

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

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17 Running title: Peptidoglycan analysis of *Streptomyces coelicolor*

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19 Keywords: Cell wall; Streptomyces; Mass spectrometry; Multicellular growth; Sporulation;
20 Programmed Cell Death

21

22 **ABSTRACT**

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

39

40 **IMPORTANCE**

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

49 muropeptide that accumulates predominantly in the spores, and may provide clues towards
50 spore development.
51

52 **INTRODUCTION**

53 Peptidoglycan (PG) is a major component of the bacterial cell wall. It forms a physical
54 boundary that maintains cell shape, protects cellular integrity against the osmotic pressure
55 and acts as a scaffold for large protein assemblies and exopolymers (1). The cell wall is a
56 highly dynamic macromolecule that is continuously constructed and deconstructed to allow
57 for cell growth and to meet environmental demands (2). PG is built up of glycan strands of
58 alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues that
59 are connected by short peptides to form a mesh-like polymer. PG biosynthesis starts with the
60 synthesis of PG precursors by the Mur enzymes in the cytoplasm and cell membrane,
61 resulting in lipid II precursor, undecaprenylpyrophosphoryl-MurNAc(GlcNAc)-pentapeptide.
62 Lipid II is transported across the cell membrane by MurJ and/or FtsW/SEDS proteins and the
63 PG is polymerized and incorporated into the existing cell wall by the activities of
64 glycosyltransferases and transpeptidases (3-5).

65 The Gram-positive model bacterium *Bacillus subtilis* grows via lateral cell-wall
66 synthesis followed by binary fission; in addition, *B. subtilis* forms heat- and desiccation-
67 resistant spores (6, 7). By contrast, the vegetative hyphae of the mycelial *Streptomyces* grow
68 by extension of the hyphal apex and cell division results in connected compartments
69 separated by cross-walls (8-10). This makes *Streptomyces* a model taxon for bacterial
70 multicellularity (11). Multicellular vegetative growth poses different challenges to
71 *Streptomyces*, including the synthesis of many chromosomes during vegetative growth and
72 separation of the nucleoids in the large multi-genomic compartments during cross-wall
73 formation (12, 13). In submerged cultures, streptomycetes typically form complex mycelial
74 networks or pellets (14). On surface-grown cultures, such as agar plates, these bacteria
75 develop a so-called aerial mycelium, whereby the vegetative or substrate mycelium is used
76 as a substrate. The aerial hyphae eventually differentiate into chains of spores, a process
77 whereby many spores are formed almost simultaneously, requiring highly complex
78 coordination of nucleoid segregation and condensation and multiple cell division (12, 15, 16).
79 Streptomycetes have an unusually complex cytoskeleton, which plays a role in polar growth

80 and cell-wall stability (17, 18). Mutants that are blocked in the vegetative growth phase are
81 referred to as bald or *bld*, for lack of the fluffy aerial hyphae (19), while those producing aerial
82 hyphae but no spores are referred to as white (*whi*), as they fail to produce grey-pigmented
83 spores (20).

84 The *Streptomyces* genome encodes a large number of cell wall-modifying enzymes,
85 such as cell wall hydrolases (autolysins), carboxypeptidases and penicillin-binding proteins
86 (PBPs), a complexity that suggests strong heterogeneity of the PG of these organisms (21,
87 22). Several concepts that were originally regarded as specific to eukaryotes also occur in
88 bacteria, such as multicellularity (11, 23, 24) and programmed cell death (25, 26).
89 Programmed cell death (PCD) likely plays a major role in the onset of morphological
90 development, required to lyse part of the vegetative mycelium to provide the nutrients for the
91 aerial hyphae (27, 28). PCD and cell-wall recycling are linked to antibiotic production in
92 *Streptomyces* (29).

93 All disaccharide peptide subunits (muropeptides) in the PG are variations on the basic
94 building block present in lipid II, which in *Streptomyces* typically consists of GlcNAc-MurNAc-
95 L-Ala-D-Glu-LL-DAP(Gly)-D-Ala-D-Ala (30, 31). Here, we have analyzed the cell wall
96 composition of vegetative mycelium and mature spores of *Streptomyces coelicolor* by LC-
97 MS, to obtain a detailed inventory of the monomers and dimers in the cell wall. This revealed
98 extensive cell-wall hydrolysis and remodeling during vegetative growth and highlights the
99 difference in cell-wall composition between vegetative hyphae and spores.

