Controlling growth and morphogenesis of the industrial enzyme producer Streptomyces lividans

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Chapter 7

General Discussion

GENERAL DISCUSSION

Enzymes play a very important role in industrial biotechnology, and where possible are applied to replace the more traditional chemical synthesis processes. This has led to what is generally referred to as "the bio-based economy". Examples of enzymes include amylases and glucose isomerases that are used in the starch industry, cellulases and xylanases in the paper industry, lipases for detergents, and peroxidases and laccases for, among others, bleaching denim. In addition, many therapeutic proteins are produced in the fermentation industry, typically in heterologous hosts, which started with the production of growth hormone in 1985. The increasing demand for energy from clean sources as a result of exhaustion of natural sources and an ever expanding world population has shifted the attention to more sustainable alternatives, namely the second generation biofuels, which are obtained via conversion of waste sugars into bioethanol and require a variety of degrading enzymes for the process.

The constant increase in the need of enzymes and new hosts for production has again brought *Streptomyces* in the spotlight. *Streptomyces* are well known as antibiotic producers, and were recently referred to as the *medicine makers* by Sir David Hopwood (Hopwood 2007). However, as saprophytic microorganisms, streptomycetes recycle all biologically occurring biopolymers, aimed at obtaining the necessary nutrients (Chater et al. 2010), whilst secreting antibiotics to compete with other microorganisms (van Wezel and McDowall 2011). The utilization of complex polysaccharides such as cellulose, chitin, starch, xylan, agar and lignin is possible due to the production of a massive arsenal of hydrolytic enzymes that are attractive for industry. Moreover, some species, such as the preferred host for the production of industrial enzymes *Streptomyces lividans*, couple high secretion to low proteolytic activity and thus prove to be a valuable alternative for the production of a variety of proteins when compared to traditional bacterial hosts (Anné et al. 2012).

Despite advances that have been made in the past, streptomycetes are still relatively unattractive production hosts. This is primarily due to their mycelial life style, which results in slow growth and mass transfer problems, with oxygen and

nutrient limitation inside the mycelia as a result (van Wezel et al. 2006). In liquid-grown cultures, such as during industrial fermentation, the intricate network of vegetative hyphae forms dense aggregates of different sizes (Pamboukian et al. 2002) with oxygen as the limiting nutrient (Celler et al. 2012). In these pellets, only the outer layer is metabolically active (Manteca et al. 2008). Moreover, the mycelial behavior causes high viscosity in the culture, problems in oxygen and nutrient transfer and limits the stirring speed to avoid cell lysis (Meyerhoff et al. 1995). Secondly, in contrast to the preferred production platforms, the molecular biological tools and expression systems are still relatively limited. Within the frame work of the ERA-Industrial Biotechnology (ERA-IB) project EPOS, funded by NWO-ACTS, new ways were explored to optimize *Streptomyces lividans* and to develop new vectors for efficient protein production.

The factors involved in pellet formation in *Streptomyces* have been partially identified, although the global mechanism remains unknown. Hyphal aggregation is linked to the formation of an extracellular matrix via cell surface proteins such as the chaplins (Claessen et al. 2003; Elliot et al. 2003) and a cellulose synthase-like protein CslA (Xu et al. 2008). The chaplins form amyloid structures (Sawyer et al. 2011; Bokhove et al. 2013) which organize into a network, perhaps in conjunction with the polysaccharide synthesized by CslA, and allow attachment of the hyphae to surfaces (de Jong et al. 2009). Deletion of either the chaplin genes or *cslA* leads to pellets of reduced size or open mycelial structures in liquid cultures (Xu et al. 2008; van Veluw et al. 2012). Another protein that influences pellet aggregation is SsgA. As a cell division protein responsible for septum localization and initiation (Jakimowicz and van Wezel 2012), its overexpression leads to hyper septation and fragmentation in liquid cultures with consequent higher enzyme production (van Wezel et al. 2006), perhaps due to the increase of the number of tips, which are active sites for secretion (Willemse et al. 2012). Changing *Streptomyces* morphology through a rational genetic approach is an attractive concept. This guided the investigation to study in more detail the function of CslA and its functional partner, a copper oxidase GlxA, and the effect on liquid morphology of null mutants in *S. lividans*.

