Morphogenesis and heterogeneity in liquid-grown *Streptomyces* cultures

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Chapter 1: General Introduction

Filamentous sporogenic bacteria of the genus *Streptomyces* are widespread inhabitants of terrestrial soils. Compared to most other bacteria, streptomycetes posses an unusually complex life cycle. The life cycle of streptomycetes begins with the germination of spores, which results in the formation of an intricate network of branched filamentous cells called hyphae. This network, referred to as the vegetative mycelium, grows at the expense of the available organic material (e.g. decaying vegetal biomass) [1, 2]. Under the influence of a number of stimuli, among which various stresses and nutrient scarcity, a developmental program is initiated. As a result, the growing colony undergoes a complex differentiation process encompassing several stages such as the activation of secondary metabolism and the programmed dismantling of portions of the mycelium [3, 4] and ultimately leading to the formation of hyphae that leave the soil environment and grow into the air [5]. These aerial hyphae further differentiate to form spores, which, following their dispersal, can give rise to new mycelia elsewhere.

Besides for reasons of fundamental interest, mainly given by features such as their incipient multicellularity and complex development [6], much of the research interest in streptomycetes stems from the astonishing biosynthetic capacity of these bacteria. Streptomycetes are among the most potent producers of valuable secondary metabolites, including numerous antibiotics, anticancer compounds and immunosuppressants [7]. This biosynthetic proficiency is coupled with the capacity to produce and secrete an arsenal of hydrolytic enzymes, which streptomycetes have evolved to degrade the diverse organic polymers on which they thrive [8]. Due to this treasure trove of valuable products, streptomycetes have a long record of use in the industrial biotech sector. Industrial processes making use of microbes are typically performed in liquid environments in large-scale bioreactors. The mode-of-growth of streptomycetes under these conditions is markedly different from that observed on solid substrates [9]. To begin with, the extent to which most strains develop in submerged cultures is limited, although a certain degree of differentiation is often observed [10, 11]. Depending on the genetic background and culture setup, the mycelium of liquid-grown streptomycetes can display a range

of different morphologies [9]. Most species, among which the model strain *Streptomyces coelicolor* and the industrially relevant *Streptomyces lividans* form dense mycelial particles called pellets. Liquid cultures of these strains contain pellets that are heterogeneous in size [12]. More specifically, at least two populations of differently-sized pellets are formed. Notably, pellet size and production have been shown to be tightly correlated in streptomycetes. In particular, most reports indicate that large pellets are best-suited for the production of antibiotics, whereas smaller pellets show an increased capacity to produce and secrete enzymes [13-16]. In light of these facts, the co-existence of differently-sized pellets appears as a highly undesirable feature of *Streptomyces* growth that translates into suboptimal fermentation properties and a potential limitation to their industrial exploitation.

The study and control of pellet size heterogeneity in streptomycetes is the subject of the research presented in this thesis. Here, the phenomena occurring between the germination of spores and the formation of pellets are characterized with the aim of understanding the factors underlying pellet size heterogeneity. Subsequently, the obtained knowledge is applied to obtain homogeneously-sized pellets of the industrial workhorse *Streptomyces lividans.* Also, the work described in this thesis addresses the fate of pellets at late stages of growth and a growth strategy representing a valuable alternative to conventional liquid cultures. Taken together, the results presented here significantly contribute to the understanding of morphogenesis in liquid-grown streptomycetes.

OUTLINE OF THE THESIS

Over the last decade, that of phenotypical heterogeneity has become an established concept in microbiology. **Chapter 2** provides a detailed overview of heterogeneity in filamentous microorganisms, with a special emphasis on streptomycetes and filamentous fungi. Heterogeneity in filamentous microbes occurs at a number of different scales: heterogeneity is evident between distinct mycelial particles, between adjacent hyphae within particles and also between adjacent compartments of individual hyphae. The mechanisms underlying these heterogeneities are discussed from the viewpoint of improving industrial exploitability.

In **Chapter 3**, the process of mycelial aggregation is described, along with its role in generating mycelial heterogeneity. Aggregation is studied using a simple yet effective approach based on the co-cultivation of *S. lividans* strains carrying distinguishable fluorescent markers. Through the use of these strains and of techniques for the high-throughput analysis of their aggregation dynamics, aggregation is shown to occur in a time-dependent manner. This experimental approach allowed to identify mutants that are impaired in aggregation and in turn to discover the

components involved in this process, which are extracellular glycans associated with the cell envelope. Importantly, the mycelial particles formed in the absence of these glycans are homogeneous in size, indicating the role of aggregation in causing heterogeneity. This chapter also shows that aggregation is common in streptomycetes and even allows different species to form multispecies particles.

Chapter 4 describes a technique to generate homogeneously-sized pellets of *Streptomyces lividans*. This was achieved using a mutant strain whose phenotype can be exploited to control aggregation. By abolishing the aggregation between distinct mycelial particles, while still allowing pellets to form at later stages of growth, homogeneously-sized pellets are obtained over a range of different sizes. These pellets are then compared in their capacity to produce and secrete a heterologous enzyme, which confirms previous observations on the interdependence of pellet size and production. Ultimately, the use of the inducible aggregation system described in this chapter led to the discovery that aggregation activates the process of cellular dismantling in liquid-grown streptomcyetes. This was concluded from the early appearance of compromised hyphae upon triggering of aggregation.

In **Chapter 5**, the analysis of submerged *Streptomyces* growth is extended by studying the process of fragmentation, namely the detachment of mycelial particles from mature pellets and the independent growth thereof. By combining the use of fluorescent *S. lividans* derivatives with the quantification of different types of mycelial particles throughout growth, this chapter shows that fragmentation coincides with nutrient exhaustion. Interestingly, the detached mycelial particles are able to efficiently aggregate to one another and establish new pellets in the presence of fresh nutrients. The results presented in this chapter provide interesting parallels between the growth dynamics of *Streptomyces* pellets and biofilms of unicellular bacteria.

In **Chapter 6**, an alternative to the conventional submerged growth of streptomycetes is described. This method consists in the entrapment of spores and their subsequent growth in microcapsules of a semi-solid gelatinous scaffold, a technique called microencapsulation. In the experiments presented in this chapter, microencapsulation is shown be applicable with a range of different *Streptomyces* strains. Microencapsulation is shown to prolong the viability of *S. lividans*, which in turn greatly enhances its capacity to produce and secrete a heterologous enzyme.

The results of the work performed in this thesis are discussed in **Chapter 7.**

Chapter 2: Multiscale heterogeneity in filamentous microbes

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Microbial cells within clonal populations can display different morphologies or carry out different tasks. This heterogeneity is beneficial at the population level and allows microbes to spread risk or separate incompatible activities. Heterogeneity is also evident in filamentous bacteria and fungi, which form mycelial networks consisting of interconnected hyphae. Here, heterogeneity is observed between clonal mycelial particles, between different zones of colonies, between adjacent hyphae and even between adjacent compartments of individual hyphae. In this review, we compare this multiscale heterogeneity in filamentous bacteria and fungi and discuss the underlying mechanisms. These mechanisms might provide targets to improve the exploitability of these organisms as cell factories in the biotech sector.

INTRODUCTION

One of the assumptions in microbiology was that cells in a monoclonal microbial population would be phenotypically indistinguishable when provided with a constant environment. Over the last two decades, however, the development of high-throughput analytical techniques has enabled microbiologists to study large numbers of cells at the individual level [17-19] and to unambiguously demonstrate that processes such as metabolism, transcription, translation and protein secretion are heterogeneous in space and time across cells [20-24, 243]. Examples of these heterogeneities have been documented in a wide range of microorganisms, including some of the best-characterized prokaryotic and eukaryotic model organisms (e.g. *Escherichia coli, Bacillus subtilis* and *Saccharomyces cerevisiae*, amongst others) [25-28]. While heterogeneity has been mostly addressed in unicellular microbes, it is also evident in multicellular species. In this review, we will focus on heterogeneities in filamentous microorganisms that are employed as cell factories in the industrial sector. We will discuss the consequences (disadvantages and benefits) of these heterogeneities and the mechanisms through which these are established.

THE CONSEQUENCES OF PHENOTYPIC HETEROGENEITY

Phenotypic heterogeneity allows microbes to withstand environmental fluctuations and carry out specialized functions at the level of single cells. In their natural habitats, microbes are confronted with rapidly changing environmental conditions. The best-known mechanism to withstand such changes is to modulate gene expression [29]. Changes in gene expression can lead to profound phenotypic changes, including cellular differentiation. Many microbes, however, ensure that a number of cells within a clonal population already possess certain defensive traits, even when the corresponding environmental stimulus is not present [30]. As a consequence of this strategy, commonly referred to as bet-hedging, only a fraction of the cells will pay the cost (e.g. reduced metabolic proficiency) associated with the expression of those genes conferring potentially useful features. Should the environmental conditions become adverse and change in their favor, these cells would already be equipped to withstand the altered conditions and are therefore more likely to survive. This behavior is beneficial to the entire population [31].
Bet-hedging and phenotypic heterogeneity have been extensively studied in *Bacillus subtilis*. This Gram-positive bacterium forms endospores when exposed to stress conditions (e.g. starvation or the presence of toxins) [32, 244]. These spores are metabolically dormant and highly resistant to extreme temperatures, desiccation and ionizing radiation. When environmental conditions suitable for growth are

restored, spores germinate to establish colonies of vegetative cells. A bet-hedging control of sporulation has two main advantages. On the one hand, it assures that not all cells commit themselves to sporulation, which is notoriously a lengthy and irreversible process [27, 34]. In this way, when only a fraction of the cells sporulates, the non-sporulating ones can quickly reinitiate growth in case the stress condition turns out to be transient, hence preventing the population from becoming outnumbered by competitors. On the other hand, since environmental changes are sometimes too harmful for the sporulation process to complete, the stochastic initiation of sporulation ensures that some cells undergo sporulation even in the absence of adverse conditions [35, 36]. Another canonical example of the benefits of microbial individuality is that of bacterial persistence.

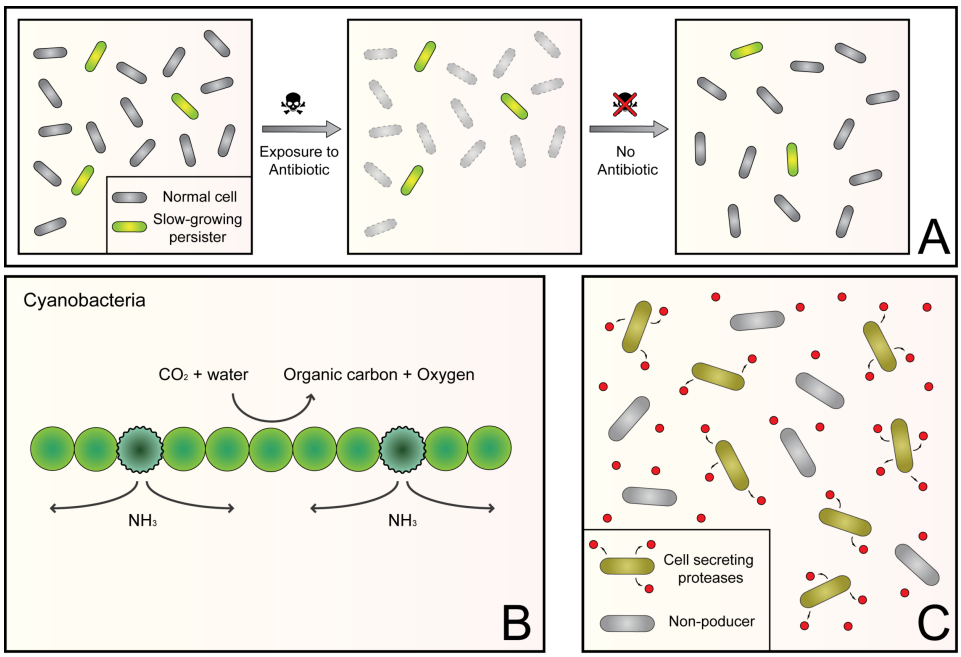

Figure 1: Examples and benefits of microbial heterogeneity. A) Bet-hedging. Microbial clonal populations often contain persisters. Such dormant cells are beneficial in conditions counter-selecting growing cells, for instance due to the presence of an antibiotic. When the normal conditions return, these persisters initiate growth, thereby restoring the population. **B) Division of labor in cyanobacteria.** Oxygenic photosynthesis and nitrogen fixation are chemically incompatible processes, since the oxygen produced through photosynthesis inactivates the nitrogenase enzyme required for nitrogen fixation. Cyanobacteria physically separate these processes by forming specialized nitrogen-fixing cells called heterocysts. The fixed nitrogen will diffuse to the neighboring cells, while heterocysts benefit from the organic carbon produced by cells engaging in photosynthesis. **C) Production of secreted molecules.** The production and secretion of molecules that benefit the entire population is often carried out by only a subset of cells. In this example, some *Bacillus subtilis* cells secrete proteases that provide hydrolyzed nutrients to the whole population under nutrient-limiting conditions. It is not clear whether the non-secreting cells participate in other ways to the overall survival of the population.

A fraction of *Escherichia coli* cells forms metabolically dormant persister cells that are able to withstand various environmental insults, such as the prolonged exposure to antibiotics [37], and to resume growth when the original conditions are restored (Fig. 1). Sub-populations of non-growing persisters that survive exposure to antibiotics have also been reported in *Salmonella* [38, 39] and *Mycobacterium* [40]. The appearance of persisters can also occur when cells are not challenged by antimicrobials [37], although stressful conditions can enhance their abundance within a population [41-43]. A similar strategy has been reported in *Saccharomyces cerevisiae*. Within clonal populations of this yeast, certain individuals are characterized by lower growth rates and concomitantly possess higher resistance to heat shock due to the accumulation of the protecting disaccharide trehalose [26]. Notably, experimentally tuning growth rates of *S. cerevisiae* using chemostat cultures has highlighted that many of the genes activated upon heat stress are also active under conditions of slow growth, which is again substantiated by the observation that cells that grow slowly are more resistant to heat stress [44].

Recent studies have shown that bet-hedging strategies culminating in phenotypic heterogeneity also become evident when microbial cells are exposed to fluctuating nutritional regimes [45-47]. This is no surprise when considering the rapidly changing nutritional conditions that microbial cells endure in their natural environments. When exposed to mixtures of carbon source, microbes typically consume them in a sequential manner. As a consequence, distinct growth phases are observed that are separated by a lag phase. During this phase, cells are believed to undergo the physiological adaptations needed for the uptake and consumption of the second carbon source. This behavior is known as diauxie [48]. The diauxic shift in *Lactococcus lactis* is explained by the fact that only a limited number of cells is able to metabolize the second carbon source. Interestingly, these cells emerge when the preferred carbon source is still present [46]. The decision to commit to the metabolism of the less preferred source depends on the metabolic state of the cell prior to the depletion of the preferred carbon source, while the number of cells enacting the shift is inversely proportional to the abundance of the first carbon source. This mechanism provides an alternative explanation for the decades-old concept of metabolic adaptation during diauxic shifts, which may also be relevant for other lag phases observed in microbiology.

While bet-hedging is beneficial in unpredictable and fluctuating environments, phenotypical heterogeneity can also be advantageous in non-fluctuating conditions, for instance when different processes have to be carried out simultaneously within a clonal population. This so-called division of labor is characterized by the coexistence of subpopulations of cells specialized in performing complementary tasks [49-51].

Under nutrient-limiting conditions, *B. subtilis* secretes subtilisin E, which degrades proteins into small peptides that are accessible to all community members [52]. Single-cell measurements have revealed that only a minority of cells produce and secrete this protease, indicating that only a few members of the clonal population pay the cost associated with its production. It is not yet clear whether this strategy represents a form of pure altruism whereby the producing cells pay the production-associated costs for the benefit of the entire population, or whether it represents a cooperative behavior in that both the producer and the recipient cells mutually benefit from each other.

Numerous are the other reported examples where some cells pay the cost for the benefit of the entire population; these include *Salmonella enterica* [53, 54], *Myxococcus xanthus* [55], and certain protozoans [56]. *M. xanthus* represents perhaps the most spectacular example of the commitment of subsets of cells within a given population to a specific function. In the presence of excess nutrients, *M. xanthus* establishes a motile group of cells called a swarm. The swarm explores the environment to forage for nutrients or predate on other bacteria [57]. Upon starvation, growth is arrested, and a developmental program is initiated that culminates in the formation of spore-bearing fruiting bodies. Three distinct subpopulations of cells contribute to the formation of fruiting bodies. While only 10% of cells differentiate into spores, roughly 30% form peripheral rods on the outer surface of the fruiting body, while the remaining fraction undergoes programmed cell lysis [58-60]. The fact that such a major fraction of the population undergoes PCD is a remarkable example of social behavior, with the lysing cells providing nutrients and energy for sporulation to complete and in turn ensure the propagation of their genome [60, 61].

In some microorganisms, division of labor is employed as a strategy to perform incompatible metabolic processes [62-64]. The best-known example of this strategy is the spatial segregation of nitrogen fixation and photosynthesis in cyanobacteria [65] (Fig. 1). The nitrogenase enzyme required for nitrogen fixation is sensitive to oxygen, the product of photosynthesis. For this reason, some cyanobacteria generate specialized cells called heterocysts, which are specialized in nitrogen fixation while lacking the oxygenic photosystem [66]. Heterocysts also have a different cell wall composition that contributes to the exclusion of oxygen to protect the nitrogenase enzyme [67]. On the other hand, non-heterocystous cyanobacteria separate photosynthesis and nitrogen fixation by temporally segregating the two incompatible processes [68].

MECHANISMS UNDERLYING INDIVIDUALITY IN UNICELLULAR MICROBES

When compared to higher multicellular eukaryotes, the regulation of gene expression in microorganisms (especially in prokaryotes) appears to be controlled by only a handful of mechanisms. As a consequence, one would expect any given gene to be expressed at a similar level in isogenic microbial cells exposed to the same environmental conditions. However, it is well accepted that bacterial gene expression is subject to intrinsic noise. In a pioneering study that paved the road for the nascent field of microbial heterogeneity, cells of *E. coli* were engineered to express two distinguishable fluorescent proteins under control of the same promoter. Major differences in the expression of these reporters were detected both within and between cells, indicating that gene expression is subject to intrinsic fluctuations [25]. It is interesting to note that the level of transcription negatively correlates with the heterogeneity in fluorescence emission, indicating that intrinsic noise is more pronounced at low transcriptional levels. Extensive work has shown that a considerable degree of heterogeneity between microbial cells originates from the fact that transcription and translation occur in so called pulses or "bursts" [69-72]. Since these stochastic pulses are asynchronous between cells, distinct subpopulations can evolve and coexist. A number of studies on *B. subtilis* have shown how pulsating genetic circuits control processes such as the development of competence, the onset of sporulation, and the response to environmental stresses [73-76]. Notably, single cell measurements in both prokaryotic and eukaryotic microbes have revealed disparities in the degree of transcriptional noise between different genes within a single cell. These disparities are seemingly not arbitrary, as the transcription of housekeeping genes is generally less noisy than that of genes associated with stress or dispensable metabolic functions [77-79].

Phenotypic heterogeneity between cells can also originate from transcriptional differences caused by cellular processes. Several studies have shown that physiological factors such as growth rate and cell cycle stage can substantially influence gene expression [80, 81]. Single-cell studies have recently shed light on the importance of the feedback of growth in causing heterogeneity. For example, fluctuations in the expression of metabolic genes can lead to fluctuations in the growth rate of individual cells, which in turn not only perturb the expression of other metabolic genes, but also of unrelated gene networks [82-84]. Deterministic choices can also be a source of cell-to-cell heterogeneity. For example, metal ion scarcity leads to a growth arrest in newborn daughter cells of *S. cerevisiae* (i.e. cells which have not budded yet). As a consequence, two populations of cells emerge: older dividing cells and younger non-dividing cells [85]. This is explained by the fact that the vacuole, which is the reservoir for metals, is not propagated to daughter cells, while it is maintained in the mother cells which keep dividing. This strategy results

in higher fitness under zinc-limiting conditions than in a mutant strain where vacuole segregation occurs homogeneously. In the latter case, zinc is diluted in fact to an extent that eventually impedes cellular division.

The generation of phenotypic heterogeneity and multi-stability have also been the subject of numerous mathematical models. Such models are important for better understanding the principles behind bet-hedging and provide predictive value that can be tested experimentally. Recent models include the stochastic nature of cellular processes and provide a powerful framework for understanding phenotypic switching between different cellular states. We here wish to refer to some excellent papers for readers interested to learn more about this aspect (see e.g. [245-248], and references therein).

HETEROGENEITY IN FILAMENTOUS ORGANISMS

The features of phenotypic heterogeneity discussed in the previous sections relate to unicellular microorganisms, the cells of which are, at least under most conditions, spatially separated from one-another. In contrast, many multicellular microbes such as filamentous actinomycetes and fungi grow by means of interconnected filaments that only physically separate into unicellular propagules during the reproductive phase [6]. From a morphological perspective, the mode of growth of filamentous actinomycetes is similar to that of filamentous fungi. This is the reason why, despite bearing the structural features of bacteria, actinomycetes were originally believed to be fungi [86]. In contrast to most unicellular organisms, filamentous fungi and actinomycetes possess a complex life cycle characterized by distinct developmental stages and the co-existence of different specialized cells. Both kinds of microorganisms propagate via spores, dormant cells equipped to withstand harsh environmental conditions [1, 87]. Spores germinate under favorable conditions, leading to germ tubes that elongate to form thread-like cells called hyphae. Hyphae of filamentous fungi and actinomycetes have a diameter of about 2-10 µm and 0.5-2 µm, respectively. They elongate at their tip (or apex), while new hyphae emerge subapically by branching [88, 89]. The combination of apical growth and branching yields an interwoven cellular network called a mycelium. The growing vegetative mycelium (also called substrate mycelium) colonizes the environment by radiating leading hyphae from peripheral regions of the colony. The encountered polymeric substrates are degraded by means of secreted hydrolytic enzymes and the degradation products are internalized by the cells to serve as nutrients [1]. When nutrients become scarce, colonies of filamentous microbes develop into complex multicellular consortia of different cell types [249-251]. For instance, while the peripheral regions of *Streptomyces* colonies proceed with

vegetative growth, more central and non-growing parts of the colony undergo an ordered process of chemical and morphological differentiation [3, 91]. Such chemical differentiation is responsible for the production of various secondary metabolites, many of which are exploited for commercial use [1, 7, 92] (*see below*). Coinciding with this chemical differentiation, specialized aerial hyphae emerge on the colony surface. The aerial hyphae of actinomycetes differentiate into chains of unigenomic spores, while those of fungi form more elaborate asexual (e.g. condidiophores) or sexual reproductive structures (e.g. mushrooms).

When grown in close proximity to certain yeasts (e.g *S. cerevisiae*) or when faced with conditions of nutrient scarcity, some streptomycetes (i.e. the best-studied and industrially the most relevant representatives of the actinomycetes) form so-called "exploring" cells. These cells can travel over nutrient-void abiotic surfaces and promote the spreading of colonies over large surfaces [93]. Despite being morphologically similar to aerial hyphae in that they do not branch, exploratory hyphae miss the hydrophobic coating which is distinctive of aerial structures, and hence represent a new cellular type with features of both vegetative and aerial hyphae. This functional differentiation is reminiscent of a bet-hedging strategy, with explorer cells allowing dispersal in those cases in which sporulation might be too costly or take too long to complete [94].

Although the distinction in structure and function between vegetative and reproductive hyphae has been known for many decades [249], we are now beginning to understand that heterogeneity is also evident in mycelial aggregates growing in liquid environments, between zones of mycelia, between adjacent hyphae within a colony zone and even between compartments of a single hypha. In the following sections we will first describe the methodologies used to study heterogeneity in filamentous microbial populations before discussing intra- and inter-hyphal heterogeneity in the vegetative mycelium of streptomycetes and filamentous fungi on solid substrates. We will then discuss heterogeneity in liquid-grown mycelia and its effect on production performances of commercially valuable products.

TECHNIQUES FOR STUDYING PHENOTYPIC HETEROGENEITY IN FILAMENTOUS MICROBES

The analysis of heterogeneity relies on techniques that enable the qualitative and quantitative assessment of physiological traits at the single cell level. Microfluidics and flow-cytometry approaches have contributed strongly to the study of phenotypic heterogeneity in unicellular microbes [18, 21, 22]. Microfluidics systems use miniaturized growth chambers that allow growth of various cell types in a finely

controlled microenvironment [252]. Because of this feature, differences observed between cells can exclusively be attributed to intrinsic cellular heterogeneity and not to varying environmental conditions. Most microfluidics devices can easily be accommodated in various type of microscopes which, in turn, allows to finely track growth of single cells, but also to use fluorescent reporters (i.e. fluorescent proteins and dyes). Different concepts have been developed in recent years, encompassing a large range of sizes and designs [253-256]. The use of microfluidics for studying filamentous organisms is limited [257], which relates to the fact that mycelia typically form large multicellular structures formed by hyphae growing and branching in three dimensions. As a result, the mycelium easily grows out of the crafted chambers. Microfluidic approaches would be feasible only by confining growth to two dimensions, which could however dramatically affect the physiology of the mycelium.

While microfluidic approaches are valuable for studying the behaviour of individual cells, flow cytometry allows for the rapid analysis of large numbers of cells. Multiple parameters are analysed, including cell size, granularity, and fluorescence. As in the case of microfluidics, suspended cells or cell aggregates (mainly encountered in filamentous microbes) sense a constant environment in well-mixed submerged cultures, which allows to directly designate the observed heterogeneity as an intrinsic property of the system under analysis. Notably, conventional flow cytometers are not suitable for the analysis of mycelial particles, due to the large size of these structures. However, a number of cytometric apparatuses are nowadays available that were specifically developed for large objects and have been successfully used to study differences between mycelial particles within populations of filamentous microbes [12, 134, 150]. These approaches are however limited to the discrimination of heterogeneity *between* distinct particles and lack the resolution to study heterogeneity *within* individual particles. To study heterogeneities at a lower scale (e.g. between distinct filaments in individual particles), fluorescence microscopy-based approaches are most commonly used. Alternatively, laser capture microdissection (LCM) can be used to collect individual mycelial sections or even individual filaments, which can be subsequently analysed in a comprehensive manner using -omics or next generation sequencing techniques [134, 258].
One other technique to study heterogeneity in filamentous microbes is nanoscale secondary ion mass spectrometry (nanoSIMS). nanoSIMS provides information on the molecular and isotopic compositions of various types of biological samples with a high spatial resolution [259, 260]**.** This technique has recently been used to detect differences in carbon assimilation between adjacent cells of the non-branching actinomycete *Microthrix parvicella* [261]. NanoSIMS can thus be used to characterize metabolic differences between cellular compartments along hyphae.

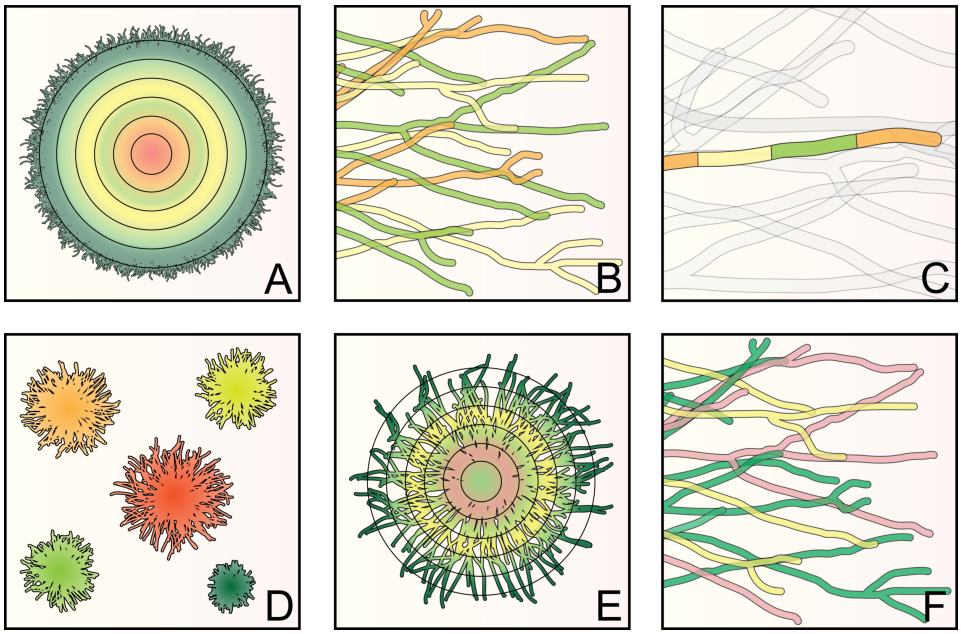

Figure 2. Multiscale heterogeneity in filamentous microorganisms. Heterogeneity has been demonstrated at different organizational levels in solid (A-C) and liquid-grown (D-F) cultures in filamentous microbes. Inter-zonal heterogeneity (A) is characterized by spatial differences in production (and/or secretion) of molecules in different zones of the colony. Heterogeneity is also evident between adjacent hyphae in the same region of the colony (B), and even between separate compartments of the same hypha (C). In liquid-grown environments, microcolonies (or pellets) are heterogeneous in size, coinciding with differences in gene expression and protein secretion (D). Heterogeneity is also evident within pellets (E), some of which may be caused by the scarcity of nutrients and oxygen in the central parts of these structures. As in solid-grown cultures, intercompartmental heterogeneity is observed within hyphae in liquid-grown cultures (F).

HETEROGENEITY IN THE VEGETATIVE MYCELIUM OF FILAMENTOUS ORGANISMS ON SOLID SUBSTRATES

The vegetative mycelium of fungi and streptomycetes simultaneously performs a large number of different tasks. Besides producing and secreting enzymes for nutrient assimilation, mycelia transport nutrients and chemically differentiate to produce a plethora of secondary metabolites [1, 7, 91]. Given that many of these metabolites are of great value to industry, much attention has traditionally been focused on the optimization of production performances in filamentous microbes. However, research in this direction has often been performed using "blind" screening procedures rather than strain optimization strategies based on a deep knowledge of the producing organism [95]. What has for instance been largely ignored so far is where the production of all these compounds occurs within the mycelium, and how approaches to increase productivity correlate with changes in the localization of production. The vegetative mycelium of several *Streptomyces* species is heterogeneous with respect to cellular morphology and physiology.

More specifically, the vegetative growth of streptomycetes has been found to encompass two phases during which different cell types are formed [3, 96]. The young mycelium that is established after spore germination is highly compartmentalized. The approximately 1-µm-wide compartments are thought to be separated by membrane structures and/or thin peptidoglycan-containing septa [97, 98]. This first compartmentalized mycelium, called MI mycelium, undergoes an ordered process of dismantling, which is followed by a second growth phase during which a multinucleated mycelium is established (MII). The cellular compartments in this mycelium are significantly larger than those formed in the MI mycelium [96]. Following growth, the MII mycelium undergoes a new round of dismantling, while the remaining viable hyphae form reproductive aerial hyphae that grow into the air [3, 99]. Both death rounds are the effect of a regulated cell suicide process, which bears close analogies to that of apoptosis in eukaryotic cells. This resemblance is illustrated by indicators such as the disruption of the cell wall and the cell membrane, the degradation of DNA and the release of the cytoplasmic content into the extracellular medium [4]. A proteomic characterization of the first apoptotic process in *S. coelicolor* has highlighted that the majority of proteins involved in this process localize at the cell wall, which thus seems to represent the first target to be dismantled during PCD [100]. Other proteins participating in cell dismantling are enzymes involved in the metabolism of fatty acids, various hydrolases, catabolic enzymes, and proteases. The activity of these enzymes is accompanied by an increase in membrane permeability and the subsequent leakage of cytosolic components into the extracellular medium. While the process of cellular dismantling has been observed and described in several streptomycetes, virtually nothing is known about its regulation and how it spatially and temporarily correlates with other processes, such as antibiotic production.

Heterogeneity in the vegetative mycelium of filamentous fungi grown on solid substrates occurs between zones of a colony, between neighboring hyphae within a zone and between compartments of a single hypha (Fig. 2). The first reports on inter-zonal heterogeneity focused on protein secretion within the vegetative mycelium of *Aspergillus niger* and *Phanerochaete chrysosporium.* Secretion of the starch-degrading enzyme glucoamylase was found to be spatially confined to the peripheral zone of *A. niger* [101], while lignin peroxidases were found to be released within the central zone of colonies of *P. chrysosporium* [262, 263]*.* Later studies revealed that each zone of an *A. niger* colony has its own secretome composition [104]. For instance, 6 and 10 proteins are at least 4-fold more and less abundant, respectively, in the outer zone when compared to an intermediate zone. Interestingly, zonal differences in expression in *A. niger* colonies can be explained by both medium-dependent and medium-independent mechanisms [105]. The concentration and nature of the carbon source determines about half of the variation in

gene expression, whereas the other half is attributed to differentiation processes in the vegetative mycelium [105]. The nature of these differentiation processes is not yet known.

Growth at the outer zone of a fungal colony is supported by nutrients in the substrate while the carbon source is exhausted in the central parts of the colony (clearly, the same holds for the mycelia of bacterial species). Here, the hyphae switch from growth on exogenous to endogenous carbon [264]. This is accompanied by vacuolization, reduced growth rate, and a decrease of the hyphal diameter. Vacuolar degradation produces sufficient endogenous carbon to support the formation of so-called secondary hyphae. In contrast to streptomycetes, the endogenous carbon source is not released extracellularly and then internalized by other hyphae, but it is transported to the tips of the newly formed filaments. This mechanism secures the nutrients for the fungus rather than enabling competing microbes to absorb them from the environment. Yet, the autolysis of hyphae with the release of nutrients in the medium may also take place in starving zones of colonies [107]. Future studies are needed to reveal which strategy of nutrient recycling is the most dominant in the fungal mycelium.

Enzyme secretion was initially believed to only occur in growing fungal hyphae [108], whereas it is nowadays clear that it can also occur in non-growing zones of a colony [105, 109]. How proteins are released into the culture medium by non-growing hyphae is not yet understood knowing that pores in the hyphal cell walls are too small to enable proteins to freely diffuse [108, 110]. In the case of growing hyphae, such pores are not needed since proteins to be released in the culture medium can co-migrate with the newly synthesized cell wall polysaccharides that are extruded at the tips of growing hyphae and pushed from the inner to the outer part of the cell wall by the turgor pressure and the addition of new cell wall material. Notably, although both growing and non-growing colony zones can secrete proteins in the culture medium, not every zone does so. The sub-peripheral zone of *A. niger* colonies is able to sporulate when environmental conditions are favorable to enable this differentiation process. This zone does not secrete proteins even when sporulation does not take place [109]. A strain of *A. niger* in which the sporulation gene *flbA* is deleted is no longer able to asexually reproduce and secretes proteins throughout the whole mycelium [109]. The *flbA* deletion strain also shows a more complex secretome consisting of a number of proteins that are not secreted by the wild-type strain. Together, these observations indicate that sporulation inhibits protein secretion in fungal colonies. From a functional perspective, this appears as coherent behavior. Once hyphae engage in sporulation, it would be inefficient to invest energy in the secretion of enzymes involved in vegetative growth.

To further study the phenomenon of sporulation inhibited protein secretion, the impact of deletion of *fluG* in *A. niger* was studied [265]. This gene is at the start of the sporulation program in *Aspergillus nidulans.* Yet, the *fluG* mutant strain of *A. niger* was shown not to be affected in sporulation. However, in contrast to wild-type *A. niger*, the deletion strain shows breakdown of starch under the whole colony. From these and other data it was concluded that FluG is a repressor of secretion in the sporulation zone.

Immuno-localization showed that not every hypha within the outer zone of the *A. niger* colony secretes glucoamylase [101]. Indeed, two types of hyphae were shown to exist in this zone; hyphae that highly and hyphae that lowly express the glucoamylase gene [112]. This heterogeneity in expression was also observed for other genes encoding hydrolytic enzymes [113]. In fact, those hyphae that highly express one of the hydrolase genes were also found to highly express the other hydrolase-encoding genes. In addition, they possess a higher transcriptional and translational activity when compared to hyphae that show lower expression. Nevertheless, both types of hyphae show a similar growth speed, indicating that for secretion to take place a higher transcriptional and translational activity is needed. Our recent findings show that the hyphae showing lower transcriptional and translational activity are also more resistant to heat stress (M Tegelaar, R Bleichrodt and HAB Wösten, unpublished data). Thus, hyphae seem to show a division of labor strategy at the periphery of *Aspergillus* colonies.

Division of labor is also evident between hyphal compartments of *A. niger* [266]. In this fungus, apical compartments are self-sustaining in growth. This was concluded from the finding that the growth rate in these compartments remains unaffected when they are mechanically detached from the rest of the hypha. Interestingly, the first subapical compartments (up to eight) function as a backup system for growth by forming new branches upon damage of the apical compartment [266]. This backup system appears crucial in nature considering the fact that fungal colonies continuously explore substrates that may locally be hostile for growth. By forming sub-apical branches that do not grow parallel to the damaged hypha, but rather grow away from it, the organism can avoid a second confrontation with the source of damage (i.e. a competing organism or a nutrient void zone).

MULTILEVEL HETEROGENEITY IN LIQUID ENVIRONMENTS

Fungi and streptomycetes produce respectively about 42% and 32% of the more than 23,000 known microbial bio-active compounds (i.e. compounds with anti-fungal, antibacterial, antiviral, antitumor, cytotoxic and immunosuppressive activity) [1, 7, 115]. They also possess a remarkable capacity to produce and efficiently secrete various hydrolytic enzymes that allow them to degrade almost any naturally occurring polymer [116, 117]. The ability of streptomycetes and filamentous fungi to produce this treasure trove of commercially-valuable compounds and enzymes has led to their large-scale industrial exploitation [7]. In industry, microbes are typically grown in large bioreactors. This choice is dictated by the fact that these systems provide the most reproducible and efficient manner to obtain high growth and production rates, which are achieved through parametric control and the efficient provision of nutrients and oxygen to cells. Notably, the continuous and often vigorous mixing of the culture medium creates a more homogenous environment for the mycelia when compared to growth on solid substrates. Yet, gradients can still exist, especially with the large volumes that are characteristic of industrial fermentation processes. Notably, heterogeneity in process parameters (e.g. pH, temperature, concentration of biomass and nutrients) can result in physiological heterogeneity and the occurrence of culture segregation in a number of microorganisms [118, 119]. In addition, fluctuating aeration regimes have been shown to decrease product formation both in streptomycetes and filamentous fungi [267, 268].

The mode-of-growth of streptomycetes and filamentous fungi in bioreactors is markedly different when compared to that observed on solid substrates. Depending on the strain and culture setup, the mycelium of these filamentous microbes can display a range of different morphologies [9, 122, 123]. Many species, among which the industrial cell factories *S. lividans* and *A. niger,* can form dense mycelial particles called pellets (also micro-colonies for filamentous fungi). These particles can have a diameter larger than 1 mm, with the pellets formed by *Aspergillus* being generally larger than those of streptomycetes [12, 269]. This mode-of-growth promotes physiological heterogeneity due to the differential diffusion of oxygen, nutrients and metabolic (by)products. One of the consequences of growth in dense pellets is that hyphae in the central part of these particles are typically starved due to the limited availability of oxygen and nutrients [125, 126, 128, 129]. The impact of nutrient and oxygen limitation on pelleted growth is also evident in other multicellular communities (e.g. biofilms) formed by single or multiple species [130, 131]. The interplay between environmentally-determined heterogeneity and actively regulated development is however still obscure. In this context, it is interesting to mention that cells residing within a biofilm structure have been found to be

more heterogeneous as opposed to planktonic cells. In experiments with *Pseudomonas aeruginosa*, phenotypical variation was found to arise when cells were cultured in the form of biofilms. Although the factors inducing this heterogeneity are unknown, a recombination-dependent system was found to provide the source of genotypic variation leading to the observed phenotypes. Furthermore, cells that had gained mutations after residing in biofilms displayed more variation in swimming capability and enhanced resistance to a number of environmental insults including oxidative stress and exposure to antimicrobials [132].

In addition to differential responses to environmental cues, deterministic choices may also stimulate heterogeneous growth in liquid environments. Most *Streptomyces* strains do not sporulate in liquid-grown cultures; nevertheless, a certain degree of developmental and physiological heterogeneity is evident throughout the mycelium. As on solid substrates, the mycelial structure changes throughout growth, and is characterized by frequent compartmentalization at early time points [3, 10]. Following a round of cellular dismantling, a multinucleated mycelium is established which contains fewer compartments. By this time, the production of antibiotics becomes noticeable. Contrary to growth on solid substrates, neither is this newly established mycelium dismantled, nor does sporulation occur. Gene expression profiling indicated however that the majority of transcripts identified on solid substrates are also present in liquid-grown cultures, including activators of secondary metabolism and development [133]. Together, these findings indicate that heterogeneity is common in liquid-grown streptomycetes.

Another form of heterogeneity was discovered in pellet-forming streptomycetes and fungi by analyzing large numbers of pellets with a flow cytometry approach. This revealed that cultures of both filamentous fungi and streptomycetes contain at least two normally distributed populations of pellets that differ in size [12, 134, 269] (Fig. 2). This heterogeneity is observed in a range of strains and growth media, suggesting that it is inherent to the mode-of-growth of these organisms. Interestingly, gene expression in micro-colonies can also be described as a bimodal distribution. For instance, two populations of *A. niger* micro-colonies exist in submerged cultures; one highly and one lowly expressing the glucoamylase gene [134]. In *Streptomyces coelicolor*, 37 proteins were found to be significantly different in abundance between the populations of large and small pellets. While 17 of these proteins are significantly overrepresented in large pellets as opposed to the small ones, 20 are significantly underrepresented [12]. Several of the proteins that are over- or underrepresented could be assigned to specific functional classes, with a number of stress-related proteins being overrepresented in the population of large pellets. The protein that is most strongly enhanced (around 30-fold) in the larger pellets is EgtD, a protein involved in the biosynthesis of the rare amino acid

ergothioneine. The synthesis of this molecule is rare in microbes, with a higher incidence in actinobacteria (including mycobacteria) and filamentous fungi. The role of ergothioneine in these organisms is still obscure, but it has antioxidant properties, which suggests that it might be involved in a stress-response mechanism. Other stress-related proteins being overrepresented in the population of large pellets include polypeptides encoded by genes in the *osdR* locus, including the gene for the universal stress protein (USP) (SCO0200) [12]. Recent studies revealed that *osdR* controls development and oxidative stress, and is functionally similar to DosR, the oxygen-sensitive dormancy response regulator in *Mycobacterium tuberculosis* [135]. It is interesting to mention that the classes of genes being differently expressed in *Streptomyces* pellets are known to be subject to transcriptional noise in other microbes (*see above*).

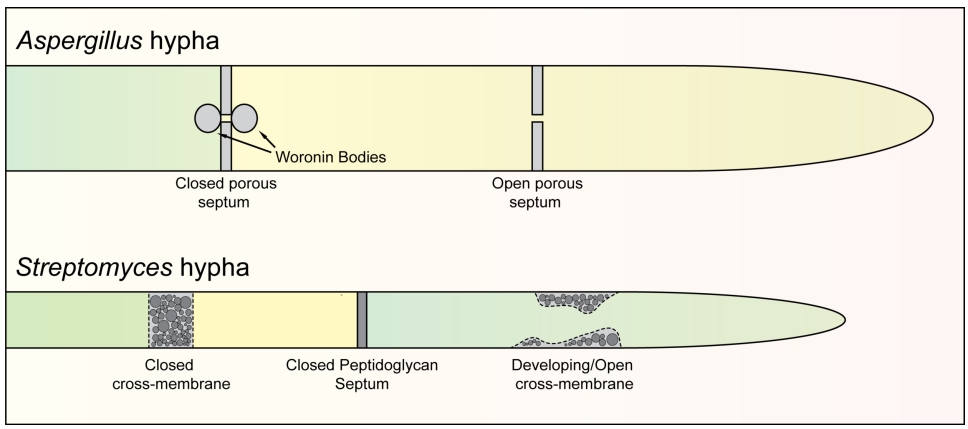

Figure 3. Compartmentalization is crucial for generating heterogeneity in filamentous organisms. Filamentous fungi and streptomycetes form hyphae that are compertmentalized by septa. In addition to septa, streptomycetes also form so-called cross-membranes for compartmentalization. In fungi, septa can be either open or closed depending on the presence of so called "Woronin bodies". Closed septa prevent molecules to freely diffuse between adjacent compartments. In streptomycetes, extended cross-membrane structures prevent cytoplasmic streaming, thereby generating heterogeneity within filaments.

MECHANISMS UNDERLYING HETEROGENEITY IN FILAMENTOUS MICROBES

Inter-hyphal and inter-compartmental heterogeneity

The hyphae of streptomycetes and the higher fungi (i.e. ascomycetes and basidiomycetes) are compartmentalized by cross-walls (also called septa). In streptomycetes, some of these cross-walls have channels, which potentially would allow streaming of the cytoplasmic content, although this has never been demonstrated directly [98, 136-138]. In addition to cross-walls, recent work has shown that extended membranous structures are able to spatially and functionally organize the vegetative mycelium of streptomycetes [98, 137] (Fig. 3).

These cross-membranes are responsible for the formation of the alternating pattern of viable and dead hyphae in the early MI mycelium and also block the diffusion of cytoplasmic proteins in 29% of the cases. Cross-membranes might thus maintain heterogeneity between compartments of the same cell by preventing molecules to mix by diffusion or streaming.

