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Recombinant protein production and streptomycetes

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ABSTRACT

The biopharmaceutical market has come a long way since 1982, when the first biopharmaceutical product, recombinant human insulin, was launched. Just over 200 biopharma products have already gained approval. The global market for biopharmaceuticals which is currently valued at over US$99 billion has been growing at an impressive compound annual growth rate over the previous years. To produce these biopharmaceuticals and other industrially important heterologous proteins, different prokaryotic and eukaryotic expression systems are used. All expression systems have some advantages as well as some disadvantages that should be considered in selecting which one to use. Choosing the best one requires evaluating the options – from yield to glycosylation, to proper folding, to economics of scale-up. No host cell from which all the proteins can be universally expressed in large quantities has been found so far. Therefore, it is important to provide a variety of host–vector expression systems in order to increase the opportunities to screen for the most suitable expression conditions or host cell. In this overview, we focus on *Streptomyces lividans*, a Gram-positive bacterium with a proven excellence in secretion capacity, as host for heterologous protein production. We will discuss its advantages and disadvantages, and how with systems biology approaches strains can be developed to better producing cell factories.

1. Introduction

Optimization of the production/secretion of proteins of interest is crucial for the development of biotechnological processes to obtain proteins for a variety of purposes, these being scientific, therapeutic, diagnostic, environmental, food-related, agricultural, vaccine production, etc. A lot is already known about the mechanism of protein secretion in bacteria in general, different secretion routes have been described and elements of the secretion pathways investigated. However, when it comes to the secretion of heterologous proteins, hardly anything is known about specificity, why some proteins are better secreted than others, how the cell deals with stress induced by the heterologous proteins, and how the induced stress cascade influences the cell physiology and its performance during the production process.

As a consequence, considerable time and expenses are spent to maximize the yield and quality of proteins produced in heterologous hosts, in most instances so far on empirical grounds. The choice of an expression system has a major impact on both product development lead times and product costs. The production host of choice, so far, is *Escherichia coli* for which many strains and expression systems are available. However, one size does not fit all: *E. coli* is not always suitable for the production of proteins, either because it is not able to carry out appropriate post-translational modifications (e.g., glycosylation), or because it does not facilitate their folding into a native (i.e., functional) configuration, or it gives only low expression levels. Post-translational modifications can be obtained by use of more expensive eukaryotic host production systems, while inappropriate folding can often be overcome by secreting proteins from the cytoplasm. Secretion has three major potential advantages over intracellular accumulation: the secreted target protein is usually natively folded (Pozidis et al., 2001); yields can be as high as or higher than that obtained from intracellular *E. coli* host/vector systems; there is a reduced requirement for expensive extraction and purification procedures, with reduced risks of contamination with host proteins and nucleic acids, and contamination with endotoxin is avoided, when the host is not Gram-negative.

Abbreviations: CDS, coding sequence; FBA, flux balance analysis; MBA, metabolic flux analysis; ORF, open reading frame; PMF, proton-motive force; Psp, phage shock protein; SP, signal peptide; SPase, signal peptidase; SRP, signal recognition particle; Tat, twin-arginine-dependent translocation.

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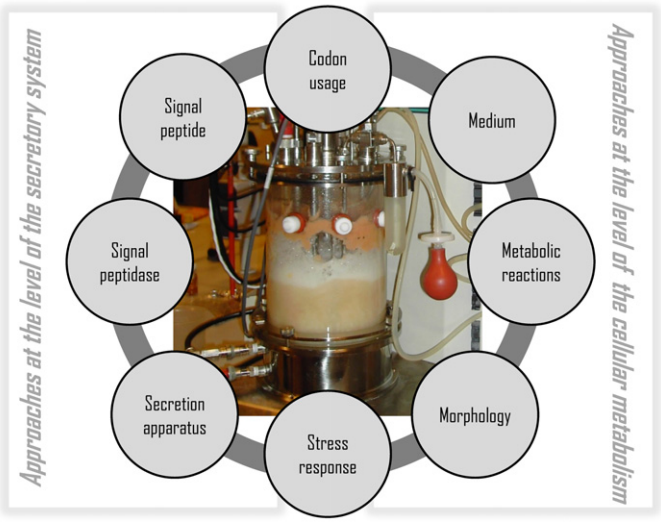

Fig. 1. Overview of potential targets for optimization of heterologous protein secretion by *Streptomyces lividans*. Each target is discussed in the text. This list is, however, not exhaustive.

Although various microbial protein secretion systems have been developed, all currently available systems have limitations that restrict their use and value as a tool for the biotech industry. These systems include secretion into the periplasm of *E. coli* or its extracellular environment (Mücke et al., 2009); production in the yeast *Pichia pastoris* (Cregg et al., 2009) or *Saccharomyces cerevisiae* (Idiris et al., 2010); production in Gram-positive bacteria such as *Bacillus subtilis* (Pohl and Harwood, 2010) and in *Streptomyces lividans* (Vrancken and Anné, 2009) in which cases the secreted proteins are directly released into the culture medium. In all instances, the yields are with some exceptions relatively low compared to that of the intracellular *E. coli* production system (mg rather than g per liter). Several approaches have been attempted to optimize the different systems (Fu et al., 2007; Jacobs et al., 2008; Huang et al., 2008; Yoon et al., 2010) and this with varying success. In this review we will focus on *S. lividans* as host for heterologous protein production. Besides an overview about the potentiality of this system, we will discuss approaches that can be followed attempting to improve yield. A schematic overview of the approaches discussed are outlined in Fig. 1.

