Controlling growth and morphogenesis of the industrial enzyme producer Streptomyces lividans

Mangiameli, G.

Chapter 2

Host-vector optimization in *Streptomyces lividans* for heterologous protein production

Giulia Mangiameli and Erik Vijgenboom

Manuscript in preparation

ABSTRACT

The growing demand for sustainable production of goods has led to an increased need for enzymes and production hosts in the last decades. Bacteria are usually the preferred hosts because of their easy genetic manipulation and fast growth. Among them, *Streptomyces* is recognized as a highly attractive enzyme production host with several advantages over other bacterial hosts but also with some constraints that require improvement. In this review, the efforts made in the last years to remove the disadvantages related to its mycelial growth and to widen the selection of expression vectors and consequently improve *Streptomyces* for heterologous protein production, will be presented.

INTRODUCTION

Streptomyces spp. belong to the family of *Actinomycetaceae* and are Gram positive, soil dwelling bacteria with a filamentous structure and a complex morphological development which has a strong resemblance with that of filamentous fungi. They are primarily known as important producers of secondary metabolites including antibacterial, anticancer, immunosuppressive, antihelmintic and antifungal agents (Hopwood 2007). In fact, 60% of the currently used antibiotics are produced by this organism. In addition, *Streptomyces* is a producer of commercially valuable proteins naturally secreted to adapt to a changing environment and to different nutritional sources (Chater et al. 2010). In particular, it produces enzymes for the degradation of many organic polymers such as cellulose, chitin and lignin, which are very much needed for the production of second generation biofuels (Jing 2010; Amore et al. 2012; Noda et al. 2013).

The application of recombinant proteins in industrial and pharmaceutical processes has grown steadily in the last decades and has become an indispensable part of the manufacturing of many products. While in the past these proteins were isolated from classical sources like plants and animals, at present they are mainly expressed in heterologous hosts, which allows a faster and safer production with improved quality and stability. More than 200 peptides and recombinant proteins have been approved so far by the FDA and they have a large variety of applications, such as in the production or modification of biofuels, textiles, leather, paper, detergents, polymers and plastics, human and animal health, medicine, diagnostics, food and nutrition. The total market of industrial enzymes, such as laccases, amylases, cellulases and proteases, was estimated in 2012 at € 2,8 billion p.a. (Novozyme report 2012).

For the production of industrial relevant proteins, yeasts and filamentous fungi are used for more than 50% of the proteins while bacteria for 30% and animals and plants are preferred in the remaining cases. Bacterial hosts include *Escherichia coli*, the most used because of its fast growth and high level of protein expression, *Bacillus* species such as *Bacillus subtilis* and *Bacillus licheniformis* and the less

frequently used *Ralstonia eutropha* and *Pseudomonas fluorescens* (Demain and Vaishnav 2009). Nevertheless, none of these systems proved to be an optimal and universal production platform. Within academic and industrial research, the design of alternative and efficient hosts continues to be a theme of interest.

Streptomyces and in particular *Streptomyces lividans* have been successfully tested for the production of proteins including human therapeutics and industrially relevant enzymes (Vrancken and Anné 2009; Anné et al. 2012). The highest production yields are assessed to be over 500 mg/L, including proteins with low or zero expression in other traditional bacterial systems (Sianidis et al. 2006). The latter makes *Streptomyces* a valuable if not the only alternative. Despite the encouraging perspectives, there are several constraints against the utilization of this organism on a larger industrial scale. From a host point of view, the main bottleneck is its mycelial growth, which results in rather dense clumps of mycelium in liquid cultures. This morphology strongly influences the overall efficiency of the fermentation, and the secretion and integrity of the desired final product. Furthermore, the very limited choice of stable vectors with strong promoters for expression of heterologous proteins is often a drawback for the selection of *Streptomyces* as host.

The classical method for strain improvement was represented in the past by random mutagenesis. For example, cycles of UV mutagenesis and screening led to remarkable yield improvements in the case of the industrial penicillin producer *Penicillium chrysogenum*, with an improvement of 100,000 fold compared to the original Fleming strain (Rokem et al. 2007). However, the labor intensity and the accumulation of unknown or unwanted mutations called for a different approach. The improved genetic tools for targeted mutation and the overall increase in understanding the morphology and physiology of bacterial and fungal production platforms contributed to the development of a rational genetic approach.

Although directed mutagenesis can be applied to improve morphology to increase the protein secretion capacity and/or modify metabolic pathways, at least one more issue needs to be resolved. To obtain high yields of heterologous proteins, optimized expression vectors are required that combine strong transcriptional and

translational elements with good secretion signals. This review will focus on the recent developments in the application of *Streptomyces* as a platform for heterologous protein production from the perspective of host-vector optimization.

HOST OPTIMIZATION

From the host perspective, optimization can take place at different levels. At the morphology level, the best phenotype for the production of the desired product can be selected through directed genetics. Improving secretion together with controlling proteolysis are also efficient tools to increase the total yield of protein produced. Finally, the control of fermentation parameters and medium composition can further boost protein production.

Morphology

Mycelial growth

The mycelial behavior of *Streptomyces* presents a serious challenge for the wider use in fermentation and protein production.

In the soil, *Streptomyces* develops from a spore to a dense branched network of vegetative mycelium, a syncytial network in which cell division is not essential and cross walls are occasionally observed. In contrast to other bacteria which grow through binary fission, *Streptomyces* shows an apical polarized growth, with extension and cell wall synthesis at the hyphal tips. The hyphal tip is a hub for the recruitment of proteins involved in growth and has been recently renamed as TIP Organizing Center (TIPOC) (Holmes et al. 2013). The molecular assembler Scy acts as a protein scaffold by recruiting, among others, DivIVA, which directs apical growth (Flärdh et al. 2012). DivIVA has a preference for negatively curved membranes (Lenarcic et al. 2009) and accumulates in multiprotein foci called polarisomes at the tips (Hempel et al. 2012). It is active through a splitting mechanisms (Richards et al. 2012), in which an existing polarisome breaks off, leaving behind a smaller aggregate that marks the site for branching along the hyphae. The disassembly of the foci seems to

be dependent on a phosphorylation mechanism via the protein kinase AfsK (Hempel et al. 2012). These studies show that TIPOC is not only required for apical growth but also establishes the branching points including new tips in the hyphae.