Septa of filamentous fungi consist of invaginations of the cell wall that are aligned with the plasma membrane. Septa have a central pore of 50–500 nm [139, 140] that allows streaming of cytosol and even organelles, thus enabling cytoplasmic mixing throughout the mycelium. Yet, the pores of *Aspergillus* can be reversibly opened and closed by peroxisome-derived organelles called Woronin bodies [136] (Fig. 3). The absence of Woronin bodies prevents septal closure, thereby abolishing the possibility to maintain long-term heterogeneity in cytosolic composition between neighboring compartments and/or hyphae [136]. It should be noted that even an open septum can maintain differences in cytosolic composition due to differential gene expression. In this case, however, heterogeneity can be only maintained in a minutes time-frame. Yet, this may be sufficient for some developmental processes to be initiated [271, 272]. Together, an arrest or reduction in cytoplasmic streaming between adjacent compartments can maintain long term heterogeneity in RNA and protein composition. Notably, the plugging of septa via Woronin bodies has no effect on inter-compartmental transport of glucose [271]. This is explained by the fact that *Aspergillus* uses permeases to enable the selective transport of metabolites. In this scenario, inter-compartmental and inter-hyphal heterogeneous distributions are only obtained for those components that cannot cross the selective plasma membrane of septa (e.g. large proteins, ribosomes, organelles, and metabolites that lack a permease in the plasma membrane lining the septal cross wall).

Inter-pellet heterogeneity

The aggregation of distinct particles is a driving factor for generating size heterogeneity between pellets. Aggregation in streptomycetes is mediated by extracellular glycans on the surface of germlings and young mycelia [142]. These glycans are produced under control of the *cslA/glxA* operon and the *mat* cluster [15, 143-146]. The structure of the glycan produced by CslA and GlxA is still unknown, while the polymer produced by the Mat proteins is poly-β-(1,6)-N-acetylglucosamine (PNAG) [273]. Abolishing the formation of these glycans yields particles whose size is no longer bimodally distributed and that are hence more homogeneous in size. Aggregation is a critical factor in generating size heterogeneity also in filamentous fungi. In this case, aggregation is a two-step process. The first phase involves the aggregation of ungerminated spores and is followed by a second aggrega-

tion phase that occurs between germlings [148]. Mutants of *A. niger* affected in the formation of spore-associated pigments yield more homogeneously-distributed pellets [269]. The underlying mechanism is not known but one wonders whether filamentous microbes make use of size heterogeneity to optimally adapt to the environment. Micro-colonies of different size might experience environmental stimuli differently and may thus differently react to these cues.

PARALLELS AND DIFFERENCES BETWEEN UNICELLULAR AND MULTICELLULAR SYSTEMS

The cellular architecture of filamentous microbes generates layers of complexity that are rarely observed in unicellular species and that result in the multiscale heterogeneity discussed in this review. As a result of this complexity, some of the well-described aspects of phenotypic heterogeneity have not yet been characterized in filamentous microbes. Mechanisms analogous to those reported in unicellular systems, such as intrinsic noise in transcription and translation, are inherent to the behaviour of their machinery, and therefore a likely source of heterogeneity in any biological system, including filamentous microbes. Unique to filamentous microorganisms is the syncytial nature of mycelia. The distribution of DNA (i.e. nuclei in fungi or chromosomes in bacteria) may not only differ between compartments, but also within compartments. This, in turn, would result in some regions possessing more copies of a given gene. Such dosage effects are known to bear a profound effect on decision making in a number of cellular systems [274-278].

Additionally, the positioning of nuclei can also result in differential gene expression within single compartments. For instance, paired nuclei in compartments of the mushroom-forming fungus *Schizophyllum commune* can migrate away from each other, resulting in changes in gene expression [279]. Thus, while some of the mechanisms involved in generating heterogeneity could be similar between filamentous and unicellular microbes, some factors (e.g. the presence of inter-compartmental streaming and multinucleate compartments) are probably unique to filamentous microorganisms.

Differences in the mechanisms through which heterogeneities arise might also differ between filamentous bacteria and filamentous fungi. Not only is gene regulation different between bacteria and fungi, but also their sizes differ. The cellular volume of a fungal filament is roughly 100 times larger than that of a streptomycete given the 10-fold larger diameter of a fungal hypha. This may affect the concentration of various intracellular species. As a consequence, noise dynamics might differ in these systems. However, no quantitative measurement of mole-

cules known to generate heterogeneity exist for what concerns filamentous microbes, thus hindering a direct comparison.

One of the most remarkable aspects of microbial phenotypic heterogeneity is its beneficial role in increasing population fitness in the face of changing environmental conditions (see section 2). It is currently unknown whether this is also true for filamentous microbes. Mycelial heterogeneity may be beneficial in terrestrial soils, where spatial and temporal variations exist in for instance the availability of nutrients and oxygen, temperature, pH, and the amount of growth-inhibiting compounds [280]. Considering the saprophytic lifestyle of most filamentous microbes and the close proximity of hyphae within a colony, one would predict a benefit for segregating functions across the colony. This would be particularly useful for acquiring nutrients or secreting costly compounds that ultimately become available to all surrounding hyphae. In this scenario, inter-hyphal heterogeneities in the secretion of enzymes (as those observed in *A. niger*) might very well reflect a division of labour strategy, in that only a subset of hyphae commit to the production of extracellular hydrolases, thereby liberating nutrients that can be taken up by both producing and non-producing hyphae. Another example where heterogeneity could provide fitness benefits to the colony is in the production of antibiotics. However, this awaits further experimental evidence.

CONCLUSIONS AND FUTURE PERSPECTIVES

Striking parallels exist between filamentous fungi and actinomycetes with respect to morphology, heterogeneity and the architecture of mycelia. Despite the increasing number of studies, we have only started to dissect the mechanisms underlying heterogeneity in these organisms. While cytoplasmic streaming in the fungal mycelium has been known for many decades, it has only recently been reported in streptomycetes [137]. Selective blocking of this process, either via Woronin bodies in fungi or membranous structures in actinomycetes, leads to physiological differences between adjacent compartments and zones of the colony. One of the outstanding questions to address is how the external and internal signals are processed and translated into changes in cytoplasmic streaming and phenotypic heterogeneity. We believe that the developments in the field of microscopy will enable us to obtain unprecedented insight into the molecular functioning of these compartment-separating structures within hyphae.

In this review we have described the different forms of heterogeneity that have been reported in filamentous fungi and streptomycetes. Interestingly, apart from inter-colony, inter-zonal, inter-hyphal and inter-compartmental heterogeneity

one may expect the existence of intra-compartmental heterogeneity. Such heterogeneity may be promoted by increasing the compartmental length and reducing the number of nuclei (fungi) or chromosomes (filamentous bacteria). Alternatively, RNAs and pathways that determine the fate of RNA could be spatially and temporally localized in subcellular compartments. More knowledge about the dynamics of nucleic acids in filaments is thus of the utmost importance to advance our understanding of heterogeneity in filamentous microbes.

While it is evident that heterogeneity is beneficial to filamentous microbes in natural environments, this feature is undesirable in industry for two reasons. First, heterogeneity decreases controllability of the fermentation process, and secondly, several lines of evidence indicate that morphology and specific productivity appear to be tightly coupled. For instance, production performance can be increased by reducing morphological heterogeneity (size distribution of pellets) in *Streptomyces* cultures [13, 15, 16, 149]. Generally speaking, smaller micro-colonies are preferable for the production of enzymes, while bigger ones are better suited for the production of antibiotics [9] Promoting increased septation in *S. lividans* results in a reduced pellet size and in turn in increased enzyme secretion [16]. Interfering with mycelial aggregation also results in smaller mycelial particles and increased protein secretion [15]. While some of these phenotypes have solely been explained as the result of the increased growth rates of smaller particles, part of the increased production may be due to the reduced size heterogeneity. Likewise, increased homogeneity could also stimulate antibiotic production, given that mutants of *Saccharopolyspora erythraea* that on average form larger pellets than the parental strain also produce more erythromycin [13]. At the same time, we cannot exclude that hyphae within mycelia of liquid-grown cultures differentiate to fulfil specific functions. In this case, heterogeneous cultures may be more productive. In light of this, it is critical to better understand the molecular mechanisms underlying heterogeneity in filamentous organisms, a quest that might be facilitated in the near future by the increasing power of next-generation sequencing technologies applied at the single cell level and the further advancement of high-end microscopy. Only once these mechanisms will have been unraveled, will we be able to tackle heterogeneity in non-natural settings, with the alluring prospect of enhanced production performances in the biotech sector.

Chapter 3: Aggregation of germlings is a major contributing factor towards mycelial heterogeneity of *Streptomyces*

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ABSTRACT

Streptomycetes are filamentous bacteria that produce numerous valuable compounds, including the majority of clinically used antibiotics. At an industrial scale, most of these compounds are produced in bioreactors. Growth of streptomycetes under these conditions is characterized by the formation of complex mycelial particles, whose sizes follow a bimodal distribution. Given the correlation between specific productivity and morphology, this size heterogeneity poses a potential drawback in industry. Recent work indicates that mycelial morphology is controlled by a number of genes that encode proteins required for the synthesis of cell surface-associated glycans. Using a quantifiable system based on fluorescent markers, we here show that these glycans mediate aggregation between germlings and young mycelia, yielding mycelial particles that originate from many different individuals. We also demonstrate that at later time points aggregation between distinct particles is no longer detectable. Notably, the absence of the corresponding glycan synthases yields mycelia that are homogeneous in size, indicating that mycelial aggregation is a driving factor towards size heterogeneity. Given that aggregation is widespread between streptomycetes and can also occur between different strains, our work paves the way to improve *Streptomyces* as a cell factory for the production of known metabolites, but also to possibly discover new ones.

INTRODUCTION

Streptomycetes are filamentous bacteria that are renowned for their ability to produce several types of bioactive compounds. Standing out among these are a plethora of antibiotics, but also numerous antiviral compounds, anticancer agents and molecules that suppress the immune system [1, 7]. Due to their saprophytic lifestyle, streptomycetes also show a remarkable capacity to produce and efficiently secrete various hydrolytic enzymes that allow them to degrade almost any naturally occurring polymer [8]. The extensive industrial use of streptomycetes does not come as a surprise when these factors are taken into account. In contrast to most other bacteria, streptomycetes grow by forming thread-like cells called hyphae [150]. These hyphae extend at their tip, while new hyphae emerge via subapical branching of pre-existing ones. This mode-of-growth leads to the construction of complex networks of interconnected hyphae called mycelia. When grown in liquid, these mycelia display markedly different morphologies [9]. Some strains, including the industrial workhorse *Streptomyces lividans*, predominantly form dense mycelial particles called pellets. In contrast, other strains form so-called mats, which are loosely entangled and scarcely dense mycelial networks [9]. Notably, a strong correlation exists between morphology and productivity in streptomycetes [9, 13, 16]. While growth as pellets is preferred for the production of antibiotics, it is suboptimal for the production of enzymes. For efficient enzyme production mycelial mats are more suitable, which relates to the fact that nutrients are more easily accessible to all hyphae. In fact, hyphae in the central part of pellets often suffer from nutrient stress leading to a phase of programmed cell death [10, 11]. To further complicate matters, recent work from our lab demonstrated that liquid-grown *Streptomyces* cultures are heterogeneous and contain at least two populations of mycelial particles that differ in size [12]. This size heterogeneity was observed across a range of different streptomycetes and growth media, suggesting that it has a genetic basis.

In the past few years, several genetic determinants involved in mycelial architecture have been identified in *S. lividans*. These include among others the recently discovered *mat* genes [15], as well as *cslA, glxA* and *dtpA* [143, 146, 150]. The common feature of these genes is that they encode proteins that are involved in the synthesis of extracellular glycans. Failure to synthesize these glycans abolishes pellet formation and leads to mycelia with an open, mat-like morphology [9]. This indicates that extracellular glycans play a pivotal role in shaping *Streptomyces* pellets, although the underlying mechanism is not known yet. One of the outstanding questions is whether these glycans solely mediate hypha-hypha interactions *within* individual particles or whether they also mediate aggregation of hyphae *between* distinct mycelia. The latter process, hereinafter referred to as aggregation, gener-

ates larger mycelial structures composed of different individuals. Aggregation has been well characterized in filamentous fungi, such as the ascomycete *Aspergillus niger* [148]. Here, aggregation was shown to be a two-step process that starts immediately after inoculation with the clustering of conidia. A second aggregation step coincides with the onset of germination, during which groups of germinated conidia aggregate to give larger particles. In streptomycetes, the clumping of mycelial particles has been noticed in *Streptomyces aureofaciens* [122], although the extent of aggregation and factors involved in this process have remained obscure.

In this study we show that aggregation is restricted to the very early stages of growth, leading to the formation of mycelial networks that originate from different individuals. A qualitative and quantitative image analysis approach using fluorescent reporter strains demonstrated that aggregation depends on *cslA*, *glxA* and *matAB*. Excitingly, the absence of these genes yields mycelial particles that originate from individual spores, and which, as a consequence, leads to a homogeneously distributed population. We also show that aggregation in streptomycetes can occur between different wild-type species. Taken together, our data provide the basis to enhance the industrial exploitation of these prolific bacteria.

RESULTS

A quantifiable system to analyse particle aggregation in *Streptomyces lividans*

To study aggregation in liquid-grown cultures, fluorescent strains of *S. lividans* 1326 were created that constitutively express *eGFP* or *mCherry* by placing these genes under control of the *gap1* promoter of *S. coelicolor*. The reporter strains were co-cultivated in TSBS or NMMP medium, resulting in the formation of pellets [12]. When spores of both fluorescent strains were mixed, pellets obtained after 24 h of growth were invariably composed of green and red fluorescent hyphae (Fig. 1A, B). Aggregates of fluorescent germlings were already visible 6 h after inoculation (Fig. 1C, D), indicating that aggregation is an early event (see below). Quantification of aggregation using the COPAS, a flow cytometer for large particles [134], revealed that > 99.8% of pellets contained both red and green fluorescent hyphae, irrespective of the used medium (Fig. 1E, Table 1).

Aggregation is mediated via germlings

The early stages of aggregation of *S. lividans* were monitored using light microscopy (Fig. 2). In the first 2 h after inoculation, only individual spores were detected in the medium (Fig. S1).

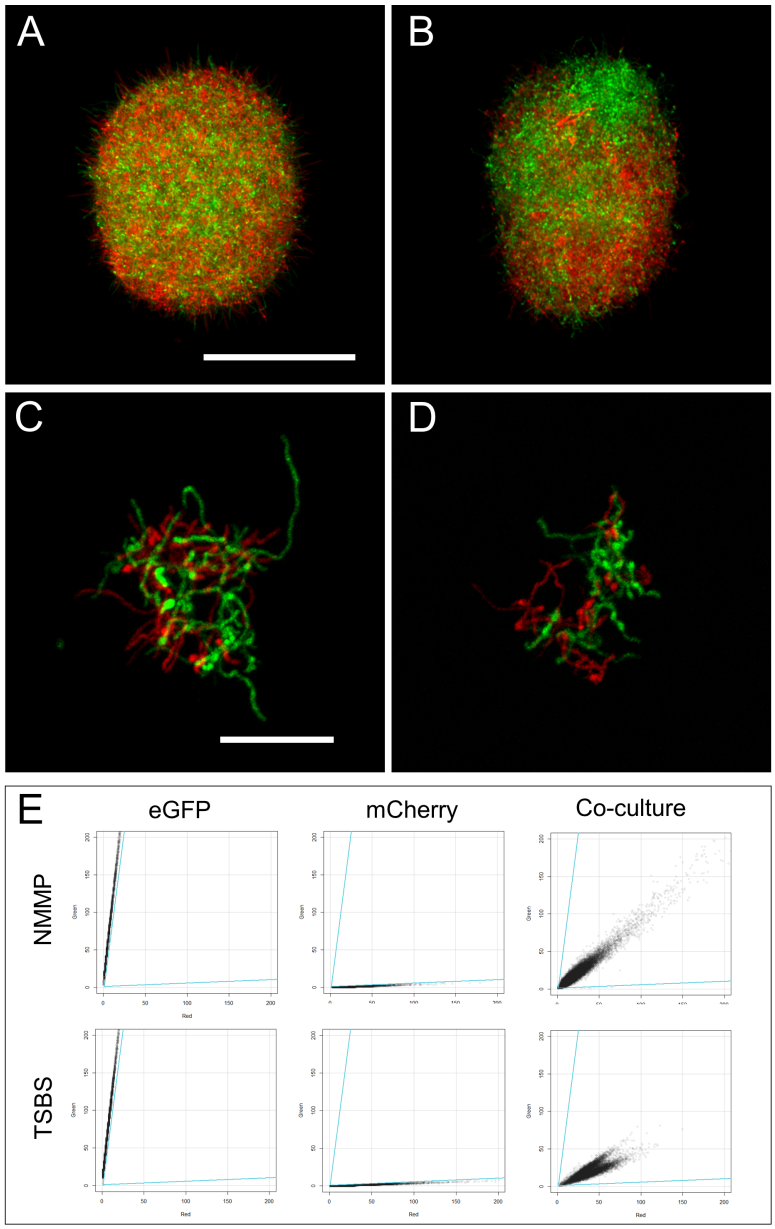

Figure 1. Analysis of aggregation of fluorescent *Streptomyces lividans* strains. Spores of strains expressing eGFP (*S. lividans* pGreen) or mCherry (*S. lividans* pRed) were mixed at the onset of growth in NMMP (A, C) or TSBS (B, D). After 24 h of growth, pellets are composed of both types of fluorescent hyphae (A, B). Aggregates of fluorescent germlings are already detected 6 hours after inoculation (C, D). **E. Quantitative analysis of aggregation using particle sorting.** Each plot represents the fluorescence intensities of pellets in the red (X-axis) and green (Y-axis) channel. The two panels on the left show the green:red ratios of pellets of the eGFP-expressing strain in NMMP and TSBS medium, while the middle two panels reveal the green:red ratios of pellets of the strain expressing mCherry. The panels on the right represent the fluorescence intensities of pellets in the co-cultivations of both fluorescent strains, which indicate that almost all pellets express both reporters. Scale bars represent 100 µm (A, B) or 20 µm (C, D).

Time (hours)	TSBS	NMMP
0	>99%	>99%
2	>99%	>99%
4	>99%	>99%
6	>99%	>99%
8	98.3 ± 0.89	93.0 ± 0.33
10	31.2 ± 1.3	29.1 ± 2.2
12	22.1 ± 7.8	10.6 ± 2.4

Table 1. Time-dependent aggregation of fluorescent strains in TSBS and NMMP medium. Values represent the percentage of particles expressing both fluorescences when mixed after 0-12 hours of growth.

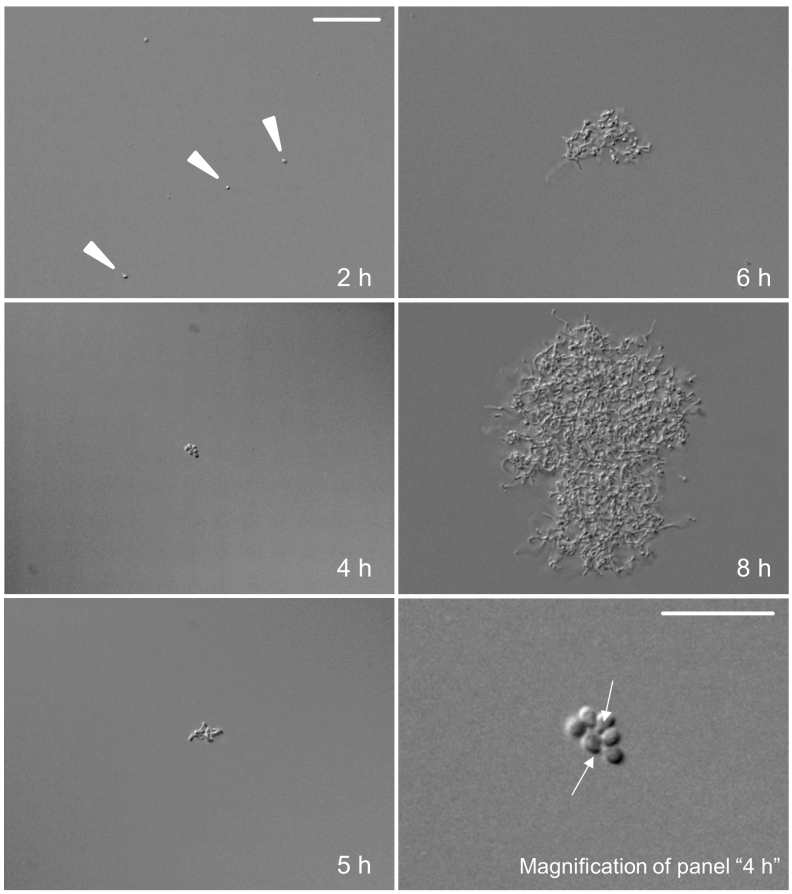

Figure 2. Microscopy analysis of early stages of growth and aggregation. *Wild-type* spores of *Streptomyces lividans* were inoculated in TSBS medium. Only individual spores (arrowheads) are observed 2 h after inoculation. Aggregation becomes visible after 4 h of growth, coinciding with the appearance of germ tubes (see also bottom-right panel). Further aggregation and growth leads to the formation of particles that increase in size over time. The bottom-right panel shows a magnification of the aggregate visible at 4 hours of growth. Arrows indicate germ tubes. Scale bars represent 20 µm in all panels, exception made for the bottom-right panel, in which the scalebar represents 5 µm.

Germination of spores after 4 h coincided with small aggregates that increased in size over time. Germ tubes were consistently visible in these aggregates (see magnification Fig. 2 and Fig. S2), implying that they are required for aggregation. In agreement, individual spores that had not initiated germination were not part of aggregates (Fig. S3). To more closely study whether aggregation specifically involves germlings or can also occur between mature mycelia, co-cultivations were performed of the red and green fluorescent strains that had been grown separately for 2, 4, 6, 8, 10 or 12 h. Analysis of the mixed cultures 24 h after the inoculation of spores indicated that virtually all pellets were composed of red and green fluorescent hyphae if the strains were mixed after 2, 4, 6 and 8 hours of growth (Fig. 3, Table 1). Conversely, when the strains were mixed after 10 or 12 h, aggregation became increasingly rare, as most pellets were only red or green fluorescent (Table 1). Notably, while pellets contained intertwined red and green fluorescent hyphae when the cultures were mixed after 2 h of growth, a patched pattern of green and red mycelial parts was observed in pellets when the cultures were mixed after 8 h of growth (Fig. S4). To exclude that compounds accumulating in the culture medium reduced aggregation at late time points, we mixed washed mycelia of the fluorescent strains in fresh medium after 12 hours of growth. After washing, 3.5±0.6% of particles aggregated in NMMP medium, while in TSBS medium 16.1±6.1% of particles contained both fluorescences (Fig. S5). Given that these values are similar to those observed without washing, these data indicate that aggregation is not hampered by compounds accumulating in the culture broth.

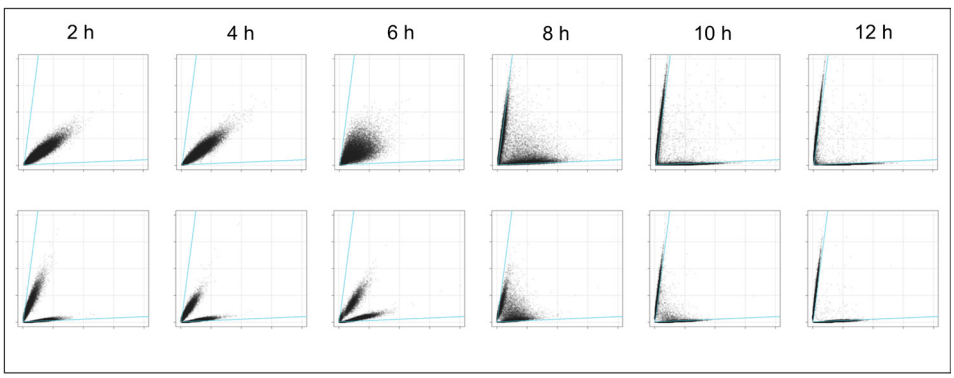

Figure 3. Quantitative analysis of time-dependent aggregation. Fluorescence profiles of 24-hour-old pellets obtained from co-cultures of *Streptomyces lividans* pRed and *Streptomyces lividans* pGreen that had initially been grown separately for 2, 4, 6, 8, 10, or 12 h. Each plot represents the fluorescence intensities of pellets in the red (X-axis) and green (Y-axis) channel determined by particle analysis. The growth media were NMMP (top) and TSBS (bottom). Note that aggregation is strongly reduced when the fluorescent strains have initially been grown separately for more than 8 h.

Genes involved in glycan synthesis are required for germling aggregation

Given the crucial role of extracellular glycans in pellet morphology, we studied whether these polymers also mediate germling aggregation. We therefore created reporter strains of the *S. lividans cslA, glxA* and *matAB* mutants, all of which are affected in glycan biosynthesis [15, 143]. Aggregation of the *cslA* and *glxA* mutants was strongly affected in both NMMP and TSBS, as indicated by the presence of particles that only showed green or red fluorescence (Fig. 4, Figures S6-S9). Quantification revealed that 20.2±3.6% and 3.5±0.7% of particles of the *cslA* mutant contained both red and green fluorescent hyphae in NMMP and TSBS medium, respectively. These values were comparable to those of the *glxA* mutant, where 23.0±3.9% (TSBS) and 0.57±0.2% (NMMP) of all particles were aggregated. Aggregation in the *matAB* mutant was also greatly reduced (Fig. 4), with only 4.8±1.7% (NMMP) and 2.3±0.2% (TSBS) of particles displaying both red and green fluorescent hyphae. These results indicate that proteins involved in glycan biosynthesis play a crucial role in the aggregation of germlings. Considering that the majority of aggregation occurs within the first 12 h of growth (*see above*), we asked whether the expression of genes involved in glycan biosynthesis was changed after 12 h of growth. We therefore isolated RNA from mycelia grown in NMMP and TSBS medium after 12 and 24 h and performed RT-PCR using primers directed against *cslA, glxA, matA* and *matB*. Expression of these genes was detected in both media at both time points (Fig. S10). This implies that the reduced aggregation after 12 hours is probably not caused by changes in the abundance of these glycan synthases.

Germling aggregation leads to size heterogeneity in a medium-dependent manner

Mycelia of the wild-type strain and its *cslA*, *glxA* and *matAB* mutant derivatives were analysed using automated image analysis to assess how aggregation affects the size distribution of mycelial particles. This approach enabled the analysis of particles that are too small for detection with the COPAS (see Methods). Mathematical analysis revealed that the size distributions of all strains grown for 12 h in NMMP was better explained by assuming the presence of at least two populations of particles that differ in size (Fig. 5A, Table 2). The size distribution of the wild-type strain was very similar to that of the *cslA* and *matAB* mutants, in which the number of small particles exceeded that of the bigger particles, as deduced from the skewed participation fractions (Table 2). In contrast, the *glxA* mutant in NMMP medium was more heterogeneous in size, with 60.7% of the particles being part of the population of small particles. Notably, the degree of heterogeneity in the size of wild-type pellets was considerably higher in TSBS medium

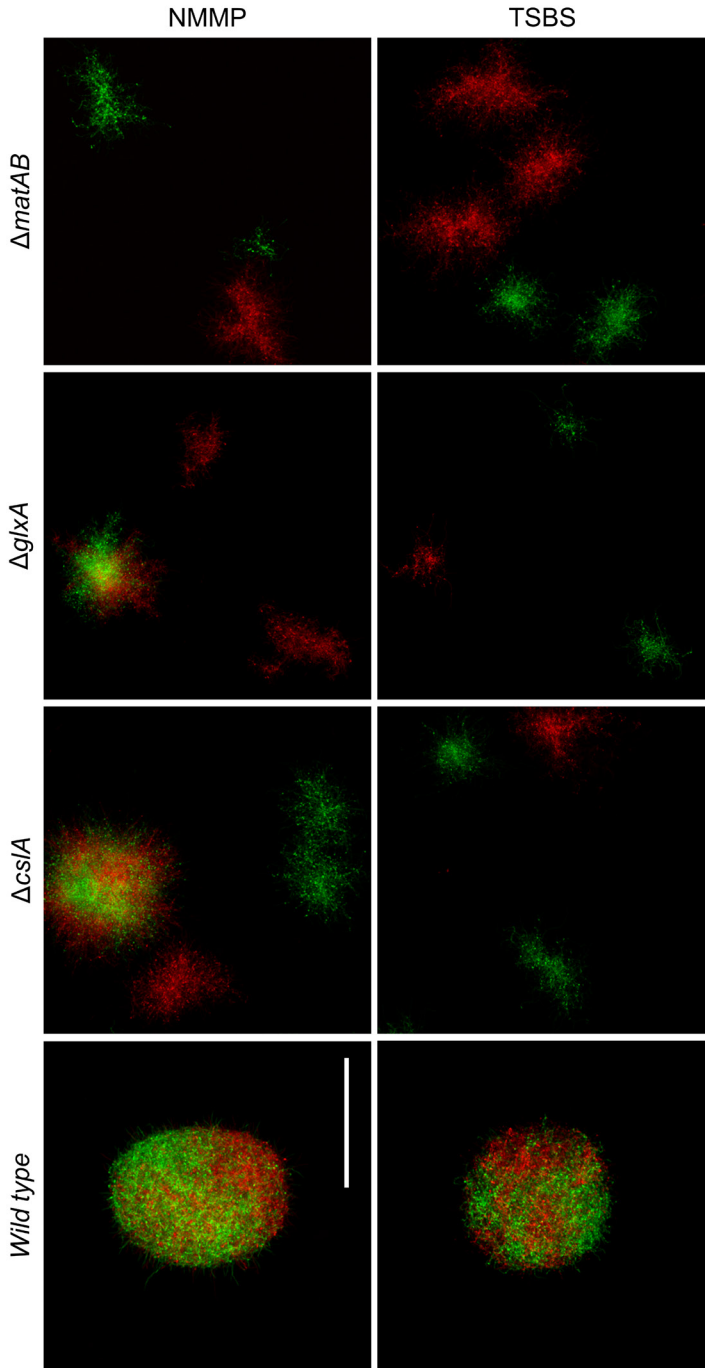

Figure 4. Aggregation depends on glycan synthases. Microscopy images of particles obtained 24 h after mixing spores of red- or green-fluorescent derivatives of the *Streptomyces lividans wild-type* (WT), *cslA, glxA* or *matAB* mutants. Note that the majority of particles only have either green or red fluorescence in the absence of *cslA*, *glxA,* or *matAB* in both NMMP (top) and TSBS (bottom). The scale bar represents 100 µm (*wild-type*) or 200 µm (deletion mutants).

compared to NMMP (Fig. 5B). This is concluded from the fact that in this case 38% and 62% of particles belonged to the population of small and large particles, respectively (Table 2). In contrast, TSBS-grown cultures of the *cslA, glxA* and *matAB* deletion strains revealed a dramatic reduction in particle size heterogeneity. In all cases, the population of small particles accounted for the majority of mycelia in these cultures, with participation fractions of 93%, 99% and 84% for the *cslA, glxA* and *matAB* deletion strains, respectively (Table 2). Taken together, these results demonstrate that aggregation leads to heterogeneity in a medium-dependent manner.

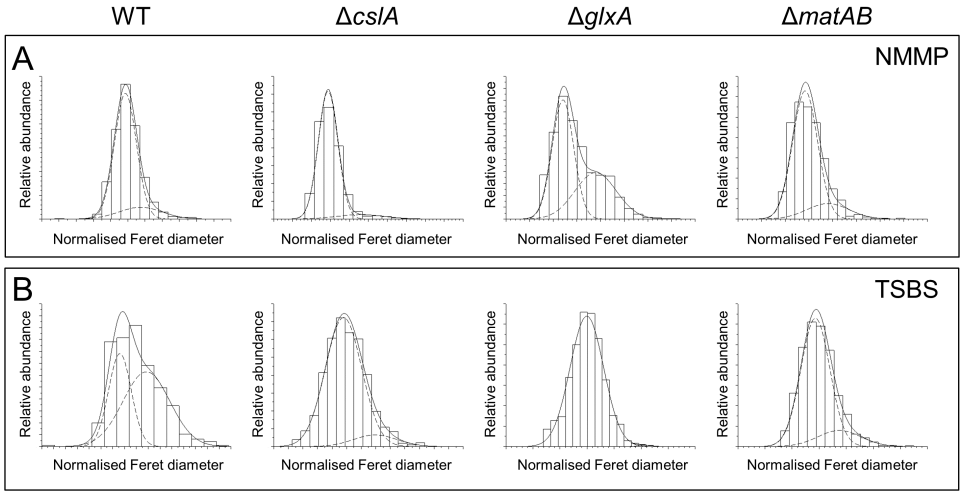

Figure 5. Quantitative analysis of particle size heterogeneity in the absence of *cslA, glxA* and *matAB*. Normalized particle size distributions of the *Streptomyces lividans wild-type* strain, the *cslA* deletion strain, the *glxA* deletion strain and the *matAB* deletion strain in NMMP (A) and TSBS (B) after 12 h of growth. The x-axis represents the normalized Feret diameter, while the y-axis represents the relative abundance. The two dashed lines in each plot represent the two normally distributed populations of particles fitted to the actual measured distribution (solid line). Size heterogeneity is strongly diminished in the absence of the *cslA*, *glxA* and *matAB* genes in TSBS medium.

	NMMP		TSBS	
	Small particles	**Large particles**	**Small particles**	**Large particles**
WT	89.5 ± 7.1	10.5 ± 7.1	34.7 ± 4.2	65.3 ± 4.2
Δ*cslA*	94.9 ± 3.6	5.1 ± 3.6	93.2 ± 2.4	6.8 ± 2.4
Δ*glxA*	60.7 ± 4.9	39.3 ± 4.9	99.0 ± 0.8	1.0 ± 0.8
Δ*matAB*	81.1 ± 2.7	18.9 ± 2.7	84.0 ± 0.6	16.0 ± 0.6

Table 2. Participation fraction of the two populations of particles in liquid-grown cultures of *Streptomyces lividans* strains. Values represent the average participation factors and standard deviations of two independent biological replicates.

Mycelial aggregation of different streptomycetes yields multi-species pellets

To determine whether aggregation is a common trait in streptomycetes, spores of different wild-type strains, i.e. *S. lividans, S. coelicolor, S. scabies* and *S. albus,* were inoculated in NMMP medium, after which the cultures were analysed via light microscopy. Aggregates of germinated spores were detected in all strains 6 h after inoculation (Fig. 6). In the case of *S. lividans* and *S. coelicolor* the aggregates appeared rather compact, unlike those of *S. scabies* and *S. albus* that had a more open morphology. To assess whether aggregation also occurs between different species, fluorescent wild-type strains of *S. lividans*, *S. coelicolor* and *S. scabies* were co-cultured in NMMP and in TSBS. The strains of *S. coelicolor* and *S. scabies* used in this experiment expressed eGFP and mCherry, respectively, whereas *S. lividans* was engineered to produce both fluorescent proteins and was false-colored cyan. Notably, all pellets after 24 h of growth were composed of the three different strains, irrespective of the medium (Fig. 7). These results not only indicate that germling aggregation is common in streptomycetes, but also shows that it occurs between different *Streptomyces* species.

DISCUSSION

Streptomycetes are among the biggest players in the fermentative production of antibiotics, hydrolytic enzymes and numerous other compounds with biological activity. The hallmark of the submerged growth of these microorganisms, the preferred mode of growth under industrial settings, is the formation of interconnected clumps of mycelium with varying sizes [12, 151-153]. The constraints imposed by this heterogeneity are most evident when one takes account of the strong link between morphology and production, which is a well-established concept in the *Streptomyces* field [9, 13, 16]. We here show that the aggregation of germlings is an important factor leading to size heterogeneity. Our findings open new avenues to improve *Streptomyces* as a cell factory in the biotech industry.

Aggregation in *Streptomyces*

In this work we present direct evidence for aggregation of independent particles in liquid-grown *Streptomyces* cultures. Whereas we mainly focus on the model strain *S. lividans* to provide insight into this phenomenon, we show that aggregation is common to a wide range of streptomycetes, and also occurs between the mycelia of different co-cultured *Streptomyces* species. About two-thirds of all *Streptomyces* genomes contain the *matAB* genes [15], including all species used in this work, whereas the *cslA/glxA* operon is present in all streptomycetes [154].

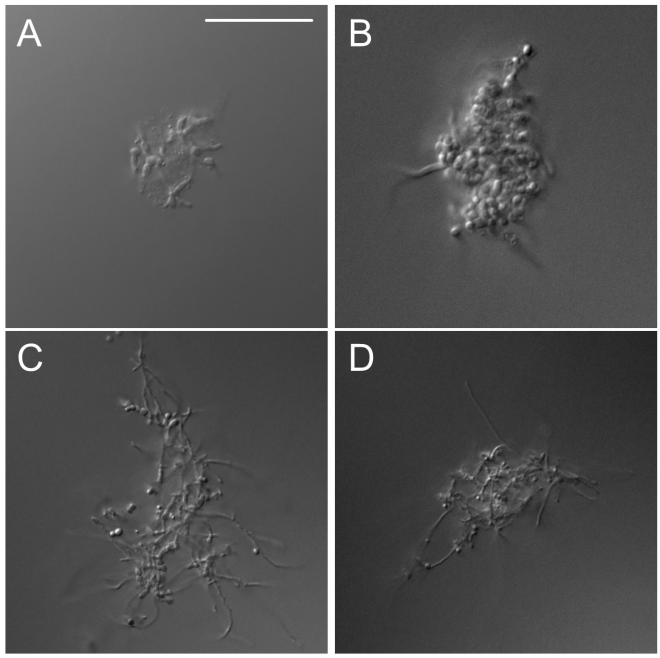

Figure 6. Germling aggregation is common in *Streptomyces*. Microscopy images of aggregates of *Streptomyces lividans* (A), *Streptomyces coelicolor* (B), *Streptomyces scabies* (C) and *Streptomyces albus* (D) taken 6 h after inoculation in NMMP medium. The scale bar represents 10 µm.

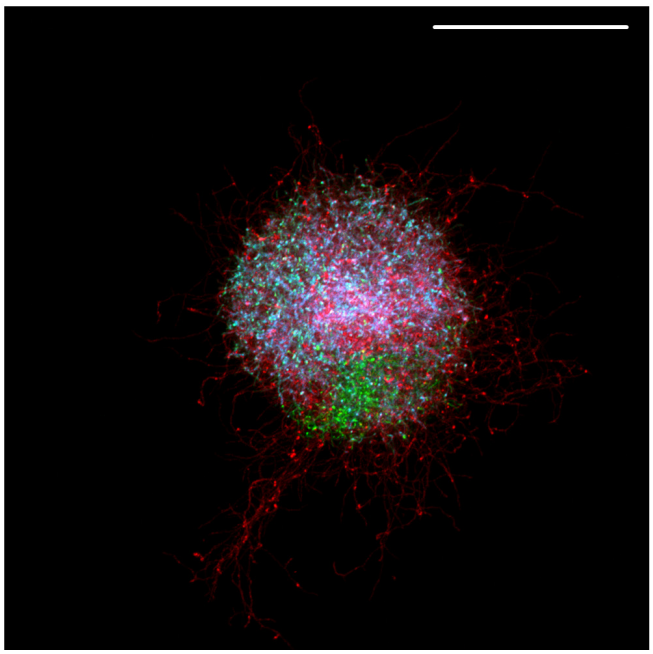

Figure 7. Formation of multi-species *Streptomyces* pellets. Germlings of *S. coelicolor* M512 (green), *S. scabies* (red) and *S. lividans* (false-colored in cyan) aggregate to form multi-species pellets. The image shows a representative pellet obtained after 24 h of growth of the three strains in NMMP medium. The scale bar represents 100 µm.

The broad conservation of these genes across the streptomycetes could explain why aggregation of germlings is so common within this genus. Unlike in filamentous fungi such as *Aspergillus niger* and *Aspergillus fumigatus* [155, 156], we did not find evidence for aggregation of ungerminated spores in streptomycetes. Spores of streptomycetes are covered with amyloid fibrils that render the spore surface hydrophobic [157, 158]. Apparently, this hydrophobicity is not a driving factor for aggregation, as aggregation was only detected after the visible emergence of germ tubes. The proteins that make up these amyloidal structures at the spore surface, called rodlins and chaplins, were previously shown to mediate attachment to abiotic surfaces [144, 158].

As aggregation of germlings proceeds, aggregates may also connect to one another leading to the formation of larger mycelial particles. Interestingly, the greater part of aggregation takes place between 4 and 10 h after the inoculation of spores. These aggregation dynamics are in line with observations performed in a pioneering study on *S. aureofaciens,* which displayed aggregated filaments after approximately 8 h of growth [122]. Given that aggregation is not impeded by molecules accumulating in the culture medium and that the glycans involved in aggregation are constitutively produced, we assume that this time-restricted process is caused by the size of the interacting particles, which may become too large to remain connected due to increasing collision forces or shear stress imposed upon them. As shown in this work, for aggregation to occur between different germlings, extracellular glycans associated with the cell surface are of crucial importance. Notably, aggregation in filamentous fungi is also mediated by glycans. In *A. fumigatus*, for instance, an α-(1-3) glucan becomes exposed upon swelling of spores and mediates conidial aggregation [155]. In *S. lividans*, the glycans produced by CslA and MatAB are key contributors mediating aggregation. The most dramatic phenotype in the absence of *cslA* and the *matAB* genes is observed in TSBS-grown cultures, where aggregation was largely abolished in both deletion mutants. Conversely, in NMMP-grown cultures aggregated particles were detected, although significantly fewer than for the parental strain. These results suggest that other adhesives may contribute to aggregation in NMMP-grown cultures. Differences in the composition of a so-called extracellular matrix under various conditions are in fact not uncommon, and have also been observed in other bacteria during the formation of biofilms [159, 160]. A wide range of molecules normally composes the extracellular matrix of bacteria, which in addition to glycans may contain proteins, lipids and nucleic acids [150, 161-163]. In this respect it is interesting to mention that pellet formation resembles the formation of a free-floating biofilm or floc [164, 165]. In agreement with this, a previous report suggested that extracellular DNA is involved in maintenance of pellet morphology in *S. coelicolor* [166].

Besides providing the first direct indication of aggregation between distinct particles in streptomycetes, our work also demonstrates that this phenomenon can occur between different *Streptomyces* species, resulting in the formation of multi-species pellets. These "synthetic" communities may be useful for the discovery of novel metabolites. Several recent examples indicate that co-cultivations may lead to increased levels of known secondary metabolites, but also to the production of novel compounds [167-170]. Notably, physical adhesion was found to be essential to elicit the biosynthesis of interaction-specific compounds in co-cultures of *Aspergillus nidulans* and *Streptomyces hygroscopicus* [167]. We anticipate that the use of aggregation between germlings of different streptomycetes may represent a useful strategy to discover novel compounds. We are currently using this approach to study whether novel metabolites can be produced by these synthetic communities.

Factors leading to size heterogeneity

Our results highlight the importance of extracellular glycans for the aggregation of independent particles, which ultimately provides the basis for mycelial size heterogeneity in *Streptomyces*. Germling aggregation leads to a range of particles that not only increase in size due to growth, but also due to the incorporation of germlings or small mycelia into pre-existing particles. In addition, larger-sized particles have an increased chance to capture new germlings by accidental collision, thereby further skewing the differences between small and large particles. In contrast, particles of the *cslA* and *matAB* mutant strains only increase in size due to growth, which may explain why they are more homogeneous in size. The fact that mycelia likely grow faster in rich medium can explain why pellets of the *S. lividans* wild-type strain are earlier heterogeneous in size in TSBS medium, compared to the minimal NMMP medium. However, we also cannot exclude that other surface-associated molecules that are differentially produced in both conditions may contribute to generating size heterogeneity. One surprising outcome was the observation that the *glxA* mutant strain is heterogeneous in size in NMMP medium after 12 h of growth, while all other strains were homogenous. More specifically, the fraction of large particles in the *glxA* mutant was considerably larger compared to that of the *cslA* mutant (Fig. 5 and Fig. S11). Notably, these larger particles were significantly denser than those being part of the population of small particles (Fig. S11). One main difference between the *glxA* and *cslA* mutant is the fact that the *glxA* mutant is still capable of synthesizing the glycan produced by CslA, on which GlxA would normally act. Perhaps this residual glycan, although not processed by GlxA, is still capable of inducing some mycelial aggregation leading to the formation of denser particles that comprise the observed second distinct population.

Opposite to aggregation, another factor that directly influences size heterogeneity is fragmentation. By fragmentation we mean the detachment of viable mycelial parts from existing particles. This process could be particularly significant for strains such as the *cslA* and *matAB* mutants, which no longer form dense pellets, and as a consequence are more susceptible to shear stress. Such detached fragments directly lead to a change in the distribution of particles in the culture. By making use of the fluorescent strains described in this paper, we have gathered preliminary evidence for fragmentation (B. Zacchetti and D. Claessen, unpublished results). Whether this process already occurs within the 12-hour timeframe described here remains to be elucidated. We hypothesize that these combined factors provide the basis for the formation of two distinct populations of pellets. We are now building a mathematical model to gain more insight into how these complex processes lead to heterogeneity. As mentioned previously, a strong link exists between mycelial morphology and production of secondary metabolites and enzymes in streptomycetes. The observation that the deletion of *cslA* and the *matAB* genes not only reduces the size of mycelial particles, but also their heterogeneity makes them interesting targets to improve enzyme production. In agreement with this, we recently showed that a *S. lividans* strain lacking the *matAB* genes produces more tyrosinase than the wild-type strain [15]. Given the propensity of many streptomycetes to aggregate and grow heterogeneously, we expect that targeting these processes may be valuable for production purposes in other *Streptomyces* species.

MATERIALS AND METHODS

Strains and culture conditions

The *E. coli* and *Streptomyces* strains used in this study are listed in Supplementary Table S1. *E. coli* strains were grown at 37°C in LB medium, supplemented with antibiotics if necessary. For growth of *E. coli* strains carrying a plasmid with an hygromycin resistance cassette, modified LB medium was used, which contains 1.25 gl^{-1} NaCl instead of 10 gl^{-1}. *Streptomyces* strains were grown at 30°C on solid MS agar plates. For growth in liquid, 250 ml flasks equipped with coils were used, each containing 100 ml TSBS medium (TSB + 10% sucrose) or NMMP medium with glucose as carbon source [171]. Cultures were inoculated with 10^6 spores ml^{-1} (both when single or two strains were used) and were grown at 30°C in an orbital shaker at 180 rpm.

