Brick in the Wall: the role of the actinobacterial cell wall in antibiotic resistance, phylogeny and development

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CHAPTER 3

High-resolution analysis of the peptidoglycan composition in *Streptomyces coelicolor*

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Chapter published as:
Journal of Bacteriology (2018) 200:e00290-18. (with Cover image)

Supplemental information available at:
https://jb.asm.org/content/jb/suppl/2018/09/11/JB.00290-18.DCSupplemental/zjb999094886s1.pdf

ABSTRACT

The bacterial cell wall maintains cell shape and protects against bursting by the turgor. A major constituent of the cell wall is peptidoglycan (PG), which is continuously modified to allow cell growth and differentiation through the concerted activity of biosynthetic and hydrolytic enzymes. Streptomycetes are Gram-positive bacteria with a complex multicellular life style alternating between mycelial growth and the formation of reproductive spores. This involves cell-wall remodeling at apical sites of the hyphae during cell elongation and autolytic degradation of the vegetative mycelium during the onset of development and antibiotic production. Here, we show that there are distinct differences in the cross-linking and maturation of the PG between exponentially growing vegetative hyphae and the aerial hyphae that undergo sporulation. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis identified over 80 different muropeptides, revealing that major PG hydrolysis takes place over the course of mycelial growth. Half of the dimers lacked one of the disaccharide units in transition-phase cells, most likely due to autolytic activity. De-acetylation of MurNAc to MurN was particularly pronounced in spores, and strongly reduced in sporulation mutants with a deletion of *bldD* or *whiG*, suggesting that MurN is developmentally regulated. Taken together, our work highlights dynamic and growth phase-dependent construction and remodeling of PG in *Streptomyces*.

IMPORTANCE

Streptomycetes are bacteria with a complex lifestyle, which are model organisms for bacterial multicellularity. From a single spore, a large multigenomic multicellular mycelium is formed, which differentiates to form spores. Programmed cell death is an important event during the onset of morphological differentiation. In this work, we provide new insights into the changes in the peptidoglycan composition and over time, highlighting changes over the course of development and between growing mycelia and spores. This revealed dynamic changes in the peptidoglycan when the mycelia aged, with extensive PG hydrolysis and, in particular, an increase in the proportion of 3-3-cross-links. Additionally, we identified a muropeptide that is highly abundant specifically in spores, which may relate to dormancy and germination.

INTRODUCTION

Peptidoglycan (PG) is a major component of the bacterial cell wall. It forms a physical boundary that maintains cell shape, protects cellular integrity against the osmotic pressure and acts as a scaffold for large protein assemblies and exopolymers (Vollmer *et al.*, 2008a). The cell wall is a highly dynamic macromolecule that is continuously constructed and deconstructed to allow for cell growth and to meet environmental demands (Kysela *et al.*, 2016). PG is built up of glycan strands of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues that are connected by short peptides to form a mesh-like polymer. PG biosynthesis starts with the synthesis of PG precursors by the Mur enzymes in the cytoplasm and cell membrane, resulting in Lipid II precursor, undecaprenylpyrophosphoryl-MurNAc(GlcNAc)-pentapeptide. Lipid II is transported across the cell membrane by MurJ and/or FtsW/SEDS proteins and the PG is polymerized and incorporated into the existing cell wall by the activities of glycosyltransferases and transpeptidases (Egan *et al.*, 2017, Leclercq *et al.*, 2017, Sham *et al.*, 2014).

The Gram-positive model bacterium *Bacillus subtilis* grows via lateral cell-wall synthesis followed by binary fission; in addition, *B. subtilis* forms heat- and desiccation-resistant spores (Popham, 2002, Keep *et al.*, 2006). In contrast, the vegetative hyphae of the mycelial *Streptomyces* grow by extension of the hyphal apex and cell division results in connected compartments separated by cross-walls (Flärdh & Buttner, 2009, Barka *et al.*, 2016, Celler *et al.*, 2016). This makes *Streptomyces* a model taxon for bacterial multicellularity (Claessen *et al.*, 2014). Multicellular vegetative growth poses different challenges to *Streptomyces*, including the synthesis of many chromosomes during vegetative growth and separation of the nucleoids in the large multi-genomic compartments during cross-wall formation (Jakimowicz & van Wezel, 2012, Wolanski *et al.*, 2011). In submerged cultures, streptomycetes typically form complex mycelial networks or pellets (van Dissel *et al.*, 2014). On surface-grown cultures, such as agar plates, these bacteria develop a so-called aerial mycelium, whereby the vegetative or substrate mycelium is used as a substrate. The aerial hyphae eventually differentiate into chains of spores, a process whereby many spores are formed almost simultaneously, requiring highly complex coordination of nucleoid segregation and condensation and multiple cell division (Jakimowicz & van Wezel, 2012, McCormick, 2009, Noens *et al.*, 2005). Streptomycetes have an unusually complex cytoskeleton, which plays a role in polar growth and cell-wall stability (Celler *et al.*, 2013, Holmes *et al.*, 2013). Mutants that are blocked in the vegetative growth phase are referred to as bald or *bld*, for lack of the fluffy aerial hyphae (Merrick, 1976), while those producing aerial hyphae but no spores are referred to as white (*whi*), as they fail to produce grey-pigmented spores (Chater, 1972)

The *Streptomyces* genome encodes a large number of cell wall-modifying enzymes, such as cell wall hydrolases (autolysins), carboxypeptidases and penicillin-binding proteins (PBPs), a complexity that suggests strong heterogeneity of the PG of these organisms (Haiser *et al.*, 2009, Peters *et al.*, 2016). Several concepts that were originally regarded as specific to eukaryotes also occur in bacteria, such as multicellularity (Lyons & Kolter, 2015, Claessen *et al.*, 2014, Shapiro, 1988), and programmed cell death (Hochman, 1997, Rice & Bayles, 2003). Programmed cell death (PCD) likely plays a major role in the onset of morphological development, required to lyse part of the vegetative mycelium to provide the nutrients for the aerial hyphae (Manteca *et al.*, 2005, Miguelez *et al.*, 1999). PCD and cell-wall recycling are

linked to antibiotic production in *Streptomyces* (Urem *et al.*, 2016).

