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

Aart, L.T. van der

CHAPTER 1

General Introduction

Lizah T. van der Aart & Gilles P. van Wezel

Streptomyces biology

Streptomyces are multicellular, Gram-positive bacteria in the phylum of actinobacteria which produce a high amount of bioactive natural products of which the expression is tightly coordinated with the lifecycle. During growth of *Streptomyces*, cell growth and division are two separate processes. Exponential growth of vegetative hyphae is achieved by apical growth and mycelial branching, driven by DivIVA (Flärdh, 2010). Cell division does not physically separate cells at this stage, instead long hyphae are formed which are separated by occasional cross-walls (Jakimowicz & van Wezel, 2012, Celler *et al.*, 2016). Canonical division occurs during sporulation, which requires all components of the divisome. At this stage, the long aerial hyphae are separated by up to a hundred septa and spores are formed by a highly coordinated process of cell division and DNA segregation (Zhang *et al.*, 2016a). One of the most renowned properties of streptomycetes is that they can growth without cell division, thus uniquely enabling the deletion of the cell division gene *ftsZ* (McCormick *et al.*, 1994). These remarkable properties make streptomycetes a unique model for the study of growth and cell division (McCormick, 2009).

Streptomyces are well-known for the production of a wide arrange of antibiotics, immune suppressants and anti-cancer compounds (Berdy, 2012, Hopwood, 2007, Barka *et al.*, 2016). The production of this high amount of specialized metabolites is commonly attributed to the lifecycle of these bacteria, consisting of growth of an intricate hyphal network, development of reproductive spores and an event of programmed cell death where old mycelia are broken down and become available in the direct environment (Figure 1). The production of secondary metabolites occurs at the onset of morphological differentiation from vegetative to reproductive growth (van der Heul *et al.*, 2018, van Wezel & McDowall, 2011).The hunt for novel antimicrobial compounds has led to the isolation of huge numbers of actinobacteria from soil and aquatic environments, leading to the discovery of more than 600 type strains of streptomycetes (Labeda *et al.*, 2017, Hopwood, 2007). These were then screened for the production of novel bioactive molecules, resulting in many different medicines from actinobacterial sources. This strategy was very successful and supported the discovery of a large amount of secondary metabolites in the past, but the amount of novel antibiotics found by this same process has decreased to a rate that this is no longer feasible (Kolter & van Wezel, 2016). A breakthrough in the field occurred when the first *Streptomyces* genomes were sequenced: it became clear that *Streptomyces* are capable of producing ten times more secondary metabolites than previously discovered (Ikeda *et al.*, 2003, Bentley *et al.*, 2002). This sparked interest in novel approaches, such as ‘awakening’ silent gene clusters (Rutledge & Challis, 2015, Zhu *et al.*, 2014a, Bentley *et al.*, 2002). Research to find novel antimicrobials is mostly driven with the natural environment and life cycle as a starting point. Scientists do not just ask ‘what’ is being produced, but also ‘why’ and ‘when’. Having the biology in mind, researchers have several different approaches: Firstly, competition between bacteria can be mimicked by co-cultivation. For example, when the filamentous fungus *Aspergillus niger* and the filamentous bacteria *Streptomyces coelicolor* are cultured together, the combination of strains produce metabolites neither organism would produce when grown solitary (Wu *et al.*, 2015). This suggests that secondary metabolites can be produced as a signal to predation or to a social cue (van der Meij *et al.*, 2017). Secondly, when strains are grown on different media they behave in a different manner, which is one of the reasons that growth on 7 different media is an essential part of *Streptomyces* taxonomy (Shirling & Gottlieb, 1966).

Rich or poor growth conditions, or the presence of specific eliciting compounds such *N*-acetyl glucosamine, can make all the difference in terms of antibiotic production (Swiatek *et al.*, 2012, Zhu *et al.*, 2014b, Rigali *et al.*, 2008). Thirdly, novel bacteria are increasingly isolated from underexplored and extreme habitats are used in order to look for more novel species with the potential to produce novel secondary metabolites (Bull *et al.*, 2016). This approach has led to the isolation of the new family of *Salinispora* sp. and a large amount of novel secondary metabolites (Ahmed *et al.*, 2013, Ziemert *et al.*, 2014, Maldonado *et al.*, 2005).

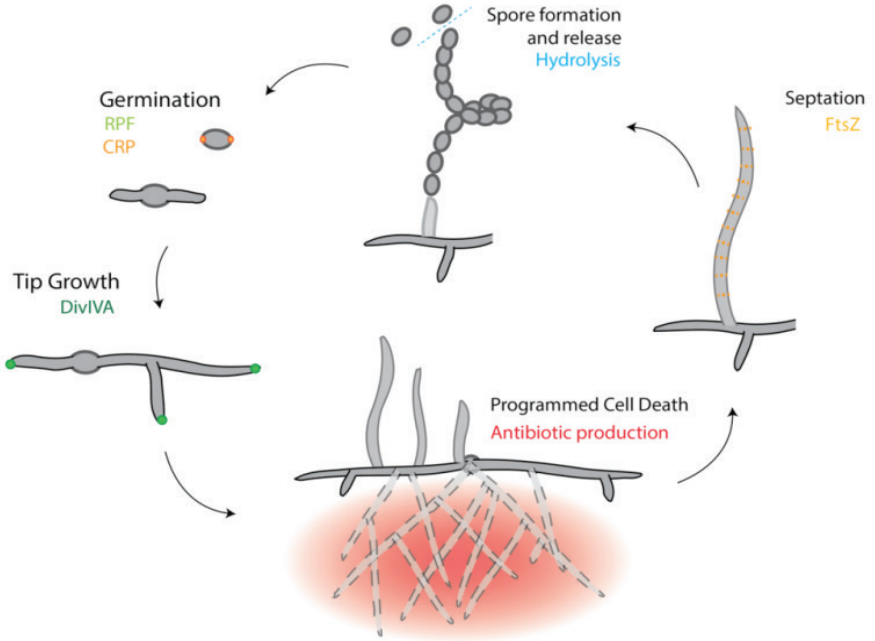

Figure 1. Schematic representation of the *Streptomyces* life cycle. Germination of spores is controlled by the cAMP receptor protein CRP and supported by RPF like proteins and muramidases. Vegetative hyphae then grow by tip growth, driven by DivIVA and eventually form a complex multicellular vegetative mycelium. When nutrients are exhausted, an event of PCD occurs simultaneously with antibiotic production and the growth of aerial hyphae. Spores are formed by simultaneous constriction of hundreds of FtsZ-rings, which are later released in order for life to start anew.