Constructs and transformation

Constructs and oligonucleotides used in this work are listed in Supplementary Table S2 and Supplementary Table S3, respectively. To create pGreen, the promoter region of *gap1* (SCO1947) of *S. coelicolor* A3(2) M145 was amplified via PCR as a BglII-NdeI 450 bp fragment using primers Gap1-FW and Gap1-RV. This promoter has a strong constitutive activity in *S. lividans* (Mangiameli and Vijgenboom, unpublished). The promoter fragment was ligated as a BglII-NdeI fragment into pIJ8630, containing the *eGFP* gene [172]. To create pRed, the integrative vector pMS82 was used, which carries an hygromycin resistance cassette [173]. The *gap1* promoter was amplified via PCR using primers Gap1-FW* and Gap1-RV, thereby introducing KpnI and NdeI sites. The *mCherry* gene was amplified as an NdeI-HindIII fragment using vector pRSET-B as a template [R. Tsien] and primers mCherry-FW and mCherry-RV. The two PCR products were ligated in vector pIJ2925 [174], after which the *gap1-mCherry* fragment was excised with KpnI and HindIII and blunted with T4 polymerase, according to the manufacturer's protocol. The fragment was subsequently cloned into the EcoRV site of pMS82. Restriction analysis and sequencing confirmed the insertion and orientation of the fragment, with the *mCherry* coding sequence running in the opposite orientation relative to the hygromycin resistance gene. The reporter constructs were introduced in *Streptomyces* via conjugation as described [171].

RNA Isolation and RT-PCR

Total RNA was isolated in duplicate from mycelia grown for 12 or 24 h in TSBS or NMMP medium using the RNeasy Mini Kit in combination with the RNAprotect Bacteria Reagent (Qiagen), according to the instructions of the manufacturer. Following RNA extraction, 250 ng of RNA was used to generate cDNA followed by PCR amplification using the SuperScript III One-Step RT-PCR System (Invitrogen). The program for cDNA synthesis and amplification was as follows: cDNA synthesis (55°C, 30 min); 30 cycles of 94°C for 15 s (denaturation), 60°C for 30 s (annealing), and 68°C for 30 s (extension), followed by a final extension for 5 min at 68°C. Specific primers for the RT-PCR are shown in Supplementary Table S3. As a control, primers directed against the 16S rRNA were used as described previously [175].

Flow cytometry

5 ml samples were harvested from liquid NMMP or TSBS cultures and fixed in 4% formaldehyde for 20 min on ice. Biomass was pelleted at 1500 rpm for 10 min, washed twice with phosphate-buffered saline (PBS), and stored at -20° until further use.

Particles were analysed using a COPAS Plus profiler (Union Biometrica) equipped with a 1 mm nozzle as described [12, 164], excluding data with an extinction below 25, which correspond to hyphal fragments and debris. All experiments were performed in duplicate, and for each replicate at least 10,000 pellets were analysed.

To calculate the number of pellets expressing both eGFP and mCherry, the green to red ratio was calculated of all pellets in the single cultivations of the fluorescent strains. The lowest value in the eGFP-expressing strain and the highest value in the mCherry-expressing strain were used as boundaries between which pellets were considered to express both reporter proteins. For fluorescence measurements, the photomultipliers were set at 800 and 900 Volts to detect the green and red signals, respectively. To compensate for the fluorescence bleed-through, the red signal was multiplied by a factor of 0.04 and subtracted from the green signal. The opposite was done for the red signal, with the green signal being multiplied by a factor of 0.05.

Microscopy

Confocal microscopy was performed using a Zeiss Observer Microscope. Samples were excited with laser light at wavelengths of 488 and 543 nm to detect eGFP and mCherry, respectively. Fluorescence emissions were monitored in the region between 505-545 nm for eGFP, while a 560 nm longpass filter was used to detect mCherry. Capture settings were such that no bleed through could be detected. The pinhole size was adjusted to one airy unit (94 micron) for optimal contrast images, which results in 4.5 µm thick coupes. All detailed fluorescence microscopy pictures (i.e. those in Figures 1, 4 and 7) represent Z-projections of stacks with a thickness of 2.2 µm (optimized for optical section thickness).

To quantify aggregation in mutants of *S. lividans*, whole microscopy wells (1 µ-Slide 2 x 9 well, Ibidi GmbH) containing samples of co-cultivations were imaged as a mosaic of 5x5 tiles. The eGFP and mCherry fluorescence emissions were merged to give 8-bit greyscale pictures that were used to detect regions containing fluorescent particles. The mean green and red fluorescence component were subsequently measured in the corresponding regions in RGB pictures. Data were used to quantify the number of particles showing green and/or red fluorescence. Particles with a green to red emission ratio between 1.2 and 0.15 were counted as aggregated, while those with a ratio above 1.2 or below 0.15 were counted as green or red, respectively. For each sample, at least 500 particles were analysed in duplicate. Examples of the images used for quantification are presented as Supplementary Figures S6-S9. Light microscopy was performed using a Zeiss Axioscope A1 upright microscope

with an Axiocam MRc5 camera at a resolution of 37.5 nm/pixel. Automated acquisition of pictures for size analysis of streptomycetes was performed using a brightfield Zeiss Observer microscope.

Size distribution analysis

12 hours after the inoculation of spores, 10 ml samples were harvested from the culture flasks. The biomass was concentrated via centrifugation at 1000 rpm for 30 mins at 4°C and resuspended in Phosphate Buffer Saline (PBS). Micrographs of samples were obtained via automated image acquisition and analysed using ImageJ (Joost Willemse, Ferhat Büke, manuscript in preparation). For size distribution analysis, the maximum Feret diameter was taken as the value representing the size of particles. For each measurement, 1000 particles were analysed to provide a sufficiently large statistical pool for further analysis. Incorrectly analysed particles (e.g. out-of-focus mycelia) were manually removed from the datasets. The size distribution analysis was performed as described [112]. Briefly, the datasets representing the normalized maximum Feret diameter of particles were fit by a probability distribution assuming two normal distributions. This model determines five parameters: the participation fraction (p), two means (μ1; μ2), and two standard deviations (σ1; σ2). For each parameter, the 95 % confidence interval (CI) estimate was obtained by refitting with the model after bootstrapping (1000 replicates) using the open source Scilab language. Datasets with non-overlapping CIs of the mean and $0.025 < p < 0.975$ were considered to be derived from a culture with two populations of pellets.

ACKNOWLEDGEMENTS

We are grateful to R. Tsien for providing plasmid pRSET-B.

Chapter 4: Control of pellet size homogeneity in *Streptomyces lividans* by exploiting the copper-dependent morphogenesis pathway

Boris Zacchetti, Erik Vijgenboom and Dennis Claessen

ABSTRACT

Streptomycetes are used as cell factories for the production of various secondary metabolites and hydrolytic enzymes. At the industrial scale, the vast majority of these compounds are produced in submerged cultures in large-scale fermenters. The growth of streptomycetes under these conditions is characterized by the formation of dense mycelial particles called pellets. A considerable degree of heterogeneity exists within pellets. While these structures consist of actively growing hyphae at the periphery, their central core is characterized by the occurrence of a cellular dismantling process referred to as programmed cell death. Interestingly, heterogeneity is also evident between pellets, which differ in size and function. This size heterogeneity is caused by the stochastic aggregation of germlings during the early stages of growth, a process yielding mycelial particles that consists of various individuals. In this work, we describe an innovative approach to generate homogeneously-sized pellets of the industrial workhorse *Streptomyces lividans.* This approach is based on preventing the time-limited process of inter-particle aggregation and inducing the formation of pellets at a later stage of growth. We demonstrate that homogeneous pellets with sizes ranging between 167 and 276 µm can be obtained and show that smaller pellets are more suitable for enzyme production than large ones. In addition, we report the role of mycelial aggregation in stimulating the activation of programmed cell death during the early stages of submerged growth. Altogether, our data demonstrate that inter-particle aggregation not only causes heterogeneity between pellets, but also physiological heterogeneity within pellets.

INTRODUCTION

Soil actinomycetes belonging to the genus *Streptomyces* are among the microbial workhorses employed in the sector of industrial biotechnology. These filamentous bacteria are renowned as the source of the vast majority of clinically-relevant antibiotics as well as of numerous antifungal and anticancer compounds [1, 7]. Streptomycetes also produce and efficiently secrete several hydrolytic enzymes tailored for the extracellular hydrolysis of polymers such as starch, cellulose, chitin, mannan and xylan [8]. In industry, the majority of these products are obtained in submerged cultures carried out in large scale fermenters. Under these conditions, most streptomycetes form large multicellular aggregates called pellets [9]. Different types of heterogeneity exist between the filamentous cells of streptomycetes (hyphae) inside pellets. While hyphae at the periphery of these structures are mostly viable and actively growing, those present in the core suffer from stresses generally believed to be caused by the limited access of nutrients and oxygen [9]. As a consequence of these stresses, but also of developmental processes, the hyphae in the central part of pellets are dismantled in an orderly process similar to programmed cell death (PCD) of eukaryotic cells [10, 11, 133, 179]. Relatively little is known about the mechanisms underlying PCD in streptomycetes and the triggers thereof.

Besides the one observed *within* pellets, heterogeneity is also evident *between* pellets. More specifically, liquid cultures of streptomycetes contain at least two distinct populations of pellets that differ in size and function [12, 13]. Notably, the size of pellets correlates to production performances in streptomycetes. More specifically, small pellets are preferred for the production of enzymes, whereas large pellets are optimal for the production of antibiotics [13-15]. We have recently shown that the size heterogeneity between pellets is caused by the aggregation of germlings (newly germinated spores) and small mycelial particles into larger assemblies, a phenomenon mediated by cell surface-associated glycans [142]. *Streptomyces* strains that are impaired in the production of these glycans do not form pellets and grow as mycelial particles characterized by an open morphology [146]. The absence of extracellular glycans also abolishes the aggregation between distinct particles during the early stages of growth and results in mycelia that are homogenous in size [142]. This is explained by the fact that in the absence of inter-particle aggregation, mycelia are formed that originate from individual spores and which apparently grow at a similar rate. In *Streptomyces lividans*, two loci have been identified to be involved in the biosynthesis of these glycans. One is the recently discovered *mat* cluster, required for the formation of poly-β-1,6-N-acetylglucosamine (PNAG) [15, 147]. A second locus, containing the transcriptionally-coupled *cslA* and *glxA* genes, is involved in the synthesis and deposition of a cellulose-like

polymer synthesized at the hyphal tips [143-145]. CslA is a family 2 glycosyltransferase, while GlxA is a membrane-associated cuproenzyme. In order to exert its function GlxA requires a copper atom in its active site, which under physiological conditions is delivered by the copper chaperone Sco. Sco is also involved in providing copper to other proteins, including the aa_3-type cytochrome c oxidase Cox [180].

In this work we present a strategy that enabled us to generate homogeneously-sized pellets of *S. lividans* and exploit this approach to correlate enzyme production to pellet size. To this end, we made use of the *sco* deletion mutant. This strain is conditionally impaired in making pellets due to its inability to provide copper to GlxA. However, the addition of micromolar amounts of copper to cultures of this mutant leads to the restoration of GlxA maturation and to the formation of pellets in turn. Since inter-particle aggregation is time-limited [142, 181], we obtained homogenously-sized pellets by adding copper after the early growth phase during which this phenomenon occurs. Using this strategy, we show that pellets can be obtained within a range of different sizes and confirm that smaller pellets are better suited for enzyme production when compared to larger ones. In addition, we report that mycelial aggregation stimulates programmed cell death at early stages of growth. Altogether, these results not only demonstrate that aggregation is the major cause of the size heterogeneity between pellets, but also that it is responsible for generating physiological heterogeneity within pellets.

RESULTS

A versatile system to reduce pellet size heterogeneity in *S. lividans*

We have recently demonstrated that inter-particle aggregation is involved in causing pellet size heterogeneity in *S. lividans* [142]. Since aggregation is limited to the early stages of growth, we anticipated that we could exploit the copper-responsive phenotype of the *sco* mutant [146] to prevent inter-particle aggregation and thereby reduce size heterogeneity, while still being able to generate pellets at later stages of growth. We first verified the copper-dependent morphology and aggregation dynamics of the *sco* mutant by creating derivatives of this strain that constitutively express eGFP or mCherry (using the constructs pGreen and pRed*, respectively) and subsequently co-cultivated these strains in liquid TSBS medium either in the presence or absence of 10 µM $CuSO_4$ (Fig. 1). Without additional copper, we observed small mycelial particles with an open-morphology, the large majority of which only displayed either fluorescence (Fig. 1). Conversely, dense pellets were formed in the presence of copper, most of which contained both green and red fluorescent hyphae (93.5±3.2% of all particles compared to 12.4± 3.2% in the

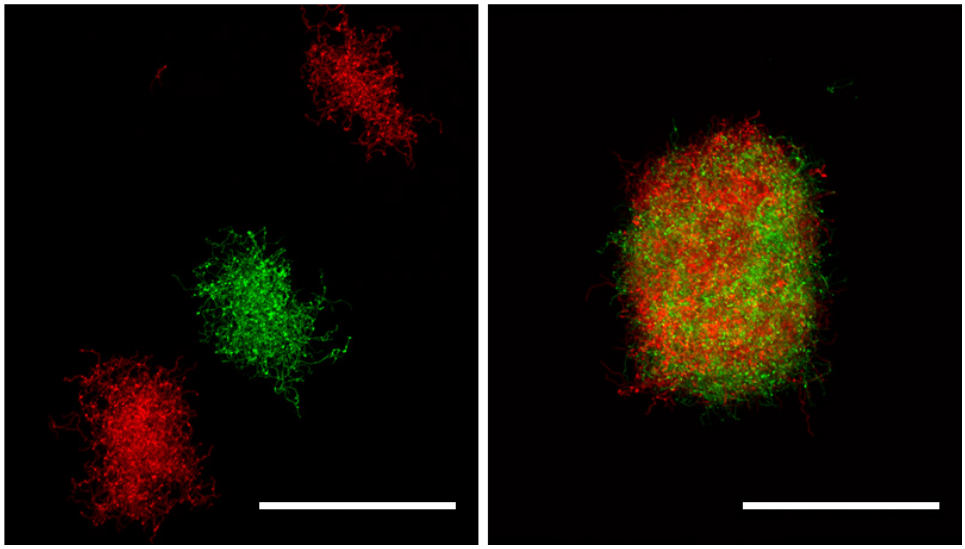

Figure 1. Aggregation of the *sco* mutant of *Streptomyces lividans* depends on copper. Derivatives of the *sco* mutant expressing eGFP or mCherry form small mycelial particles that do not aggregate in TSBS medium (left). When 10 µm $CuS0_4$ is added to the culture, inter-particle aggregation and pellet formation is restored, yielding particles that contain both types of fluorescent hyphae (right). The scale bars represent 150 µm.

Seed culture volume	Participation factor main population	Heterogeneity
10 ml	97.9 ± 1.8	NO
5 ml	99.8 ± 0.1	NO
1 ml	99.5 ± 0.2	NO
500 µl	99.3 ± 0.5	NO
No transfer (+ Cu 0 h)	97.6 ± 2.2	NO
No transfer (+ Cu 18 h)	99 ± 0.7	NO

Table 1. Participation factor and analysis of heterogeneity of pellets of *S. lividans Δsco* in TSBS medium. Pellets were analyzed 24 hours after the addition of different amounts of seed cultures (lacking copper) to copper-containing TSBS medium. The last two rows represent analyses performed on 24-hour-old pellets present in seed cultures to which copper was added either at the onset or after 18 hours of growth.

absence of copper (Fig. 1). Having verified the copper-responsive phenotype in the *sco* mutant, we devised a strategy that would allow us to obtain pellets derived from single spores, which we anticipated would be uniform in size. To this end, we first prepared pre-cultures of the *sco* mutant by inoculating its spores in TSBS medium without copper, i.e. under non-aggregating conditions. After 24 hours of growth, we added 1 ml of these pre-cultures to 49 ml of fresh TSBS supplemented with 10 µM $CuSO_4$. The presence of copper restored pelleted growth in the inoculated mycelia, which at this point were however unable to aggregate with one-another. Notably, image analysis of pellets obtained after 24 hours of growth in the copper-containing medium revealed that their size was normally distributed (Fig. 2, Table 1). This is markedly different in the wild-type strain, which forms pellets

with a bimodal size distribution independent of the growth conditions [12]. We then tested the potential of this system to generate pellets of different sizes, based on the assumption that in the absence of inter-particle aggregation the number of growing pellets equals that of inoculated spores. Consequently, the size that can be reached by pellets is limited by the extent to which these can grow, which in turn largely depends on the availability of nutrients. To test this hypothesis, we suspended different amounts of pre-cultures of the *sco* mutant in TSBS medium containing 10 µM $CuSO_4$. After 24 hours of growth, comparable amounts of biomass were obtained in these diluted cultures irrespective of the amount of transferred pre-culture (Table 2). As shown in Fig. 3A and Table 3 the sizes of pellets obtained in this manner were inversely correlated to the amount of inoculated mycelium and were significantly different between samples ($p<0.0001$).

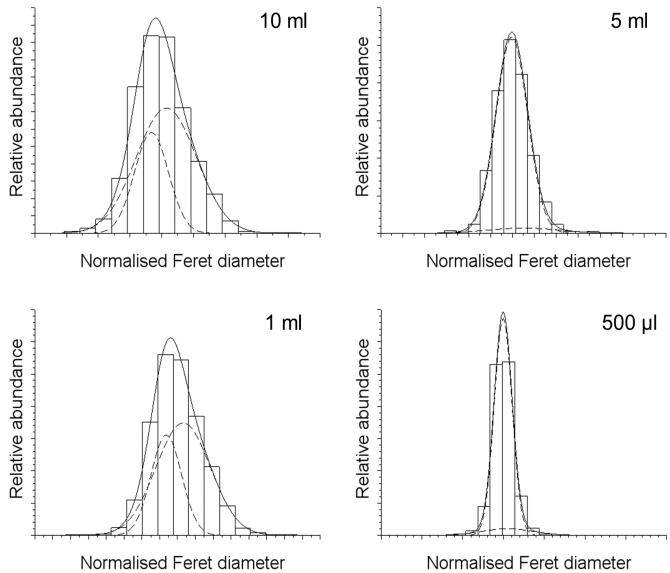

Figure 2. Analysis of particle size hetereogeneity of pellets formed by the *Streptomyces lividans sco* mutant in copper-containing TSBS medium. Normalized particle size distributions of the *S. lividans sco* mutant in TSBS medium. The x-axis represents the normalized Feret diameter, while the y-axis represents the relative abundance. The two dashed lines highlight the two normally distributed populations of particles fitted to the actual measured size distribution (solid line).

Seed culture volume	Dry weight (g l^{-1})
10 ml	5.28 ± 0.01
5 ml	5.84 ± 0.31
1ml	5.91 ± 0.37
500 µl	4.51 ± 0.34

Table 2. Dry weight measurements of 48 hour-old cultures of the *S. lividans sco* mutant forming homogeneously-sized pellets. The volumes represent the seed culture volume added to the copper-containing TSBS medium.

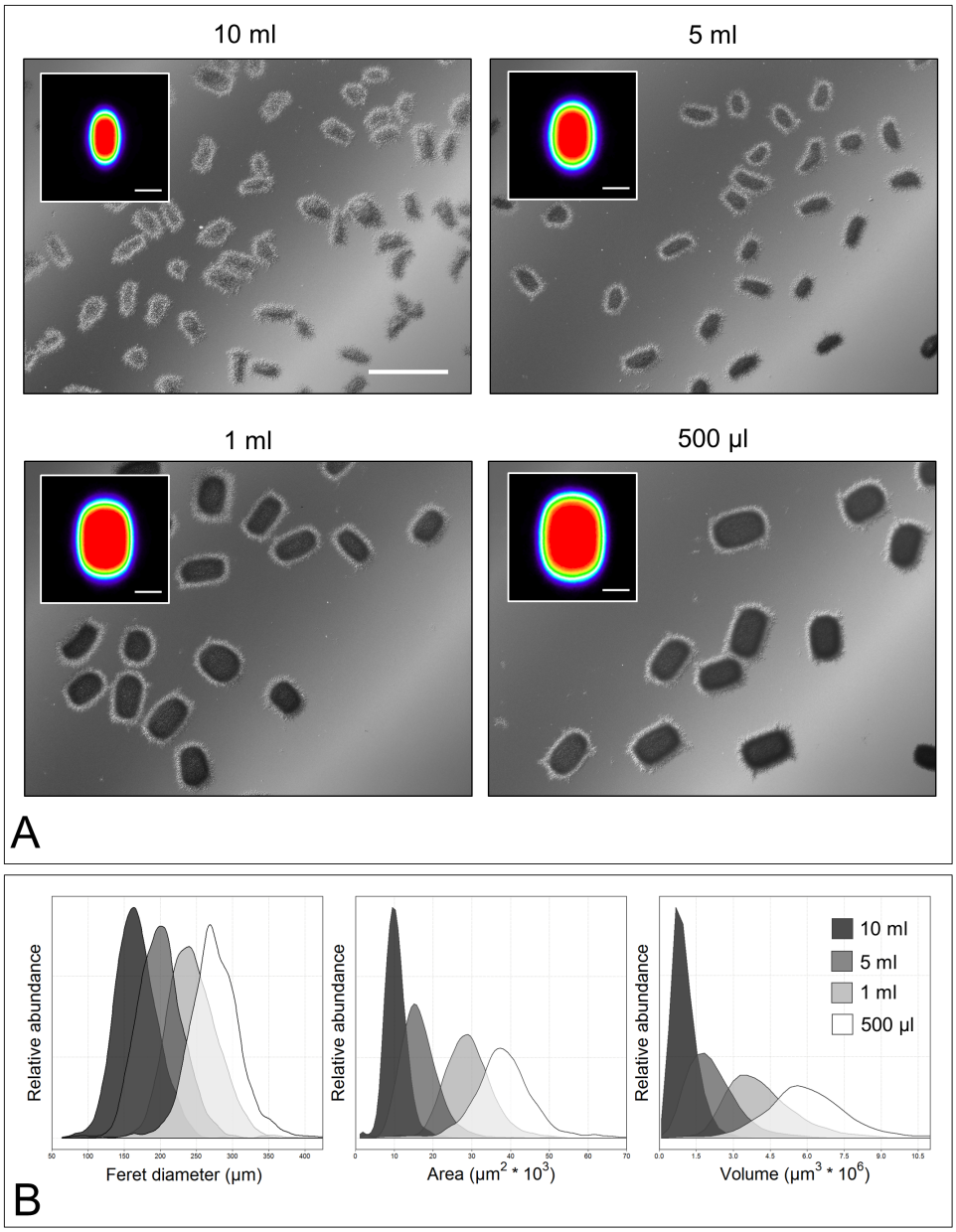

Figure 3. Size analysis of pellets formed by the *sco* mutant of *Streptomyces lividans* in copper-containing TSBS medium. Phase contrast micrographs and size heatmaps (inlays) of pellets obtained after diluting the biomass contained in 10 ml, 5 ml, 1 ml or 500 µl of precultures in 50 ml fresh TSBS medium complemented with 10 µM $CuSO_4$ (A). The color palette used in the heatmaps was chosen such that 95% of all pellets are represented in the red area. (B) Plots representing the Feret diameter, the area and the volume of the differently-sized pellets formed by the *sco* mutant. The scale bars represent 500 and 100 µm in the micrographs and inlays, respectively.

Seed culture volume	Feret diameter (µm)	Area µm²*10³
10 ml	167.1 ± 29.1	10474 ± 3087
5 ml	200.4 ± 32.6	16651 ± 5470
1ml	244.9 ± 34.6	29193 ± 6413
500 µl	275.9 ± 37.8	45995 ± 11540
No transfer (+ Cu 0 h)	151.2 ± 27.5	12071 ± 9446
No transfer (+ Cu 18 h)	107.2 ± 42.1	3278 ± 1998

Table 3. Average Feret diameter and surface area of pellets in cultures of *S. lividans Δsco* in TSBS medium. Pellets were analyzed 24 hours after the addition of different amounts of seed cultures (lacking copper) to copper-containing TSBS medium. The last two rows represent analyses performed on 24-hour-old pellets present in seed cultures to which copper was added either at the onset or after 18 hours of growth.

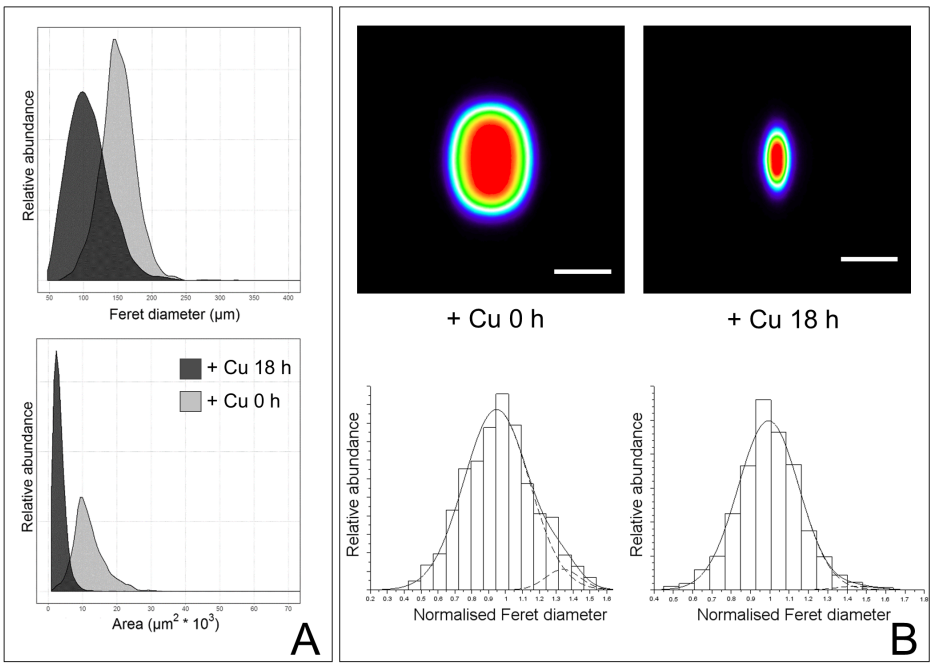

Figure 4. Size analysis of pellets formed by the *sco* mutant of *Streptomyces lividans* in TSBS medium supplemented with copper after 0 or 18 hours of growth. (A) Plots representing the Feret diameter and the area of 24-hour-old pellets formed by the *sco* mutant in TSBS medium supplemented with copper after 0 (light grey) or 18 hours (dark grey). (B) Size heatmaps (top) and normalized size distribution analyses (bottom) of the pellets formed by the *sco* mutant in TSBS medium. The color palette used in the heatmaps was chosen such that 95% of all pellets are represented in the red area. In the size distribution analysis plots, the x-axis represents the normalized Feret diameter, while the y-axis represents the relative abundance of pellets. The two dashed lines highlight the two normally distributed populations of particles fitted to the actual measured size distribution (solid line). Note that pellets formed by the *sco* mutant are homogenous in size. The scalebars represent 100 µm.

The largest pellets, with an average diameter of 276 µm, were obtained when 500 µl of pre-culture were transferred, while the smallest ones were on average 167 µm in diameter after the transfer of 10 ml pre-culture. Excitingly, all pellet sizes were homogenously distributed, indicating that a transient arrest in aggregation had abolished size heterogeneity (Fig. 3). Altogether, these results indicate that the copper-responsive phenotype of the *sco* mutant can be exploited to obtain homogenously distributed pellets of distinct sizes.

The addition of copper partially complements the morphology of the sco mutant in liquid cultures

While the addition of copper restored pellet formation in the *sco* mutant, the sizes of pellets were relatively small compared to those formed by the wild-type strain, and more homogeneous in size (Fig. 4, Tables 1 and 3) [142, 181]. This prompted us to compare the number of spores participating in pellet formation in the wild-type strain and the complemented *sco* mutant. To this end, we quantified the number of particles in liquid cultures (see materials and methods for details) and related it to the number of inoculated spores. Surprisingly, when we calculated the average number of spores contributing to the formation of pellets in the two contexts, we found that whereas only 14±2 spores form one pellet in the *sco* mutant, this value is as high as 946±433 for the wild-type strain. Taken together, these data indicate that the addition of copper partially restores pellet formation in the *sco* mutant.

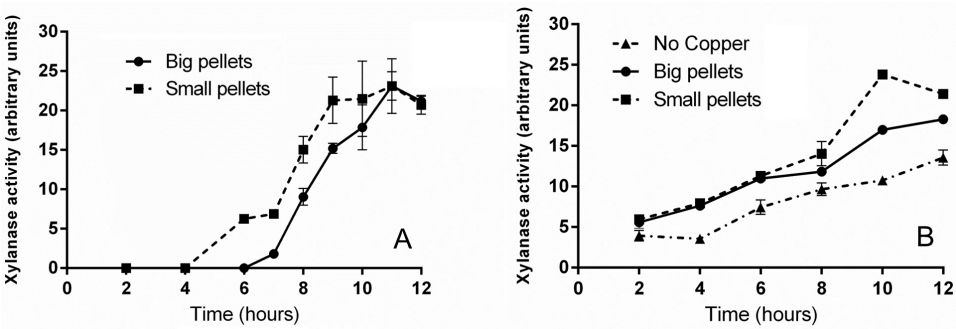

Figure 5. The role of pellet size on the production of a heterologous xylanase. (A) Comparison of xylanase activity in cultures containing pellets with average diameters of 275 (big) and 167 µm (small), and which had an age of 48 hours (see text for further details). The extracellular xylanase activity was measured for 12 hours after resuspension of mycelium into the copper-containing TSBS medium (B). Comparison of xylanase activity in cultures containing pellets with average diameters of 151 (big) and 107 (small) µm, and which had an age of 24 hours. The *sco* strain grown in the absence of copper served as a control. The xylanase activity is higher in cultures containing small pellets, regardless of their age.

The role of pellet size on the production of heterologous xylanase

Previous research has indicated that small pellets of streptomycetes are better suited for the production of enzymes as opposed to larger ones [15, 16]. However, size heterogeneity has never been taken into consideration. We therefore generated homogeneously sized pellets with average diameters of 167 and 276 µm. These 48-hours-old pellets were then used to compare the production of a heterologous xylanase after their transfer into fresh TSBS medium supplemented with 10 um $CuSO_4$. Notably, the production of xylanase occurred earlier in the small pellets and was significantly higher between 6 and 9 hours after the transfer of cultures (Fig. 5A, $p<0.05$). Using this experimental setup, however, we detected abundant mycelial fragmentation during the phase where xylanase activity was quantified (Fig. 6; see also Chapter 5 of this thesis), which complicated correlating pellet size to productivity in a conclusive manner. We therefore modified the experimental setup and used younger pellets obtained by pre-growing the *sco* mutant either in the presence of copper at time 0 h or with copper added at 18 hours of growth. In these experiments, both kinds of pellets were compared in their production efficiency after only 24 hours of total growth, as opposed to those used in the experiment presented above (which had been grown for 48 hours in total). The size difference between these pellets was less pronounced when compared to that between older pellets, but still significant ($p<0.0001$, Fig. 4A). The average diameter of the small pellets was 107 µm, while that of the larger pellets was 151 µm. Also, these younger pellets were homogeneous in size and did not fragment throughout the production phase (not shown). Using this experimental setup, comparable results were obtained to those detected with older pellets, reinforcing the notion that smaller pellets are better suited for the production of enzymes (Fig. 5B).

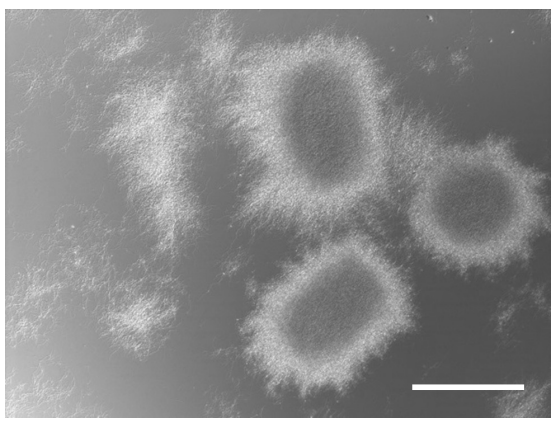

Figure 6. Fragmentation in aging *Streptomyces* pellets. Morphology of mycelium of the *sco* mutant after 48 hours of growth. In addition to pellets, detached mycelial fragments are evident in the culture. The scale bar represents 200 µm.

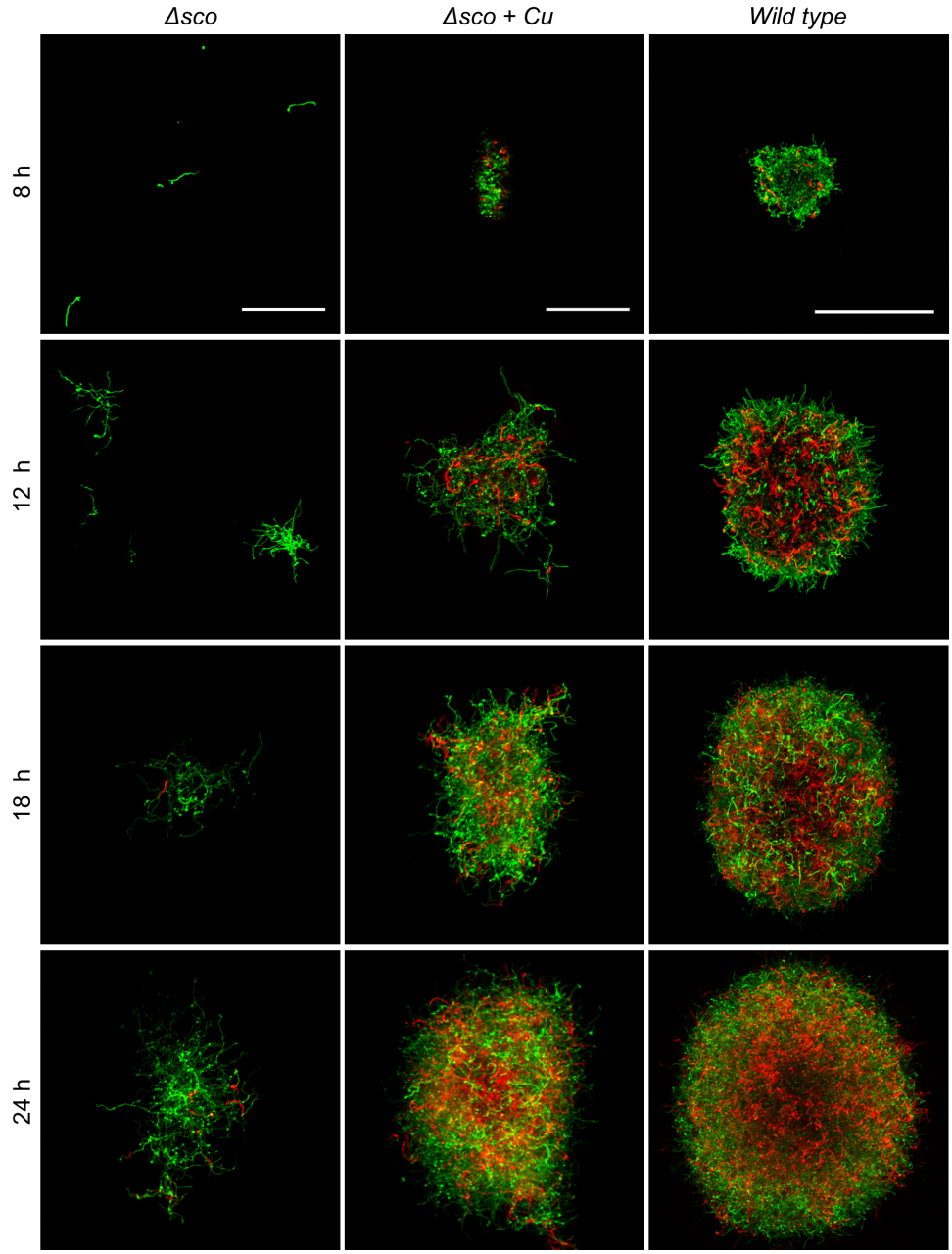

Figure 7. Aggregation triggers programmed cell death in *Streptomyces lividans*. Mycelium of the *sco* mutant of *S. lividans* grown in TSBS medium (left panels) or in TSBS medium supplemented with 10 µm $CuSO_4$ (middle panels) was stained with Syto9 (green) and propidium iodide (red) to detect living and dead mycelium, respectively. As a control, pellets of the *wild-type* strain formed in TSBS medium without additional copper were used (right panels). Dead hyphae are already visible after 8 hours of growth of the *sco* mutant grown in the presence of copper (top middle panel), and in the *wild-type* strain grown without copper (top right panel). In the absence of copper, dead hyphae in the *sco* mutant become evident only after 18 hours of growth. These results imply that aggregation induces programmed cell death. The scalebars represent 50 (*sco* mutant) and 100 µm (*wild-type* strain), respectively.

Early aggregation stimulates the dismantling of hyphae

The copper-responsive phenotype of the *sco* mutant not only offers a switch to gain control over morphology but can also be exploited to characterize the physiological response of mycelia to aggregation. As a proof-of-concept for this principle, we monitored how aggregation affects the appearance of compromised hyphae during the submerged growth of *S. lividans*. We performed this by staining mycelia of the *sco* mutant with Syto9 and Propidium Iodide (PI) in cultures grown either with or without added copper (Fig. 7). During the first 12 hours of growth only green fluorescent spores and germlings were detected in the absence of added copper, with red fluorescent hyphae only becoming evident after 18 hours of growth. In the presence of copper, however, aggregates were formed which already contained red fluorescent hyphae after 8 hours of growth. The portion of mycelium staining with PI increased over time, along with the size of pellets. To exclude that the apparent loss in viability had been caused by the addition of copper rather than by aggregation, we performed a control experiment using the wild-type strain grown in TSBS medium without copper. Notably, the onset of PI staining in the wild-type strain was comparable to that of the *sco* mutant grown in presence of copper (Fig. 7). Taken together, these results show that aggregation triggers an early cellular dismantling process in *S. lividans.*

DISCUSSION

Streptomycetes are largely employed in the biotech sector as producers of various antibiotics and hydrolytic enzymes. Despite their astonishing biosynthetic capacity, these bacteria possess rather unattractive fermentation properties. More specifically, the filamentous mode-of-growth of streptomycetes constraints the ability to maximize product yields, which is in part caused by the high viscosity of the culture broth and the presence of non-viable biomass portions [9]. To further complicate matters, liquid-grown cultures of various streptomycetes contain pellets that are heterogeneous in size [12]. This trait is highly undesirable, given the correlation between pellet size and production [13-15]. In this study we present a system to generate homogeneously sized pellets of the industrial workhorse *Streptomyces lividans*. To the best of our knowledge this is the first report of the finely-tuned pellet size control in a filamentous microorganism. Furthermore, our observations unveil the role of aggregation in promoting the formation of non-viable mycelial parts in submerged *Streptomyces* cultures.

Obtaining homogeneously-sized *Streptomyces* pelets by blocking inter-particle aggregation

The experiments described in this chapter indicate that pellets derived from single spores have sizes that are normally distributed. This agrees well with our previous observations [142] and unambiguously demonstrates that inter-particle aggregation causes pellet size heterogeneity in submerged cultures of *S. lividans*. Besides the fundamental interest, the system employed here enabled us to compare enzyme production in pellets of different sizes and to confirm previous observations on the relationship between pellet size and production. It should be noted that conversely to previous reports, the comparison carried out in this work was between homogeneously sized pellets. Our experiments confirm that cultures containing small pellets accumulate a heterologous xylanase more rapidly than those containing larger pellets. The faster accumulation of products is a desirable feature of any industrial process, since reduced production times correspond to higher production capacities with fixed equipment costs.

In this work, we made use the the *sco* mutant to provide a proof-of-concept that preventing inter-particle aggregation is crucial to generate homogeneous pellets. Our results provide a solid basis for the development of industrial processes making use of streptomycetes, which should not be limited to the use of the *sco* mutant. In our experiments using this strain, a number of unexpected outcomes complicates in fact drawing firm conclusions on the interdependence of pellet size and overall physiology. These regard for example the only partial complementation of this strain upon addition of copper. Pellets formed by the ''complemented'' *sco* mutant are considerably smaller than those of the wild-type strain and were found to be also less heterogeneous, indicating that aggregation was only partially restored. Reinforcing this notion, a reduced number of germlings was found in these pellets as opposed to those of the wild type strain. This is consistent with a model in which aggregation only provides the basis for heterogeneity, which is subsequently caused by other factors such as different growth rates at the level of single pellets (see thesis discussion for a further explanation). We also found that the open growing particles of the *sco* mutant formed in the absence of copper produce less xylanase than the pellet forming counterpearts. This contrasts with the notion that non-pelleting strains, such as the *matAB* mutant and a strain overexpressing the cell division activator protein SsgA grow faster and produce enzymes more efficiently [9, 16]. We hypothesize that this is caused by the involvement of Sco in the maturation of proteins other than GlxA, most notably the Cytochrome c Oxidase. Although the activity of Cox is dispensable for growth, the *cox* mutant of *S. lividans* is retarded in growth when compared to the wild-type strain [180]. Altogether, these facts highlight the need for alternative strategies

to gain control over inter-particle aggregation in liquid grown Streptomycetes. Notably, one easily tunable parameter that greatly influences this occurrence in submerged cultures is the degree of mechanical stress [181]. Industrial fermenters are typically stirred at low speed during early growth stages, being high levels of dissolved oxygen still available to the growing cells. However, low stirring speeds might scarcely counteract inter-particle aggregation. In this scenario, utilizing higher stirring speeds during the early stages of growth could be favorable with the aim of reducing mycelial aggregation, hence the rise of heterogeneity.

The role of aggregation in causing PCD

One intriguing outcome of the work presented here is the identification of aggregation as a trigger for the initiation of what appears as programmed cell death (PCD). By making use of the phenotypical switch offered by the *sco* mutant we were able to show the early staining of hyphae with PI upon induction of aggregation. Notably, the size of aggregates at 8 and 12 hours is well below the supposed critical size above which transport limitation phenomena are believed to occur [9], indicating that the nutrient and oxygen limitations that accompany pelleted growth only partially explain the presence of dead cells inside pellets [125, 127]. Our observations suggest that a contact-mediated mechanism might be responsible for the onset of PCD. We expect this to be relevant in natural environments where cell-cell contact could serve as a quorum sensing mechanism involved in the initiation of development. Clearly, further work is needed to unravel the fundamental aspects related to this phenomenon.

MATERIALS AND METHODS

Strains and culture conditions

The strains used in this study are listed in Table S1. All strains were grown at 30°C in 50 ml TSBS medium (TSB + 10 % sucrose) [171] in 125 ml Erlenmeyer flasks equipped with coils in an orbital shaker at 160 rpm. All cultures were inoculated with 10^6 spores ml^{-1} (for co-cultures of fluorescent strains $5*10^5$ spores of each strain were used per ml). To obtain homogeneously-sized pellets, the *sco* mutant of *Streptomyces lividans* 1326 was used [180]. A pre-culture was grown in TSBS medium; after 18 h $CuSO_4$ (Merck Millipore) was added to the pre-culture to obtain a final concentration of 10 µM. 6 hours after the addition of copper (hence after 24 hours of growth in total), different amounts of the pre-culture were used to inoculate fresh TSBS medium containing 10 µM $CuSO_4$, to obtain a final volume of 50 ml. For transferred volumes of 0.5 and 1 ml, the pre-culture was added directly to 49,5 ml

and 49 ml of fresh medium, respectively. Larger pre-culture volumes (5 and 10 ml) were first centrifuged at room temperature in 15 ml sterile tubes at 2000 rpm for 10 minutes and thoroughly suspended in a total volume of 10 ml TSBS + 10 µM $CuSO_4$ before being added to 40 ml fresh medium. These cultures were further grown for 24 hours.

Dry weight measurements were performed with freeze-dried mycelium obtained from 10 ml culture broth, after having washed the biomass twice with 10 ml milliQ water. For the quantification of Colony Forming Units (CFUs), samples of liquid-grown cultures were diluted in MQ water before being plated on MS agar plates. To this end, 1 ml of the diluted samples was evenly distributed on the surface by gently swirling the plates, after which these were allowed to dry in the fume hood for 30 min. Colonies were counted after 24 hours of growth at 30◦C. For production experiments, 100 mg of pre-grown biomass in the form of differently-sized pellets (dry weight, calculated by growing cultures in the same conditions and determining the biomass concentration) were brought into 50 ml fresh TSBS medium supplemented with 10 µm $CuSO_4$.

Constructs

The constructs used in this work are listed in Table S2. These constructs were introduced in *Streptomyces* via conjugation as previously described [171].

Microscopy and size distribution analysis

The quantification of aggregation using microscopy was performed as described [142]. For the visualization of viable and dead mycelium, samples were stained with Syto-9 and propidium iodide (PI) (Invitrogen). To this end, Syto-9 and PI were added to samples prior to imaging at a final concentration of 5 µM and 15 µM, respectively. Stained samples were excited at 488 and 543 nm to detect Syto-9 and PI, respectively. The fluorescence emission of Syto-9 was monitored in the regionbetween 505-545 nm, while a long-pass filter at 560 nm was used to detect PI. The size distribution heat maps were created using ImageJ V1.48f. Briefly, the regions of interest (ROI) corresponding to pellets were pasted as binary masks on images consisting of 600*600 pixels, with their center of mass positioned in the middle of the image and the maximum Feret diameter aligned vertically. The obtained images were subsequently summed to create a stacked image. The color palette was chosen such that the region where at least 95% of the pellets are found appears red. The collection of samples and analysis of pellet size distributions was performed as described [142]. A statistical analysis of the significance of differences in pellet size and enzyme production between different conditions

was performed using a one-way analysis of variance (ANOVA), followed by a Tukey or Bonferroni test, all of which were performed using Graphpad Prism V6.01.

