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Chapter 2

Morphogenesis of *Streptomyces* in submerged cultures

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ABSTRACT

Members of the genus *Streptomyces* are mycelial bacteria that undergo a complex multicellular life cycle and propagate via sporulation. Streptomycetes are important industrial microorganisms, as they produce a plethora of medically relevant natural products, including the majority of clinically important antibiotics, as well as a wide range of enzymes with industrial application. While development of *Streptomyces* in surface-grown cultures is well studied, relatively little is known of the parameters that determine morphogenesis in submerged cultures. Here, growth is characterized by the formation of mycelial networks and pellets. From the perspective of industrial fermentations, such mycelial growth is unattractive, as it is associated with slow growth, heterogeneous cultures and high viscosity. Here, we review the current insights into the genetic and environmental factors that determine mycelial growth and morphology in liquid-grown cultures. The genetic factors include cell-matrix proteins and extracellular polymers, morphoproteins with specific roles in liquid culture morphogenesis, with the SsgA-like proteins as well-studied examples, and programmed cell death. Environmental factors refer in particular to those dictated by process engineering, such as growth media and reactor set-up. These insights are then integrated to provide perspectives as to how this knowledge can be applied to improve streptomycetes for industrial applications.

1. INTRODUCTION

Streptomycetes are filamentous bacteria that belong to the phylum of actinobacteria. These medically and industrially highly relevant microorganisms are producers of over half of the antibiotics used in the clinic today as well as of a plethora of other natural products, such as anticancer, immunosuppressive, antifungal and antihelmentic agents (Baltz, 2007, Baltz, 2008, Olano *et al.*, 2009, Hopwood, 2007). Furthermore, streptomycetes are saprophytic bacteria that grow on almost any natural polymer, and as such are a rich source of industrial enzymes (Bhosale *et al.*, 1996, Tokiwa & Calabia, 2004, Vrancken & Anne, 2009, Yikmis & Steinbüchel, 2012). Unlike most other bacteria, streptomycetes are non-planktonic and grow as a mycelium consisting of a network of closely interwoven hyphae. Exponential growth is thereby achieved by a combination of tip extension and branching. The multigenomic hyphae are divided by occasional cross-walls, which makes *Streptomyces* a rare example of a multicellular prokaryote (Claessen *et al.*, 2014). When nutrient availability becomes limiting, streptomycetes initiate a complex developmental program, which leads to morphological and chemical differentiation (Chater & Losick, 1997, Flärdh & Buttner, 2009). At this stage, aerial hyphae are formed that are coated with water-repellent proteins to allow them to break through the aqueous soil surface and grow into the air (Wösten & Willey, 2000, Claessen *et al.*, 2006). Eventually the aerial hyphae differentiate into chains of unigenomic spores, following a spectacular cell division process whereby some one hundred septa are produced in a very short time span (Schwedock *et al.*, 1997, Jakimowicz & van Wezel, 2012). Genes that are involved in the onset of aerial mycelium formation are designated bald (*bld*) genes, characterized by the bald appearance of mutants due to their failure to produce the fluffy aerial hyphae (Merrick, 1976), and those that are essential for the formation of grey-pigmented spores are called white (*whi*) genes, characterized by the white appearance of mutants due to the lack of the WhiE spore pigment (Chater, 1972). It is important to note that most of the developmental regulators function by controlling transcription or translation. In this review, we primarily focus on the genes that control morphogenesis in submerged culture. For detailed information on the genes that control aerial hyphae formation and sporulation in surface-grown cultures we refer to excellent reviews elsewhere (Chater & Losick, 1997, Kelemen & Buttner, 1998, Flärdh & Buttner, 2009).

The timing of antibiotic production is tightly controlled with the life cycle, and many antibiotics are produced at a time correlated to the onset of morphological differentiation (Bibb, 2005, van Wezel & McDowall, 2011, Liu *et al.*, 2013a). Mutants that are blocked in development (so-called *bld* mutants) therefore typically fail to produce antibiotics (Bibb, 2005). The onset of development and antibiotic production coincides with the autolytic dismantling of the vegetative mycelium, necessary to provide nutrients as building blocks for the aerial mycelium, in a process strongly resembling programmed cell death (Fernández & Sánchez, 2002, Manteca *et al.*, 2006b).

Industry-level production of secondary metabolites and enzymes occurs in bioreactors, and industrial exploitation of streptomycetes is hampered by the formation of large mycelial

networks or clumps, which is unattractive from the perspective of process engineering (Braun & Vecht-Lifshitz, 1991, Hodgson, 2000, van Wezel *et al.*, 2009). Compared to fermentations with unicellular microorganisms such as *Saccharomyces cerevisiae*, *Escherichia coli* or *Bacillus subtilis*, the more complex morphology of streptomycetes puts constraints on the ability to maximize product yields (Wucherpfennig *et al.*, 2010). Entanglement of mycelia increases the viscosity of the broth, which lowers the transfer rates of nutrients and gasses, and because many strains have the tendency to aggregate into pellets a part of the biomass might be shielded from the supply of nutrients altogether. To further complicate matters, optimal productivity is tied to morphology in a product-specific manner, meaning that often less favored conditions have to be accepted for optimal productivity (Martin & Bushell, 1996, Wardell *et al.*, 2002, van Wezel *et al.*, 2006, Anné *et al.*, 2014).

We are only beginning to unravel the mechanisms that control morphogenesis of streptomycetes, and this is particularly true for mycelial growth in submerged cultures. At the same time, understanding the correlation between morphogenesis and productivity is of critical importance for the exploitation of streptomycetes in the industrial domain. In this review we present an overview of the current literature on the morphogenesis of streptomycetes in liquid-grown cultures and look at how this may be translated to better match morphology and productivity during industrial fermentations.

2. MORPHOGENESIS IN SUBMERGED CULTURES

2.1 Hyphal growth

Active growth of streptomycetes typically starts with spore germination. The spore is not only a means for dispersal but also serves as a way to survive a period of adverse environmental conditions. Once the conditions become favorable for growth, spores typically establish two germ tubes at the polar sides, which grow out to become young vegetative hyphae. The molecular steps responsible for the emergence of germ tubes have not yet been identified, and surprisingly little is known about this germination process. One reason may be that it is difficult to differentiate between early signaling events for the onset of germination and essential metabolic and housekeeping activities that relate to early growth. Spore germination is controlled by the cyclic-AMP receptor protein Crp (Derouaux et al., 2004, Piette et al., 2005), but Crp also controls antibiotic production (Gao et al., 2012). A major consequence of the absence of Crp was the production of a very thick spore wall, which was identified as a likely cause for the germination delay (Piette et al., 2005). The correlation between cell-wall hydrolysis and the speed of germination was further supported by the delayed germination in the absence of the cell-wall hydrolase RpfA (Haiser et al., 2009). Another cell wall-associated protein that relates to germination is NepA, originally identified as a protein that localizes to the 'subapical stem', which connects vegetative with aerial hyphae during early development (Dalton et al., 2007). Deletion of nepA results in altered germination, which in particular occurs in a more synchronized

manner (de Jong et al., 2009a).

After germination, the hyphae grow out to form a branched network of hyphae, the vegetative mycelium. Studies in streptomycetes with compounds that are incorporated into newly synthesized peptidoglycan, such as labelled vancomycin or N-acetylglucosamine, revealed that peptidoglycan synthesis primarily occurs at hyphal tips and - therefore by definition - also at emerging branches (Gray *et al.*, 1990, Daniel & Errington, 2003). Penicillins particularly target apical sites of the hyphae, and less the lateral walls, and the latter may therefore be regarded as a relatively inert murein. During normal growth of streptomycetes cross-walls are formed in the hyphae, which do not lead to cell fission, thus resulting in long multinucleoid compartments (Chater & Losick, 1997, Flärdh & van Wezel, 2003, Jakimowicz & van Wezel, 2012). Branches are formed at sites behind the tip, and

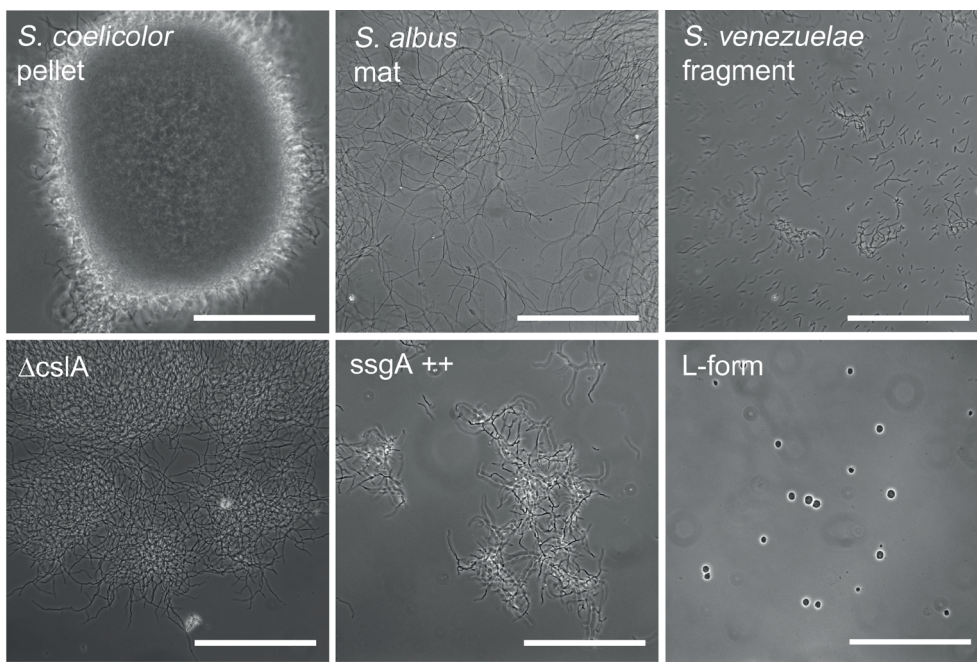

Figure 1. Distinct morphologies of Streptomyces species in submerged cultures. Top row: *Streptomyces* strains representing different morphologies, namely pellets (*S. coelicolor*), mycelial mats (*S. albus*) and fragments (*S. venezuelae*). Bottom row: changes in morphology due to genetic factors. Images show *cslA* null mutant (left) and SsgA overexpressing strains (middle) of *S. coelicolor*, and L-form cells derived from *S. viridifaciens*. Scale bar, 100 µm.

frequently but not always adjacent to crosswalls, leading to the establishment of new cell poles, and the combination of apical growth and branching ensures exponential growth (Flärdh *et al.*, 2012).

