# Identification and characterization of developmental genes in streptomyces

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# VII

**Summary and Discussion**
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## SUMMARY AND DISCUSSION

Cell division in *Streptomyces* differs a lot from the situation in most bacteria, which is due to the mycelial instead of planktonic life style. Firstly, two different types of cell division occur: cytokinesis and cell fission only occur during sporulation, while in the vegetative stage cell division results in the formation of cross walls which dissect the hyphae into large compartments (for an overview see Chapter I). Therefore, streptomycetes are multicellular, which is again in contrast to most bacteria (Claessen *et al.*, 2014). In fact, the concept of multicellularity was first mentioned only some 25 years ago (Shapiro, 1988). Secondly, the way cell division is controlled is also very different. In the model organisms *E. coli* and *B. subtilis*, cell division is primarily negatively controlled, aiming at preventing cell division at places away from midcell, and avoiding damage to the nucleoid. In other words, cell division is controlled such that the septum is formed only at the middle of the cell and when the DNA is duplicated and safely out of the way. This immediately highlights a major conceptual difference, as the long hyphae of streptomycetes do not have a clear midcell position. Instead, during *Streptomyces* sporulation, the actinomycete-specific protein SsgA marks future septation sites and somehow ensures the localization of SsgB to the septum sites, which then in turn recruits the cell division scaffold protein FtsZ (Willemse *et al.*, 2011); subsequently FtsZ then forms a characteristic pattern that is seen as spectacular ladders in the sporogenic hyphae (Schwedock *et al.*, 1997). During cell division, SsgB remains at the septum and keeps interacting with and stabilizing FtsZ, forming the same ladder-like pattern. Thus, SsgB is a unique example of a divisome component that localizes prior to FtsZ.

All data suggest that SsgA is mainly responsible for the initial activation of cell division, and indeed overexpression of the protein during early growth results in what can only be described as metamorphosis of the vegetative hyphae, which then resemble sporogenic aerial hyphae and even at times sporulate (notably in liquid-grown cultures; (van Wezel *et al.*, 2000b; van Wezel *et al.*, 2000a)). However, many of the components required for proper sporulation are missing - including the sporulation-specific SsgB protein - and the process is at best erratic. SsgA-like proteins (SALPs) do not show significant sequence similarity to other proteins in the databases to give away clues as to how they may function, but structural analysis revealed strong structural similarity of SsgB to mitochondrial guide RNA binding proteins (Xu *et al.*, 2009). However, interaction with FtsZ, the structural model and experimental evidence suggest that SsgB does not interact with nucleic acids. Instead, it lo-

calizes close to the membrane.

At the start of this PhD work, major questions we sought to address were, how does SsgB interact with the membrane, and what other actinomycete-specific proteins are there to control the localization of the septum in time and space in aerial hyphae? And how is damage to the chromosomes prevented during synchronous multiple cell division in multinucleoid hyphae? One obvious place to look is in the *dcw* cluster which contains primarily genes related to cell wall synthesis and cell division. The function of several of the genes between *ftsZ* (SCO2082) and *divIVA* (SCO2077) was unknown, despite the fact that for example *ylmD* and *ylmE* are downstream of - and most likely in operon with *ftsZ* in many Gram positive bacteria, including *Streptomyces*. All four genes in this region, namely *ylmD, ylmE, sepF* and *ylmG* (SCO2081-SCO2078), were therefore analyzed in more detail.