Quantification of xylanase activity

The extracellular activity of xylanase was measured with a method adapted from [182]. Briefly, 10 µl of supernatant samples were added to 25 µl of a 0.4% xylan solution in 50 mM phosphate-citrate buffer at pH 6.4 [183] and kept at 50° C for 40 min, after which 140 µl of freshly prepared BCA-$CuSO_4$ (2,2-bicinchoninic acid) solution (Pierce™ BCA Protein Assay Kit, ThermoFisher Scientific) was added and let to react for 30' at 80°C. The increase in the amount of reducing ends in the xylan molecule is a measure of the enzymatic activity and was quantified by determining the absorbance at 562 nm. Given that BCA also reacts with other reducing sugars and proteins, controls were performed in the absence of xylan (supernatant with water) or supernatant (xylan with water). The xylanase activity was calculated by subtracting the absorbance in these controls from that of the reaction mixture.

Chapter 5: Dynamics of pellet fragmentation and aggregation in liquid-grown cultures of *Streptomyces lividans*

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ABSTRACT

Streptomycetes are extensively used for the production of valuable products, including various antibiotics and industrial enzymes. The preferred way to grow these bacteria in industrial settings is in large-scale fermenters. Growth of streptomycetes under these conditions is characterized by the formation of complex mycelial particles called pellets. While the process of pellet formation is well characterized, little is known about their disintegration. Here, we use a qualitative and quantitative approach to show that pellet fragmentation in *Streptomyces lividans* is initiated when cultures enter the stationary phase, which coincides with a remarkable change in pellet architecture. Unlike young pellets, aging pellets have a less dense structure and are characterized by the appearance of filaments protruding from their outer edges. These morphological changes are accompanied by a dramatic increase in the number of mycelial fragments in the culture broth. In the presence of fresh nutrients, these fragments are able to aggregate with other small fragments, but not with disintegrating pellets, to form new mycelial particles. Altogether, our work indicates that fragmentation might represent an escape mechanism from the environmental stress caused by nutrient scarcity, with striking similarities to the disassembly of bacterial biofilms.

INTRODUCTION

Streptomycetes are sporogenic, soil-dwelling bacteria that belong to the phylum *Actinobacteria*. Unlike unicellular bacteria, streptomycetes form so-called mycelia, which are intricate networks of interconnected filamentous cells. As producers of a plethora of valuable secondary metabolites (e.g. antibiotics) and hydrolytic enzymes, streptomycetes are extensively used in the field of industrial biotechnology [1, 8]. For their industrial exploitation, streptomycetes are typically grown in large-scale bioreactors, where they display morphologies that not only differ between different strains, but also greatly depend on the growth conditions [9, 122, 123]. Typically, morphologies vary from small fragments to large mycelial particles with a diameter of several hundred microns. For instance, the model strain *Streptomyces lividans,* an industrial workhorse for enzyme production*,* forms dense mycelial structures called pellets.

Previous research showed that the size of these pellets is highly heterogeneous [12], which poses constraints in terms of industrial exploitability. An important factor driving the size heterogeneity of pellets is the stochastic aggregation of germinating spores, which thereby lose their individuality [142]. Aggregation is mediated by at least two glycans associated with the outer cellular surface. These glycans are produced under control of the *cslA/glxA* operon and the *mat* cluster [15, 143-146]. Whereas the structure of the glycan produced by CslA and GlxA has not yet been resolved, that produced by the Mat proteins was recently shown to be poly-β-(1,6)-N-acetylglucosamine (PNAG) [147]. PNAG is not uniquely present in streptomycetes, but is a commonly identified constituent of the extracellular matrix of bacterial biofilms, where this polymer contributes to the adherence of cells to one another [184, 185]. In addition to genetic factors, environmental factors and culture set-up also influence the growth and morphology of streptomycetes [9]. One poorly-studied process affecting the morphology of mycelia is that of fragmentation. Fragmentation consists in the detachment of mycelial portions from pre-existing particles, an occurrence which has been observed in streptomycetes after prolonged periods of growth in fermenters or under temperature induced stress [11, 100, 186]. Notably, very little is known about the timing of this process in relation to growth and morphology, the fate of the detached fragments, and the role that fragmentation has on culture heterogeneity.

Here, we systematically analyzed the dynamics of fragmentation using a qualitative and quantitative approach. We first show that the architecture of pellets changes when cultures enter the stationary phase. This morphological switch coincides with the detachment of small viable fragments that we show to be able to aggregate with one-another and to form new pellets in presence of fresh nutri-

ents. These results not only enhance our understanding of pellet morphogenesis and growth dynamics in *Streptomyces*, but also show that fragmentation is strikingly similar to biofilm dispersal.

RESULTS

Qualitative and quantitative analysis of fragmentation in *Streptomyces lividans*

With the aim to investigate the onset of fragmentation in liquid-grown cultures, *Streptomyces lividans* 66 was grown in seed cultures for 24, 48 or 72 h. We then used 2 or 10 ml of these seed cultures to inoculate new TSBS cultures, which were subsequently grown for 24 h before being analyzed. Microscopy revealed that transferring mycelium from seed cultures after 24 h yielded pellets with an average Feret diameter of 533 and 483 µm for inoculated volumes of 2 and 10 ml, respectively (Fig. 1, Fig. S1, Table 1). These pellets were significantly larger than those formed after 48 h in the seed cultures, and which had not been transferred to fresh medium.

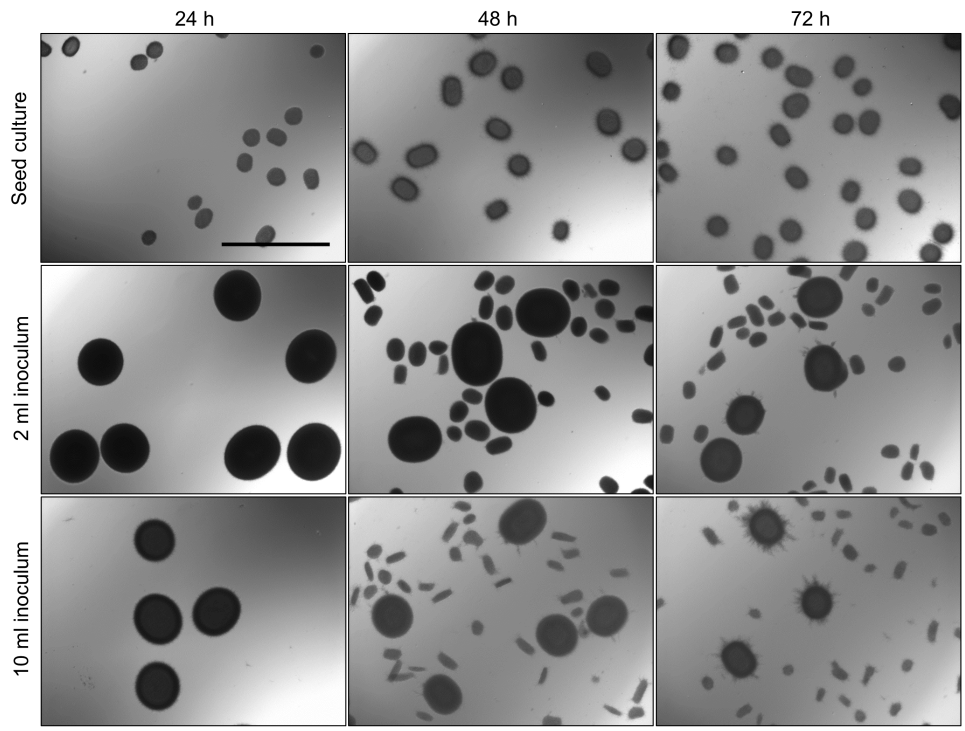

Figure 1. Morphological analysis of pellets of *Streptomyces lividans* 66 in TSBS cultures. The morphology of pellets in seed cultures after 24 (left), 48 (middle) and 72 (right) h of growth in TSBS medium is shown in the top panels. The other panels show micrographs of pellets following the transfer of 2 (middle panels) or 10 ml (bottom panels) of seed cultures in fresh TSBS medium and subsequent growth for 24 h. The scale bar represents 1 mm.

Seed culture age (Feret diameter in µm)	Transferred seed culture volume	Feret diameter in µm
24 h (194 ± 28.3)	2 ml	533 ± 53.4
	10 ml	483 ± 55.2
48 h (278 ± 42.1)	2 ml	250 ± 102
	10 ml	216 ± 100
72 h (233 ± 28.3)	2 ml	184 ± 79.7
	10 ml	154 ± 78.6

Table 1. Average Feret diameter of pellets in 24-hours old liquid cultures, which had been inoculated with 2 or 10 ml of seed cultures of different ages. The average Feret diameter of pellets in the seed cultures is shown inside parentheses.

When 48- or 72-h old seed cultures were used to inoculate new cultures, two populations of pellets with distinct sizes became evident after 24 h of growth; in addition to large pellets (with an average diameter larger than 350 µm), many small pellets with a diameter below 250 µm were found. Notably, the size of small and large pellets in the diluted cultures was reduced when a larger seed culture volume was used as the inoculum, independent of its age (Fig.1, Fig. S1, Table 1). The appearance of small pellets when using seed cultures that were 48- or 72-h old prompted us to investigate whether these originated as a result of the detachment of mycelial fragments from pre-existing pellets in the seed cultures. This hypothesis was reinforced by the observation that the outer edge of pellets after 48 or 72 h of growth was visibly less compact than that of pellets that had grown for 24 h (Fig. 2, Fig. S2). In particular, many filaments were found to protrude from the outer edge of older pellets. To shed light on this, we collected the filtrates of seed cultures after passage through a series of filters of which the last one had a diameter of 5 µm (see Material and Methods). Interestingly, filtrates of seed cultures grown for 24 h contained approximately the same number of CFUs in the 100 and 5 µm filtrates ($1.67*10^5$ ml^{-1} and $1.62*10^5$ ml^{-1} respectively, Table 2).

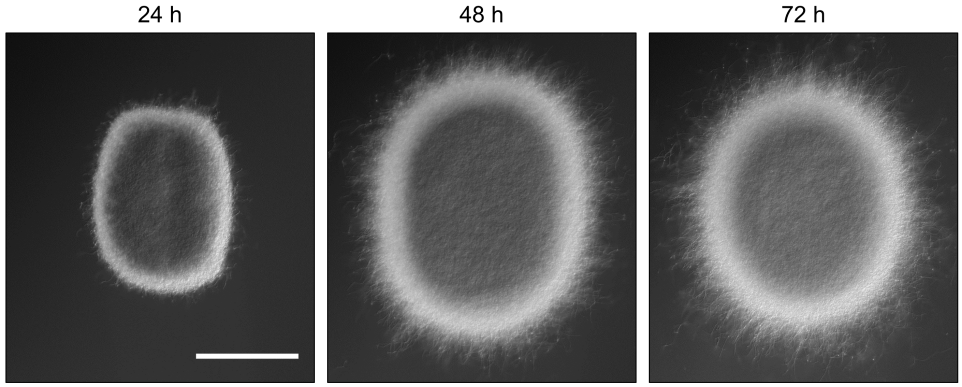

Figure 2. Morphological changes of aging *Streptomyces lividans* pellets. Representative pellets of *Streptomyces lividans* 66 after 24, 48 and 72 h of growth in TSBS cultures. The scale bar represents 100 µm.

This indicates that the vast majority of particles in the filtrates at this stage was smaller than 5 µm. A microscopic analysis revealed that the large majority of these CFUs were viable ungerminated spores, which had been used for inoculation (Fig. 3). This assumption is based on the fact that *S. lividans* has never been observed to sporulate in TSBS cultures. A markedly different situation was observed when the seed cultures were filtered after 48 h of growth. The number of CFUs in the filtrates had increased by an order of magnitude to $1.77*10^6$ ml^{-1} and $1.62*10^6$ ml^{-1}

Culture age (h)	Pore size (µm)	CFUs ml^{-1}
24	100	$1,67 \pm 0.33 * 10^5$
24	5	$1,63 \pm 0.51 * 10^5$
48	100	$1,77 \pm 0.33 * 10^6$
48	5	$1,62 \pm 0.53 * 10^6$
72	100	$2.16 \pm 0.32 * 10^6$
72	5	$1.91 \pm 0.28 * 10^6$

Table 2. Number of Colony Forming Units (CFU) in the filtrates of liquid-grown cultures of different ages. The used filters had an average pore size of 100 and 5 µm. The numbers were obtained by counting the Colony Forming Units (CFUs) after 48 hours of growth on MS agar medium.

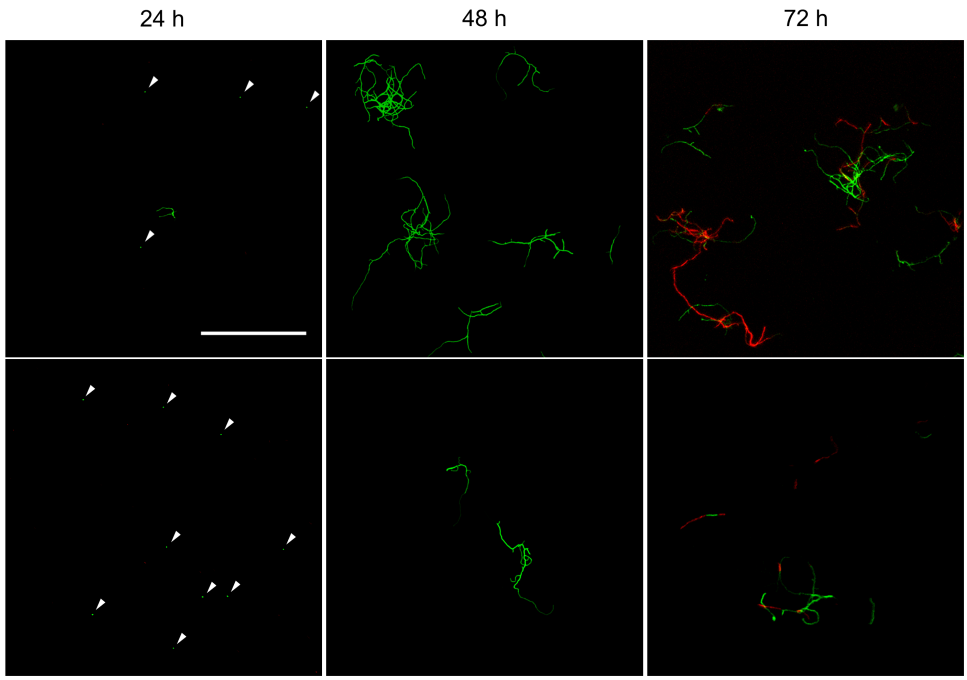

Figure 3. Morphological analysis of filtrates of seed cultures. Filtrates were obtained through the sequential filtering of TSBS cultures which had been grown for 24, 48 or 72 h, through cell strainers with a pore size of 100 (top panels), 40 and 5 µm (lower panels). Prior to imaging, samples were stained with Syto-9 (green) and propidium iodide (red) for the visualization of viable and dead mycelium, respectively. The scale bar represents 100 µm.

when using 100 and 5 µm filters, respectively (Table 2). Notably, the number of CFUs at this stage was higher than that of the inoculated spores, strongly implying that fragmentation had occurred. In agreement with this, a microscopic analysis of the filtrates revealed that small, viable mycelial fragments represented the vast majority of particles in the filtrate. After 72 h, filtered seed cultures also contained a large number of dead hyphae, in addition to viable fragments (Fig. 3).

Fragmentation coincides with nutrient exhaustion in submerged cultures of *Streptomyces lividans*

To better analyze the occurrence of fragmentation throughout growth, we devised an experiment in which all particles smaller than 100 µm (spores and fragments) were first removed via filtration from seed cultures that had been grown for 16, 24, 32 or 40 h (see Material and Methods). The remaining particles were then brought back in the same medium and allowed to further grow for 8 h, after which cultures were again filtered with a 100 µm filter. The number of viable particles in the filtrates was determined by counting CFUs on solid MS agar plates. Using this method, we could quantify the occurrence of fragmentation within time frames of 8 h. $2{,}6*10^2$ CFUs ml^{-1} were found in the filtrate when 16-h old pellets were used. The number of CFUs increased more than 100-fold to $3.2*10^4$ and $7.0*10^4$ CFUs ml^{-1} with pellets of 32 and 40 h, respectively (Fig. 4, Table 3). Consistent with these observations, when the particles in the filtrates were brought into fresh TSBS medium and the biomass quantified that derived from their growth, a comparable trend was observed (Table 3).

Notably, the steep increase in the number of viable particles in the filtrate correlated with the entrance of cultures into the stationary phase (Fig. 4). This prompted us to investigate whether the exhaustion of nutrients correlated directly with the onset of fragmentation. If true, we reasoned that fragmentation would occur earlier in cultures containing less nutrients. We therefore generated seed cultures of *S. lividans* in normal or half-strength TSBS (½ x TSBS) medium, which were used to inoculate fresh TSBS cultures after 12, 18, 24, 30 or 36 h.

Culture age (h)	CFUs ml^{-1}	Dry weigth (g L^{-1})
16	$2.6 \pm 0.1 * 10^2$	N.D.
24	$1.7 \pm 0.3 * 10^3$	0.3 ± 0.1
32	$3.2 \pm 0.2 * 10^4$	3.1 ± 0.2
40	$7.0 \pm 0.6 * 10^4$	3.7 ± 0.2

Table 3. Number of viable fragments released in 8 hours time-frames by pellets collected from liquid-grown cultures of different ages. The numbers were obtained by counting the Colony Forming Units (CFUs) after 48 hours of growth on MS medium. The biomass (dry weight) obtained after inoculating the released fragments in fresh TSBS medium after 24 hours is indicated in g L^{-1}.

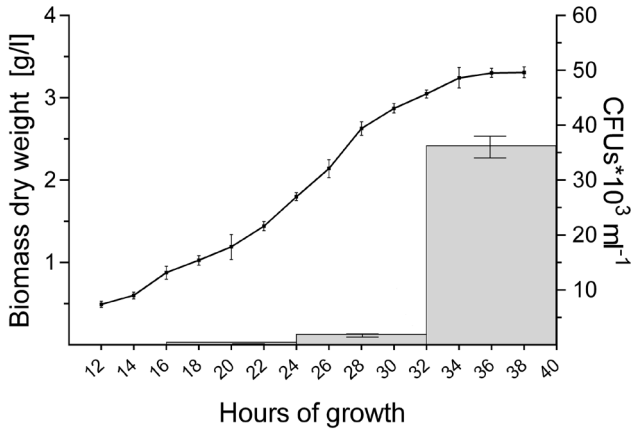

Figure 4. Abundant pellet fragmentation coincides with the entrance of cultures into the stationary phase. Growth curve of *Streptomyces lividans* 66 in TSBS medium, as determined by dry weight measurements over time (line). The grey bars represent the number of CFUs released by the growing pellets over time (see also Table 4).

Fragmentation in half-strength TSBS medium occurred at least 12 h earlier than in normal TSBS medium, as inferred from the earlier detection of small pellets in the diluted cultures (see arrowheads in Fig. S3).

Detached fragments are able to aggregate with one another, but not with mature pellets

In the previous section we have shown that the onset of fragmentation correlates with the entrance of cultures into the stationary phase. To test whether the detached fragments can aggregate with one another to form new composite pellets, we used fluorescent strains of *S. lividans* transformed with pGREEN [142] and pRED*, constitutively expressing eGFP and mCherry, respectively. The seed cultures, containing equal amounts of each fluorescent strain, were diluted after 24, 48 or 72 h in fresh TSBS medium. Consistently with our previous results, we noticed that cultures inoculated with 24-h old seed cultures only contained large pellets, while many smaller pellets were found when 48- and 72-h old seed cultures were used (Fig. S4). Notably, all large and small pellets contained mycelium of both fluorescent strains. This suggests that the fragments contained hyphae of both fluorescent strains at the moment of detachment, or alternatively that detached hyphae expressing either fluorescent protein were able to aggregate to form composite pellets. Visualization of the detached fragments collected from seed cultures of the co-cultured fluorescent strains indicated that they were invariably either green or red fluorescent (Fig. S5). This is consistent with a model in which the detached fragments are able to aggregate with one-another when forming new pellets.

To test this more directly, cultures of the fluorescent *S. lividans* strains were grown first separately for 48 and 72 h. Equal amounts of each culture were subsequently transferred into fresh medium to give co-cultures and further grown for 24 h. Again, both large and small pellets occurred in these co-cultures. Fluorescence microscopy revealed that that 95.3±3.8% and 94.2±3.1% of the small pellets contained both red and green hyphae (Fig. 5) in the co-cultures performed with 48- and 72-h old seed cultures, respectively. Conversely, the large pellets were exclusively green or red fluorescent, showing that aggregation between small detached fragments and pre-existing mature pellets did not occur. To validate this observation, we repeated this experiment with a non-fluorescent wild-type strain and the strain constitutively expressing eGFP (Fig. S6), which would facilitate the detection of fluorescent hyphae associated with non-fluorescent pellets. However, no signs of adhesion between fragments and pre-existing pellets was observed. Altogether these results demonstrate that, when provided with fresh nutrients, the small detached fragments are able to aggregate with one another but not with pre-existing pellets.

Shear stress limits aggregation between large particles

Studies addressing the impact of mechanical stress on the morphology of streptomycetes in liquid-grown cultures have demonstrated that high shear stress typically leads to the formation of small mycelial particles, while large pellets are observed with less shear [187, 188]. Given that we only detected fragmentation after 30 h of growth, we reasoned that mechanical forces exert their influence on morphology largely by counteracting the aggregation between larger particles, rather than promoting fragmentation throughout growth. To test this hypothesis, we performed an experiment in which we separately grew the fluorescent derivatives of *S. lividans* for 12 h, when the aggregation between distinct particles is known to become negligible [142]. We then mixed these cultures in flasks either with or without a metal coil, a stratagem commonly used to regulate the degree of shear stress [171]. Consistently with earlier observations, the presence of the metal coil prevented aggregation between pre-existing particles, as concluded from the observation that only 3.7±1.0% of pellets were either red or green fluorescent (Fig. 6). In co-cultures performed without coils, however, we found that 68.7±0.4% of pellets contained distinct patches of red and green fluorescent mycelium. On average, these composite pellets were larger (352.3±87.7 µm) than those formed in higher shear stress conditions (179.8±37.4 µm). Taken together, these results indicate that mechanical stress plays a role in shaping pellets by limiting the aggregation between large aggregates.

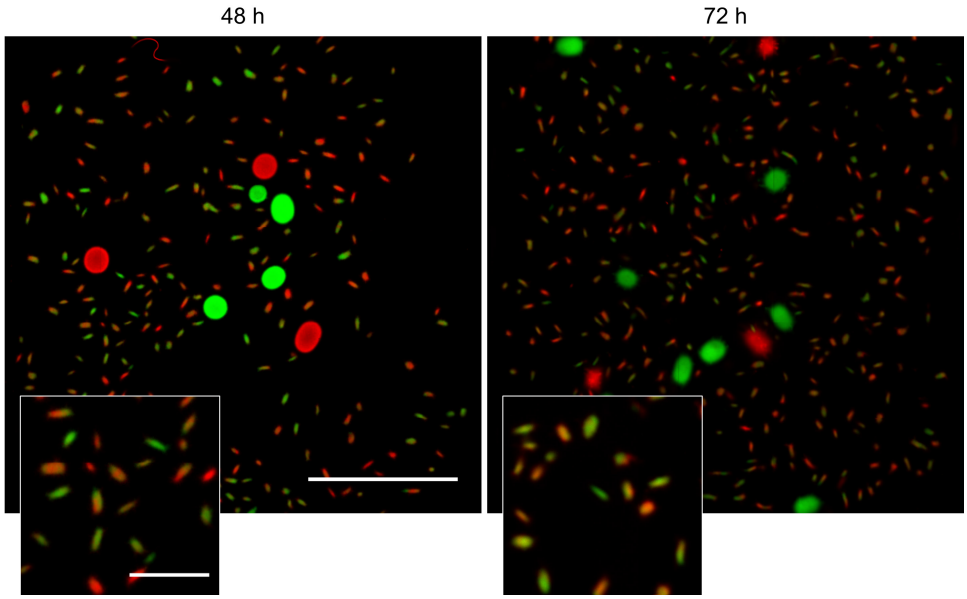

Figure 5: Detached fragments are able to aggregate with one-another during the formation of new pellets. Micrographs of pellets in co-cultures of red- and green-fluorescent derivatives of *Streptomyces lividans* 66 obtained by mixing separate cultures of these strains after 48 (left) and 72 h (right) of growth. The inlays show that the small (new) pellets are composed of both red and green fluorescent mycelium. The scale bars represent 2 mm and 200 µm in the overview pictures and inlays, respectively.

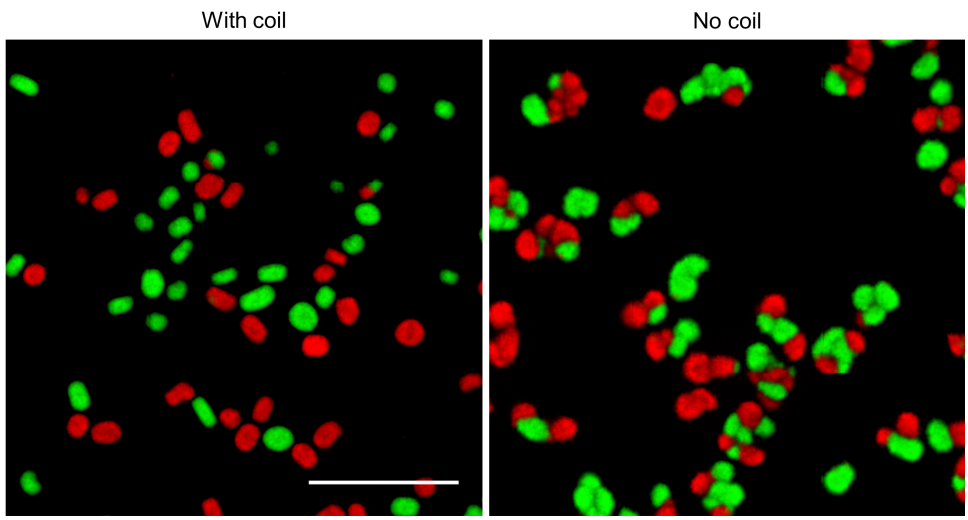

Figure 6. Shear stress limits aggregation between large particles. Micrographs of pellets in co-cultures of the red- and green-fluorescent derivatives of *Streptomyces lividans* 66. These co-cultures were obtained by mixing separate cultures of the fluorescent strains after 12 h of growth and allowing these to grow for 24 h in flasks with (left) or without (right) a shear-inducing coil. The scale bar represents 1 mm.

DISCUSSION

Over the past years we have witnessed a dramatic increase in our understanding of the physiology and growth dynamics of liquid-grown streptomycetes [9]. Dissecting the factors that influence the submerged morphology of these bacteria is of crucial importance to improve their industrial exploitation, given the correlation between morphology and production [13, 16]. We here performed a systematic analysis of fragmentation in submerged cultures of the industrial workhorse *S. lividans* and show that this process is initiated when cultures enter a stationary phase. Notably, when fresh nutrients are provided, the released fragments are able to establish new pellets. This behavior is strikingly similar to the dispersal of biofilms, a strategy that allows the inhabitants of these multicellular communities to colonize other niches when the environmental conditions become unfavorable (e.g. nutrient starvation, presence of toxins).

The similarities between *Streptomyces* pellets and biofilms are remarkable also from a broader perspective (Fig. 7), as previously suggested [166]. As in biofilms, the formation of pellets depends on the ability to synthesize extracellular polymeric substances (EPSs) [189, 190]. *S. lividans* produces at least two EPSs, which are a glycan containing β-(1-4)-glycosidic bonds produced under control of the *cslA-glxA* operon, and PNAG, which is synthesized by the MatAB proteins [15, 143, 147]. PNAG is a widespread component of bacterial biofilms, e.g. those formed by *Staphylococcus*, but also many other species [185, 191]. In *Streptomyces*, these glycans mediate the adhesion between hyphae either belonging to the same or to distinct particles, a feature that causes the deletion mutants of *cslA*, *glxA or matAB* to grow as individual particles with an open morphology [142]. In *S. lividans*, the aggregation of distinct individuals occurs during the early stages of growth. This process has a duration that depends on the degree of mechanical stress in liquid cultures. Here we show that when the level of shear is reduced, larger particles are able to further aggregate, after which they continue to grow as a larger whole. When a threshold size is reached by these particles that impedes further aggregation, they further increase in size as a result of hyphal elongation and eventually form pellets. Pellets may also become fortified at this stage by other extracellular polymers, including amyloid fibrils, extracellular DNA and hyaluronic acid [166]. The involvement of these components in strengthening the adhesion between cells is commonly observed during the development and maturation of various bacterial biofilms [189, 192].

Opposite to pellet formation is the process of their disassembly, commonly referred to as fragmentation. In this study, we provide compelling evidence that fragmentation only commences when cultures enter their stationary phase.

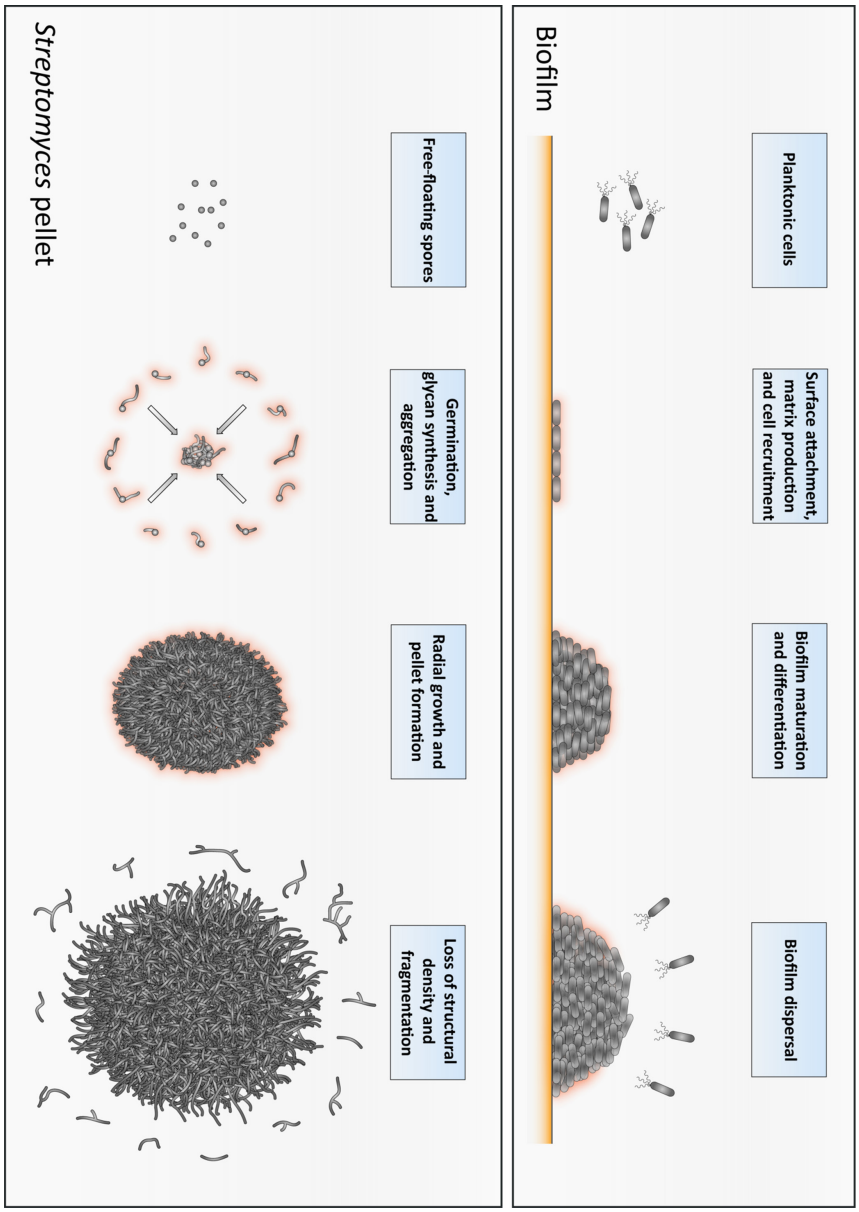

Figure 7. Similarities between the formation and development of biofilms and of *Streptomyces* pellets. (Top) At the onset of biofilm formation, planktonic cells differentiate into non-motile, matrix-producing cells that attach to a surface and recruit other cells by means of their adhesiveness. This leads to the formation of a large community of cells that are embedded in the extracellular matrix. When the conditions become unfavorable, such as when nutrients and other resources become limiting and waste products accumulate, the process of biofilm disassembly is initiated. In this way, cells are released from the biofilm microstructure to disperse to more favorable locations. (Bottom) The growth cycle of *Streptomyces* pellets begins with the germination of spores. The adhesive glycans that cover the surface of the so-called germlings cause them to rapidly adhere to one-another and to other small aggregates. The aggregates further expand radially utilizing the available nutrients to eventually form dense pellets. When the stationary phase of growth is reached, the outer edge of pellets becomes fluffier due to the appearance of protruding hyphae, which detach as fragments. When provided with fresh nutrients, these fragments are able to establish new pellets.

From this moment onwards, small mycelial fragments are found in the culture broth and their number increases rapidly over time. Initially, the majority of these fragments are viable, able to aggregate with one another and to form new pellets when provided with fresh nutrients. In the absence of fresh nutrients, however, these fragments eventually die. Interestingly, the onset of fragmentation coincides with a change in the morphology of pellets. More specifically, numerous hyphae protrude from the periphery of aging pellets (see Fig. 2 and S2). The adhesive forces between hyphae may be weakened at this stage, making these filaments prone to fragmentation as a result of mechanical stress. Given that fragmentation is initiated when cultures enter the stationary phase, we speculate that this process may also be stimulated by the organism itself, similarly to the ordered process of biofilm disassembly [193-195]. Processes related to programmed cell death (PCD), which are known to occur in aging *Streptomyces* pellets, could contribute to the active detachment of fragments [10, 11]. Furthermore, several organisms employ specific hydrolases to degrade the endogenous extracellular matrix and promote the dispersal of cells. An interesting example to mention in this context is the PNAG-hydrolase Dispersin B, produced by *Actinobacillus actinomycetemcomitans* [196]. Dispersin B is also able to counteract the deposition of PNAG on the hyphal surface of *Streptomyces* pellets [147]. Interestingly, a putative homologue of dispersin B is present in *S. lividans.* It thus seems conceivable that pellet-forming streptomycetes could also actively contribute to the disassembly of pellets and the release of fragments as a strategy to maximize the chances of survival in hostile environments.

Altogether, our work provides new valuable insights into the dynamics of pellet formation and disassembly in the industrial workhorse *S. lividans*. Besides the fundamental interest, we envision that these results are useful for a better understanding and more rational optimization of production processes. In particular, the notion that inoculating mycelium of different ages results in differently sized populations of pellets can be very important for the accumulation of biomass in industrial processes, especially when a particular morphology is preferred for the production of the desired product.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The strains used in this study are listed in Table S1. Strains were grown on MS agar medium [171] at 30˚C to prepare spore solutions and to quantify the amount of colony forming units in liquid-grown cultures. Liquid cultivations were performed in a total end volume of 100 ml (thus seed culture volumes of 2 or 10 ml were added to 98 and 90 ml fresh medium, respectively). The used liquid media were TSBS [171] or ½ x TSBS medium (15 g/l tryptic soya broth, 10% sucrose). Cultures were grown in 250 ml unbaffled Erlenmeyer flasks equipped with metal coils, unless differently stated. Cultures were grown at 30˚C in an orbital shaker with a 2-inch stroke at 160 rpm. All seed cultures were inoculated with 10^6 spores ml^{-1}. For co-cultivations, 5 x 10^5 spores ml^{-1} of each fluorescent strain were used. Dry weight measurements were performed with the freeze-dried mycelium obtained from 10 ml culture broth, after having washed the biomass twice with 10 ml milliQ water.

Filtration and analysis of seed cultures

Seed cultures were grown for 24, 48 and 72 h before being sequentially filtered through 100, 40 and 5 µm filters (Falcon® Cell Strainer 100 µm Nylon, Falcon® Cell Strainer 40 µm Nylon, PluriSelect pluriStrainer® 5 µm). The 40 µm filtration step was necessary to prevent clogging of the 5 µm filter. The 48- and 72-h samples were inspected without further preparation, whereas for the 24-h sample 15 ml of the filtrate were concentrated via centrifugation at 1000 rpm for 30 min at 4° C and subsequently analyzed.

Quantification of fragmentation

Large aggregates and pellets present in 50 ml seed cultures at 16, 24, 32 or 40 h, were filtered using a 100 µm filter (Falcon® Cell Strainer 100 µm Nylon). Small particles potentially entrapped in the filtered biomass were removed by four consecutive washing steps, for which 25 ml sterile milliQ water were allowed to pass through the filter. The filtered aggregates and pellets were subsequently brought into sterile Erlenmeyer flasks by rinsing the filter bottom up with 100 ml spent medium, which was obtained by filter-sterilizing the original culture medium using a filtration device equipped with a 0.22 µm filter (Sarstedt Filtropur® V50 500 ml). The cultures were then grown for another 8 h, after which they were again filtered through a 100 µm filter. At this stage pellets were discarded, while the filtrate was used to quantify the number of Colony Forming Units (CFUs, see

below), or to inoculate new TSBS cultures, for which 50 ml filtrates were mixed with 50 ml fresh TSBS medium.

Quantification of Colony Forming Units (CFUs) in liquid-grown cultures

Samples of liquid-grown cultures or filtrates thereof were diluted in MQ water before being plated on MS agar plates. To this end, 1 ml of the diluted samples was evenly distributed on the surface by gently swirling the plates, after which these were allowed to dry in the fume hood for 30 mins. Colonies were counted after 48 h of growth at 30˚C.

Construction of the reporter plasmid pRed*

To create pRed*, the *gap1* (SCO1947) promoter of *Streptomyces coelicolor* A3(2) M145 and the *mCherry* gene were amplified by PCR with the Gap1-FW primer (GATAGATCTCCGAGGGCTTCGAGACC) and the mCherry-RV primer (TTTGCGGCCGCTTACTTGTACAGCTCGTCCAT). A pIJ2925-derivative carrying these elements was used as a template [142]. The obtained PCR product was then ligated as a *Bgl*II-*Not*I fragment into pIJ8630 [172]. The obtained reporter construct was introduced in *S. lividans* 66 via conjugation [171].

Microscopy settings

Fluorescence and light microscopy, including whole-slide image analysis were performed as described previously [142, 197]. At least 300 pellets were analyzed for each sample, both to determine Feret diameters as well as the percentages of aggregated pellets in co-cultures of fluorescent strains. For the visualization of viable and dead mycelium, samples were stained with Syto-9 and propidium iodide (PI) (Invitrogen). To this end, Syto-9 and PI were added to the samples prior to imaging at a final concentration of 5 µM and 15 µM, respectively. Stained samples were excited at 488 and 543 nm to detect Syto-9 and PI, respectively. The fluorescence emission of Syto-9 was monitored in the region between 505-545 nm, while a long-pass filter at 560 nm was used to detect PI. The fluorescence micrographs presented in Fig. S5 were background corrected by setting the fluorescence signal outside spores and hyphae to 0, as to obtain a sufficiently dark background. These corrections, as well as all image-analysis based quantifications, were performed using ImageJ version 1.48f.

ACKNOWLEDGEMENTS

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Chapter 6: Microencapsulation extends mycelial viability of *Streptomyces lividans* 66 and increases enzyme production

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ABSTRACT

Filamentous bacteria of the genus *Streptomyces* produce a large arsenal of industrially relevant antibiotics and enzymes. The industrial production of these molecules occurs in large fermenters, where many streptomycetes form dense mycelial networks called pellets. Pellets are characterized by slow growth and inefficient nutrient transfer and therefore regarded as undesirable from the perspective of henhancing productivity. Although non-pelleting strains have increased growth rates, their morphology also leads to a dramatic increase in the viscosity of the culture broth, which negatively impacts the process dynamics. Here, we applied immobilization of *Streptomyces lividans* 66 using alginate as semi-solid matrix. This alginate-mediated micro-encapsulation increased the production of the extracellular enzyme tyrosinase more than three-fold. The increased production was accompanied by extended viability of the mycelium and a dramatic reduction in the release of intracellular proteins into the culture broth. Our data demonstrate the utility of micro-encapsulation as a powerful technique to achieve higher yields and lower downstream-processing costs of streptomycetes-derived products.

INTRODUCTION

Filamentous organisms are widely used in the field of industrial biotechnology. Of particular relevance are the streptomycetes, multicellular bacteria that produce a vast array of useful metabolites, including over half of the clinically relevant antibiotics, various antitumor agents, immunosuppressants, anthelminthics, antifungals, herbicides and insecticides [1, 92]. In addition, streptomycetes produce and secrete a wealth of extracellular hydrolases, which they employ to degrade the big majority of natural occurring polymers [8]. Streptomycetes grow as filaments (hyphae) that occasionally branch and establish extended cellular networks called mycelia. Growth under industrial settings is characterized by the formation of dense mycelial networks called pellets [142, 198], a phenomenon posing significant drawbacks in terms of industrial applicability. More specifically, pellets only actively grow at the periphery, which limits productivity [15, 199]. Recent work has indicated that it is possible to circumvent pellet formation in *Streptomyces lividans* by interfering with the biosynthesis of extracellular glycans produced by the *cslA-glxA* and *matAB* loci [15, 143]. These glycans mediate the adherence of hyphae, hence leading to the formation of dense clumps and pellets [142]. Deletion mutants of these genes do not form pellets and grow in a dispersed manner. This increases growth and enzyme production rates [15], but comes with the offset of a higher viscosity of the culture broth (our unpublished data). To further complicate matters, pelleted growth appears to be essential at least for the production of some antibiotics [9, 13, 14]. All in all, the mycelial mode-of-growth of streptomycetes results in production processes that are characterized by a complex rheology. This translates into suboptimal mass-transfer properties, heat transfer problems, mechanical and oxidative stress [9, 198, 200].

An attractive alternative to classical fermentations is offered by micro-encapsulation, via the immobilization of cells in a semi-solid scaffold, often sodium alginate [201]. The behavior of a number of micro-organisms has been characterized in this immobilized state, which was found to bear several advantages. In comparison to free-living cells, immobilized cells are better protected from harsh environmental conditions [202-205] and enhanced productivity has been reported [206, 207]. Additionally, immobilized cells are readily recycled or filtered, which reduces the yield loss associated with the accumulation of biomass and facilitates downstream processing [208]. In this study, we report that micro-encapsulation of the industrial workhorse *Streptomyces lividans* enhances heterologous production and purity of the extracellular enzyme tyrosinase. Our data indicate that microencapsulation provides protection against shear stress, thereby maintaining mycelial viability and integrity. This in turn stimulates production and reduces contaminations with proteins released by cell lysis.

RESULTS

Growth of streptomycetes in calcium-alginate microcapsules extends the viability of the mycelium

To study the effect of microencapsulation on the growth of streptomycetes, spores of *S. lividans*, *S. coelicolor*, *S. venezuelae* and *S. griseus* were immobilized in alginate microcapsules (see Materials and Methods). Following the immobilization step, the encapsulated spores were grown in liquid YEME or $NMMP_{mod}$. After 48 h, abundant mycelial growth was detected for all strains in both media (Fig. 1 and Fig. S1). In YEME medium, the encapsulated mycelium of all strains formed highly compact mycelial clumps, while portions of the mycelium that had started to grow out of the microcapsules adopted a more relaxed morphology, whereby individual hyphae could be discerned (Fig. S1). In $NMMP_{mod}$ medium, the encapsulated mycelium formed less compact clumps (Fig. 1). The mycelium of all strains started to grow outside of the microcapsules after 48 h of growth, and became visible as 'spikes' protruding from their edges. With the exception of *S. coelicolor,* non-encapsulated mycelium was found in all strains in the liquid during the late stages of growth (Fig. 1 and Fig S2). This phenomenon was particularly evident in *S. griseus* and *S. venezuelae* at 48 h of growth, while it became apparent in *S. lividans* at 96 h of growth (Fig. S2). The less compact morphology of the encapsulated mycelium in $NMMP_{mod}$ compared to YEME medium, which we anticipated to be beneficial for enzyme production, was a reason to further only use $NMMP_{mod}$ medium. We also focused on *S. lividans*, given the prominent role of this strain for the industrial production of enzymes. To analyze how encapsulation affects mycelial viability, we performed a LIVE/DEAD analysis on the encapsulated and free-living mycelium (Fig. 2).

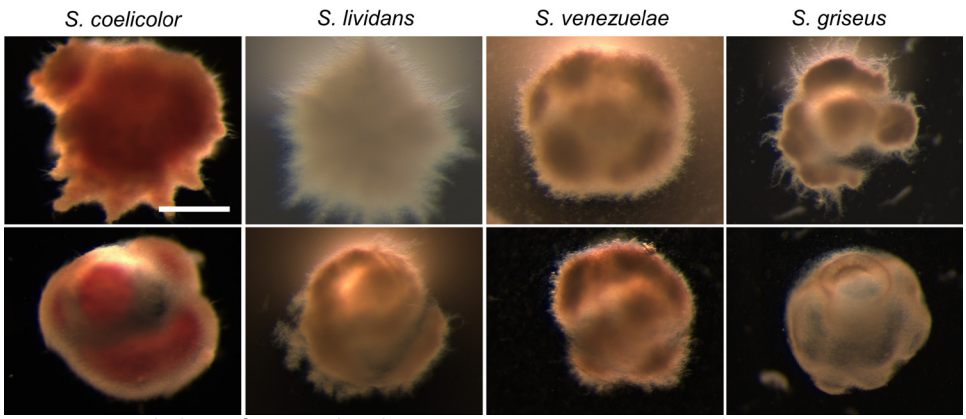


Figure 1. Morphology of encapsulated streptomycetes in $NMMP_{mod}$ medium. Microscopy images of microcapsules containing mycelium of *Streptomyces coelicolor*, *Streptomyces lividans*, *Streptomyces venezuelae* and *Streptomyces griseus* grown in $NMMP_{mod}$ medium at 48 (top panel) and 96 h (lower panel). The scale bar corresponds to 200 µm.