Subsequent growth and morphology of the mycelial mass is in part species and strain-dependent. Three types of morphologies are generally distinguished in liquid-grown cultures; (1) freely dispersed mycelia, which predominantly behave like single cells with

high mass transfer properties; (2) open mycelial networks, also often called mycelial mats, which generally have good mass transfer characteristics, but increase the viscosity of the media; and (3) pellets that do not increase the viscosity significantly, but often contain a nutrient-deprived center (Paul & Thomas, 1998), Fig. 1). The wide range of morphological phenotypes, which often co-exist in the reactor, is due to the large number of variables that influence the ability of the mycelia to grow, branch, aggregate and fragment (see Section 5). The dominant type is genetically determined, and differs considerably between species and strains. For instance, *Streptomyces venezuelae* typically forms highly fragmented mycelia and sporulates in liquid cultures, *Streptomyces clavuligerus* forms mycelial mats, while *Streptomyces coelicolor* mostly forms large and dense pellets (Bewick *et al.*, 1975, van Wezel *et al.*, 2006). These differences already reveal that general predictions are difficult to make, although some genetic determinants influence morphology regardless of the strain (see below).

2.2. Submerged sporulation

Whereas major differences in mycelial architecture are observed between different streptomycetes, further complexity is caused by the capacity of some strains to form spores in liquid environments. The first report of submerged sporulation was made as early as in 1947 (Erikson, 1947). However, in 1983 Kendrick and Ensign provided a groundbreaking study on the morphology and on sporulation of *Streptomyces griseus* B-2682 in submerged culture (Kendrick & Ensign, 1983). This led to the identification of several streptomycetes that produce submerged spores, including *Streptomyces granaticolor, S. griseus*, *Streptomyces roseosporus* and *S. venezuelae* (Kendrick & Ensign, 1983, Daza *et al.*, 1989, Glazebrook *et al.*, 1990). These can be further subdivided into streptomycetes that only sporulate in nutrient-limiting media, such as *S. griseus* (Kendrick & Ensign, 1983), and those that produce submerged spores in nearly all media, such as *S. venezuelae* (Glazebrook *et al.*, 1990). Although until recently it was believed that the ability to produce spores in liquid cultures was something like a rarity, the possibility cannot be ruled out that in principle all streptomycetes can do so under specific conditions; indeed, a recent survey revealed that submerged sporulation is likely much more widespread than originally anticipated, with half of a random selection of over 50 streptomycetes sporulating in submerged culture at least under some growth conditions (Girard *et al.*, 2013). Interestingly, addition of high concentrations of calcium to liquid-grown cultures of *S. coelicolor* and *Streptomyces lividans* induces the occasional formation of spore-like compartments. Since phosphate starvation is an important trigger for submerged sporulation, this calcium effect was explained by the reduction of the phosphate pool (Kendrick & Ensign, 1983, Daza *et al.*, 1989, Glazebrook *et al.*, 1990), although further analysis is required to corroborate this.

There is a clear transition in the vegetative hyphae prior to submerged sporulation: the hyphae thicken, and widened club-like structures or ‘pre-conidia’ are produced at the apical sites of the hyphae (Biró *et al.*, 1980, Rueda *et al.*, 2001a). Comparison of thin sections

of aerial and submerged spores by transmission electron microscopy (TEM) showed that the cell walls of surface-grown spores are thicker than those of submerged spores, with a width of approximately 40 nm and 25 nm, respectively (Kendrick & Ensign, 1983). However, aerial and submerged sporogenic hyphae of *Streptomyces braziliensis* by TEM show strong similarity (Rueda *et al.*, 2001b), with the main difference in the appearance of the sheath around the hyphae, which was thinner and less structured in sporogenic vegetative hyphae, perhaps due to a difference in the rodlet layer (Gebbink *et al.*, 2005).

While sporulation of streptomycetes is typically studied in surface-grown cultures, the study of sporulation in submerged cultures is an attractive alternative for several reasons. First of all, culturing time is much shorter, and synchronous sporulation can be more readily achieved. Sporulation of *S. griseus* is induced by transferring the strain from rich to nutrient-limited media, whereby sporogenic hyphae become evident within a few hours and then continue to elongate until septation occurs at approximately 10 h, with spores maturing over a subsequent period of 10-12 hours (Kwak & Kendrick, 1996).

In addition to the advantage of synchronization of cultures, submerged development also readily facilitates global expression profiling by systems biology approaches like transcriptome, proteome or metabolome analysis. This is exemplified by recent studies on developmental mutants in *S. venezuelae* (Bibb *et al.*, 2012, Bush *et al.*, 2013). Buttner and colleagues are currently developing *S. venezuelae* as model system for morphological differentiation for its ability to readily sporulate in both minimal and rich liquid media, and developmental (*bld, whi*) mutants that were studied previously in *S. coelicolor*, are being recreated in this interesting background to facilitate '-omics' approaches (Mark Buttner and Maureen Bibb, pers. comm.). Submerged sporulation also allows discriminating between genes involved in the control of aerial hyphae formation and those required for sporulation-specific cell division. After all, the former is not relevant in submerged cultures, and presumably genes required for erection of aerial hyphae should not interfere with the ability to initiate sporulation-specific cell division, while the cell division process itself is likely very similar during submerged and solid culture sporulation. Thus, submerged sporulation should be a particularly good model system for studies on developmental cell division.

2.3. A special case: Streptomyces L-forms

Mycelial growth is a hallmark feature of streptomycetes. Production of secondary metabolites, such as antibiotics or antitumor agents, is often linked to the inherent capacity to form mycelial pellets. However, streptomycetes can also be forced to produce single cells. Treatment of mycelia with lysozyme results in the formation of protoplasts, which are identical-sized spherical cells without a cell wall used for cell fusion and plasmid transformation (Hopwood *et al.*, 1977, Bibb *et al.*, 1978)1977, Bibb<style face="italic"> et al.</style>, 1978. While protoplasts cannot propagate, streptomycetes can also form so-called L-forms (Innes & Allan, 2001). Selection of L-forms occurs by growth in the presence of lysozyme, which degrades the peptidoglycan, and penicillin, which inhibits *de novo*

peptidoglycan synthesis. Subsequent cultivations in osmotically balanced media can lead to the acquisition of mutations that allow these cells to propagate without their cell wall, even in the absence of the inducing agents (i.e. penicillin and lysozyme;(Innes & Allan, 2001, Leaver *et al.*, 2009, Mercier *et al.*, 2013). L-forms have been shown to associate with plants acting as biocontrol agents (Amijee *et al.*, 1992, Innes & Allan, 2001). The absence of a cell wall allows these pleomorphic cells to invade spaces that would otherwise be inaccessible, such as the extracellular space within plant tissue, or even inside plant cells (Paton & Innes, 1991). Biocontrol activity was shown for *Pseudomonas* and *Bacillus* species (Amijee *et al.*, 1992, Walker *et al.*, 2002, Waterhouse *et al.*, 1996), but could also be true for streptomycetes, which naturally produce a large arsenal of antifungal and antimicrobial compounds (Hopwood, 2007).

L-forms have been generated in a wide-range of unrelated bacterial species, including, amongst others, *Escherichia coli* (Glover *et al.*, 2009), *Bacillus subtilis* (Leaver & Errington, 2005), and *Listeria monocytogenes* (Dell'Era *et al.*, 2009), and also in several *Streptomyces* species, including *Streptomyces hygroscopicus, S. griseus, Streptomyces levoris* and *Streptomyces viridifaciens* (Gumpert, 1982, Gumpert, 1983, Innes & Allan, 2001); Fig. 1). L-form growth is largely driven by changes in the cell surface area to volume ratio of these cells, and is characterized by blebbing, tubulation, vesiculation and fission (Errington, 2013, Mercier *et al.*, 2013). Interestingly, division of phospholipid vesicles, which to some extent resemble empty L-forms, could merely be driven by changes in lipid composition (Peterlin *et al.*, 2009). Also, cell division of L-forms is stimulated by increased fatty acid synthesis (Mercier *et al.*, 2013), and does not require the canonical cell division machinery (Leaver *et al.*, 2009). As such, L-form proliferation could mimic how primordial cells propagated before the cell wall was invented.

