Molecular characterization of copper-dependent enzymes involved in Streptomyces morphology

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General Discussion

The microbial world consists of millions of organisms with a wonderful variability of morphologies. They can range from single-celled individuals, such as yeasts and many bacterial species, to chains of cells and large multicellular communities, such as bacterial biofilms. One of the most sophisticated groups of bacteria is formed by the streptomycetes. These saprophytic bacteria thrive predominantly in soil habitats, where they colonize dead and living organic material. Unlike most other bacteria, streptomycetes grow by forming thread-like structures called hyphae, that together form a branched mycelial network. This filamentous growth enables the bacterium to burrow into neighbouring materials that would otherwise be inaccessible. After a period of vegetative growth, the onset of morphological differentiation is triggered by a lack of nutrients. This leads to the formation of a reproductive aerial mycelium, that ultimately gives rise to the formation of millions of spores that can withstand harsh environmental conditions.

Morphological differentiation is coupled to the production of a large arsenal of antibiotics to protect the non-motile *Streptomyces* colony from predation by other microbes. From an applied perspective we are interested in streptomycetes due to their ability to synthesize antibiotics and many other secondary metabolites used in medicine, biotechnology and agriculture, including antitumor, antifungal and anthelmintic compounds (Hopwood, 2007). In addition, as saprophytes these bacteria produce a large number of hydrolytic enzymes, which can be employed for industrial use (Chater *et al.*, 2010). However, optimal growth and production in bioreactors is complicated by the filamentous mode-of-growth of the bacteria, that results in the formation of complex mycelial structures. Especially macromolecules on the cell surface are important for bacterial morphology. The subject of this thesis was the formation of these extracellular macromolecules and their influence on morphogenesis, with a special emphasis on glycan production by genes of the *cslA-glxA* gene cluster. The

new knowledge derived from my work may lead to improved production of secondary metabolites or enzymes by streptomycetes.

Heterogeneity in Streptomyces pellets

Growth of streptomycetes in bioreactors results in the formation of intertwined clumps of hyphae called pellets. *Streptomyces* pellets can reach sizes over a millimetre in diameter and are therefore too large for analysis with regular flow cytometers. In Chapter 3 for the first time a Complex Object Parametric Analyser and Sorter (COPAS) was used to quantitatively analyse pellet sizes of different streptomycetes. Interestingly, we observed the presence of two populations of pellets in liquid-grown cultures of *Streptomyces lividans, Streptomyces coelicolor, Streptomyces scabies,* and *Streptomyces griseus*, that differ in size and probably also in function (see below). The average size of the particles belonging to the population of small mycelia had a constant mean diameter of approximately 260 µm. In contrast, the particles belonging to the population of large mycelia showed more variation in size, with *S. scabies* having pellets with the largest mean diameter of 557 µm. The diameter of the population of small mycelia of *S. coelicolor* was not affected by culture age and medium composition, whereas the size of the larger pellets did vary. Our data suggest that especially in the population of larger pellets the particle diameter is dependent on external parameters. These parameters may include the composition of the growth medium, pH, viscosity, surface tension, agitation speed, dissolved oxygen levels, temperature and inoculum (Tough and Prosser, 1996; Celler *et al.*, 2012; van Dissel *et al.*, 2014).

Proteomic comparison of large and small *S. coelicolor* pellets revealed an overrepresentation of proteins involved in antibiotic production in the population of large pellets (Chapter 3). This is consistent with the concept that larger mycelial structures are better for antibiotic production (Martin and Bushell, 1996). The overrepresentation of stress-related proteins in the larger pellets may be explained by the reduced availability of oxygen or nutrients in the centre of large pellets, which may in fact be triggers to induce antibiotic production. These stress-related proteins include the response regulator SCO0204 and the universal stress protein USP (SCO0200), which is part of the SCO0204 regulon (Urem *et al.* manuscript in preparation). Low levels of oxygen or nutrients in the centre of these larger mycelia might constrain growth and primary metabolism. In agreement, the small pellets contained more proteins involved in active growth, such as those involved in DNA metabolism and organization. Given that small pellets probably grow faster than large pellets makes them more suitable for the production of industrial relevant enzymes. This would be consistent with the increased growth speed and enzyme production observed upon induced fragmentation by the cell division activator protein SsgA (van Wezel *et al.*, 2006).