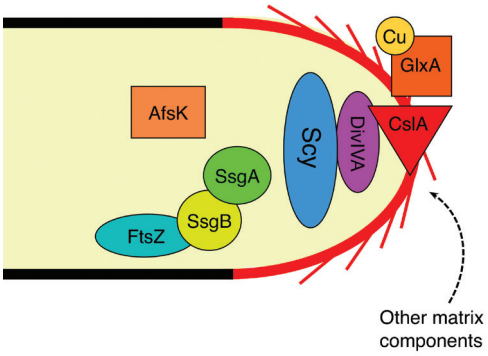

Figure 1. A schematic summary of proteins present in the tip that are required for tip growth, septum formation, aerial growth and attachment/penetration of solids such as agar.
The Tip Organizing Center TIPOC is made by the protein scaffold Scy and the polar growth determinant DivIVA, whose regulation is modulated by AfsK. CslA interacts with DivIVA at the very front of the tip and is responsible for the synthesis of a chitin-like glycan, together with its functional patner GlxA. The glycan is responsible of aerial development and attachment to solid substrates. Other matrix components such as chaplins, rodlins and SapB take part in the formation of the matrix during development and erection of the aerial mycelium. The proteins belonging to the cell division machinery, SsgA, SsgB and FtsZ, are also shown in the picture, although their interaction with the TIPOC remains to be elucidated.

Following an external signal such as nutrient depletion, the vegetative mycelium is broken down in concert with the erection of new hyphae, the so-called aerial mycelium. During this process, an important role is played by proteins which lower the surface tension at the air-water interface: the lantibiotic-like peptide SapB (de Jong et al. 2012), the rodlin proteins RdlA and RdlB (Claessen et al. 2002), together with the eight chaplins ChpA-H (Claessen et al. 2003; Elliot et al. 2003). They coat the hyphae providing a hydrophobic sheat known as the rodlin layer that is essential for aerial growth (Claessen et al. 2004). Recently, the extracellular matrix was also shown to be essential for attachment to solid substrates. A chitin-like extracellular polysaccharide synthesized by the concerted action of the glycosyl transferase CslA and the radical copper oxidase GlxA, both present at the hyphal tips, is required for attachment to and growth into substances as agar (Chapter 3).

After the erection of the aerial mycelium, multiple processes such as septation and chromosome segregation occur, giving rise to spore formation. The family of SsgA-like proteins (SALPs) plays an important role in the process of cell division prior to sporulation (Noens et al. 2005; Traag and van Wezel 2008), including the recruitment of FtsZ and the formation of the cell divisome (Willemse et al. 2011). SsgA in particular seems to have a central role in septum formation (van Wezel et al. 2000a; van Wezel et al. 2006).

In submerged cultures, many Streptomycetes grow vegetatively and only a small number had been recognised as forming submerged spores, with *Streptomyces griseus* and *Streptomyces venezuelae* as well known examples (Kendrick and Ensign 1983; Glazebrook et al. 1990). However, a recent investigation showed that morphological development in submerged culture is much more common than originally anticipated, with half of a collection of randomly selected streptomycetes producing submerged spores (Girard et al. 2013). A specific signature consisting of six amino acids in the SsgA protein allowed prediction of the ability to produce submerged spores, thus dividing the phylogenetic tree of streptomycetes into an LSp (liquid sporulation) branch and an NSLp (no liquid sporulation) branch. The signature also correlates to a single amino acid residue in SsgB, with Thr128 corresponding to the LSp phenotype, and Gln128 to NLSp phenotype.

The mycelia of streptomycetes form aggregates of various sizes that can be categorized according to their diameter into pellets (950 μm), clumps (600 μm), branching and not branching hyphae (Pamboukian et al. 2002) (Fig 2A). No differentiation in liquid cultures was reported until two different growth phases were detected in *S. coelicolor* submerged cultures (Manteca et al. 2008). A first phase starts with the germination of the spores, followed by radial growth of the pellet and growth arrest due to cell death in the inner part, after which a second phase starts with new growth from the inside and ends when the maximum diameter of the pellet is reached. A link between development and metabolite production was also demonstrated, with exclusive production of the antibiotics undecylprodigiosin and actinorhodin during the second growth phase. The exact process behind pellet aggregation is still

largely obscure. Two mechanisms have been proposed: a coagulating one, with the agglomeration of multiple germinating spores, and a non-coagulating one, with the independent germination of separate single spores (Metz and Kossen 1977). Pellets can also arise from hyphal aggregates and fragments of broken down mycelium (Whitaker 1992).

Recently, two populations of pellets differing in size have been described in *Streptomyces* liquid cultures (van Veluw et al. 2012), in line with the heterogeneity observed in other microbial strains. The large size population is dependent on the strain and parameters such as medium composition and age of the culture, while the small size population is strain and medium independent and remains constant through time. Moreover, the deletion of the chaplins and CslA affects the size of the larger pellets, demonstrating the involvement of the cell surface proteins and matrix components in the aggregation process. The smaller pellets are not affected by these deletions and therefore seem to be an intrinsic property of *Streptomyces* growth, independent of the extracellular matrix components.

In other microorganisms, different substances have been described to be involved in cell aggregation. Surface polysaccharides are involved in spores aggregation of *Phanerochaete chrysosporium* in liquid cultures (Gerin et al. 1993). They are also implicated in biofilm formation, cell aggregation and pathogenicity in various bacteria (Zogaj et al. 2003; White et al. 2003; Latgé 2007; Saldaña et al. 2009; Lenardon et al. 2010). Hyaluronic acid is an example of a polysaccharide responsible for biofilm formation and virulence as shown in *Streptococcus pyogenes* (Cho and Caparon 2005), while extracellular DNA is a main component of the extracellular matrix in *Pseudomonas aeruginosa* (Whitchurch et al. 2002). Other cell aggregation processes require specific molecular recognition mediated through outer membrane proteins like shown for the TraA protein of Myxobacteria (reviewed in Wall 2013).

In *Streptomyces*, pellet aggregation seems to be affected by different factors. An amine oxidase (HyaS) associated to the vegetative mycelium has been identified as an enzyme required for aggregation in liquid cultures (Koebsch et al. 2009). Deletion of hyaS led to fluffy clumps with protruding hyphae (Fig 2B). Moreover,

overexpressing SsgA led to a highly fragmenting phenotype in liquid cultures (Fig 2C). The independent deletion of the synthase and the oxidase responsible for the synthesis of a chitin-like polysaccharide associated to the cell wall resulted in the formation of nonpelleting mycelium in various liquid media (Chapter 3 and Fig 2D). Other studies related *Streptomyces* pelletting behavior to biofilm formation, with a crucial role for substances such as DNA, hyaluronic acid and calcium (Kim and Kim 2004).

