Chapter 1

General Introduction and Outline of Chapter Contents

GENERAL INTRODUCTION

Streptomycetes are Gram-positive multicellular soil-dwelling bacteria which are commercially used as natural producers of antibiotics, anticancer agents and immunosuppressants, as well as many industrial enzymes (Hopwood 2007). Similarly to fungi, they carry out a complex developmental life cycle, forming highly structured multicellular colonies composed of physiologically distinct hyphae (Miguelez *et al.* 2000). This life cycle starts with the germination of a spore, from which one or more germ tubes emerge. A complex mycelial network is formed as the hyphae grow and branch to form what is called the vegetative or substrate mycelium (Chater and Losick 1997). Growth occurs by tip extension, during which newly synthesized wall material is incorporated at the hyphal apex (Gray *et al*. 1990). In Streptomyces, the combination of tip growth and branching results in exponential growth of the organism (Jakimowicz and van Wezel 2012).

Nutrient depletion and other stresses (heat, drought, competing microbes) are considered the major triggers for the onset of aerial mycelium formation in the soil and also on solid-grown cultures in the laboratory environment. At this point, hydrophobic vegetative hyphae break through the moist surface of the soil or agar and grow into the air (Claessen *et al.* 2006). Multiple cell division occurs, whereby up to 100 septa are formed more or less simultaneously, and the multigenomic aerial hyphae are converted into chains of unigenomic spores. These spores then disseminate to start the formation of new colonies in more favorable conditions.

In liquid-grown cultures, on the other hand, most streptomycetes only grow vegetatively. Hyphae grow and branch, taking on a variety of morphological shapes, from dense pellets to small fragmented mycelia. In general, the morphology of liquid-culture pellets is a determining factor for productivity during industrial fermentations of filamentous organisms (van Wezel *et al.* 2009), with larger clumps favoring antibiotic production in *Saccharopolyspora erythraea* (Wardell *et al.* 2002), and fragmentation stimulating enzyme production by *Streptomyces lividans* (van Wezel *et al.* 2006). The understanding and control of mycelial morphology is thus key for optimization of industrial fermentations (Celler *et al.* 2012). With this in mind, the purpose of this work was to combine different approaches and techniques to arrive at a better understanding of *Streptomyces* morphogenesis and development, with the ultimate aim to improve *Streptomyces* as a production platform in industrial biotechnology (Figure 1).

A natural starting point for the study of morphogenesis is the cytoskeleton, or cellular scaffolding, which helps to maintain and change cell shape, but also plays an important role