Transcriptional regulation of aerial mycelium formation is due to '*bld*' genes, named after the bald phenotype of a *Streptomyces* without aerial mycelia (Merrick, 1976). The *bld* mutants do not form aerial hypae but are often also disturbed in carbon utilization and antibiotic production, emphasizing the relation between nutrient utilization, antibiotic production and development (Pope *et al.*, 1996). Of the *bld* genes, *bldD* is a global transcription factor that represses most developmental genes during vegetative growth, including *bldN, whiG, sigH* and *ssgB* (den Hengst *et al.*, 2010, Elliot *et al.*, 2001). Especially interesting is the indirect regulation by *bldA* which specifies a leucyl-tRNA for the translation of UUA codons, which is extremely rare in the GC-rich streptomycetes. The BldA tRNA posttranslationally controls genes involved in development and antibiotic production (Lawlor *et al.*, 1987, Leskiw *et al.*, 1991). The *whi* genes are named after the white phenotype of *Streptomyces*

that fail to produce grey-pigmented spores (Chater *et al.*, 1989), and typically encode transcriptional regulators that regulate processes related to the onset of sporulation. WhiG is a sigma factor that controls the early stage of spore formation, with null mutants forming straight aerial hyphae without septa. WhiB controls the onset of sporulation and is the archetypal member of the Wbl (WhiB-like) proteins (Molle *et al.*, 2000). WhiB and WhiA co-control sporulation, and *whiA* and *whiB* mutants have very similar phenotypes, producing long, curly aerial hyphae without spores (Ainsa *et al.*, 2000, Ainsa *et al.*, 1999). Sporulation is further regulated by the SsgA-like proteins (SALPS), which are small proteins (125-142 aa) with an average amino acid similarity of 30-40% between them (Traag & van Wezel, 2008). All sporulating actinobacteria have at least one SALP and the amount of SALPS increases with the complexity of the organism (Keijser *et al.*, 2003). Deletion mutants lacking *ssgA* have sporulation defects, while overexpression of this gene causes an increase of the amount of germ tubes from a spore and a higher branching frequency (van Wezel *et al.*, 2006, van Wezel *et al.*, 2000a).

When nutrients become scarce, the vegetative mycelium undergoes a large event of Programmed Cell Death (PCD), resulting in breakdown of the vegetative or substrate hyphae for the benefit of the growth of aerial hyphae (Tenconi *et al.*, 2018, Manteca *et al.*, 2006, Miguelez *et al.*, 2000). During PCD, a large part of the vegetative cell wall is hydrolyzed in order to reuse cell-wall derived aminosugars, and in particular *N*-acetyl Glucosamine (GlcNAc), or to recycle for *de novo* cell wall synthesis (Urem *et al.*, 2016, Rigali *et al.*, 2008). At the time of PCD, streptomycetes are in a sensitive position when their own vegetative mycelium is partly hydrolyzed, as nutrients are being released at a time of scarcity. This will attract other bacteria via chemotaxis, and the only way for a slow growing bacterium to defend itself is by producing bioactive compounds, and notably antibiotics. Prodigionines, DNA-damaging agents which serve as anti-cancer compounds, are produced as a molecular trigger to eradicate most of the vegetative mycelium and this way provide nutrients for the surviving, reproductive cells (Tenconi *et al.*, 2018). Understanding cell growth and development, especially that of PCD, will greatly contribute to our understanding of antibiotic production and lead to the discovery of novel antibiotics.

The Bacterial Cell Wall

At first, bacterial shape might seem a trivial feature, at most an easy way to distinguish one species from the other. However, cell shape is subject to heavy environmental pressure and each shape requires a different type of regulation from cell wall biosynthetic machineries (Kysela *et al.*, 2016). In Gram-positive bacteria, cell shape is maintained by the peptidoglycan (PG) layer. PG forms a dynamic macromolecule that is actively remodeled to enable cell growth and differentiation through a tightly regulated interplay of hydrolytic and biosynthetic enzymes (Egan *et al.*, 2017). The chemical structure of PG is extremely well conserved, consisting of a glycan chain backbone of interchanging *N*-acetyl glucosamine (GlcNAc) and *N*-acetyl muramic acid (MurNAc). The glycan chain is cross-linked by peptide chains of non-ribosomally synthesized L- and D- amino acids, which in turn are linked to the lactate group of MurNAc. Although the chemical composition is conserved, the structure (degree of cross-linking/glycan strand length) can vary widely, depending on the species, growth phase and environment (Vollmer *et al.*, 2008a). PG architecture research is an important tool for bacterial biologists in finding mechanisms for cell shape determination. This has shown that the helical shape of Helicobacter pylori is maintained

by regulated cross-linking and hydrolysis (Sycuro *et al.*, 2012).

In Gram-positive bacteria, growth can be regulated in two different manners, namely via lateral growth or tip growth, depending on the phylogeny. Well-known representatives are firmictues (lateral growth) and actinobacteria (tip growth). These bacterial families can be discerned based on features such as G+C content of the DNA, lateral growth vs tip growth and the production of endospores as opposed to external spores. Growth and division in firmicutes and actinobacteria is shown in Figure 2. Firmicutes grow by lateral growth: the cytoskeletal protein MreB forms filaments around the lateral wall of the bacteria and supports PG incorporation and cross-linking, elongating the cell (Hussain *et al.*, 2018). In contrast, actinobacteria grow by tip extension where new PG material is built in solely at the tips of rods or hyphae, a mechanism independent of MreB or FtsZ (Flärdh, 2010, Mazza *et al.*, 2006, Flärdh & Buttner, 2009). Tip growth in actinobacteria is driven by DivIVA (Flärdh *et al.*, 2012). This protein localizes at sites with a negative membrane curvature, and recruits the cell wall construction machinery (Hamoen & Errington, 2003). In *Streptomyces*, DivIVA supports the growth of long hyphae. The signal or mechanism by which branching sites are decided is unknown.