All disaccharide peptide subunits (muropeptides) in the PG are variations on the basic building block present in Lipid II, which in *Streptomyces* typically consists of GlcNAc-MurNAc-L-Ala-D-Glu-LL-diaminopimelate(Gly)-D-Ala-D-Ala (Hugonnet *et al.*, 2014, Schleifer & Kandler, 1972). Here, we have analyzed the cell wall composition of vegetative mycelium and mature spores of *Streptomyces coelicolor* by liquid chromatography-mass spectrometry (LC-MS), to obtain a detailed inventory of the monomers and dimers in the cell wall. This revealed extensive cell wall hydrolysis and remodeling during vegetative growth and highlights the difference in cell wall composition between vegetative hyphae and spores.

MATERIAL AND METHODS

Bacterial strain and culturing conditions

Streptomyces coelicolor A3(2) M145 (Kieser et al., 2000), and its *bldD*, J774 (cysA15 pheA1 mthB2 bldD53 NF SCP2* (Merrick, 1976) and *whiG*, J2400 (M145 *whiG*::hyg (Flärdh et al., 1999)) mutants, were obtained from the John Innes Centre strain collection. All media and methods for handling *Streptomyces* are described in the *Streptomyces* laboratory manual (Kieser et al., 2000). Spores were collected from Soy Flour Mannitol (SFM) agar plates. Liquid cultures were grown shaking at 30°C in a flask with a spring, using normal minimal medium with phosphate (NMM+) supplemented with 1% (w/v) mannitol as the sole carbon source; polyethylene glycol (PEG) was omitted to avoid interference with the MS identification. Cultures were inoculated with spores at a density of 10^6 CFU/ML. A growth curve was constructed from dry-weight measurements by freeze-drying washed biomass obtained from 10 mL of culture broth (three biological replicates). To facilitate the harvest of mycelium from agar plates, they were grown on cellophane slips, after which the biomass was scraped of the cellophane. Spores were collected from SFM agar plates by adding 0.01% (w/v) SDS to facilitate spore release from the aerial mycelium, scraping them off with a cotton ball and drawing the solution with a syringe. Spores were filtered with a cotton filter to separate spores from residual mycelium.

PG extraction

Cells were lyophilized for a biomass measurement, 10 mg biomass was directly used for PG isolation. PG was isolated according to (Kühner *et al.*, 2014), using 2 mL screw-cap tubes for the entire isolation. Biomass was first boiled in 0.25% SDS in 0.1 M Tris/HCl pH 6.8, thoroughly washed, sonicated, treated with DNase, RNase and trypsin, inactivation of proteins by boiling and washing with water. Wall teichoic acids were removed with 1 M HCl. PG was digested with mutanolysin and lysozyme (Arbeloa *et al.*, 2004). Muropeptides were reduced with sodium borohydride and the pH was adjusted to 3.5-4.5 with phosphoric acid.

To validate the method, we compared it to the method described previously (Bui *et al.*, 2012). For this, *S. coelicolor* mycelia were grown in 1 L NMM+ media for 24 h. After washing of the mycelia, pellets were resuspended in boiling 5% (w/v) SDS and stirred vigorously for 20 min. Instead of sonicating the cells, they were disrupted using glass beads, followed by removal of the teichoic acids with an HF treatment at 4°C as described.

LC-MS analysis of monomers

The LC-MS setup consisted of a Waters Acquity UPLC system (Waters, Milford, MA, USA) and a LTQ Orbitrap XL Hybrid Ion Trap-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an Ion Max electrospray source.

Chromatographic separation was performed on an Acquity UPLC HSS T3 C_{18} column (1.8 μm, 100 Å, 2.1 × 100 mm). Mobile phase A consist of 99.9% H_2O and 0,1% Formic Acid and mobile phase B consists of 95% Acetonitrile, 4.9% H_2O and 0,1% Formic Acid. All solvents used were of LC-MS grade or better. The flow rate was set to 0.5 ml/min. The binary gradient program consisted of 1 min 98% A, 12 min from 98% A to 85% A, and 2 min from 85% A to 0% A. The column was then flushed for 3 min with 100% B, the gradient was then set to 98% A and the column was equili-

brated for 8 min. The column temperature was set to 30°C and the injection volume used was 5 μL. The temperature of the autosampler tray was set to 8°C. Samples were run in triplicates.

MS/MS was done both on the full chromatogram by data dependent MS/MS and on specific peaks by selecting the mass of interest. Data dependent acquisition was performed on the most intense detected peaks, the activation type was Collision Induced Dissociation (CID). Selected MS/MS was performed when the resolution of a data dependent acquisition lacked decisive information. MS/MS experiments in the ion trap were carried out with relative collision energy of 35% and the trapping of product ions were carried out with a q-value of 0.25, and the product ions were analyzed in the ion trap., data was collected in the positive ESI mode with a scan range of *m*/*z* 500–3000 in high range mode. The resolution was set to 15.000 (at m/z 400).