100

101 **RESULTS**

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

112 To allow analyzing a large number of samples simultaneously and in a reasonable
113 time frame, we adapted a method for PG purification (32) for use in *S. coelicolor*. The
114 advantage of this method is that it requires only a small amount of input biomass and much
115 faster sample handling. For this, 10 mg of lyophilized cell-wall material was isolated by
116 boiling cells in 0.25% SDS in 2 ml microcentrifuge tubes, and secondary cell-wall polymers
117 such as teichoic acids were removed by treatment for 4 h with hydrochloric acid (HCl) (see
118 Materials and Methods section for details). As a control for the validity of the method, it was
119 compared to a more elaborate method that is used more routinely (33). In the latter method,
120 biomass from 1 L culture of *S. coelicolor* was boiled in 5% SDS and subsequently treated for
121 48 h with hydrofluoric acid (HF) to remove teichoic acids. Comparison of the two methods
122 revealed comparable outcomes between the two methods in peak detection (Table S5). This
123 validated the rapid method based on 0.25% SDS and HCl, which was therefore used in this
124 study.

125 The isolated PG was digested with mutanolysin (32, 34) and the muropeptide
126 composition was analyzed by liquid chromatography linked to mass spectrometry (LC-MS).
127 Peaks were identified in the m/z range from 500-3000 Da, whereby different m/z in co-eluting
128 peaks were further characterized by MS/MS. The eluted m/z values were compared to a

129 dataset of theoretical masses of predicted muropeptides. Table 1 shows a summary of the
130 monomers and dimers that were detected during growth in liquid media, and Table 2 a
131 summary of muropeptides during growth on solid media. The full datasets are given in
132 Tables S1-S4. We identified several modifications, including the amidation of D-iGlu to D-
133 iGln at position 2 of the stem peptide, deacetylation of MurNAc to MurN, removal of amino
134 acids to generate mono-, di-, tri- and tetrapeptides, loss of LL-DAP-bound glycine, and the
135 presence of Gly (instead of Ala) at position 1,4 or 5. The loss of GlcNac or GlcNAc-MurNAc
136 indicates hydrolysis (Figure 2).

137 For all amino acid positions in the pentapeptide chain, the position is indicated as [n],
138 whereby n is the number in the chain (with [1] the position closest to the PG backbone, i.e.
139 the MurNac residue, and [5] the last aa residue).

140

141 **Growth phase-dependent changes in the PG composition**

142 The muropeptide that is incorporated from Lipid II by glycosyltransferases contains a
143 pentapeptide with a Gly residue linked to LL-DAP in aa position 3 (LL-DAP[3]). In many
144 bacteria pentapeptides are short-lived muropeptides that occur mostly at sites where *de novo*
145 cell-wall synthesis takes place, *i.e.* during growth and division (35, 36). This is reflected by
146 the high abundance of pentapeptides in the samples obtained from exponentially growing
147 cells, with a pentapeptide content of 21% during early exponential growth (18 h), as
148 compared to 14% and 11% during late exponential growth (24 h), transition phase (36 h) and
149 stationary phase (48 h), respectively. Conversely, tripeptides increased over time, from 24%
150 during early exponential phase to 32% in transition-phase cultures.

151 Addition of Gly to the medium and, in consequence, incorporation of Gly in the PG
152 can cause changes in morphology (37, 38). This property has been applied to facilitate
153 lysozyme-mediated formation of protoplasts in *Streptomyces*, used for protoplast
154 transformation methods (39-41). In *S. coelicolor*, Gly can be found instead of D-Ala[1], D-
155 Ala[4] or D-Ala[5] in the pentapeptide chain. During liquid growth, tetrapeptides carrying
156 Gly[4] increased from 3% during early growth to 8% during the latest time points. The relative

157 abundance of pentapeptides carrying Gly at position 5 (4-5%) did not vary over time. On
158 solid-grown cultures, the Gly content of the peptidoglycan was around 1%, which is
159 significantly lower than in liquid-grown cultures.

