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

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CHAPTER 7

General Discussion

The bacterial cell wall is an extremely dynamic macromolecule subject to constant construction and deconstruction depending on the requirements that are imposed by cell growth. New cell wall material is incorporated in a manner which still supports cell structure and survives the turgor pressure – there is no room for weak spots. Correct regulation of cell wall synthesis is essential for several processes in *Streptomyces*. After dormancy, spores need to germinate in a favorable environment, a process tightly regulated by the cAMP receptor protein CRP and Resuscitation Promoting Factors (RPFs) (Piette *et al.*, 2005, Sexton *et al.*, 2015). After germination, DivIVA driven apical growth drives the formation of a large vegetative mycelial structure (Flärdh *et al.*, 2012). During vegetative growth, cross-membranes and vegetative septa are formed to compartmentalize the vegetative mycelium, an SsgB-independent but FtsZ-dependent process (Celler *et al.*, 2016, Yague *et al.*, 2016). Later, spores are formed by a process where SsgB recruits FtsZ, resulting in Z-rings that are spaced by approximately 1 μM, followed by septation and eventually spores (Willemse *et al.*, 2011). After spore dispersal, the life cycle starts anew. This complexity and the necessity for regulation is expressed in the 7-11 SsgA-like proteins in streptomycetes that serve to control processes relating to cell wall remodeling, including germination, branching and sporulation (Traag & van Wezel, 2008), in the in the nine penicillin binding proteins (PBPs) and seven putative L,D-transpeptidases (LDTs) which together serve to cross-link the peptidoglycan, and in the 50-60 genes predicted to be involved in peptidoglycan hydrolysis (Haiser *et al.*, 2009). This regulation and modification is visible in the diverse muropeptide pattern, which shows more than 60 separate muropeptides, as shown in this thesis. As one of the most well-conserved cellular entities, the bacterial cell wall is a major target for antibiotics, and many of these are produced by actinobacteria (Barka *et al.*, 2016). To protect themselves against their own antibiotics, *Streptomyces* carry antibiotic resistance mechanisms that vary from modifying the function of a single protein, like D-cycloserine resistance, to a complete cluster of genes that modifies a part of the cell wall, as seen in vancomycin resistance (Schaberle *et al.*, 2011). Not surprisingly, there are many aspects of *Streptomyces* biology that have not been resolved. In this thesis, I investigated how the actinobacterial cell wall contributes to species differentiation, cell growth and antibiotic resistance.

Cell wall research in actinobacteria finds its origins in the taxonomic separation of different species. Beginning with the pioneering work by Lechevalier and Lechevalier, the composition of the cell wall has served as a useful tool for actinomycete classification (Lechevalier *et al.*, 1971, Cummins, 1962). They showed that all actinomycete cell walls contain *N*-acetyl-glucosamine, *N*-acetyl-muramic acid, alanine and glutamic acid. Diagnostic differences between different actinomycetes species can be found in the presence of glycine, lysine and either LL- or LD(*meso*)-diaminopimelic acid, together with a set of diagnostic sugars: arabinose, galactose, xylose and madurose. In their analysis, the authors also detected the sugars arabinose and galactose, which form the arabinogalactan layer of *Mycobacterium* and *Nocardia* species.

Chapter 2 shows a polyphasic taxonomic approach to the characterization of *Streptomyces roseifaciens*, a novel species of Streptomyces originally isolated from the QinLing mountains in China (Zhu *et al.*, 2014a, Zhu *et al.*, 2014b). This *Streptomyces* species is a morphological outlier of the *Streptomyces*, as this does not

make long spore chains, but verticillate spores which branch out of the side of aerial hyphae (Hatano *et al.*, 2003). On analyzing the distribution of the SsgA-like proteins (SALPS), it became apparent that verticillate *Streptomyces* typically lack SsgE. In *Streptomyces coelicolor*, *ssgE* deletion mutants predominately produce single spores, rather than spore chains, suggesting a role in spore chain morphology. This correlates well with the fact that verticillate streptomycetes have an entirely different way of producing spore chains, namely many short chains at the lateral side of the aerial hyphae instead of conversion of the aerial hyphae in a single long spore chain.