In *Bacillus subtilis*, localization of the septum at midcell is controlled by the Min and Noc systems. The Min complex prevents cell division away from mid-cell, while the nucleoid occlusion system Noc ensures that the chromosomes are well segregated prior to septum synthesis, to avoid DNA damage (Errington *et al.*, 2003, Lutkenhaus, 2007, Margolin, 2005, Wu & Errington, 2011). In most unicellular bacteria, FtsZ is tethered to the membrane by FtsA. The FtsZA complex forms filaments, which encircle the cell at the division site referred to as an FtsZ-ring. The FtsZA complex then recruits PG synthases such as PBPs to build a septum that divides the cell in two (Bisson-Filho *et al.*, 2017). In *Streptomyces*, canonical cell division that requires the divisome proteins exclusively occurs in aerial hyphae. Here, FtsZ is actively recruited to septal sites by SsgB in an SsgA-dependent manner (Willemse *et al.*, 2011). The SsgB-FtsZ complex is tethered to the membrane by SepG (Zhang *et al.*, 2016a). Thus, in streptomycetes, cell division is positively controlled, in contrast to the Min-dependent negative control systems found in many other bacteria.

Regulation of both growth and division is fundamentally different in Actinobacteria and Firmicutes, not a single regulatory protein from Firmicutes has the same function in Actinobacteria. Together, this shows that the Actinobacteria are an especially interesting model organism to investigate cell growth and division.

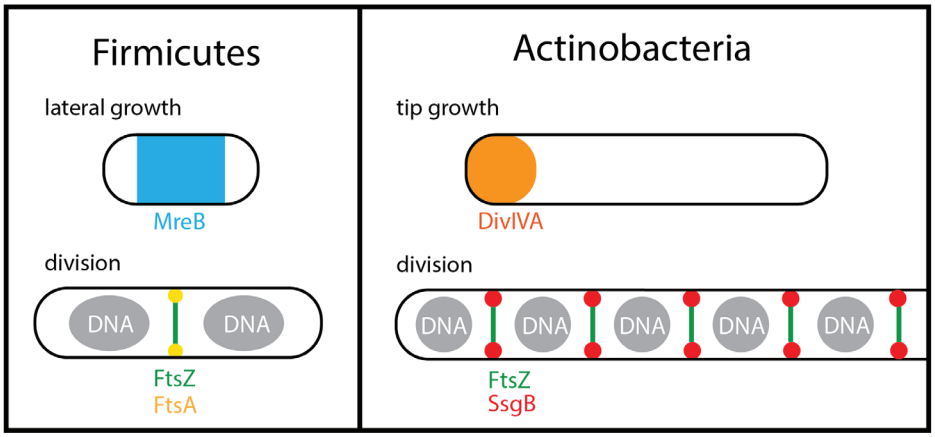

Figure 2. **Growth and division in *Firmicutes* and *Actinobacteria* is regulated differently.** *Firmicutes* elongate along the lateral wall, driven by MreB. Actinobacteria grow at the tip, driven by DivIVA. *Firmicutes* have a single division site in the center of the cell, where the FtsZ/A complex constricts the cell. Actinobacteria can have several division planes in a single cell during spore formation, regulated by positive control of SsgA and SsgB.

Cell wall construction

Bacterial peptidoglycan construction is a highly conserved and regulated process. The amino acids in the pentapeptide chain consist of interchanging L- and D-amino acids. This structure assists in resistance against non-specific peptidases, who would normally cleave chains of L-amino acids (Cava *et al*., 2011). The production of D-amino acids is performed by specialized racemases, which convert L-amino acids into D-Amino acids (Radkov & Moe, 2014). The alanine racemase (Alr) converts L-Ala to D-Ala, an essential part of the peptidoglycan. An *alr* knock-out mutant of *S. coelicolor* causes the strain to become dependent on exogenous D-Ala (Tassoni *et al*., 2017, Walsh, 1989). Lipid II, the building brick of PG, is constructed in the cytoplasms by a set of exceptionally well-conserved Mur-proteins, as shown in Figure 3. Mur-proteins ligate glycans and amino acids in a step-by-step manner, MurA and MurB produce undecaprenol (UDP) MurNAc from UDP-GlcNAc. MurC, MurD and MurE then together ensure the attachment of L-Ala, D-Glu and LL-DAP to the D-lactate group of MurNAc (Smith, 2006). The dipeptide D-Ala-D-Ala is ligated by the D-Ala-D-Ala ligase Ddl, and subsequently attached to the growing peptide chain by MurF. MraY connects the peptidoglycan precursors to the lipid-associated bactoprenyl-phosphate to yield Lipid I. Then MurG attaches GlcNAc and FemX connects Gly to LL-DAP, finishing the Lipid II molecule. The entire structure is then flipped across the lipid membrane by FtsW and/or MurJ. FtsW was shown *in vitro* to have affinity to Lipid II and for flipping activity (Mohammadi *et al*., 2014, Mohammadi *et al*., 2011). But this protein appeared to be inactive during *in vivo* studies. MurJ is an essential membrane protein of which a deletion causes accumulation of Lipid II in the cytoplasm and cell lysis. While *in vitro* data supports FtsW as a flippase, *in vivo* data prefers MurJ (Meeske *et al*., 2015, Sham *et al*., 2014, Mohammadi *et al*., 2011). Finally, Lipid II is displayed on the cell surface and can be incorporated into the mature murein by glycosyltransferases and transpeptidases, among which RodA, PBPs and LDT. Figure 3 features cytoplasmic production of Lipid II in *S. coelicolor*.