160

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

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

172

173 **PG hydrolysis increases as the culture ages**

174 PG hydrolysis is associated with processes such as separation of daughter cells after cell
175 division and autolysis, and mutants of bacteria that fail to produce PG amidases grow in
176 chains of connected cells (42, 43). On solid media, vegetative hyphae of *Streptomyces*
177 undergo programmed cell death (PCD) and hydrolysis. In liquid-grown cultures, cell death
178 occurs in the center of dense pellets. During spore maturation, spores are separated
179 hydrolytically from one another. Some streptomycetes sporulate in submerged culture, but
180 this is not the case for *S. coelicolor* (44). Our data show that as growth proceeds in
181 submerged cultures, the *S. coelicolor* peptidoglycan progressively loses GlcNAc and
182 GlcNAc-MurNAc moieties (Table 1), as a result of N-acetylglucosaminidase activity. The
183 proportion of dimers lacking GlcNAc-MurNAc thereby increases in time from 24% at 18 h to
184 56% at 48 h. Figure 3C shows MS/MS profiles of a TriTri-dimer with a single set of glycans.

185 During growth on solid media the trend was inversed. This may be due to the different
186 developmental stages, whereby 24 h corresponds to early developmental events and PCD,
187 48 h to aerial growth and sporulation at 72 h. This analysis shows the relative abundance of
188 muropeptides of the total amount of biomass, when hydrolysis has occurred at the vegetative
189 mycelium. During later stages of growth on agar plates a large amount of aerial hyphae is
190 formed, and this can therefore not be compared directly to samples that only contain
191 vegetative hyphae (Table 2).

192

193 **Deacetylation of MurNAc is associated with mycelial aging and sporulation**

194 Modifications to the glycan strands are commonly linked to lysozyme resistance (45). In
195 particular, N-deacetylation of PG strands is widespread among bacteria, which can occur
196 both at GlcNAc and at MurNAc (46). In the case of *S. coelicolor*, the only glycan modification
197 is the deacetylation of MurNAc to MurN. Our data show that this modification becomes more
198 prominent as the vegetative mycelium ages, from 5% during early growth to 8% during later
199 growth stages. On agar plates, 3.7% of the monomers was deacetylated at 24 h, 4.4% at 48
200 h and 6.1% at 72 h.

201 The PG composition of spores and vegetative mycelia was compared to get more
202 insights into the possible correlations between PG composition and important processes
203 such as dormancy and germination. Muropeptides in spores were strongly biased for
204 tetrapeptides, making up 44% of the monomers, as compared to 23-25% of the vegetative
205 PG. Conversely, pentapeptides were found in much lower amounts in spores (5% of the
206 monomers), as compared to 10-22% in vegetative hyphae. The muropeptide that stood out in
207 the analysis of the spore PG was a tripeptide which lacks GlcNAc and contains a
208 deacetylated MurNAc, called MurN-Tri (Figure 2). In spores, MurN-Tri made up 3.5% of the
209 monomers, whereas the less modified muropeptide, GlcNAcMurN-Tri only made up 0.2% of
210 the monomers.

211 To further investigate this interesting phenomenon, and show the applicability of our
212 work for the analysis of developmental mutants, we analyzed *bldD* and *whiG* mutants. The

213 *bldD* gene product is a global transcription factor that controls the transcription of many
214 developmental genes and is therefore blocked in an early stage of morphogenesis (47), while
215 the *whiG* gene product is a σ factor that controls early events of aerial growth (48). The
216 monomer profile of *S. coelicolor* M145 and its *bldD* and *whiG* mutants are summarized in
217 Table 3. For the wild-type strain M145, 24 h represents vegetative growth, 48 h aerial growth
218 and 72 h spore formation. In line with the notion that MurN-Tri accumulates particularly in
219 spores, the *bldD* mutant accumulated hardly any MurN-Tri (0-0.2%) over the course of time
220 and the *whiG* mutant 0.4%, 0.6% and 1.3% after 24 h, 48 h, and 72 h. respectively. In
221 contrast, the wild-type strain M145 had 0.6%, 1.7% and 3.1% MurN-Tri at these time points,
222 respectively, strongly suggesting that MurN-Tri accumulates in a sporulation-specific
223 manner.

