# Chapter 8

**General Discussion and Conclusions**

## GENERAL DISCUSSION

Examining *Streptomyces* under the microscope, irrespective of scale, reveals a beautiful organism. From spore, to individual hyphae, to colony - in liquid broth or on solid media - the fractal-like symmetry of branching growth, colourful hues of secreted products, and fluorescent flashes of dynamically localizing proteins are all signs of the intricacies of *Streptomyces* biology. These microbes have an extraordinary ability to adapt to a diversity of ecosystems and thrive alongside a variety of competing and coexisting organisms. To do so, they have evolved a complex multicellular lifecycle, including several steps of morphological differentiation, with development tightly linked to antibiotic production (van Wezel and McDowall 2011; McCormick and Flärdh 2012).

Although the streptomycetes produce numerous commercially-important secondary metabolites, enzymes, and other secreted products (Hopwood 2007; Horinouchi 2007), the relationship between growth and morphology, on the one hand, and biomass accumulation and productivity, on the other, is complicated and poorly understood. In liquid cultures, *Streptomyces* grow by a balance of tip extension, branching, and fragmentation, creating an extensive mycelial network. Within this mycelium, programmed cell death in certain hyphal parts (and not others) occurs to provide nutrients to the growing organism (Manteca *et al*. 2008); this stage of development is linked with antibiotic production. A major scientific challenge lies in understanding how growth parameters are controlled in response to nutritional conditions, and how this affects the efficiency of production and secretion of proteins and antibiotics. Studies have demonstrated that morphology is key, but morphology varies from species to species. Besides genetics and physiological parameters, environmental triggers and growth conditions also play a large role in determining how a mycelium will grow. In effect, antibiotic and enzyme production results from a combination of species-specific genetic make-up and the environment (natural or laboratory) in which the organism is found. The work presented in this thesis aims to arrive at a better understanding of *Streptomyces* morphogenesis and development, and how these processes link to productivity.

To better understand how protein secretion relates to growth, we performed localization studies on the twin-arginine transport, or Tat, protein export machinery. Live imaging of fluorescent fusions revealed that the three subunits (TatA, TatB, and TatC) of the Tat complex translocate dynamically throughout hyphae before colocalizing, often roughly 2 µm behind the tip. To facilitate quantitative analysis of focal trajectories, we therefore developed a single particle tracking package specially suited for application to diffusion-limited foci in

long *Streptomyces* hyphae. We tested the package against other algorithms by participating in the IEEE International Symposium on Biomedical Imaging (ISBI) 2012 Single Particle Tracking Challenge (http://bioimageanalysis.org/track/). Although no algorithm performed best for all competition data sets, the superior performance of some algorithms over others appeared to stem mainly from careful parameter tuning and making the best possible use of prior knowledge about the data. The outcome of the challenge and an analysis of participating algorithms has been submitted for publication (Chenouard *et al. submitted*).

We applied our particle tracking package to better understand the dynamics of TatA, the most abundant subunit of the Tat machinery, and found that TatA undergoes different motion regimes during localization, with small units, likely consisting of tetramers, oligomerizing during the localization sequence before colocalizing with TatBC to form a functional translocation complex (Chapter 3). Use of the tracking package enabled quantitative analysis of the localization dynamics, which previously could only be described qualitatively. Analysis of other dynamically localizing proteins (for example, the cell division protein SsgA) in this manner should provide more insight their function in time and space during hyphal growth.

Work on this thesis coincided with the opening of the Netherlands Centre for Electron Nanoscopy (NeCEN) in 2012. It was my good fortunate to be the first to use the new 300 keV Titan microscope to study of *Streptomyces* ultrastructure at an unprecedented resolution. In the past years, the possibility to image biological samples at high keV has been revolutionizing the field of bacterial cell biology. 3D reconstructions, or tomograms, of tilt series images collected provide an inside-view of cryo-fixed cells at nanometer resolution, avoiding the artifacts of chemical fixatives. Building on this technology, we developed cryo-correlative light and electron tomography (cryo-CLET) for use in *Streptomyces*. Cryo-CLET enables labeling and identification of cellular structures at low resolution with cryo-fluorescent light microscopy, and then imaging at NeCEN to obtain a high resolution tomogram at the location of interest.