Whereas cell wall research has started as a taxonomic indicator, current cell wall research is performed to generate insight in bacterial cell growth and development. In Chapter 3 the cell wall composition of *S. coelicolor* was analyzed in growing vegetative hyphae and of spores to look for trends and changes in peptidoglycan composition, and more importantly, to get a hint on the mechanisms of tip growth. Therefore, the muropeptide profile was analyzed by LC-MS, which allows direct detection of the masses associated with different muropeptides, even with overlapping retention times. This study identified over 60 different muropeptides over the course of growth and development. Especially interesting was the high amount of 3-3 cross-links, produced by L, D-transpeptidases (LDTs). LDTs are recognized as transpeptidases that function similarly to Penicillin Binding Proteins (PBPs), although cross-linking at a different point. Importantly, PBPs require the presence of pentapeptides to form a 3-4 cross-link, whereas LDTs require tetrapeptides to form a 3-3 cross-link. The abundance of 3-3 cross-links is especially high in actinobacteria (which grow at the tip) and LDTs are suggested to remodel the cell wall in the post-tip area (Baranowski *et al.*, 2018). The work described in Chapter 3 also revealed that the amount of 3-3 cross-links increases as vegetative mycelium ages, and hyphae grow longer. Cell wall hydrolysis was clearly visible in our data, especially vegetative mycelium showed a high amount of dimers lacking a set of glycans, indicating constant cell wall hydrolysis. In addition, we show that a single muropeptide increases in abundance over the course of development, namely a MurN-tri peptide, which lacks GlcNAc and carries a de-acetylated MurNAc. Possibly, this muropeptide has no function itself but is the result of hydrolysis or spore separation.

The muropeptide profile of *S. coelicolor* showed that the most apparent difference between the cell wall composition of vegetative mycelium and spores relates to the abundance of certain muropeptides, while no muropeptides were uniquely associated with either vegetative mycelium or spores. To investigate this further, we looked back at the taxonomy of streptomycetes. The presence of either LL-DAP, *meso*-DAP or both is indicative of the genus. This proves invaluable to distinguish *Streptomyces* from *Kitasatosporae*, which are very similar in respect to morphology and 16S rRNA and resulted in the inclusion of *Kitasatospora* within the genus *Streptomycetes* (Kim et al., 2004). However, *Kitasatosporae* have different SsgA-like proteins, and lack orthologues of *mbl, bldB* and *whiJ*, suggesting that development is regulated in a different manner (Girard *et al.*, 2014). The main diagnostic difference between the cell walls of the two genera within the *Streptomycetaceae* is that *Streptomyces* cell walls contain LL-DAP, whereas *Kitasatospora* cell walls carry *meso*-DAP in the vegetative mycelium and LL-DAP in the spores (Takahashi, 2017, Girard *et al.*, 2014). DAP is a key component of the peptidoglycan, as this amino acid is at the connecting part of cross-links between peptide chains, either a 3-3 or a 3-4 cross-link. We sus-

pected that the difference in stereochemistry could be related to a difference in the peptidoglycan household. Indeed, the work in Chapter 4 shows that two species of *Kitasatospora*, *K. setae* and *K. viridifaciens*, heavily remodel the peptidoglycan between vegetative growth and reproductive growth. It is almost as if the muropeptide profiles from vegetative mycelium and spores had been obtained from different organisms. This shows that not only the DAP isometry is different between two stages of development, but the entire PG architecture changes radically. The regulation of incorporation of either *meso*- or LL-DAP is unclear, as is the function of this chemical difference between vegetative mycelium and spores.

As mentioned above, the bacterial cell wall is a very important target for antimicrobial compounds. Vancomycin is a glycopeptide antibiotic, mostly used as a last-resort compound, when infections of Gram-positive bacteria are unresponsive to other antibiotics. Chapter 5 discusses the vancomycin resistance cluster of *S. coelicolor,* which largely resembles resistance clusters as found in vancomycin resistant *Enterococci* (Hong et al., 2004). Vancomycin targets the D-Ala-D-Ala terminus of Lipid II, before this is incorporated into the mature peptidoglycan. Resistance against vancomycin is gained by replacing the D-Ala-D-Ala terminus by D-Ala-D-Lac, performed by a combination of seven genes. A two-component regulatory system, VanR and VanS recognize vancomycin in the environment, VanH produces D-Lactic acid, VanA ligates D-Alanine and D-Lactic acid and VanX is a dipeptidase that cleaves D-Ala-D-Ala. We show that the addition of D-Alanine increases the sensitivity of bacteria to vancomycin due to the bifunctional activity of the D-Alanine-D-Lactate ligase VanA. VanA is in essence a modified version of the D-Alanine-D-Alanine ligase Ddl and VanA is able to ligate D-Ala and D-Ala when this is available in the environment. This effect becomes especially clear in a knock-out mutant of *vanX* with the addition of exogenous D-Ala. When the amount of D-Ala in the environment is high, VanA produces D-Ala-D-Ala and when this is not broken down by VanX, the strain regains sensitivity to vancomycin. This emphasizes that antibiotic resistance mechanisms do not function perfectly, but serve to function good enough as to secure survival. A small molecule screen should facilitate the discovery of drugs that target VanX. If such a drug is found, it may be an efficient enhancer of the bioactivity of vancomycin.