224

225

226 DISCUSSION

227 In this study we have analyzed changes in the composition of the peptidoglycan during
228 growth and development of *Streptomyces coelicolor*. The different masses were thereby
229 identified by MS and MS/MS analysis, which allowed detailed identification of the subunits,
230 including dimers that are cross-linked by either 3-3 or 3-4 cross-links between the peptide
231 moieties. Our data show that the *Streptomyces* peptidoglycan composition is changing
232 dynamically, whereby major peptidoglycan recycling was seen, whereby over half of all
233 GlcNAc-MurNac dimers were hydrolyzed in late-exponential cultures.

234 L,D-transpeptidases (LDTs) are especially prevalent in the actinobacterial genera
235 *Mycobacterium*, *Corynebacterium* and *Streptomyces*. Suggestively, these bacteria have a
236 much higher percentage of 3-3 cross-links, with an abundance of at least 30% 3-3-cross links
237 in investigated actinobacterial peptidoglycan as compared to bacteria with lateral cell-wall
238 growth such as *E. coli* (<10%) and *E. faecium* (3%) (30, 49, 50). LDTs attach to D-Ala[4] and
239 form a cross-link between glycine and LL-DAP[3]. D-Ala[4] is considered a donor for this type
240 of cross-link (51). An interesting feature of these two mechanisms is that 3-4 cross-links can

241 only be formed when a pentapeptide is present to display the D-Ala[5] donor, whereas 3-3
242 cross-links can be formed with a tetrapeptide as a donor strand. Dimers in vegetative (liquid-
243 grown) cells carry 36.5% 3-3 cross-links at 18 h of growth, increasing to 48% at 24 h, 54.5%
244 at 36 h and 57.3% at 48 h. Between these stages of growth, the main structural difference is
245 the length of hyphae compared to growing tips. The data agrees with the idea that 3-3 cross-
246 links could be required to remodel the cell wall beyond the tip-complex, using available
247 tetrapeptides contrary to newly constructed pentapeptides (52-55).

248 A major event associated with lytic degradation of the cells is programmed cell death
249 (PCD). PCD is likely a major hallmark of multicellularity (11), and has been described in the
250 biofilm-forming *Streptococcus* (56) and *Bacillus* (57), in Myxobacteria that form fruiting
251 bodies (58), in the filamentous cyanobacteria (59, 60), and in the branching *Streptomyces*
252 (28, 61, 62). In streptomycetes, cell-wall hydrolases support developmental processes like
253 branching and germination (21). Additionally, PCD and the autolytic release of GlcNAc from
254 the cell wall is an important signal for the onset of morphological differentiation and antibiotic
255 production in streptomycetes (29, 63). Our data show an exceptionally high amount of dimers
256 which carry a cross-linked set of peptides but a single set of glycans, from 25% of dimers in
257 18 h old liquid cultures to 56% at 48 h old cultures. The increase in abundance of dimers
258 lacking a set of glycans is especially prevalent in liquid-grown mycelia, while the overall
259 increase in hydrolyzed dimers is not as high in mycelia grown on solid media. It should ne
260 noted that on agar plates also aerial hyphae are formed, which are not subject to the
261 extensive lysis seen in vegetative hyphae, and this may reduce the relative content of these
262 glycan-less peptides.

263 We have also analyzed changes in the PG that correlate to sporulation. One question
264 that remains to be answered is how future sites of branching in the hyphae or germination in
265 the spores are marked, and oen interesting possibility the cell wall may be changed as a
266 marker for the start of future *de novo* PG synthesis. After all, even after very long storage of
267 spores, germination still occurs at the spore 'poles', suggesting that physical marks to the
268 PG, such as rare modifications, may occur. A previous study showed that mutation of the

269 gene *dacA* that encodes D-alanyl-D-alanine carboxypeptidase disrupts spore maturation and
270 germination, where one could influence the other. This indicates that either pentapeptides
271 inhibit spore maturation, or that a high amount of tetrapeptides is important (64). Indeed, we
272 report a high amount of tetrapeptides in spores, 48% of the monomers. A necessity for
273 tetrapeptides could be linked to the formation of 3-3 cross-links, which require tetrapeptides,
274 contrary to pentapeptides, as a substrate. Indeed, spores carry 3-3 cross-links in 35% of the
275 dimers, which probably strongly contribute to structural stability. Interestingly, a relatively
276 high amount of MurN-Tri (3.5%) was identified in the spore PG, while this molecule was
277 almost completely absent in *bldD* mutants, which are arrested in the vegetative growth
278 phase. A small amount of MurN-Tri (0.4-1.2%) was found in *whiG* mutants, which do develop
279 aerial hyphae but do not sporulate. It will be interesting to see what the biological significance
280 is of the overrepresentation of MurN-Tri in aerial and spore PG. This underlines the
281 importance of analyzing the cell wall of different culture types, as it reveals novel features
282 that may play a key role in development.