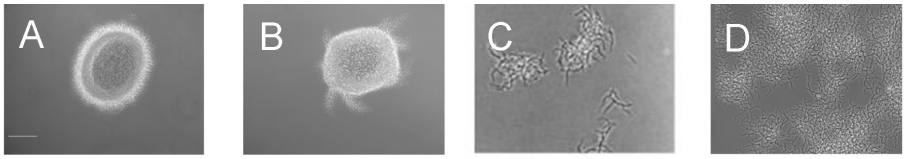

Figure 2. Different morphologies of *S. lividans* 1326 in liquid cultures: pellets (A), pellets with branching hyphae (B), fragmenting mycelium (C), open mycelium (D). Scale bar: 100 μm.

The mycelial nature of these organisms strongly affects fermentation. On the one hand, large pellets suffer from transfer limitation of oxygen and nutrients as they are made of distinct layers, with actively growing hyphae in the outer part and progressively less active cells towards the center (Celler et al. 2012). On the other hand, a dispersed mycelium causes high viscosity of the medium, presenting problems in maintaining a homogeneous and well mixed culture, with the formation of stagnant zones and nutrient gradients, requiring high stirring speeds and expensive downstream processes. Different approaches to overcome filamentous growth, including physical or genetic methods, will be presented in the next section.

Controlled growth and morphology during fermentation

Different strategies have been established to enhance protein production through the control of growth and morphology during industrial fermentation. However, with the production of enzymes and antibiotics by *Streptomyces* as an example (discussed in more detail below), the preferred morphological behavior depends very much on the combination between production host and product of interest.

The first studies focused mainly on antibiotic production. In *Penicillium*

chrysogenum, only pellets with diameters less than 400 μm were considered as actively metabolizing (Schügerl et al. 1983). In *Saccharopolyspora erythraea*, there is a critical pellet diameter (80–90 μm) below which production of erythromycin was abolished (Martin & Bushell, 1996). The correlation between morphology and antibiotic productivity in this organism was further investigated, with the best production obtained in variants with enhanced strength and reduced branching rates (Wardell et al. 2002). In *Streptomyces*, an alteration from long to short hyphae corresponded to a block in antibiotic production (Kuznetsov et al. 1992). It is generally believed that pellet and clump formation is fundamental to obtain good production of secondary metabolites (Vecht-Lifshitz et al. 1992; Sarrà et al. 1997), supporting the hypothesis that antibiotics are produced at a fixed distance from the hyphal end. Nevertheless, shake-flask cultures of *Streptomyces hygroscopicus* with glass beads reduced pellet size with simultaneous increase of the geldanamycin production (Dobson et al. 2008).

It is unclear how these observations can be applied for the production of proteins . In the case of filamentous fungi, production of enzymes is generally favored by a small/loose pellet morphology, which allows better oxygen and nutrient transfer. This is the case, for example, for the production of glucoamylase by *Aspergillus niger* (Papagianni and Moo-Young 2002) or to obtain acid phosphatase from *Neurospora crassa* (Wen Su and Jun He 1997).

In order to control the morphology of filamentous microorganisms in liquid cultures, different approaches can be applied, for example by altering the inoculum,the pH or the stirring speed (reviewed in Papagianni 2004). A low inoculum typically leads to pellet formation, while a dense inoculum results in dispersed growth. The initial pH of the medium (or in precultures) plays a role influencing the aggregation properties of the surface of the hyphae, with a more open mycelium at lower pH values. A strong agitation in stirred tank bioreactors forms free filaments rather than pellets but the maximum rate is limited as it causes physical damage to the mycelium. The use of microparticles affects the growth of filamentous organisms in liquid, changing the morphology from pellets to single hyphae (Walisko et al. 2012). The shear stress and consequent oxygenation of cultures grown in different types of flasks

affects morphology as well as production to a great extend (Gamboa-Suasnavart et al. 2011). The smaller pellet morphology obtained in baffled and coiled flask resulted in three-time higher production and higher glycosylation of the APA antigen from *Mycobacterium tuberculosis* in *S. lividans* when compared to standard flasks. This was successfully scaled up by changing the power input in a small scale bioreactor (Gamboa-Suasnavart et al. 2013).

Nevertheless, physical methods show some limitations as they create a mix of different morphologies and do not allow a precise control. Therefore, a rational genetic approach can be used to favor a specific phenotype in submerged cultures.

In *Streptomyces*, a specific case is represented by the overexpression of SsgA, a protein belonging to the SALP family and involved in septation during cell division and peptidoglycan maintenance (van Wezel et al. 2006). Enhanced expression of this protein led to a fragmenting phenotype in three species (*S. coelicolor, S. lividans* and *S. roseosporus*), clearly underlying a correlation between septation and the degree of fragmentation (see Fig. 2C for *S. lividans*). A fragmented phenotype presents a potential improvement for large scale liquid cultivations in terms of mass and nutrients transfer. In fact, the *S. lividans* SsgA overexpressing strain showed higher growth rates and a halved fermentation time, coupled to an increase in enzyme production when tyrosinase, a phenoloxidase, was used as a reporter. The effect on antibiotic production is less predictable. While enhanced expression of SsgA strongly enhanced the production of prodiginines, it completely blocked production of actinorhodin. This may be explained by the fact that undecylprodigiosin is produced during an earlier growth phase than actinorhodin, and that SsgA locks streptomycetes in an earlier phase of the life cycle (van Wezel et al. 2009).

Extending a rational genetic approach for a wider use is strongly depending on the current knowledge about genetic control and regulatory pathways. The *S. lividans* SsgA overexpressing strain is a clear example of scientific and industrial success towards the building of *Streptomyces* as a valuable alternative for commercial protein production (van Wezel and Vijgenboom 2003). Therefore, it is worthwhile to invest more research time to extend the knowhow on morphology related processes.

Secretion

Secretion pathways in Streptomyces

From a downstream processing point of view, heterologously expressed proteins can be best secreted into the fermentation broth. Therefore, increasing the secretion capacity is an important parameter towards optimization of the yield. The Sec pathway is the major secretion pathway in bacteria, and translocates unfolded proteins over the membrane (recently reviewed in (du Plessis et al. 2011). The Sec translocase is a highly flexible transmembrane channel constituted by a SecYEG heterotrimeric complex, associated to the motor protein SecA (Zimmer et al. 2008; du Plessis et al. 2009). Two different energy sources are used to create the mechanical motion: ATP and the proton motive force (PMF) (Driessen 1992). In *Streptomyces*, Sec-substrate proteins bind co-translationally to the signal recognition protein (SRP) prior translocation (Palacín et al. 2003). The substrate is gradually bound and released from the complex until the complete expulsion, followed by cleavage of the signal sequence.