The production of secondary metabolites by streptomycetes is often linked to the complex pattern of morphological development (van Wezel & McDowall, 2011). Surprisingly, stable L-forms of *S. viridifaciens* were still able to produce tetracycline, in addition to another uncharacterized green-pigmented metabolite (Innes & Allan, 2001). However, compared to the parental form the yields were relatively low. Nevertheless, the capacity of L-forms to produce secondary metabolites including antibiotics highlights their potential use as biocontrol agents.

3. MOLECULAR CONTROL OF LIQUID-CULTURE MORPHOGENESIS

3.1. The tip-organizing center and the cytoskeleton

During apical growth, DivIVA localizes close to the growing tip and its pivotal role in the control of apical growth is highlighted by the fact that it is essential or growth, while its overexpression leads to drastic changes in hyphal morphology including hyper-branching (Flärdh *et al.*, 2012, Hempel *et al.*, 2012). In *Bacillus subtilis*, DivIVA controls septum-site determination by interacting with the MinCD cell division inhibitor complex (Edwards &

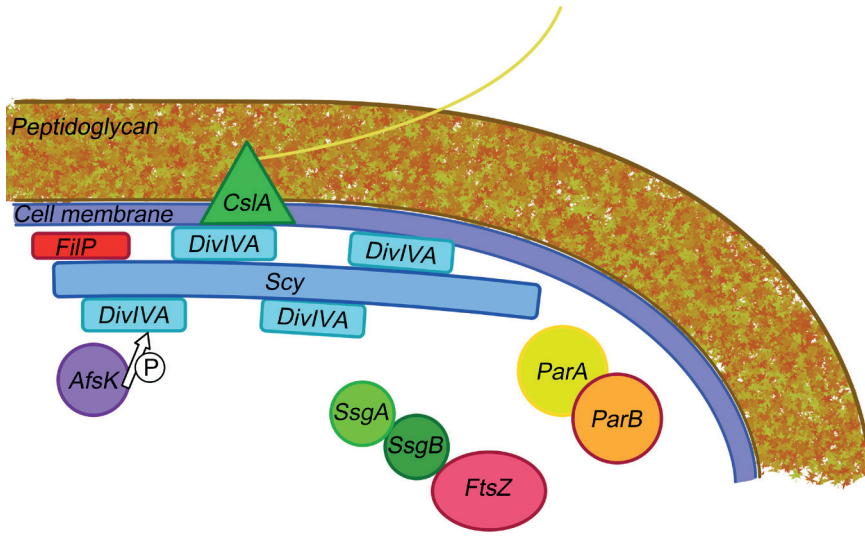

Figure 2. Components of the tip organizing center (TIPOC) of S. coelicolor. The TIPOC is a multi-protein complex that coordinates tip growth, cell wall synthesis, DNA replication and segregation, and cell division. DivIVA is required for peptidoglycan synthesis and interacts directly with the cytoskeletal protein Scy. The latter assists in assembly of the complex. Other members include the cellulose synthase-like protein CslA, the cytoskeletal element FilP and SsgA, which controls processes requiring cell wall remodeling. The TIPOC interacts with the proteins involved in chromosome segregation (ParA and ParB), and probably with those involved in cell division (SsgA, SsgB and FtsZ). AfsK negatively controls the activity of DivIVA by phosphorylation. For further details and references see the text. Adapted from (Holmes *et al.*, 2013)

Errington, 1997). However, streptomycetes lack a Min system, and DivIVA has instead taken up a (yet not fully understood) role in apical growth.

DivIVA is part of a larger complex of proteins that collectively have been dubbed tip-organizing center (TIPOC; Fig. 2; (Holmes *et al.*, 2013). In recent years several proteins and protein complexes have been identified that play a role in tip growth and DNA replication. These include the *Streptomyces* cytoskeletal element Scy (Holmes *et al.*, 2013), the twin-arginine transport (Tat) secretion system (Willemse *et al.*, 2012), the cell-wall remodeling protein SsgA (Noens *et al.*, 2007) and the cellulose synthase-like protein CslA (Xu *et al.*, 2008). Furthermore, new chromosomes are also replicated close to but distinctly away from the tip in so-called replisomes (Wolánski *et al.*, 2011). The TIPOC likely ensures that all apical processes, such as DNA replication and cell wall synthesis, are carried out in coordinated fashion (Ditkowski *et al.*, 2013, Fuchino *et al.*, 2013) and that DNA is not damaged by the cell-wall synthetic machinery (K. Celler and GPvW, unpublished). The extracellular cellulose-like polymer synthesized by CslA might form an additional protective layer at the outside of the hyphal tips, thereby preventing cell damage (Chater *et al.*, 2010).

Recent evidence indicates that DivIVA is phosphorylated by the Ser/Thr protein kinase AfsK (Hempel *et al.*, 2012). The C-terminal part of DivIVA has multiple sites for phosphorylation, and the level of phosphorylation increases dramatically when cell-wall

synthesis is arrested (Hempel *et al.*, 2012). Increased phosphorylation coincided with the disappearance of DivIVA from the hyphal tips, followed by the emergence of new lateral branches. Under normal growth conditions, branches are formed by a so-called tip-splitting mechanism, in which new foci of DivIVA originate from existing foci (Richards *et al.*, 2012). Consistent with the observation that DivIVA is required for apical growth, it is recruited to branch sites to allow the start of apical growth.

The cytoskeleton of streptomycetes is highly complex, with likely over ten different cytostructural proteins (recently reviewed in (Celler *et al.*, 2013). The Scy protein acts in close collaboration with DivIVA in establishing growth polarity (Holmes *et al.*, 2013). In contrast to *divIVA*, *scy* can be deleted, which has a pronounced effect of hyphal morphology. Notably, in the absence of Scy hyphal tips were often branching, leading to a tip-splitting phenotype and aberrant hyphal geometry. The Scy protein is a large 1326 amino acid (aa) protein with a high propensity to form coiled-coil structures. *In vitro* experiments indicated that this protein forms long filaments, which potentially act as a scaffold for the assembly of the TIPOC. Indeed, Scy not only interacts with DivIVA, but also with the chromosome-partitioning protein ParA (Ditkowski *et al.*, 2013) and with the intermediate filament-like protein FilP, encoded by a gene immediately downstream of *scy* (Bagchi *et al.*, 2008, Holmes *et al.*, 2013). FilP also interacts with DivIVA, which indicates that these three proteins together form a large polar assembly that likely plays a role in the spatial and temporal control of apical growth (Fig. 2). Interestingly, during sporulation of *B. subtilis* DivIVA interacts with the chromosome segregation machinery, to aid in positioning the oriC region of the chromosome at the cell pole, in preparation for polar division (Thomaides *et al.*, 2001). Considering the polar interaction with ParA, this functionality of DivIVA is retained in streptomycetes.

3.2. Extracellular polymers and pellet morphology

Multicellular structures are typically held together by an extracellular matrix (Branda *et al.*, 2005, McCrate *et al.*, 2013, Vlamakis *et al.*, 2013). Although the composition of these matrices are diverse between different organisms, they typically contain, amongst others, proteins, polysaccharides, and extracellular DNA (White *et al.*, 2002, Zogaj *et al.*, 2003, Gebbink *et al.*, 2005, Claessen *et al.*, 2014). The matrix contributes to structural integrity of the multicellular community, while simultaneously providing protection against various stresses (Scher *et al.*, 2005, Romero *et al.*, 2010, DePas *et al.*, 2013). While matrices are usually mentioned in the context of biofilms, streptomycetes also make extracellular substances that contribute to morphology. Kim and Kim (2004) already demonstrated that pellets of *S. coelicolor* were susceptible to DNase treatment. In addition to extracellular DNA (eDNA), a role for hyaluronic acid in pellet integrity was proposed. Interfering with these matrix components made pellets fragile, leading to their (partial) disintegration (Kim & Kim, 2004). These data lead to a model in which an extracellular matrix, consisting of at least eDNA and hyaluronic acid, contributes to morphology of *Streptomyces* pellets by acting as

an adhesive. The eDNA component of this matrix is probably released in the environment during programmed cell death occurring in the central part of the pellet, and trapped within the pellet core.

Another component of such an extracellular matrix is the polymer produced by the cellulose synthase-like protein CslA (Xu *et al.*, 2008, de Jong *et al.*, 2009b). CslA was discovered as an interaction partner of DivIVA (Xu *et al.*, 2008). CslA is conserved in streptomycetes, and synthesizes a polymer consisting of β-(1-4) glycosidic bonds, consistent with a cellulose-like polymer, at hyphal tips and branch sites. The exact nature of the polysaccharide is still unclear. Deletion of the *cslA* gene has a pronounced effect on the morphology of liquid-grown mycelia, with a much more dispersed growth than wild-type cells (Fig. 1; (Xu *et al.*, 2008). This suggests that the polymer produced by CslA contributes to pellet architecture, perhaps by acting as an adhesive. Interestingly, deletion of the downstream-located gene *glxA*, which encodes a putative galactose-like oxidase, also results in an open mycelial morphology (our unpublished data), and GlxA may modify the CslA-synthesized polysaccharide. Indeed, both genes are transcriptionally coupled under most growth condition (Xu *et al.*, 2008, Liman *et al.*, 2013).