The size of pellets increases by tip growth and branching, while fragmentation and lysis leads

to a decrease in their size. Because these processes occur simultaneously in a bioreactor, pellets are heterogeneous in size. In filamentous fungi pellets originate from aggregated spores and germlings (Lin *et al.*, 2008), which implies that surface properties of spores and hyphae affect pellet size (van Veluw *et al.*, 2013). In streptomycetes the secreted chaplin proteins are known to decorate spores with a hydrophobic layer (Claessen *et al.*, 2003; Elliot *et al.*, 2003), while the cellulose synthase-like protein CslA produces an extracellular glycan at hyphal tips that could become part of the spore surface (Xu *et al.*, 2008). Deletion of *cslA* or the genes encoding for the chaplins resulted in a large decrease in the average size of mycelia, although sizes were still heterogenic (Chapter 3). Size heterogeneity was also observed when cultures were inoculated with pre-grown mycelia or synchronously germinated spores. These results show that, similarly to filamentous fungi, cell surface properties are crucial for pellet architecture, although their influence in spore and germling aggregation is not the only factor giving rise to two differentially sized populations of pellets (Chapter 3). This infers that other factors explain the heterogeneity in mycelial size, with shear force leading to random fragmentation being a likely candidate.

Structural cell-surface components involved in pellet architecture

Pellet integrity is challenged by the strong shear forces in bioreactors. This is especially important in pellets that have initiated programmed cell death, which leads to the partial degradation of pre-existing hyphae in the central part of these structures (Rioseras *et al.*, 2014). This may be compensated for by the synthesis of a number of glycans, such as those produced by CslA, that could form an adhesive, extracellular matrix that contributes to pellet integrity. Recently, the *mat* gene cluster was identified as a locus that is required for pellet growth of streptomycetes (van Dissel *et al.*, 2015). Homology of the *mat* cluster with the *ica* operon of *Staphylococcus* spp. suggests that it encodes a polysaccharide synthase complex that produces a polysaccharide containing β-(1,6)-linked *N*-acetylglucosamine molecules (van Dissel *et al.*, 2015). A third secreted polysaccharide possibly involved in pellet integrity is hyaluronic acid (Kim and Kim, 2004), a glucosaminoglycan consisting of repeating units of D-glucuronic acid and D-*N*-acetylglucosamine, linked via alternating β-(1,4)- and β-(1,3)-glycosidic bonds. Treatment of pellets with hyaluronidase makes the structures very fragile (Kim and Kim, 2004), although the purity and specificity of the enzyme preparation that was used is unknown. The SLI_5327-SLI_5330 gene cluster is a candidate for synthesis of hyaluronic acid in *S. lividans*. Deletion of this gene cluster, however, had no major effect on pellet morphology under the conditions tested (our unpublished results).

In addition to glycans, an extracellular pellet matrix may also contain other macromolecules. Many biofilm-forming bacteria secrete DNA in the environment that contributes to biofilm architecture (Whitchurch *et al.*, 2002). Likewise, extracellular DNA has been suggested to be involved in shaping *Streptomyces* pellets (Kim and Kim, 2004). Moreover, matrices often

contain adhesive proteins, including amyloid-forming proteins such as curli in *Escherichia coli* and TasA in *Bacillus subtilis* (López *et al.*, 2010). The effect on *Streptomyces* pellet morphology caused by deletion of the amyloid-forming chaplin proteins (Chapter 3), illustrates the similarity in extracellular matrix between pellets and bacterial biofilms.