The twin arginine translocation (Tat) presents a second secretion pathway. Firstly discovered in the thylakoid membrane of chloroplasts, it has been subsequently identified in the membrane of many bacteria and studied extensively in *E. coli*. For a detailed overview see Palmer and Berks (2012). The route takes its name from the two arginines present in the recognition sequence, which play a crucial role during recognition. The ability of the Tat pathway to translocate folded substrates makes it the preferred route for a number of proteins: (1) those who require the insertion of complex cofactors, (2) proteins that need to avoid competition of metal ions for binding to their cofactor site, (3) the transport of hetero-oligomeric complexes and (4) substrates that need accessory proteins in the cytoplasm for folding or maturation or cannot be kept unfolded after translation.

Homologues of *E. coli* Tat proteins, TatA, TatB and TatC, have been identified and characterized in *S. lividans* (Hicks et al. 2006). TatB and TatC have a key role in triggering the assembly of the complex and binding the substrate, while TatA is abundantly present in the membrane, oligomerizing into a ring shaped channel

suggested to act as a translocon (De Keersmaeker et al. 2005; De Keersmaeker et al. 2007). TatA is recruited by PMF, which is used as the only energy source. After transport, the protein is cleaved by a signal peptidase and the complex dissociates.

Recently, it was shown that during normal growth the Tat complex localises near the tips of growing hyphae (Willemse et al. 2012). The Tat complex shows a highly dynamic localization pattern, with more foci present along the hyphae including the tips. The assembly of the TatA complex was followed in time and space via a single particle tracking technique (Celler et al. 2013). Three different stages were identified in the assembly: an inert state followed by movement along the hyphae and wobbling prior final localization at about 2 μm from the tip. All these results confirm that Tat secretion occurs at the hyphal tips, in agreement to what is observed in fungi. The tip localization of TatABC suggests that increasing the number of apical sites and reducing branching and hyphal length would be a good strategy for enhancing the secretion of Tat-secreted enzymes. Indeed, increased fragmentation of the hyphae (which effectively increases the number of apical sites) resulted in enhanced secretion of the Tat substrate tyrosinase (van Wezel et al. 2006), which points at an intimate relation between morphology and secretion in *Streptomyces*. This example shows that better understanding of the behaviour, dynamics and localization of the proteins involved in secretion may lead to rational design of production hosts.

In contrast with most bacteria, substrate prediction programs revealed an exceptionally high number of Tat substrates in *S. coelicolor* genome: 129 proteins predicted by TATscan (Li et al. 2005) against 22 in *E. coli* (Dilks et al. 2003). The major role of this pathway in this organism is confirmed by proteomic studies on *tat* mutants in *S. coelicolor* (Widdick et al., 2006), with 25 out of 43 proteins verified as Tat-targeted. Moreover, 63 proteins were identified as Tat substrates in *S. lividans* (Guimond and Morosoli 2008), among which only 7 were expected from *in silico* prediction, and 47 out of the 73 proteins predicted from proteomic experiments in *S. scabies* were confirmed to be secreted through this pathway (Joshi et al. 2010). In addition, *S. lividans* mutants Δ*tatB* and Δ*tatC* showed a retarded development on solid media and a dispersed growth in liquid (Schaerlaekens 2004), underscoring that

proteins essential for morphological development are secreted via the Tat pathway. The evidence suggests a different and more important role of the Tat pathway in *Streptomyces* than in other bacteria. This assumes a particular relevance in case of production of recombinant proteins that might need to be exported in an active form or that fail to be secreted through the Sec system.

Enhancing heterologous protein production through secretion improvement

Reaching elevated yields of heterologous proteins is often impeded by the saturation of the secretion system. This paragraph will give a brief overview of the various strategies tested to enhance production levels through modification of the secretion capacity.

Enhancing secretion by simultaneous overexpression of the three components of the Tat pathway (TatA, TatB, TatC) proved to be an efficient tool in the case of xylanase C (XlnC) production in *S. lividans*, enhancing secretion of five fold (De Keersmaeker et al. 2006). The same strategy has been successfully tested in other bacteria such as *Corynebacterium glutamicum* (Kikuchi et al. 2009) and might therefore present a general way to improve protein secretion in various systems. The overexpression of Sec components could also present an alternative to increase protein secretion. However, the relative high number of proteins required for a functional Sec system seems to be a drawback. Only the overexpression of the core components SecYEG has been reported in *E. coli* (Douville et al. 1995), resulting in a 30 fold increase in translocation of the preprotein proOmpA.

Several experiments have shown that cross talk seems to exist between the Sec and Tat pathways, leading to an increase in secretion from one route when the other one is inactivated. This has been exploited in the case of the *S. lividans* TatB mutant**,** in which the Sec secretion of the human interleukin 10 (hIL10) showed a 15-fold increase compared to the wild type (Schaerlaekens et al. 2004). The opposite effect on Sec secretion has been observed in the TatABC overexpressing strain, with a strong reduction of XlnB secretion (De Keersmaeker et al. 2006). How the two secretion pathways communicate and how this is regulated is not understood.

Another interesting example of improvement of the secretion efficiency is presented by the overexpression of the phage shock protein A (PspA). Phage-shock protein response is a cell response activated in situation of stress such as phage infection, which causes dissipation of the proton motive force and alteration of redox state (reviewed in Joly et al. 2010). PspA, the main effector protein, reduces membrane depolarization and damage by blocking the proton leakage by a still unknown mechanism. A block in protein secretion is known to activate the response, although the exact nature of this interaction is still unclear. Recently, a PspA-TatA complex has been purified and analyzed in *E. coli*, suggesting an involvement of PspA in the control of membrane stress at active translocons (Mehner et al. 2012). The overexpression of PspA increased Tat dependent secretion in the case of XlnC and heterologous eGFP in *S. lividans*, while a limited effect (20%) was observed for the Sec pathway (Vrancken et al. 2007). This result is expected as the Tat translocation is solely dependent on the PMF, while the Sec translocation requires also hydrolysis of ATP. An increase in protein production through the Tat pathway in a *pspA* overexpressing strain is in accordance with experiments in *E. coli*, where the secretion of the exoglucanase (Exg) of *Cellulomonas fimi* was achieved, avoiding the previously reported saturation of the Sec system (Wang et al. 2011).

Proteolysis and product integrity

Proteolysis is a general mechanism to regulate levels of native proteins, removing the defective ones or inactivating them and recycling amino acids. In case of protein overexpression, proteolysis can be triggered due to folding problems and hamper final expression levels and protein integrity. The involvement of proteases in stress response after induction of heterologous protein expression has been well described in *E. coli* (Gill et al. 2000) and the use of protease-deficient mutants reduces protein degradation (Jiang et al. 2002).