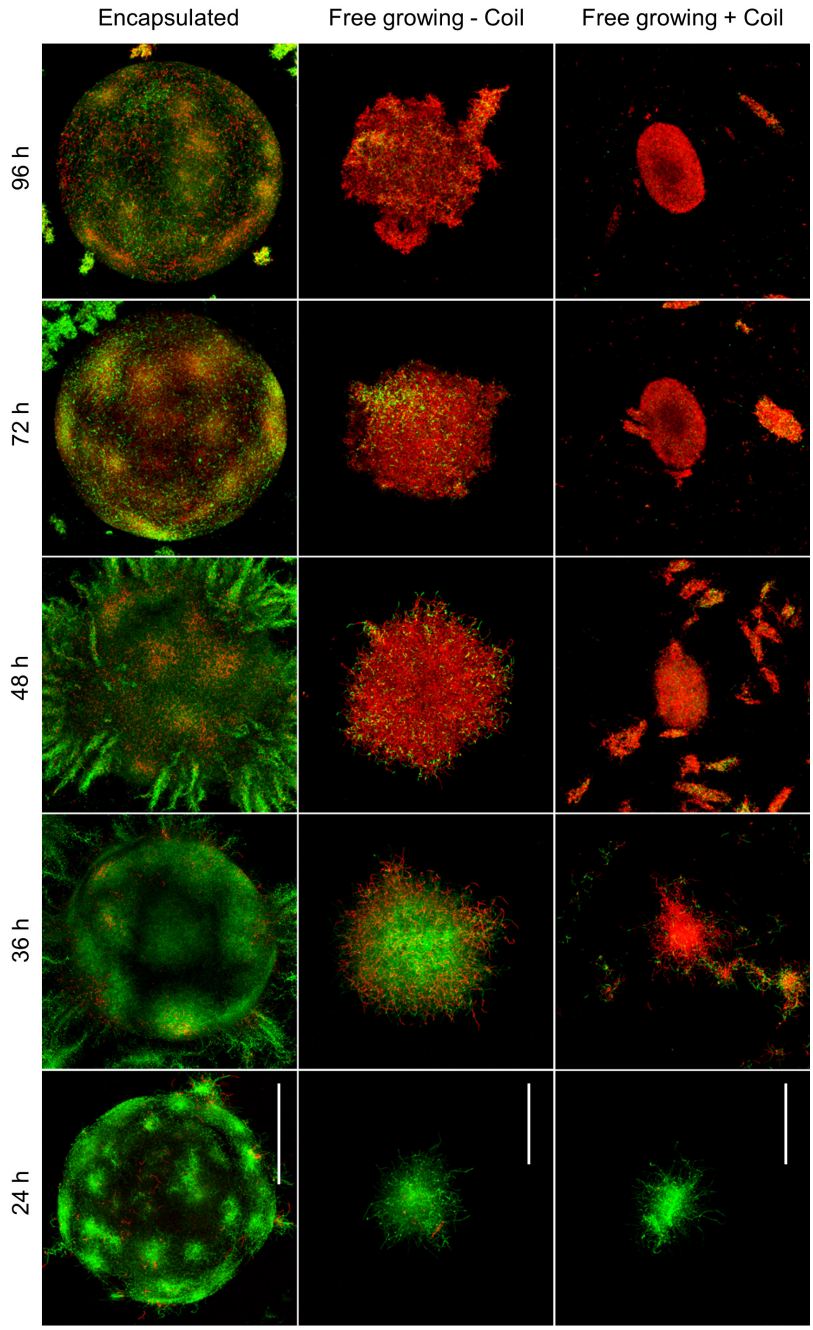


Figure 2. Microencapsulation reduces mycelial damage. LIVE/DEAD staining of *Streptomyces lividans* grown in microcapsules (top row), or non-encapsulated in the absence (middle row) or presence (bottom row) of a metal coil. Mycelium stained with Syto9 (green) represents viable mycelium, while propidium iodide-stained mycelium (red) is damaged. Whereas abundant viable mycelium is visible in the encapsulated state at 48 h of growth, the non-encapsulated mycelium appears highly damaged. Note that the mycelium grown in the presence of the coil appears already damaged after 36 h. Scale bars represent 200 µm (top panel) or 100 µm (middle and bottom panel).

Interestingly, a major fraction of the mycelium present in, or associated with the microcapsules after 48 and 72 h of growth was viable, as derived from the pronounced staining with Syto-9. In contrast, the presence of damaged mycelium, indicated by the red PI stain, occurred significantly earlier in the free-growing mycelium, either cultivated in the presence or absence of a metal coil (used to counteract aggregation and induce fragmentation by shear). When free-growing mycelium of *S. lividans* was cultivated in the absence of a metal coil in the culture flask, we noticed that the occurrence of damaged mycelium was evident after 48 h of growth, and apparently delayed in comparison to the mycelium suffering from coil-induced shear.

Microencapsulation increases the heterologous production of tyrosinase

To test the effect of microencapsulation on heterologous enzyme production, we introduced plasmid pIJ703 into *S. lividans* 66 [209]. This plasmid contains the *melC2* gene of *Streptomyces antibioticus,* encoding an extracellular tyrosinase that is secreted via the twin arginine translocation pathway [210]. Transformants were selected based on their ability to form the pigmented compound melanin; one of these, hereinafter called *S. lividans* pIJ703, was selected for further analysis. *S. lividans* pIJ703 was encapsulated, after which tyrosinase production was assayed and compared to the non-encapsulated controls (Fig. 3). Significantly enhanced activity (*P*-value < 0.0001) was detected in the supernatant between 40 and 54 hours of growth when *S. lividans* pIJ703 was grown in microcapsules, with a more than three-fold increase in comparison to the non-encapsulated strain. The highest tyrosinase activity in the supernatants of the encapsulated strain peaked after 48 h of growth, followed by a slow and gradual decrease. However, tyrosinase activity

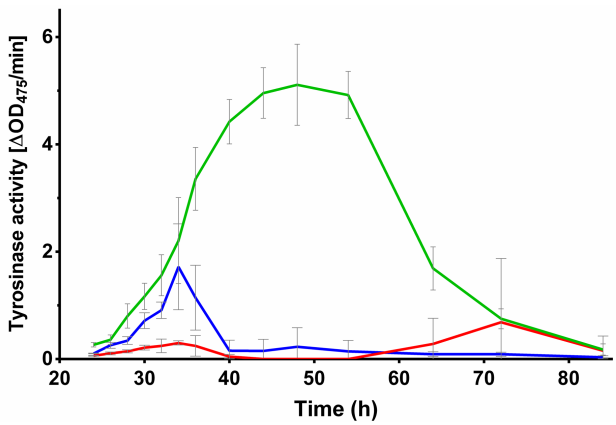


Figure 3. Microencapsulation increases tyrosinase activity in the supernatant. Lines represent the tyrosinase activity present in the supernatant of *Streptomyces lividans pIJ703* grown encapsulated (green), or non-encapsulated in the absence (red) or presence (blue) of a coil. The highest activity was observed in the culture broth of the encapsulated mycelium after 48 h of growth.

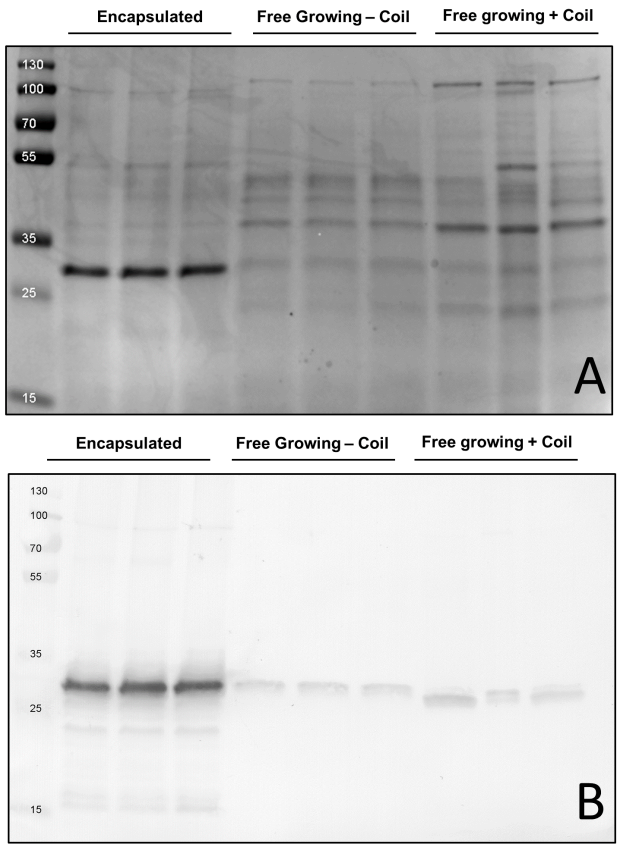


Figure 4. Microencapsulation increases tyrosinase purity in the culture broth. (A) SDS-page showing the protein profiles in the supernatants of cultures of *S. lividans pIJ703*, grown encapsulated (lanes 2-4), or non-encapsulated in the absence (lanes 5-7) and presence (lanes 8-10) of a metal coil. All cultures were performed in triplicate. Molecular weight markers (lane 1) are indicated in kDa. (B) Western analysis of the abovementioned supernatants using an anti-Tyrosinase antibody. Molecular weight markers are indicated in kDa.

was still detectable at 72 h of growth. In the non-encapsulated state, the tyrosinase activity peaked at approximately 34 h of growth, after which a rapid decline was detected. After 50 h, tyrosinase activity was barely detectable. Given that the growth rate of the encapsulated mycelium could not be assessed, we measured glucose consumption over time (Fig. S3). The consumption of glucose did not show significant differences between the three culture types, suggesting that the mycelia grew at comparable rates. We also qualitatively analyzed the extracellular proteins present in the culture supernatants (Fig. 4A). An SDS-PAGE analysis indicated that the supernatant of the encapsulated strain contained an abundant protein with an apparent molecular weight equal to that of tyrosinase (~ 30 kDa) after 48 h of growth, corresponding to the time point at which the highest tyrosinase activity was detected. Conversely, the protein profiles in the supernatants of the non-encapsulated control cultures were more complex and showed a large number of proteins (Fig. 4A).

Western analysis was used to verify that the dominant protein in the supernatant of the encapsulated strain was in fact tyrosinase (Fig. 4B). Consistent with the measured activities in the supernatant, only small amounts of tyrosinase were detected in the non-encapsulated strains. Taken together, these data demonstrate that encapsulation enhances the production of heterologously expressed tyrosinase.

DISCUSSION

Streptomycetes are proficient producers of enzymes and antibiotics. For industrial production processes, these organisms are usually grown as liquid-grown cultures in large scale fermenters. The growth of streptomycetes under these conditions is marked by the formation of mycelial particles that consist of interconnected hyphae [12, 142]. Industrial fermenters are typically stirred at high speeds to provide homogeneous mixing but also to ensure that sufficient oxygen and nutrients are available to the growing biomass. This vigorous mixing comes at the cost of severe shear stress, which can cause fragmentation and lysis of the mycelium [211-213]. The concomitant release of intracellular contents into the culture broth thereby complicates product purification [214].

We here show that microencapsulation is a valuable and alternative approach circumventing some of the negative aspects of classical fermentations, consistent with earlier observations [207]. Microencapsulation physically separates a large fraction of the mycelium from the liquid environment, with the exception of the small mycelial fragments that grow out of the capsules at late time points. While calcium-alginate is permeable to small molecules [215], the encapsulated mycelium is protected from extrinsic mechanical stress. Our experiments demonstrate that the viability of the mycelium is prolonged inside microcapsules, which is in agreement with earlier observations in *S. coelicolor* [207]. We conclude that this effect is obtained by reducing the degree of shear stress encountered by the mycelium. The earlier occurrence of dead mycelium in non-encapsulated cultures performed with metal coils as compared to those without is also indicative of this fact. Besides extending viability, microencapsulation dramatically increased the production and purity of heterologously produced tyrosinase. The higher production was not only evident from the amount of this protein in the supernatant, but also from measurements of its specific activity throughout growth. More specifically, the amount of active tyrosinase was more than three-fold increased when the mycelium was grown inside microcapsules. Shear-induced cell lysis can be a major cause for the dissipation of substrate in streptomycetes [212]. This, accompanied by the observation that the trend in carbon consumption was similar under all conditions, suggests that the encapsulated mycelium

invests more energy in production rather than in other processes, such as those related to cell repair and maintenance. The higher purity of the extracellular tyrosinase not only supports that micro-encapsulation reduces cell lysis, but also poses another major benefit: the decreased number of contaminants facilitates product purification and increases the efficiency of the production process. Our work serves as proof-of-concept showing that the microencapsulation of streptomycetes prolongs mycelial viability and enhances the production and purity of enzymes. One issue to overcome is the need to scale up to allow larger scale production with encapsulated strains. Considering this, we anticipate that our approach might be particularly suitable for the production of high-value natural products and enzymes by streptomycetes and possibly other filamentous organisms.

MATERIALS AND METHODS

Strains and culture conditions

Streptomyces coelicolor A3(2) M145 [216], *Streptomyces lividans* 66 [209] and *Streptomyces venezuelae* diversa were obtained from the John Innes Centre strain collection, and *Streptomyces griseus* DSM40236 from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). MS agar plates [171] were used to prepare spore suspensions of *Streptomyces* strains and to determine colony forming units (CFU) for the spore stocks. For liquid-grown cultures, YEME medium [171] or a modified NMMP medium ($NMMP_{mod}$) were used. The buffer system of $NMMP_{mod}$ was optimized to avoid the detrimental effect of phosphates on the integrity of the alginate microcapsules [217]. For the preparation of 1 liter $NMMP_{mod}$ medium, 100 ml 0.25 M TES buffer (pH 7.2), 10 ml 0.1M Na-K buffer (pH 6.8), 25 ml 20% glucose and 65 ml milliQ water were added to 800 ml NMMP basis [171]. For experiments using strains containing the tyrosinase-expressing plasmid pIJ703 [210], 10 µM $CuSO_4$ were added to the growth medium. All cultures were grown in a total volume of 100 ml of liquid medium contained in 250 ml Erlenmeyer flasks. Cultures were grown in an orbital shaker set at 30° C and 160 rpm. Unless differently stated, all experiments were performed in duplicate. For microencapsulation experiments, on average 75 viable spores were incorporated into every capsule with an average diameter of 415 µm. To this end, spores were suspended in sterile liquid sodium alginate and thoroughly mixed before the preparation of microcapsules. A total of 5 ml of alginate microcapsules was used to inoculate 100 ml of medium (*see below*). We calculated the equivalent number of spores, which we used to inoculate all other cultures, and which corresponded to 10^5 spores per ml of medium.

Microscopy

A Leica MZ12 stereo microscope was used for the visualization of microcapsules and encapsulated mycelium. For the visualization of live and dead mycelium, samples were stained with Syto-9 and propidium iodide (PI) (Invitrogen). To this end, pellets and microcapsules were briefly sedimented via centrifugation (10 min at 2000 rpm at room temperature) and resuspended in PBS, to which Syto-9 and PI were added to a final concentration of 5 µM and 15 µM respectively. After mixing and incubating for 10 minutes in the dark at 30°C, samples were analyzed using a Zeiss LSM 5 EXCITER confocal microscope. Stained samples were excited at 488 and 543 nm for Syto-9 and PI, respectively. The fluorescence emission of Syto-9 was monitored in the region between 505-545 nm, while a long-pass filter of 560 nm was used to detect PI [218]. The pictures shown in Fig. 2 represent Z-stacks of at least 15 layers in the specimen with a slice thickness of 7 µm for microcapsules and 4 µm for pellets.

Encapsulation of *Streptomyces* spores in calcium alginate

Sodium alginate (Sigma-Aldrich, CAS:9005-38-3) was dissolved under constant stirring for 1 hour in milliQ water to obtain a 2% solution. To remove undissolved micro-particles and other contaminants, the obtained solution was passed through two different filters. The first filter had a pore size of 1.2 µm (GE Healthcare, CatNo:1822-047) and was used in a vacuum filtration apparatus (PALL Magnetic Filter Funnel). The filtrate was then filter-sterilized using a syringe filter with a pore size of 0.22 µm (Sarstedt). For the production of calcium-alginate microcapsules, a home-made device was used similar to that described in [219]. A schematic representation is shown in Fig. S4. This apparatus is based on a coaxial gas-flow extrusion principle, with sterile air as the used gas. The air flow was regulated via a controller (Kytola, Model E) and was set at 3 liters per minute, thus yielding alginate particles with an average diameter of 415 µm (± 12.3 µm; based on analyzing 150 particles). A constant alginate flow was obtained by using a syringe pump (Fisher-Scientific) set at 30 ml/hour. The microcapsules were produced by dispersing the extruded alginate using a home-made nozzle that allowed co-axial laminar flow. While the alginate constitutes the inner sheath, air is flown in a co-axial fashion and determines the rate of formation of the alginate drops. This allowed control over the volume of the falling droplets, which were collected into a gently stirred 200 mM $CaCl_2$ solution. The cross-links formed through sodium/calcium ion exchange almost instantly transformed the liquid drops into gel-like microcapsules. The alginate microcapsules were left to harden in the stirring 200 mM $CaCl_2$ solution for 5 minutes. The obtained suspension of calcium-alginate microcapsules was then filtered using a vacuum filtration apparatus (PALL, GE

Healthcare filters as above), after which the microcapsules were washed three times with 500 ml sterile demi-water.

Glucose measurement assay

Glucose concentrations were determined using a commercial kit (Megazyme, HK/G6P-DH), according to the instructions of the manufacturer.

Tyrosinase activity assay

The specific activity of tyrosinase produced by *S. lividans* harboring pIJ703 was determined by following the conversion of L-3,4-dihydroxyphenylalanine spectrophotometrically at a wavelength of 475 nm, as described earlier [220]. Graphpad Prism 6.01 was used for performing a one-way analysis of variance (ANOVA) followed by a Bonferroni correction.

SDS-PAGE and Western blot analyses

Supernatants of liquid-grown cultures were harvested after 48 h of growth. The culture samples were first centrifuged for 10 min at 5000 rpm and 4° C, after which the supernatants were filtered through 0.22 µm syringe filters (Sarstedt), to remove any possible contaminants (e.g whole cells, spores). Extracellular proteins were concentrated via acetone precipitation. Briefly, 1.2 ml of cold acetone (-20°C) were added to 300 µl of supernatant sample. Following thorough mixing, the sample was kept at -20°C for 1 hour and subsequently centrifuged at 13,000 rpm for 10 min at 0°C. Subsequently, the liquid was removed without disturbing the protein pellet, after which 500 µl of cold acetone were added. After a second centrifugation step, the acetone was removed and the pellet was dried at 37 °C for 10 min. The obtained protein pellets were dissolved in 30 µl of 10 mM Tris-HCl buffer (pH 8.0). A Bradford analysis was used to determine the protein concentrations in the obtained samples, and 2 µg of proteins were used for separation by SDS–PAGE on precast 12 % miniprotean TGX Gels (BioRad) at 205 V, 200 mA for approximately 50 min. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare) and incubated overnight with anti-Tyrosinase polyclonal antibodies (1:25,000 dilution). Following 1 hour of incubation with goat anti-rabbit alkaline phosphatase, detection was carried out with NBT/BCIP.

Chapter 7: General Discussion

Industrial biotechnology makes use of living systems to produce chemicals, materials and energy. In this sector, microbial cells are most often the platform of choice for production processes, as these are generally easier to handle than other cell types (e.g. mammalian cells) and more robust under industrial conditions. Industrially produced microbial products fall within the categories of fine chemicals, pharmaceuticals, food additives and supplements, colorants, vitamins, pesticides, bioplastics, solvents, bio-plastics, bulk chemicals and biofuels. Antibiotics and their intermediates are among the most relevant fine chemicals, with a world market of about 40 billion $. Generally speaking, there are two alternative strategies that can be pursued to produce a compound in microbes. The molecule can either be produced by the endogenous host or, alternatively, the genes for its biosynthesis can be introduced into organisms that are better suited for industrial production, a strategy referred to as heterologous expression. Through heterologous expression a large arsenal of products are being produced in a handful of well-characterized organisms. Among the preferred heterologous hosts are the bacteria *Escherichia coli* and *Bacillus subtilis*, the fungi *Aspergillus niger* and *Trichoderma reseei* and the yeast *Saccharomyces cerevisiae*. Heterologous expression is however not always a feasible approach, in particular when host-specific factors are necessary for production, or when complex biosynthetic pathways have to be introduced in the heterologous host. Well-known examples of compounds that are difficult to produce heterologously are the antibiotics, most of which are derived from soil bacteria called streptomycetes [1, 7]. The production of antibiotics relates to the complex growth fashion of these soil bacteria.

Unlike most other bacteria, streptomycetes form complex mycelial networks consisting of branched filaments called hyphae. Being proficient saprophytes, streptomycetes also produce a treasure-trove of industrially relevant hydrolases that enable them to degrade almost all naturally-occurring polymers, including polysaccharides (e.g. starch, pectin, and chitin) and proteins [8]. Upon a number of environmental stimuli (e.g. nutrient exhaustion), streptomycetes form specialized hyphae that grow into the air and that ultimately develop into spores. During this morphological differentiation, streptomycetes undergo a process of programmed cellular lysis [221]. More specifically, the central zones of *Streptomyces* colonies are

subject to an orderly process of cellular dismantling during which valuable nutrients are released into the extracellular milieu. These nutrients are recycled by the streptomycete to allow the sporulation process to complete. At this stage, antibiotics are produced to prevent possible competitors from utilizing the released nutrients.

In industry, most compounds are produced by microorganisms in liquid cultures in large-scale fermenters. Under these conditions, streptomycetes form mycelial particles of varying densities, ranging from small particles with an open structure to larger and densely-packed structures called pellets. In aging cultures, detached hyphal fragments also become evident, which dramatically increase the viscosity of the medium and negatively impact the downstream processing [11, 100, 186]. Notably, morphology and production are correlated in streptomycetes. This means that a particular morphology is preferred, and sometimes even required for the efficient production of a certain product [13, 15, 16, 222]. More specifically, the presence of large pellets is beneficial for the production of antibiotics [13, 222], while smaller mycelial particles are better suited for enzyme production [15, 16]. Given that different morphologies usually co-exist in bioreactors, the heterogenous nature of mycelia represents a bottleneck for the industrial exploitation of streptomycetes [12]. The work presented in this thesis focuses on the mycelial heterogeneity of liquid-grown streptomycetes and on strategies to overcome this occurrence. The results presented here may be of significant value to improve the exploitation of these prolific bacteria as cell factories.

THE ROLE OF AGGREGATION IN SHAPING LIQUID-GROWN *STREPTOMYCES* MYCELIA

In the last fifteen years, we have witnessed a dramatic improvement in our understanding of the genetic factors affecting morphology in liquid-grown streptomycetes. Early reports on this topic highlighted the importance of components of the cell division machinery, with *ssgA* as an outstanding example [223]. SsgA is the first characterized member of a class of proteins specific for actinomycetes with a role in growth and cell division. Overexpression of this gene increases the septation rate and the propensity of the mycelium to fragment, yielding mycelial particles that are considerably smaller than those of the wild-type strain. Together with the decreased particle size, the *ssgA*-overexpressing strain also grows significantly faster and produces heterologous enzymes at a faster rate [16]. Despite these advantages, the overexpression of *ssgA* causes hyperseptation in both vegetative and aerial hyphae, thus conflicting with the control systems involved in maintaining physiological differences between the two cell types [223]. An in-depth analysis

of transcription in an *ssgA* mutant background also indicated that this gene is not only involved in cell division, but also in other key processes such as overall growth, development and protein secretion [224].

More recently, it was established that extracellular macromolecules associated with the cell wall are also key determinants of pellet formation. For instance, genes participating in the synthesis of extracellular glycans have been found to be essential for the formation of pellets in submerged cultures [12, 15, 145, 147]. More specifically, two different types of glycans with complementary functions have been identified. These are a cellulose-like glycan produced under control of the *cslA-glxA* operon [143-145] and poly-N-acetylglucosamine (PNAG), produced under control of the recently discovered *mat* cluster [15, 147]. Interestingly, these glycans play separate roles in the adhesion of *Streptomyces* hyphae to surfaces. Whereas the cellulose-like polymer produced by *cslA-glxA* mediates adhesion to hydrophobic surfaces [144], PNAG is involved in mediating adhesion to hydrophilic surfaces [147]. In addition to glycans, the chaplins, small proteins forming an hydrophobic sheath around *Streptomyces* hyphae [225, 226], have been shown to contribute to submerged morphology [12]. Also, other reports have demonstrated the involvement of extracellular DNA and hyaluronic acid in maintaining pellet architecture, further reinforcing the notion that extracellular components are crucial to morphology [186].

Chapter 3 of this thesis highlights the critical role of extracellular glycans in mediating the aggregation of distinct mycelial particles in liquid-grown streptomycetes. Although inter-particle aggregation had previously been described in filamentous fungi [227], evidence for this process was lacking in the *Streptomyces* field. By making use of fluorescent strains expressing distinguishable fluorophores and high-throughput analytical techniques, aggregation was characterized in the industrial model strain *Streptomyces lividans*. Contrary to filamentous fungi [227], aggregation in *S. lividans* is only evident after spores have started to germinate (i.e. when visible germ tubes have been formed). At this stage multiple germlings aggregate with one another to form larger structures that continue to grow as a larger whole. Interestingly, this early aggregation step depends on the size of the aggregating particles and the degree of shear in liquid cultures. Indeed, experiments reported in Chapter 5 show that reducing the level of shear in liquid cultures enables larger particles to adhere to each other. The components mediating inter-particle aggregation are those produced by the *cslA-glxA* operon and the *matAB* cluster. Notably, these glycans not only mediate the contact between hyphae of the same particle, but also aggregation between different particles. In other words, mutants lacking functional copies of *cslA*, *glxA* or *matAB* possess an "open" mycelial structure and grow as separate individuals. Contrary to those of the wild-type strain, the particles formed by non-aggregating mutants (i.e. those

of the *cslA* and *matAB* mutants) are also homogeneous in size. More specifically, while the mycelia formed by the wild-type strain belong to two distinct populations that differ in size, those formed by the mutants are normally distributed. This indicates that aggregation is a driving factor causing size heterogeneity. Aggregation is also reported to occur between different species, resulting in the establishment of multi-species pellets. This discovery is of great importance when considering the role of interspecies interactions in promoting chemical diversity. For instance, co-cultivation strategies of streptomycetes and filamentous fungi have been shown to elicit the production of molecules that are not observed when these organisms are cultivated separately [167, 168]. Notably, despite the large number of known bioactive compounds produced by streptomycetes, the biosynthetic potential of these microorganisms might still be greatly underestimated. Analyses of the increasing number of available genome sequences indicate in fact that these contain the genetic information for the biosynthesis of numerous compounds that are never detected under standard laboratory conditions [216, 228]. In this scenario, the observation that commonly used *Streptomyces* strains can develop intimate interactions in submerged cultures indicates that co-cultivation approaches can be pursued to expand chemical diversity and possibly lead to the discovery of novel compounds with biological activity.

Although aggregation is reported to be crucial in causing heterogeneity, no mechanistic insight is presented on how the size heterogeneity of pellets is actually established. In light of the knowledge gathered in this thesis and other recently published studies [9, 15], a hypothesis can however be formulated. Aggregation seemingly represents the step at which uniformly sized particles (germlings) are bound together in a stochastic manner. This yields mycelial aggregates of different sizes, but which in principle are still normally distributed. Small differences in the size of aggregates, however, could lead to markedly different growth dynamics. Previous work on filamentous fungi has demonstrated that larger particles suffer from oxygen and nutrient depletion in their central part [125, 127, 229]. This restricts active growth in large particles to peripheral hyphae, which results in a growth mode conventionally called “cube root” [200, 230, 231]. In contrast, smaller particles are still able to grow exponentially, since they are not subject to any limitation in their access to nutrients and oxygen. In other words, it can be hypothesized that a threshold particle size exists below which exponential growth occurs and above which cube root growth dynamics come into place. Accordingly, once particles reach this threshold size, they will switch from exponential to cube-root growth. The interplay between these different growth dynamics could ultimately lead to two populations of pellets. Notably, this interpretation would not only explain why aggregation is involved in causing heterogeneity, but also why this phenomenon is influenced by the medium composition (see Chapter 3).

Although pellets ultimately become heterogeneous in size under both conditions [12], the aggregates of *S. lividans* are in fact still homogeneous in size after 12 hours of growth in minimal media, while they are already heterogeneous in rich media. This difference likely relates to the higher growth rate in the rich medium, which causes earlier heterogeneity as a consequence. Notably, varying degrees of cell lysis inside aggregates (see Chapter 4) could further impact differences in growth rates between pellets and the formation of distinct populations.

Chapter 4 describes a technique to obtain homogeneously sized *Streptomyces* pellets. To this end, the *sco* mutant of *S. lividans* was used. Sco is a copper chaperone required to provide GlxA with a copper atom, which is needed for the maturation of this protein [146]. Without Sco, GlxA is inactive, resulting in a phenotype analogous to that of *glxA* and *cslA* mutants. Notably, GlxA maturation can be restored in the *sco* mutant through the addition of micromolar amounts of copper to the growth medium. In other words, the addition of copper restores both pellet formation and inter-particle aggregation in the *sco* mutant. In the work presented in this Chapter, this copper-dependent morphological switch is exploited to induce pellet formation while impeding inter-particle aggregation. The rationale behind this strategy was to obtain pellets that are derived from individual spores, and which as a consequence would be uniform in size. Using this approach, homogeneously-sized pellets were obtained with controllable diameters varying between 167 and 276 µm. This allowed the direct investigation of the effect of pellet size on production of a heterologously produced xylanase. Notably, smaller pellets were found to accumulate this enzyme more quickly than larger pellets. This is consistent with earlier reports showing that smaller mycelial particles are better enzyme producers than large pellets [15, 16]. Contrary to the previous reports, however, the pellets described in this work have a uniform size distribution. Nevertheless, drawing firm conclusions on the interdependence of morphology and overall physiology based on the results obtained using the *sco* mutant is complicated by the fact that the phenotype of this strain is not fully rescued by the addition of copper. This is concluded from the measured reduction in inter-particle aggregation and the reduced number of spores in pellets of the complemented mutant, which as a consequence shows smaller pellets and decreased size heterogeneity. Additionally, the *sco* mutant displays different growth kinetics and grows markedly slower than the *glxA* strain (Erik Vijgenboom, personal communication). These differences might relate, at least partially, to the fact that Sco not only assists in the maturation of GlxA, but also that of the Cytochrome C oxidase Cox [180]. This oxidase is involved in the energy metabolism of the mycelium, which suggests that the uncomplemented strain might have less energy and in turn grow more slowly. Although the data presented in Chapter 4 do not fully resolve the relationship between morphology and production, they allowed to identify

an important and previously unknown link between aggregation and cell death. This was demonstrated by following the growth and viability of the *sco* mutant in the absence or presence of copper in submerged cultures. While in the absence of copper (and hence in the absence of aggregation) only viable hyphae were observed in liquid-grown cultures until 18 hours of growth, the copper-induced aggregation resulted in the formation of dead hyphae within aggregates as early as at 8 hours of growth. The detection of compromised hyphae was also evident in the wild-type strain after 8 hours of growth, indicating that it was not an effect of the addition of copper. These observations indicate that a contact-mediated mechanism might be responsible for the onset of development and PCD in streptomycetes. Programmed cell death has been previously characterized during the development of streptomycetes in solid and liquid cultures [3, 4, 10, 99], although it has never been shown to occur at such early stages of growth.

THE ROLE OF FRAGMENTATION IN THE MORPHOGENESIS OF LIQUID-GROWN STREPTOMYCETES

While aggregation has been demonstrated to be essential for pellet formation and the occurrence of culture heterogeneity, little was known about the process of pellet disassembly. This process has mostly been characterized in filamentous fungi used in the biotech sector [232-235]. In these systems, hyphal fragmentation can occur either via the breakage of loose hyphae or via the so-called “shaving” of hyphae from the periphery of pellets. Also, a general trend has been described concerning the size of mycelia in submerged cultures, with an increase during the early stages of growth followed by a decrease in size related to aging of the mycelia (also observed in streptomycetes) [12]. Interestingly, this trend does not seem to be influenced by the agitation rate of cultures (hence the degree of mechanical stress) although higher impeller speeds generally result in the presence of smaller mycelial particles.

Chapter 5 presents an in-depth analysis of pellet fragmentation in *S. lividans*. In pilot experiments addressing this phenomenon, pellets of different ages were first transferred to fresh medium and the morphology and growth of the derivating mycelial particles were subsequently monitored. This approach unambiguously revealed that fragmentation only becomes evident in aging mycelia and that it is initiated upon the entrance of cultures into the stationary phase. Further experiments described in this Chapter demonstrated that the detached fragments are able to aggregate to one-another when inoculated in fresh medium, but mostly die when they remain in the spent medium. Interestingly, this second aggregation step only involves the newly released mycelial fragments but not the

pre-existing pellets. These discoveries might be relevant for industrial processes, especially when considering that bioreactors are usually inoculated with pregrown biomass. In particular, the notion that inoculating mycelium of different ages results in differently-sized populations of pellets can be very important for the accumulation of biomass in industrial processes, especially when a particular morphology is sought to enhance production.

THE GROWTH OF STREPTOMYCETES IN MICROCAPSULES

From an application viewpoint, the formation of pellets is an unattractive feature of *Streptomyces* growth [9]. The large size of these structures causes mass-transfer problems, which prevent oxygen and nutrients from reaching their central parts. As a consequence, but also due to the onset of programmed developmental processes, cells undergo a process of dismantling which negatively influences the overall growth of cultures [10] (see also Chapter 4). These problems can be avoided by using non-aggregating *Streptomyces* strains [9, 15, 144, 146], which bear however a negative impact on the rheological properties of cultures (e.g. high viscosity). In light of these observations, in Chapter 6 an alternative cultivation technique was studied that circumvents many of these disadvantages. This technique, called micro-encapsulation, has already been used in a range of microorganisms to enhance growth rates and production [202-207]. Upon micro-encapsulation, living cells are embedded in µm-scale capsules of a gelatinous scaffold (most commonly calcium alginate) and subsequently grown in submerged cultures. These microcapsules allow the free diffusion of gases and nutrients while protecting cells from the external environment [215].

The work described in Chapter 6 demonstrates that micro-encapsulation greatly extends mycelial viability in *S. lividans*. Moreover, micro-encapsulation leads to enhanced extracellular activity and concentration (indicating enhanced production, secretion, or both) of the reporter enzyme tyrosinase. Although this aspect was not investigated in detail, the prolonged viability and increased enzyme titer most likely stem from the decreased exposure of the mycelium to mechanical stresses that are characteristic of liquid-shaken cultures. Together with an increase in the amount and activity of tyrosinase in the culture supernatant, the amount of other proteins in the supernatant was reduced when using encapsulated mycelium. This increased tyrosinase purity most likely related to the reduced degree of mechanical stress perceived by the mycelium upon micro-encapsulation.

In addition to the encouraging results presented in this work, micro-encapsulation can be envisioned to be utilized for the achievement of other profitable goals. As a matter of fact, micro-encapsulation bears the potential to stimulate antibiotic production, as it has for instance been observed for *S. coelicolor* [207], but also *S. lividans*. More specifically, the production of the blue-pigmented antibiotic actinorhodin was observed when *S. lividans* was encapsulated (our unpublished results). Notably, *S. lividans* contains the genetic elements required for the production of this antibiotic, but usually fails to produce it under common laboratory conditions. This discovery indicates that also other biosynthetic clusters remaining silent under laboratory conditions might be activated by making use of this technique.

A REVISED DESCRIPTIVE MODEL FOR PELLET FORMATION AND FRAGMENTATION IN *STREPTOMYCES LIVIDANS*

Given the prominent role of streptomycetes in the industrial sector and the influence of submerged morphology on the production performance of these organisms, considerable research effort has been made to develop predictive models for their growth. In particular, a number of models have been constructed to predict submerged morphology, but which mainly focus on the effect of environmental factors [236-240] In more recent years, more advanced *in silico* models were developed. The model developed by Celler and co-workers is able to predict the three-dimensional morphology of liquid-grown mycelia based on parameters as oxygen diffusion, hyphal growth, branching, fragmentation, cross-wall formation and inter-particle collisions [198]. Clearly, for such modelling approaches to function, a good understanding is required of the genetic determinants of morphogenesis, as well as an integrated approach including the experimental validation of the modelling procedure. The results described in this thesis enable to further advance a revised descriptive model of the morphological changes accompanying *Streptomyces* growth in liquid cultures. Although this model is based on experimental data mainly obtained using *S. lividans*, it may also be relevant for other pellet-forming streptomycetes. This description partially complements and renovates the one previously proposed [9].

Following the inoculation of liquid cultures, the first visible signs of growth are observed after approximately 4 hours, coinciding with the germination of spores. Germination occurs via the formation of one or two germ tubes at the polar sites of spores; these germ tubes subsequently elongate to form young vegetative hyphae. Interestingly, a portion of the inoculated spores does not germinate despite being viable and can be found in the medium at later stages of growth (see Chapter 5). As shown in Chapter 3, germination leads to the onset of ag-

gregation, whereby germlings assemble into larger aggregates. Spores that germinate at a later stage can either aggregate with each other or with pre-formed aggregates. In addition, growing aggregates can adhere to one-another until approximately 12 hours after the inoculation of cultures. Interestingly, the duration of this aggregation phase is dependent on the degree of mechanical stress. With a lower degree of shear, the duration of the aggregation phase is in fact extended and larger pellets are formed (Chapter 5). The aggregation between larger particles is seemingly determined by their physical properties, in particular their shape and mass, which influence the ability to endure mechanical stresses. Briefly, when two mycelial particles collide, a first contact is established between their surfaces. This contact must be maintained until further growth consolidates their adhesion (e.g. the hyphae of the two individuals grow intertwined). When this first contact occurs between small particles, abundant exposed surface is present in relation to the overall mass of the aggregate. Conversely, large particles possess a reduced surface-to-volume ratio, which causes the nascent aggregate to less efficiently endure mechanical stress. In other words, the larger the size of the aggregating particles, the more easily their contact will be disrupted due to shear. This means that mycelial particles ultimately reach a size that prevents them from further aggregating. Once this threshold size has been reached, aggregates further increase in size as a result of hyphal elongation and branching and eventually form large pellets. Although the presence of dead hyphae is already observed at the onset of aggregation in *S. lividans* (see Chapter 4), nutrient and oxygen limitation at the core of pellets begin to occur at this stage. It is in fact known that a critical size exists above which the central parts of pellets become starved for nutrients and oxygen [125, 127, 229], either due to the sequestration of these elements from the hyphae growing at the outer edges of these particles or because of the impaired diffusion through the mycelium. As a result of these events, a core of dead cells becomes evident inside pellets. Ultimately, when nutrients become exhausted, cultures reach a stationary phase and fragmentation begins to occur. As shown in Chapter 5, the small mycelial fragments that detach from mature pellets are able to aggregate to one another in fresh medium. However, in cultures that are not supplemented with fresh nutrients these fragments will ultimately die.

OUTLOOK AND FUTURE PERSPECTIVES

The work described in this thesis opens new avenues for the rational optimization of streptomycetes as cell factories. In particular, the discovery of inter-particle aggregation as a key determinant of pellet size heterogeneity makes this phenomenon the most obvious target to tackle with the aim of gaining control of submerged morphogenesis. Besides the strategy presented in Chapter 4, two

possible approaches can be envisioned to counteract inter-particle aggregation. One way to achieve this, or to at least reduce the number of germlings forming pellets, is to increase the degree of mechanical stress during the early stages of growth, until particles have reached a size that prevents them from adhering to each other. This could be obtained by temporarily increasing the stirring speed of cultures, thereby intensifying mechanical stress. This strategy is however not devoid of risks, as a too vigorous stirring may physically damage the mycelia, with concomitant cell lysis and reduced product yields.

An alternative approach would be to modulate the expression of the glycans mediating inter-particle aggregation (those produced by *cslA*/*glxA* or the *mat* genes) [142, 147] and prevent their expression in the first 16-20 hours of growth as to yield particles that originate from individual spores. To this end, the *matAB* genes are the preferred candidates, given that the polymer produced by CslA, besides mediating aggregation, is also involved in providing rigidity to hyphal tips [144]. This approach has already been attempted, but bears the disadvantage that pellets showed scarce density, seemingly because the promoter being used activates transcription too late during growth (Dino van Dissel, PhD thesis). A similar strategy could be implemented using either natural or synthetic promoters that activate transcription at an earlier stage of growth. Alternatively, an inducible system can be employed, although this might be too costly under industrial conditions.

Another major outcome of the work presented in this thesis is that aggregation is responsible for the accelerated cellular development resulting in cell lysis, as documented by the fact that lysed hyphae can already be discerned within aggregates of *S. lividans* at 8 hours of growth. This observation is reinforced by the fact that modulating aggregation using the *sco* mutant of *S. lividans* allows to distinguish between fully viable, non-aggregated particles and aggregates containing dead cells. Interestingly, this phenomenon might partly explain why open growing *Streptomyces* strains generally possess higher growth rates than pelleting strains, besides for reasons related to the better diffusion of nutrients and oxygen [15]. Research can be performed with the aim to obtain mutants with reduced aggregation-induced lysis as to maximize cell viability and production in turn. For instance, experiments can be performed combining the use of random mutagenesis and that of strains constitutively expressing unstable variants of GFP or related fluorescent proteins [241]. In this way, mutants can be chosen by selecting, most likely via cytometry approaches, for high fluorescence in young mycelial aggregates. The rationale behind this is that since only the actively growing cells will give a detectable fluorescence (being the instability of the fluorophore), fluorescence will inversely correlate with viability inside aggregates. This kind of research might allow to obtain strains with an abolished early stage cellular lysis and

to consequently enhance biomass and product accumulation in submerged cultures. Cellular lysis might still occur in these mutants as a result of the oxygen and nutrient limitations discussed above, although these would only become evident in larger (older) mycelial particles.

Another possible line of research indicated by the work presented in this thesis would be to gain control on the process of pellet fragmentation. As described in Chapter 5, pellet fragmentation in *S. lividans* bears striking similarities with the dispersal of bacterial biofilms. Biofilm dispersal is in many cases dependent on the active hydrolysis of the extracellular matrix, which is carried out by secreted hydrolases. In this context it is relevant to mention that the genome of *S. lividans* encodes a putative hydrolase with significant similarity to dispersin B of *Actinobacillus actinomycetemcomitans*. Dispersin B is known to hydrolyze poly-N-acetylglucosamine (PNAG), a widespread component of the extracellular matrix of biofilms. Importantly, Dispersin B was recently shown to efficiently solubilize the extracellular PNAG layer covering *Streptomyces* germlings [147]. In light of these facts, research can be performed to dissect the role of this protein in submerged morphogenesis, in particular to see whether changes in the timing and nature of pellet fragmentation can be achieved by impeding or modulating its production throughout growth. Although it is unclear how pellet size and fragmentation might be related in young cultures (note that fragmentation only occurs once the stationary growth phase is reached) it is interesting to mention that in *Streptomyces coelicolor* the homologue of this protein is significantly more abundant in large pellets [12]. Additionally, gaining control on the process of pellet fragmentation might be profitable when young cultures are diluted, as to increase the capacity of young mycelia to form new small particles (possibly better suited for enzyme production). On the other hand, impeding fragmentation at the late stages of growth might be beneficial with the aim of maintaining pellet architecture in aging cultures and in turn to enhance the accumulation of secondary metabolites for which the presence of pellets is a requirement (e.g. antibiotics).

As previously mentioned, this thesis is focused on the study of pellet size heterogeneities in liquid-grown streptomycetes. Heterogeneities exist however at a number of different scales (see Chapter 2 for further details). With respect to filamentous fungi, the only kind of heterogeneity which has not yet been reported in liquid-grown streptomycetes is that between adjacent hyphae within pellets. It is clearly of great interest to see whether these heterogeneities exist, and if so to address them with the aim of homogenizing production throughout the mycelium. Notably, the mechanisms through which heterogeneities arise between hyphae seem to be dependent on hyphal compartmentalization, which can occur either by means of peptidoglycan septa or membranous cross membranes [98, 137, 223, 242].

Although the control of septation is quite well understood, the process whereby cross membranes are established is unknown. Also, it still remains obscure what the interplay of these heterogeneities is for example with developmental processes. Further studies are needed to address these unsolved questions.

Other unanswered questions relate to the exact function in time and space of those components reported here and elsewhere [12, 15, 166] to be involved in the submerged morphogenesis of *Streptomyces*. For instance, whereas the phenotype of *cslA*, *glxA* and *matAB* strains is analogous for what concerns inter-particle aggregation efficiency [142] (percentage of aggregated particles in co-cultures of fluorescent strains) and overall morphology, these glycans play separate roles in the adhesion of *Streptomyces* hyphae to surfaces [144, 147] (see above). This being said, it is unclear how these components cooperate in mediating inter-particle and inter-hyphal adhesion. This is even more puzzling when considering that the overexpression of the *mat* cluster is sufficient to rescue pellet formation in *cslA* mutants of *S. lividans* [147]. Other extracellular components (chaplins, extracellular DNA and hyaluronic acid) are seemingly involved in maintaining pellet architecture at later stages of growth. As a matter of fact, DNA might be released by lysing cells during the PCD process. Also, preliminary experiments have shown that a *Streptomyces coelicolor* strain lacking the main chaplins is not capable of maintaining pellet morphology upon prolonged growth at low pH (Boris Zacchetti, Marloes Petrus, Dennis Claessen, unpublished observations), which strongly implies an involvement of these proteins in the maintenance of pellet architecture.