The pentapeptide chain in the peptidoglycan consists of interchanging L- and D-amino acids, of the MurNAc-bound D-Lac, L-Ala, D-Glu, LL-DAP and D-Ala-D-Ala (Vollmer *et al.*, 2008a). This interchange serves to deter unspecific peptidases as regular peptidases only target L-amino acids, and a relatively simple mirror image of a peptidase could target a chain of D-amino acids (Radkov & Moe, 2014). A chain of interchanging L- and D- amino acids requires highly specific endopeptidases, such a phenomenon is also found in lantibiotics (Knerr & van der Donk, 2013). In Chapter 6 we have explored the role of the alanine racemase Alr, which is essential for growth, and mutants can only be produced in the presence of D-Ala. This feature was used to show the *in vitro* bioactivity of the enzyme; *alr* null mutants cannot grow when media are supplemented with L-Ala, but if the amino acid is preincubated with Alr enzyme, the mixtures contained sufficient D-Ala to support growth. This clearly showed the *in vitro* racemase bioactivity of the enzyme, and this enzyme prep was subsequently used to determine its crystal structure. The Alr protein is pyridoxal phosphate-dependent and forms a heterodimer. The crystal structure of the protein is solved both with and without its inhibitor D-cycloserine (DCS), a D-Ala analogue that acts as a sui-

cide substrate for Alr. We expected that the DCS producer *Streptomyces lavendulae* might have significant changes in its Alr homologue, but comparison of the crystal structures of the Alr orthologues of *S. coelicolor* and *S. lavendulae* did not reveal any changes that could directly be related to obvious differences in substrate specificity. We therefore suspect that the D-alanine-D-alanine ligase (Ddl) plays an additional role in DCS resistance, and this idea needs to be worked out further.

OUTLOOK

The work presented in this thesis features different approaches to characterize actinomycetes and their cell wall, from the small scale of protein isolation and characterization, peptidoglycan architecture analysis, to taxonomy and antibiotic resistance mechanisms. Each of the research directions in this thesis leaves questions unanswered. In the case of the verticillate *Streptomyces*, we have identified a different set of SALPS from *Streptomyces* with canonical spores, but we do not know how sporulation along the lateral wall is regulated. If this is resolved, it will deliver important cues as to how sporulation-specific cell division is controlled in time and space. The cell wall analysis of Streptomyces showed which muropeptides are available in a complete culture at different moments in time, but a muropeptide analysis requires an entire culture so we do not yet have spatial knowledge of different PG modifications. Analysis of *Kitasatospora* PG showed that vegetative PG had *meso*-DAP and spore PG LL-DAP. We can only wonder whether the difference in stereochemistry acts as a signal, or whether the stereochemistry is functional at all. The work on vancomycin resistance showed how a deletion of *vanX* increased sensitivity in Vancomycin-resistant strains by a thousand fold, showing major potential for a solution to the high amount of vancomycin resistant infections. Here, the missing component is a chemical inhibitor of the VanX protein. This thesis describes a working fluorescence-based assay to screen for VanX-inhibitors, perhaps this can inspire future scientists to continue the research.

We are currently at a time where genome sequencing becomes commonplace, among others due to ever-decreasing cost, and chemical toolboxes are expanding with new high-throughput methods for cell wall analysis. Imaging is becoming more informative and broadly applicable with fluorescent dyes such as the fluorescent D-amino acid HADA, that binds to PG at the position of D-Ala and allows scientists to localize sites of cell wall remodeling (Kuru *et al*., 2015). This combination of genomics, biochemical tools and biological insights provides scientists with the perfect opportunity to work with understudied organisms and to investigate how the different modes of growth are executed and controlled. Future work might show why *Kitasatosporae* have different stereoisomers of DAP at different developmental stages, how sporulation is regulated in verticillate *Streptomyces*, or how *Streptomyces* know where septal sites in aerial mycelia need to be in order to produce a long chain of equal sized spores. Exiting times are ahead!