Data analysis

Chromatograms were evaluated using the free software package MZmine (http://mzmine.sourceforge.net/ (Pluskal *et al.*, 2010)) to detect peaks, deconvolute the data and align the peaks. Only peaks corresponding with a mass corresponding to a muropeptide were saved, other data was discarded. The online tool MetaboAnalyst (Xia *et al.*, 2015) was used to normalize the data by the sum of the total peak areas, then normalize the data by log transformation. The normalized peak areas were exported and a final table which shows peak areas as percentage of the whole was produced in Microsoft Excel.

Muropeptide identification

The basic structure of the peptidoglycan of *S. coelicolor* has been published previously (Hugonnet *et al.*, 2014). Combinations of modifications were predicted and the masses were calculated using ChemDraw Professional (PerkinElmer). When a major peak had an unexpected mass, MS/MS helped resolve the structure. MS/MS was used to identify differences in cross-linking and to confirm predicted structures.

RESULTS

To assess how growth and development translate to variations in the PG composition, we isolated the PG and analyzed the muropeptide profile of spores and of vegetative hyphae during different phases of growth in liquid-grown cultures. In submerged cultures, *S. coelicolor* does not sporulate, while it forms aerial hyphae and spores on solid media. Vegetative mycelia of *S. coelicolor* M145 were harvested from cultures grown in liquid minimal media (NMM+). Samples taken after 18 h and 24 h represented exponential growth, while samples taken after 36 h and 48 h represented mycelia in transition phase (Figure 1A,B). Samples from solid-grown cultures were taken at 24 h to represent vegetative growth, 48 h, representing growth of aerial hyphae and 72 h, when the strain has formed spores (Figure 1C). Spores were harvested from SFM agar plates and filtered to exclude mycelia fragments.

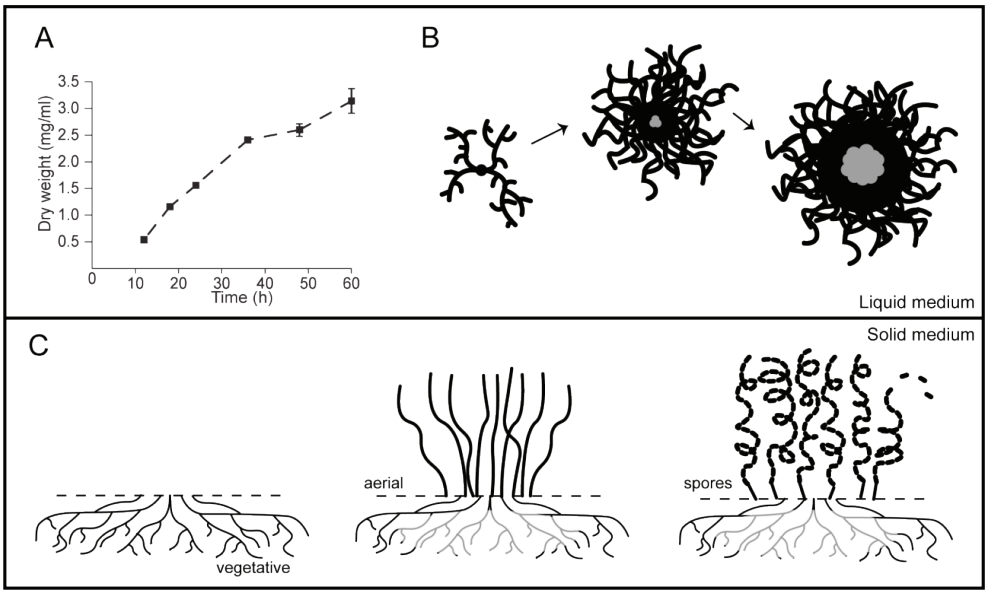

Figure 1. Growth of *S. coelicolor* in liquid and solid medium. (A) Growth curve on NMM+ medium based on triplicate dry weight measurements. (B) Pellet morphology in liquid medium. After spore germination, hyphae emerge through top growth and branching that form an intricate network or pellet. The center of the pellets eventually lyses due to PCD (gray). (C) Growth on solid medium, starting with development of vegetative mycelium, after which aerial hyphae grows into the air, coinciding with an event of PCD at the vegetative mycelium, indicated by grey hyphae. Spores are generated by septation of the aerial hyphae.

To allow analyzing a large number of samples simultaneously and in a reasonable time frame, we adapted a method for rapid PG purification (Kühner *et al.*, 2014) for *S. coelicolor*. The advantage of this method is that it requires only a small amount of imput biomass and much faster sample handling. For this, 10 mg of lyophilized cell wall material was isolated by boiling cells in 0.25% SDS in 2-ml microcentrifuge tubes, and secondary cell-wall polymers such as teichoic acids were removed by treatment for 4 h with hydrochloric acid (HCl) (see Materials and Methods section for details). As a control for the validity of the method, it was compared to a more elab-

orate method that is used routinely (Bui *et al.*, 2012). In the latter method, biomass from a 1-liter culture of *S. coelicolor* was boiled in 5% SDS and subsequently treated for 48 hours with hydrofluoric acid (HF) to remove teichoic acids. Comparison of the two methods revealed comparable outcomes between the two methods in peak detection (Table S5). This validated the more rapid method using 0.25% SDS and HCl, which was therefore used in this study.

The isolated PG was digested with mutanolysin (Kühner *et al.*, 2014, Glauner, 1988) and the muropeptide composition was analyzed by LC-MS. Peaks were identified in the *m/z* range from 500-3000 Da, whereby different *m/z*'s in co-eluting peaks were further characterized by MS/MS. The eluted *m/z* values were compared to a dataset of theoretical masses of predicted muropeptides. Table 1 shows a summary of the monomers and dimers that were detected during growth in liquid medium, and Table 2 shows a summary of muropeptides on solid medium. The full datasets are given in Tables S1,S2, S3 and S4. We identified several modifications, including the amidation of D-iGlu to D-iGln at position 2 of the stem peptide, deacytelation of MurNAc to MurN, removal of amino acids to generate mono-, di-, tri- and tetrapeptides, loss of LL-DAP-bound glycine, and the presence of Gly (instead of Ala) at position 1,4 or 5. The loss of GlcNac or GlcNAc-MurNAc indicates hydrolysis (Figure 2). For all amino acids in the pentapeptide chain, the position is indicated as [n], whereby n is the number in the chain (with [1] the position closest to the PG backbone, i.e., the MurNAc residue, and [5] being the last amino acid residue.