YlmG is a relatively small 95 aa protein with transmembrane domains at the N- and C-terminus. Deletion of *sepG* caused largely delayed and also impaired sporulation, pointing out its role in sporulation-specific cell division (Chapter II). Due to its clear role in the control of septum formation, *ylmG* was renamed *sepG*. Strikingly, in the absence of *sepG*, SsgB localized temporarily in foci, with foci formed in a 'flash'-like manner, similarly to SsgA (Joost Willemse & Gilles van Wezel, unpublished data). This suggests that the initial SsgB localization can occur in the absence of SepG, but that it failed to localize to septum sites. FRET imaging data showed direct interaction between SepG and SsgB, which strongly suggests a model whereby SepG forms the membrane anchor for SsgB to ensure the localization of SsgB to future septum sites. Therefore, SepG was identified as a new member of the positive cell division control system in *Streptomyces*. However, SepG did not remain at the septum and is therefore not a component of the divisome. Instead, the protein follows the spore-wall synthetic machinery. There it most likely plays a second role, namely in ensuring that the nucleoid is compacted to avoid DNA damage by spore wall (and septum) synthesis. Indeed, during spore maturation, SepG forms a ring structure close to the spore wall, enveloping the chromosomes. Conversely, deletion of *sepG* led to doughnut-shaped chromosomes, suggesting that SepG plays a role in nucleoid compaction during sporulation. The precise role of SepG in a nucleoid-occlusion-like mechanism requires further investigation.

SepF, encoded by the gene upstream of *sepG*, was previously shown to tether the Z-ring to the membrane in *B. subtilis* and promote FtsZ protofilament formation (Hamoen *et al.*, 2006; Ishikawa *et al.*, 2006). Like in *B. subtilis*, in *S. coelicolor* SepF also localized to septum sites and formed the septal ladder-like

pattern typical of *Streptomyces* divisome proteins (Chapter III). We failed to delete the *sepF* gene, despite many different attempts, but work by the group of Joe McCormick showed that in their genetic background such mutants were viable, displaying a sporulation-deficient phenotype very similar to that of *ftsZ* null mutants (Joe McCormick, unpublished data). This suggests that SepF is required for FtsZ localization but also its polymerization, as the phenotype is more destructive than that of *ssgB* mutants (which still form occasional septa). In other words, while SsgB recruits FtsZ and also stimulates FtsZ filament formation (Willemse *et al.*, 2011), SepF is essential for FtsZ polymerization.

Besides *sepF* itself, *Streptomyces* have two *sepF*-like genes, SCO1749 and SCO5967, encoding SflA and SflB (for Sep̲F̲-l̲ike proteins), respectively. Our study showed that SflA and SflB play different roles than SepF, with deletion of *sflAB* leading to extensive branching of the spore chains. Conversely, over-expression of SflA or SflB largely blocked aerial hyphae formation, suggesting they act as negative regulators of *Streptomyces* development. The data can perhaps be reconciled by a repressing effect on apical growth of the aerial hyphae: deletion then results in more tips, while overexpression reduces tip growth altogether. Furthermore, the colonies of *sflA-* or *sflB*-overexpressing strains lost the ability to adhere to the agar surface, again suggesting an effect on hyphal branching, albeit in this case of the vegetative hyphae (no aerial hyphae were formed by these colonies). It should thereby be noted that *divIVA*, which is essential for tip growth of vegetative hyphae (Flärdh, 2003a), lies only two genes downstream of *sepF*. Taken together, our data suggest that SflA and SflB control branching of *Streptomyces* hyphae. Still, we cannot rule out that these effects are mediated directly or indirectly via the control of SepF. SflB directly interacts with SepF in two-hybrid studies, and surprisingly localized in a ladder-like pattern except close to the cell wall, where septum formation is initiated. This implies that SflB and SepF form heterocomplexes, aimed perhaps at inhibition of SepF function. Additionally, SflA localizes along the lateral membrane of the aerial hyphae. More detailed imaging analysis is required, but a model wherein SflA and SflB together inhibit premature polymerization of SepF at all sites other than the site of septum initiation - and therefore inhibit premature division - is feasible. That would imply the existence of a negative control system besides the SsgAB-mediated positive control. Finally, the roles of SflAB on both division and branching may be explained by a single activity if SepF controls the localization and/or function of DivIVA. However, no data are currently available to support this highly speculative concept.