Nederlandse Samenvatting

Industriële biotechnologie maakt gebruik van levende systemen om chemicaliën, materialen en energie te produceren. Voor productieprocessen worden vaak microbiële cellen gebruikt, omdat ze in het algemeen makkelijker te hanteren zijn dan andere celtypen (bijvoorbeeld zoogdiercellen). Verder zijn microbiële cellen robuuster onder industriële omstandigheden. Onder andere chemicaliën, farmaceutische producten, levensmiddelenadditieven en supplementen, kleurstoffen, vitaminen, pesticiden, bioplastics, oplosmiddelen en biobrandstoffen worden door micro-organismen geproduceerd. Over het algemeen worden twee strategieën gebruikt om een bepaald product te maken. Het molecuul kan ofwel door de endogene stam worden geproduceerd of de biosynthese genen worden overgebracht naar een organisme dat beter geschikt is voor industriële productie. Deze laatste strategie wordt heterologe expressie genoemd. Door gebruik te maken van heterologe expressie wordt tegenwoordig een groot arsenaal aan producten verkregen in relatief een klein aantal, goed bestudeerde organismen. De meest gebruikte industriële micro-organismen zijn de bacteriën *Escherichia coli* en *Bacillus subtilis*, de schimmels *Aspergillus niger* en *Trichoderma reesei* en de gist *Saccharomyces cerevisiae*. Ondanks de soms grote voordelen is heterologe expressie niet altijd mogelijk, bijvoorbeeld in gevallen wanneer specifieke factoren nodig zijn voor expressie of wanneer complexe biosyntheseroutes moeten worden overgebracht naar een productiestam. Bekende voorbeelden van producten die op deze manier moeilijk zijn te verkrijgen zijn antibiotica, waarvan de meeste zijn afgeleid van draadachtige bodembacteriën, streptomyceten genaamd [1, 7]. Antibiotica behoren bij de meest relevante fijne chemicaliën, met een wereldmarkt van ongeveer 40 miljard dollar per jaar. In streptomyceten is de productie van antibiotica nauw gekoppeld aan de complexe manier van groei. Streptomyceten vormen ingewikkelde netwerken, of mycelia, bestaand uit vertakte filamenten die hyfen worden genoemd. Naast antibiotica produceren streptomyceten ook een groot aantal industrieel relevante enzymen die hen in staat stellen bijna alle natuurlijk voorkomende polymeren af te breken, waaronder polysacchariden (bijv. zetmeel, pectine en chitine) en eiwitten [8]. Onder invloed van bepaalde omgevingsstimuli (bijvoorbeeld een tekort aan voedingsstoffen), vormen streptomyceten gespecialiseerde hyfen die de lucht ingroeien om uiteindelijk sporen te kunnen maken. Tijdens deze zogenaamde morfologische differentiatie ondergaan streptomycet-

en een proces van geprogrammeerde cellulaire lysis [221]. Tijdens dit proces worden delen van het eerder gevormde mycelium op een gecoördineerde manier afgebroken, waardoor waardevolle voedingsstoffen vrijkomen in het extracellulaire milieu. Deze voedingsstoffen zijn belangrijk voor streptomyceten om het sporulatieproces te kunnen voltooien. Door op dit moment antibiotica te produceren wordt voorkomen dat andere micro-organismen de loskomende voedingsstoffen kunnen gebruiken.

In de industrie worden de meeste producten gemaakt in grote vloeibare cultures in zogenaamde bioreactoren. Onder deze omstandigheden vormen streptomyceten deeltjes met verschillende dichtheden, variërend van kleine deeltjes met een open structuur tot grotere en sterk opeengepakte structuren die pellets worden genoemd. Daarnaast zijn er met name in latere groeistadia ook veel losse fragmenten zichtbaar. Deze fragmenten verhogen de viscositeit van het medium, wat een negatief effect heeft op het productieproces. Zoals reeds vermeld zijn morfologie en productie nauw aan elkaar gekoppeld in streptomyceten [11, 100, 186]. Dit betekent dat een bepaalde morfologie de voorkeur heeft en soms zelfs is vereist voor productie [13, 15, 16, 222]. Zo zijn grote pellets over het algemeen nodig voor de efficiënte productie van antibiotica [13, 222], terwijl juist kleinere deeltjes meer geschikt zijn voor de productie van enzymen [15, 16]. Doordat verschillende morfologieën naast elkaar voorkomen in vloeibare cultures, is de industriële toepassing van streptomyceten nog niet optimaal [12]. Het werk dat in dit proefschrift wordt beschreven is gericht op de heterogeniteit van in vloeistof gekweekte streptomyceten en op strategieën om deze heterogeniteit te voorkomen. De resultaten in dit proefschrift zijn van grote waarde om het gebruik van deze bacteriën in de industrie te verbeteren.

DE ROL VAN AGGREGATIE IN DE MORFOGENESE VAN *STREPTOMYCES*

In de afgelopen vijftien jaar is ons begrip over de genetische factoren die de morfologie van streptomyceten in vloeibare culturen beïnvloeden sterk verbeterd. Zo heeft het proces van celdeling een grote invloed op de morfologie, waarbij *ssgA* een belangrijk functie vervult [223]. SsgA behoort tot een familie eiwitten die specifiek is voor actinomyceten, en waarvan de leden allemaal groei en celdeling beïnvloeden. Overexpressie van *ssgA* stimuleert celdeling, waardoor fragmentatie toeneemt en de gevormde deeltjes kleiner zijn dan in de wild-type stam. Naast de afgenomen deeltjesgrootte groeit de stam die meer *ssgA* produceert ook sneller dan de ouderstam en maakt deze meer enzymen [16]. Ondanks deze voordelen is de overexpressie van *ssgA* de oorzaak van hyperseptatie zowel in de vegetatieve als de luchthyphen, en dus in tegenspraak met de controlesystemen die de fysi-

ologische verschillen tussen de twee celtypes handhaven [223]. Een globale analyse van genexpressie in een *ssgA*-deletie achtergrond gaf ook aan dat SsgA niet alleen bij celdeling is betrokken, maar ook bij andere belangrijke processen zoals groei, ontwikkeling en eiwitsecretie [224]. Recentelijk is het aangetoond dat celwand-geassocieerde macromoleculen ook belangrijk zijn voor de vorming van pellets. De genen die betrokken zijn bij de synthese van extracellulaire glycanen bleken essentieel te zijn voor dit proces [12, 15, 145, 147]. Er zijn twee verschillende glycanen geïdentificeerd die complementaire functies lijken te spelen. Dit zijn een cellulose-achtige glycan dat geproduceerd wordt door de eiwitten die gecodeerd zijn in het *cslA*-*glxA* operon [143-145], en poly-N-acetylglucosamine (PNAG), dat geproduceerd wordt onder invloed van de genen in het onlangs ontdekte *mat* cluster [15, 147]. Opvallend is dat deze glycanen verschillende functies spelen bij het hechten van *Streptomyces* aan oppervlakken. Terwijl het cellulose-achtige polymeer betrokken is bij de hechting aan hydrofobe oppervlakken [144], is PNAG betrokken bij het hechten aan hydrofiele oppervlakken [147]. Naast deze glycanen lijken ook de chaplins, kleine eiwitten die een hydrofobe laag kunnen vormen rond hyfen [225, 226], een belangrijke rol te hebben in de totstandkoming van de morfologie van streptomyceten in vloeibare cultures [12]. Werk van anderen heeft ook een rol aangetoond voor extracellulair DNA en hyaluronzuur bij de totstandkoming van de pellets [186], waardoor het idee verder wordt versterkt dat extracellulaire componenten cruciaal zijn voor morfologie.

In Hoofdstuk 3 van dit proefschrift wordt beschreven hoe extracellulaire glycanen betrokken zijn bij het proces van aggregatie tussen verschillende mycelium deeltjes. Hoewel aggregatie tussen deeltjes al beschreven was in filamenteuze schimmels [227], ontbrak het bewijs voor dit proces in het *Streptomyces* veld. Door gebruik te maken van onderscheidbare fluorescente stammen en high-throughput analysetechnieken, is het proces van aggregatie gekarakteriseerd in de industriële modelstam *Streptomyces lividans*. In tegenstelling tot schimmels [227] wordt aggregatie in *S. lividans* alleen zichtbaar nadat sporen zijn begonnen te ontkiemen (dat wil zeggen wanneer zichtbare kiembuizen zijn gevormd). In dit stadium aggregeren meerdere gekiemde sporen met elkaar en vormen grotere structuren die als één geheel blijven groeien. Interessant is dat deze vroege aggregatiestap sterk afhangt van de grootte van de aggregerende deeltjes en de mate van mechanische stress in vloeibare cultures. Uit experimenten die in hoofdstuk 5 staan beschreven blijkt dat deeltjes beter aan elkaar blijven hechten bij een lage schuifspanning. De glycanen die verantwoordelijk zijn voor aggregatie worden geproduceerd door de eiwitten die gecodeerd zijn in het *cslA*-*glxA*-operon en het *matAB*-cluster. Deze glycanen zorgen niet alleen voor een stevig contact tussen hyfen van hetzelfde deeltje, maar ook voor aggregatie tussen verschillende deeltjes. Met andere woorden, mutanten zonder functionele kopieën van *cslA*, *glxA* of *matAB* hebben

een “open” myceliumstructuur en groeien als losse individuen. In tegenstelling tot het wild-type zijn deeltjes van deze mutanten (dat wil zeggen die van de *cslA*- en *matAB*-mutanten) ook homogeen in grootte. Terwijl het wild-type twee verschillende populaties van mycelia vormt die in grootte verschillen, behoren mycelia die door de mutanten zijn gevormd tot één populatie met een normale verdeling. Dit geeft aan dat aggregatie een belangrijke factor is die de heterogeniteit van pellets veroorzaakt. Aggregatie wordt ook in verschillende soorten streptomyceten waargenomen, wat gebruikt kan worden voor het maken van pellets bestaande uit meerdere soorten. Deze toepassing kan leiden tot het ontdekken van nieuwe moleculen die ontstaan door interacties tussen verschillende soorten. Zo is aangetoond dat de co-cultivatie van streptomyceten en filamenteuze schimmels de productie kan stimuleren van moleculen die nooit gedetecteerd worden wanneer deze organismen apart van elkaar groeien [167, 168]. Ondanks het grote aantal bekende bioactieve moleculen geproduceerd door streptomyceten, is het biosynthetische potentieel van deze micro-organismen nog sterk onderschat. Analyses van het toenemende aantal beschikbare genoomsequenties geven aan dat deze organismen de genetische informatie bevatten voor de biosynthese van een groot aantal moleculen die nog nooit onder standaard laboratoriumomstandigheden zijn gevonden [216, 228]. Door dergelijke experimenten kunnen we in de toekomst wellicht moleculen vinden met een nieuwe biologische activiteit.

Hoewel aggregatie belangrijk is voor het ontstaan van heterogeniteit, wordt er in dit proefschrift geen mechanistisch inzicht gegeven over de manier waarop heterogeniteit uiteindelijk tot stand komt. Echter, op basis van de in dit proefschrift beschreven data en elders opgedane kennis [9, 15], kan een hypothese worden geformuleerd. Aggregatie is een stochastisch proces waarbij deeltjes met uniforme groottes (kiemen) aan elkaar kleven. Dit levert mycelium aggregaten van verschillende groottes op, die in principe nog steeds normaal verdeeld zijn. Kleine verschillen in de grootte van aggregaten zouden echter tot duidelijk verschillen kunnen leiden in de groeidynamiek. Eerder werk in filamenteuze schimmels heeft aangetoond dat de kern van grotere deeltjes vrij snel een tekort heeft aan zuurstof en voedingsstoffen [125, 127, 229]. Dit beperkt actieve groei tot de hyfen aan de buitenkant van pellets, wat leidt tot een groeimodus die “cube root” wordt genoemd [200, 230, 231]. Kleinere deeltjes daarentegen kunnen dan nog steeds exponentieel groeien, omdat ze aan geen beperking onderhevig zijn. Met andere woorden, men kan denken dat er een drempelgrootte bestaat waaronder exponentiële groei plaatsvindt en waarboven “cube root” groei optreedt. Zodra deeltjes deze drempelwaarde bereiken, zullen ze overschakelen van exponentiële naar “cube root” groei. Het samenspel van deze twee groeidynamieken kan uiteindelijk tot twee populaties pellets leiden. Deze interpretatie zou niet alleen verklaren waarom aggregatie betrokken is bij het optreden van heterogeniteit, maar ook

waarom dit fenomeen door de mediumsamenstelling (zie hoofdstuk 3) wordt beïnvloed. Hoewel pellets in zowel rijke als minimale media heterogeen worden in grootte [12], zijn de aggregaten van *S. lividans* na 12 uur groei in minimale media nog steeds homogeen, terwijl ze al heterogeen zijn in rijke media. Dit verschil heeft waarschijnlijk te maken met de hogere groeisnelheid in het rijke medium, waardoor heterogeniteit eerder ontstaat. Daarnaast kan de mate van lysis in de kern van aggregaten (zie hoofdstuk 4) de verschillen in groeisnelheid en het ontstaan van verschillende populaties verder beïnvloeden.

Hoofdstuk 4 beschrijft een techniek om homogene *Streptomyces* pellets te verkrijgen. Hiervoor werd gebruik gemaakt van de zogenaamde *sco*-mutant van *S. lividans*. Sco is een koper-chaperone die GlxA van een koperatoom voorziet, wat nodig is voor het matureren van dit eiwit [146]. In de afwezigheid van Sco is GlxA inactief leidend tot een fenotype dat vergelijkbaar is met dat van de *glxA* en *cslA*-mutanten. Opvallend is dat de maturatie van GlxA in de *sco*-mutant kan worden hersteld door de toevoeging van micromolaire hoeveelheden koper. Door deze toevoeging wordt zowel de vorming van pellets als de aggregatie tussen deeltjes herstelt in de *sco* mutant. Deze koperafhankelijke morfologische schakelaar wordt hier benut om de vorming van pellets aan te schakelen op het moment dat aggregatie tussen deeltjes niet meer optreedt. De redenering achter deze strategie is om pellets te verkrijgen die vanuit individuele sporen zijn ontstaan en dientengevolge uniform in grootte zijn. Met behulp van deze benadering kunnen pellets met een homogene grootte worden verkregen, met diameters die variëren tussen 167 en 276 µm. Dit maakt een directe analyse mogelijk naar het verband tussen de grootte van pellets en productie, waarbij in dit geval gekeken is naar een heteroloog geproduceerd xylanase. Opmerkelijk bleken de kleinere pellets het enzym sneller te produceren dan grotere pellets. Dit komt overeen met eerdere artikelen waarin het is aangetoond dat kleinere deeltjes beter zijn voor het produceren van enzymen dan grote pellets [15, 16]. In tegenstelling tot deze eerdere artikelen hebben de pellets die in dit hoofdstuk van dit proefschrift worden beschreven een uniforme grootteverdeling.

Omdat het fenotype van de hier gebruikte *sco* mutant echter niet volledig wordt hersteld door de toevoeging van koper, is het trekken van keiharde conclusies over het verband tussen morfologie en productie gecompliceerd. Zo is er in de aanwezigheid van koper nog steeds een verminderde aggregatie tussen deeltjes, wat leidt tot kleinere pellets en verminderde heterogeniteit. Bovendien heeft de *sco* mutant een andere groeikinetiek vergeleken met de ouderstam en groeit aanzienlijk langzamer dan de *glxA* mutant (Erik Vijgenboom, persoonlijke communicatie). Deze verschillen worden wellicht veroorzaakt door het feit dat Sco niet alleen betrokken is bij het matureren van GlxA, maar ook van het Cytochrome C

oxidase enzym Cox [180]. Dit oxidase speelt een belangrijke rol in het energiemetabolisme in het mycelium, wat suggereert dat de *sco* mutant mogelijk minder energie ter beschikking heeft en daardoor langzamer groeit. Hoewel de experimenten in hoofdstuk 4 de relatie tussen morfologie en productie niet volledig verklaren, lieten ze een belangrijke en tot nu toe onbekende link zien tussen aggregatie en celdood. Dit werd aangetoond door de groei en levensvatbaarheid van de *sco*-mutant te volgen in zowel aan- als afwezigheid van koper. Terwijl in de afwezigheid van koper (en daarom de afwezigheid van aggregatie) alleen levensvatbare hyfen werden waargenomen tot 18 uur, leidde de koper-geïnduceerde aggregatie tot het ontstaan van dode hyfen in aggregaten, die al na 8 uur zichtbaar werden. De detectie van beschadigde hyfen was ook al duidelijk in de wildtype stam na 8 uur (in de afwezigheid van koper), wat aangeeft dat dit geen effect was van de kopertoevoeging. Deze waarnemingen duiden op de aanwezigheid van een contact-afhankelijk mechanisme voor de activatie van ontwikkeling en geprogrammeerde celdood in streptomyceten. Geprogrammeerde celdood is al eerder beschreven tijdens de ontwikkeling van streptomyceten in vaste en vloeibare cultures [3, 4, 10, 99], hoewel tot dusver nooit is aangetoond dat het al in dit vroege stadium van groei kan voorkomen.

DE ROL VAN FRAGMENTATIE IN DE MORFOGENESE VAN *STREPTOMYCES*

Zoals hierboven beschreven is aggregatie van belang voor de vorming van pellets en het ontstaan van heterogeniteit. Een andere proces dat bijdraagt aan heterogeniteit, en waarover tot dusver weinig bekend is, is fragmentatie. Dit proces is redelijk goed beschreven in schimmels die gebruikt worden in de biotechnologiesector [232-235]. In schimmels kan fragmentatie zowel optreden via het breken van losse hyfen of via het zogenaamde “scheren” van hyphae aan de buitenkant van pellets . Ook is in schimmels een algemeen patroon beschreven met betrekking tot de grootte van mycelia, waarbij in de vroege groeifase een toename plaatsvindt gevolgd door een afname in grootte gerelateerd aan de veroudering van mycelia (ook waargenomen in streptomyceten) [12]. Het is interessant om te zien dat dit patroon schijnbaar niet wordt beïnvloed door de roersnelheid van de culture (en daardoor de mate van mechanische stress), hoewel hogere snelheden in het algemeen wel resulteren in kleinere myceliumdeeltjes.

Hoofdstuk 5 toont een uitgebreide analyse van pellet fragmentatie in de modelstam *S. lividans*. Tijdens een aantal initiële experimenten werden pellets van verschillende leeftijden in vers medium overgebracht, waarna de morfologie en groei van de deeltjes werden gevolgd. Deze aanpak liet zien dat fragmentatie alleen optreedt in oude mycelia op het moment dat cultures in de stationaire fase komen.

Vervolgexperimenten beschreven in dit hoofdstuk tonen aan dat de losgelaten fragmenten opnieuw kunnen aggregeren wanneer ze in vers medium worden overgebracht, maar afsterven wanneer ze in het gebruikte medium blijven. Opvallend is dat de aggregatie van fragmenten alleen plaatsvindt met andere losse fragmenten, maar niet met al bestaande pellets. Deze ontdekkingen zijn relevant voor industriële toepassingen, vooral met betrekking tot het feit dat grote bioreactoren vaak geïnoculeerd worden met een voorculture. Met name relevant is de ontdekking dat de leeftijd van het overgebrachte mycelium van invloed is op de grootte van de daarop nieuw gevormde pellets en de accumulatie van biomassa, vooral als een bepaalde morfologie de voorkeur heeft om productie te maximaliseren.

MICROENCAPSULATIE EN GROEI VAN *STREPTOMYCES*

Vanuit het perspectief van industriële toepassing is de vorming van pellets een onaantrekkelijk kenmerk van de groei van streptomyceten [9]. De grote omvang van pellets zorgt voor problemen met massaoverdracht die ertoe leiden dat zuurstof en voedingsstoffen maar moeilijk de centrale delen van deze structuren kunnen bereiken. Mede als gevolg hiervan en van geprogrammeerde ontwikkelingsprocessen worden mycelia na verloop van tijd deels ontmanteld, wat de algehele groei negatief beïnvloedt [10] (zie ook hoofdstuk 4). Deze problemen kunnen worden vermeden door niet-aggregerende *Streptomyces* stammen te gebruiken [9, 15, 144, 146]. Echter, dergelijke niet-aggregerende stammen hebben een negatieve invloed op de reologische eigenschappen van de cultures, waar een hoge viscositeit een bekend voorbeeld van is. In het licht van deze waarnemingen werd in hoofdstuk 6 een alternatieve kweektechniek bestudeerd die veel van deze nadelen omzeilt. Deze techniek, die micro-encapsulatie wordt genoemd, is al in veel micro-organismen gebruikt om de groeisnelheid en productie te verbeteren [202-207]. Middels micro-encapsulatie worden levende cellen ingebed in µm-schaal capsules (meestal gemaakt uit calciumalginaat) en vervolgens gekweekt in vloeibare cultures. Deze microcapsules zorgen ervoor dat gassen en voedingsstoffen vrij kunnen verspreiden, terwijl cellen tegen de externe omgeving worden beschermd [215].

Het in Hoofdstuk 6 beschreven werk toont aan dat micro-encapsulatie de levensvatbaarheid van S. *lividans* enorm kan vergroten. Bovendien leidt micro-encapsulatie tot een verhoogde extracellulaire concentratie en activiteit van het reporter-enzym tyrosinase, wat duidt op een hogere productie en/of secretie. Hoewel dit aspect niet in detail werd onderzocht, worden de verlengde levensvatbaarheid en verhoogde enzymproductie waarschijnlijk veroorzaakt doordat het mycelium minder onderhevig is aan de mechanische stress van schudcultures.

Samen met de toename in de hoeveelheid en activiteit van tyrosinase in het supernatant, lijkt de hoeveelheid andere eiwitten in het supernatant juist verminderd te zijn wanneer mycelium ingebed wordt in capsules. Deze verhoogde zuiverheid komt mogelijk door een afname van de lysis, die normaal (deels) veroorzaakt wordt door de mechanische stress in cultures.

Naast de hierboven beschreven toepassing kan micro-encapsulatie mogelijk ook worden gebruikt om andere winstgevende doelen te bereiken. Micro-encapsulatie kan bijvoorbeeld de productie van antibiotica stimuleren, zoals is waargenomen in *S. coelicolor* [207], maar ook S. *lividans*. Zo werd de productie van het blauw-gepigmenteerde antibioticum actinorhodine waargenomen wanneer *S. lividans* werd gegroeid in capsules (onze niet-gepubliceerde resultaten). Hoewel *S. lividans* de genetische informatie heeft die nodig is voor de productie van dit antibioticum, wordt actinorhodine meestal niet geproduceerd tijdens gebruikelijke laboratoriumomstandigheden. Deze vinding wijst erop dat door gebruik te maken van micro-encapsulatie wellicht ook andere "slapende" biosynthetische genclusters kunnen worden geactiveerd.

EEN NIEUW MODEL VOOR DE VORMING VAN PELLETS EN FRAGMENTATIE IN *STREPTOMYCES LIVIDANS*

Gezien de prominente rol van streptomyceten in de industriële sector en de invloed van morfologie op de productiviteit, is in de afgelopen jaren veel onderzoek verricht om modellen te ontwikkelen die de morfogenese van streptomyceten in vloeistof kunnen voorspellen. Er zijn met name modellen ontworpen die zich voornamelijk richten op de effecten van omgevingsfactoren [236-240]. Meer recentelijk zijn geavanceerdere *in silico* modellen ontwikkeld. Het door Celler en collega's ontwikkelde model is in staat de driedimensionale morfologie van in vloeistof gegroeide mycelia te voorspellen op basis van parameters als de mate van zuurstofdiffusie, groeisnelheid van hyfen, de mate van vertakking en fragmentatie en botsingen tussen deeltjes [198]. Het is duidelijk dat dergelijke modelleerbenaderingen alleen kunnen functioneren als er een goed begrip is van de genetische determinanten van morfogenese, en wanneer een geïntegreerde benadering wordt gebruikt die ook een experimentele validatie van de modelleringsprocedure bevat. De resultaten beschreven in dit proefschrift stellen ons in staat een verbeterd model van de morfogenese van streptomycetes te beschrijven. Hoewel dit model gebaseerd is op experimentele studies met *S. lividans*, kan het ook relevant zijn voor andere pellet-vormende streptomyceten. Deze beschrijving is gedeeltelijk complementair aan en vervangt eerder voorgestelde modellen [9].

Na het inoculeren van vloeibare cultures, worden met het kiemen van sporen de eerste zichtbare tekenen van groei na ongeveer 4 uur waargenomen. Hoewel ze levensvatbaar zijn, zal een deel van de sporen dat gebruikt is voor het inoculeren niet ontkiemen (zie hoofdstuk 5). Zoals in hoofdstuk 3 is aangetoond leidt kieming tot het ontstaan van aggregatie, waarbij gekiemde sporen aan andere gekiemde sporen of al grotere aggregaten plakken. Sporen die later ontkiemen kunnen zowel aan elkaar of aan reeds gevormde aggregaten hechten. De groeiende aggregaten kunnen verder aggregeren tot ongeveer 12 uur na de inoculatie. Interessant is dat de duur van deze aggregatiefase afhankelijk is van de mate van mechanische stress. Bij een verminderde schuifspanning wordt de duur van de aggregatiefase verlengd, wat tot gevolg heeft dat grotere pellets gevormd kunnen worden (hoofdstuk 5). De aggregatie tussen grotere deeltjes wordt blijkbaar bepaald door hun fysische eigenschappen, in het bijzonder hun vorm en massa, die de weerstand tegen mechanische spanningen beïnvloeden. Kort gezegd, wanneer twee deeltjes tegen elkaar botsen, wordt er een initieel contact tot stand gebracht middels hun oppervlakken. Dit contact moet worden behouden totdat verdere groei de hechting kan verstevigen (bijvoorbeeld door middel van verstrengelde groei van de hyfen van beide deeltjes). Wanneer dit contact tussen kleine deeltjes optreedt, is een relatief groot oppervlak aanwezig in verhouding tot de totale massa van het aggregaat. Bij grotere deeltjes is deze oppervlakte-massa verhouding minder gunstig, waardoor het aggregaat makkelijker uit elkaar valt door de mechanische stress in de culture. Dit betekent dat myceliumdeeltjes uiteindelijk een grootte bereiken die voorkomt dat ze verder gaan aggregeren. Zodra deze drempelwaarde wordt bereikt, nemen aggregaten alleen nog toe in omvang door groei om zo uiteindelijk grote pellets te vormen. Hoewel de aanwezigheid van dode hyfen al wordt waargenomen aan het begin van de aggregatiefase in *S. lividans* (zie hoofdstuk 4), ontstaat in dit stadium ook een tekort aan voedingsstoffen en zuurstof in de kern van pellets [125, 127, 229]. Dit tekort wordt veroorzaakt doordat de hyfen aan de buitenkant van pellets deze stoffen consumeren, al dan niet gecombineerd met de verstoorde diffusie door de hoge dichtheid van het mycelium. Als gevolg van deze verschijnselen wordt een kern van dode cellen zichtbaar in pellets. Als na verloop van tijd de voedingsstoffen uitgeput raken, bereiken cultures de stationaire fase en begint fragmentatie op te treden. Zoals in Hoofdstuk 5 wordt aangetoond kunnen de kleine myceliumfragmenten, die van grote pellets loskomen, opnieuw met elkaar aggregeren in vers medium. Daarentegen zullen deze fragmenten uiteindelijk afsterven wanneer cultures niet worden aangevuld met verse voedingsstoffen.

VOORUITZICHTEN EN TOEKOMSTPERSPECTIEVEN

De kennis opgedaan in dit proefschrift is van groot belang met het oog op een rationele optimalisatie van streptomyceten als celfabrieken. Gezien de belangrijke rol die aggregatie heeft bij het ontstaan van heterogeniteit, lijkt dit proces het meest voor de hand liggend om te moduleren om uiteindelijk morfogenese beter te kunnen sturen. Naast de strategie beschreven in hoofdstuk 4, kunnen twee mogelijke benaderingen worden bedacht om de aggregatie tussen deeltjes tegen te gaan. Eén manier om dit doel te bereiken is door de mechanische stress te verhogen tijdens de eerste groeifase, tot het moment dat deeltjes een grootte hebben bereikt die voorkomt dat ze aan elkaar kunnen hechten. Dit kan relatief eenvoudig worden verkregen door de roersnelheid van cultures tijdelijk te verhogen, waardoor de mechanische stress verhoogd wordt. Deze strategie is echter niet geheel zonder risico's, omdat te veel mechanische stress kan leiden tot fysieke beschadiging van de mycelia, met mogelijke lysis en verminderde productopbrengsten als gevolg.

Een alternatieve benadering zou zijn om de productie te moduleren van glycanen die betrokken zijn bij de aggregatie tussen deeltjes [142, 147] , door de expressie van de betrokken genen in de eerste groeifase (tot 16-20 uur) uit te schakelen. Voor dit doel hebben de *matAB* genen de voorkeur boven *cslA* of *glxA*, aangezien het door CslA-geproduceerde polymeer ook betrokken is bij het verstevigen van de uiteinden van de hyfen [144]. Deze benadering werd al onderzocht, maar heeft als nadeel dat pellets een lagere dichtheid vertonen, schijnbaar omdat de hierbij gebruikte promoter pas actief wordt in een vergevorderde groeifase (Dino van Dissel, proefschrift) . Een volgende stap zou zijn om natuurlijke of synthetische promoters te gebruiken die transcriptie al eerder activeren. Als alternatief kan een induceerbaar systeem worden gebruikt, hoewel dit te kostbaar kan zijn onder industriële omstandigheden.

Een andere opvallende uitkomst van het hier beschreven onderzoek is dat aggregatie een belangrijke schakelaar is die het proces van geprogrammeerde celdood kan activeren. Deze waarneming is gebaseerd op de vinding dat de inductie van aggregatie van de S. *lividans sco* mutant leidt tot mycelia die al snel dode hyfen bevatten. Dit zou ook deels kunnen verklaren waarom niet-aggregerende *Streptomyces* stammen over het algemeen hogere groeisnelheden vertonen, naast redenen die verband houden met de verhoogde diffusie van voedingsstoffen en zuurstof [15]. Een vervolgstudie zou zich kunnen richten op het vinden van mutanten met een verminderde aggregatie-geïnduceerde celdood. Dergelijke mutanten kunnen worden gevonden door het toepassen van een random mutagenese screen op een stam die constitutief een onstabiele GFP-variant produceert [241].

Op deze manier kunnen mutanten worden geselecteerd, bijvoorbeeld door middel van een flow cytrometrische aanpak, die een hoge fluorescentie laten zien in jonge aggregaten. Door de instabiliteit van deze GFP variant zullen alleen de actief groeiende hyfen fluorescent zijn, waardoor de mate van fluorescentie evenredig is met de hoeveelheid levende hyfen in aggregaten. Deze aanpak zou kunnen leiden tot het vinden van stammen met een verminderde lysis en met een mogelijk superieure accumulatie van biomassa en product. Hoewel lysis nog steeds kan optreden in deze mutanten als gevolg van de hierboven besproken zuurstof- en voedingsstofbeperkingen, zal dit effecten pas duidelijk zichtbaar zijn in grotere (oudere) myceliumdeeltjes. Een andere mogelijke onderzoekslijn, voortkomend uit dit proefschrift, zou zich kunnen richten op het mechanisme dat ten grondslag ligt aan mycelium fragmentatie. Zoals in hoofdstuk 5 beschreven is, vertoont fragmentatie opvallende overeenkomsten met de verspreiding van cellen uit bacteriële biofilms, een proces dat in veel gevallen afhankelijk is van de actieve hydrolyse van de extracellulaire matrix. In deze context is het interessant om te noemen dat het genoom van *S. lividans* een gen voor een vermoedelijk hydrolase bevat welke significante gelijkenis vertoont met Dispersin B van *Actinobacillus actinomycetemcomitans*. Van Dispersin B is het bekend dat het poly-N-acetylglucosamine (PNAG) hydrolyseert, dat een veel voorkomende component van biofilms is, en dat ook aanwezig is op aggregerende hyfen van *Streptomyces* [147]. Inderdaad blijkt Dispersin B heel efficiënt deze extracellulaire PNAG-laag te kunnen oplossen die *Streptomyces* kiembuizen bedekt [147]. Het is daarom interessant om de rol van dit homologe Dispersin B eiwit in morfogenese te karakteriseren, en te kijken of veranderingen in de timing en mate van fragmentatie optreden door de expressie van dit eiwit te moduleren. Hoewel het onduidelijk is hoe pelletgrootte en fragmentatie elkaar beïnvloeden in jonge culturen, is het interessant te vermelden dat in *Streptomyces coelicolor* de Dispersin B homoloog significant meer in grote pellets voorkomt [12]. Een goede controle over het fragmentatie proces zou winstgevend kunnen zijn wanneer jonge culturen verdund worden, om bijvoorbeeld het vermogen van jonge mycelia te vergroten om nieuwe kleine deeltjes (beter geschikt voor enzymproductie) te vormen. Aan de andere kant kan het voorkomen van fragmentatie in latere groeistadia gunstig zijn om de architectuur en grootte van pellets te behouden, wat de productie van secundaire metabolieten (waarvoor de aanwezigheid van pellets vereist is) ten goede kan komen.

Zoals eerder goenoemd, is dit proefschrift gericht op het bestuderen van heterogeniteit in de grootte van pellets. Heterogeniteit is echter niet beperkt tot verschillen in pelletgroottes, maar komt ook voor op andere niveaus (zie hoofdstuk 2 voor meer informatie). In vergelijking tot schimmels is de enige vorm van heterogeniteit die nog niet in streptomyceten is bestudeerd, degene mogelijk aanwezig tussen

aangrenzende hyfen binnen pellets. Het is duidelijk van groot belang om te zien of deze vorm van heterogeniteit bestaat en te bestuderen wat het effect hiervan is op productiviteit. De mechanismen waardoor heterogeniteit tussen aangrenzende hyfen kan ontstaan berusten op afsluitbare compartimenten in hyfen, wat bewerkstelligd wordt door middel van septa of zogenaamde cross-membranes [98, 137, 223, 242]. Hoewel de controle van septatie vrij goed is begrepen, is het proces waarbij cross-membranes worden gevormd nog onbekend. Verdere studies zijn hard nodig om deze onopgeloste vragen te kunnen beantwoorden.

Andere onbeantwoorde vragen betreffen de exacte functie van die hier en elders beschreven [12, 15, 166] componenten betrokken bij *Streptomyces* morfogenese. Hoewel het fenotype van *cslA*-, *glxA*- en *matAB*-stammen vergelijkbaar is met betrekking tot de mate van aggregatie tussen deeltjes en algemene morfologie [142], spelen deze glycanen verschillende rollen bij het hechten van *Streptomyces* hyfen aan oppervlakken (zie boven) [144, 147]. Het is echter onduidelijk of, en hoe deze componenten samenwerken om adhesie tot stand te brengen. Daarnaast spelen andere extracellulaire componenten (chaplins, extracellulair DNA en hyaluronzuur) een belangrijk rol in de totstandkoming van de pelletarchitectuur. Zo hebben pilot experimenten aangetoond dat een *Streptomyces coelicolor* stam waarin de belangrijkste chaplins werden verwijderd, niet in staat is pelletmorfologie te behouden na langdurige groei bij lage pH (Boris Zacchetti, Marloes Petrus, Dennis Claessen, niet-gepubliceerde resultaten), wat sterk wijst op de betrokkenheid van deze eiwitten bij het vormen van pellets.

References

1. Barka, E.A. et al. Taxonomy, physiology, and natural products of *Actinobacteria*. *Microbiol Mol Biol Rev* **80**, 1-43 (2016).

2. Chater, K.F. Recent advances in understanding Streptomyces. *F1000Res* **5**, 2795 (2016).

3. Manteca, A., Fernandez, M. & Sanchez, J. A death round affecting a young compartmentalized mycelium precedes aerial mycelium dismantling in confluent surface cultures of *Streptomyces antibioticus*. *Microbiology* **151**, 3689-3697 (2005).

4. Manteca, A., Fernandez, M. & Sanchez, J. Cytological and biochemical evidence for an early cell dismantling event in surface cultures of *Streptomyces antibioticus*. *Res Microbiol* **157**, 143-152 (2006).

5. Claessen, D., de Jong, W., Dijkhuizen, L. & Wösten, H.A.B. Regulation of *Streptomyces* development: reach for the sky! *Trends Microbiol* **14**, 313-319 (2006).

6. Claessen, D., Rozen, D.E., Kuipers, O.P., Søgaard-Andersen, L. & van Wezel, G.P. Bacterial solutions to multicellularity: a tale of biofilms, filaments and fruiting bodies. *Nat Rev Microbiol* **12**, 115-124 (2014).

7. Hopwood, D.A. *Streptomyces* in nature and medicine: the antibiotic makers. (Oxford University Press, USA, New York; 2007).

8. Vrancken, K. & Anné, J. Secretory production of recombinant proteins by *Streptomyces*. *Future Microbiol* **4**, 181-188 (2009).

9. van Dissel, D., Claessen, D. & van Wezel, G.P. Morphogenesis of streptomyces in submerged cultures. *Adv Appl Microbiol* **89**, 1-45 (2014).

10. Manteca, A., Alvarez, R., Salazar, N., Yagüe, P. & Sanchez, J. Mycelium differentiation and antibiotic production in submerged cultures of *Streptomyces coelicolor*. *Appl Environ Microbiol* **74**, 3877-3886 (2008).

11. Rioseras, B., López-García, M.T., Yagüe, P., Sánchez, J. & Manteca, A. Mycelium differentiation and development of *Streptomyces coelicolor* in lab-scale bioreactors: Programmed cell death, differentiation, and lysis are closely linked to undecylprodigiosin and actinorhodin production. *Bioresour Technol* **151**, 191-198 (2013).

12. van Veluw, G.J. et al. Analysis of two distinct mycelial populations in liquid-grown *Streptomyces* cultures using a flow cytometry-based proteomics approach. *Appl Microbiol Biotechnol* **96**, 1301-1312 (2012).

13. Wardell, J.N., Stocks, S.M., Thomas, C.R. & Bushell, M.E. Decreasing the hyphal branching rate of *Saccharopolyspora erythraea* NRRL 2338 leads to increased resistance to breakage and increased antibiotic production. *Biotechnol Bioeng* **78**, 141-146 (2002).

14. Pickup, K.M. & Bushell, M.E. Non-fragmenting variants of *Streptomyces* hyphae have enhanced activity of an enzyme (phospho-*N*-acetylmuramyl pentapeptide translocase) in peptidoglycan biosynthesis. *J Ferment Bioeng* **79**, 247-251 (1995).

15. van Dissel, D., Claessen, D., Roth, M. & van Wezel, G.P. A novel locus for mycelial aggregation forms a gateway to improved *Streptomyces* cell factories. *Microb Cell Fact* **14**, 44 (2015).

16. van Wezel, G.P. et al. Unlocking *Streptomyces* spp. for use as sustainable industrial production platforms by morphological engineering. *Appl Environ Microbiol* **72**, 5283-5288 (2006).

17. Brehm-Stecher, B.F. & Johnson, E.A. Single-cell microbiology: tools, technologies, and applications. *Microbiol Mol Biol Rev* **68**, 538-559, table of contents (2004).

18. Davis, K.M. & Isberg, R.R. Defining heterogeneity within bacterial populations via single cell approaches. *Bioessays* **38**, 782-790 (2016).

19. Binder, D. et al. Homogenizing bacterial cell factories: analysis and engineering of phenotypic heterogeneity. *Metab Eng* **42**, 145-156 (2017).

20. Veening, J.W., Smits, W.K. & Kuipers, O.P. Bistability, epigenetics, and bet-hedging inbacteria. *Annu Rev Microbiol* **62**, 193-210 (2008).

21. Ackermann, M. A functional perspective on phenotypic heterogeneity in microorganisms. *Nat Rev Microbiol* **13**, 497-508 (2015).

22. Avery, S.V. Microbial cell individuality and the underlying sources of heterogeneity. *Nat Rev Microbiol* **4**, 577-587 (2006).

23. Smits, W.K., Kuipers, O.P. & Veening, J.W. Phenotypic variation in bacteria: the role of feedback regulation. *Nat Rev Microbiol* **4**, 259-271 (2006).

24. van Boxtel, C., van Heerden, J.H., Nordholt, N., Schmidt, P. & Bruggeman, F.J. Taking chances and making mistakes: non-genetic phenotypic heterogeneity and its consequences for surviving in dynamic environments. *J R Soc Interface* **14** (2017).

25. Elowitz, M.B., Levine, A.J., Siggia, E.D. & Swain, P.S. Stochastic gene expression in a single cell. *Science* **297**, 1183-1186 (2002).

26. Levy, S.F., Ziv, N. & Siegal, M.L. Bet hedging in yeast by heterogeneous, age-correlated expression of a stress protectant. *PLoS Biol* **10**, e1001325 (2012).

27. Chastanet, A. et al. Broadly heterogeneous activation of the master regulator for sporulation in *Bacillus subtilis*. *Proc Natl Acad Sci U S A* **107**, 8486-8491 (2010).

28. Maamar, H., Raj, A. & Dubnau, D. Noise in gene expression determines cell fate in *Bacillus subtilis*. *Science* **317**, 526-529 (2007).

29. Jacob, F. & Monod, J. Genetic regulatory mechanisms in the synthesis of proteins. *J Mol Biol* **3**, 318-356 (1961).

30. Philippi, T. & Seger, J. Hedging one's evolutionary bets, revisited. *Trends Ecol Evol* **4**, 41-44 (1989).

31. Grimbergen, A.J., Siebring, J., Solopova, A. & Kuipers, O.P. Microbial bet-hedging: the power of being different. *Curr Opin Microbiol* **25**, 67-72 (2015).

32. Errington, J. Regulation of endospore formation in *Bacillus subtilis*. *Nat Rev Microbiol* **1**, 117-126 (2003).

33. Higgins, D. & Dworkin, J. Recent progress in *Bacillus subtilis* sporulation. *FEMS Microbiol Rev* **36**, 131-148 (2012).

34. Russell, J.R., Cabeen, M.T., Wiggins, P.A., Paulsson, J. & Losick, R. Noise in a phosphorelay drives stochastic entry into sporulation in *Bacillus subtilis*. *EMBO J* **36**, 2856-2869 (2017).

35. Siebring, J. et al. Repeated triggering of sporulation in *Bacillus subtilis* selects against a protein that affects the timing of cell division. *ISME J* **8**, 77-87 (2014).

36. Veening, J.W. et al. Bet-hedging and epigenetic inheritance in bacterial cell development. *Proc Natl Acad Sci U S A* **105**, 4393-4398 (2008).

37. Balaban, N.Q., Merrin, J., Chait, R., Kowalik, L. & Leibler, S. Bacterial persistence as a phenotypic switch. *Science* **305**, 1622-1625 (2004).

38. Claudi, B. et al. Phenotypic variation of *Salmonella* in host tissues delays eradication by antimicrobial chemotherapy. *Cell* **158**, 722-733 (2014).

39. Helaine, S. et al. Internalization of *Salmonella* by macrophages induces formation of nonreplicating persisters. *Science* **343**, 204-208 (2014).

40. Manina, G., Dhar, N. & McKinney, J.D. Stress and host immunity amplify *Mycobacterium tuberculosis* phenotypic heterogeneity and induce nongrowing metabolically active forms. *Cell Host Microbe* **17**, 32-46 (2015).

41. Dörr, T., Vulić, M. & Lewis, K. Ciprofloxacin causes persister formation by inducing the TisB toxin in *Escherichia coli*. *PLoS Biol* **8**, e1000317 (2010).

42. Johnson, P.J.T. & Levin, B.R. Pharmacodynamics, population dynamics, and the evolution of persistence in *Staphylococcus aureus*. *PLoS Genet* **9**, e1003123 (2013).

43. Mulcahy, L.R., Burns, J.L., Lory, S. & Lewis, K. Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. *J Bacteriol* **192**, 6191-6199 (2010).

44. Lu, C., Brauer, M.J. & Botstein, D. Slow growth induces heat-shock resistance in normal and respiratory-deficient yeast. *Mol Biol Cell* **20**, 891-903 (2009).

45. Kotte, O., Volkmer, B., Radzikowski, J.L. & Heinemann, M. Phenotypic bistability in *Escherichia coli*'s central carbon metabolism. *Mol Syst Biol* **10**, 736 (2014).

46. Solopova, A. et al. Bet-hedging during bacterial diauxic shift. *Proc Natl Acad Sci U S A* **111**, 7427-7432 (2014).

47. van Heerden, J.H. et al. Lost in transition: start-up of glycolysis yields subpopulations of nongrowing cells. *Science* **343**, 1245114 (2014).

48. Monod, J. The growth of bacterial cultures. *Annual Review of Microbiology* **3**, 371-394 (1949).

49. van Gestel, J., Vlamakis, H. & Kolter, R. From cell differentiation to cell collectives: *Bacillus subtilis* uses division of labor to migrate. *PLoS Biol* **13**, e1002141 (2015).

50. van Gestel, J., Vlamakis, H. & Kolter, R. Division of labor in biofilms: the ecology of cell differentiation. *Microbiol Spectr* **3**, MB-0002-2014 (2015).

51. Zhang, Z., Claessen, D. & Rozen, D.E. Understanding microbial divisions of labor. *Front Microbiol* **7**, 2070 (2016).

52. Veening, J.W. et al. Transient heterogeneity in extracellular protease production by *Bacillus subtilis*. *Mol Syst Biol* **4**, 184 (2008).

53. Arnoldini, M. et al. Bistable expression of virulence genes in *Salmonella* leads to the formation of an antibiotic-tolerant subpopulation. *PLoS Biol* **12**, e1001928 (2014).