Growth phase dependent changes in the PG composition

The muropeptide that is incorporated from Lipid II by glycosyltransferases contains a pentapeptide with a Gly residue linked to LL-DAP [3]. In many bacteria pentapeptides are short-lived muropeptides that occur mostly at sites where *de novo* cell-wall synthesis takes place, *i.e.* during growth and division (Kuru *et al.*, 2015, Morales Angeles *et al.*, 2017). This is reflected by the high abundance of pentapeptides in the samples obtained from exponentially growing cells, with a pentapeptide content of 21% during early exponential growth (18 h), as compared to 14% and 11% during late exponential growth (24 h), transition phase (36 h) and stationary phase (48 h), respetively. Conversely, tripeptides increased over time, from 24% during early exponential phase to 32% in transition-phase cultures. Addition of Gly to the medium and, in consequence, incorporation of Gly in the peptidoglycan can cause changes in morphology (Hammes *et al.*, 1973, Takacs *et al.*, 2013). This property was applied to facilitate lysozyme-mediated formation of protoplasts in *Streptomyces*, used for protoplast transformation methods (Okanishi *et al.*, 1974, Hopwood *et al.*, 1977, Kieser *et al.*, 2000). In S.coelicolor, Gly can be found instead of Ala at position 1, 4 or 5 in the pentapeptide chain. During liquid growth, tetrapeptides carrying Gly at position 4 increased from 3% during early growth to 8% during the latest time points. The relative abundance of pentapeptides carrying Gly at position 5 (4-5%) did not vary over time. On solid-grown cultures, the Gly content of the peptidoglycan was around 1%, which is significantly lower than in liquid-grown cultures.

Table 1. Relative abundance(%)[a] of muropeptides in vegetative cells from liquid NMM+.

Muropeptide[a]	**Abundance (%)[b] in *S. coelicolor* M145**			
Monomers	**18h**	**24h**	**36h**	**48h**
Mono	1.6	2.1	3.3	3.3
Di	14.2	15.5	14.5	13.2
Tri	27.4	32.2	35.1	35.8
Tetra	26.7	24.4	23.9	23.9
Tetra[Gly4][c]	3.5	5.3	6.9	8.2
Penta	22.7	16.9	13.1	12.9
Penta[Gly5][c]	4.7	4.8	4.7	4.4
D-Glutamine	67	62	61.1	63.7
Deacetylated	3.9	6.0	7.9	8.0
MurN-Tri	0.1	0.7	1.2	2.3
GlcNAc-MurN-Tri	1.8	2.2	2.6	2.1
Dimers	**18h**	**24h**	**36h**	**48h**
TriTri (3-3)	4.1	4.8	6.5	7.0
TriTri - MurNAcGlcNAc	8.7	14.8	23.7	34.3
TriTetra(3-3)	23.9	24.2	22.3	16.9
TriTetra(3-4)	1.0	8.7	8.2	6.1
TriTetra - MurNAcGlcNAc	9.6	15.1	16.1	16.2
TetraTetra(3-4)	23.3	13.5	10.1	8.6
TetraTetra - MurNAcGlcNAc	6.0	7.3	4.8	5.6
TetraPenta (3-4)	24.6	9.1	5.6	3.0
MurN	1.8	1.2	1.5	1.2
-GlcNac	0.3	0.6	1.1	1.2
missing MurNAcGlcNAc	24.3	37.2	44.6	56.1
Proportion(%) of 3-3 cross-links	36.5	48.0	54.5	57.3

[a]Monomers and dimers are treated as separate datasets.
[b]Relative abundance is calculated as the ratio of the peak area over the sum of all peak areas recognized in the chromatogram.
[c] Gly detected instead of Ala

Table 2. Relative abundance(%)[a] of muropeptides over growth on solid SFM-medium and spores.

Muropeptide[a]	**Abundance (%)**[b] **in *S. coelicolor* M145**			
Monomers	**24h**	**48h**	**72h**	**spores**
Mono	3.6	4.3	4.1	4.5
Di	21.6	17.6	17.9	13.1
Tri	29.6	34.3	34.2	28.1
Tetra	25.4	29.5	32.0	48.3
Tetra[Gly4][c]	0.9	1.1	1.0	2.3
Penta	16.8	9.9	7.2	5.3
Penta[Gly5][c]	1.2	1.4	1.3	4.0
Deacatylated	3.7	4.4	6.1	4.5
D-Glutamine	76.2	80.3	82.9	74.0
Missing GlcNAc	1.5	3.4	5.0	4.8
MurN-Tri	0.6	1.7	3.1	3.5
GlcNAc-MurN-Tri	1.9	1.4	1.6	0.1
Dimers	**24h**	**48h**	**72h**	**spores**
Tri-Tri (3-3)	7.4	10.5	12.6	4.9
Tri-Tri - MurNacGlcNac	0.6	0.6	0.3	7.1
Tri-Tetra(3-3)	20.4	22.2	21.8	19.1
Tri-Tetra(3-4)	9.7	12.7	11.8	4.7
Tri-Tetra - MurNacGlcNac	13.3	14.5	13.0	6.3
Tetra-Tetra(3-4)	13.3	15.8	15.7	38.9
Tetra-Tetra - MurNacGlcNac	17.3	13.7	13.2	17.1
Tetra-Penta (3-4)	12.7	7.3	5.4	0.7
MurN	1.0	0.3	1.2	0.4
-GlcNAc	0.4	0.2	0.4	0.1
missing MurNAcGlcNAc	31.1	28.7	26.5	30.4
Proportion(%) of 3-3 cross-links	43.8	47.8	51.1	35.1