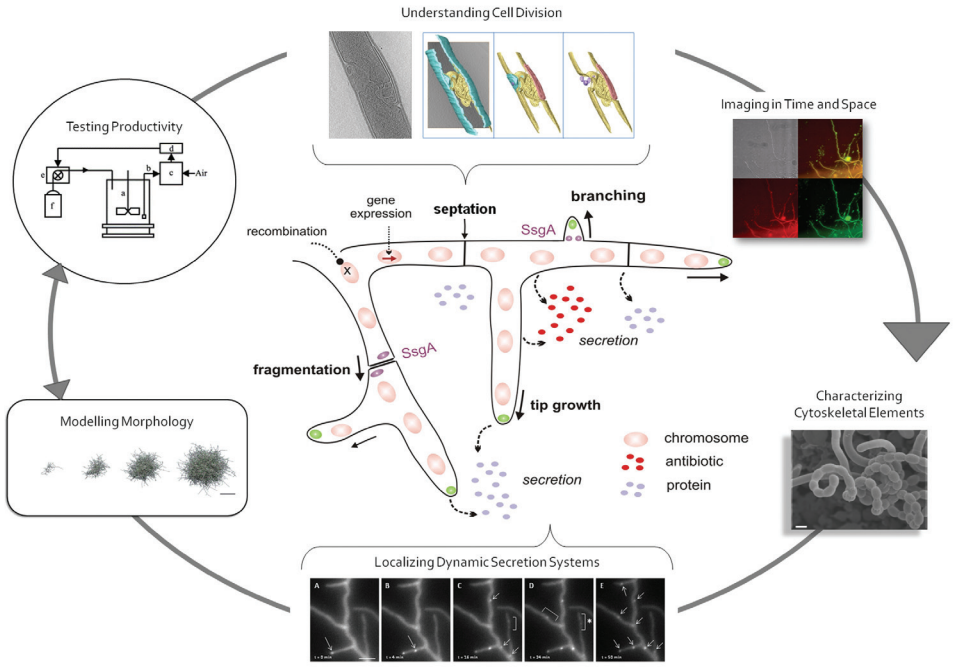

Figure 1**. **The systems biology of growth and production.

The basis of morphogenesis is the cytoskeleton, a dynamic collection of proteins which helps to maintain and control cell shape, but also influences intracellular trafficking and cell division. As the Streptomyces mycelium grows, branching frequency, tip growth rate and fragmentation of hyphae are influenced and controlled by the combination of cytoskeletal proteins and growth conditions. Novel imaging techniques, such as cryo-correlative light and electron microscopy and specifically tomography, can provide fundamental insight into vegetative cell division and its effect on development within a pellet. At the same time, fluorescent live imaging in time and space is an invaluable tool to better understand and localize dynamic secretion systems. Combining the multi-scale information gained can lead to improved morphological models and provide a test drive for the fermentation process. The aim is to improve Streptomyces as a production platform in industrial biotechnology.

in many dynamic cellular processes such as intracellular trafficking and cell division. Until recent years, the cytoskeleton was believed to be a feature unique to eukaryotic organisms, but this perspective was overturned when homologs of all three known eukaryotic cytoskeletal elements - actin, tubulin, and intermediate filaments (IFs) - were identified in prokaryotes and implicated in major cell functions, including growth, morphogenesis, cell division, DNA partitioning and cell motility (Graumann 2007; Cabeen and Jacobs-Wagner 2010). In addition, new elements have been identified which have no apparent eukaryotic counterparts, such as the Walker A-Type ATPases or the bactofilins (Koonin 1993; Kühn *et al.* 2010).

Multidimensional imaging techniques have contributed to new insights and demonstrated the astonishing complexity of the bacterial intracellular world, demonstrating cytoskeletal proteins forming filaments or patches, often rapidly translocating in a directed manner to respond to the many changing functions of the cell. The recent advances made towards understanding the role of cytoskeletal proteins in controlling bacterial shape and division, with particular emphasis on modern imaging approaches, are reviewed in **CHAPTER 2**.

Experiments to identify the localization of proteins in cells, such as for the cytoskeletal proteins described above, is often performed by tagging the protein with a fluorescent reporter such as eGFP (green fluorescent protein). Nowadays, *in vivo* multidimensional fluorescence microscopy enables the collection of very large data sets showing the directed spatial relocation of dynamic proteins in three-dimensional time lapse movies. Automated analysis and particle tracking are necessary to facilitate physical parameter estimation and large-scale analysis of localization experiments and/or object dynamics. To tackle this problem, we developed a particle tracking algorithm to specifically deal with tracking of fluorescently-tagged proteins in *Streptomyces*. It involves the application of a series of image filtering methods for optimal foci detection with subsequent nearest-neighbour tracking to link detections into tracks that can be analyzed. The algorithm was submitted to the Particle Tracking Challenge of the IEEE International Symposium on Biomedical Imaging (ISBI) in 2012 (Chenouard *et al.*, *submitted for publication*). Using the software allowed us to provide a mathematical tracking analysis of diffusion-limited foci in vegetative and aerial hyphae of *Streptomyces*.