54. Diard, M. et al. Stabilization of cooperative virulence by the expression of an avirulent phenotype. *Nature* **494**, 353-356 (2013).

55. Velicer, G.J., Kroos, L. & Lenski, R.E. Developmental cheating in the social bacterium *Myxococcus xanthus. Nature* **404**, 598-601 (2000).

56. Strassmann, J.E., Zhu, Y. & Queller, D.C. Altruism and social cheating in the social amoeba *Dictyostelium discoideum*. *Nature* **408**, 965-967 (2000).

57. Reichenbach, H. The ecology of the myxobacteria. *Environ Microbiol* **1**, 15-21 (1999).

58. O'Connor, K.A. & Zusman, D.R. Development in *Myxococcus xanthus* involves differentiation into two cell types, peripheral rods and spores. *J Bacteriol* **173**, 3318-3333 (1991).

59. Nariya, H. & Inouye, M. MazF, an mRNA interferase, mediates programmed cell death during multicellular *Myxococcus* development. *Cell* **132**, 55-66 (2008).

60. Wireman, J.W. & Dworkin, M. Developmentally induced autolysis during fruiting body formation by *Myxococcus xanthus*. *J Bacteriol* **129**, 798-802 (1977).

61. Berleman, J.E., Chumley, T., Cheung, P. & Kirby, J.R. Rippling is a predatory behavior in *Myxococcus xanthus*. *J Bacteriol* **188**, 5888-5895 (2006).

62. Levine, J.H., Lin, Y. & Elowitz, M.B. Functional roles of pulsing in genetic circuits. *Science* **342**, 1193-1200 (2013).

63. Johnson, D.R., Goldschmidt, F., Lilja, E.E. & Ackermann, M. Metabolic specialization and the assembly of microbial communities. *ISME J* **6**, 1985-1991 (2012).

64. de Lorenzo, V., Sekowska, A. & Danchin, A. Chemical reactivity drives spatiotemporal organisation of bacterial metabolism. *FEMS Microbiol Rev* **39**, 96-119 (2015).

65. Mitsui, A. et al. Strategy by which nitrogen-fixing unicellular cyanobacteria grow photoautotrophically. *Nature* **323**, 720-722 (1986).

66. Adams, D.G. Heterocyst formation in cyanobacteria. *Curr Opin Microbiol* **3**, 618-624 (2000).

67. Kumar, K., Mella-Herrera, R.A. & Golden, J.W. Cyanobacterial heterocysts. *Cold Spring Harb Perspect Biol* **2**, a000315 (2010).

68. Berman-Frank, I. et al. Segregation of nitrogen fixation and oxygenic photosynthesis in the marine cyanobacterium *Trichodesmium*. *Science* **294**, 1534-1537 (2001).

69. Golding, I., Paulsson, J., Zawilski, S.M. & Cox, E.C. Real-time kinetics of gene activity in individual bacteria. *Cell* **123**, 1025-1036 (2005).

70. Cai, L., Friedman, N. & Xie, X.S. Stochastic protein expression in individual cells at the single molecule level. *Nature* **440**, 358-362 (2006).

71. Ozbudak, E.M., Thattai, M., Kurtser, I., Grossman, A.D. & van Oudenaarden, A. Regulation of noise in the expression of a single gene. *Nat Genet* **31**, 69-73 (2002).

72. Blake, W.J., Kærn, M., Cantor, C.R. & Collins, J.J. Noise in eukaryotic gene expression. *Nature* **422**, 633-637 (2003).

73. Süel, G.M., Kulkarni, R.P., Dworkin, J., Garcia-Ojalvo, J. & Elowitz, M.B. Tunability and noise dependence in differentiation dynamics. *Science* **315**, 1716-1719 (2007).

74. Locke, J.C., Young, J.W., Fontes, M., Hernandez Jimenez, M.J. & Elowitz, M.B. Stochastic pulse regulation in bacterial stress response. *Science* **334**, 366-369 (2011).

75. Young, J.W., Locke, J.C.W. & Elowitz, M.B. Rate of environmental change determines stress response specificity. *Proc Natl Acad Sci U S A* **110**, 4140-4145 (2013).

76. Levine, J.H., Fontes, M.E., Dworkin, J. & Elowitz, M.B. Pulsed feedback defers cellular differentiation. *PLoS Biol* **10**, e1001252 (2012).

77. Silander, O.K. et al. A genome-wide analysis of promoter-mediated phenotypic noise in *Escherichia coli*. *PLoS Genet* **8**, e1002443 (2012).

78. Newman, J.R. et al. Single-cell proteomic analysis of S. cerevisiae reveals the architecture of biological noise. *Nature* **441**, 840-846 (2006).

79. Taniguchi, Y. et al. Quantifying *E. coli* proteome and transcriptome with single-molecule sensitivity in single cells. *Science* **329**, 533-538 (2010).

80. Slavov, N. & Botstein, D. Decoupling nutrient signaling from growth rate causes aerobic glycolysis and deregulation of cell size and gene expression. *Mol Biol Cell* **24**, 157-168 (2013).

81. Berthoumieux, S. et al. Shared control of gene expression in bacteria by transcription factors and global physiology of the cell. *Mol Syst Biol* **9**, 634 (2013).

82. Kiviet, D.J. et al. Stochasticity of metabolism and growth at the single-cell level. *Nature* **514**, 376-379 (2014).

83. Klumpp, S. & Hwa, T. Bacterial growth: global effects on gene expression, growth feedback and proteome partition. *Curr Opin Biotechnol* **28**, 96-102 (2014).

84. Tan, C., Marguet, P. & You, L. Emergent bistability by a growth-modulating positive feedback circuit. *Nat Chem Biol* **5**, 842-848 (2009).

85. Avraham, N., Soifer, I., Carmi, M. & Barkai, N. Increasing population growth by asymmetric segregation of a limiting resource during cell division. *Mol Syst Biol* **9**, 656 (2013).

86. Goodfellow, M., Mordarski, M. & Williams, S.T. The biology of the actinomycetes. (Academic Press, London; 1983).

87. Walker, G.N. & White, N.A. Fungi: biology and applications, Edn. 3rd. (Wiley, 2005).

88. Flärdh, K. Cell polarity and the control of apical growth in *Streptomyces*. *Curr Opin Microbiol* **13**, 758-765 (2010).

89. Riquelme, M. Tip growth in filamentous fungi: a road trip to the apex. *Annu Rev Microbiol* **67**, 587-609 (2013).

90. Chater, K.F. Taking a genetic scalpel to the *Streptomyces* colony. *Microbiology* **144**, 1465-1478 (1998).

91. Borkovich, K.A. & Ebbole, D.J. Cellular and molecular biology of filamentous fungi. (ASM Press, 2010).

92. Bérdy, J. Bioactive microbial metabolites. *J Antibiot (Tokyo)* **58**, 1-26 (2005).

93. Jones, S.E. et al. *Streptomyces* exploration is triggered by fungal interactions and volatile signals. *Elife* **6** (2017).

94. Jones, S.E. & Elliot, M.A. *Streptomyces* exploration: competition, volatile communication and new bacterial behaviours. *Trends Microbiol* **25**, 522-531 (2017).

95. Papagianni, M. Fungal morphology and metabolite production in submerged mycelial processes. *Biotech Adv* **22**, 189-259 (2004).

96. Manteca, A., Fernandez, M. & Sanchez, J. Mycelium development in *Streptomyces antibioticus* ATCC11891 occurs in an orderly pattern which determines multiphase growth curves. *BMC Microbiol* **5**, 51 (2005).

97. Yagüe, P., López-García, M.T., Rioseras, B., Sánchez, J. & Manteca, A. Pre-sporulation stages of *Streptomyces* differentiation: state-of-the-art and future perspectives. *FEMS Microbiol Lett* **342**, 79-88 (2013).

98. Yagüe, P. et al. Subcompartmentalization by cross-membranes during early growth of *Streptomyces* hyphae. *Nat Commun* **7**, 12467 (2016).

99. Manteca, A., Claessen, D., Lopez-Iglesias, C. & Sanchez, J. Aerial hyphae in surface cultures of *Streptomyces lividans* and *Streptomyces coelicolor* originate from viable segments surviving an early programmed cell death event. *FEMS Microbiol Lett* **274**, 118-125 (2007).

100. Manteca, A., Sanchez, J., Jung, H.R., Schwämmle, V. & Jensen, O.N. Quantitative proteomic analysis of *Streptomyces coelicolor* development demonstrates that onset of secondary metabolism coincides with hyphae differentiation. *Mol Cell Proteomics* **9**, 1423-1436 (2010).

101. Wösten, H.A.B., Moukha, S.M., Sietsma, J.H. & Wessels, J.G.H. Localization of growth and secretion of proteins in Aspergillus niger. *J Gen Microbiol* **137**, 2017-2023 (1991).

102. Moukha, S.M., Wösten, H.A.B., Asther, M. & Wessels, J.G.H. *In situ* localization of the secretion of lignin peroxidases in colonies of *Phanerochaete chrysosporium* using a sandwiched mode of culture. *J Gen Microbiol* **139**, 969-978 (1993).

103. Moukha, S.M., Wösten, H.A.B., Mylius, E.J., Asther, M. & Wessels, J.G.H. Spatial and temporal accumulation of mRNAs encoding two common lignin peroxidases in *Phanerochaete chrysosporium*. *J Bacteriol* **175**, 3672-3678 (1993).

104. Krijgsheld, P. et al. Spatially resolving the secretome within the mycelium of the cell factory *Aspergillus niger*. *J Proteome Res* **11**, 2807-2818 (2012).

105. Levin, A.M. et al. Spatial differentiation in the vegetative mycelium of *Aspergillus niger*. *Eukaryot Cell* **6**, 2311-2322 (2007).

106. Pollack, J.K., Li, Z.J. & Marten, M.R. Fungal mycelia show lag time before re-growth on endogenous carbon. *Biotechnol Bioeng* **100**, 458-465 (2008).

107. Perez-Leblic, M.I., Reyes, F., Martinez, M.J. & Lahoz, R. Cell wall degradation in the autolysis of filamentous fungi. *Mycopathologia* **80**, 147-155 (1982).

108. Wessels, J.G.H. Wall growth, protein excretion and morphogenesis in fungi. *New Phytol* **123**, 397-413 (1993).

109. Krijgsheld, P. et al. Deletion of *flbA* results in increased secretome complexity and reduced secretion heterogeneity in colonies of *Aspergillus niger*. *J Proteome Res* **12**, 1808-1819 (2013).

110. Wessels, J.G.H. A steady-state model for apical wall growth in fungi. *Acta Bot Neerl* **37**, 3-16 (1988).

111. Wang, F.F. et al. FluG affects secretion in colonies of *Aspergillus niger*. *Antonie Van Leeuwenhoek* **107**, 225-240 (2015).

112. Vinck, A. et al. Hyphal differentiation in the exploring mycelium of *Aspergillus niger*. *Mol Microbiol* **58**, 693-699 (2005).

113. Vinck, A. et al. Heterogenic expression of genes encoding secreted proteins at the periphery of *Aspergillus niger* colonies. *Environ Microbiol* **13**, 216-225 (2011).

114. Tegelaar, M. & Wösten, H.A.B. Functional distinction of hyphal compartments. *Sci Rep* **7** (2017).

115. Lazzarini, A., Cavaletti, L., Toppo, G. & Marinelli, F. Rare genera of actinomycetes as potential producers of new antibiotics. *Antonie Van Leeuwenhoek* **78**, 399-405 (2000).

116. Anné, J., Maldonado, B., Van Impe, J., Van Mellaert, L. & Bernaerts, K. Recombinant protein production and streptomycetes. *J Biotechnol* **158**, 159-167 (2012).

117. Hoffmeister, D. & Keller, N.P. Natural products of filamentous fungi: enzymes, genes, and their regulation. *Nat Prod Rep* **24**, 393-416 (2007).

118. Takors, R. Scale-up of microbial processes: impacts, tools and open questions. *J Biotechnol* **160**, 3-9 (2012).

119. Delvigne, F., Boxus, M., Ingels, S. & Thonart, P. Bioreactor mixing efficiency modulates the activity of a prpoS::GFP reporter gene in E. coli. *Microb Cell Fact* **8**, 15 (2009).

120. Yegneswaran, P.K., Gray, M.R. & Thompson, B.G. Effect of dissolved oxygen control on growth and antibiotic production in *Streptomyces clavuligerus* fermentations. *Biotechnol Prog* **7**, 246-250 (1991).

121. Larsson, G. & Enfors, S.O. Studies of insufficient mixing in bioreactors: effects of limiting oxygen concentrations and short term oxygen starvation on *Penicillium chrysogenum*. *Bioprocess Eng* **3**, 123-127 (1998).

122. Tresner, H.D., Hayes, J.A. & Backus, E.J. Morphology of submerged growth of streptomycetes as a taxonomic aid. I. Morphological development of *Streptomyces aureofaciens* in agitated liquid media. *Appl Microbiol* **15**, 1185-1191 (1967).

123. Braun, S. & Vecht-Lifshitz, S.E. Mycelial morphology and metabolite production. *Trends Biotechnol* **9**, 63-68 (1991).

124. van Veluw, G.J. et al. Heterogeneity in liquid shaken cultures of *Aspergillus niger* inoculated with melanised conidia or conidia of pigmented mutants. *Stud Mycol* **74**, 47-57 (2013).

125. Bizukojc, M. & Gonciarz, J. Influence of oxygen on lovastatin biosynthesis by *Aspergillus terreus* ATCC 20542 quantitatively studied on the level of individual pellets. *Bioprocess Biosyst Eng* **38**, 1251-1266 (2015).

126. Clark, D.S. Submerged citric acid fermentation of ferrocyanide-treated beet molasses: morphology of pellets of *Aspergillus niger*. *Can J Microbiol* **8**, 133-136 (1962).

127. Wittier, R., Baumgartl, H., Lübbers, D.W. & Schügerl, K. Investigations of oxygen transfer into *Penicillium chrysogenum* pellets by microprobe measurements. *Biotechnol Bioeng* **28**, 1024-1036 (1986).

128. Gerlach, S.R. et al. Influence of reactor systems on the morphology of *Aspergillus awamori*. Application of neural network and cluster analysis for characterization of fungal morphology. *Process Biochem* **33**, 601-615 (1998).

129. Driouch, H., Hänsch, R., Wucherpfennig, T., Krull, R. & Wittmann, C. Improved enzyme production by bio-pellets of *Aspergillus niger*: targeted morphology engineering using titanate microparticles. *Biotechnol Bioeng* **109**, 462-471 (2012).

130. von Ohle, C. et al. Real-time microsensor measurement of local metabolic activities in ex vivo dental biofilms exposed to sucrose and treated with chlorhexidine. *Appl Environ Microbiol* **76**, 2326-2334 (2010).

131. Kragh, K.N. et al. Role of multicellular aggregates in biofilm formation. *MBio* **7**, e00237 (2016).

132. Boles, B.R., Thoendel, M. & Singh, P.K. Self-generated diversity produces "insurance effects" in biofilm communities. *Proc Natl Acad Sci U S A* **101**, 16630-16635 (2004).

133. Yagüe, P. et al. Transcriptomic analysis of liquid non-sporulating *Streptomyces coelicolor* cultures demonstrates the existence of a complex differentiation comparable to that occurring in solid sporulating cultures. *PLoS One* **9**, e86296 (2014).

134. de Bekker, C., van Veluw, G.J., Vinck, A., Wiebenga, L.A. & Wösten, H.A.B. Heterogeneity of *Aspergillus niger* microcolonies in liquid shaken cultures. *Appl Environ Microbiol* **77**, 1263-1267 (2011).

135. Urem, M. et al. OsdR of *Streptomyces coelicolor* and the dormancy regulator DevR of *Mycobacterium tuberculosis* control overlapping regulons. *mSystems* **1** (2016).

136. Bleichrodt, R.J. et al. Hyphal heterogeneity in *Aspergillus oryzae* is the result of dynamic closure of septa by Woronin bodies. *Mol Microbiol* **86**, 1334-1344 (2012).

137. Celler, K., Koning, R.I., Willemse, J., Koster, A.J. & van Wezel, G.P. Cross-membranes orchestrate compartmentalization and morphogenesis in *Streptomyces*. *Nat Commun* **7**, ncomms11836 (2016).

138. Jakimowicz, D. & van Wezel, G.P. Cell division and DNA segregation in *Streptomyces*: how to build a septum in the middle of nowhere? *Mol Microbiol* **85**, 393-404 (2012).

139. Shatkin, A.J. & Tatum, E.L. Electron microscopy of *Neurospora crassa* mycelia. *J Biophys Biochem Cy* **6**, 423-& (1959).

140. Moore, R.T. & Mcalear, J.H. Fine structure of mycota. 7. Observations on septa of ascomycetes and basidiomycetes. *Am J Bot* **49**, 86-& (1962).

141. Bleichrodt, R.J., Vinck, A., Read, N.D. & Wösten, H.A.B. Selective transport between heterogeneous hyphal compartments via the plasma membrane lining septal walls of *Aspergillus niger*. *Fungal Genet Biol* **82**, 193-200 (2015).

142. Zacchetti, B. et al. Aggregation of germlings is a major contributing factor towards mycelial heterogeneity of *Streptomyces*. *Sci Rep* **6**, 27045 (2016).

143. Chaplin, A.K. et al. GlxA is a new structural member of the radical copper oxidase family and is required for glycan deposition at hyphal tips and morphogenesis of *Streptomyces lividans*. *Biochem J* **469**, 433-444 (2015).

144. de Jong, W., Wösten, H.A.B., Dijkhuizen, L. & Claessen, D. Attachment of *Streptomyces coelicolor* is mediated by amyloidal fimbriae that are anchored to the cell surface via cellulose. *Mol Microbiol* **73**, 1128-1140 (2009).

145. Xu, H., Chater, K.F., Deng, Z. & Tao, M. A cellulose synthase-like protein involved in hyphal tip growth and morphological differentiation in *Streptomyces*. *J Bacteriol* **190**, 4971-4978 (2008).

146. Petrus, M.L.C. et al. The DyP-type peroxidase DtpA is a Tat-substrate required for GlxA maturation and morphogenesis in *Streptomyces*. *Open Biol* **6**, 150149 (2016).

147. van Dissel, D. et al. Production of poly-β-1,6-N-acetylglucosamine by MatAB is required for hyphal aggregation and hydrophilic surface adhesion by *Streptomyces*. *Microb Cell* **5**, 269-279 (2018).

148. Grimm, L.H. et al. Kinetic studies on the aggregation of *Aspergillus niger* conidia. *Biotechnol Bioeng* **87**, 213-218 (2004).

149. Wang, H., Zhao, G. & Ding, X. Morphology engineering of *Streptomyces coelicolor* M145 by sub-inhibitory concentrations of antibiotics. *Sci Rep* **7**, 13226 (2017).

150. Petrus, M.L.C., van Veluw, G.J., Wösten, H.A.B. & Claessen, D. Sorting of *Streptomyces* cell pellets using a complex parametric analyzer and sorter. *J Vis Exp* **84**, e51178 (2014).

151. Nieminen, L., Webb, S., Smith, M.C.M. & Hoskisson, P.A. A flexible mathematical model platform for studying branching networks: experimentally validated using the model actinomycete, *Streptomyces coelicolor*. *PLOS One* **8**, e54316 (2013).

152. Reichl, U., King, R. & Gilles, E.D. Characterization of pellet morphology during submerged growth of Streptomyces tendae by image analysis. *Biotechnol Bioeng* **39**, 164-170 (1992).

153. Tough, A.J. & Prosser, J.I. Experimental verification of a mathematical model for pelleted growth of *Streptomyces coelicolor* A3(2) in submerged batch culture. *Microbiology* **142**, 639-648 (1996).

154. Liman, R., Facey, P.D., van Keulen, G., Dyson, P.J. & Del Sol, R. A laterally acquired galactose oxidase-like gene is required for aerial development during osmotic stress in *Streptomyces coelicolor*. *PLoS One* **8**, e54112 (2013).

155. Fontaine, T. et al. Cell wall α(1-3) glucans induce the aggregation of germinating conidia of *Aspergillus fumigatus*. *Fungal Genet Biol* **47**, 707-712 (2010).

156. Metz, B. & Kossen, N.W.F. The growth of molds in the form of pellets – a literature review. *Biotechnol Bioeng* **19**, 781-799 (1977).

157. Claessen, D. et al. The formation of the rodlet layer of streptomycetes is the result of the interplay between rodlins and chaplins. *Mol Microbiol* **53**, 433-443 (2004).

158. Claessen, D. et al. Two novel homologous proteins of *Streptomyces coelicolor* and *Streptomyces lividans* are involved in the formation of the rodlet layer and mediate attachment to a hydrophobic surface. *Mol Microbiol* **44**, 1483-1492 (2002).

159. Cairns, L.S., Hobley, L. & Stanley-Wall, N.R. Biofilm formation by *Bacillus subtilis*: new insights into regulatory strategies and assembly mechanisms. *Mol Microbiol* **93**, 587-598 (2014).

160. Uhlich, G.A., Chen, C.Y., Cottrell, B.J. & Nguyen, L.H. Growth media and temperature effects on biofilm formation by serotype O157:H7 and non-O157 Shiga toxin-producing *Escherichia coli*. *FEMS Microbiol Lett* **354**, 133-141 (2014).

161. Speziale, P., Pietrocola, G., Foster, T.J. & Geoghegan, J.A. Protein-based biofilm matrices in Staphylococci. *Front Cell Infect Microbiol* **4**, 171 (2014).

162. Barnes, A.M., Ballering, K.S., Leibman, R.S., Wells, C.L. & Dunny, G.M. *Enterococcus faecalis* produces abundant extracellular structures containing DNA in the absence of cell lysis during early biofilm formation. *mBio* **3**, e00193-00112 (2012).

163. Hobley, L., Harkins, C., MacPhee, C.E. & Stanley-Wall, N.R. Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes. *FEMS Microbiol Rev* **39**, 649-669 (2015).

164. Petrus, M.L.C. & Claessen, D. Pivotal roles for *Streptomyces* cell surface polymers in morphological differentiation, attachment and mycelial architecture. *Antonie van Leeuwenhoek* **106**, 127-139 (2014).

165. Martins, A.M.P., Picioreanu, C., Heijnen, J.J. & Van Loosdrecht, M.C.M. Three-dimensional dual-morphotype species modeling of activated sludge flocs. *Environ Sci Technol* **38**, 5632-5641 (2004).

166. Kim, Y.M. & Kim, J.H. Formation and dispersion of mycelial pellets of *Streptomyces coelicolor* A3(2). *J Microbiol* **42**, 64-67 (2004).

167. Schroeckh, V. et al. Intimate bacterial-fungal interaction triggers biosynthesis of archetypal polyketides in *Aspergillus nidulans*. *Proc Natl Acad Sci U S A* **106**, 14558-14563 (2009).

168. Wu, C. et al. Expanding the chemical space for natural products by *Aspergillus-Streptomyces* co-cultivation and biotransformation. *Sci Rep* **5**, 10868 (2015).

169. Abrudan, M.I. et al. Socially mediated induction and suppression of antibiosis during bacterial coexistence. *Proc Natl Acad Sci U S A* (2015).

170. Moody, S.C. Microbial co-culture: harnessing intermicrobial signaling for the production of novel antimicrobials. *Future Microbiol* **9**, 575-578 (2014).

171. Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F. & Hopwood, D.A. Practical *Streptomyces* genetics. (The John Innes Foundation, Norwich; 2000).

172. Sun, J., Kelemen, G.H., Fernández-Abalos, J.M. & Bibb, M.J. Green fluorescent protein as a reporter for spatial and temporal gene expression in *Streptomyces coelicolor* A3(2). *Microbiology* **145**, 2221-2227 (1999).

173. Gregory, M.A., Till, R. & Smith, M.C.M. Integration site for *Streptomyces* phage phiBT1 and development of site-specific integrating vectors. *J Bacteriol* **185**, 5320-5323 (2003).

174. Janssen, G.R. & Bibb, M.J. Derivatives of pUC18 that have *Bgl*II sites flanking a modified multiple cloning site and that retain the ability to identify recombinant clones by visual screening of *Escherichia coli* colonies. *Gene* **124**, 133-134 (1993).

175. Hindra, Pak, P. & Elliot, M.A. Regulation of a novel gene cluster involved in secondary metabolite production in *Streptomyces coelicolor*. *J Bacteriol* **192**, 4973-4982 (2010).

176. Hanahan, D. Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557-580 (1983).

177. MacNeil, D.J. et al. Complex organization of the *Streptomyces avermitilis* genes encoding the avermectin polyketide synthase. *Gene* **115**, 119-125 (1992).

178. Floriano, B. & Bibb, M.J. *afsR* is a pleiotropic but conditionally required regulatory gene for antibiotic production in *Streptomyces coelicolor* A3(2). *Mol Microbiol* **21**, 385-396 (1996).

179. Yagüe, P., Manteca, A., Simon, A., Diaz-Garcia, M.E. & Sanchez, J. A new method for monitoring programmed cell death and differentiation in submerged cultures of *Streptomyces. Appl Environ Microbiol* (2010).

180. Blundell, K.L.I.M., Wilson, M.T., Svistunenko, D.A., Vijgenboom, E. & Worrall, J.A.R. Morphological development and cytochrome c oxidase activity in *Streptomyces lividans* are dependent on the action of a copper bound Sco protein. *Open Biol* **3**, 120163 (2013).

181. Zacchetti, B., Smits, P. & Claessen, D. Dynamics of pellet fragmentation and aggregation in liquid-grown cultures of *Streptomyces lividans*. *Front Microbiol* **9**, 943 (2018).

182. Kenealy, W.R. & Jeffries, T.W. Rapid 2,2'-bicinchoninic-based xylanase assay compatible with high throughput screening. *Biotechnol Lett* **25**, 1619-1623 (2003).

183. Gomori, G. Preparation of buffers for use in enzyme studies. *Meth Enzymol* **1**, 138–146 (1955).

184. Pace, J.L., Rupp, M.E. & Finch, R. Biofilms, infection, and antimicrobial therapy. (CRC Press, 2009).

185. Jefferson, K.K. (ed. Ullrich M) 175-186 (Caister Academic, Norfolk, UK; 2009).

186. Kim, J.H. & Hancock, I.C. Pellet forming and fragmentation in liquid culture of *Streptomyces griseus*. *Biotechnol Lett* **22**, 189-192 (2000).

187. Tamura, S., Park, Y., Toriyama, M. & Okabe, M. Change of mycelial morphology in tylosin production by batch culture of *Streptomyces fradiae* under various shear conditions. *J Ferment Bioeng* **83**, 523-528 (1997).

188. Xia, X., Lin, S.J., Xia, X.X., Cong, F.S. & Zhong, J.J. Significance of agitation-induced shear stress on mycelium morphology and lavendamycin production by engineered *Streptomyces flocculus*. *Appl Microbiol Biotechnol* **98**, 4399-4407 (2014).

189. Flemming, H.C. & Wingender, J. The biofilm matrix. *Nat Rev Microbiol* **8**, 623-633 (2010).

190. Flemming, H.C., Neu, T.R. & Wozniak, D.J. The EPS matrix: the "house of biofilm cells". *J Bacteriol* **189**, 7945-7947 (2007).

191. Götz, F. *Staphylococcus* and biofilms. *Mol Microbiol* **43**, 1367-1378 (2002).

192. Abee, T., Kovács, Á.T., Kuipers, O.P. & van der Veen, S. Biofilm formation and dispersal in Gram-positive bacteria. *Curr Opin Biotechnol* **22**, 172-179 (2011).

193. Kaplan, J.B. Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. *J Dent Res* **89**, 205-218 (2010).

194. McDougald, D., Rice, S.A., Barraud, N., Steinberg, P.D. & Kjelleberg, S. Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. *Nat Rev Microbiol* **10**, 39-50 (2011).

195. Guilhen, C., Forestier, C. & Balestrino, D. Biofilm dispersal: multiple elaborate strategies for dissemination of bacteria with unique properties. *Mol Microbiol* **105**, 188-210 (2017).

196. Kaplan, J.B., Ragunath, C., Ramasubbu, N. & Fine, D.H. Detachment of *Actinobacillus actinomycetemcomitans* biofilm cells by an endogenous β-hexosaminidase activity. *J Bacteriol* **185**, 4693-4698 (2003).

197. Willemse, J. et al. SParticle, an algorithm for the analysis of filamentous microorganisms in submerged cultures. *Antonie Van Leeuwenhoek* **111**, 171-182 (2018).

198. Celler, K., Picioreanu, C., van Loosdrecht, M.C.M. & van Wezel, G.P. Structured morphological modeling as a framework for rational strain design of *Streptomyces* species. *Antonie van Leeuwenhoek* **102**, 409-423 (2012).

199. Liu, G., Chater, K.F., Chandra, G., Niu, G. & Tan, H. Molecular regulation of antibiotic biosynthesis in streptomyces. *Microbiol Mol Biol Rev* **77**, 112-143 (2013).

200. Nielsen, J. Modelling the morphology of filamentous microorganisms. *Trends Biotechnol* **14**, 438-443 (1996).

201. Park, J.K. & Chang, H.N. Microencapsulation of microbial cells. *Biotechnol Adv* **18**, 303-319 (2000).

202. Buzas, Z., Dallmann, K. & Szajani, B. Influenc of pH on the growth and ethanol production of free and immobilized Saccharomyces cerevisiae cells. *Biotechnol Bioeng* **34**, 882-884 (1989).

203. Russo, A., Basaglia, M., Tola, E. & Casella, S. Survival, root colonisation and biocontrol capacities of Pseudomonas fluorescens F113 LacZY in dry alginate microbeads. *J Ind Microbiol Biotechnol* **27**, 337-342 (2001).

204. McCabe, B.K., Kuek, C., Gordon, G.L. & Phillips, M.W. Production of beta-glucosidase using immobilised Piromyces sp. KSX1 and Orpinomyces sp. 478P1 in repeat-batch culture. *J Ind Microbiol Biotechnol* **30**, 205-209 (2003).

205. Briceno, G. et al. Removal of the insecticide diazinon from liquid media by free and immobilized Streptomyces sp. isolated from agricultural soil. *J Basic Microbiol* **55**, 293-302 (2015).

206. Anisha, G.S. & Prema, P. Cell immobilization technique for the enhanced production of α-galactosidase by *Streptomyces griseoloalbus*. *Bioresour Technol* **99**, 3325-3330 (2008).

207. López-García, M.T., Rioseras, B., Yagüe, P., Álvarez, J.R. & Manteca, Á. Cell immobilization of *Streptomyces coelicolor*: effect on differentiation and actinorhodin production. *Int Microbiol* **17**, 75-80 (2014).

208. Najafpour, G., Younesi, H. & Syahidah Ku Ismail, K. Ethanol fermentation in an immobilized cell reactor using Saccharomyces cerevisiae. *Bioresour Technol* **92**, 251-260 (2004).

209. Cruz-Morales, P. et al. The genome sequence of *Streptomyces lividans* 66 reveals a novel tRNA-dependent peptide biosynthetic system within a metal-related genomic island. *Genome Biol Evol* **5**, 1165-1175 (2013).

210. Katz, E., Thompson, C.J. & Hopwood, D.A. Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. *J Gen Microbiol* **129**, 2703-2714 (1983).

211. Large, K.P., Ison, A.P. & Williams, D.J. The effect of agitation rate on lipid utilisation and clavulanic acid production in *Streptomyces clavuligerus*. *J Biotechnol* **63**, 111-119 (1998).

212. Roubos, J.A., Krabben, P., Luiten, R.G.M., Verbruggen, H.B. & Heijnen, J.J. A quantitative approach to characterizing cell lysis caused by mechanical agitation of *Streptomyces clavuligerus*. *Biotechnol Prog* **17**, 336-347 (2001).

213. Mehmood, N. et al. Relation between pristinamycins production by *Streptomyces pristinaespiralis*, power dissipation and volumetric gas–liquid mass transfer coefficient, kLa. *Process Biochem* **45**, 1779-1786 (2010).

214. Ohta, N., Park, Y.S., Yahiro, K. & Okabe, M. Comparison of neomycin production from *Streptomyces fradiae* cultivation using soybean oil as the sole carbon source in an air-lift bioreactor and a stirred-tank reactor. *J Ferment Bioeng* **79**, 443-448 (1995).

215. Gaserod, O., Sannes, A. & Skjak-Braek, G. Microcapsules of alginate-chitosan. II. A study of capsule stability and permeability. *Biomaterials* **20**, 773-783 (1999).

216. Bentley, S.D. et al. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **417**, 141-147 (2002).

217. Bhujbal, S.V., Paredes-Juarez, G.A., Niclou, S.P. & de Vos, P. Factors influencing the mechanical stability of alginate beads applicable for immunoisolation of mammalian cells. *J Mech Behav Biomed Mater* **37**, 196-208 (2014).

218. Willemse, J. & van Wezel, G.P. Imaging of *Streptomyces coelicolor* A3(2) with reduced autofluorescence reveals a novel stage of FtsZ localization. *PLoS One* **4**, e4242 (2009).

219. Kontturi, L.S. et al. A laboratory-scale device for the straightforward production of uniform, small sized cell microcapsules with long-term cell viability. *J Control Release* **152**, 376-381 (2011).

220. Lerch, K. & Ettinger, L. Purification and characterization of a tyrosinase from Streptomyces glaucescens. *Eur J Biochem* **31**, 427-437 (1972).

221. Manteca, A. & Sanchez, J. *Streptomyces* development in colonies and soils. *Appl Environ Microbiol* **75**, 2920-2924 (2009).

222. Martin, S.M. & Bushell, M.E. Effect of hyphal micromorphology on bioreactor performance of antibiotic-producing *Saccharopolyspora erythraea* cultures. *Microbiology* **142**, 1783-1788 (1996).

223. van Wezel, G.P. et al. *ssgA* is essential for sporulation of *Streptomyces coelicolor* A3(2) and affects hyphal development by stimulating septum formation. *J Bacteriol* **182**, 5653-5662 (2000).

224. Noens, E.E.E. et al. Loss of the controlled localization of growth stage-specific cell-wall synthesis pleiotropically affects developmental gene expression in an *ssgA* mutant of *Streptomyces coelicolor. Mol Microbiol* **64**, 1244-1259 (2007).

225. Claessen, D. et al. A novel class of secreted hydrophobic proteins is involved in aerial hyphae formation in *Streptomyces coelicolor* by forming amyloid-like fibrils. *Genes Dev* **17**, 1714-1726 (2003).

226. Elliot, M.A. et al. The chaplins: a family of hydrophobic cell-surface proteins involved in aerial mycelium formation in *Streptomyces coelicolor. Genes Dev* **17**, 1727-1740 (2003).

227. Kelly, S., Grimm, L.H., Jonas, R., Hempel, D.C. & Krull, R. Investigations of the morphogenesis of filamentous microorganisms. *Eng Life Sci* **6**, 475-480 (2006).

228. Omura, S. et al. Genome sequence of an industrial microorganism *Streptomyces avermitilis*: deducing the ability of producing secondary metabolites. *Proc Natl Acad Sci U S A* **98**, 12215-12220 (2001).

229. Hille, A., Neu, T.R., Hempel, D.C. & Horn, H. Oxygen profiles and biomass distribution in biopellets of *Aspergillus niger. Biotechnol Bioeng* **92**, 614-623 (2005).

230. Marshall, K.C. & Alexander, M. Growth characteristics of fungi and actinomycetes. *J Bacteriol* **80**, 412-416 (1960).

231. Trinci, A.P.J. Kinetics of the growth of mycelial pellets of *Aspergillus nidulans*. *Arch Mikrobiol* **73**, 353-367 (1970).

232. Cui, Y.Q., van der Lans, R.G.J.M. & Luyben, K.C.A.M. Effect of agitation intensities on fungal morphology of submerged fermentation. *Biotechnol Bioeng* **55**, 715-726 (1997).

233. Kelly, S., Grimm, L.H., Bendig, C., Hempel, D.C. & Krull, R. Effects of fluid dynamic induced shear stress on fungal growth and morphology. *Process Biochem* **41**, 2113-2117 (2006).

234. Ayazi Shamlou, P., Makagiansar, H.Y., Ison, A.P., Lilly, M.D. & Thomas, C.R. Turbulent breakage of filamentous microorganisms in submerged culture in mechanically stirred bioreactors. *Chem Eng Sci* **49**, 2621-2631 (1994).

235. Heydarian, S.M., Ison, A.P., Lilly, M.D. & Ayazi Shamlou, P.A. Turbulent breakage of filamentous bacteria in mechanically agitated batch culture. *Chem Eng Sci* **55**, 1775-1784 (2000).

236. Yang, H., King, R., Reichl, U. & Gilles, E.D. Mathematical model for apical growth, septation, and branching of mycelial microorganisms. *Biotechnol Bioeng* **39**, 49-58 (1992).

237. Liu, G., Xing, M. & Han, Q. A population-based morphologically structured model for hyphal growth and product formation in streptomycin fermentation. *World J Microb Biot* **21**, 1329-1338 (2005).

238. Meyerhoff, J., Tiller, V. & Bellgardt, K.-H. Two mathematical models for the development of a single microbial pellet. *Bioprocess Eng* **12**, 305-313 (1995).

239. Nielsen, J. & Villadsen, J. Modelling of microbial kinetics. *Chem Eng Sci* **47**, 4225-4270 (1992).

240. Tough, A.J., Pulham, J. & Prosser, J.I. A mathematical model for the growth of mycelial pellet populations. *Biotechnol Bioeng* **46**, 561-572 (1995).

241. Andersen, J.B. et al. New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl Environ Microbiol* **64**, 2240-2246 (1998).

242. Willemse, J., Borst, J.W., de Waal, E., Bisseling, T. & van Wezel, G.P. Positive control of cell division: FtsZ is recruited by SsgB during sporulation of *Streptomyces*. *Genes Dev* **25**, 89-99(2011).

243. Wösten, H.A.B., van Veluw, G.J., de Bekker, C. & Krijgsheld, P. Heterogeneity in the mycelium: implications for the use of fungi as cell factories. *Biotechnol. Lett.* **35**, 1155-1164 (2013).

244. Higgins, D. & Dworkin, J. Recent progress in *Bacillus subtilis* sporulation. *FEMS Microbiol. Rev.* **36**, 131-148 (2012).

245. Jia, C., Qian, M., Kang, Y. & Jiang, D. Modeling stochastic phenotype switching and bet-hedging in bacteria: stochastic nonlinear dynamics and critical state identification. *Quantitative Biology* **2**, 110-125 (2014).

246. Henson, M.A. Dynamic modeling of microbial cell populations. *Curr. Opin. Biotechnol.* **14**, 460-467 (2003).

247. Wilkinson, D.J. Stochastic modelling for quantitative description of heterogeneous biological systems. *Nat. Rev. Genet.* **10**, 122-133 (2009).

248. Meister, A., Du, C., Li, Y.H. & Wong, W.H. Modeling stochastic noise in gene regulatory systems. *Quant Biol* **2**, 1-29 (2014).

249. Chater, K.F. Taking a genetic scalpel to the *Streptomyces* colony. *Microbiol-sgm* **144**, 1465-1478 (1998).

250. Kues, U. & Liu, Y. Fruiting body production in basidiomycetes. *Appl. Microbiol. Biotechnol.* **54**, 141-152 (2000).

251. Krijgsheld, P. et al. Development in Aspergillus. *Studies in Mycology*, 1-29 (2013).

252. Delvigne, F. et al. Taking control over microbial populations: Current approaches for exploiting biological noise in bioprocesses. *Biotechnology Journal* **12** (2017).

253. Reece, A. et al. Microfluidic techniques for high throughput single cell analysis. *Curr. Opin. Biotechnol.* **40**, 90-96 (2016).

254. Grunberger, A., Wiechert, W. & Kohlheyer, D. Single-cell microfluidics: opportunity for bioprocess development. *Curr. Opin. Biotechnol.* **29**, 15-23 (2014).

255. Hol, F.J.H. & Dekker, C. Zooming in to see the bigger picture: Microfluidic and nanofabrication tools to study bacteria. *Science* **346**, 438-+ (2014).

256. Wu, F. & Dekker, C. Nanofabricated structures and microfluidic devices for bacteria: from techniques to biology. *Chem Soc Rev* **45**, 268-280 (2016).

257. Grünberger, A. et al. Simple microfluidics for complex organisms: A microfluidic chip system for growth and morphogenesis studies of filamentous fungi. *17th International Conference on Miniaturized Systems for Chemistry and Life Sciences, Microtas 2013.* **2**, 1273-1275 (2013).

258. de Bekker, C., Bruning, O., Jonker, M.J., Breit, T.M. & Wosten, H.A.B. Single cell transcriptomics of neighboring hyphae of Aspergillus niger. *Genome Biology* **12** (2011).

259. Nunez, J., Renslow, R., Cliff, J.B., 3rd & Anderton, C.R. NanoSIMS for biological applications: Current practices and analyses. *Biointerphases* **13**, 03B301 (2017).

260. He, C., Fong, L.G., Young, S.G. & Jiang, H. NanoSIMS imaging: an approach for visualizing and quantifying lipids in cells and tissues. *J Investig Med* **65**, 669-672 (2017).

261. Sheik, A.R. et al. *In situ* phenotypic heterogeneity among single cells of the filamentous bacterium *Candidatus* Microthrix parvicella. *ISME J* **10**, 1274-1279 (2016).

262. Moukha, S.M., Wösten, H.A.B., Mylius, E.J., Asther, M. & Wessels, J.G.H. Spatial and temporal accumulation of mRNAs encoding two common lignin peroxidases in *Phanerochaete chrysosporium*. *J. Bacteriol.* **175**, 3672-3678 (1993).

263. Moukha, S.M., Wösten, H.A.B., Asther, M. & Wessels, J.G.H. *In situ* localization of the secretion of lignin peroxidases in colonies of *Phanerochaete chrysosporium* using a sandwiched mode of culture. *Journal of General Microbiology* **139**, 969-978 (1993).

264. Pollack, J.K., Li, Z.J. & Marten, M.R. Fungal mycelia show lag time before re-growth on endogenous carbon. *Biotechnol. Bioeng.* **100**, 458-465 (2008).

265. Wang, F.F. et al. FluG affects secretion in colonies of *Aspergillus niger*. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology* **107**, 225-240 (2015).

266. Tegelaar, M. & Wösten, H.A.B. Functional distinction of hyphal compartments. *Scientific Reports* **7** (2017).

267. Yegneswaran, P.K., Gray, M.R. & Thompson, B.G. Effect of dissolved oxygen control on growth and antibiotic production in *Streptomyces clavuligerus* fermentations. *Biotechnol. Prog.* **7**, 246-250 (1991).

268. Larsson, G. & Enfors, S.O. Studies of insufficient mixing in bioreactors: effects of limiting oxygen concentrations and short term oxygen starvation on *Penicillium chrysogenum*. *Bioprocess Eng.* **3**, 123-127 (1998).

269. van Veluw, G.J. et al. Heterogeneity in liquid shaken cultures of *Aspergillus niger* inoculated with melanised conidia or conidia of pigmented mutants. *Studies in Mycology* **74**, 47-57 (2013).

270. Wittier, R., Baumgartl, H., Lübbers, D.W. & Schügerl, K. Investigations of oxygen transfer into *Penicillium chrysogenum* pellets by microprobe measurements. *Biotechnol. Bioeng.* **28**, 1024-1036 (1986).

271. Bleichrodt, R.J., Vinck, A., Read, N.D. & Wösten, H.A.B. Selective transport between heterogeneous hyphal compartments via the plasma membrane lining septal walls of *Aspergillus niger*. *Fungal Genet. Biol.* **82**, 193-200 (2015).

272. Bleichrodt, R.J., Hulsman, M., Wösten, H.A.B. & Reinders, M.J.T. Switching from a unicellular to multicellular organization in an *Aspergillus niger* hypha. *Mbio* **6** (2015).

273. van Dissel, D. et al. Production of poly-β-1,6-N-acetylglucosamine by MatAB is required for hyphal aggregation and hydrophilic surface adhesion by *Streptomyces*. *Microbial Cell* (2018).

274. Narula, J. et al. Chromosomal Arrangement of Phosphorelay Genes Couples Sporulation and DNA Replication. *Cell* **162**, 328-337 (2015).

275. Soler-Bistue, A. et al. Genomic Location of the Major Ribosomal Protein Gene Locus Determines Vibrio cholerae Global Growth and Infectivity. *PLoS Genet.* **11** (2015).

276. Chai, Y.R., Norman, T., Kolter, R. & Losick, R. Evidence that metabolism and chromosome copy number control mutually exclusive cell fates in Bacillus subtilis. *EMBO J.* **30**, 1402-1413 (2011).

277. Slager, J., Kjos, M., Attaiech, L. & Veening, J.W. Antibiotic-induced replication stress triggers bacterial competence by increasing gene dosage near the origin. *Cell* **157**, 395-406 (2014).

278. Veening, J.W., Smits, W.K., Hamoen, L.W. & Kuipers, O.P. Single cell analysis of gene expression patterns of competence development and initiation of sporulation in Bacillus subtilis grown on chemically defined media. *J. Appl. Microbiol.* **101**, 531-541 (2006).

279. Schuurs, T.A., Dalstra, H.J.P., Scheer, J.M.J. & Wessels, J.G.H. Positioning of nuclei in the secondary mycelium of Schizophyllum commune in relation to differential gene expression. *Fungal Genet. Biol.* **23**, 150-161 (1998).

280. Stoyan, H., De-Polli, H., Bohm, S., Robertson, G.P. & Paul, E.A. Spatial heterogeneity of soil respiration and related properties at the plant scale. *Plant Soil* **222**, 203-214 (2000).