So far, it is not clear how all these extracellular components collaborate in the establishment and maintenance of pellet integrity. Since chaplins and the cellulose-like structure produced by CslA were shown to cooperate in the attachment of hyphae to hydrophobic surfaces (de Jong *et al.*, 2009) it is likely that this also happens in the process of pellet formation. In addition, one can easily imagine interaction between the different polysaccharides produced in the pellet matrix. For example, it is shown that fibril association and crystallinity of the cellulosic network produced by *Glucanoacetobacter xylinus* strains is influenced by the presence of non-cellulosic exopolysaccharides (Fang and Catchmark, 2015). Furthermore, diverse covalent bonds are present between different polysaccharides in the arabinogalactan-peptidoglycan complex in the mycobacterial cell envelope (Crick *et al.*, 2001). Since the glycan produced by CslA is directly produced in the growing tip (Chapter 4; Xu *et al.*, 2008), it might serve as a scaffold to efficiently organize other macromolecules in the extracellular matrix. However, this awaits further experimental evidence.

Structural cell-surface components in aerial growth

Structural cell-surface molecules are also important for morphological differentiation on solid substrates. Vegetative hyphae of streptomycetes elongate via tip extensions at the cell pole, while new hyphae emerge via branching of sub-apical compartments. Hydrostatic pressure acting on the extension zones drives the hyphal elongation process at the tips, which are the sites where the cell wall is constantly being remodelled (Prosser and Tough, 1991). This means that growing hyphal tips are relatively weak compared to the remainder of the hyphae that are surrounded by mature peptidoglycan. To control cell integrity during growth many proteins function together in the so-called tip-organizing centre (Holmes *et al.*, 2013). One of the crucial proteins is the polar localized protein DivIVA that plays an essential role in apical growth by orchestration of the cell wall synthesis process (Flärdh, 2003a; Flärdh *et al.*, 2012). Protein-protein interactions showed that DivIVA binds to CslA, suggesting that the glycan produced by CslA is required during cell wall synthesis. Its putative function is to provide protection to the growing tips (Xu *et al.*, 2008; Chater *et al.*, 2010). Although deletion mutants of *cslA* are able to establish a vegetative mycelium, the erection of rigid hyphae into the air requires the presence of CslA (Chapter 4, (Xu *et al.*, 2008)). Immediately after the onset of aerial mycelium formation expression from the promoter upstream of *cslA* is dramatically reduced (Liman *et al.*, 2013).

In this thesis also the morphological role of the radical copper oxidase GlxA was extensively

studied. The *glxA* gene is located immediately downstream of *cslA* in an operon-like manner that is highly conserved in streptomycetes (Liman *et al.*, 2013). Both CslA and GlxA are required for hyphal tip staining with the glycan-binding stain calcofluor white, suggesting that GlxA cooperates with CslA in the synthesis, anchoring and/or modification of a glycan at the growing tips (Chapter 4). This is strongly supported by the observations that *glxA* deletion mutants grow as dispersed mycelia in liquid cultures and are unable to produce an aerial mycelium as was shown for *cslA* deletion mutants (Chapter 4 and 5 and (Xu *et al.*, 2008; Liman *et al.*, 2013)). Finally, deletion of either *cslA* or *glxA* reduces the attachment to hydrophobic surfaces in liquid standing cultures (Chapter 6). Thus, the *glxA* mutant is a phenocopy of the *cslA* mutant under various conditions. The crystal structure of GlxA contains tunnels that are sufficiently large for a polysaccharide strand to stretch from the protein surface all the way to the active site of the enzyme (Chapter 4). We envision a scenario whereby the nascent glycan produced by CslA is fed into one of these tunnels, orientated in the substrate pocket, oxidised and then released through another tunnel. Notably, small differences in development can be observed between the *cslA* and *glxA* mutants under specific conditions. For instance, the *cslA* mutant is severely delayed in aerial growth on MYM medium, while the *glxA* mutant fails to form aerial hyphae altogether (Chapter 6). This hints at a CslA-independent role for GlxA in development, which is supported by the identification of a second promoter in the *cslA* coding sequence that drives transcription of the *glxA* gene independent of *cslA* (Liman *et al.*, 2013). On the other hand, the phenotype of the *cslA* deletion mutant is more severe on relatively low osmolyte media, suggesting that at least under those conditions GlxA is not essential for CslA function (Liman *et al.*, 2013).