CslA is required for the hyphal attachment to surfaces (de Jong *et al.*, 2009b). This attachment coincides with the formation of an extracellular matrix, which is characterized by fimbrial structures that protrude from the cell surface of the adhering hyphae. Notably, while the absence of CslA had no visible effect on the number of fimbriae, their connection to the cell surface was considerably weakened. Further characterization of these fimbriae indicated that they were largely composed of bundled amyloid fibrils of so-called chaplin proteins (de Jong *et al.*, 2009b). Without chaplins, much thinner fibrils were observed that were susceptible to treatment with cellulase. This enzyme could also release wild-type fimbriae from the cell surface. This led to a model in which the CslA-produced polysaccharide provides a scaffold for fimbriae formation, while also contributing to their anchoring. It is tempting to speculate that the formation of pellets is also mediated via attachment and aggregation. Rather than connecting hyphae to surfaces, fimbriae would now mediate interactions between adjacent hypha, leading to a compact pellet structure. Consistent with this idea is the observation that the formation of pellets is not only disturbed without *cslA*, but also in the absence of chaplins (M.L.C. Petrus and D.C., unpublished data).

3.3. Proteins that control liquid-culture morphogenesis

Presently, there is relatively little known of the proteins that are specifically involved in the control of submerged sporulation. Indeed, scanning the literature shows that of the close to 500 publications on the topic of sporulation of *Streptomyces*, and fewer than 20 of those are primarily dedicated to the biology of submerged sporulation (PubMed search as of February 2014). The first studies into proteins that control submerged sporulation were done in the mid 90s of the previous century. Comparison of protein expression profiles between liquid-grown cultures prior to and at the onset of submerged sporulation identified

a 52 kDa sporulation-specific protein, designated EshA (for extension of sporogenic hyphae), as a cyclic nucleotide binding protein that is expressed during the first 12 hours of submerged sporulation and that is required for growth of sporogenic hyphae at an early stage of morphogenesis of *S. griseus* (Kwak & Kendrick, 1996). Interestingly, while *eshA* null mutants were inhibited in the elongation of sporogenic hyphae from new branch points in submerged culture, spore chains were instead formed ectopically in vegetative hyphae, apparently by accelerating septation and spore maturation at the pre-existing vegetative filaments (Kwak & Kendrick, 1996). This suggests that EshA is required for growth of sporogenic hyphae but not for sporulation *per se*. Saito, Ochi and colleagues demonstrated that EshA also plays a role in the control of antibiotic production, whereby deletion of *eshA* inhibits production of actinorhodin but not of prodigionines in *S. coelicolor* (Kawamoto *et al.*, 2001)2001 and streptomycin production in *S. griseus* (Saito *et al.*, 2003b). Furthermore, *eshA* is conditionally required for sporulation on surface-grown cultures of *S. griseus*, but not for *S. coelicolor* (Saito *et al.*, 2003b). Interestingly, EshA forms larger protein complexes, potentially forming icosahedron-like structures. While the protein and its orthologue MMPI of *Mycobacterium leprae* were reported to be membrane-associated (Winter *et al.*, 1995, Kwak *et al.*, 2001), consistent with the presence of putative transmembrane helices, at least the multimeric complexes were primarily identified in the cytoplasm of *S. griseus* (Saito *et al.*, 2003b). While the exact role of EshA is still unclear, large amounts of dNTPs accumulate in *eshA* null mutants, coinciding - and consistent - with strongly reduced rates of DNA synthesis, in particular at a time coinciding with the onset of development (Saito *et al.*, 2003b). It therefore seems likely that EshA plays a role in the activation of DNA synthesis during the onset of sporulation-specific cell division. It should be noted that *eshA* lies immediately upstream of the genes for synthesis of the volatile organic compound (VOC) 2-methylisoborneol (Wang & Cane, 2008), and analysis in String (www.string-embl.de) reveals strong phylogenetic linkage to the gene encoding the germacradienol/geosmin synthase GeoA, which synthesizes the VOC geosmin (Gust *et al.*, 2003a). It is yet unclear what the functional relevance is of this surprising linkage between EshA and VOC biosynthetic genes in streptomycetes.

Another protein with major impact on liquid-culture morphology is HyaS, which affects pellet morphology and integrity (Koebsch *et al.*, 2009). This protein is conserved in streptomycetes, and produced in liquid-grown cultures. HyaS associates with substrate hyphae and induces tight fusion-like contacts between hyphae (Koebsch *et al.*, 2009). Deletion of *hyaS* in *S. lividans* resulted in irregularly shaped pellets, which were less dense than those of the parental strain. Interestingly, the C-terminal part of the HyaS protein possesses amine oxidase activity, which is required for normal pellet morphology. Koebsch and colleagues speculate that this enzyme activity might induce cross-linking with other hyphae-associated protein(s) or compounds, in a similar manner as the eukaryotic cell surface-located lysyl oxidases are involved in matrix remodeling (Lucero & Kagan, 2006).

On searching for proteins that were able to suppress hypersporulation of a spontaneous

S. griseus mutant at high copy number, Kawamoto and Ensign identified SsgA as an important submerged sporulation-related protein (Kawamoto & Ensign, 1995). It was soon discovered that SsgA functions by stimulating fragmentation of hyphae by activating septum formation (Kawamoto *et al.*, 1997), and SsgA is required for both solid- and liquid-culture sporulation of streptomycetes (Jiang & Kendrick, 2000, van Wezel *et al.*, 2000a, Yamazaki *et al.*, 2003). On solid media, *SsgA* null mutants display a conditional "white" (non-sporulating) phenotype, as they are able to produce spores on mannitol-containing medium, but not in the presence of glucose (Jiang & Kendrick, 2000, van Wezel *et al.*, 2000a). Although many early developmental (*bld*) mutants are carbon source-dependent (Merrick, 1976, Pope *et al.*, 1996), such dependence is very rare among *whi* mutants and this may reflect the fact that SsgA also controls submerged sporulation by sporogenic vegetative hyphae. The function of SsgA is discussed in detail in the next section.

It is likely that more genes are involved in the control of morphogenesis. For example, non-pelleting mutants were obtained after selection for such a phenotype in continuous cultures (Roth *et al.*, 1985), and previous work identified several spontaneous mutants of *S. griseus* that were affected specifically in submerged sporulation (Kawamoto & Ensign, 1995, Kwak & Kendrick, 1996). Apparently, such mutants are readily obtained, and many have not yet been characterized, strongly suggesting that much is yet to be learned about proteins that control submerged morphogenesis.

3.4. Surface modification of Streptomyces spores

Streptomyces spores formed in submerged cultures are decorated by a pattern of pairwise aligned rods, called the rodlet layer (Claessen *et al.*, 2004). This layer, which apparently forms the same mosaic as that found on aerial spores, renders the surface of spores hydrophobic. Assembly of the rodlet layer involves two classes of proteins, rodlins (Claessen *et al.*, 2002) and chaplins (Claessen *et al.*, 2003, Elliot *et al.*, 2003). The chaplin proteins form the main building blocks of the rodlet layer, by assembling into thin fibrils that are aligned by the rodlin proteins into wild-type rodlets (Petrus & Claessen, 2014). Indeed, without rodlins the chaplin fibrils are randomly deposited on the spore surface. Recent evidence indicates that chaplins self-assemble into an asymmetric fibrillar membrane when confronted with a hydrophobic-hydrophilic interface (Bokhove *et al.*, 2013, Ekkers *et al.*, 2014). The hydrophilic side of this membrane is relatively smooth, while the hydrophobic side has a fibrillar appearance. While such an interface is present when hyphae grow out of the aqueous environment into the hydrophobic air, it is absent in sporogenic hyphae formed in liquid, such as those of *S. griseus* or *S. venezuelae*. This strongly implies that other factors contribute to the assembly process, at least in liquid environments. This is not uncommon for other fibril-forming proteins, including the fungal equivalents of the chaplins, called hydrophobins (Wösten, 2001). Here, the assembly of the SC3 hydrophobin from the filamentous fungus *Schizophyllum commune* is stimulated by schizophyllan, one of the glycans present in the cell wall (Scholtmeijer *et al.*, 2009). Also, SC3 assembly could

be induced when the concentration of the monomers was increased. In fact, this makes it tempting to speculate that the schizophyllan binds to hydrophobin monomers, which locally increases the concentration thereby initiating self-assembly. Notably, the polymer produced by CslA at the hyphal tip could have a similar role, which in particular in liquid environments could be critical (Xu *et al.*, 2008, de Jong *et al.*, 2009b, Chater *et al.*, 2010). However, this awaits further experimental evidence.

4. THE SSGA-LIKE PROTEINS

4.1. SsgA-like proteins and morpho-taxonomy of actinomycetes

SsgA is a small 15 kDa protein that has so far only been found in the streptomycetaceae *Streptomyces* and *Kitasatospora*. Homologues of SsgA - the SsgA-like proteins or SALPs - are found in all of what may be considered as morphologically complex actinomycetes, with a suggestive correlation between the number of SALPs and the number of spores produced per spore chain: species producing single spores (*e.g. Micromonospora, Salinispora*) typically have a single SALP, those producing short spore chains (*e.g. Saccharopolyspora*) typically have two SALPs and those forming spore chains (*Streptomyces*) or sporangia (*Frankia*) have multiple SALPs (Girard *et al.*, 2013, Traag & Wezel, 2008). Members of the SALP family of proteins are typically between 130-145 aa long, with 30-50% aa identity between the different family members. *S. coelicolor* contains seven SALPs (SsgA-G; (Noens *et al.*, 2005), of which SsgA, SsgB and SsgG play a role in septum-site localization. SsgB is the archetypal SALP and functions by recruiting FtsZ to septum sites during the onset of sporulation-specific cell division (see below). The crystal structure of SsgB from *Thermobifida fusca* (Xu *et al.*, 2009) revealed a bell-shaped trimer with - surprisingly - strong similarity to the structure of mitochondrial RNA binding proteins MRP1 and MRP2 (Schumacher *et al.*, 2006) and ssDNA binding protein PBF-2 (Desveaux et al., 2002). Recently, a novel structural homologue of SsgB was identified in the spirochete *Borrelia burgdorferi*, a pathogen that causes lyme borreliosis (Bhattacharjee *et al.*, 2013). The *B. burgdorferi* OspE protein recruits the complement regulator FH onto the bacterial cell wall, which then results in immune evasion (Bhattacharjee *et al.*, 2013). Suggestively, as discussed below SsgB also functions by recruiting a protein, in this case FtsZ to the site of cell division (Willemse *et al.*, 2011).