Moreover, analysis of global transcript profiles by RNA-seq of *cslA* and *glxA* mutants identified novel proteins potentially involved in morphogenesis. Considering their major impact on mycelial morphology, a pilot study was initiated to study the effect of the *ssg* genes in *S. lividans*, to see if this could potentially be an attractive target for altering mycelial morphology and protein production. Therefore, several *ssg* genes for SsgA-like proteins (SsgA-G) were deleted to analyze the phenotype of the mutants and compare it to the data obtained in *S. coelicolor* (Traag and van Wezel 2008). This showed similarities but also some noteworthy differences between *S. lividans* and *S. coelicolor*, which should be analyzed in more detail in the future.

The data presented in this thesis show that both CslA and GlxA are required for the synthesis and correct deposition at the hyphal tips of a polysaccharide involved in matrix formation, aerial development on rich media, agar invasion and pellet aggregation. That means the proteins have a major impact on the morphology of the mycelia. We were able to detect thin fibrils of the polysaccharide with a fluorescent chitin binding protein as probe, which suggests that the glycan may at least in part consist of chitin. This represents a novel aspect in *Streptomyces* morphology, and is a feature more akin to the fungal cell wall than to that of bacteria (Bowman and Free 2006). The localization of CslA and GlxA to in particular the hyphal tips again stresses that this may be a hub for the expression proteins involved in growth and development (Holmes et al. 2013). RNA-Seq analysis of the *cslA* and *glxA* null mutants revealed that a cluster of 17 adjacent genes (SCO4242-SCO4260 and SLI_4479-SLI_4495) showed a two- to five- fold increase in the mutants. The function of these genes in bacteria is not known, but their products show similarity with phage tail proteins and a role in virulence and biofilm formation has been suggested in some species (Chen et al. 2010; Ghosh et al. 2012; Du et al. 2012). It is intriguing that one or more of these proteins may assemble into amyloid-like fibrils, which have an important role in *Streptomyces* morphogenesis (Claessen et al. 2003; de Jong et al. 2009; Gras and Claessen, 2014). In a recent study in our laboratory on the spontaneous nonpelleting mutant *S. lividans* 1326MR, phage tail proteins were also among the most significantly deregulated proteins (D. van Dissel and G.P. van

Wezel, unpublished), and this further supports the notion that these proteins may be pivotal for maintaining the typical pellet morphology. More extensive analysis of these proteins is required to establish their precise function and if indeed these proteins may be a target for strain engineering. One important aspect of this work that awaits to be resolved is the exact nature of the polysaccharide produced by the concerted action of CslA and GlxA. Fibrils that were suggested to consist of cellulose were previously identified as dependent on CslA in *S. coelicolor* (de Jong et al. 2009) and, although it is known that bacteria can synthesize polysaccharides with different composition according to the growth conditions (Lee et al. 2001), characterizing the polysaccharide in detail will be of great interest for both basic and applied research. Moreover, it will be compelling to unravel the links between CslA, GlxA and all the proteins described so far as involved in matrix formation, aerial development and pellet aggregation, namely the chaplins, SapB and HyaS, in addition to identifying the specific role of the phage tail related proteins in this organism.

In addition to the study of genes involved in matrix formation, several genes belonging to the SALP group were also analyzed for their involvement in cell division and morphology. Mutants of the *ssg* genes *ssgA*, *ssgB*, *ssgC*, *ssgD* and *ssgE* were studied in *S. lividans* and compared to the corresponding *S. coelicolor* mutants, demonstrating functional overlaps but also some differences between the function of the genes in the two strains. This awaits further analysis.