As discussed in Chapter 2 the formation of an aerial mycelium also strongly depends on the secretion of the lantibiotic-like peptide SapB and the chaplin proteins, both of which act as surfactants to lower the water surface tension enabling aerial growth (Claessen *et al.,* 2003; Willey *et al.,* 2006; Capstick *et al.,* 2007). Together with the rodlin proteins, the chaplins have a second role in development by assembling into a hydrophobic layer surrounding the aerial hyphae and spores (Claessen *et al.*, 2002 and 2003; Elliot *et al.*, 2003). The capacity to initiate aerial growth relates to three crucial factors: the turgor pressure inside the hyphae, the rigidity of the hyphal wall and the surface tension barrier at the medium-air interface. Deletion of *cslA* or *glxA* probably affects the rigidity of the vegetative hyphal wall, deletion of the chaplin genes or the *ram* gene cluster (required for SapB biosynthesis) prevents the required reduction in surface tension, while changes in the medium composition influences all three factors. Considering the combined effect of these factors underlying aerial growth may explain why the block in development of the *cslA* mutant (less rigid hyphae) could be rescued by the addition of chaplins (thereby further reducing the surface tension; Xu *et al.*, 2008), why a reduction in osmolarity (thereby increasing turgor pressure and (slightly) reducing surface tension) enables the *glxA* mutant (with less rigid walls) to establish an aerial mycelium (Liman *et al.*, 2013), why SapB is not essential in low osmolyte media

(during which hyphae can generate more turgor pressure) and why *cslA* deletion mutants are able to erect aerial hyphae on some media, while they cannot on others (Chapter 4 and 5). Thereby, the structural cell wall components that are involved in hyphal rigidity also seem to contribute to aerial mycelium formation, while others like the polysaccharides produced by the *mat* cluster are only important for pellet formation.

The role of copper in morphogenesis

S. lividans displays a distinct dependence on copper (Cu) to fully initiate morphological development. The work in this thesis shows that GlxA is the principle extracytoplasmic cuproenzyme that is essential for morphogenesis. Cu plays an essential role in the activity of GlxA as it forms one of the two redox centres in the active site of the protein. The other redox centre is formed by a nearby cross-link between a Cys residue and one of the Cu-coordinating Tyr residues (Chapter 4). *In vitro* data showed that the Tyr-Cys cross-link is only formed in the presence of Cu (Chapter 5). With the combined redox centres GlxA can access three oxidation states: Cu(I)-Tyr-Cys (reduced), Cu(II)-Tyr-Cys (semi-reduced) and Cu(II)-Tyr-Cys• (fully oxidized). GlxA should therefore be able to catalyse the two-electron oxidation of a substrate (Chapter 4).

The GlxA crystal structure shows a lid-like structure that protects both the redox centres in the active site and the substrate binding pocket (Chapter 4). However, this raised the question how GlxA acquires and incorporates Cu. Using Western Blot analysis, the acquisition of Cu by GlxA could be detected, due to a change in electrophoretic mobility when the Cu dependent Tyr-Cys cross-link is formed (Chapter 5). Further analysis showed that *in vivo* GlxA maturation depends on the extracytoplasmic copper chaperone Sco. Sco was already known to deliver Cu to the active site of an aa_3-type of cytochrome c oxidase (CcO). However, the lack of aerial mycelium formation in the *sco* deletion mutant could not be explained by its role in Cu transfer to CcO, as the *cco* mutant develops normally (Blundell *et al.*, 2013; Blundell *et al.*, 2014). GlxA being a second target of Sco fits with the identical defects of both mutants including the lack of aerial growth on solid media, and the absence of pellet formation in liquid cultures (Chapter 5). Only when copper is available in high concentrations GlxA will not rely on the presence of Sco, thereby enabling the *sco* deletion mutant to erect aerial hyphae and to create small pellets (Chapter 5).