283

284 CONCLUSIONS

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

296 modifications, in both time and space, should provide further insights into the role of the cell
297 wall in the control of growth and development of streptomycetes.

298 **EXPERIMENTAL PROCEDURES**

299 **Bacterial strain and culturing conditions**

300 *Streptomyces coelicolor* A3(2) M145 (41), *bldD* mutant J774 (*cysA15 pheA1 mthB2*
301 *bldD53* NF SCP2* (19)) and *whiG* mutant J2400 (*whiG::hyg* (65)), were obtained from the
302 John Innes Centre strain collection. All media and methods for handling *Streptomyces* are
303 described in the *Streptomyces* laboratory manual (41). Spores were collected from Soy Flour
304 Mannitol (SFM) agar plates. Liquid cultures were grown shaking at 30°C in a flask with a
305 spring, using normal minimal medium with phosphate (NMM+) supplemented with 1% (w/v)
306 mannitol as the sole carbon source; polyethylene glycol (PEG) was omitted to avoid
307 interference with the MS identification. Cultures were inoculated with spores at a density of
308 10⁶ CFU/ML. A growth curve was constructed from dry-weight measurements by freeze-
309 drying washed biomass obtained from 10 ml of culture broth (three biological replicates). To
310 facilitate the harvest of mycelium from agar plates, they were grown on cellophane slips,
311 after which the biomass was scraped of the cellophane. Spores were collected from SFM
312 agar plates by adding 0.01% (w/v) SDS to facilitate spore release from the aerial mycelium,
313 scraping them off with a cotton ball and drawing the solution with a syringe. Spores were
314 filtered with a cotton filter to separate spores from residual mycelium.

315

316 **PG extraction**

317 Cells were lyophilized for a biomass measurement, 10 mg biomass was directly used for PG
318 isolation. PG was isolated according to (32), using 2 mL screw-cap tubes for the entire
319 isolation. Biomass was first boiled in 0.25% SDS in 0.1 M Tris/HCl pH 6.8, thoroughly
320 washed, sonicated, treated with DNase, RNase and trypsin, inactivation of proteins by boiling
321 and washing with water. Wall teichoic acids were removed with 1 M HCl. PG was digested
322 with mutanolysin and lysozyme (66). Muropeptides were reduced with sodium borohydride
323 and the pH was adjusted to 3.5-4.5 with phosphoric acid.

324 To validate the method, we compared it to the method described previously (33). For
325 this, *S. coelicolor* mycelia were grown in 1 L NMM+ media for 24 h. After washing of the

326 mycelia, pellets were resuspended in boiling 5% (w/v) SDS and stirred vigorously for 20 min.
327 Instead of sonicating the cells, they were disrupted using glass beads, followed by removal of
328 the teichoic acids with an HF treatment at 4°C as described.

329

330 **LC-MS analysis of monomers**

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

334 Chromatographic separation was performed on an Acquity UPLC HSS T3 C₁₈ column (1.8
335 µm, 100 Å, 2.1 × 100 mm). Mobile phase A consist of 99.9% H₂O and 0,1% Formic Acid and
336 mobile phase B consists of 95% Acetonitrile, 4.9% H₂O and 0,1% Formic Acid. All solvents
337 used were of LC-MS grade or better. The flow rate was set to 0.5 ml/min. The binary gradient
338 program consisted of 1 min 98% A, 12 min from 98% A to 85% A, and 2 min from 85% A to
339 0% A. The column was then flushed for 3 min with 100% B, the gradient was then set to 98%
340 A and the column was equilibrated for 8 min. The column temperature was set to 30°C and
341 the injection volume used was 5 µL. The temperature of the autosampler tray was set to 8°C.
342 Samples were run in triplicates.