[a]Monomers and dimers are treated as separate datasets.
[b]Relative abundance is calculated as the ratio of the peak area over the sum of all peak areas recognized in the chromatogram.
[c] Gly detected instead of Ala

The abundance of 3-3 cross-links increases over time

Two types of cross-links are formed via separate mechanisms, namely the canonical D,D-transpeptidases (PBPs) producing 3-4 (D,D) cross-links between LL-DAP[3] and D-Ala[4] and L,D-transpeptidases that form 3-3 (L,D) cross-links between two LL-DAP[3] residues (Figure 2). These types of peptidoglycan cross-linking can be distinguished based on differences in retention time and their MS/MS fragmentation patterns. Dimers containing a tripeptide and a tetrapeptide (TetraTri) may have either cross-link, giving rise to isomeric forms that elute at different retention times, allowing for assessment by MS/MS (Figures 3A and 3B). In *S. coelicolor*, the ratio of 3-3 cross-linking increased over time towards transition phase; the relative abundance increased from 37% of the total amount of dimers at 18 h (exponential phase) to 57% of all dimers at 48 h (Figures 3A and 3B).

PG hydrolysis increases as the culture ages

PG hydrolysis is associated with processes such as separation of daughter cells after cell division and autolysis, and in *E. coli* and other species deletion mutants lacking PG amidases grow in chains of unseparated cells (Heidrich *et al.*, 2002, Vollmer *et al.*, 2008b). On solid media, vegetative hyphae of *Streptomyces* undergo programmed cell death (PCD) and hydrolysis. During spore maturation, spores are separated hydrolytically from one another. Some streptomycetes sporulate in submerged culture, but this is not the case for *S. coelicolor* (Girard *et al.*, 2013). Our data show that as growth proceeds in submerged cultures, the *S. coelicolor* peptidoglycan progressively loses GlcNAc and GlcNAc-MurNAc moieties (Table 1), as a result of N-acetylglucosamidase activity. The proportion of dimers lacking GlcNAc-MurNAc thereby increases in time from 24% at 18 h to 56% at 48 h. Figure 3C shows MS/MS profiles of a TriTri-dimer with a single set of glycans. During growth on solid media the trend was inversed. This may be due to the different developmental stages, whereby 24 h corresponds to early developmental events and PCD, 48 h to aerial growth and sporulation at 72 h. This analysis shows the relative abundance of muropeptides of the total amount of biomass, when hydrolysis has occurred at the vegetative mycelium, while the strain has simultaneously produces a large amount of aerial hyphae, the relative abundance of muropeptides of the entire culture could be misleading (Table 2).

Deacetylation of MurNAc is associated with mycelial aging and sporulation

Modifications to the glycan strand are commonly linked to lysozyme resistance (Meyrand *et al.*, 2007). In particular, N-deacetylation of PG strands is widespread among bacteria, which can occur both at GlcNAc and at MurNAc (Vollmer, 2008). In the case of *S. coelicolor*, the only glycan modification is the deacetylation of MurNAc to MurN. Our data show that this modification becomes more prominent as the vegetative mycelium ages, from 5% during early growth to 8% during later growth stages. On agar plates, 3.7% of the monomers was deacetylated at 24 h, 4.4% at 48 h and 6.1% at 72h. The PG composition of spores and vegetative mycelia was compared to get more insights into the possible correlations between PG composition and important processes such as dormancy and germination. Muropeptides in spores were strongly biased for tetrapeptides, making up 44% of the monomers, as compared to 23-25% of the vegetative PG. Conversely, pentapeptides were found in much lower