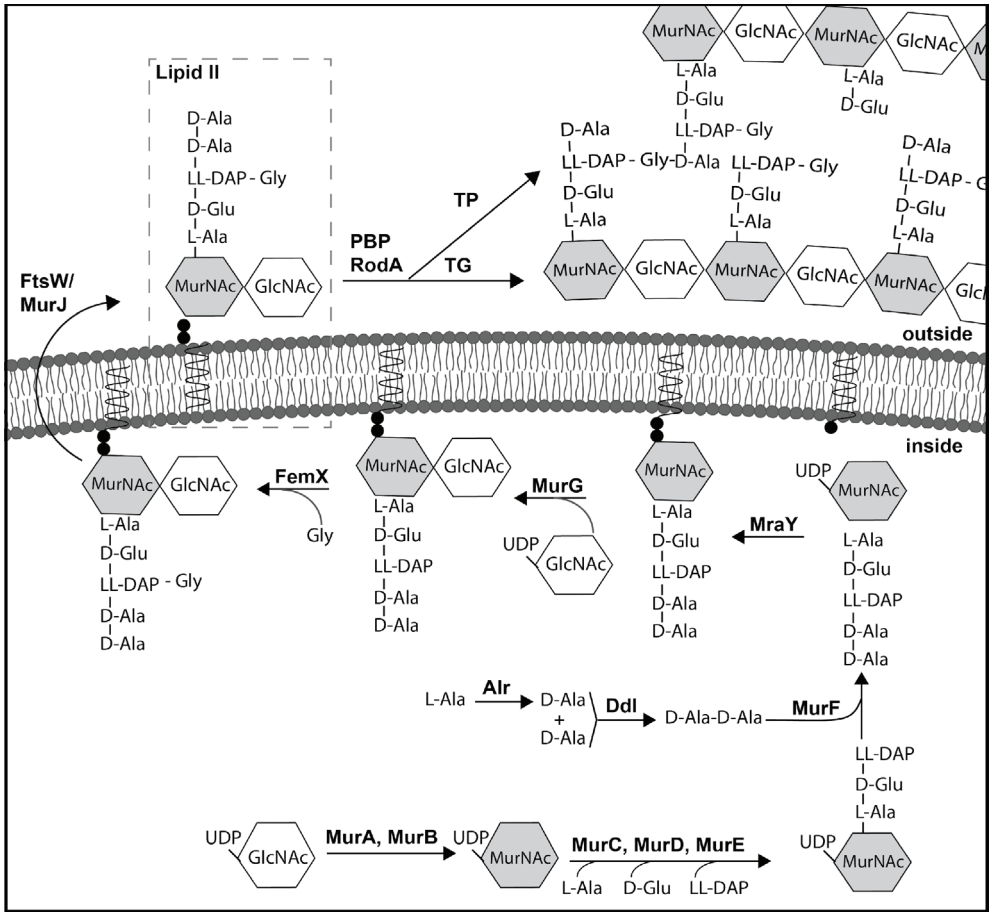

Figure 3. Construction of Lipid II is performed by Mur-genes in the cytoplasm. UDP-GlcNAc is transferred into UDP-MurNAc. To MurNAc, the first three amino acids, L-Ala, D-Glu and LL-DAP are attached by MurC, MurD and MurE, respectively. L-Ala is modified to D-Ala by Alr, after which D-Ala and D-Ala are ligated by Ddl. Then, D-Ala-D-Ala is connected to the growing PG precursor by MurF. Following this, MurY connects the PG precursor to C_{55}-phosphate, and thus connects it to the lipid membrane. MurG ligates UDP-GlcNAc to MurNAc and finally FemX attaches a single Gly to LL-DAP. This structure is flipped over the lipid membrane by either or both FtsW and MurJ. Lipid II is later cross-linked by RodA, LDTs and PBPs.

Peptidoglycan cross-linking

Lipid II construction is highly conserved among bacteria, with minor modifications at the point of the interpeptide bridge (Schleifer & Kandler, 1972). The cross-linking process afterwards is dependent on cell shape and growth mode (Vollmer *et al.*, 2008a). The two major modes of growth, lateral and tip growth, are regulated in a different way and have different mechanistic constraints. During lateral growth, the cell wall expands along the lateral wall of a cell, where new tips are generated only during septation. During tip growth, only the bacterial tip extends. Mechanistically, this means that in laterally growing cells, 'old' cell wall material is located at the tip, whereas in tip growing bacteria the 'old' cell wall material is located along the lateral wall (Kysela *et al.*, 2016). Bacteria that grow at the apex only generate novel peptidoglycan at the spherical tip and require remodeling along the wall of the hyphae to generate a cylindrical cell wall. A lack of remodeling enzymes leads to a bloated, round cells (Baranowski *et al.*, 2018). Here, we discuss PG cross-linking in tip growing bacteria.

After Lipid II translocation over the cell membrane, the new building blocks are incorporated into glycan strands, which are connected to the growing murein by proteins with glycosyltransferase (GT) activity, such as RodA and bifunctional Penicillin Binding Proteins (PBPs) (Emami *et al.*, 2017, Meeske *et al.*, 2016, Leclercq *et al.*, 2017). The peptide chain is cross-linked by transpeptidase (TP) activity by PBPs and L, D-transpeptidases (LDTs). PBPs have been discovered early on due to their ability to bind (and hence sensitivity to) penicillin, and only later it became clear that these proteins are mostly responsible for cell wall construction. Class A PBPs have bifunctional activity as both GT and TP, class B PBPs only have TP activity. PBPs form a 3-4 cross-link, conveniently named for creating a link between the 3rd and 4th amino acid in the pentapeptide chain of muropeptides (Daniel *et al.*, 2000). PBPs require at least one pentapeptide as a donor strand to form a cross-link, this donor strand loses the terminal D-Ala[5] and PBPs cross-link two muropeptides via the Gly cross-bridge of the acceptor strand. The requirement for pentapeptides demands that PBPs are present at the site of active peptidoglycan construction (Egan *et al.*, 2015).

PBPs are mostly responsible for PG cross-linking in firmicutes and bacteria with lateral cell wall growth. LDTs are penicillin-insensitive proteins that are able to replace a part of the function of PBP's by cross-linking two peptide chains at LL-DAP[3] and LL-DAP[3], creating a 3-3 cross-link (Mainardi *et al.*, 2005, Mainardi *et al.*, 2002). 3-3 cross-links are especially prevalent in micro-organisms that grow at the apex and have been linked to penicillin-insensitivity. Many actinobacteria contain a high amount of 3-3 cross links, such as *Mycobacterium* (30-80%), *Corynebacterium* (38%) and *Streptomyces* (30-60%) (Lavollay *et al.*, 2011, Hugonnet *et al.*, 2014, Lavollay *et al.*, 2009, van der Aart *et al.*, 2018). In contrast, bacteria with lateral cell wall growth like *E.coli* (>10%) and *E.faecium* (>3%) have a much lower amount of 3-3 cross-links (Pisabarro *et al.*, 1985, Sacco *et al.*, 2010). LDTs attach to tetrapeptides and form a cross-link between glycine and LL-DAP[3]. Here, the D-Ala[4] is considered a donor for the cross-link. The requirement of tetrapeptides in contrast to pentapeptides allow LDTs to remodel the cell wall after in the post-apical area. In mycobacteria, 3-4 cross-linking by PBPs occurs solely at the tips, at the site of peptidoglycan construction. Along the lateral wall, where the peptidoglycan is more highly modified and has a higher amount of tetrapeptides, LDTs produce 3-3 cross-links to remodel the peptidoglycan (Figure 4) (Baranowski *et al.*, 2018).