343 MS/MS was done both on the full chromatogram by data dependent MS/MS and on
344 specific peaks by selecting the mass of interest. Data dependent acquisition was performed
345 on the most intense detected peaks, the activation type was Collision Induced Dissociation
346 (CID). Selected MS/MS was performed when the resolution of a data dependent acquisition
347 lacked decisive information. MS/MS experiments in the ion trap were carried out with relative
348 collision energy of 35% and the trapping of product ions were carried out with a q-value of
349 0.25, and the product ions were analyzed in the ion trap., data was collected in the positive
350 ESI mode with a scan range of *m/z* 500–3000 in high range mode. The resolution was set to
351 15.000 (at *m/z* 400).

352

353 **Data analysis**

354 Chromatograms were evaluated using the free software package MZmine
355 (<http://mzmine.sourceforge.net/> (67)) to detect peaks, deconvolute the data and align the
356 peaks. Only peaks corresponding with a mass corresponding to a muropeptide were
357 saved, other data was discarded. The online tool MetaboAnalyst (68) was used to
358 normalize the data by the sum of the total peak areas, then normalize the data by log
359 transformation. The normalized peak areas were exported and a final table which shows
360 peak areas as percentage of the whole was produced in Microsoft Excel.

361

362 **Muropeptide identification**

363 The basic structure of the peptidoglycan of *S. coelicolor* has been published previously (30).
364 Combinations of modifications were predicted and the masses were calculated using
365 ChemDraw Professional (PerkinElmer). When a major peak had an unexpected mass,
366 MS/MS helped resolve the structure. MS/MS was used to identify differences in cross-linking
367 and to confirm predicted structures.

368

369 **Acknowledgments**

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

372

373 **Conflict of interest statement**

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

375

376 **Author contributions**

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

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558 **LEGENDS**

559

560 **Figure 1.** Growth of *S. coelicolor* in liquid media (top) and on solid media (bottom). A: Growth
561 curve on NMM+ medium based on triplicate dry-weight measurements. B: Pellet morphology
562 in liquid media. After spore germination, hyphae emerge through top growth and branching
563 that form an intricate network or pellet. The center of pellets eventually lyses due to PCD
564 (light grey). C: Growth on solid media, starting with the development of vegetative mycelium
565 from a single spore; after the onset of development, the vegetative hyphae differentiate into
566 aerial hyphae that grow into the air, coinciding with lysis of the vegetative mycelium (zone of
567 lysis represented in light grey). Chains of spores are generated by septation of the aerial
568 hyphae.

569

570 **Figure 2.** Summary of structures of main monomers and dimers observed in PG from *S.*
571 *coelicolor*. Modification to the PG include: alteration of the length of the amino acid chain;
572 [Gly1], L-Ala is replaced by Gly; [Glu], where Glutamic acid (Glu) is present instead of D-
573 Glutamine (Gln); [Gly4], where D-Ala(4) is replaced by Gly; [Gly5], where D-Ala(5) is
574 replaced by Gly. Specific for dimers: (3-3) shows a cross-link between LL-DAP(3) to LL-
575 DAP(3) with a Gly-bridge; (3-4) shows a cross-link between LL-DAP(3) and D-Ala(4) with a
576 Gly-bridge; (-MurNAcGlcNAc) shows hydrolysis of a set of sugars.

577

578 **Figure 3.** MS/MS fragmentations of TetraTri dimers with either 3-3 cross-link (A) or 3-4
579 cross-link (B). Differentiation between these two types of cross-links is possible at the point
580 of asymmetry, at Gly attached to LL-DAP. The 3-3 cross-linked dimer (A) fragments into
581 masses of 966.0 m/z and 941.3 m/z, which can be found in the respective MS/MS spectrum.
582 The 3-4 cross-linked dimer (B) fragments into masses of 1037.4 m/z and 870.5 m/z. These
583 masses are found in the MS/MS spectrum. Boxed MS/MS spectra show a magnification of
584 masses between m/z 850 and 1050 to show masses present in lower abundance. (C) a TriTri

585 dimer lacking GlcNAcMurNAc with an M+H of 1355.6, diagnostic fragments are given in the
586 proposed structures.

587

588

589 **Table 1.** Relative abundance(%)^a of mucopeptides in vegetative cells from liquid NMM+.