This very powerful and novel combination of technologies allowed us to discover a vast intracellular membrane system within hyphae, and establish that in fact the DNA and membranes are mutually exclusive (Chapter 4). As the Min and NOC control systems, which act to ensure that division occurs at mid-cell in rod-shaped bacteria, are missing in *Streptomyces sp*., it was until now not clear how and where crosswall formation is initiated and completed in the multinucleoid hyphae. The work published in this thesis shows that most likely intracellular membranes fulfill this role, and mediate division-site selection and nucleoid occlusion in the vegetative mycelium. During growth and development, membranes, consisting of a heterogeneous distribution of lipids, dynamically localize to the

division site, creating a large cell-delimiting barrier to ensure that septum formation can occur without damage to the chromosomes. Additionally, cross-membranes fully colocalize with - and are dependent on - the division scaffold protein FtsZ, demonstrating that a subtle interplay exists between membrane and cell wall formation during the initiation of cell division.

This study illustrates the power of cryo-electron tomography and correlative techniques for elucidating the ultrastructural details of cell-cycle processes. Excitingly, we are only at the tip of the iceberg in terms of what can be discovered with these technologies. Improvement in cryo-light resolution, as well as fluorescence signal enhancement of reporters (such as eGFP) under cryo-conditions should achieve a long-term goal, namely the localization of protein complexes directly in high resolution images, and on native samples. Development of live-fluorescent imaging - prior to plunge freezing of samples for tomography - will make possible the capture of dynamic events as they are taking place. With these technologies, high resolution structural information can be gained about cell-cycle processes, creating macromolecular landscapes of the unperturbed cell.

Cryo-electron tomography has recently been applied to the study of cytoskeletal elements in bacteria (Briegel *et al.* 2006; Ingerson-Mahar *et al.* 2010; Pilhofer *et al*. 2011; Swulius *et al.* 2011). Control of bacterial shape is the task of the cytoskeleton, and numerous structural proteins cooperate to ensure that the elaborate shapes of bacteria are maintained throughout growth and division. In *Streptomyces coelicolor*, coiled-coil rich proteins DivIVA (Flärdh 2003), FilP (Bagchi *et al.* 2008; Fuchino *et al.* 2013) and Scy (Walshaw *et al.* 2010; Holmes *et al.* 2012) have been characterized, and their function in controlling polarized growth and hyphal integrity described. Cytoskeletal proteins and their interaction partners, however, not only play a role in establishing cell shape - they also have roles in cell division (FtsZ and the SsgA-like proteins), growth (MreB in rod-shaped bacteria; DivIVA in *Streptomyces*), DNA partitioning (ParA) and motility (in motile bacteria). Study of the cytoskeleton is a dynamic field, with new discoveries made by high resolution light and electron microscopy techniques rapidly changing the way we perceive bacteria (reviewed in Chapter 2).

Perhaps due to their complicated mycelial lifestyle and developmental program, *Streptomyces sp*. encode for many more coiled-coil rich proteins. In this work, I focused on novel genes for coiled-coil and associated proteins: SCO2259 and the adjacent gene SCO2260 as well as SCO3285 and SCO3286 (Chapter 7). SCO2259 and SCO3285 encode large proteins with coiled-coil motifs, while SCO3286 encodes for a flotillin domain protein, associated with lipid raft formation in eukaryotes. In *B. subtilis* flotillins seem to generate