2. Characteristics of *Streptomyces* spp.

Streptomycetes are Gram-positive bacteria with a high GC-content. They have a large genome as shown by the available whole genome sequences of *Streptomyces coelicolor* A3(2) (Bentley et al., 2002), *Streptomyces avermitilis* (Ikeda et al., 2003), *Streptomyces griseus* (Ohnishi et al., 2008) and *Streptomyces scabies* (Yaxley et al., 2009; Bignell et al., 2010). Predominantly found in soil and decaying vegetation, streptomycetes have a complex life cycle, growing as substrate and aerial mycelia that finally form exospores. At an industrial scale, streptomycetes are grown submerged in fermentors, where their vegetative mycelium forms a network of hyphae.

Streptomycetes exhibit a unique metabolic diversity and enzymatic capabilities. The compounds they produce as secondary metabolites are valuable for industrial and pharmaceutical purposes. These include antibiotics for human and veterinary medicine or applied in agriculture, anti-cancer and immunosuppressive agents, other pharmacologically active compounds and anti-parasitic agents, herbicides. Since the natural habitat of streptomycetes is the soil, they need to exploit complex organic material in the soil for nutrient acquisition. Therefore, it is obvious that these soil bacteria can secrete a large variety of hydrolytic enzymes. This was confirmed by the analysis of the genome sequence of *S. coelicolor* (Bentley et al., 2002), which encodes 819 potentially secreted proteins including hydrolases, proteases/peptidases, chitinases/chitosanases, cellulases/endoglucanases, amylases and pectate lyases. Several of the enzymes produced by streptomycetes are economically valuable, and are widespread used for a variety of applications in medicine, food industries, and textile and leather industries and also in analytical processes. As a consequence, several of these are produced at an industrial scale, where they are secreted into the culture medium at high concentration driven by a potent secretion apparatus. This efficient secretion system can also be applied for the production of heterologous proteins (see Table 1 for some recent examples).

3. Protein secretion pathways in *Streptomyces* spp.

The Sec pathway constitutes the main protein secretion pathway in *Streptomyces*. Proteins secreted via this pathway are initially synthesized as preproteins with an N-terminal hydrophobic signal peptide (SP) with a positively charged N-domain followed by a longer, hydrophobic H-domain and a C-terminal part containing at the end three amino acids which form the signal peptidase recognition site. For a more detailed overview of protein secretion in bacteria, see e.g., Pohl and Harwood (2010), du Plessis et al. (2011). See also Fig. 2 for a schematic representation of the Sec- and Tat-dependent protein secretion pathways.

It is generally believed that the signal peptide (SP) is required for targeting the preprotein to the Sec translocon consisting of SecY, SecE and SecG, which mediates the translocation of the proteins. During or after translocation, the SP is cleaved off and degraded, and the mature protein is then released in the culture medium. Recent data suggest, however, that the mature domain of the preprotein adds a significant contribution to the targeting, rendering the signal peptide less critical in bacterial secretory proteins than generally assumed. They should rather function as allosteric activators of the translocase that makes signal peptides essential for protein secretion (Gouridis et al., 2009). Once preproteins are bound to the translocase, the ATPase motor SecA drives stepwise the translocation of the unfolded proteins across the membrane (Papanikou et al., 2007). During or shortly after translocation, secretory preproteins are processed by a type I signal peptidase (SPase I) removing the signal peptide.

Streptomycetes contain for 4 different SPases I (SipX, SipW, SipY, SipZ) with different, but overlapping substrate specificity (Geukens et al., 2001). None of the corresponding genes was found to be essential for cell viability by themselves, but SipY seems to be the most important SPase I (Palacín et al., 2002).

Streptomyces spp. also have the cotranslational secretion system brought about by the RNA and protein components of the signal recognition particle (SRP) pathway including a ribonucleoprotein composed of Ffh (fifty-four homolog), a short RNA and the SRP receptor FtsY. FtsY facilitates the cotranslational protein targeting by recruiting SRP–protein complex to SecYEG. For *S. lividans* the involvement of SRP in targeting secretory proteins has been experimentally shown (Palacín et al., 2003). Besides its role in protein transportation, data showed that *S. coelicolor* FtsY is necessary for normal production of actinorhodin and sporulation; and its N-terminus was indispensable for this function (Shen et al., 2008).

Streptomycetes contain also a functional twin-arginine-dependent translocation (Tat) pathway (Schaerlaekens et al., 2004a; Widdick et al., 2006; Joshi et al., 2010). The Tat machinery exports fully folded proteins across the cytoplasmic membrane and the energy for translocation is provided by the proton-motive force (PMF). Similarly to Gram-negative bacteria, in actinomycetes

Table 1
Recent examples of heterologous (mammalian and microbial) proteins produced using *S. lividans*.