	<i>S. coelicolor</i> M145			
Monomers^b	18 h	24 h	36 h	48 h
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
	<i>S. coelicolor</i> M145			
Dimers^b	18 h	24 h	36 h	48 h
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

590 ^aRelative abundance is calculated as the ratio of the peak area over the sum of all peak591 areas recognized in the chromatogram. ^bMonomers and dimers are treated as separate592 datasets. ^cGly detected instead of Ala

593

594 **Table 2.** Relative abundance(%)^a of muropeptides in mycelia and spores of *S. coelicolor*
 595 M145 harvested after growth on SFM agar plates.

Monomers ^b	<i>S. coelicolor</i> M145			
	24 h	48 h	72 h	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
Deacetylated	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
	<i>S. coelicolor</i> M145			
Dimers ^b	24 h	48 h	72 h	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

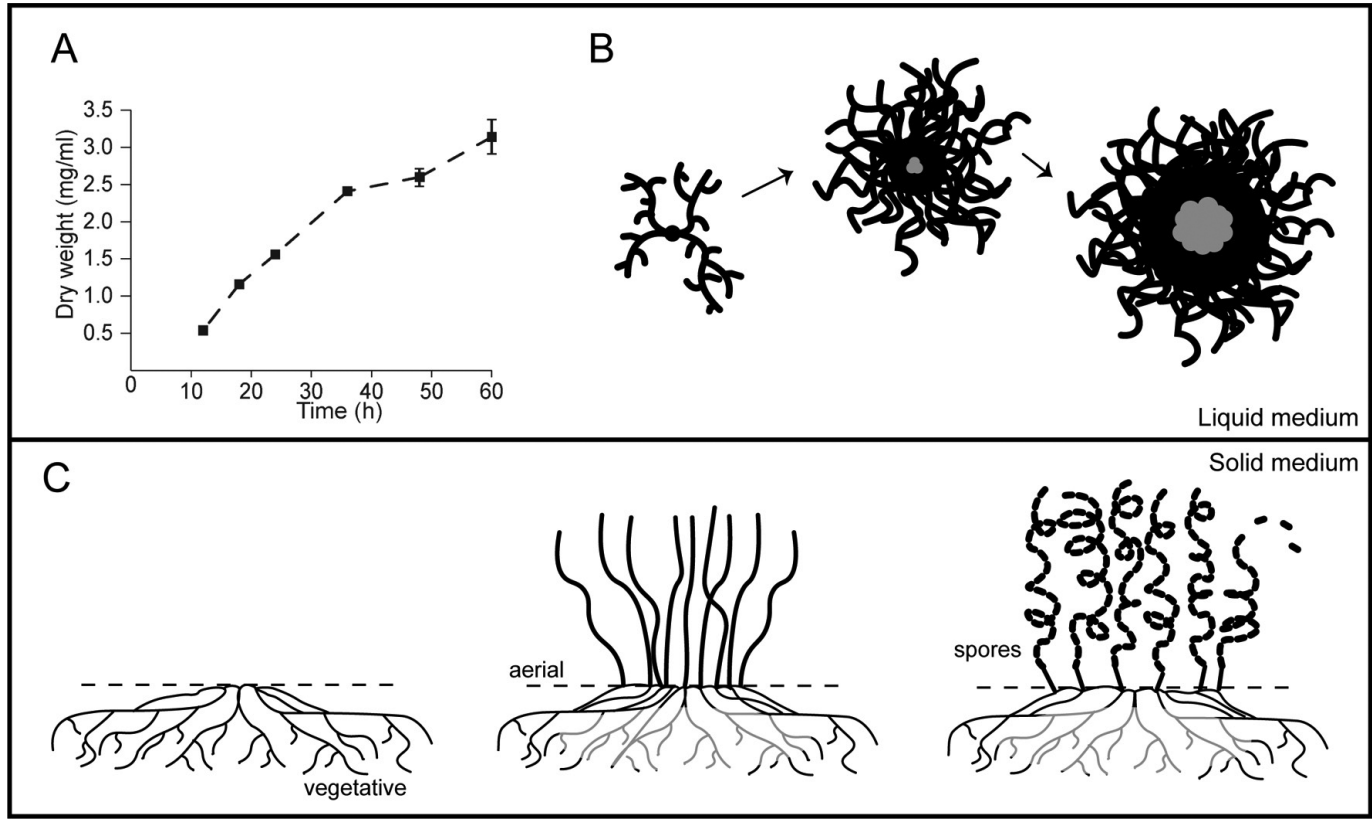
596 ^aRelative abundance is calculated as the ratio of the peak area over the sum of all peak
 597 areas recognized in the chromatogram. ^bMonomers and dimers are treated as separate
 598 datasets. ^cGly detected instead of Ala