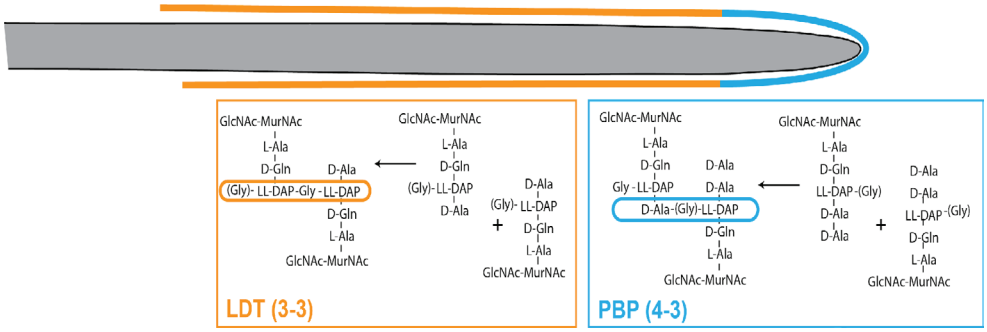

Figure 4. Peptidoglycan cross-linking at the tip and the lateral wall. In *Streptomyces*, cell wall construction takes place at the tips, with cell wall modifications along the lateral wall. At the tip, there is a high abundance of pentapeptides, the necessary donors for PBP's to form a 4-3 cross-link. Further from the tip complex, pentapeptides are modified by D,D-carboxypeptidases and PBP's, presenting a higher amount of tetrapeptides on the cell surface. At this point, LDT's modify the peptidoglycan by forming 3-3 cross-links, using tetrapeptides as a donor muropeptide.

Programmed Cell Death and cell wall hydrolysis

Over the course of growth, new peptidoglycan is continuously generated and broken down. Cell wall hydrolysis has several roles in the life of *Streptomyces*: hydrolyzing PG to allow incorporation of new glycan strands, hydrolyzing the cell wall material between mature spores to allow single spores to disperse, breaking down the thick spore wall during germination and the developmental step of PCD (Haiser *et al.*, 2009). The function of different modifying proteins is summarized in Figure 5. The *Streptomyces coelicolor* genome contains a high amount of hydrolysis-associated genes, consisting of 60 different genes encoding the four functional groups of amidases, carboxypeptidases, endopeptidases and *N*-acetylglucosaminidases (Haiser *et al.*, 2009). The large number of cell-wall modifying enzymes suggests a high degree of redundancy, in line with the multi-stage lifecycle of *Streptomyces*.

Amidases hydrolyze the lactyl bond between MurNAc and L-Ala, these enzymes are found to be essential for cell separation, as a knockdown of amidases or FtsE/FtsX will generate filaments in *E. coli* (Uehara *et al.*, 2010, Heidrich *et al.*, 2001). FtsE and FtsX are suggested to act as ATP-binding cassette transporters (ABC transport proteins), which transport amidases across the membrane (Yang *et al.*, 2011a). In *E.coli*, FtsE and FtsX function in association with FtsZ, in order to separate daughter cells after septum formation. An *ftsX* knockout mutant in *Streptomyces* still showed peptidoglycan linkage between spores, suggesting incomplete hydrolysis of the peptidoglycan (Noens, 2007). The *S. coelicolor* peptidoglycan contains a large amount of dimers which have a cross-linked set of amino acids but has lost one set of glycans, reflecting high activity of PG amidases (van der Aart *et al.*, 2018). This effect was especially pronounced in vegetative cells grown in liquid media. As pellets grow and develop, the shift from exponential to stationary growth occurs with a round of PCD, during which a large part of the cell wall in the centre is hydrolyzed (Manteca *et al.*, 2008).

Carboxypeptidases cleave the terminal amino acid from muropeptides, D,D-carboxypeptidases cleave the 5th D-Ala from a pentapeptide, while L,D-carboxypeptidases cleave the 4th D-Ala from a tetrapeptide. D,D-carboxypeptidases play a key role in spore wall maturation in *S. coelicolor* (Rioseras *et al.*, 2016), while the spore

cell walls mostly consist of tetrapeptides that lack a terminal D-Ala (van der Aart *et al.*, 2018). The product of D,D-carboxypeptidases, namely tetrapeptides, are required as donor strands for 3-3 cross links by L,D-transpeptidases. In *Streptomyces*, the role of D,D-carboxypeptidases is partly associated with 3-3 cross-linking.

Lysozyme-like proteins, muramidases and glucosamidases cleave the glycan strands between GlcNAc and MurNAc. In bacteria, muramidase or glycosamidase activity is especially important during the initiation of spore germination. In actinobacteria, 'resuscitation promoting factors' (RPFs) play a major role in spore germination and resuscitation of dormant cells (Keep *et al.*, 2006). RPFs were first described as a growth factor for non-culturable *Micrococcus luteus,* where minute amounts of protein can resuscitate dormant cells (Mukamolova et al., 2002). Later it was shown that RPFs bind to the peptidoglycan and locally act as muralytic enzymes, similar to lysozyme (Mukamolova *et al.*, 2006). Interestingly, RPFs can also resuscitate dormant cells of mycobacteria (Nikitushkin et al., 2013), which shows their potential as target for drug development, particularly because dormant cells are a major problem in terms of drug treatment. In correspondence with this data, RPFs contribute to germination of *Streptomyces* spores, which can also be considered to be in a state of dormancy (Sexton *et al.*, 2015). RpfA and another muramidase, SCO5466, are controlled by the cAMP-receptor regulon (Piette *et al.*, 2005). In *Streptomyces*, cAMP accumulates during germination and binds to the cAMP-receptor, an important transcriptional regulator (Susstrunk *et al.*, 1998). Mutants lacking the cAMP receptor protein CRP have a much thicker spore wall, suggesting that control mechanisms for spore wall synthesis are impaired (Piette *et al.*, 2005). As a result, it takes much longer for spores to germinate. The germination defect of the *crp* null mutant is explained by the hydrolases encompassed by the *crp*-regulon, among which SCO5466 and RpfA (Piette *et al.*, 2005).