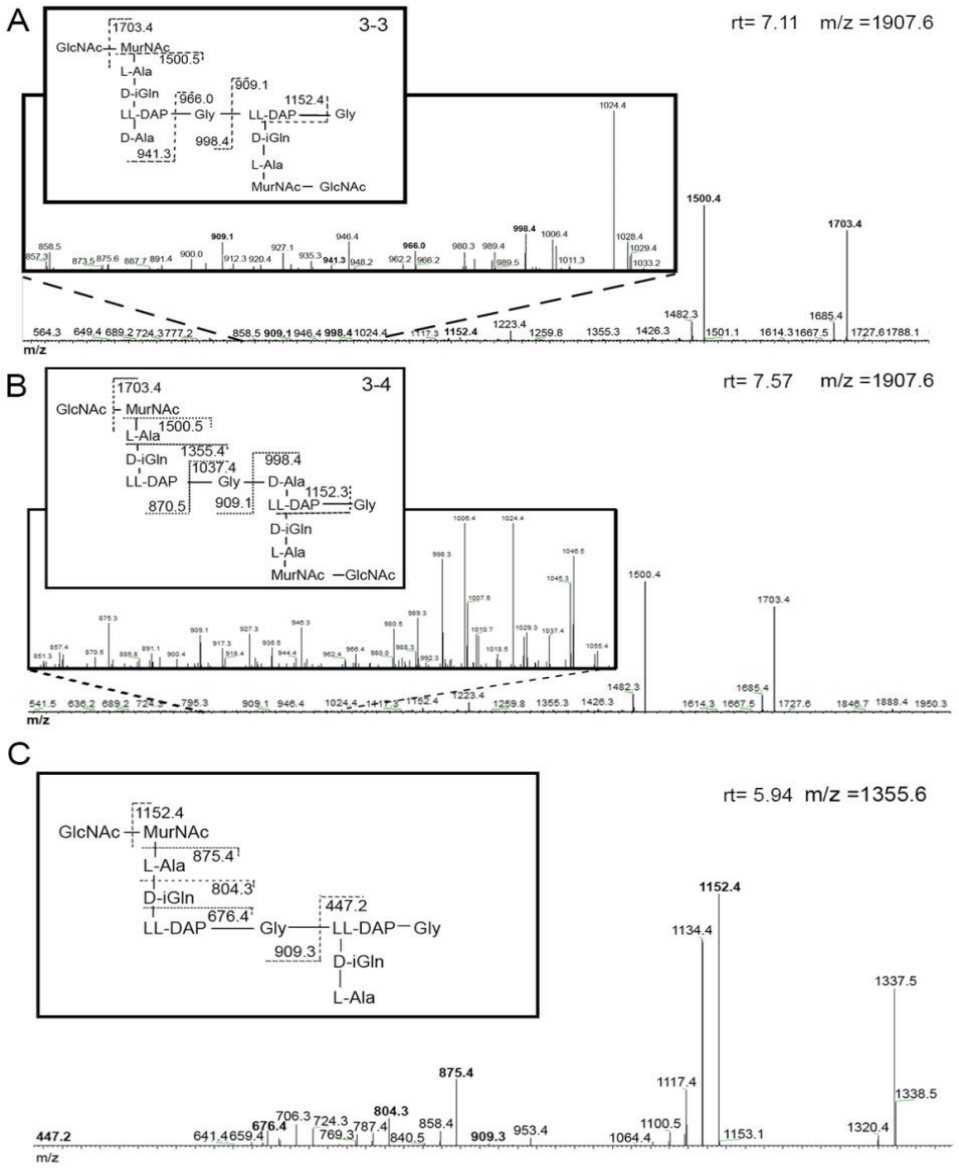

Figure 2. MS/MS fragmentations of TetraTri dimers with either 3-3 cross-link (A) or 3-4 cross-link (B). Differentiation between these two types of cross-links is possible at the point of asymmetry, at Gly attached to LL-DAP. The 3-3 cross-linked dimer (A) fragments into masses of 966.0 m/z and 941.3 m/z, which can be found in the respective MS/MS spectrum. The 3-4 cross-linked dimer (B) fragments into masses of 1037.4 m/z and 870.5 m/z. These masses are found in the MS/MS spectrum. Boxed MS/MS spectra show a magnification of masses between m/z 850 and 1050 to show masses present in lower abundance. (C) a TriTri dimer lacking GlcNAcMurNAc with an M+H of 1355.6, diagnostic fragments are given in the proposed structures.

amounts in spores (5% of the monomers), as compared to 10-22% in vegetative hyphae. A previous study showed that mutation of the gene *dacA* that encodes D-alanyl-D-alanine carboxypeptidas disrupts spore maturation and germination, where one could influence the other. This indicates that either pentapeptides inhibit spore maturation, or that a high amount of tetrapeptides is important (Rioseras *et al*., 2016). The muropeptide that stood out in the analysis of the spore PG was a tripeptide which lacks GlcNAc and contains a deacetylated MurNAc, called MurN-Tri (Figure 2). In spores, MurN-Tri made up 3.5% of the monomers, whereas the less modified muropeptide, GlcNAcMurN-Tri only made up 0.2% of the monomers.

To further investigate this interesting phenomenon, we analyzed two developmental mutants, namely the *bldD* and *whiG* mutants. The *bldD* gene product is a global transcription factor that controls the transcription of many developmental genes and is therefore blocked in an early stage of morphogenesis (den Hengst *et al*., 2010), while the *whiG* gene product is a σ factor that controls early events of aerial growth. The monomer profile of *S. coelicolor* M145 and its *bldD* and *whiG* mutants are summarized in Table 3. For the wild-type strain M145, 24 h represents vegetative growth, 48 h aerial growth and 72 h spore formation. In line with the notion that MurN-Tri accumulates particularly in spores, the *bldD* mutant accumulated hardly any MurN-Tri (0-0.2%) over the course of time and the *whiG* mutant 0.4%, 0.6% and 1.3% after 24 h, 48 h, and 72 h. respectively. In contrast, M145 had 0.6%, 1.7% and 3.1% MurN-Tri at these time points, strongly suggesting that MurN-Tri accumulates in a sporulation-specific manner.

Table 3. Summary of relative abundance (%)[a] of monomers from developmental *bldD*- and *whiG*-mutants and the parent strain, M145.

		Mono	Di	Tri	Tetra	Penta	De-acetyl-ated	MurN-Tri	GlcNAc-MurN-Tri
ΔbldD	24h	4.5	25.7	28.0	23.0	10.8	6.5	0.0	5.3
	48h	4.3	26.3	38.3	23.4	11.1	8.5	0.2	6.6
	72h	4.3	27.2	40.9	19.9	9.5	7.6	0.2	5.8
ΔwhiG	24h	3.5	23.2	27.0	32.5	15.2	3.0	0.4	1.3
	48h	3.6	17.5	44.3	25.5	7.9	5.0	0.6	3.2
	72h	4.1	18.5	48.8	20.9	6.9	6.2	1.3	3.8
M145	24h	3.6	21.6	29.6	25.4	16.8	3.7	0.6	1.9
	48h	4.3	17.6	34.3	29.5	9.9	4.4	1.7	1.4
	72h	4.1	17.9	34.2	32.0	7.2	6.1	3.1	1.6
spores		4.5%	13.1	28.1	48.3	5.3	4.5	3.5	0.1

[a]Relative abundance is calculated as the ratio of the peak area over the sum of all peak areas recognized in the chromatogram.