Among streptomycetes, *S. lividans* has a lower level of endogenous secreted protease activity when compared to other strains. However, extracellular (Lichenstein

et al. 1992; Butler et al. 1995), intracellular (Butler et al. 1994) and mycelium-associated proteases (Binnie et al. 1995) are present. In *S. lividans*, a proteasome-deficient mutant proved to enhance protein production (Hong et al. 2005). The proteasome is an intracellular, self-compartimentalizing protease with confined proteolytic activity. It was first discovered in eukaryotes, followed by archea and bacteria belonging to order of *Actinomycetales* such as *Streptomyces* (De Mot et al. 1999). A comprehensive review covering the different domains of life has been recently published (Maupin-Furlow 2012). The physiological role in bacteria is unclear, as no cellular targets have been identified and no physiological changes have been observed in the deficient mutants, neither in *Mycobacterium* (Knipfer and Shrader 1997) nor in *S. coelicolor* (Nagy et al. 2003) or *S. lividans* (Hong et al. 2005). However, the level of protein expression increased in *S. lividans* proteasome mutant compared to wild type strain in case of the human tumor necrosis factor receptor II (shuTNFRII) and salmon calcitonin (sCT), while no effect was demonstrated on shuTNFRI (Hong et al. 2005). These results suggest that protein expression may benefit from deleting the proteasome genes in the host.

In bacteria, proteins are tagged and directed to the proteasome by prokaryotic ubiquitin-like protein (Pup), similar to the ubiquitin system in eukaryotes (Burns and Darwin 2010). The pupylation mechanism is active in *S. coelicolor*, with 20 potential targets identified (P. Mazodier, J.L. Pernodet, personal communication). Therefore the pupylation system may be an interesting target for increasing heterologous protein production.

Despite the increase in protein expression obtained in a proteasome-deficient mutant, few studies describing the influence of the deletion of other proteolytic enzymes have been published so far. Several protease mutants of *S. lividans* were studied for their protein secretion but none of them showed a better protein secretion level than the wild type (Arias et al. 2007).

Fermentation

Optimizing fermentation of *Streptomyces* for its use at industrial level requires insights in more aspects than the already discussed growth and morphology related issues (vide supra). *Streptomyces* is generally cultivated in batch fermentation in stirred tank reactors with a blade turbine impeller. Usually, a dispersed mycelium is the predominant form in these cultures while pellets are formed in shake flasks. In addition to genetic engineering (van Wezel et al. 2006), bioreactor operating conditions such as agitation and aeration influence to a great extent growth, morphology, oxygen and nutrient transfer. The impact of hydrodynamics on *Streptomyces* cultures in shaking flasks and stirred bioreactors has been recently reviewed (Olmos et al. 2012).

A major limiting aspect for fermentation of filamentous organisms is the transfer of oxygen and nutrients. Especially for a strictly aerobic microorganism as *Streptomyces*, oxygen availability is a limiting step because of its diffusion at the gas-liquid interface and transfer to the center of the pellet. Due to the great interest in *Streptomyces* as an antibiotic producer, most of the fermentation studies are focused on the effects of oxygen on secondary metabolism rather than on production of primary metabolites or heterologous proteins. Antibiotic production is coupled with an increase in oxygen consumption and a higher dissolved oxygen tension enhances production (Olmos et al. 2012). Concerning primary metabolism, it has been demonstrated that in *S. lividans* strain producing mTNFα, the dissolved oxygen percentage dropped to approximately 10% compared to the 30% for the WT strain (D'Huys et al. 2011), demonstrating also in this case a higher oxygen consumption as seen for antibiotic production. This is in agreement with studies in *E. coli*, where more oxygen is needed for protein production (Özkan et al. 2005), while a positive effect of hypoxic conditions has been described for *Pichia pastoris* (Baumann et al. 2010).

One way to facilitate oxygen transfer is to increase the agitation rate, but this affects the rheology of the cultures, with fragmentation of the clumps, increase in viscosity, cell damage and even lysis. The dependence of morphology on the agitation

rate has been reported for *S. fradiae*, where a decrease in shear stress caused a shift in morphology from free filaments to pellets (Tamura et al. 1997). Cell lysis was also studied in correlation to the power input, a parameter that depends on agitation rate, airflow and reactor geometry. The effect of power input and consequent shear stress has been quantified for *S. clavuligerus* in batch cultivations with defined media, supplemented with different carbon and nitrogen sources (Roubos et al. 2001). The study led to the conclusion that this strain is extremely shear-sensitive and that cell lyses occurs even at low agitation rates. The identification of threshold values below which no major lysis was observed was strictly dependent on the medium composition, with mycelium grown on glutamate and maltose or succinate as the most sensitive to lysis. Moreover, the power input can also influence the morphology of the cultured strain and the production of post-translationally modified proteins. This is the case for the production of *Mycobacterium tuberculosis* APA protein in *S. lividans*, where a controlled agitation rate led to a higher degree of mannosylation compared to cultures in shake flasks (Gamboa-Suasnavart et al. 2013).

As an alternative to increasing the agitation rate, pure oxygen can be supplied during fermentation to increase the productivity without affecting the morphology of the culture. This is the case, for example, in the production of the immunosuppressant rapamycin in *S. hygroscopicus* (Yen and Hsiao 2013) where, despite a high requirement of oxygen, a high agitation rate resulted in pellet damage and lower production. Nevertheless, supplying pure oxygen is expensive and costs need to be carefully evaluated for commercial applications. It remains to be seen if this approach also works for protein production.

Beside the control of the bioreactor parameters, medium composition and nutrient dosing influence the efficiency of fermentation and protein production. This was demonstrated in early studies (Payne et al. 1990) where a continuous feeding of both glucose and tryptone led to a 25-fold increase in production for a *Flavobacterium* hydrolase in *S. lividans*, with an enhanced specific activity. Complex media increase protein production (Pozidis et al. 2001) and are usually preferred by industry for their low cost.

Various studies have dealt with the importance of the amino acid composition of the medium for different protein production platforms as *Saccharomyces cerevisiae* (Görgens et al. 2005b) and *Pichia stipitis* (Görgens et al. 2005a). The same approach has been applied to *Streptomyces*, unraveling the importance of amino acid supplementation in heterologous protein production. A systematic study based on a statistical set up identified aspartate, phenylalanine and methioinine as the essential amino acids for the production of the recombinant human interleukin-3 (rHuIL-3) in a glucose-based medium (Nowruzi et al. 2008). Moreover, a metabolite analysis in *S. lividans* producing mTNFα (Kassama et al. 2010) identified seventeen metabolites at a higher level in the protein producing strain. Their role as energy source and in maintaining the membrane potential necessary for secretion was postulated.