Cell wall biosynthesis and hydrolysis are two inseparable processes, as suggested by the 'make before break' model, first suggested by Koch and Holtje (Höltje, 1993, Koch, 1990). This model, also called the 3-for-1 model, proposes that trimers replace monomers, suggested that after a connection is made, old cell wall material has to be hydrolyzed to allow for expansion of the glycan strand.

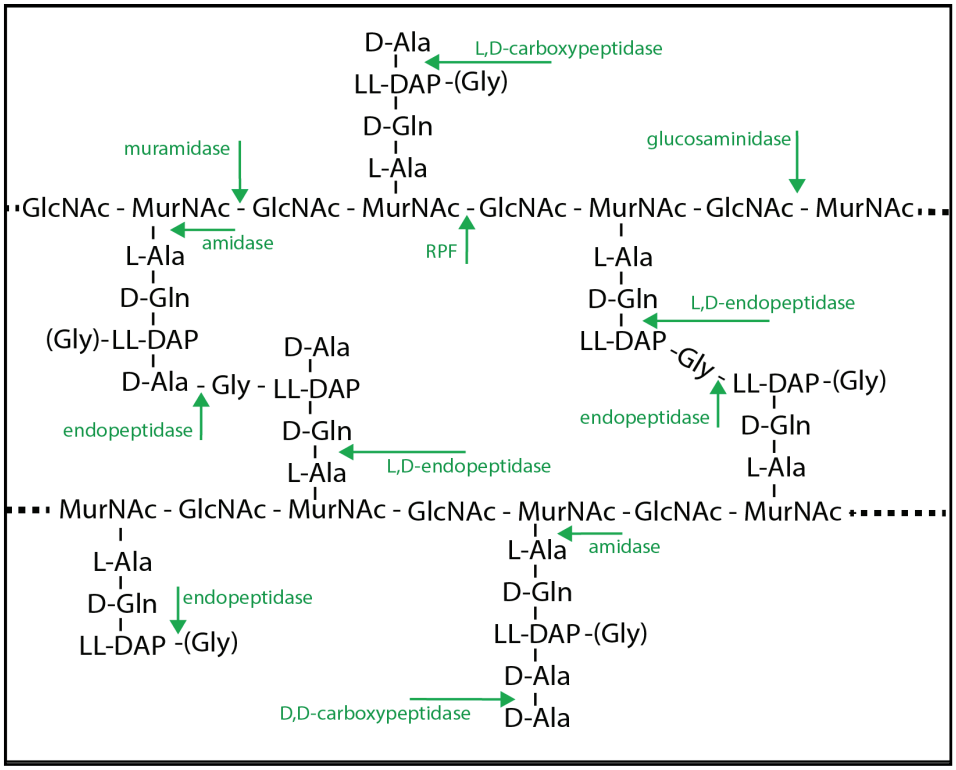

Figure 5. Hydrolysis of the *Streptomyces* peptidoglycan. The peptidoglycan structure of S. coelicolor is shown, with LL-DAP at position 3 and Gly which forms the cross-link between two peptide chains. N-acetylglucosaminidases (glucosaminidases) cleave between GlcNAc and MurNAc, N-acetylmuramidases (muramidases) cleave between MurNAc and GlcNAc. RPFs have a muramidase activity. N-acetylmuramyl-L-alanine amidases (amidases) hydrolyze at the site of the lactyl group between MurNAc and L-Ala. L,D-endopeptidases cleave the site between an L-, and D-amino acid in the peptide chain. D,D-, and L,D-carboxypeptidases remove C-terminal D- or L-amino acids. The acetyl group of MurNAc can be removed by a deacetylase, to generate MurN.

The bacterial cell wall and antibiotic resistance

The bacterial cell wall is a favourite target for antibiotics, as it is highly conserved between bacteria, it is conveniently located on the outside of the cell and is essential for growth and the maintenance of turgor pressure. A major advantage for clinical application is that cell wall-targeting antibiotics generally have no cytotoxicity as human cells lack peptidoglycan, and more than 50% of our clinical antibiotics target the bacterial cell wall and cell wall synthesis (Schneider & Sahl, 2010). The PG precursor Lipid II is a highly conserved structure; prior to cross-linking to the mature PG, the lipid tail is lost and can be recycled (Scheffers & Tol, 2015). Due to the essential nature of Lipid II, it is difficult for bacteria to generate resistance against cell wall and Lipid II targeting antibiotics.

In order to prevent antibiotic production from becoming a suicide mission, actinomycetes carry many antibiotic resistance genes, in addition to potential to produce antibiotics (Marshall *et al.*, 1998). Resistance mechanisms found in actinobacteria are also found in clinical isolates, suggesting that genes carrying antibiotic resistance mechanisms readily travel across the bacterial realm, driven by an increase of fitness for the recipient (Perry & Wright, 2013). Here, we focus on (self) resistance against three cell wall antibiotics, vancomycin, D-cycloserine (DCS) and penicillin, and we discuss two new cell wall antibiotics of which resistance mechanisms are still largely unknown, daptomycin and teixobactin.