SsgB is extremely well conserved in streptomycetes, with typically a maximum of one amino acid change between the orthologues, while at the same time the homology between orthologues in different genera is low (around 40% amino acid identity). This unique feature was used as a novel taxonomic analysis of actinomycetes to complement 16S rRNA-based taxonomy (Girard *et al.*, 2013). Phylogenetic analysis of the SsgA and SsgB proteins in streptomycetes showed that on the basis of the conservation of these proteins, streptomycetes fall apart into two subclasses, which are also distinct in terms of liquid-culture morphogenesis. The first class consists of species that produce mycelial clumps but fail to produce submerged spores, which cluster in the NLSp (No Liquid-culture

Sporulation) branch, and the second form the LSp (Liquid-culture Sporulation) branch of the streptomycetes. Strikingly, *Streptomyces* species of the LSp type have an SsgB orthologue with a Thr128, while those of the NLSp type have an SsgB with Gln128. The exception to the rule is *S. avermitilis*, which (as far as we know) does not sporulate in submerged cultures, but contains SsgB variant T128. This apparently correlates with the absence in *S. avermitilis* of SsgG, which is functionally related to SsgB (GPvW, unpublished).

4.2. How does SsgA control hyphal morphogenesis?

SsgA localizes to sites where cell wall remodeling is required and in both vegetative and aerial hyphae, namely at sites for germination, branching and septum formation (Noens *et al.*, 2007). SsgA activates all of these processes, although the precise mechanism is not always clear. In terms of germination, SsgA-overexpressing strains have been shown to form on average around 2.5 germ tubes per spore (against 2.0 germ tubes per spores for the wild-type strain and 1.7 for *ssgA* null mutants), whereby even five or more germ tubes emerge from a single spore (Noens *et al.*, 2007). Secondly, enhanced expression of the protein stimulates branching, whereby many short branches are formed that fail to grow out to normal length (van Wezel *et al.*, 2000a). The best-studied activity of SsgA relates to its ability to activate cell division, and over-expression of SsgA results in a large number of very thick septa produced in vegetative hyphae (van Wezel *et al.*, 2000a). Thus, SsgA activates cell-wall remodeling processes, perhaps via physical modification of the peptidoglycan. The latter is among others suggested by the strongly increased sensitivity of SsgA-overexpressing cells to lysis (GPvW, unpublished).

The effect of SsgA on hyphal morphology is highly pleiotropic, as underlined by two further observations. Firstly, enhanced expression of SsgA does not only stimulate cell division, but enforces pleiotropic changes of the morphology of the hyphae. Hyphae become

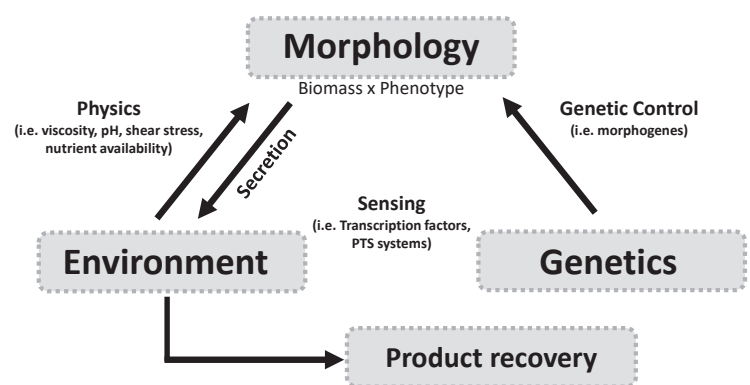

*Figure 3. Streptomyces morphology is determined by environmental and genetic determinants***.** An important factor for the behavior and productivity of streptomycetes in bioreactors is morphology, which is influenced by physical and genetic parameters. In turn, morphology and growth affect the environmental conditions, such as rheology and nutrient composition, and reactor conditions and morphology affect the global gene expression profile.

twice as wide as normal vegetative hyphae (around 1 µm instead of 0.5 µm), giving the appearance of aerial hyphae, and submerged sporulation is observed in *S. coelicolor*, which normally only sporulates in surface-grown cultures (van Wezel *et al.*, 2000a). Secondly, microarray studies revealed that some 1000 genes were more than two-fold deregulated in an *ssgA* null mutant of *S. coelicolor*, and most notably almost all developmental genes (*bld*, *whi*), as well as *divIVA, ftsI*, *chp* and *rdl* for the chaplin and rodlin spore coat proteins, genes for the components of the Sec and Tat secretion systems and many genes involved in DNA segregation and topology (Noens *et al.*, 2007). The remarkable upregulation of these genes indicates a major disturbance in the control of development and secretion.

Finally, SsgA also has a major impact on antibiotic production, although it is likely that this is due to its influence on morphology. Colonies that over-express the SsgA protein fail to produce actinorhodin, while production of prodigionines (Red) is strongly enhanced (van Wezel *et al.*, 2000b). In fact, in batch fermentations, Red production is some 20-50 times enhanced as compared to the wild-type strain (van Wezel *et al.*, 2006). The most logical explanation is that SsgA induces fragmentation and fast growth, which is detrimental for the production of antibiotics that are produced later during growth, such as Act (Gramajo *et al.*, 1993). Red production occurs during vegetative growth (Takano *et al.*, 1992), and (perhaps as a consequence) benefits from fast and fragmented growth (van Wezel *et al.*, 2006).

4.3. SsgA and SsgB control the localization of FtsZ

Like SsgA, SsgB is also required for sporulation (Keijser *et al.*, 2003)2003 and it is part of the cell division complex (divisome) during sporulation-specific cell division. These studies were done in surface-grown cultures, but most likely translate to submerged sporulation. However, this needs to be experimentally validated. During sporulation, SsgB functions by actively recruiting FtsZ, which forms the contractile cell division ring (Bi & Lutkenhaus, 1991), to division sites. SsgB localizes to future division sites prior to FtsZ, and live imaging showed that soon after the appearance of SsgB foci, also FtsZ arrives at these sites, after which they fully colocalize during the entire division process (Willemse *et al.*, 2011). SsgB interacts with FtsZ and activates polymerization of FtsZ protofilaments *in vitro*, resulting in 450 nm long FtsZ filaments(Willemse *et al.*, 2011). Different modes of action have been described for proteins involved in FtsZ filament formation, such as ZipA, which stimulates the formation of filament networks (RayChaudhuri, 1999), or ZapA, which promotes bundling of the filaments (Gueiros-Filho & Losick, 2002, Low *et al.*, 2004). The activity of SsgB is mechanistically most similar to that of ZipA.

The next step in understanding the role of the SALPs in the control of cell division in *Streptomyces* is to find out how SsgB itself is localized. SsgA plays a role in this process during sporulation in solid-grown cultures, and the two proteins transiently interact prior to the start of division. Little is known of how cell division is controlled during vegetative growth. SsgA in fact acts by triggering an aerial-type cell division in vegetative hyphae, leading to cell fission (fragmentation, submerged sporulation); this likely requires an intact divisome, while

vegetative cell division takes place in the absence of canonical cell division proteins like FtsI and FtsW (Mistry *et al.*, 2008, McCormick, 2009)2008, McCormick, 2009. We anticipate that the (size of the nucleoid) may play an important role in spatially determining the sites for division, as control systems should be in place that prevent septum formation over nonsegregated chromosomes, as is the case in all bacterial systems (Wu & Errington, 2012). This idea waits further experimental testing.

5. ENVIRONMENTAL AND REACTOR CONDITIONS

So far, we have mainly focused on the genetic factors influencing morphology of streptomycetes in liquid-grown environments. However, mycelial morphology and development is also strongly influenced by environmental factors and by the reactor set-up (Fig. 3). Such factors include nutrients (Naeimpoor & Mavituna, 2000, Jonsbu *et al.*, 2002), pH (Glazebrook *et al.*, 1992), viscosity (O'Cleirigh *et al.*, 2005), agitation (Belmar-Beiny & Thomas, 1991, Ayazi Shamlou *et al.*, 1994, Cui *et al.*, 1997, Heydarian *et al.*, 1999), dissolved oxygen (DO) levels (Vecht-Lifshitz *et al.*, 1990) and surface tension (Vecht-Lifshitz *et al.*, 1989). Here, we will discuss the environmental factors that affect pellet morphology.

5.1. Culture heterogeneity

Heterogeneity is a common trait in microbial communities, which probably contributes to increased fitness (Smits *et al.*, 2006). However, in industrial settings heterogeneity is an unwanted feature, because it contributes to an unpredictable outcome of the fermentation process. Mycelia growing in flasks or bioreactors are highly heterogeneous in terms of morphology. One cause of heterogeneity is asynchronous initiation of germination and subsequent outgrowth of spores (Hardisson *et al.*, 1978). Analysis of *Streptomyces antibioticus* indicated that approximately 20% of the spores showed no visible signs of germination 5 hr after inducing this process (Hardisson *et al.*, 1978). The asynchrony might result from substances that are released during spore germination, which would inhibit germination of neighboring spores (Grund & Ensign, 1985, Aoki *et al.*, 2011).