DISCUSSION

The *Streptomyces* cell wall is essential for structural rigidity and, in a pinch, a source of nutrients (Rigali *et al.*, 2008). Here, we analyzed the peptidoglycan composition over the course of growth and development. All masses are identified by MS and MS/MS, by which we were able to identify between dimers which were cross-linked by either 3-3- or 3-4 cross-links. A large event of peptidoglycan recycling was recognized in the hydrolysis of GlcNAc-MurNac from up to 56% of the dimers in late-exponential cultures. One muropeptide is linked to development, which was confirmed in developmental mutants. In conclusion, *Streptomyces* peptidoglycan is dynamic, being constantly constructed and deconstructed while maintaining cell shape and solidity.

Implications for the biology of actinobacteria

Sporulation is a key feature of *Streptomyces* biology and the spore wall is a major line of defense against environmental stresses, allowing the bacteria to survive under adverse conditions such as heat and cold stress, osmotic pressure, starvation or drought (van der Meij *et al.*, 2017, Okoro *et al.*, 2009). The spores are spread to a new environment, where they germinate as soon as the right conditions are met and start a new mycelium. Not much is known about the environmental and genetic factors that control the onset of germination. In terms of the genetics, we have previously shown that mutants deficient for the cAMP-receptor protein Crp have a much thicker spore wall, presumably due to reduced expression of cell-wall hydrolases and consequently germinate slowly (Piette *et al.*, 2005). Additionally, Crp is an positive regulator of secondary metabolite production where the expression of Crp increases undecylprodigiosin production (Gao *et al.*, 2012). Conversely, strains over-expressing the cell-division activator protein SsgA show an increase in the number of germ tubes per spore (Noens *et al.*, 2007), with on average three germ tubes emerging from a single spore (instead of the two in wild-type spores and significantly less than that in *ssgA* mutants). It is yet unclear how future sites of branching in the hyphae or germination in the spores are marked. However, even after very long storage of spores, germination still occurs at the spore 'poles', suggesting physical marks to the PG, such as rare modifications. Yet, in our analysis, we did not find muropeptide moieties such as muramic d-lactam, which is uniquely found in the spore-cortex of *B. subtilis*, but which is absent in vegetatively growing cells (Popham *et al.*, 1996). The only major difference was the relatively high amount of MurN-Tri in the spore PG, which was hardly seen in *bldD* mutants, and also much less prominent in the *whiG* mutant. This strongly suggests that incorporation of MurN-Tri directly relates to sporulation. It will be interesting to see why this moiety is overrepresented in the spore PG. At the same time, this also shows the importance of analyzing the cell wall of different culture types as it reveals novel features that may play a key role in development. L,D-transpeptidases (LDTs) are especially prevalent in the actinobacterial genera *Mycobacterium*, *Corynebacterium* and *Streptomyces*, and, suggestively, these bacteria have a much higher percentage of 3-3 cross-links, with an abundance of at least 30% 3-3-cross links in investigated actinobacterial peptidoglycan as compared to bacteria with lateral cell-wall growth such as *E. coli* (<10%) and *E. faecium* (3%) (Cameron *et al.*, 2014, Hugonnet *et al.*, 2014, Lavollay *et al.*, 2009). LDTs attach to D-Ala[4] and form a cross-link between glycine and LL-DAP[3]. D-Ala[4] is considered a donor for this type of cross-link (Mainardi *et al.*, 2005). An interesting

feature of these two mechanisms is that 3-4 cross-links can only be formed when a pentapeptide is present to display the D-Ala[5] donor, whereas 3-3 cross-links can be formed with a tetrapeptide as a donor strand. The data agrees with the idea that 3-3 cross-links could be required for remodeling of the cell wall beyond the tip-complex, using available tetrapeptides contrary to newly constructed pentapeptides (Lavollay *et al.*, 2008, Sanders *et al.*, 2014, Sacco *et al.*, 2010, Baranowski *et al.*, 2018).

The cell wall and programmed cell death

Evidence is accumulating that, like eukaryotes, bacteria undergo a process of programmed cell death (PCD). Bacterial multicellularity implies a lifestyle involving cellular heterogeneity and the occasional sacrifice of selected cells for the benefit of survival of the colony (Zhang *et al.*, 2016b, Yague *et al.*, 2010, Yague *et al.*, 2013). PCD is likely a major hallmark of multicellularity (Claessen *et al.*, 2014), and has been described in the biofilm-film forming *Streptococcus* (Guiral *et al.*, 2005) and *Bacillus* (Engelberg-Kulka *et al.*, 2006), in Myxobacteria that form fruiting bodies (Sogaard-Andersen & Yang, 2008), in the filamentous cyanobacteria (Bornikoel *et al.*, 2017, Ning *et al.*, 2002), and in the branching *Streptomyces* (Manteca *et al.*, 2006, Miguelez *et al.*, 1999, Tenconi *et al.*, 2018). In streptomycetes, cell-wall hydrolases support developmental processes like branching and germination (Haiser *et al.*, 2009). Additionally, PCD is likely an important event during the onset of development from a vegetative lifestyle to a reproductive lifestyle, as autolytic degradation of the cell wall is intrinsically linked with the onset of antibiotic production and spore formation (Tenconi *et al.*, 2018). Conceptually, GlcNAc accumulates at high concentrations around colonies during PCD, and GlcNAc is an important signaling molecule for the onset of morphological differentiation and antibiotic production in streptomycetes (Rigali *et al.*, 2008, Urem *et al.*, 2016). The linkage of PCD and antibiotic production is logical from a biological perspective; autolytic dismantling of the vegetative (substrate) mycelium generates building blocks in a nutrient-depleted soil, which will inevitably attract motile competitors. Antibiotics likely serve to fend off these competitors and protect the food source. Thus, cell-wall hydrolysis may facilitate the correct timing of development.