Vancomycin

The glycopeptide antibiotic vancomycin targets the D-Ala-D-Ala terminus of Lipid II as it is exposed on the cell surface, this way preventing it from being incorporated into the mature peptidoglycan. Notably, vancomycin is incapable of passing the outer membrane of Gram-negative bacteria and only targets Gram-positives. Vancomycin and the related drug Balhimycin, are both produced by *Amycolatopsis orientalis* and *Amycolatopsis balhimicina*, respectively, and both these species are resistant against the glycopeptide antibiotic they produce (Marshall *et al.*, 1998, Schaberle *et al.*, 2011). Vancomycin-resistant bacteria replace the terminal D-Ala-D-Ala by D-Ala-D-Lac in order to decrease vancomycin susceptibility by a thousand-fold. Vancomycin resistance is gained by a conserved set of genes of which the most important ones are: the two-component regulatory system consisting of VanR and VanS; VanH, which converts pyruvate into D-Lac; VanA, a D-Ala-D-Lac ligase; VanX, a dipeptidase which cleaves D-Ala-D-Ala and VanY, a carboxypeptidase which cleaves D-Ala from a pentapeptide precursor (Hong *et al.*, 2004, Arthur *et al.*, 1994). These vancomycin resistance genes occur in the producing bacteria and in clinical isolated of Enterococcus, posing a large problem for the medical healthcare (Decousser *et al.*, 2003, Courvalin, 2006).

D-cycloserine

D-cycloserine (DCS) is a cyclic analogue of D-Ala produced by *Streptomyces lavendulae* (Noda et al., 2004a). This antibiotic binds to proteins which would otherwise bind to D-Ala, such as Alr and Ddl, and this way prevents the formation of a functional peptidoglycan layer. In order for the producing bacteria to become resistant against the antibiotic it produces, it has modified both Alr and Ddl to decrease affinity to DCS (Noda *et al.*, 2004b). This modification is different from the resistance mechanism in Mycobacteria, which heavily upregulates Alr in order to gain resistance (Caceres *et al.*, 1997).

Penicillin

Penicillin structurally resembles the D-Ala-D-Ala terminus of Lipid II, the recognition site of PBPs, and in this way functions as a suicide substrate for PBP's (Park & Strominger, 1957). As a general rule, penicillin resistance is not gained by changing the function of PBPs, but by producing β-lactamases, which readily break down β-lactam antibiotics like penicillins and cephalosporins (Ogawara *et al.*, 1999). The life of β-lactam antibiotics in the clinic has been extended by the discovery of clavulanic acid, produced by *Streptomyces clavuligerus* (Reading & Cole, 1977). However, PBPs are not the only proteins with GT and TP activity. It has recently been demonstrated that RodA is a penicillin-insensitive glycosyltransferase, of which the overexpression can relieve the absence of Class A PBPs (Emami *et al.*, 2017, Meeske *et al.*, 2016). While PBPs are the major TPs for laterally expanding bacteria, polar growing bacteria rely on LDTs for 30-70% of the cross-links (Lavollay *et al.*, 2008). LDTs in *Streptomyces* recognize the terminus of a tetrapeptide, consisting of LL-DAP and D-Ala, and are insensitive to Penicillin. Structural studies of LDTs have shown that carbapenem antibiotics, originally developed for Gram-negative infections, readily targets LDTs and stay functional in combination with β-lactamase activity (Iannazzo *et al.*, 2016). Potentially, carbapenems could be optimized to target only LDTs, this way producing an antibiotic which specifically targets mycobacteria. It remains open for discussion whether LDTs are developed to support tip growth, or to support antibiotic resistance (Mainardi *et al.*, 2008).

Teixobactin

Recently, the novel cell wall antibiotic Teixobactin has been discovered that uniquely binds to two different targets in the cell wall, which strongly reduces the chance of resistance development (Ling *et al.*, 2015). Teixobactin is a Lipid II targeting antibiotic isolated from a Gram-negative soil bacterium *Elefteria terrae*, which binds to the pyrophosphate-sugar moiety of undecaprenyl-bound cell wall precursors Lipid II and Lipid III. Lipid III is a precursor for teichoic acids, and this dual activity against PG and teichoic acid synthesis, combined with the fact that it does not bind to mature murein, makes this an especially interesting antibiotic (Homma *et al.*, 2016). Teixobactin is active against several Gram-positive bacteria including mycobacteria and staphylococci. Initial studies by the author showed no sign of development of resistance mechanisms by serial passaging of *S. aureus* with the compound, and the mechanism of self-resistance mechanism in the producing strain is also unknown. It is worth noting that vancomycin resistance in *Staphylococci* was also considered negligible during initial studies on vancomycin resistance in 1981 (Griffith, 1981). Vancomycin resistant enterococci were isolated from clinical infections in 1988 (Leclercq *et al.*, 1988).

Daptomycin

Daptomycin is a membrane-targeting lipopeptide antibiotic with activity against Gram-positive bacteria and is clinically important to ward multidrug resistant infections, especially methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant enterococci (VRE). Daptomycin is produced by *Streptomyces roseosporus,* and like for teixobactin, the self-resistance mechanism is yet unknown (McHenney *et al.*, 1998). Daptomycin functions by depolarizing the lipid membrane (Taylor & Palmer, 2016). Resistance against daptomycin is unusual in clinical settings, but is thought to relate to the charge or fluidity of the lipid membrane, or an

increase in the amount of teichoic acids, which prevent daptomycin from accessing the cell membrane (Gomez Casanova *et al.*, 2017).

Bacteria have been producing antibiotics for half a billion years, and resistance mechanisms co-evolved. This is emphasized by a recent expedition to a cave in Mexico, which had been sealed for 4 million years. Researchers had sampled the microbial community in this cave and encountered bacteria which showed resistance against many different clinical antibiotics, of which some species showed resistance against 14 antibiotics in total (Bhullar *et al.*, 2012). As we isolate many more different species of bacteria and collect more full genome sequences every day, we expect to find novel antibiotics and also resistance mechanisms in for example rare actinobacteria,. Antibiotic resistance research will continue to show us amazing features of bacterial versatility.