S. lividans	Signal peptide [vector]	Protein [source]	Size	Yield	Reference
TK24	*vsi* [pCBS2mTNFa+2]	mTNF-α [mouse]	36 kDa	200–300 mg/L	Pozidis et al. (2001)
TK24	*gpp* [pSGLgpp]	IL-4R [human]	24 kDa	10 mg/L	Zhang et al. (2004)
1326	*Long synthetic signal peptide* [pIJ699]	Rat α Integrin CD11b A [rat]	21 kDa	8 mg/L	Ayadi et al. (2007)
TK24	*melC1* [pMGA]	Glucagon [human]	3.5 kDa	24 mg/L	Qi et al. (2008)
TK24	*cagA* [pIMB4]	IL-6 [human]	~20 kDa	0.61 mg/L	Zhu et al. (2011)
IAF10-164 (*)	*celA* [pIAF811-A.8]	Antigenic protein [*Mycobacterium tuberculosis*]	19 kDa 38 kDa	200 mg/L 80 mg/L	Tremblay et al. (2002)
1326	Native [pIJ6021]	L-amino acid oxidase [*Rhodococcus opacus*]	54.2 kDa	0.18 U/mL	Geueke and Hummel (2003)
JI66	*xys1* [pIJ702]	Xylanase [*Aspergillus nidulans*]	22 kDa	19 U/mL	Díaz et al. (2004)
1326	Native [pIJ486]	Glycoprotein [*Mycobacterium tuberculosis*]	45/47 kDa	5 mg/L	Lara et al. (2004)
1326	SP of PLD from *Stv. cinnamoneum* [pUC702]	Phospholipase D [*Stv. cinnamoneum*]	56 kDa	118 mg/L	Ogino et al., 2004
1326	SP of PLD from *Stv. cinnamoneum* [pUC702]	phospholipase D [*Stv. cinnamoneum*]	56 kDa	118 mg/L	Ogino et al., 2004
JT46	Native [pAE053]	transglutaminase [*Streptomyces platensis*]	38 kDa	2.2 U/mL	Lin et al. (2004)
TK24	*vsi* [pIJ486]	Xyloglucanase [*Jonesia spp.*]	100 kDa	100–150 mg/L	Sianidis et al. (2006)
TK24	*dag* [pRAGA1]	APA protein [*Mycobacterium tuberculosis*]	45/47 kDa	80 mg/L	Vallin et al. (2006)
TK24	*vsi* [pOW15]	streptokinase [*Streptococcus equisimilis*]	47 kDa	15 mg/L	Pimienta et al. (2007)
JI66	SP from *phoA* [pIJ702]	Alkaline phosphatase [*Thermus thermophilus*]	54.7 kDa	266.9 U/mL	Díaz et al. (2008)
JT46	Native [pAE053]	Transglutaminase [*Streptoverticillium platensis*]	38 kDa	2.54–5.36 U/mL	Lin et al. (2008)
msiK⁻ (*)	Native [pIAFD95A]	Thermostable lipase [*metagenomics isolate*]	32.6 kDa	11.3 mg/L	Meilleur et al. (2009)
1326	SP of PLD from *Stv. cinnamoneum* [pUC702]	β-1,4-endoglucanase; β-glucosidase [*Thermobifida fusca* YX]		114 mg/L, 230 mg/L	Noda et al. (2010)
1326	SP of PLD from *Stv. cinnamoneum* [pUC702]	Transglutaminase [*Stv. cinnamoneum*]		64.3 mg/L	Noda et al. (2010)

cagA: signal sequence of C-1027 apoprotein in *S. globisporus* C-1027, with replacement of rare leucine codon; cellulase A signal sequence; *dag*: *S. coelicolor* agarase signal sequence; *gpp*: signal sequence of *S. globisporus*; *melC1:* signal sequence of activator protein in *S. lividans* melanin operon; *phoA*: alkaline phosphatase coding gene from *T. thermophilus;* PLD: phospholipase D; *Stv*: *Streptoverticillium*; *vsi: Streptomyces venezuelae* CBS762.70 subtilisin inhibitor signal sequence; *celA*: *xys1: S. halstedii* xylanase Xys1 (spS), a direct in-frame fusion between the *Streptomyces xysA* signal peptide coding sequence; SP: signal peptide; mTNF-α: mouse tumour necrosis factor alpha; IL-6: interleukin-6; (*): a cellulase- and xylanase-negative mutant of *S. lividans* 1326.

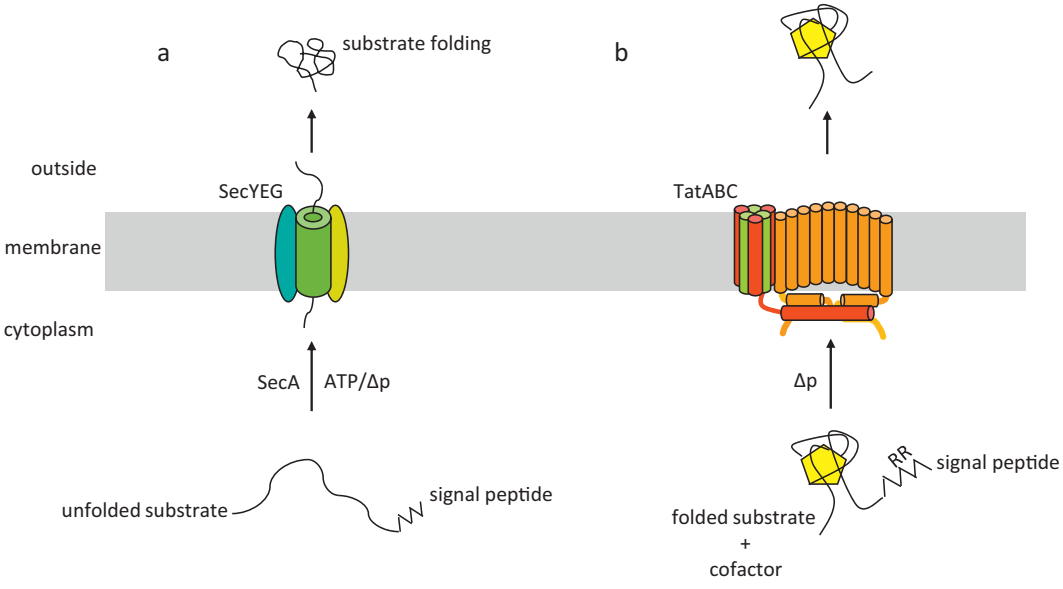

Fig. 2. Schematic representation of Sec (a) and Tat (b) protein transport systems. (Figure adapted from Palmer et al., 2005.)