Once the septum is formed by the divisome, which is highly conserved also in *Streptomyces* (Flärdh and van Wezel, 2003; Jakimowicz and van Wezel,

2012; McCormick, 2009), sporulation progresses and eventually a thick spore wall is produced. Peptidoglycan synthesis includes several stages (recently reviewed in (Pinho *et al.*, 2013)): the biosynthesis of the cell-wall precursors UDP-*N*-acetylmuramic acid (UDP-MurNAc) and UDP-*N*-acetylglucosamine (UDP-GlcNAc); addition of the pentapeptide chain to UDP-MurNAc to produce UDP-MurNAc-pentapeptide; coupling of MurNAc-pentapeptide to bactoprenol to form lipid I followed by further addition of GlcNAc to form lipid II; and transport over the cytoplasmic membrane by FtsW (Mohammadi *et al.*, 2011) followed by polymerization through cross-bridges to form mature peptidoglycan. In terms of peptidoglycan (PG) synthesis, a challenging issue for streptomycetes is to ensure the timely accumulation of large amounts of PG precursors that are required for septum and spore-wall synthesis. Considering the amount of PG precursors required, synthesis most likely has to occur *in situ* in the aerial hyphae rather than through long-distance transport. Our data suggest that YlmD and YlmE, which show similarity to alanine racemase and laccase, respectively, plays a role in such precursor supply (Chapter IV). D-Ala-D-Ala produced by the enzyme D-Ala-D-Ala ligase (Ddl) is the final unit of the pentapeptide chain of UDP-MurNAc-pentapeptide and the racemase domain in YlmE suggested a possible role in precursor synthesis. However, YlmE failed to show alanine racemase activity *in vitro* and could not take over the function of alanine racemase (Alr) during vegetative growth *in vivo*, suggesting that it either has a different function or requires a co-factor for function. Still, deletion of either *ylmD* or *ylmE* resulted in a sporulation defect, with defective and mislocalized peptidoglycan and cell membrane synthesis in *ylmD* and in particular in *ylmE* mutants during sporulation. Furthermore, higher concentrations of D-Ala partially restored sporulation to *ylmE* mutants, and the sensitivity of *ylmE* mutant cells to D-cycloserine increased. Earlier research showed similar hypersensitivity to DCS in *alr* mutants (Caceres *et al.*, 1997; Noda *et al.*, 2004; Peteroy *et al.*, 2000). Therefore, the sporulation defect of *ylmE* mutants may indeed be attributed at least in part to the lack of D-Ala precursor supply.

Another cell division-related gene cluster was discovered by genome sequencing of a hypersporulating mutant of *Streptomyces griseus*, whereby SNP analysis identified a mutation that changed the 7th codon of *lcmA* to a stop codon, thereby affecting the entire *lcmABC* operon (Chapter V). The orthologs in *S. coelicolor* are SCO1385-SCO1387, and suggestively, those in *B. subtilis* lie immediately adjacent to *divIB* in the *dcw* cluster. Mutation or deletion of *lcm* genes, which all encode membrane proteins, led to production of thin-walled spores with increased sensitivity to heat treatment, especially in mutants with

either the nonsense mutation *lcmA\** or lacking the entire *lcmABC* cluster. The mutants also showed accelerated growth and development on solid media, perhaps due to the fact that the thinner spore-wall allows faster germination. Such a correlation between spore-wall thickness and germination had been seen previously for *crp* mutants (Piette *et al.*, 2005). LcmA, LcmB and LcmC all localize to the septum at sites of septum closure, and mutants frequently produce unfinished septa. This is consistent with a function of the Lcm proteins in the last stages of spore maturation. Interestingly, the phylogenetic linkage of the *lcm* genes to genes of the glycine cleavage (*gcv*) system in many bacteria prompted NMR-based metabolome analysis of spore content; this indeed showed significant changes in primary metabolism in *lcmABC* null mutants as compared to wild-type spores, although not in glycine accumulation, which was however seen for a *gcv* mutant that was used as the control. The precise implications of the altered metabolic profile in the spores are as yet unclear.