Nevertheless, no clear connections with metabolism were demonstrated until recently, when the amino acid uptake in fed batch cultures for *S. lividans* producing mTNFα was described, representing the first link between biomass and protein production (D'Huys et al. 2011). The analysis of the wild type strain in minimal medium supplemented with a complex mixture of amino acids allowed the identification of two distinct growth phases: the first based on glutamate and aspartate consumption, and the second with glucose as the limiting substrate. Due to the unusual excess of nitrogen and glucose during fermentation, a lot of by-products as pyruvate, α-ketoglutarate, succinate and alanine were detected. In addition, lactic acid was produced during the entire growth, probably as a result of the limitation in oxygen in the central part of the pellets, resulting in a microaerobic environment. On the other hand, the analysis of the mTNFα producing strain showed a lower growth rate, formation of bigger clumps and a shift in metabolism. This was represented by an increase in lactate and a decrease in by-product formation as a consequence of the increase in pellet size, with a less active metabolizing biomass.

The metabolic network of *S. coelicolor* had been investigated at genome scale, allowing the identification of 121 genes essential for metabolism and the simulation of growth and antibiotic production in media with different carbon and nitrogen sources (Borodina et al. 2005). To expand the knowledge obtained in this study, a

genome scale metabolic model based on flux analysis in different limiting conditions (glucose-ammonium, amino acids, organic acids and alanine) was investigated (D'Huys et al. 2012). Depletion of glutamate and aspartate diminished growth rate and biomass formation with consequent increase in protein production. It is clear from these observations that a complex equilibrium exists in the metabolism of expression strains and that the type and availability of carbon and nitrogen sources need to be optimized to promote heterologous protein production at the expense of biomass formation or undesired metabolites.

To conclude, a general protocol for optimal fermentation parameters has not been written yet. Improvement of the process needs to be done experimentally on a case by case basis and strongly depends on multiple factors such as host, morphology, hydrodynamics parameters, medium composition and metabolism.

EXPRESSION VECTOR OPTIMIZATION

An efficient expression vector is an indispensable element to be combined with an engineered host for a maximum level of heterologous protein production. As for strain optimization, the design of an expression vector has to take into consideration all the possible processes that can be improved to get to a better protein production, with transcription, translation, posttranslational modification and secretion as the main ones. However, issues such as the selection used to maintain the plasmid, which is nowadays mainly done by antibiotic resistance, should be taken into account as well. The progresses in design of recombinant expression systems in actinomycetes have been reviewed (Nakashima et al. 2005). An overview of all the important elements is given in the following section.

Copy number

The vectors used for genetic manipulation in *Streptomyces* can be classified into two categories: integrative and multicopy (Kieser et al. 2000). The integrative vectors provide higher stability through integration into the genome using specific phage attachment sites. Based on the pioneering work of Maggie Smith, Keith Chater and

colleagues, in particular the attachment site for ΦC31 was exploited. This integration system found its way, among others, into the Eli Lilly vector pSET152 (Bierman et al. 1992) which is one of the most used vectors worldwide and revolutionized the field. Another frequently used vector system for genomic integration is based on pSAM2 (Pernodet et al. 1984; Smokvina et al. 1990). Although the integration should result in a single copy in the genome, expression from such vectors is often higher than expected, which may be explained by the fact that tandem integrations occur, with up to 10 integrations events at the same time (Combes et al. 2002). For industrial applications, integrative vectors may have the disadvantage of requiring antibiotic resistance markers. Therefore, strategies for genomic integration based on homologous recombination are a valuable alternative. Recently, major advances have been made in markerless integration of DNA intro the genome of streptomycetes (Siegl and Luzhetskyy 2012).

The autonomously replicating vectors occur at high, medium or low copy, depending on the *ori*. Industry typically uses high copy number vectors, which in streptomycetes are based on the vector pIJ101, such as pIJ702 (Katz et al. 1983) and pIJ486 (Ward et al. 1986), with up to 300 copies per chromosome. *E. coli-Streptomyces* shuttle vectors are also available, and usually preferred to simplify cloning and amplification, although there are major stability issues. The multi-copy shuttle vector pWHM3 is so unstable that it is used for gene disruption strategies as it is lost at an extremely high frequency when antibiotic pressure is relieved (van Wezel et al. 2005). Recently, novel high copy shuttle vectors derived from pIJ101 have been constructed for protein overexpression: pL97 and pL98 were tested in protein production using *eGFP* and *redD* as reporters (Sun et al. 2012), while pZRJ362 produced a four fold increase in the enzyme activity of the endoglucanase Cel6A from *Thermobifida fusca* when *S. lividans* is compared to *Pichia pastoris* (Li et al. 2013).

Selection markers

The choice of a selection marker is usually restricted to antibiotics. However, *Streptomyces* is naturally resistant to several of them (chloramphenicol, tetracycline

and β-lactams) limiting considerably the choice. Moreover, the necessity of keeping a constant antibiotic selection pressure is undesirable for large-scale industrial processes due to the risk of contaminating the final product. Recently, a promising alternative represented by a toxin-antitoxin system has been functionally tested in *Streptomyces* (Sevillano et al. 2012). The system consists of two small proteins, which act as a toxin-antitoxin complex in which the first one is inactivated by the latter one. This mechanism is wide spread among bacteria and archaea, with up to 33 TA systems identified in *E. coli*, although their specific function in nature remains unclear. In *Streptomyces*, three different systems have been identified *in silico*. Among them, the proteins encoded by SCO2235/2236 in *S. coelicolor* have been studied and re-named YefM and YoeB by analogy to the YefM/YoeB system in *E. coli* (Sevillano et al. 2012). An *S. lividans* Δ*yefM/yoeB* null mutant strain carrying a copy of the toxin integrated in the genome and a copy of the antitoxin on a mulitcopy plasmid was tested for stable protein expression (Sevillano et al. 2013). High level of both xylanase and amylase were obtained. The expression system proved to be stable and to keep its expression level even after storage of the mycelium/spores, demonstrating the potential of this system as an alternative to antibiotic selection.

Promoters

A strong promoter resulting in a high level of transcript is an essential element in the design of an expression vector for optimal protein production. Very few promoters are known to be strong in *Streptomyces* and those that have been studied can be divided into constitutive and inducible (Table 1).