including *S. lividans* and other streptomycetes, TatA, TatB and TatC are the essential components for this pathway. TatA and TatB are sequence-related proteins with a common structure: each is predicted to comprise a membrane-spanning α-helix at the amino-terminus, immediately followed by an amphipathic helix located at the cytoplasmic side of the membrane and a C-terminal region of variable length. The TatC protein is highly hydrophobic and is predicted to have 6 transmembrane helices, with the N- and C-termini at the cytoplasmic face of the membrane (Palmer and Berks, 2003). Signal peptides that target proteins to the Tat pathway resemble Sec signal peptides, but with a conserved S/T-R-R-x-F-L-K consensus motif, where the twin arginines are invariant and normally essential for efficient export by the Tat pathway (Stanley et al., 2000). However, the Tat-specific signal sequence with two arginine residues may not be an absolute prerequisite for the Tat pathway (Watanabe et al., 2009). Whereas for most bacteria with an identified Tat pathway, only few proteins are transported via this pathway, for *Streptomyces* species investigated the *in silico* Tat substrate prediction programs (TATFIND 1.4 and TatP) revealed an exceptionally high number of Tat substrates: 145 of the 7,825 ORFs of *S. coelicolor*, reduced to 129 using TATscan (Li et al., 2005), of which 25 have been experimentally proven (Widdick et al., 2006) and 145 of the 7576 ORFs for *S. avermitilis* using TATFIND 1.4. Using an agarase reporter assay, it was shown that the signal peptides of 47 candidate *S. scabies* Tat substrates were able to mediate Tat-dependent export, of which 2 were not identified by TATFIND 1.4 due to the presence of His, Ile or Phe at the +1 position (Joshi et al., 2010). Based on these data, one assumes that there are more than 100 Tat substrates in *S. scabies*.

4. Heterologous protein secretion in *Streptomyces* spp.

The host of choice for heterologous protein secretion using *Streptomyces* is *S. lividans*. The main reasons are its limited restriction–modification system as such avoiding the requirement to use non-methylated DNA for transformation or conjugation, and its low endogenous protease activity, when compared to many other streptomycetes (Butler et al., 1996; Nakashima et al., 2005). A wide variety of host–vector systems have been developed, many of which are based on plasmid pIJ101, such as pIJ702 and pIJ486 (Kieser et al., 2000). Experience with *S. lividans* for the production of heterologous proteins gave a mixed picture: proteins of different size and origin could sometimes successfully be secreted and yields (500 mg/L and more) of homologous proteins could be approached. In some cases, proteins which could hardly or not produced in *B. subtilis* or *E. coli* such as e.g., xyloglucanase from *Jonesia* sp. (Sianidis et al., 2006) and CelA from a thermophilic bacterium (Ó. Friðjónsson, unpublished results) were successfully produced with *S. lividans*. In other cases, however, only low yields could be obtained (Table 1), a phenomenon also experienced with other expression systems. Reasons for these yield differences are so far unknown, making predictions about yield impossible. While streptomycetes have a high GC (>70%), and as a consequence a high GC codon bias, CDSs with a lower GC content could nevertheless also efficiently be expressed, and could therefore not account for failure in some cases. This could be further confirmed by the fact that adaptation of the heterologous gene to the host codon usage does not necessarily lead to improved production (Vrancken and Anné, 2009), although in some cases it did (Li et al., 2009). From results shown in Table 1, it is obvious that secretion by *S. lividans* of proteins from prokaryotic origin is in general more successful. In most instances, the Sec-dependent pathway has been used and this with variable success. In some instances, the Tat-dependent pathway was required such as for the secretion of eGFP (Vrancken et al., 2007). A comparison between the efficiency of both pathways showed that in case the Sec pathway could be used, when the Sec-dependent signal peptide was replaced by a Tat-dependent signal peptide, results were in general pitifully low (Schaerlaekens et al., 2004b; Pimienta et al., 2007).

To express the gene of interest, primarily strong, constitutive promoters have been used. Examples are the promoter from *vsi* gene of *Streptomyces venezuelae* CBS762.70 (Lammertyn et al., 1997), *ermE*-up of *Streptomyces erythraeus* (Bibb et al., 1994), metalloendopeptidase promoter from *Streptomyces cinnamoneus* TH-2 (Hatanaka et al., 2008) and *act1* of *S. coelicolor* CH999 (Rowe et al., 1998). Inducible promoters are also available such as from the gene of *Rhodococcus rhodochrous* J1 nitrilase (Herai et al., 2004) or of *Mycobacterium smegmatis* acetoamidase (Triccas et al., 1998) and *S. lividans tipA* (Murakami et al., 1989).

5. Approaches to improve heterologous protein secretion

To increase the secretion yield of heterologous proteins, different approaches have been attempted with different degrees of success, similarly for different host systems. Below a number of existing and potentially promising strategies are discussed.