In turn, Sco receives its Cu ion from the extracytoplasmic Cu chaperone ECuC in a unidirectional manner (Blundell *et al.*, 2014). The transfer takes place in the cuprous form (Cu(I)) of the ion, but the oxidation state after transfer is unknown (Blundell *et al.*, 2014). Interestingly, transfer from Sco towards CcO required the cupric (Cu(II)) oxidation state of the metal (Blundell *et al.*, 2013), while the required oxidation state for incorporation in GlxA is not known. Both the morphological defects and reduced CcO activity are less pronounced

in the *ecuc* mutant compared to the *sco* mutant, suggesting that Sco can scavenge Cu(II) directly from extracytoplasmic pools (Chapter 5). Efficient loading probably does require ECuC as a Cu(I) donor and the subsequent action of an oxidizing molecule or protein to create Cu(II). The nature of this molecule or protein is unknown.

sco (SLI_4214) and *ecuc* (SLI_4213) are the first two genes of an operon that also contains a gene encoding a putative Cu transport protein (SLI_4212) as well as *dtpA* for a Dyp-type peroxidase (SLI_4211, Chapter 5). Interestingly, deletion of *dtpA* also results in a developmental arrest coinciding with impaired GlxA maturation (Chapter 5). The morphological defects of *dtpA* mutants can be overcome by high levels of Cu, as is the case for the *sco* mutant (Chapter 5). The physiological role of Dyp-type peroxidases is still relatively unclear, although they are widely distributed amongst fungi and bacteria (Singh and Eltis, 2015). One possibility would be that GlxA and DtpA act in a coupled assay, whereby DtpA activates or stabilizes GlxA via protein-protein interactions or via the removal of H_2O_2 produced by GlxA. Such a cooperation of a cognate peroxidase with an oxidase has been reported in some fungal systems (Hamilton *et al.*, 1978; Kersten, 1990; Takano *et al.*, 2010). We showed that DtpA can act as a peroxidase in the presence of active GlxA *in vitro* (Chapter 5). *In vivo,* these proteins could potentially also function together as GlxA is an extracellular membrane-associated protein (Chapter 4), while also DtpA is secreted into the extracellular environment via the twin-arginine translocation pathway (Chapter 5). This transport pathway is known for the secretion of folded proteins, especially when these proteins contain a cofactor, such as the haem in DtpA. However, no strong physical interaction between GlxA and DtpA could be observed *in vitro*, and DtpA did not boost the activity of GlxA in a peroxidase coupled enzymatic assay compared with the regularly used horse radish peroxidase (Chapter 5).

Alternatively, DtpA could function in the Cu-trafficking pathway towards GlxA. We could speculate that DtpA is the oxidizing protein that creates Cu(II) from Sco-bound Cu(I). This would not only explain why GlxA maturation is impaired in a comparable manner to the *sco* mutant (Chapter 5), but also why the CcO activity is reduced in a *dtpA* mutant in *S. coelicolor* (Fujimoto *et al.*, 2012). Sco proteins are known to be able to bind both Cu(I) and Cu(II) and it is not unlikely that Cu transfer to acceptor proteins is dependent on the oxidation state of the metal (Banci *et al.*, 2007). A role for DtpA in oxidizing metal ions would be very similar to the function of the Dyp-type peroxidase EfeB, which oxidizes Fe(II) to Fe(III) before cellular uptake (Miethke *et al.*, 2013).

Cu is not the only redox-active metal that is important for morphology in streptomycetes. Especially the role of iron (Fe) has gained a lot of interest since this metal plays essential roles in house-keeping functions, such as DNA replication, protein synthesis and respiration (Cornelis and Andrews, 2010; Lambert *et al.*, 2014). Many microbes secrete iron-chelating

molecules called siderophores, which are important for the acquisition of the poorly soluble Fe(III). The siderophore desferrioxamine (DFO) has recently been shown to be essential for growth of *S. coelicolor* on medium with low levels of Fe (Lambert *et al.*, 2014). In addition, many of the classical *bld* mutants, which are unable to erect an aerial mycelium under certain conditions, show altered DFO-mediated Fe utilization (Lambert *et al.*, 2014). The availability of Fe might also influence the Cu-dependent morphology pathway, given the presence of the Fe-containing haem group in DtpA. This thesis describes a range of new *bld* mutants, all of which are affected in Cu utilization and trafficking. Taken together, this implicates an essential role for metals in morphological differentiation. One question that remains to be answered is how Cu is sequestered and transported into the cell. The only known copper-acquisition compound in nature is Methanobactin, which is produced and secreted by the methanobacterium *Methylosinus trichosporum* (Kim *et al.*, 2004) . Whether streptomycetes produce comparable molecules that can acquire copper remains to be discovered.