Besides the analysis of genes involved in the control of morphogenesis, a search was also initiated for new strong and constitutive promoters for use in *Streptomyces* expression vectors that should allow higher level expression of industrial enzyme genes. Through the analysis of in-house transcription data (RNA-Seq and microarray data obtained previously), three promoters were identified, namely $P_{SCO1947}$, $P_{SCO4253}$ and $P_{SCO3484}$, with higher or comparable strength than the benchmark P_{ermE} and validated for the production in *S. lividans* of a small laccase SLAC from *S. coelicolor*. This represents an improvement in respect to reported studies, which failed to produce promoters stronger than the currently known ones (Seghezzi et al. 2011).

Based on the data described in this thesis as well as in the literature, advances can be envisioned for both morphology and expression systems. As mentioned previously, *Streptomyces* are able to decompose various substrates such as chitin, cellulose, lignin and xylan to obtain nutrients (Chater et al. 2010). Their capacity as hosts would be therefore exploited at best in the production of these proteins, with clear advantages, for example, in the production of second generation biofuels.

The most effective change in morphology has so far been achieved by the overexpression of the SsgA protein, which enhances cell division resulting in mycelial fragmentation, which leads to enhanced expression and secretion of proteins (van Wezel et al. 2006). However, other mutants with an open mycelial morphology might present good alternatives, either in terms of producing higher yield per kg of biomass, as well as in improving the secretion efficiency. This is among others achieved by increasing the number of apical sites, as this is the place in the mycelia where secretion may primarily take place (Willemse et al. 2012). Targeting cell surface (Claessen et al. 2006) or cytoskeleton-related genes (Celler et al. 2013) should be considered and in particular the application of *cslA* and *glxA* need to be analyzed further. For example, while deletion mutants produce less dense pellets, we do not have sufficient insight into the stress resistance of the hyphae that lack the CslA-produced polysaccharide. Such stress resistance is also an important factor during industrial fermentations as extensive lysis in the often heavily stirred cultures would be detrimental for the production process.

New ways need to be found to create a morphology that circumvents viscosity issues while allowing high growth rate and maintaining production and secretion capacity. Preliminary studies have shown that null mutants of *sco1*, encoding the copper chaperon suggested to deliver the metal to GlxA, show the same open mycelial phenotype as *glxA* null mutants, while the addition of copper to the medium restores pellet growth. Perhaps positioning *glxA* under the control of an inducible promoter is an option as it will allow temporally changing the expression of the gene without disrupting the production of the structural polysaccharide during growth phases where this is undesirable.

In terms of expression vectors, the development of tailor-made promoter-expression combinations for expression of genes in *Streptomyces* is continuing to be an important issue. Such an expression system should encompass a stable multi-copy vector, a strong constitutive or inducible promoter, an efficient ribosome binding site such as that for the elongation factor EF-Tu (Vijgenboom et al. 1994) and a signal sequence for efficient secretion, such as *vsi* or *xlnC* (Schaerlaekens et al. 2004; Pimienta et al. 2007). In terms of promoter sequences, this thesis revealed the possible applicability of the promoters $P_{SCO1947}$, $P_{SCO3484}$ and $P_{SCO4253}$ for strong and constitutive gene expression. To further improve their transcriptional activity, random mutagenesis and/or SELEX methods (Zimmermann et al. 2010) should be investigated. Thus, the thesis presents new ideas towards developing enabling technologies for the improved production of enzymes by *Streptomyces* in general and by *S. lividans* in particular. The availability of the *S. lividans* genome sequence is thereby very useful for among others genomics-based global strain improvement approaches. For example, production hosts have been generated by random strain improvement programs over the years, by companies that use *Streptomyces* for industrial enzyme production. Comparison of these strains by genomics approaches should reveal new genes that correlate to the achieved improved productivity. Such novel insight in combination with a morphology-optimized host and excellent expression systems should make streptomycetes a preferred industrial platform for heterologous protein production.