5.1. Screening for and modulation of proper signal peptide

The signal peptide is a primary requirement for protein secretion, but an appropriate signal peptide does not guarantee an efficient secretion of a particular (heterologous) protein. So far, with a few exceptions, Sec-dependent signal peptides have been chosen. Identification of efficient SPs for different target proteins has been approached in several ways, similarly for different microorganisms. These include charge variation of the N-region (Lammertyn and Anné, 1998; Lammertyn et al., 1998), the screening of a signal peptide library (Degering et al., 2010; Brockmeier et al., 2006), sequence optimization (Ravn et al., 2003; Caspers et al., 2010), directed evolution (Rakestraw et al., 2009), conservation of the signal peptidase cleavage site (Lammertyn et al., 1997). Secretion optimization based on the selection and modification of the signal peptide has been proven to be very effective, but improvement remained limited to a specific protein or a small set of proteins. It is yet impossible to predict which signal peptide, or modifications thereof, will result in improved secretion for a given extracellular target protein. All together, these findings support the notion that, at the present time, the ideal combination has still to be investigated on empirical grounds. It is very likely that the same holds true for the Tat-dependent secretion, but so far, apart from a few publications (Li et al., 2006), not much has been investigated in this respect. Noteworthy is that the production of xylanase, encoded by *xlnB1*, a protein consisting of two discrete structural and functional units, and resulting in two discrete xylanases (XlnB1 and XlB2) could be increased, when the Tat- and Sec-dependent secretion pathways were simultaneously used. Therefore, Sec- and Tat-dependent SPs were cloned in front of XlnB1 and XlnB2, respectively (Gauthier et al., 2005). Whether this approach is more widely applicable for other proteins has still to be proven.

5.2. Overproduction of components involved in protein secretion

5.2.1. Signal peptidases

Signal peptidases cleave off the signal peptide during the translocation process, and are indispensable to free the mature protein from the cytoplasmic membrane. For different bacteria including *Bacillus* (van Dijl et al., 1992; Malten et al., 2005) and *S. lividans* (Geukens, 2002) "proof-of-principle"-experiments were reported demonstrating that SPase overproduction, but also deletion in *B. subtilis* (Tjalsma et al., 1998; Bron et al., 1998) and *S. lividans* (Palacín et al., 2002), can be a valuable approach for

improved secretion. As indicated above, *S. lividans* contains 4 different type I SPases (SipW, SipX, SipY and SipZ) (Parro et al., 1999) with overlapping, non-identical substrate specificity (Palacín et al., 2002; Geukens et al., 2004). SipY seemingly is of major importance for efficient preprotein processing. Most interestingly, it has been shown that preproteins do not preferentially bind to a particular SPase, suggesting that the *S. lividans* SPases compete for binding preproteins (Geukens et al., 2004). In addition, no real correlation between the SPase-binding affinity and preprotein-processing efficiency seems to be present. As such, overexpression of a SPase which efficiently cleaves the precursor may result in increased secretion of a particular protein. Deletion of a SPase which binds efficiently, but cleaves the substrate poorly allows the binding of the precursor to a better processing SPase as such resulting in improved secretion of that particular protein. The finding that SPases compete for binding preproteins and have different substrate-processing efficiencies *in vivo*, opens perspectives for the rational engineering of improved secretion of heterologous proteins.

5.2.2. Tat translocon

The Tat system has been proven to play an important role in protein secretion and, therefore, it has been suggested that it could be an alternative pathway for proteins that failed to be produced via the Sec pathway. However, it has been shown that in certain cases the Tat system is less efficient in protein translocation than the Sec-dependent pathway, and that it can become saturated when proteins are overproduced (Brüser, 2007). To improve translocation, besides selecting an alternative Tat-dependent signal peptide, heterologous translocons have been tested as well as the overexpression of Tat components (Barrett et al., 2003). As shown for the Tat-dependent protein xylanase C, TatABC overexpression in *S. lividans* resulted in a fivefold increase of xylanase C production (De Keersmaeker et al., 2006). This positive effect of TatABC overexpression on protein secretion is not limited to the above-mentioned example, but is also applicable for other bacteria and other proteins. For example, when TatABC was overexpressed in *Corynebacterium glutamicum*, the amounts of pro-protein glutaminase could be increased more than 30-fold, also depending on the signal peptide used (Kikuchi et al., 2009).