CONCLUSIONS

We have provided a detailed analysis of the peptidoglycan of *Streptomyces* mycelia and spores, and developed a reliable and fast method to compare larger numbers of samples. Our data show significant changes over time, among which changes in the amino acid chain, hydrolysis of dimers, and the accumulation of the rare MurN-Tri specifically in the spores. The cell wall likely plays a major role in the development of streptomycetes, with implications for germination and the switch to development and antibiotic production (via PCD-released cell wall components). The dynamic process that controls the remodeling of the cell wall during tip growth is poorly understood, but we anticipate that the local cell-wall structure at sites of growth and branching may well be different from that in older (non-growing) hyphae. This is consistent with the changes we observed over time, between the younger and older mycelia. Detailed localization of cell-wall modifying enzymes and of specific cell-wall modifications, in both time and space, is required to further reveal the role of the cell wall in the control of growth and development of streptomycetes.

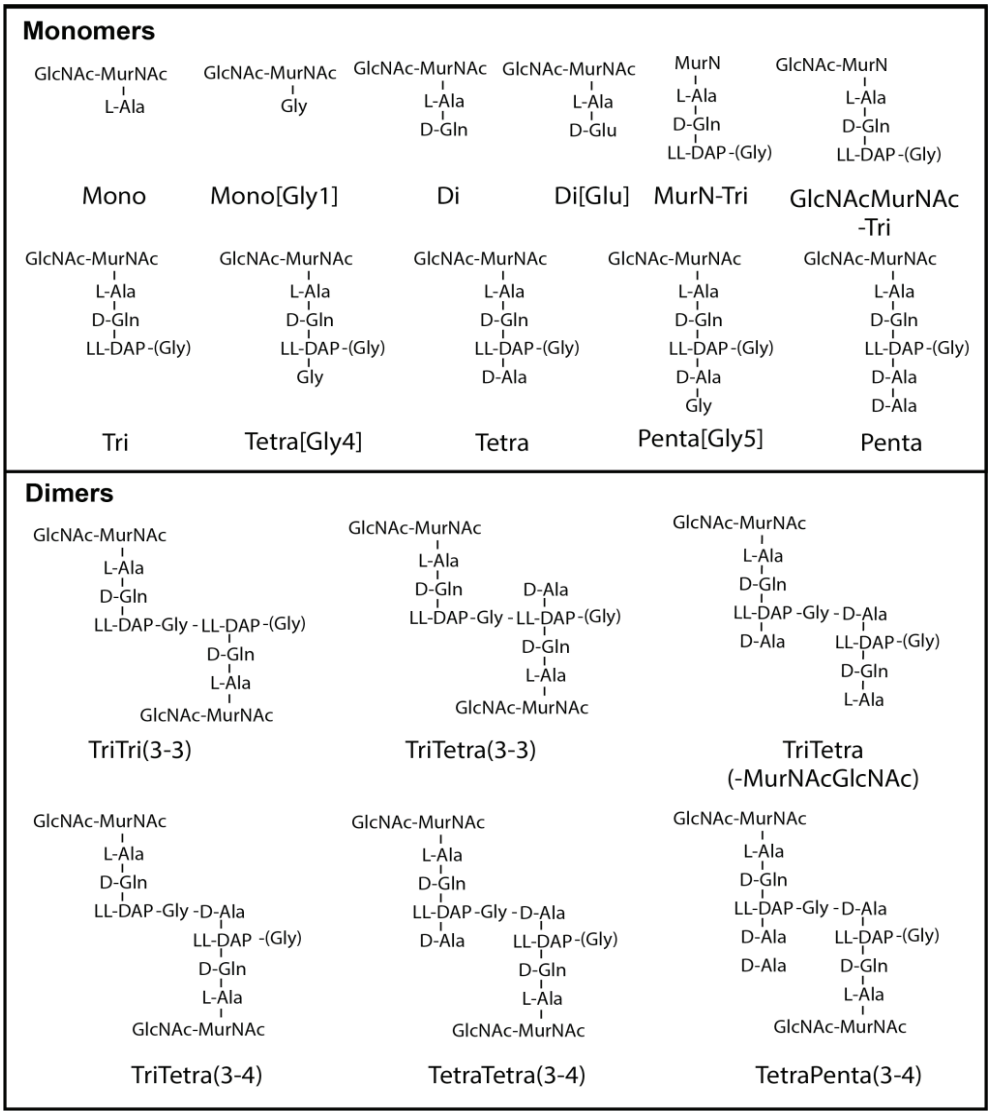

Figure 3. Summary of structures of main monomers and dimers observed in PG from *S. coelicolor*. Modification to the PG include: alteration of the length of the amino acid chain; [Gly1], L-Ala is replaced by Gly; [Glu], where Glutamic acid (Glu) is present instead of D-Glutamine (Gln); [Gly4], where D-Ala(4) is replaced by Gly; [Gly5], where D-Ala(5) is replaced by Gly. Specific for dimers: (3-3) shows a cross-link between LL-DAP(3) to LL-DAP(3) with a Gly-bridge; (3-4) shows a cross-link between LL-DAP(3) and D-Ala(4) with a Gly-bridge; (-MurNAcGlcNAc) shows hydrolysis of a set of sugars.

Acknowledgments

This work is part of the profile area Antibiotics of the Faculty of Sciences of Leiden University.

Conflict of interest statement

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

LvdA performed the experiments with the help of GS. LvdA and GvW conceived the study. LvdA, AH, TH and GvW wrote the article with the help of WV. All authors approved the final manuscript.