The inducible promoters have the advantage that production can be started when sufficient biomass is available, although the addition of an inducer is undesirable for industrial applications. Constitutive promoters such as the frequently used P_{ermE} and P_{vsi} do not show the same strength under all conditions and in all hosts and therefore are far from optimal. The addition of thiostrepton when using the inducible promoter P_{tipA} gives collateral expression of undesired proteins in *Streptomyces* genome that can saturate the expression machinery and thus have a negative effect on the production

Table 1 List of promoters in *Streptomyces* expression vectors

Gene	Product	Host	Reference
vsi	subtilisin inhibitor	*Streptomyces venezuelae*	(Lammertyn et al. 1997)
ermE	resistance gene to erythromycin	*Saccharopolyspora erythraea*	(Bibb et al. 1985)
ssmp	metalloendopeptidase promoter	*Streptomyces cinnamoneus*	(Hatanaka et al. 2008)
pld	phospholipase D	*Streptoverticillium cinnamoneum*	(Noda et al. 2010)
actI	actinorhodin biosynthetic gene cluster	*Streptomyces coelicolor*	(Rowe et al. 1998)
dagA	agarase gene	*Streptomyces coelicolor*	(Parro and Mellado 1993)
nitA	nitrilase inducible by ε-caprolactam	*Rhodococcus rhodochrous*	(Herai et al. 2004)
amdS	inducible acetamidase promoter	*Mycobacterium smegmatis*	(Triccas et al. 1998)
tipA	thiostrepton induced promoter	*Streptomyces lividans*	(Murakami et al. 1989)
xysA	xylanase A, xylose inducible	*Streptomyces halstedii JM8*	(Adham et al. 2001)
tc	tetracycline inducible promoter	*Streptomyces coelicolor*	(Rodríguez-García et al. 2005)

of the desired protein/enzyme. Further investigation and characterization of new promoters is therefore a must for the design of optimal expression vectors.

To characterize strong promoters, extensive studies were carried out in the past in bacteria as *E. coli*, leading to the identification of essential elements for binding of the RNA polymerase, namely the -35 box, the -10 box for recognition by σ factors, the region upstream (between roughly -60/-35) for binding of the α subunit of the RNAP and other regions that determine the local structure of the DNA (reviewed in (Hook-Barnard & Hinton, 2007). However, the specific elements involved in promoter strength in *Streptomyces* are still largely unknown. More than a hundred sequences directly upstream from mapped transcriptional start sites have been compared (Strohl 1992; Bourn and Babb 1995), showing a wide variety of features with only a small group of *E. coli*-like promoters, while the majority did not resemble any known prokaryotic promoters. In *Streptomyces*, the work is complicated

by the existence of a large number of σ factors, such as the 66 σ factors encoded by the *S. coelicolor* genome, most of which are so-called ECF σ factors for extracellular functions (Bentley et al. 2002). This large number sharply contrasts with the seven σ factors found in *E. coli* (Pérez-Rueda and Collado-Vides 2000). Some of the σ factors are developmentally controlled, like *bldN* and *sigF* (Kelemen et al. 1996; Bibb et al. 2000) or activated in response to different stimuli (rewied in Gruber & Gross 2003).

Despite the interest in *Streptomyces* as an industrial host, few studies have been published in the last years on the identification of new strong promoters. An interesting study was provided by the Virolle group (Seghezzi et al. 2011), who created a synthetic library of randomized promoters based on the promoter for the household σ factor RpoD (σ^{70} or σ^{HrdB}). The results showed an over-representation of the guanine nucleotide in strong promoters in the -10 region and the -35 region, in addition to a motif related to an "extended -10 region", an imperfect repetition particularly important in *E. coli* for recognition and positioning of the RNA polymerase. The abundance of guanine residues can be explained by the high GC-content in *Streptomyces* strains, which might have led to an evolution driven towards highly G-rich strong promoters. The synthetically derived consensus sequence shows remarkable sequence similarity to P_{ermE} although none of the identified clones showed higher strength.

Recently, a combination of rational and random mutagenesis of the promoter of *kasO*, which encodes the pathway-specific transcriptional activator gene for the cryptic type-I polyketide Cpk, led to the design of a stronger version of the promoter when compared to the original P_{kasO} or P_{ermE} (Wang et al. 2013). The promoter is recognized by the σ^{HrdB} and repressed by the concerted action of two regulatory proteins, ScbR and ScbR2. The ScbR/ScbR2 binding site was deleted by rationally truncating the sequence up to an optimal length, while the second repressor binding site for ScbR2 was situated in an essential part of the sequence and was therefore inactivated by random mutagenesis. The recombinant promoter showed a higher strength both in *E. coli* and *Streptomyces* with luciferase and the antibiotic actinorhodin used as reporters, respectively. Such promoter optimization strategies

could be followed up for other vector-promoter systems. Moreover, this study also underlined the importance of the 20 nucleotides upstream of the -35 region (where the α subunit of the RNAP binds) and of the 18 nucleotide spacer between the -35 and -10 region, as substantial modifications in nucleotide composition or length strongly affected promoter activity.

Strong transcriptional elements from species other than streptomycetes can be tested as well for their efficiency. The mostly used expression system in *E. coli* is the T7 system based on the T7 bacteriophage polymerase and promoter. An *S. lividans* expression strain based on the same system has been created, combining the ability of transcribing large fragments of DNA with a high copy number pIJ101-derivative vector (Lussier et al. 2010). Nevertheless, no applications are reported in literature for protein expression at present.

In addition to a high level of transcription, the control of gene expression is sometimes also desirable for protein expression, for instance when the product has a negative effect on growth or is even lethal. An example of such control elements are riboswitches. Riboswitches are sequences present at the 5' end of a transcript and made of a ligand-binding/sensor domain and an expression platform. Upon binding of specific ligands, they adopt an altered conformation resulting in activation or repression of transcription or translation (Serganov and Nudler 2013). In *Streptomyces*, recent work has shown the applicability of the synthetic design of such sequences. The efficiency of at least two of the six tested theophylline-dependent riboswitches derived from *B. subtilis* was demonstrated in combination with three different promoters (P_{gal2}, P_{ermE} and SF14). Gene expression could be activated in a dose-dependent manner up to 260 fold, confirming the technique as a promising tool to regulate protein expression in *Streptomyces* (Rudolph et al. 2013).

In this thesis, a rational approach towards the identification of strong, constitutive promoters was tested based on RNA-Seq and microarray data (Chapter 5). With this approach, three sequences were identified with strength higher or comparable to P_{ermE} when tested for the production of a small laccase in *S. lividans*. These sequences were the promoters of SCO1947 for glyceraldehyde-3-phosphate

dehydrogenase (GADPH), SCO3484 encoding a sugar binding protein and for SCO4253 for an uncharacterized protein.