The algorithm was applied to analyze the dynamic localization of TatA, the most abundant unit of the Tat (twin-arginine translocation) complex. The Tat pathway transports folded proteins across the bacterial cytoplasmic membrane and is a major route of protein export in *Streptomyces*. A better understanding of where and when secretion takes place in hyphae can lead to important insights for strain-improvement and rational process design. TatA localizes dynamically before colocalizing with TatBC to form an active translocation complex. **CHAPTER 3** provides a quantitative analysis of TatA movement, revealing the dynamics of complex assembly at different stages as well as the total time needed to arrive at the final stage in localization, roughly 2 µm behind the tip. Our easy-to-use particle tracking package should allow for the study of the dynamics of many other proteins and protein complexes in these fascinating multicellular microorganisms.

At higher magnification, the application of electron microscopy to *Streptomyces* hyphae provides a unique opportunity to visualize and increase fundamental understanding of morphogenesis, capturing the ultrastructure of tip growth, branching, and cell division

at nanometer resolution. Unlike other electron microscopy techniques, cryo-electron tomography (cryo-ET) enables the collection of structural information under conditions that are directly relevant to the native state of the cell (Subramaniam 2005). We applied cryo-ET to better understand vegetative cell division and its effect on development within a pellet. In fact, during the *Streptomyces* life cycle, two distinct forms of cell division occur. In aerial hyphae, cell division occurs by septation, where thick, double-layer sporulation septa are formed. During septation, a significant amount of cell-wall material is synthesized and remodeled during the metamorphosis of unicellular prespore compartments into mature spores (McCormick 2009). In vegetative hyphae, on the other hand, cell division occurs by the formation of crosswalls at irregular intervals of roughly 5-10 µm. These crosswalls delimit the syncytial cells, but do not constrict, and do not lead to cell fission. Much is known about aerial septation, but little was known of vegetative division. Using the new Netherlands Centre for Electron Nanoscopy (NeCEN) 300 keV Titan microscope to image hyphae at the high resolution of 10 nm, we discovered that crosswall formation is preceded by the formation of large membrane structures delimiting hyphae.

To identify the membrane components and better understand their function, we developed cryo-correlative light and electron tomography (cryo-CLET). Cryo-CLET correlates the images acquired by fluorescence light microscopy and electron tomography, enabling selective labeling and identification of cellular components as well as DNA and mapping of these lower resolution data onto high resolution tomograms. Using a combination of cryo-CLET and live fluorescent light microscopy, we discovered that the membranes, enriched in phosphatidylglyderol and cardiolipin, dynamically localize during development, and play a major role in division-site selection and nucleoid occlusion in *Streptomyces* hyphae. A better understanding of vegetative cell division can lead to new insight into mycelial differentiation and morphogenesis, as well as fragmentation. **CHAPTER 4** discusses these results.

As mentioned previously, morphology and productivity in filamentous organisms are closely connected. Morphological modeling is a valuable tool to predict optimal fermentation conditions, applicable in, for example, strain improvement approaches. Such a form of modeling should include important biological details, such as the correlation between gene expression and protein localization on the one hand, and growth and product formation on the other. Combining different modeling approaches, a structured, 3D morphological modeling framework for rational design of *Streptomyces* species was developed and is described in **CHAPTER 5**. The initial development of an interactive 3D web implementation (using HTML5 and WebGL) of the Matlab model are described in **CHAPTER 6**. The objective is to ultimately create an interactive model which can be a test-drive for the fermentation

process, where several input variables (such as bulk oxygen concentration, growth rate or morphological parameters such as distance between branches or maximum branch length) can be adjusted to arrive at an optimal production strain.

Finally, perhaps due to their filamentous growth and multicellular nature, *Streptomyces* have a particularly wide range of coiled-coil proteins, proposed to be cytoskeletal in nature. Based on sequence analysis, streptomycetes may have more than ten IF-like elements, and preliminary mutational analysis suggests specific functions for a number of these proteins in the control of cell integrity, growth, development, protein secretion and DNA segregation. **CHAPTER 7** discusses the functional analysis of two pairs of cytoskeletal genes: SCO2259, SCO2260 and SCO3285, SCO3286 and their effects on sporulation-specific cell division.

A general conclusion to this thesis is provided in **CHAPTER 8**.