Appendix I:
Supplementary Information

SUPPLEMENTARY INFORMATION BELONGING TO CHAPTER 3

***Escherichia coli* strains**	**Description**	**Reference**
DH5α	F- Φ80*lac*ZDM15 D(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(rK-, mK-) *phoA supE44 thi-1 gyrA96 relA1 λ-*	176
ET12567	F-*dam*-13::Tn9 *dcm-6 hsdM hsdR recF143 zjj-202*::Tn10 *galK2 galT22 ara14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL136 hisG4 tsx78 mtl-1 glnV44*	177
***Streptomyces* strains**		
S. coelicolor A3(2) M145	*Wild-type SCP1- SCP2-*	Laboratory stock
S. lividans 1326	*Wild-type*	Laboratory stock
S. scabies ISP5078	*Wild-type*	Gift from Prof. Loria
S. albus	*Wild-type*	Laboratory stock
S. lividans ΔcslA	*S. lividans* 1326 lacking *cslA* (marker-less)	143
S. lividans ΔglxA	*S. lividans* 1326 lacking *glxA* (marker-less)	143
S. lividans ΔmatAB	*S. lividans* 1326 lacking *matA* and *matB* (marker-less)	15
S. coelicolor M512	*Streptomyces coelicolor* A3(2) M145 lacking *redD* and *actII-ORF4*	178
S. lividans pGreen	*S. lividans* 1326 containing pGreen	This work
S. lividans pRed	*S. lividans* 1326 containing pRed	This work
S. lividans ΔcslA pGreen	*S. lividans ΔcslA* containing pGreen	This work
S. lividans ΔcslA pRed	*S. lividans ΔcslA* containing pRed	This work
S. lividans ΔglxA pGreen	*S. lividans ΔglxA* containing pGreen	This work
S. lividans ΔglxA pRed	*S. lividans ΔglxA* containing pRed	This work
S. lividans ΔmatAB pGreen	*S. lividans ΔmatAB* containing pGreen	This work
S. lividans ΔmatAB pRed	*S. lividans ΔmatAB* containing pRed	This work
S. lividans pGreen pRed	*S. lividans* 1326 containing pGreen and pRed	This work
S. coelicolor M512 pGreen	*S. coelicolor* M512 containing pGreen	This work
S. scabies ISP5078 pRed	*S. scabies* ISP5078 containing pRed	This work

Table S1. Bacterial strains used in this study.

Name	Description and relevant features	Reference
pGreen	pIJ8630 containing *eGFP* under control of the constitutive *gap1* promoter of *S. coelicolor* A3(2) M145.	This work
pRed	pMS82 containing *mCherry* under control of the constitutive *gap1* promoter of *S. coelicolor* A3(2) M145.	This work
pIJ8630	*E. coli–Streptomyces* shuttle vector containing the ϕC31 *attP-int* region for genomic integration. Contains an apramycin resistance cassette.	172
pMS82	*E. coli–Streptomyces* shuttle vector containing the ϕBT1 *attP-int* region for genomic integration. Contains an hygromycin resistance cassette.	173
pIJ2925	Derivative of pUC18 that contains two BglII sites flanking a modified MCS derived from plasmid pIJ486	174
pRSET-B	Plasmid containing the *mCherry* gene	R. Tsien

Table S2. Vectors and constructs used in this study.

Primer name	Sequence 5'-3'	Restriction site
Gap1-FW	GATAGATCTCCGAGGGCTTCGAGACC	BglII
Gap1-RV	GCCCATATGCCGATCTCCTCGTTGGTACG	NdeI
Gap1-FW*	AAAGGTACCACGCAGACCGAGGGCTTCGAG	KpnI
mCherry-FW	TAACATATGGTGAGCAAGGGCGAGGAGGATAAC	NdeI
mCherry-RV	GGGAAGCTTTTACTTGTACAGCTCGTCCATGC	HindIII
RT-cslA-FW	AGTCGCAGCAGTTCCTCTTC	
RT-cslA-RV	TTCTTGTGGCGGTGCATCTC	
RT-glxA-FW	AGTTCGAGCAGCGGATCGAG	
RT-glxA-RV	TCAGCCGCACCTTCTTGACC	
RT-matA-FW	CTCGGAGGCTGGACGAGATG	
RT-matA-RV	GGCCGCCTATTCGGGAAC	
RT-matB-FW	AGTCCGAGAAGCGCATCGACTG	
RT-matB-RV	GTCCTCGCTGTCGGTGTTGTTG	
RRNA-1	AGAGTTTGATCCTGGCTCAG	
RRNA-2	CGAACCTCGCAGATGCCTG	

Table S3. Primers used in this study.

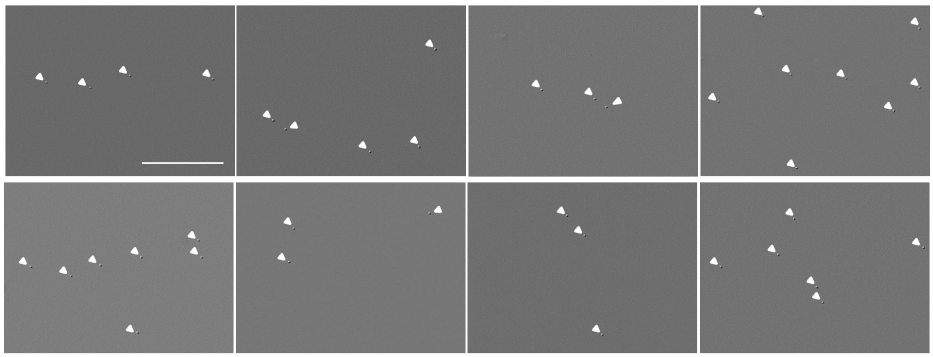


Figure S1. Collage of representative images of *Streptomyces lividans* particles in TSBS cultures after 2 h of growth. The arrowheads indicate individual spores in the culture medium. Neither spore germination nor aggregation were detected at this time point. The scale bar represents 50 µm.

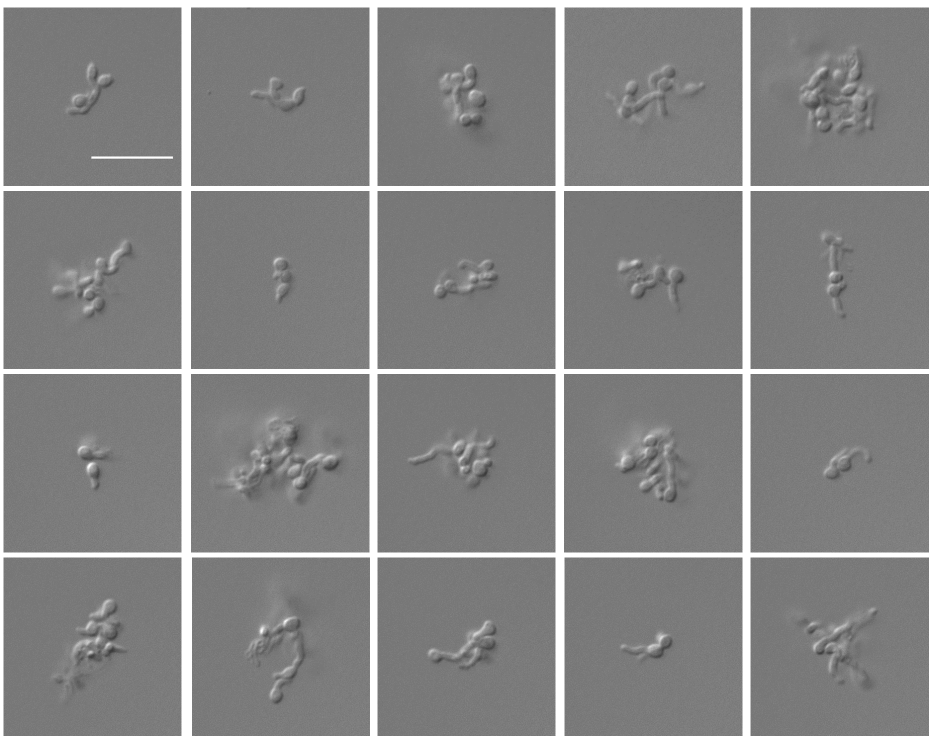


Figure S2. Collage of representative images of spore aggregates of *Streptomyces lividans* in TSBS cultures. Small aggregates invariably contain germlings. Pictures were taken after 5 h of growth. The scale bar represents 50 µm.

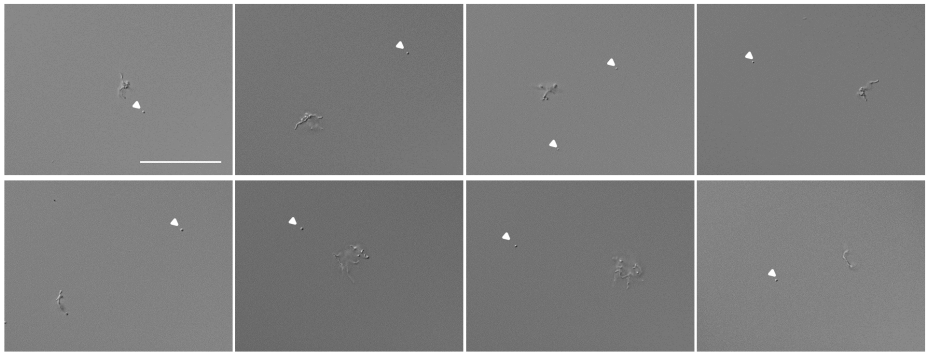


Figure S3. Collage of representative images of *Streptomyces lividans* particles in TSBS cultures after 5 h of growth. In addition to small aggregates, individual spores (arrowheads) are visible. Note that these spores show no visible germ tubes. The scale bar represents 50 µm.

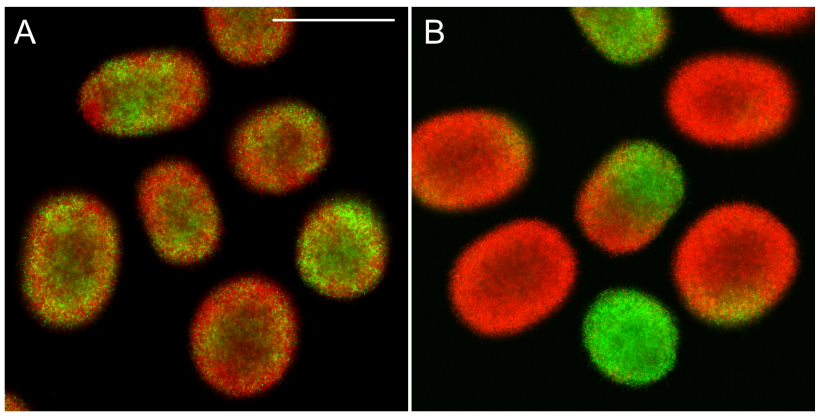


Figure S4. Fluorescent pellets of aggregated *Streptomyces lividans* strains. The strains expressing either the *eGFP* or the *mCherry* gene were grown separately for 2 (A) or 8 (B) hours prior to mixing them. Pictures of the aggregated strains were taken after 24 h of growth in NMMP medium. The scale bar represents 200 µm.

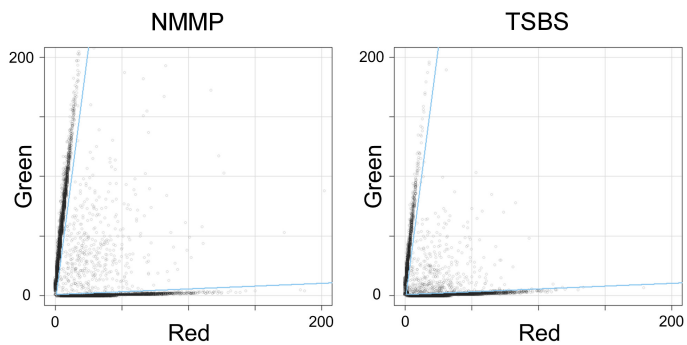


Figure S5. Time-dependent aggregation is not caused by secreted molecules accumulating in the medium. The *Streptomyces lividans* strains expressing either the *eGFP* or the *mCherry* gene were grown separately for 12 hours, after which they were mixed in fresh NMMP (left) or TSBS (right) medium. Each plot represents the fluorescence intensities of pellets in the red (X-axis) and green (Y-axis) channel, as determined by particle analysis. Quantification indicates that 3.5% and 16.1% of the particles contain both fluorescences in NMMP and TSBS medium, respectively.

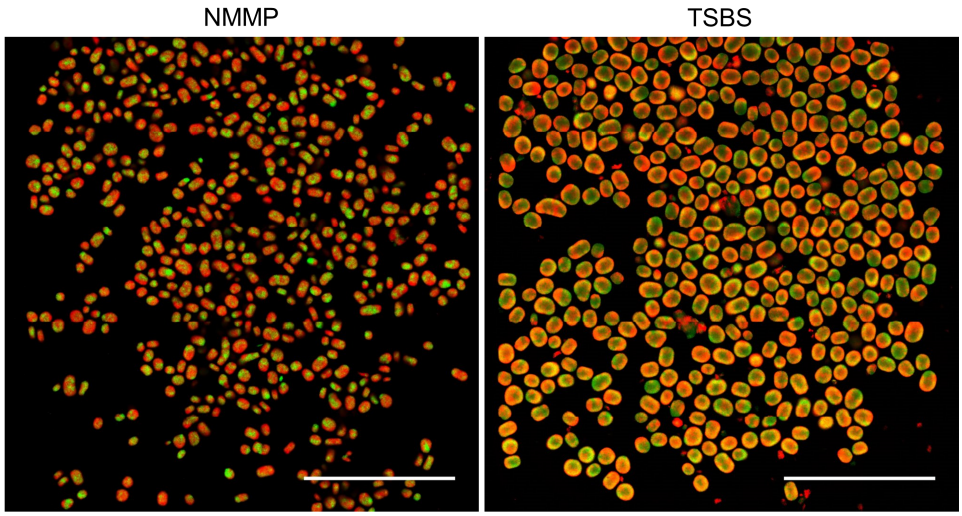


Figure S6. Analysis of aggregation of fluorescent *Streptomyces lividans wild-type* derivatives. Spores of the *wild-type* strains expressing either the *eGFP* or the *mCherry* gene were mixed at the onset of growth in NMMP (left) or TSBS (medium). After 24 h of growth, most pellets are composed of both types of fluorescent hyphae. Scale bars represent 2 mm.

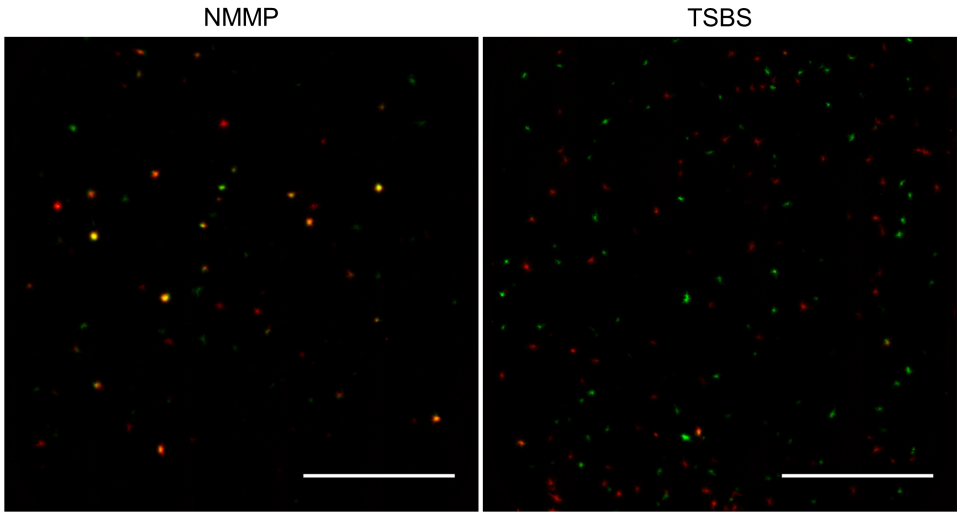


Figure S7. Aggregation depends on CslA. Spores of the *ΔcslA* derivative strains of *Streptomyces lividans* expressing either the *eGFP* or the *mCherry* gene were mixed at the onset of growth in NMMP (left) or TSBS medium (right). After 24 h of growth, the majority of particles is either green or red fluorescent in the absence of *cslA*. Scale bars represent 2 mm.

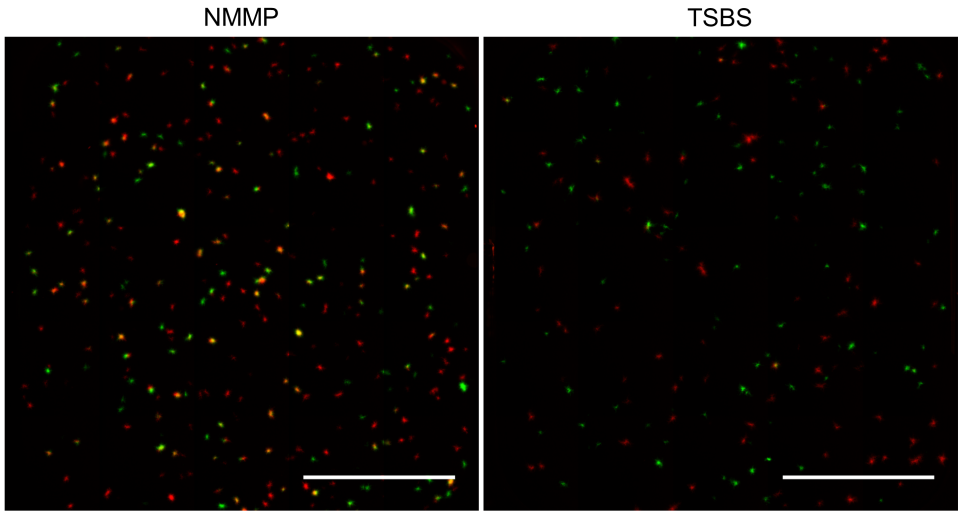


Figure S8. Aggregation depends on GlxA. Spores of the *ΔglxA* derivative strains of *Streptomyces lividans* expressing either the *eGFP* or the *mCherry* gene were mixed at the onset of growth in NMMP (left) or TSBS medium (right). After 24 h of growth, most particles are either green or red fluorescent in the absence of *glxA*. Scale bars represent 2 mm.

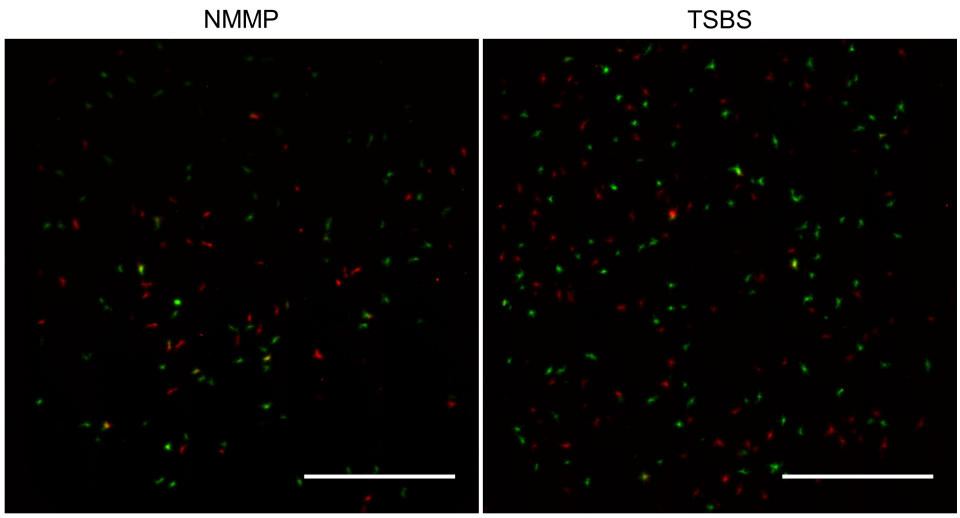


Figure S9. Aggregation depends on MatAB. Spores of the *ΔmatAB* derivative strains of *Streptomyces lividans* expressing either the *eGFP* or the *mCherry* gene were mixed at the onset of growth in NMMP (left) or TSBS medium (right). After 24 h of growth, the majority of particles is either green or red fluorescent in the absence of the *matAB* genes. Scale bars represent 2 mm.

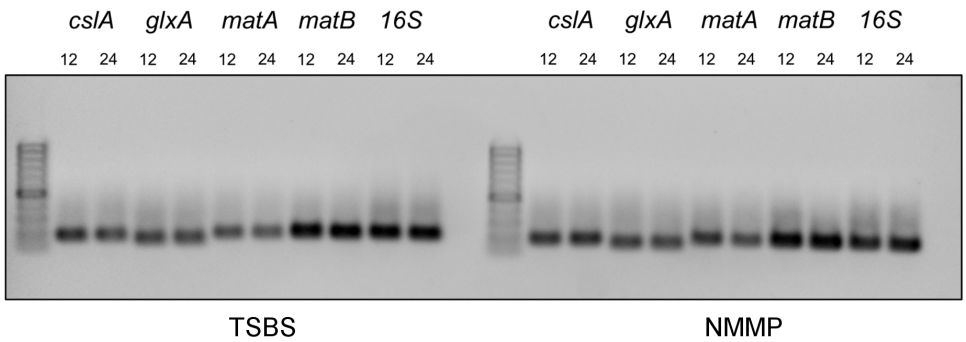


Figure S10. Genes required for glycan biosynthesis are expressed in NMMP and TSBS medium. Total RNA was isolated from NMMP and TSBS cultures after 12 and 24 hours of growth and analyzed for the presence of transcripts of the *cslA*, *glxA*, *matA* and *matB* genes using RT-PCR. 16S rRNA was used as a control. Note that all genes are expressed at both time points in both media.

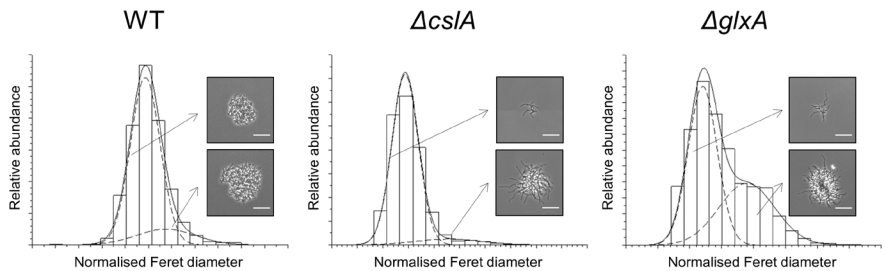


Figure S11. Particle size distributions of *Streptomyces lividans wild-type*, *ΔcslA* and *ΔglxA* strains related to mycelial morphology. After 12 h of growth in NMMP medium, particles of the *wild-type* (left) and *ΔcslA* strains (middle) are more homogenous in size than those of the *ΔglxA* strain (right). Note that most particles of the *ΔcslA* strain are small and characterized by an open morphology, while the *wild-type* strain almost exclusively forms pellets that are larger and denser. In the *ΔglxA* strain both types of mycelia are present which are more equally distributed. The scale bars represent 25 μm.

SUPPLEMENTARY INFORMATION BELONGING TO CHAPTER 4

Strain	Description	Reference
S. lividans 66	Wild-type	Laboratory stock
S. lividans Δsco1	Wild type *S. lividans* 66 lacking sco (marker less)	180
S. lividans Δsco1 pGreen	*S. lividans 66 Δsco1* containing pGreen	This work
S. lividans Δsco1 pRed*	*S. lividans 66 Δsco1* containing pRed*	This work
S. lividans Δsco1 pXyl11	*S. lividans 66 Δsco1* containing pXyl11.	E. Vijgenboom & G. Voshol unpublished

Table S1. *Streptomyces* strains used in this study.

Name	Description and relevant features	Reference
pGreen	pIJ8630 containing *eGFP* under control of the constitutive *gap1* promoter of *S. coelicolor* A3(2) M145.	142
pRed*	pIJ8630 containing *mCherry* under control of the constitutive *gap1* promoter of *S. coelicolor* A3(2) M145.	181
pXyl11	pSET152 containing a *xyl* gene under control of the constitutive *gap1* promoter of *S. coelicolor* A3(2). The xyl gene encodes for a moderate thermostable family GH11 xylanse.	E. Vijgenboom & G. Voshol, unpublished

Table S2. Constructs used in this study.

SUPPLEMENTARY INFORMATION BELONGING TO CHAPTER 5

Strain	Description	Reference
Streptomyces lividans 66	Wild-type strain	Laboratory stock
Streptomyces lividans pGreen	*S. lividans* 66 containing pGreen	142
Streptomyces lividans pRed*	*S. lividans* 66 containing pRed*	This study

Table S1. *Streptomyces* strains used in this study.

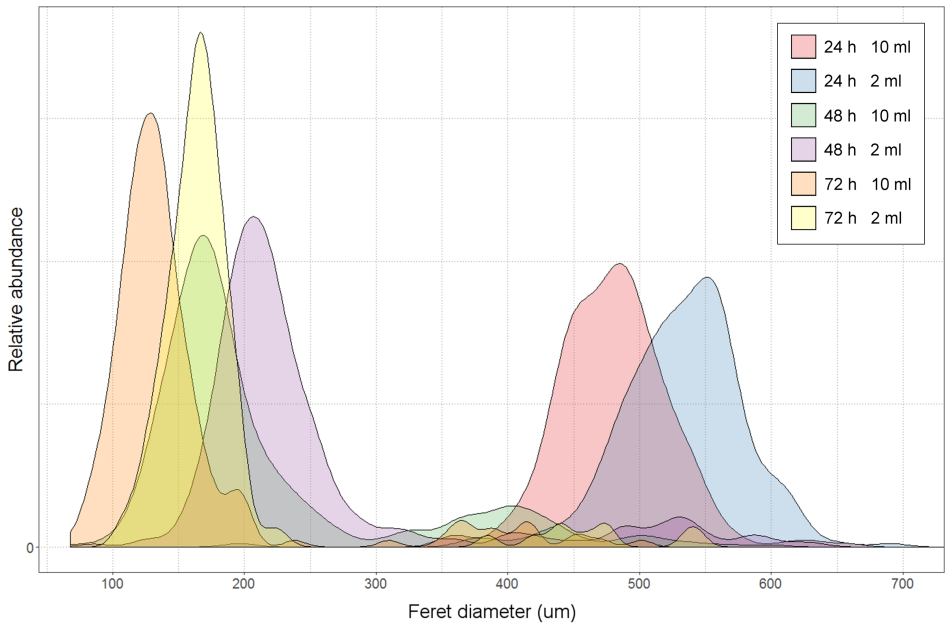


Figure S1: Size distributions of pellets in diluted cultures of *Streptomyces lividans* 66. TSBS cultures were inoculated with 2 or 10 ml of seed cultures that had been grown for 24, 48 or 72 h. The plots represent the size distribution of at least 300 pellets per sample, obtained after 24 h of growth. All sizes are indicated in micrometers.

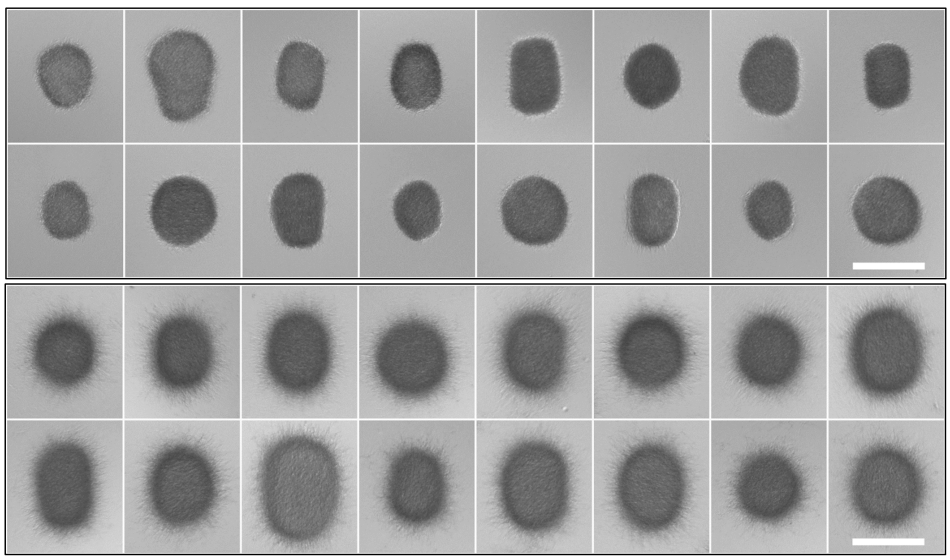


Figure S2. Morphological changes accompanying the aging of *Streptomyces* pellets. Collage of representative micrographs of pellets of *Streptomyces lividans* 66 in TSBS cultures after 24 (top) and 72 h (bottom) of growth. The scale bars represent 200 µm.

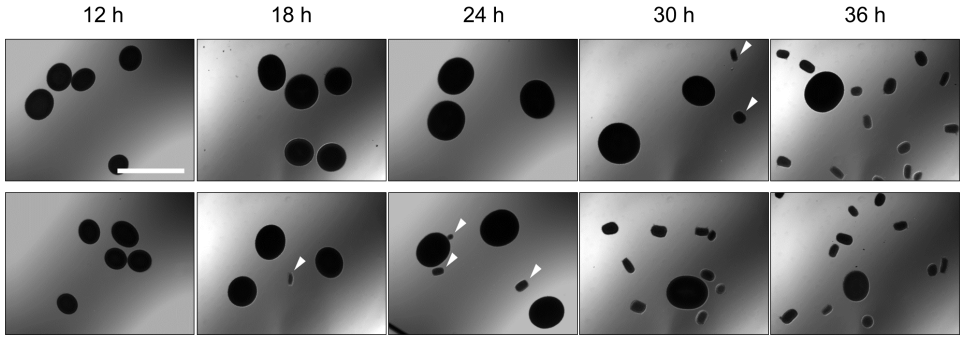


Figure S3. Fragmentation correlates with nutrient availability. Micrographs representing pellets of *Streptomyces lividans* 66 obtained by diluting seed cultures in fresh TSBS medium after 12, 18, 24, 30 and 36 h of growth. The seed cultures were prepared in normal TSBS (top panels) or in ½ x TSBS (bottom panels) medium. Note that the small pellets, derived from outgrowing fragments, are observed at least 12 h earlier in ½ x TSBS than in normal TSBS. The scale bar represents 1 mm.

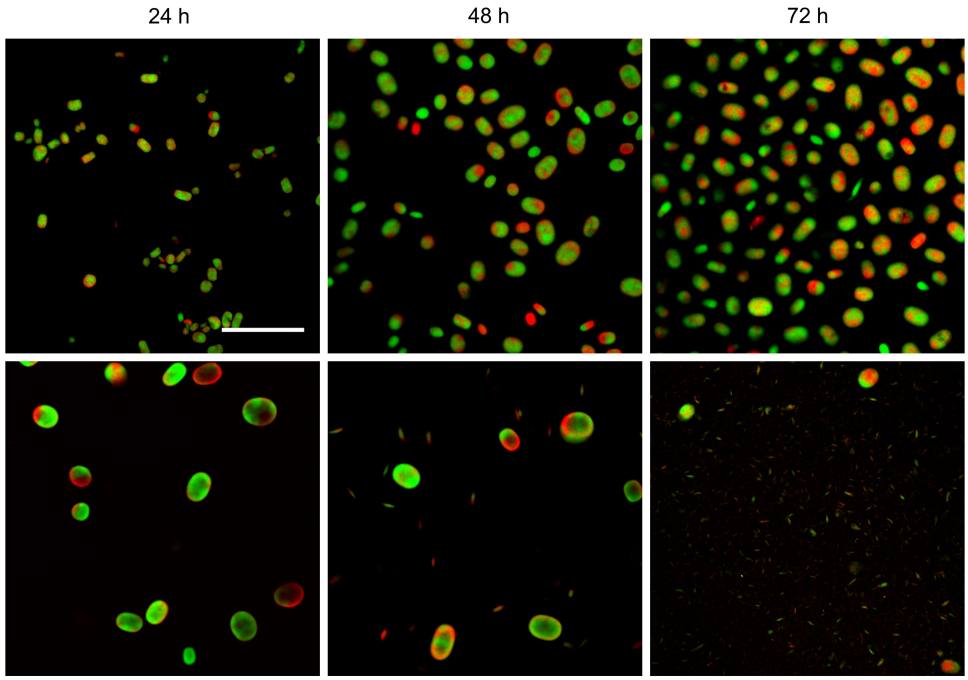


Figure S4. Morphological analysis of pellets of the fluorescent derivative strains of *Streptomyces lividans* in TSBS cultures. Seed cultures were prepared by co-culturing *S. lividans* strains constitutively expressing eGFP or mCherry in TSBS medium. The morphology of pellets in these seed cultures after 24 (left), 48 (middle) and 72 (right) h of growth are shown in the top panels. The bottom panels show micrographs of pellets following the transfer of 10 ml of the seed cultures in fresh TSBS medium and subsequent growth for 24 h. The scale bar represents 1 mm.

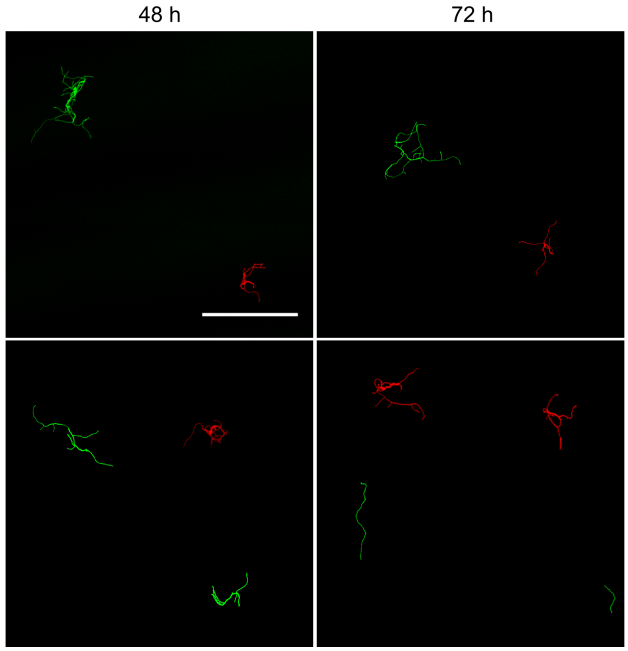
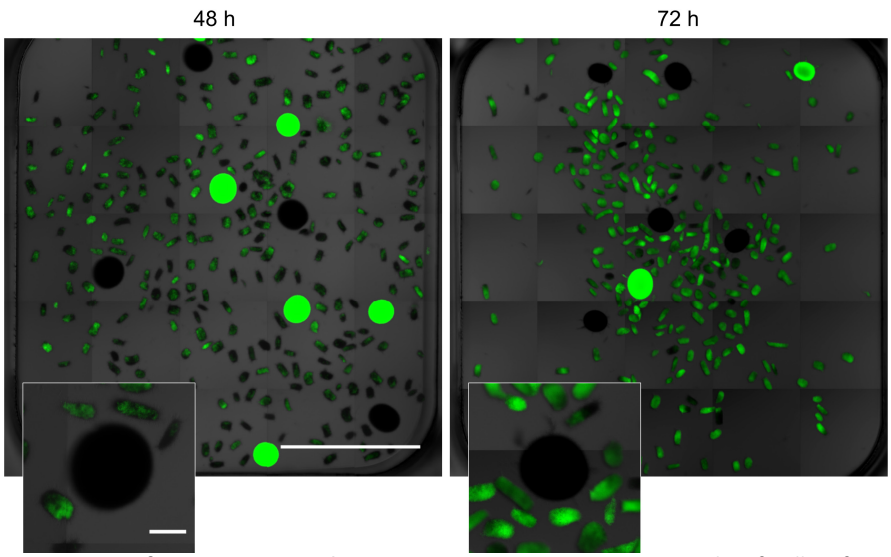


Figure S5. Visualization of detached fragments from pellets of co-cultured fluorescent *Streptomyces lividans* strains. Filtrates were obtained by the sequential filtering of TSBS cultures, which had been grown for 48 or 72 h, through cell strainers with a pore size of 100 (top panels), 40 and 5 µm (lower panels). Note that the detached fragments are either green or red fluorescent. The scale bar represents 100 µm.



Figures S6. Large, fragmenting particles are inert to aggregation. Micrographs of pellets from co-cultures of the *Streptomyces lividans wild-type* strain and its green-fluorescent derivative, obtained by mixing separate cultures of both strains after 48 (left) and 72 h (right) of growth. The inlay shows that the large *wild-type* pellets remain non-fluorescent after the transfer, indicating that small mycelial fragments do not aggregate with these large particles. The scale bars represent 2 mm and 200 µm in the overview pictures and inlays, respectively.

SUPPLEMENTARY INFORMATION BELONGING TO CHAPTER 6

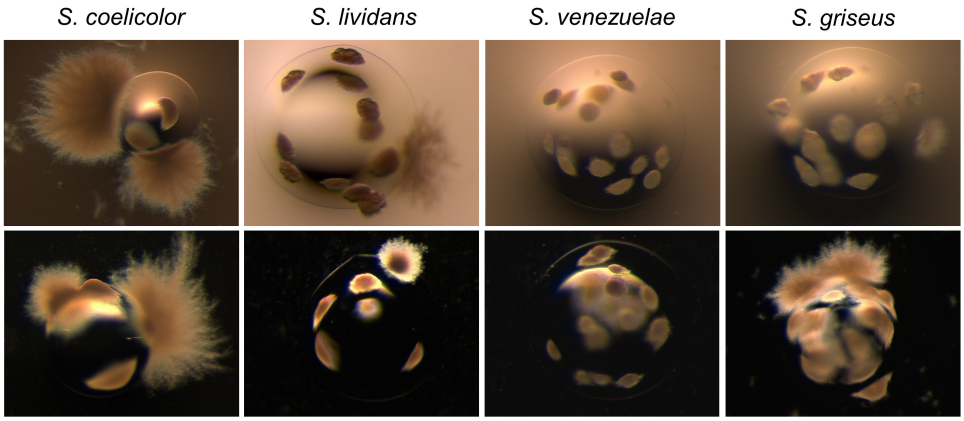


Figure S1. Morphology of encapsulated streptomycetes in $NMMP_{mod}$ medium. Microscopy images of microcapsules of *Streptomyces coelicolor*, *Streptomyces lividans*, *Streptomyces venezuelae* and *Streptomyces griseus* grown in $NMMP_{mod}$ medium at 48 (top panel) and 96 h (lower panel). The scale bar corresponds to 200 µm.

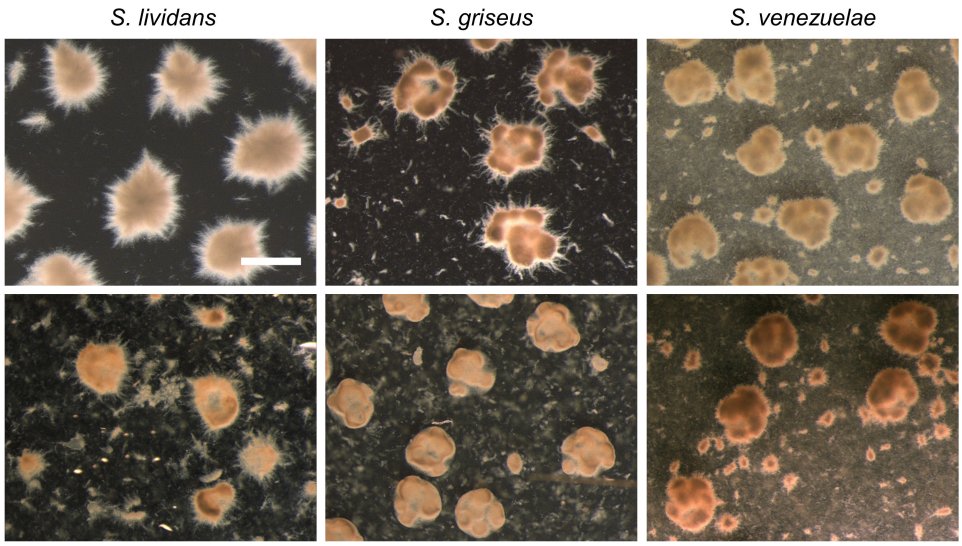


Figure S2: Growth and detachment of mycelium from microcapsules containing different streptomycetes. Overview images of the mycelium of *Streptomyces lividans*, *Streptomyces griseus* and *Streptomyces venezuelae* grown in $NMMP_{mod}$ medium for 48 (top panel) and 96 h (lower panel). Note that detached mycelial fragments are evident in the culture broth of *S. griseus* and *S. venezuelae* at 48 h. After 96 h, detached mycelial fragments are also observed in *S. lividans*. The scale bar corresponds to 500 µm.

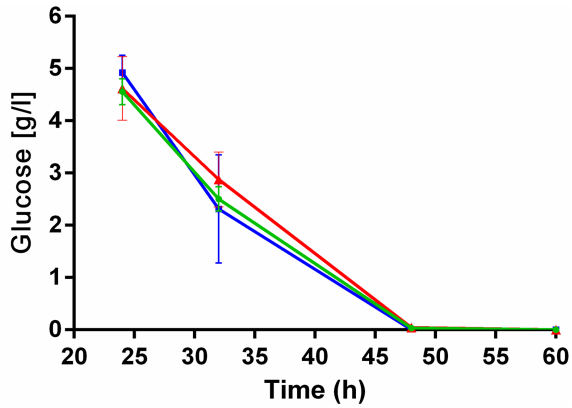


Figure S3. Glucose consumption by encapsulated and non-encapsulated mycelium. The residual glucose concentrations (in g/L) in $NMMP_{mod}$ medium are shown when *Streptomyces lividans pIJ703* is grown in micro-capsules (green), or non-encapsulated in the absence (red) and presence (blue) of a metal coil.

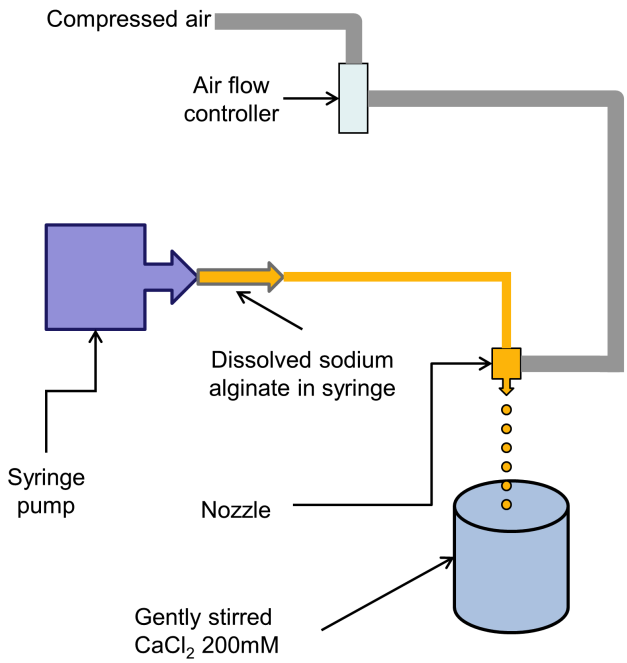


Figure S4: Schematic representation of the microencapsulation apparatus. A constant flow of dissolved alginate is maintained with a syringe pump set at 30 ml h^{-1}, while the air flow is regulated with an air flow controller set at 3 L min^{-1}. The sodium alginate droplets that detach form the nozzle fall in a gently-stirred solution of 200 mM $CaCl_2$ leading to formation of the microcapsules via ion exchange. The nozzle functions according to the coaxial gas-flow extrusion principle [219].

Curriculum Vitae

Boris Zacchetti was born on February 2nd, 1986 in Lecco, a small city in the north of Italy. In 2005, he graduated from Liceo Scientifico "Vittorio Bachelet" in Oggiono. After a year spent doing various jobs, he enrolled at the University of Milano-Bicocca to pursue a Bachelor's in Biotechnology, which he obtained in July 2010. Later that same year, he started a Master program in Industrial Biotechnology at the same University. During this program, he studied for a year at Leiden University, where he worked under the supervision of Dr. Dennis Claessen on a project aimed at characterizing interactions between filamentous microorganisms. Boris obtained his Master's degree in March 2013 and in November of the same year he accepted a PhD position at Leiden University. The work performed as a PhD student under the supervision of Dr. Dennis Claessen and Prof. Gilles van Wezel is presented in this thesis.

Boris is currently working as a postdoc under the supervision of Prof. Frank Delvigne at Gembloux Agro-Biotech, Liège University, Belgium, working on an ERA-CoBioTech project focused on singe-cell heterogeneity in industrially relevant microbes.

Publications

Zacchetti B., Wösten H.A.B., Claessen D, 2018. Multiscale heterogeneity in filamentous microbes. Biotechnology Advances. *In press.*

Delvigne F., **Zacchetti B.**, Fickers P., Fifani B., Roulling F., Lefebvre C., Neubauer, P. Junne S., 2018. Improving control in microbial cell factories: from single cell to large-scale production. FEMS Microbiology Letters. *In press.*

Zacchetti B., Smits P., Claessen D., 2018. Dynamics of pellet fragmentation and aggregation in liquid-grown cultures of *Streptomyces lividans*. Frontiers in Microbiology 9, 943.

Zacchetti B., Andrianos A., van Dissel D., de Ruiter E., van Wezel G.P., Claessen D., 2018. Microencapsulation extends mycelial viability of *Streptomyces lividans* 66 and increases enzyme production. BMC Biotechnology 18 (1), 13.

van Dissel D., Willemse J., **Zacchetti B.**, Claessen D., Pier G., van Wezel G.P., 2018. Production of poly-β-1,6-N-acetylglucosamine by MatAB is required for hyphal aggregation and hydrophilic surface adhesion by *Streptomyces*. Microbial Cell 5 (6), 269-279.

Zacchetti B., Willemse J., Recter B., van Dissel D., van Wezel G.P., Wösten H.A.B., Claessen D., 2016. Aggregation of germlings is a major contributing factor towards mycelial heterogeneity of *Streptomyces*. Scientific Reports 6, 27045.

Wu C., **Zacchetti B.**, Ram A.F.J., van Wezel G.P., Claessen D., Choi Y.H., 2015. Expanding the chemical space for natural products by *Aspergillus-Streptomyces* co-cultivation and biotransformation. Scientific Reports 5, 10868.