5.2.3. Phage shock protein (Psp)

Overexpression of proteins can result in activation of different stress responses. One of these is the Psp system, a highly conserved system in Gram-negative bacteria. In *E. coli*, the *pspA* gene is the first gene in the *pspABCDE* operon. PspA is induced upon infection by filamentous phage and responds to other extracytoplasmic stress that may reduce the energy status of the cell, such as osmotic shock, ethanol shock, extreme heat shock and protein secretion stress, the latter due to hindrance of the protein secretion pathway, both in the Tat- (DeLisa et al., 2004) and the Sec-dependent pathway (Kleerebezem et al., 1996; Antelmann et al., 2003). PspA senses membrane damage and/or a reduction of the PMF. On the other hand, *E. coli psp* mutants have defects in the maintenance of the PMF, protein export by the Sec and Tat pathways and survival in stationary phase at alkaline pH (Darwin, 2005). The consensus so far is that the Psp response plays a role in maintaining the PMF, with PpsA being the main effector. In turn, the PMF is thought to help the cell to cope with membrane-related stresses, such as overloading or blocking of the export machinery (Kleerebezem and Tommassen, 1993). Sec-dependent precursor translocation is an energy-driven process which requires both ATP hydrolysis and the PMF (Geller, 1991). The PMF may be the driving force for translocation during periods when the SecA protein, which supplies the ATPase activity, is not bound to the precursor and at later stages in the translocation process. Accordingly, it has been shown that the transport of various Sec-dependent precursors is less efficient *in vivo* and *in vitro* in the absence of PspA, while expression of the *pspA* gene stimulates efficient Sec-mediated protein export (Kleerebezem and Tommassen, 1993). Investigation of the effect of PspA overexpression on protein secretion in *S. lividans* showed that it resulted in a more than 30% increase in the total amount of secreted proteins, and this without affecting growth (Vrancken et al., 2007). Similarly as in *E. coli* (DeLisa et al., 2004), PspA overexpression in *S. lividans* showed a positive effect both on Tat-dependent and on Sec-dependent secretion. However, the effect was far more pronounced on Tat-dependent secretion, both for homologous and heterologous proteins. For example, the Tat-dependent *S. lividans* xylanase C (Faury et al., 2004) and heterologous eGFP (Vrancken et al., 2007) showed respectively an almost 4- and 3-fold increased yield, when PspA was co-overexpressed. Sec-dependent protein secretion was less affected, although an increase up to 20% was reported (Vrancken et al., 2007). This is of course not unexpected, since contrary to Sec-dependent translocation which requires ATP-hydrolysis, the Tat-dependent precursor translocation is solely dependent on the PMF (Natale et al., 2008). The export of a number of heterologous proteins and endogenous Tat substrates becomes saturated when their expression is elevated. This saturation of export might in part be related to the energy requirement of Tat translocation. It has been reported (DeLisa et al., 2003) that export saturation was exacerbated in strains deficient in *pspA*. Accordingly, expression of PspA relieves the export saturation that occurs upon high-level expression of heterologous and native Tat substrates. On the other hand, deletion of PspA did not impair Tat-dependent secretion in *S. lividans* (Vrancken, 2009). These data seem to indicate that PspA is not an absolute requirement for Tat secretion and other factors contributing to the regulation of the PMF might be able to compensate for the lack of PspA in the mutant strain, when there is overexpression of a Tat substrate. It was hypothesized that, while PspA is involved in PMF maintenance and repair under stress conditions, there is probably little influence on the PMF in unshocked cells.

5.3. Metabolomic approaches and relation to growth

To produce maximum yield of the heterologous protein of interest, the cell has to divide the available resources between cellular growth and metabolite production, and has to cope with the stress posed on the cell due to the presence of plasmids and the overproduction of a foreign protein. Several tools have become available to investigate this at the cellular and genomic level such as metabolomics, fluxomics, transcriptomics and proteomics. Although very important, few reports have attempted so far, to understand the effect of a particular recombinant protein on the metabolic burden in the cell, which can alter the central metabolism, as such affecting the quantity and quality of the heterologous protein produced. Complementary to the question on the metabolic burden *in* the cell, the role of nutrient types in protein production should be understood since this would allow sound medium optimization. Besides the medium composition and the metabolic state, the morphological development of *Streptomyces* in submerged culture can affect growth rate as well as heterologous protein production.

5.3.1. Metabolomics

The first attempts to investigate the impact of heterologous protein production on the metabolism of *S. lividans* are reported by Kassama et al. (2010) and D'Huys et al. (2011), whom both exploit metabolomic approaches. Kassama et al. (2010) set up an experimental protocol for intracellular metabolite analysis in *S. lividans*. Exploring the capabilities of two extraction methods, metabolic fingerprints for wild-type, plasmid-bearing and mouse

tumour necrosis factor alpha (mTNF-α) producing *S. lividans* TK24 were determined. Seventeen metabolites, such as alanine, leucine, and aspartate, showed significantly increased concentrations in the protein-secreting strain; yet clear links with the metabolic activity could not be made. Inspired by the fact that heterologous protein production can be improved by proper medium selection, particularly, by adding mixtures of amino acids, D'Huys et al. (2011) investigated the amino acid uptake profiles in batch fermentations of wild-type and recombinant *S. lividans* TK24 growing on an amino acid rich medium in order to better understand the relevance of individual amino acids. Complex media tend to increase protein secretion levels (Pozidis et al., 2001), however, the observed yields differ between various undefined amino acid sources (ranging from 50 to 120 mg/L, up to 300 mg/L when combining amino acids mixtures with glucose). In an attempt to replace casein peptone as amino acid source, Nowruzi et al. (2008) adopted multifactorial mixture experimental design and formulated a defined medium [containing glucose and three amino acids (Asp, Met, Phe)] supporting secretion (up to 50 mg/L) of human interleukin-3 (IL-3) by *S. lividans*. Glucose and aspartate act as a carbon and energy source. Methionine appeared to be essential to produce IL-3. Except for being a methyl donor, the exact role of methionine is not known. Phenylalanine is probably advantageous, because it contains an aromatic ring. To gain further insight in amino acid uptake metabolism, D'Huys et al. (2011) looked into the phenotypic behaviour of recombinant *S. lividans* in a complex medium. As compared to the wild-type strain, the growth rate reduced, the by-product formation pattern altered and denser clump formation could be observed for a recombinant protein-secreting strain. Furthermore, the amino acid requirements shifted, clearly pointing at an altered metabolism. Glutamate and aspartate are taken up rapidly by wild-type and recombinant strain and are generally known to be preferential carbon and energy sources. The augmented growth rate in the presence of glutamate and aspartate, however, coincided with a lowered protein production rate and yield. In other words, substrates which are readily shuttled to biomass formation should be minimally supplied after reaching a reasonable biomass amount. Other particular observation is the secretion of alanine, which is higher for the recombinant protein-secreting strain. Alanine secretion is assumed to be a salvage pathway to remove excess ammonium pointing at an imbalance between carbon and nitrogen intake fluxes in the cell. After depletion of the main carbon source (glucose, glutamate, and aspartate), a diauxic shift to organic acids and alanine is observed. Notwithstanding this diauxic growth, by-product formation should be avoided to limit fermentation times.