Future directions

Despite the various roles of CslA in morphogenesis the true identity of the produced glycan remains to be discovered. New bioinformatics tools confirm that CslA is a processive glycosyltransferase, which adds sugar moieties to the growing end of a linear polysaccharide without releasing the acceptor substrate (Chapter 6). The overall conformation and the fold of the active site of the protein are similar to those of the cellulose synthase BcsA of *Rhodobacter spaeroides* (Chapter 6). This shows that CslA combines the intracellular synthesis of a glycan directly with its transport through the cytoplasmic membrane. However, amino acid substitution of residues that interact with the sugar binding pocket or the growing glycan chain suggest an altered specificity of CslA compared to that of BcsA (Chapter 6).

In the direct vicinity of the *cslA-glxA* operon lie several genes that relate functionally to carbohydrate metabolism, including *cslZ*, for a glycosyl hydrolase of family 6 that encompasses mainly cellulases and cellobiohydrolases, *nagD* for a possible ribonucleotide monophosphatase, which are implicated in recycling UDP when liberated by the action of glycosyltransferases on UDP-activated sugars (Tremblay *et al.*, 2006), and SLI_3183 encoding a lytic polysaccharide monooxygenase (LPMO), which are enzymes known to target crystalline surfaces of polysaccharides. In Chapter 4 I showed that GlxA and CslA cooperate in the synthesis and modification of an extracellular glycan, which is likely composed of β(1,3) or β(1,4)glycosidic linkages based on calcofluor white staining assays. The best *in vitro* activity of GlxA was measured using glycoaldehyde as the substrate, which is the smallest molecule to contain both an aldehyde and a hydroxyl group (Chapter 4). Identification of polysaccharide substrates for CslZ, SLI_3183 and/or GlxA is required to further our understanding of the nature of the polymer produced, modified and degraded by the joint

action of these enzymes. Conversely, characterization of the polymer could lead to a better understanding of the chemical conversions performed by these enzymes.

Direct characterization of the glycan synthesized by CslA was hampered by the relative little amount that is produced in comparison to the abundance of peptidoglycan in the *Streptomyces* cell wall (Chapter 6). Use of a newly developed peptidoglycan-independent synthesis platform showed potential for characterization of bacterial glycans that are produced only in limited amounts in their endogenous (Gram-positive) hosts (Chapter 6). Preliminary data using this system indicated that the CslA polymer could be composed of glucose and/or *N*-acetylgalactosamine moieties (Chapter 6). The first step to fully characterize both the composition and the structure of the glycan would be to overproduce the glycan in this peptidoglycan-independent synthesis platform by increased expression of the *cslA-glxA* gene cluster using a strong promoter.

A major discovery described in this thesis is the importance of DtpA for development. Undoubtedly, work must be continued to better understand the precise function of this protein and the other described proteins involved in copper-dependent morphogenesis. Interestingly, the morphology of *sco* or *dtpA* deletion mutants shows new opportunities to manipulate bacterial growth in bioreactors by simply changing the concentration of Cu in the culture broth. Low levels of copper result in growth of a more dispersed mycelium, which probably results in faster growth and good enzyme production, while high levels of copper result in the formation of pellets, which will be beneficial for antibiotic production. The work presented in this thesis provides a foundation for the further research on the role of cell-surface components in controlling *Streptomyces* morphology. This is of fundamental importance for understanding the determinants of growth and development of this exciting multicellular model organism, and at the same time may help us to further optimize their exploitation for the industrial production of secondary metabolites and enzymes.