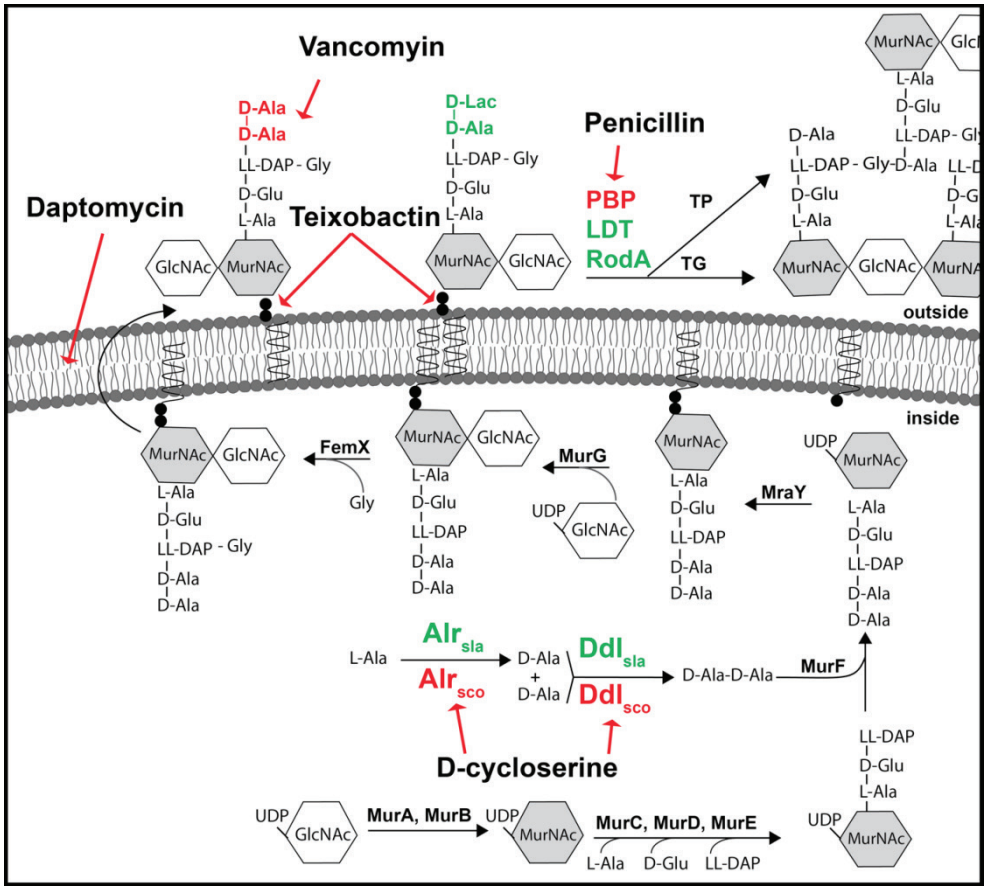

Figure 6. Summary of cell wall antibiotics and their targets. Vancomycin targets the D-Ala-D-Ala terminus of Lipid II. Vancomycin resistant strains mostly replace the terminal D-Ala by D-Lac to relieve vancomycin sensitivity. Penicillin targets PBPs, sensitivity can partially be decreased by upregulation of LDTs and RodA. D-cycloserine targets proteins with a D-Ala binding site, Alr and Ddl. Daptomycin functions by destabilizing the plasma membrane. Teixobactin targets the pyrophosphate-sugar moiety of Lipid II, both vancomycin resistant and vancomycin sensitive.

Outline of the thesis

Streptomycetes are especially interesting bacteria due to their complex morphology and production of secondary metabolites, which has led to the isolation and characterization of more than 600 type strains. Chapter 2 discusses the characterization and taxonomic classification of the novel species *Streptomyces roseofaciens*. This species of *Streptomyces* has an unusual mode of growth, producing spores perpendicular to the aerial mycelia instead of septating the aerial hyphae into long spore chains. The phenotype could be correlated to a different household of SsgA-like proteins, a set of actinobacteria-specific proteins which regulate growth and sporulation.

The complexity of the *Streptomyces* life cycle is astonishing and the consequences for cell wall synthesis are yet unclear. In Chapter 3, the peptidoglycan layer of *S. coelicolor* is analyzed over the course of growth and development. Spores were isolated separately from vegetative mycelium, so as to look for specific changes which could hint towards a germination mechanism. A high-resolution peptidoglycan analysis of *Streptomyces coelicolor* was performed by LC-MS, allowing for easy identification of all muropeptides despite potentially overlapping retention times. The analysis shows that the peptidoglycan is continuously modified and heavily broken down during PCD. An analysis of developmental mutants, Δ*bldD* which only produces vegetative mycelium and Δ*whiG* which only produces aerial hyphae, emphasizes the function of a muropeptide which was specifically abundant in spores.

Streptomyces and *Kitasatospora* are sister genera within the *Streptomycetaceae*. The genera are morphologically very similar, but can be readily distinguished based on their peptidoglycan composition. The stereoisomers of DAP, LL-DAP and MESO-DAP are a key diagnostic feature in the phylogeny of Actinobacteria, and while members of the genus *Streptomyces* carry LL-DAP, *Kitasatosporae* carry MESO-DAP in vegetative mycelia and LL-DAP in their spore walls. Chapter 4 shows an in-depth peptidoglycan analysis of two species of *Kitasatospora*, *K. setae* and *K. viridifaciens*, to lay a connection between taxonomy and cell wall homeostasis.

Antibiotic resistance is part and parcel of the ability of bacteria to produce antibacterial compounds. *S. coelicolor* carries inducible resistance genes that mediate target resistance against vancomycin, an antibiotic produces by another species of actinomycetes. When vancomycin production is initiated, the molecule is sensed by the producer strain, and resistance is switched on. That results in to the production of an altered version of Lipid II with D-Ala-D-Lac as a terminus, resulting in a resistant cell wall, contrary to when the wild-type molecule with a D-Ala-D-Ala terminus is built in. In Chapter 5, we investigated whether vancomycin resistance can be counteracted via metabolic interference with exogenous D-Ala. The experiments revealed a possible novel approach by sensitizing cells with D-Ala in the presence of a VanX inhibitor. Indeed, *vanX* null mutants had a thousand-fold lower MIC against vancomycin in the presence of D-Ala than with L-Ala.

D-amino acids are an essential part of the peptidoglycan, and the D-Ala-D-Ala terminus is a universal part of bacterial peptidoglycan, with the exception being vancomycin resistant bacteria. In Chapter 6 we biochemically and structurally characterized the alanine racemase of *S. coelicolor*. The data in this chapter show that the lack of Alr makes a strain dependent on D-Ala for growth. The crystal structure of the enzyme was resolved at a 2.8 Å resolution, and compared to other Alr enzymes, including that of a strain that is resistant to the Alr-targeting antibiotic D-cycloserine.

The implications of the work for *Streptomyces* biology are discussed in Chapter 7.