5.3.2. Morphology in submerged cultures

Clump formation is typical during submerged filamentous growth of streptomycetes. Actually, several morphological stages can be distinguished: pellets (compact, 950 μm Ø), clumps (less compact, 600 μm Ø), branched hyphae and non-branched hyphae (Pamboukian et al., 2002). It is generally accepted that cells on the boundary of pellets are physiologically different from those in the centre, e.g., cells in the pellet kernel experience oxidative and nutrient limitation. Recently, Manteca et al. (2008) visualized mycelium development of submerged cultures of *S. coelicolor* A3(2) using viability staining and fluorescence microscopy. Compartmentalized hyphae start to develop forming pellets by radial growth. In the pellet centre, relatively quickly cell death can be detected. Besides its impact on cell metabolism and hereby viability, high medium viscosity, high mixing and aeration requirements, and laborious downstream processing steered van Wezel and co-workers in the development of a morphologically engineered strain expressing increased fragmentation (van Wezel et al., 2006). Overexpression of SsgA, a member of a novel family of actinomycete-specific proteins with six or seven members in streptomycetes that relate functionally to cell division and morphogenesis (Noens et al., 2005), led to increased mycelium fragmentation in liquid culture growth as well as increased specific growth rate and secretion of the Tat-dependent tyrosinase in *S. lividans*. As a consequence, the enhanced expression might be potentially very interesting for protein production in the fermentor (van Wezel et al., US patent 09/749,185).

5.3.3. Towards metabolic flux analysis

Current results stress the fact that type and availability of substrates together with biomass growth rate and morphology affect the protein secretion efficiency and should be optimally controlled, e.g., by appropriate medium formulation and substrate dosing. Metabolite analysis can uncover shifts in the metabolome providing leads to interesting pathways or reactions to be further investigated, and underpinning efforts to identify active metabolic reactions/pathways, but it is insufficient to truly understand metabolic activity and the relevance of various medium components. Hereto, quantification of metabolic fluxes (referred to as fluxomics in a systems biology context) is required. Flux measurements quantify the distribution of resources and energy in the cell and are useful to analyze the impact of genetic alterations in the metabolic reaction network (regulatory interactions not accounted for). Nowadays, popular tools are ^{13}C-labeling based metabolic flux analysis (^{13}C MFA) and constraint-based flux balance analysis (FBA) (e.g., Wiechert, 2001; Edwards and Palsson, 2000). ^{13}C MFA and FBA studies on streptomycetes are not numerous and mostly address the link between the primary and secondary metabolism in view of understanding/increasing antibiotic production (Borodina et al., 2005a,b, 2008; Bushell et al., 2006). Also applications of ^{13}C MFA and FBA to recombinant protein-producing micro-organisms are scarce. Fürch et al. (2007) and Wittmann et al. (2007) uncovered flux re-arrangements related to precursor, energy and reductive power demands for protein synthesis in recombinant *Bacillus megaterium* and *E. coli*, respectively. Baumann et al. (2010) applied ^{13}C MFA to study the impact of oxygen availability on flux distributions in the central carbon metabolism of recombinant *P. pastoris* in order to get insight in the unexpectedly positive effect of hypoxic conditions on recombinant protein synthesis. Heyland et al. (2010) investigated recombinant protein-producing *P. pastoris* under batch and fed-batch conditions. Ow et al. (2009) observed a five times increase in the ATP maintenance flux in plasmid-bearing *E. coli* and obtained the best agreement between ^{13}C-based and FBA-based intracellular fluxes when maximizing ATP dissipation instead of biomass growth. Özkan et al. (2005) maximized recombinant protein secretion but also adjusted the ATP maintenance flux to avoid model prediction discrepancies. These sporadic studies give indications of the metabolic shift and increased energy demands in recombinant strains, but none performs a systematic investigation of the metabolic burden and exploits this knowledge for strain improvement through metabolic engineering.

Thus far, it is clear that metabolic fluxes and medium composition affect heterologous protein secretion yields. Further studies should focus on understanding the metabolic burden of heterologous protein secretion on *S. lividans* exploiting the abovementioned tools and, based on this information, search for optimal bioreactor conditions and targets for metabolic engineering to improve yield and productivity. In order to improve protein yield, metabolic resources should be maximally routed towards protein synthesis and fluxes through ATP-generating reaction should be promoted. Attaining high productivity, on the other hand, will require an optimal balancing of metabolic resources between growth and protein production.

5.4. Search for relevant genes via transcriptomics

As extensively described above, protein yield can be improved by the modulation, co-overexpression or deletion of key proteins involved in the secretion pathway such as the signal peptide, signal peptidases, Tat components. Besides these proteins, there are many more players involved in the protein production process, which can support the expression/secretion of target proteins. Whereas metabolomics and fluxomics focus on the contribution of metabolic reactions, DNA microarray analysis can be helpful to get a global view on the involvement of genes and gene products. To get a more precise view of the involvement of these actors, DNA microarray analysis can be helpful.