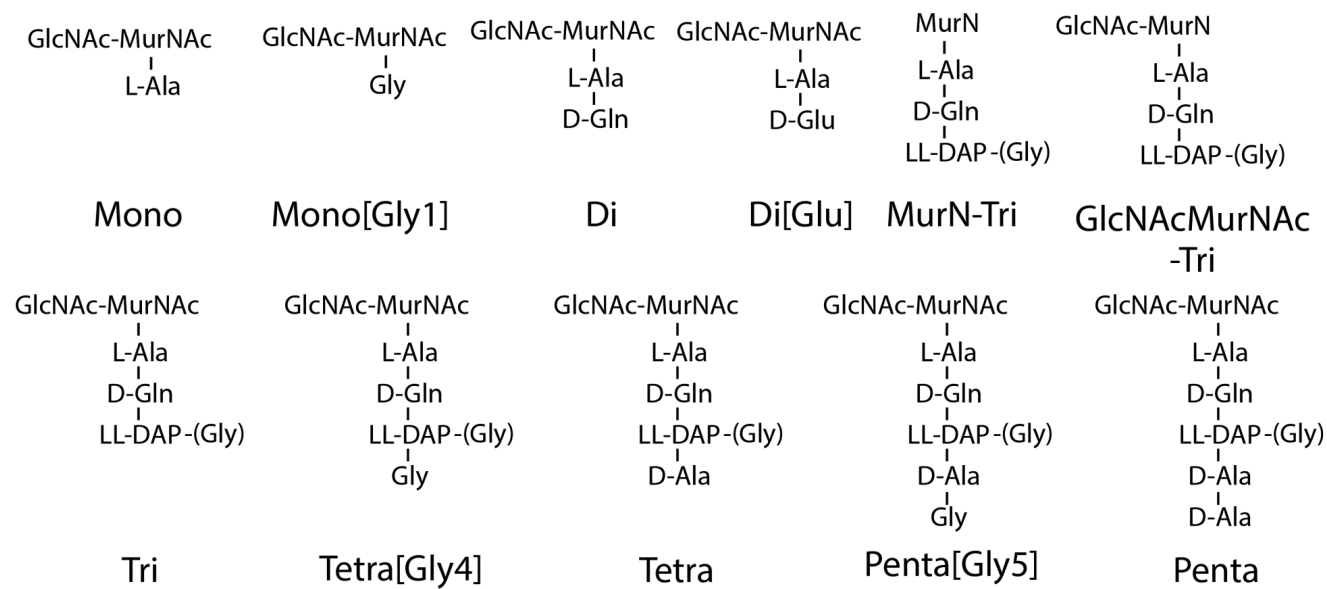
599

600 **Table 3.** Relative abundance (in %) ^a of monomers from developmental *bldD* and *whiG*
 601 mutants and the wild-type strain, *S. coelicolor* M145.

		Mono	Di	Tri	Tetra	Penta	Deacetylated	MurN-Tri	GlcNAc-MurN-Tri
<i>ΔbldD</i>	24 h	4.5	25.7	28.0	23.0	10.8	6.5	0.0	5.3
	48 h	4.3	26.3	38.3	23.4	11.1	8.5	0.2	6.6
	72 h	4.3	27.2	40.9	19.9	9.5	7.6	0.2	5.8
<i>ΔwhiG</i>	24 h	3.5	23.2	27.0	32.5	15.2	3.0	0.4	1.3
	48 h	3.6	17.5	44.3	25.5	7.9	5.0	0.6	3.2
	72 h	4.1	18.5	48.8	20.9	6.9	6.2	1.3	3.8
M145 (wt)	24 h	3.6	21.6	29.6	25.4	16.8	3.7	0.6	1.9
	48 h	4.3	17.6	34.3	29.5	9.9	4.4	1.7	1.4
	72 h	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

602 ^a Relative abundance is calculated as the ratio of the peak area over the sum of all peak
 603 areas recognized in the chromatogram.



Monomers**Dimers**