Ribosome binding sites and codon usage

The next step in the design of an expression vector is to optimize the translation of the transcript. Two of the main factors to be taken into account are: an efficient Ribosome Binding Site (RBS) and the codon usage. The RBS sequence is involved in binding and correct positioning of the ribosome. Typically, the RBS is located between 5-12 nucleotides upstream of the start codon, and has the consensus sequence AGGAGG, complementary to the sequence 5'-CCUCCU-3' at the 3' end of the 16S rRNA. In *Streptomyces*, analysis of the interaction with the ribosome showed that a high complementarity is not required (Strohl 1992). In addition to this, some genes do not show any obvious RBS in the 20 nucleotides sequence upstream of their translational start, implying that the binding to this region is not a prerequisite for ribosome translation. A well studied example is the *ermE* gene, encoding the 23S rRNA methylase of *Saccharopolyspora erythrae*, where the transcript initiates at the translational start codon without any sequence upstream for ribosome recognition (Bibb et al. 1994). In other cases, two putative translational starts are preceded by a RBS-like sequence (as for mannase, cellulase A, chitosanase, subtilisin inhibitor and esterase genes) (Morosoli et al. 2006). Evidence suggests that both RBS's might be used as a way to increase the efficiency of protein translation, with two ribosomes binding the same mRNA molecule.

In *Streptomyces* the most often used RBS is obtained from the *tuf1* gene from *S. ramocissimus*, which encodes the highly expressed elongation factor EF-Tu1. This ribosome binding site and linker have been cloned downstream of the *ermE* promoter (Motamedi et al. 1995) and use of his ribosome binding site allows very efficient recruitment of the ribosome (van Wezel et al. 2000a.

Codon usage has a strong influence on the efficiency of translation. Heterologous mRNA species have a high probability of containing rare codons when moved to another host (Gustafsson et al. 2004). This is particularly relevant in

Streptomyces, which has a high G+C content, and is reflected by a strong bias towards G or C in the second and third (wobble) codon positions. As a result expression of genes from lower G+C organisms such as *E. coli* and *B. subtilis* can be less efficient. Moreover, the translation of some rare codons such as the leucine codon TTA are strictly regulated by development (Takano et al. 2003). Codon modification has been reported to increase expression of heterologous proteins in *E. coli* between 5- and 15-fold (Gustafsson et al. 2004). Several reports on codon optimization for reporter enzyme systems such as *lux* (Craney et al. 2007), eGFP (Sun et al. 1999), *creA* (Fedoryshyn et al. 2008) or signal sequences (Zhu et al. 2011) have been published but none of them was aimed at high level protein production.

Signal peptides

The transport across the membrane is another target for modification to obtain maximal yield of heterologous proteins. In addition to the strategies discussed before in this chapter, the choice of an appropriate signal peptide has as well a strong influence on directing protein secretion. Sec signal peptides such as the one derived from the subtilisin inhibitor of *S. venezuelae* (*vsi*) and the cellulase A (*celA*) from *S. lividans* are most frequently used. The *vsi* signal peptide was successfully used for the production of high levels of TNF-α (Lammertyn et al. 1997) while *celA* signal peptide increased the production of xylanase A (XlnA) (Pagé et al. 1996). Including a Tat pathway signal sequence in the expression construct is a prerequisite when expressing proteins that require folding and/or cofactor incorporation prior to secretion, as in the case of GFP (Thomas et al. 2001; Santini et al. 2001; Vrancken et al. 2007). As mentioned above, *Streptomyces* have the highest number of predicted Tat substrates. However, results of protein secretion through this route have not always been promising. The mouse tumor factor α (TNFα) and the human interleukin-10 (hIL10) were fused to the Tat signal peptide of *xlnC* of *S. lividans* and of the tyrosinase chaperone *melC1* from *S. antibioticus*, but the levels of production were not competitive with the secretion via Sec by the *vsi* signal peptide (Schaerlaekens et al., 2004). Recently, a novel expression

vector has been tested for the production of human interleukin-6 (IL-6) in *S. lividans* (Zhu et al. 2011). The signal pepetides of MelC1 and of CagA were tested and only the latter turned out to support secretion of IL-6. Attempts to obtain better Tat secretion of XlnC by using a number of other Tat signal sequences identified by various prediction programmes were not successful. Moreover, a mutation analysis of the signal peptide of XlnC did not result in more efficient secretion, suggesting that at least this signal sequence is already optimal (Li et al. 2006). Positive results using the Tat pathway were obtained recently, when 25 degrading enzymes of *Thermobifida fusca* and *S. lividans* were analyzed for secretion in *S. lividans* (Miyazaki et al. 2013). Using a cytoplasmic enzyme, β-glucosidase from *T. fusca*, as reporter, 17 promising signal peptides for both the Sec and Tat pathway, were tested if they could drive secretion of the reporter. The Sec signal of phospholipase D was used as reference (Noda et al. 2010). Three Tat-dependent signals, including the XlnC signal peptide, showed higher enzyme secretion.

Gauthier et al. (2005) showed that secretion of xylanase B1, through the Tat and Sec pathway in one transformant resulted in the sum of the enzyme levels obtained from transformants using only one of the secretion systems. In this way, the maximum secretion capacity of the host is utilized and therefore a promising approach if applicable to other enzymes.

CONCLUSION

Generating a general *Streptomyces* host capable of efficient high level enzyme production has come a long way but is far from complete, in particular when compared to the extremely efficient vector-host combinations for production platforms such as *E. coli* and *B. subtilis*.

From a morphological point of view, a balance needs to be reached between reducing pellet size and preventing high viscosity due to fragmentation or mycelial mat production. The first steps in this process have been established and the focus can now be set on creating strains that have the ultimate submerged culture morphology. Testing and selecting different fermentation parameters will be strictly dependent on the chosen strain and desired product. Moreover, the tool box for the design of strong expression vectors has been expanded in the last decade and now contains sufficient parts to assemble expression vectors to meet the needs of a given production system.

Since the publication of the complete genome of *S. coelicolor* in 2002 (Bentley et al. 2002), a fast improvement in last generation sequencing has occurred, allowing a large number of streptomycetes genome sequences being available (http://www.genomesonline. org). This increasing volume of data has rapidly led to post-genomic and system biology studies in this organism. Integrating the results coming from these different techniques is a potent tool, on the one hand to identify novel actors in morphogenesis and, on the other hand, to study metabolism and growth during fermentation and protein production. Overall, this combinative approach might lead in the future to a substantial strain improvement for industrial purposes.

A comment on the best *Streptomyces* production host that can be assembled at this moment can be found in the General Discussion section of this Thesis.