Transcriptomics is a systems biology approach that examines the global pattern of gene expression at the mRNA level. This technique allows systematic determination of the effects of heterologous protein secretion at genomic level by identifying genes that are differentially expressed under secretion stress conditions. Relevant genes can be selected for strain engineering to reduce stress during secretion process and thus, optimize recombinant protein production in *S. lividans.*

Such genes can be identified following hybridization experiments in which expression profiles of *S. lividans* clones expressing recombinant proteins and a control strain are compared using available *S. lividans* TK24- or *S. coelicolor* A3(2)-specific microarrays (both available from Eurogentec, Liège, Belgium). Although not ideal, the latter arrays could be used to have a first impression about the expression profiles of *S. lividans*, since as investigated by BLAST analysis from the 7913 existing *S. coelicolor* probes, there is a perfect alignment for 4878 *S. coelicolor* probes to the corresponding *S. lividans* genes (M. Daukandt, C. Lambert et al., to be published – www.streptomics.org). A first successful illustration of the transcriptomics approach for gene selection in view of strain optimization is described in Vrancken (2009) using a *pspA* deletion mutant (*S. lividans pspA*::Tn*5062*), which as mentioned above did not impair Tat-dependent secretion using eGFP and XylC as model proteins. Genes were looked for which might compensate for the loss of PspA by comparing the transcriptional profile of the *S. lividans pspA* mutant and wild-type *S. lividans* TK24. Results revealed a list of genes that were differentially expressed in the *pspA* mutant compared to *S. lividans* TK24, with 67 and 117 genes that were at least twofold up- or downregulated, respectively. Among the proteins with a known or predicted function, several of these are linked to stress regulation (cold shock proteins, sigma factors), while others are involved in metabolic processes such as energy production and conversion, and general metabolism (Vrancken, 2009; Vrancken et al., to be published). The microarray analysis showed that in the *pspA* mutant there was an increased expression of *sco6996,* whose encoded protein shows some homology with the RNA polymerase sigma factor RpoE. Experiments in *E. coli* and *Salmonella* Typhimurium previously showed that there appears to be some functional overlap between PspA and RpoE (Becker et al., 2005; Egler et al., 2005). Loss of either *pspA* or *rpoE* led to a depolarization of the membrane potential, showing that both can affect the PMF. Investigation on *sco6996* overexpression on Tat-dependent secretion of XylC and eGFP resulted in an increase in yield between 10 and 20%, respectively.

Experiments are currently ongoing, in which differentially expressed genes identified by comparative transcriptomics analysis of *S. lividans* TK24 strains producing different heterologous proteins, are manipulated. As briefly described below, preliminary data look very promising and strongly evidence that this approach may be helpful in strain optimization. The expression profiles of *S. lividans* TK24 strains producing four different heterologous proteins [human/mouse tumour necrosis factor alpha (hTNFα/mTNFα, respectively)], monomeric red fluorescent protein and xyloglucanase] were compared to the corresponding control strain containing the empty vector only. Based on these analyses, one gene that showed a significant 2-fold change in the recombinant strains overproducing hTNFα and xyloglucanase was selected for strain engineering. This gene encodes a phosphoenolpyruvate carboxykinase (PEP carboxykinase) involved in the tricarboxylic acid (TCA) cycle and gluconeogenesis. Overexpression of this gene in both *S. lividans* TK24 production strains increased almost 2-fold the yield of recombinant hTNFα and xyloglucanase when compared with the initial production strains (Maldonado et al., to be published). Overall, these results show that a transcriptomics-based approach represents a useful tool for a rational optimization of heterologous protein secretion in *S. lividans*.

6. Conclusion

Recombinant protein production is an indispensable tool in many aspects of biotechnology, both for fundamental as for applied purposes. To become efficient and cost-effective, the host organism should produce the target protein in sufficient quantity and, desirably, also with the requested quality. A diverse battery of cell factories is available, each with their own advantages and disadvantages. As emphasized in this review, *Streptomyces* deserves its own place in the array of production systems. It has been shown in several cases to be a valuable alternative, or even a necessary substitute. As for other Gram-positive bacteria, and other secretory systems, a main advantage is that it secretes the proteins directly into the culture medium, eventually producing the heterologous protein in the right conformation as such avoiding expensive refolding processes. Some heterologous proteins can be expressed at commercially attractive levels (e.g., Pozidis et al., 2001, Sianidis et al., 2006), but unfortunately – like for other hosts- not all proteins can be secreted in sufficiently high amounts and yield optimization is pressing. To improve yield, several successful approaches have become available as highlighted in this review, but a general observation in these efforts for strain optimization, with the intension to increase heterologous protein yield, is that, no predictions on the success can be made so far, nor for *Streptomyces* nor for any other host cell, and that, up-to-now these efforts are based on empirical grounds. Systems biology approaches can, however, be very helpful in this respect. Based on a founded reasoning and illustrated with some first results, this review puts forward the potential or perspectives of transcriptomics, metabolomics and fluxomics in rational strain engineering. Study of differential expression of genes through transcriptomics as well as study of the metabolic burden through metabolic flux analysis will open new paths to enhance protein production in *S. lividans* (as well as in other hosts). Particularly, results of ^{13}C MFA and FBA applications will be helpful in balancing metabolic resources between growth and heterologous protein production through targeted strain and medium optimization. If more data become available, a more global view, and hence, a more rational approach will become possible.

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