a local environment favoring membrane curvature, perhaps helping to recruit cell division proteins to the division site (Dempwolff *et al.* 2012). We were therefore particularly interested in studying the putative function of SCO3286 in membrane formation. Deletion of SCO2259 and SCO3285 affected spore chain formation; in the SCO2259 mutant some spores were branching, while in the SCO3285 mutant, new cell wall material was not deposited correctly, resulting in spores not correctly aligned within chains, but rather forming zig-zags. A large phenotypic difference was observed when SCO3286, was deleted. This mutant demonstrated a dramatic increase of sporulation, with many short spore chains and few non-sporulating aerial hyphae at a time where wild-type cells only produce aerial hyphae. A similar phenotype was obtained when a different flotillin homolog, SCO3607, was disturbed in *Streptomyces albus*, suggesting that flotillins either induce sporulation where they localize or play an important role in the negative control of sporulation. Interestingly, the double mutant of SCO3286 and SCO3285 demonstrated difficulty sporulating. Localization studies of these proteins using fluorescent eGFP fusions revealed that SCO3285 localizes in young aerial hyphae and that SCO3286 forms long filaments around forming spores. These genes appear therefore to cooperate to ensure that sporulation-specific cell division can take place correctly. Our results highlight the importance of the *Streptomyces* developmental program, where many large coiled-coil rich proteins contribute to ensure that strong, viable spores are reliably formed for the survival of progeny.

To integrate the multi-scale information obtained during this work, and arrive at a better understanding of *Streptomyces* morphogenesis and development, a structured morphological model was created. The model can be applied as a framework for rational design of *Streptomyces sp.* for production purposes (Chapter 5). Since secondary metabolite production in filamentous organisms is associated with morphological differentiation (Giudici *et al.* 2004; Manteca *et al.* 2008), a structured approach, in which hyphae are divided into compartments indicating different stages of cellular differentiation, was favored. With this approach, a particular morphology (dictated by branching and cross wall formation frequency, as well as fragmentation), has a particular ''structure'' or compartmentalization. A fraction of the pellet is able to actively produce antibiotic (the hyphal compartment), a fraction to branch (the subapical compartment), and a fraction to grow (the apical compartment). Pellet composition (based on these compartment fractions) can be correlated with information gained in fermentation trials, relating morphology to productivity. The structured morphological modeling approach is a step towards rational strain improvement and process design.

Drawing inspiration from the Google Chrome Experiments (http://www.chromeexperiments.com) and the new trend of using the web as a platform for software applications, an

interactive version of the model was created in Javascript for HTML5 browser-based viewing and manipulation (Chapter 6). Basic model processes of growth and branching were coded and visualized using WebGL, a new web technology which enables visualization of Graphics Processing Unit (GPU) hardware-accelerated 3D graphics on the browser without additional software, plug-ins or extensions. Although the combination of HTML5 and WebGL is not yet suitable for scientific computing, further development should enable the shift from binary application-based modeling to dynamically delivered web application-based modeling (Taivalsaari *et al.* 2011). A proof-of-concept model was therefore coded to determine the capabilities and limitations of online modeling. Based on the promising results, future plans are to extend the model to include additional graphical user interface (GUI) parameters and experimental data to increase the biological relevance of the model. By adjusting the parameters, a user should be able to affect morphology and follow the 3D growth of a pellet over time. Such an interactive in-browser modeling framework could be a valuable tool to enhance visualization of morphological changes and their effect on natural product formation.

The work presented in this thesis is diverse and multidisciplinary in nature, combining molecular biology, light and electron microscopy, and computational modeling techniques to better approach the questions at hand. Not only are these multi-disciplinary techniques - but also multi-dimensional, spanning the whole scale of experimental observation, from whole mycelial colony, down to individual genes and their expression. Such a combined approach is fundamental for improving understanding of *Streptomyces* biology.

Each of the research directions pursued leave many questions unanswered. In the case of the cytoskeletal proteins, it will be interesting to study more proteins with flotillin domains (there are six encoded for in *Streptomyces coelicolor*) to better understand the role of lipid rafts in control of growth and development. The particle tracking package can be applied to live imaging studies of other dynamically localizing proteins (such as the aforementioned SsgA) to better understand how and where they localize during growth and morphogenesis. A major contribution of this thesis was the work done at NeCEN and the discoveries made regarding the role of intracellular membranes in *Streptomyces* vegetative division. This work must undoubtedly continue and mutants with altered lipid composition, or affected in cell division (*ssgA*- overexpressing strains or the *ftsZ* deletion strain) imaged to better understand the mechanisms behind membrane formation and localization. As more information is gained, extension of the modeling framework will be of value to provide a real test drive for the fermentation process and to pre-assess the effect of different variables on productivity.