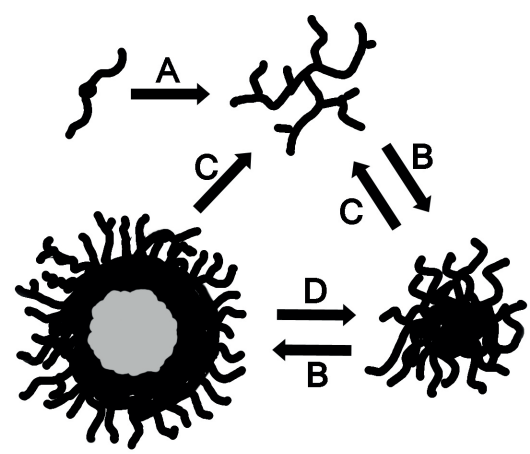

Figure 4. Proposed life cycle of a Streptomyces pellet. Germination (A) leads to the formation of a mycelial network that grows (B) into a clump. Continued growth leads to the formation of dense pellets. Clumps and pellets can also fragment (C) or disintegrate (D; in particular for pellets) to give rise to new mycelia or clumps. The grey area represents the 'dead' core of a large pellet.

Notably, heterogeneity also develops during growth. This heterogeneity is heritable and characterized by the presence of two populations of pellets that differ in size (van Veluw *et al.*, 2012, Petrus & Claessen, 2014). This heterogeneity was also observed when spore germination was synchronized, or when cultures were inoculated with precultured mycelia. Analysis of a range of different streptomycetes indicated similar behavior with two different populations, regardless of strain, culturing conditions or culture age. Interestingly, the average pellet size of the population of small pellets was rather constant throughout growth, and similar between strains (van Veluw *et al.*, 2012). In contrast, the average size of the larger pellets was variable. This indicates that environmental parameters known to influence morphology, such as flask geometry, stirring speed and medium composition in particular affect the population of large pellets (Tough & Prosser, 1996, Celler *et al.*, 2012).

5.2. Nutrients and morphology

The availability and diversity of nutrients strongly affects *Streptomyces* morphogenesis and antibiotic production (Ueda *et al.*, 2000, Bibb, 2005, Sanchez *et al.*, 2010, van Wezel & McDowall, 2011, Gubbens *et al.*, 2012). The frequency of branching of the vegetative hyphae is strongly dependent on the growth conditions, whereby nutrient-rich conditions favor branching, so as to allow acquisition of nutrients in the soil, while under nutrient-depleted conditions branching is reduced, and growth is dictated by tip extension, which favors the formation of so-called “searching hyphae” (Bushell, 1988). Both branching and cross-wall formation reduce hyphal strength (McCormick *et al.*, 1994, Wardell *et al.*, 2002). As discussed above, over-expression of SsgA leads to strongly enhanced cell division in vegetative hyphae, coinciding with fragmentation, which is often seen occurring at the septa.

The critical role of sugar metabolism on morphogenesis is underlined by the fact that mutation of any of a range of different sugar transport systems results in vegetative arrest on surface-grown cultures: in essence, they are *bld* genes (Seo *et al.*, 2012, Rigali *et al.*, 2006, Colson *et al.*, 2008, Chater *et al.*, 2010). In an attempt to create a more reproducible morphology, *Streptomyces akiyoshiensis* was grown on various carbon sources (Glazebrook *et al.*, 1992). The largest pellets were obtained by growth on lactose (over 600 µm), while growth on glucose resulted in the smallest pellets (less than 200 µm). Growth on either of these carbon sources resulted in some five-fold lower biomass as compared to growth on starch, which highlights the complex link between growth and morphology. The optimal carbon source for production varies between species. Mannitol was the best carbon source for the geosmin production by *Streptomyces halstedii* (Schrader & Blevins, 2001), a combination of fructose and mannose was best for rapamycin production by *Streptomyces hygroscopicus* (Kojima *et al.*, 1995) and glycerol was needed for good production of clavulanic acid by *Streptomyces clavuligerus* (Romero *et al.*, 1984). Media composition also had a major impact on the hyphal stability of *S. clavuligerus*, with cells cultured in media containing glutamate, glycerol and ammonia being more shear resistant as compared to

cells grown in different media (Roubos *et al.*, 2001).

5.3. Fragmentation

The mechanical forces encountered in the submerged environment leads to fragmentation of the pellets, which occurs on a stochastic basis and counter-balances the size increase of a growing pellet (Fig. 4). The mechanical forces in a reactor originate from the combination of agitation, gas hold-up and the rheology of the culture fluid (van't Riet & Tramper, 1991, Olmos *et al.*, 2013). Especially the water swirls, or eddies, which arise under turbulent flow velocities, stretch hyphae in opposite direction with fragmentation as a logical result (Ayazi Shamlou *et al.*, 1994, Heydarian *et al.*, 2000). For filamentous microorganisms the importance of understanding the relationship between the mechanical forces and growth is non-trivial because the mycelia themselves influence the rheology of the culture broth. Entanglement of the mycelia can dramatically increase the viscosity, which affects the shear stress and can reduce the transfer of heat and nutrients (Metz & Kossen, 1977). This process occurs when the biomass grows as a mat, but it has also been observed when pellets are the predominant morphology (Mehmood *et al.*, 2010).

Fragmentation can occur as small hyphal fragments detach from the periphery of the pellet, or via disintegration of pellets into multiple parts (Cui *et al.*, 1997, Kelly *et al.*, 2006). For an exposed hypha to break, the force applied must be greater than its tensile strength. Studies using a blender showed that the fungus *Penicillium notatum* is about four times more likely to break in the middle of a hypha than at the septum (Savage & Vander Brook, 1946). A correlation exists between the likelihood of hyphal breakage and the presence of vacuoles, which are hypothesized to cause localized weak spots (Paul *et al.*, 1994, Papagianni *et al.*, 1999). However, in *Streptomyces* vacuoles are rarely seen in vegetative mycelia (Wildermuth, 1970). Notably, stimulating septation via overproduction of SsgA increases fragmentation of streptomycetes, although these septa more resemble sporulation-type septa (see above). Conversely, less branching in *Saccharopolyspora erythraea* decreased fragmentation (Wardell *et al.*, 2002). Because crosswalls are often found near branch points these results suggest that they in fact represent local weak spots along the hyphae.

Disintegration of pre-existing pellets is the second mechanism by which new pellets can be established. The combined strength of the forces that keeps a pellet together is much larger than the tensile strength of individual hyphae. It seems therefore that this process can only occur when the interactions between hyphae are diminished. This can be caused by a changing environment, for example a change in pH (Glazebrook *et al.*, 1992), but also due to a lack of nutrients, oxygen or the buildup of toxins that induce lysis in the center of a pellet (Papagianni, 2004, Hille *et al.*, 2005). Notably, the susceptibility to fragmentation changes over the course of growth. Generally the pellet size seems to increase during exponential growth, but decreases when entering end-log or stationary phase (Reichl *et al.*, 1992, van Veluw *et al.*, 2012). Susceptibility of *Sacch. erythraea* to fragmentation is almost twice as

high in the stationary phase compared to exponential growing cells (Stocks & Thomas, 2001). This probably relates to programmed cell death in the center of a pellet (Manteca *et al.*, 2010, Rioseras *et al.*, 2014), consistent with the observation that in filamentous fungi pellets become hollow in the center, severely reducing stability (El-Enshasy *et al.*, 1999). This hollowing was observed in cross-sections of a pellet of *Streptomyces tendae* with a diameter of 120 µm (Braun & Vecht-Lifshitz, 1991), but it is unclear in how far this occurs in pellets of other streptomycetes.

5.4. Relationship between agitation, oxygenation, morphology and productivity

As a rule of thumb, and expectedly, more vigorous stirring leads to smaller pellet size (Ohta *et al.*, 1995, Tough & Prosser, 1996, Bellgardt, 1998). However, the morphology of *Streptomyces fradiae* showed an inverse correlation, with low or medium shear stress favoring pelleted growth, while high shear stress caused mycelia of *S. fradiae* to fragment. Interestingly, the pellets grown under low shear stress continued to increase in size, while under medium shear a decrease in size was observed after the exponential growth phase (Tamura *et al.*, 1997, Heydarian *et al.*, 1999). Because faster mixing also increases mass transfer, it typically increases growth rate and biomass accumulation (Heydarian *et al.*, 1999) and can therefore also have a major impact on the production of secondary metabolites (Heydarian *et al.*, 1999, Rosa *et al.*, 2005, Cerri & Badino, 2012). Most studies show an optimum stirring speed for production of the metabolite of interest, where initially the production increases with stirrer speed and then decreases again at very high speeds (Large *et al.*, 1998, Heydarian *et al.*, 1999, Roubos *et al.*, 2002, Mehmood *et al.*, 2010). This decrease in yield is most likely the result of cell damage caused by high shear conditions, as illustrated by the comparison of growth and lipase production of *Streptomyces fradiae* in an airlift with a stirred vessel. Leakage of lipase into culture fluid, indicative of cell damage, was exclusively observed in stirred vessels (Ohta *et al.*, 1995).