Finally, the genetic cause was examined of another exciting and long-known spontaneous mutant of *S. griseus* NRRL B2682, namely the A-factor nonproducer AFN (Biró *et al.*, 2000). For more than 40 years now, *S. griseus* has been a model strain for γ-butyrolactone (GBL)-mediated extracellular signaling via the GBL A-factor (Khokhlov *et al.*, 1973). A-factor acts by binding to the A-factor receptor protein ArpA, which results in the release of the repression by ArpA of the transcription of the global regulatory gene *adpA* (Ohnishi *et al.*, 1999; Ohnishi *et al.*, 2005). AdpA then directly activates the transcription of genes involved in development and secondary metabolism (Ohnishi *et al.*, 1999; Ohnishi *et al.*, 2005). Sequence analysis of *S. griseus* AFN revealed that in this mutant, the codon for Trp881 of the gene *afsR* was changed to a stop codon (Chapter VI). AfsR, which is phosphorylated by the important cell cycle serine/threonine kinase AfsK, was previously studied and likely involved in the response of development and antibiotic production to glucose (Umeyama *et al.*, 1999). We noticed a strong direct correlation between AfsR expression and colony size and streptomycin production in *S. griseus*, with a large colony phenotype and accelerated aerial hyphae formation as well as enhanced streptomycin production for colonies with extra copies of *afsR*. This is in apparent contrast to the data published previously (Umeyama *et al.*, 1999). Unfortunately the original *afsR* mutant of *S. griseus* was lost and could therefore not be compared. However, A-factor production was not affected in either a full deletion mutant of *afsR* or in strains in which the AFN-derived nonsense mutation was introduced, which suggests that AfsR may play its role independent of the A-factor cascade.

# Future work

Many new cell division-related genes have been identified in this work, and deletion resulted in often pleiotropic defects in sporulation or spore maturation. This offers a lot of potential for future research. However, while the phenotypic changes are sometimes drastic, it is not always easy to understand the underlying molecular basis for the defects, in other words, to find out what precisely these genes do. SepG controls sporulation-specific cell division, most likely by tethering SsgB to the membrane, while the role of SepG in nucleoid compaction is yet unexplained. *In vivo* and *in vitro* interaction studies, such as FRET interaction studies to establish the interaction with the membrane, and bacterial two-hybrid screening or pull-down assays to find interaction partners, should provide further functional insights into the function of SepG and in the control of division. The surprising and yet unexplained effect of the SepF-like proteins on apical growth and branching is also something that needs to be pursued, and colocalization studies and live imaging of SepF, SflA, SflB, DivIVA and FtsZ would shed light on their role in balancing apical growth and sporulation. Biochemical studies on ring formation by SepF and the Sfl proteins should establish whether heterocomplexes are formed and what the affinities are, to validate the model wherein the Sfl proteins serve to curb the activity of SepF.

Despite many cell biological and biochemical experiments, and their crucial roles in cell-wall synthesis, it is yet unclear how precisely the enzymes YlmD and YlmE contribute to sporulation-specific peptidoglycan synthesis. Currently, in collaboration with Raffaella Tassoni and Marcellus Ubbink (Department of Chemistry, Leiden University), extensive ligand-interaction studies are being performed using purified YlmD and YlmE proteins, in an attempt to identify the substrate for these enzymes. Live imaging techniques, including FRET studies, should provide insights into how the Lcm proteins function and interact, and what their roles are in septum synthesis and/or closure during sporulation-specific cell division. How the Lcm proteins relate to the observed changes in the spore metabolome remains to be elucidated. Finally, the observed relationship between AfsR expression level, colony size and secondary metabolism (streptomycin production) is surprising. Reverse engineering of the *afsR** mutation into wild-type *S. griseus* did not result in an AFN phenotype, and to find the responsible mutation perhaps more extensive SNP analysis, or genetic complementation with a genomic library should be attempted, both in a wild-type and *afsR** background.

This PhD study started out from the perspective that, besides the clear fundamental importance, the study of novel cell division-related genes may also

offer new leads for strain-engineering approaches, similar to earlier applications of the cell division activator SsgA. So far however, the major valorization of the work lies in generating functional insights on several well-conserved genes near *ftsZ* in the *dcw* cluster and also elsewhere on the genome. This has further deepened our understanding of how positive control of cell division is governed in *Streptomyces*, but since many of the targets are conserved in Gram-positive bacteria, will also have an impact in the broad field of bacterial cell division.