Because oxygen transfer is closely linked with agitation, the effects of the one from the other need to be distinguished (Bartholomew *et al.*, 1950, Shioya *et al.*, 1999, Rocha-Valadez *et al.*, 2007, Mehmood *et al.*, 2010). Due to its low solubility and the high energetic cost of antibiotic production, dissolved oxygen (DO) levels are often rate-limiting and oxygen depleted in the center of a pellet (Hille *et al.*, 2005, Wucherpfennig *et al.*, 2010, Olmos *et al.*, 2013). Fermentations in the presence of a saturated DO levels increased the production of cephamycin by *S. clavuligerus* more than two-fold (Yegneswaran *et al.*, 1991), increasing the DO levels using perfluorocarbon increased the production of actinorhodin by *S. coelicolor* about five-fold (Elibol & Mavituna, 1999), and extra oxygen supplied by producing haemoglobin in *Sacch. erythraea* increased the production of erythromycin (Brünker *et al.*, 1998). These results clearly demonstrate the critical role that oxygen has on productivity.

DO levels also affect pellet morphology. Vecht-Lifshitz observed a proportional decrease of pellet size when DO levels were lower (Vecht-Lifshitz *et al.*, 1990). From a biological perspective, regulation of the morphology by oxygen is may be needed to balance

the physical protection offered by the mycelium with the ability to produce secondary metabolites, which offer chemical protection. Biofilms of filamentous fungi are known to contain channels through which liquid and nutrients can flow towards the internal parts of these structures (Wimpenny *et al.*, 2000). They have recently also been identified in bacterial biofilms (Wilking *et al.*, 2013). It will therefore be interesting to see if Streptomyces pellets also possess these 'artery-like' structures.

6. MORPHOLOGY AND ANTIBIOTIC PRODUCTION

6.1. Impact of morphology on antibiotic production

The formation of pellets is a major drawback for industrial applications, as pellets represent a slow-growing morphology (Liu *et al.*, 2013b). Therefore, many efforts have focused on obtaining a more open or dispersed morphology. Addition of charged polymers like junlon or carbopol has been applied as a means to obtain a dispersed morphology (Hobbs *et al.*, 1989, Harriott & Bourret, 2003). These compounds probably interfere with the electrostatic properties of the cell wall, which prevents initial aggregation (Wargenau *et al.*, 2013). Lowering the pH also influences the surface charge of the cell wall, thereby yielding a similar effect (Braun & Vecht-Lifshitz, 1991, Wargenau *et al.*, 2011). Increasing the viscosity of the broth also induces more fragmented growth because it increases shear stress, while reducing pellet-pellet collisions, which could also lead to their aggregation (O'Cleirigh *et al.*, 2005). However, higher viscosity demands more energy input to obtain sufficient stirring, which increases production costs. For some products, mostly enzymes but also some antibiotics, dispersed growth can increase yields (van Wezel *et al.*, 2006), while for the production of the majority of the antibiotics pelleted growth is preferred (Pickup & Bushell, 1995, Martin & Bushell, 1996). The latter often leads to a situation whereby morphology is suboptimal as a compromise to maintain relatively high antibiotic yields (Braun & Vecht-Lifshitz, 1991, Martin & Bushell, 1996).

In surface-grown cultures of *Streptomyces* there is a clear link between the production of antibiotics and the developmental cycle (Bibb, 2005, van Wezel & McDowall, 2011). Well-established is the growth-phase dependence of production, where the onset of synthesis usually occurs when growth stalls (Bibb, 2005, van Wezel & McDowall, 2011). However, once activated, there apparently is no additional control; placing *redD*, the pathway-specific activator gene for production of prodigionines in *S. coelicolor*, under the control of the promoter of the gene for the global nitrogen regulator (*glnR*) or a sporulation-specific sigma factor (*sigF*), ensures that production of the antibiotic is controlled by nitrogen or produced in aerial hyphae, respectively (van Wezel *et al.*, 2000b). This implies that at least for some antibiotics, there are no metabolic limitations as to when or where they are produced, and therefore that restrictions on production imposed by growth and morphology-related control mechanisms can be overcome.

In submerged cultures, the linkage between mycelial morphology and production is

exemplified by avermectin production by *S. avermitilis*, which is highest when small dense pellets were formed (Yin *et al.*, 2008), and by the fact that a high-producing variant of *Streptomyces noursei* formed dense pellets, while the wild-type strain formed loose clumps (Jonsbu *et al.*, 2002). Pellets were a prerequisite for the production of a hybrid antibiotic by *S. lividans* (Sarrà *et al.*, 1999), and filtering of a culture of *Sacch. erythraea* revealed that small pellets with a diameter below 88 µm were unable to produce erythromycin (Martin & Bushell, 1996). As discussed in section 4, fragmentation of mycelia by enhanced expression of SsgA has a major effect on antibiotic production by *S. coelicolor*, with a block of Act production, while Red is massively upregulated, again underlining the major influence of morphology on production.

6.2. Programmed cell death and antibiotic production

Mycelial development in liquid-grown cultures may be more similar to that in surface-grown cultures than initially thought. Life/dead staining showed that the early mycelium is compartmentalized in *S. coelicolor*, similar to the initial mycelium in surface-grown cultures. A fraction of this early mycelium undergoes a process that strongly resembles programmed cell death (PCD), with two rounds of PCD occurring during the *Streptomyces* life cycle (Manteca *et al.*, 2011). After spore germination, a compartmentalized mycelium grows and then undergoes a first round of PCD, formed during early vegetative growth (Manteca *et al.*, 2011). The second round of PCD starts during the onset of development, which corresponds to the transition phase between exponential growth and stationary phase in liquid-grown cultures (Granozzi *et al.*, 1990, Manteca *et al.*, 2005). During PCD, specific nucleases are activated that are involved in degradation of chromosomal DNA (Granozzi *et al.*, 1990, Fernández & Sánchez, 2002, Manteca *et al.*, 2006a, Rioseras *et al.*, 2014). Following this PCD event, secondary mycelium emerges from the center of a pellet (Park *et al.*, 1997, Manteca *et al.*, 2008). The secondary mycelium was found to be distinct from the initial mycelia by being multinucleated, which is again similar to solid cultures (Yagüe *et al.*, 2014).

The idea that *Streptomyces* mycelia also undergo a developmental cycle in submerged cultures was suggested by the first microarray experiments done on *S. coelicolor*, which showed that the transcription of many developmental genes is switched on during the transition phase, which is the phase when growth slows down in submerged cultures (Huang *et al.*, 2001). More recently, this was also shown by proteomic comparison of young and older mycelia, with early mycelium enriched in primary metabolic enzymes while proteins involved in secondary metabolism and those associated with development and sporulation were enriched in the multinucleated secondary mycelium (Manteca *et al.*, 2010, Yagüe *et al.*, 2014). Interestingly, many developmental genes are actively transcribed in the secondary mycelium, including several *bld* genes (*i.e. bldB, bldC, bldM, bldN*), but also those involved in formation of the rodlet layer (*chpC, chpD, chpE,* and *chpH*; (Claessen *et al.*, 2004, Manteca *et al.*, 2007). The antibiotics undecylprodigiosin and actinorhodin were exclusively produced by the secondary mycelium in both solid- and liquid-grown cultures (Manteca *et*

al., 2008). Various sporulation-specific genes are upregulated in older cultures (Huang *et al.*, 2001, Yagüe *et al.*, 2014). These data strongly suggest that liquid-grown mycelia also undergo differentiation. In fact, a small fraction of the mycelium appeared to be initiating a sporulation-like process, which is rarely seen for *S. coelicolor* (Rioseras *et al.*, 2014)2014. Indeed, overexpression of *whiG* or *ssgA* induced a certain degree of submerged sporulation in submerged cultures of *S. coelicolor* (Chater *et al.*, 1989, van Wezel *et al.*, 2000a).

A direct link between PCD and antibiotic production was revealed when it was established that cell wall-derived N-acetylglucosamine (GlcNAc) acts as an important signaling molecule for the onset of development and antibiotic production in *Streptomyces* (Rigali *et al.*, 2006, Rigali *et al.*, 2008). In the competitive soil habitat, postponing sporulation is important if sufficient nutrients are available, while during starvation sporulation and ensuing dispersal are essential for survival. In nature, GlcNAc is obtained from hydrolysis of the abundant natural polymer chitin by the chitinolytic system. For bacteria GlcNAc is a favorable C- and N-source, and a major constituent of cell wall peptidoglycan. Some 13 chitinases and chitosanases have been identified in *S. coelicolor* (Delic *et al.*, 1992, Saito *et al.*, 2003a, Colson *et al.*, 2007), and GlcNAc and glutamate are preferred over glucose in fermentations of *S. coelicolo*r (van Wezel *et al.*, 2009).

Under poor nutritional conditions such as on minimal media, supplementing

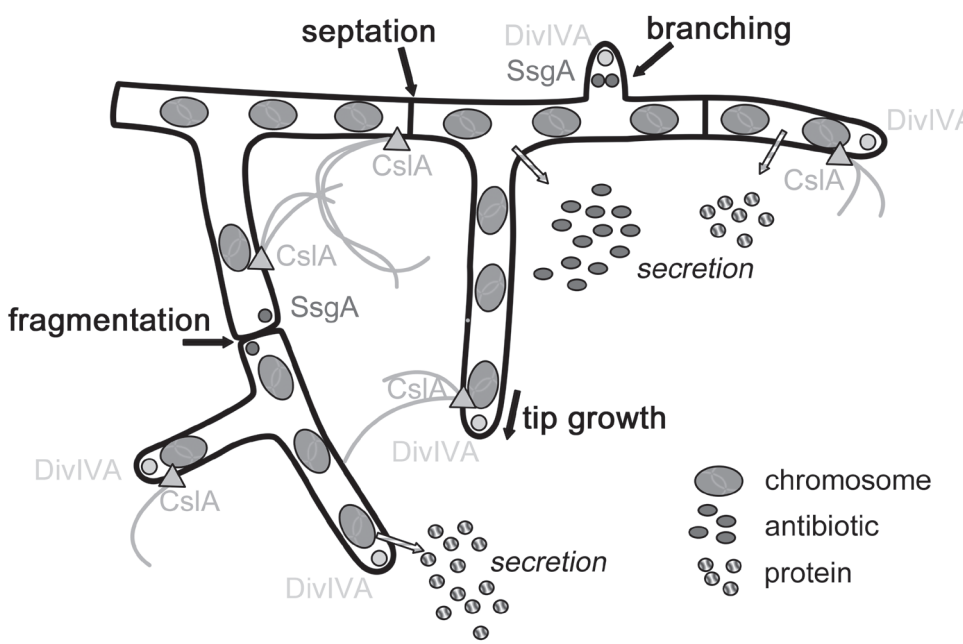

*Figure 5. Control of growth of Streptomyces hyphae during fermentation***.** Branching frequency, tip growth rate and fragmentation and aggregation are determined by the activity of morphoproteins and by growth conditions (pH, feedstock, stress). Morphology has a major impact on production and secretion. Some enzymes are secreted near the tips of the hyphae, as was shown recently for Tat substrates (Willemse *et al.*, 2012), but it is unclear if this is also true for Sec substrates. Where antibiotics and other natural products are secreted is unknown. The drawn secretion sites are hypothetical.

GlcNAc accelerates both the onset of development and antibiotic production, suggesting that under these conditions GlcNAc signals nutrient stress, resulting in accelerated development. Conversely, in rich media, higher concentrations of GlcNAc block development and antibiotic production, thus resembling conditions that promote vegetative growth (Rigali *et al.*, 2008). These growth conditions may thus resemble conditions of *feast* or *famine* in the natural environment, whereby GlcNAc would be derived from chitin in nutrient-rich soil (feast), or from the *Streptomyces* cell wall during PCD (famine), respectively. The secret appears to lie in the nature of the sugar transporters. Monomeric GlcNAc enters the cell via the NagE2 permease (Nothaft *et al.*, 2010), which is part of the PEP-dependent phosphotransferase system (PTS; (Postma *et al.*, 1993, Titgemeyer *et al.*, 1995), while chitobiose (dimeric GlcNAc), which is the subunit of chitin, enters via the ABC transporters DasABC or NgcEFG (Schlösser *et al.*, 1999, Nothaft *et al.*, 2010, Saito *et al.*, 2007, Colson *et al.*, 2008). Subsequently, internalized GlcNAc is converted by the enzymes NagA and NagB to glucosamine-6-phosphate (GlcN-6-P; (Świątek *et al.*, 2012a), a central metabolite that can then enter glycolysis (as fructose-6P) or the pathway towards peptidoglycan synthesis.

GlcNAc-derived GlcN-6-P acts as an allosteric effector of the GntR-family regulator DasR (Rigali *et al.*, 2006), a highly global regulator that controls the GlcNAc regulon (Rigali *et al.*, 2006, Świątek *et al.*, 2012a, Nazari *et al.*, 2013), but also the production of antibiotics (Rigali *et al.*, 2008) and siderophores (Craig *et al.*, 2012). GlcNAc-dependent nutritional signalling is most likely mediated through changes in the intracellular level of GlcN-6-P, which binds as a ligand to the GntR-family regulator DasR, leading to derepression of DasR-mediated control of antibiotic production (Rigali *et al.*, 2008). Recent work showed that addition of phosphorylated sugars to growth media under phosphate limitation delays the occurrence of the second round of PCD and results in vegetative arrest, also preventing antibiotic production (Tenconi *et al.*, 2012).

The pleiotropic DasR control network is well conserved in actinomycetes, and can be manipulated to activate antibiotic production. Addition of GlcNAc to cultures of streptomycetes grown on nutrient-depleted media accelerates development and enhanced antibiotic production by many streptomycetes (Rigali *et al.*, 2008). This concept can be applied to activate cryptic antibiotic gene clusters, which are not or poorly expressed under normal growth conditions (Baltz, 2008). Indeed, GlcNAc induces expression of the *cpk* gene cluster for the cryptic polyketide Cpk (Rigali *et al.*, 2008, Gottelt *et al.*, 2010). Thus, understanding of the correlation between morphogenesis and antibiotic production may be employed for drug discovery approaches.

7. OUTLOOK: THE CORRELATION BETWEEN MORPHOLOGY AND PRODUCTION

As the producers of a wide range of medically important natural products, streptomycetes are very important microorganisms for the pharmaceutical industry (Hopwood, 2007, Baltz, 2008, Olano *et al.*, 2009). Moreover, the streptomycetes also produce a plethora of

extracellular enzymes that allow them to degrade almost any naturally occurring polymer, such as cellulose, mannan, chitin, xylan, starch, glycan and agar (Vrancken & Anne, 2009, Anné *et al.*, 2014). However, as discussed in this review, streptomycetes grow as complex mycelia, which forms a major bottleneck for industrial fermentations, as mycelial growth is associated with slow growth, culture heterogeneity and high viscosity of the fermentation broth. These factors typically have an adverse effect on the yield. Heterologous expression in a host with better growth properties, such as *Bacillus* or yeast, is not an option for natural products with their very complex biosynthetic machinery and dependence on metabolic pathways, and also many actinomycete-derived enzymes require actinomycete-specific machinery for proper folding, modification and/or secretion, and can therefore not be produced in a bioactive form in other hosts.

In terms of process engineering, the focus typically lies on changes in reactor or media conditions, by for instance changing stirring speed, pH, or nutrients, which have a pronounced effect on mycelial morphology. The advantage of this approach is that production yields for a particular compound can be improved fairly quickly. The disadvantage of this approach, however, is that results are difficult to translate to other streptomycetes, which often respond differently to changed conditions. These different responses might relate to for instance changes in cell wall composition, thereby influencing processes such as fragmentation and aggregation. In this respect, better understanding of the genetic factors involved in mycelial growth and architecture are a prerequisite to find general leads to improve streptomycetes in industry. However, while detailed insights into the molecular determinants of mycelial growth are critical for *Streptomyces* strain improvement, it is good to realize that the eventual productivity is determined by many different process technological and genetic parameters, whereby the effect of those parameters on productivity also largely depends on the product to be produced.

To predict the effect of culturing conditions as well as genetic factors on morphology, many different *in silico* models have been designed in the past (Nielsen & Villadsen, 1992, Yang *et al.*, 1992, Meyerhoff *et al.*, 1995, Tough & Prosser, 1996, Liu *et al.*, 2005). The older models largely focused on the influence of environmental factors on morphology, while genetics-based modeling had not been attempted (Kossen, 2000). Two new models of *Streptomyces* growth have been developed recently, which should lead to new impetus for modeling of *Streptomyces* growth and production (Celler *et al.*, 2012, Nieminen *et al.*, 2013). Taking advantage of the incredible increase in computing power since the design of previous models, a three-dimensional *in silico* model was developed that allows visualization of growth of mycelial pellets with distinct morphologies (Celler *et al.*, 2012). As parameters, this model includes among others oxygen diffusion, hyphal growth, branching, fragmentation, cross-wall formation as well as a novel collision detection algorithm. The model was designed with industrial application in mind, allowing the user to change both physical and genetic parameters and see what the predicted effect is on pellet growth and yield. However, for such an approach to function as say an *in silico* test system for the fermentation industry,

better insight in the genetic parameters that control morphogenesis is required, as well as an iterative process of modeling and experimentation.

In recent years, progress has been made in our understanding of the factors that govern mycelial growth, such as DivIVA, Scy and CslA that coordinate tip growth, and the SsgA-like proteins that control hyphal morphology and cell division (Fig. 5). Better understanding of the genetic parameters that control growth should allow us to better control the morphology of the mycelia in the fermentation broth. For example, fragmented growth of streptomycetes can be achieved by overexpression of SsgA, a property that is applied successfully in the industrial domain (van Wezel *et al.*, 2006). For rational design of streptomycetes as production hosts we will also need to understand how morphology affects yield. Live imaging showed that the twin arginine translocation (Tat) secretion system localizes highly dynamically and directly behind the tip complex (Celler *et al.*, 2012, Willemse *et al.*, 2012), which suggested that fragmented growth and therefore increased number of apical sites, should favor secretion of Tat-dependent proteins. Indeed, secretion of the Tat substrate tyrosinase is strongly enhanced in fragmenting strains of *S. coelicolor and S. lividans* (van Wezel *et al.*, 2006). The majority of the genetic studies have focused on the micromorphology, while little is known of how these proteins eventually influence macromorphology, such as the formation of clumps or pellets. Conversely, industrial strain engineering has led to mutant strains with often very good reactor properties, but with the many mutations that have occurred during the strain improvement programs, it is hard to identify the changes that may be exploited for rational strain engineering. One important approach that has become feasible in this era of genomics and next-generation sequencing is comparing the genome sequences and the global expression profiles of several generations of one production strain. This should allow identification of genes that may form novel targets for morphological engineering. Analysis of the mutations will provide valuable biological information that might be widely applicable to actinomycete production strains. In this way the historically used black-box approach can be replaced by rational design of future production strains.

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