

Summing up particular features of protein secretion in *Streptomyces lividans*

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Abstract In recent years much attention has been given to the identification and characterisation of the key elements of the secretory machinery of *Streptomyces lividans*, a non-pathogenic filamentous Gram-positive soil bacterium, whose metabolism is relatively well characterised and capable of secreting large amounts of proteins when grown in laboratory conditions. The relevance of *S. lividans* from a commercial standpoint is due to its potential usefulness for the overproduction of secretory homologous and heterologous proteins of interest. Therefore, this review focuses on the knowledge already obtained on the *S. lividans* secretion pathways.

Keywords *Streptomyces lividans* · Secretion pathways · Secretory proteins · Leader peptides

Introduction

Streptomycetes are Gram-positive soil bacteria, whose complex morphological life cycle comprises the development of an aerial mycelium ending in the formation of spores, and during their different phases of growth endure morphological changes in combination with the production of secondary metabolites. They need to produce and secrete large quantities of proteins (Gilbert et al. 1995), such as the suitable and more prevalent hydrolytic enzymes, as well as enzyme inhibitors, in addition to antibiotics and signalling molecules, in order to adapt to their environment, largely

formed by insoluble polymers, while undergoing differentiation (Chater 1998). It is precisely this extracellular production capacity that has aroused an interest in obtaining more in-depth information on how the streptomycetes secretion machinery functions, and whether this knowledge could be exploited to engineer *Streptomyces* strains as hosts for the synthesis of homologous or heterologous proteins of commercial interest.

Streptomyces lividans is a bacterium that can be cultivated and transformed in the laboratory with relative ease because of its relaxed restriction-modification capacity, since it can take up exogenous DNA without excessive degradation. Moreover, *S. lividans* has been found to be an ideal candidate as a bacterial host for extracellular protein overproduction, owing to the extensive homology of its genome to that of the widely studied and closely related bacterium, *Streptomyces coelicolor*, and its capacity to synthesise and secrete a significant number of extracellular proteins. Thus, *S. lividans* has frequently been used in experiments as a host for the secretory production of homologous and heterologous proteins (Gilbert et al. 1995; Anné and Mellaert 1993; Binnie et al. 1997; Lammertyn et al. 1997; Parro and Mellado 1994; Van Mellaert and Anné 1994; Isiegas et al. 1999; Vrancken and Anné 2009).

The Sec pathway is the main route by which secretory proteins are released outside the cell across the cytoplasmic membrane (Driessen and Nouwen 2008). The second protein transport system found in streptomycetes is the Tat pathway that is able to transport already folded proteins across the cytoplasmic membrane (Berks et al. 2000). A third secretion system (Esx, or type VII) has been described in Gram-positive bacteria, where it seems to be required for the secretion of small extracellular proteins (Pallen et al. 2002). The function of this system in streptomycetes is currently unknown (Chater et al. 2010) and,

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therefore, cannot yet be considered as an essential system for protein secretion in streptomycetes.

Type I signal peptidases in *S. lividans*

Extracellular proteins are synthesised as pre-proteins carrying a signal peptide needed for proper targeting and translocation across the cellular membrane, and the signal peptide is removed by signal peptidases during the course of translocation or shortly after it (Van Welly et al. 2001). The majority of exported pre-proteins are processed by prokaryotic type I signal peptidases (SPases), also known as leader peptidases (Lep). Most organisms contain only one type I SPase, seemingly essential, as in *E. coli* (Dalbey and Wickner 1985; Tschantz et al. 1993) or yeast (Böhni et al. 1988); however, there are organisms containing two type I SPases such as the cyanobacterium *Synechocystis* PCC 6803 (Kaneko et al. 1996), the bacteria *Bacillus amyloliquefaciens* (Hoang and Hofemeister 1995; Meijer et al. 1995) and *Staphylococcus aureus* (Cregg et al. 1996). Most eukaryotic species also harbour two SPase isoforms (Dalbey et al. 1997). Three SPases have been found in the archaeon *Archaeoglobus fulgidus* (Klenk et al. 1997) and in *Deinococcus radiodurans* (White et al. 1999). Among the Gram-positive bacteria, seven SPases have been described for *Bacillus subtilis*, where the genes corresponding to five of these are widespread on the chromosome (van Dijl et al. 1995; Tjalsma et al. 1998), and two other genes have been found in plasmids (Meijer et al. 1995). Moreover, four adjacent genes (*sipW*, *sipX*, *sipY* and *sipZ*) encoding different type I signal peptidases have been identified in the *S. lividans* TK21 genome, where three of the *sip* genes (*sipW*, *sipX* and *sipY*) constitute an operon, and the fourth one (*sipZ*) is the first gene of another operon encompassing three additional unrelated genes (Parro et al. 1999). In accordance with the construction and in vivo phenotypical characterization of mutants in each of the four *S. lividans sip* genes, SipY appears to be the *S. lividans* SPase that plays a major role in pre-protein processing (Palacín et al. 2002).

Analysis of the *S. lividans* TK21 secretome by 2D-PAGE followed by mass-spectrometric analysis was of great assistance in identifying the *S. lividans* secretory proteins, the majority (84%) carrying typical export signals for the Sec route, and the rest being non-secretory proteins (Palacín et al. 2002). This proteomic analysis of the *S. lividans* secretome confirmed the differences in extracellular protein patterns between *S. lividans* TK21 and the different *sip* mutant strains, revealing a severe reduction in the accumulation of extracellular proteins in the *sipY* mutant compared to that of the remaining *sip* mutants, and additionally confirming the major role played by the SipY

protein in secretion (Palacín et al. 2002). The SipY mutant also experiences intracellular retention of a model secretory protein (Palomino and Mellado 2005), consequently affecting the intracellular distribution of the secretion pathway components, blocking the translocon temporarily and triggering the accumulation of the model pre-protein at the membrane.

Upon complementation of the SipY mutant with each of the Sip proteins encoded in multicopy plasmids, the SipY mutant sporulation-delayed phenotype was repaired and the analysis of extracellular proteins indicated that the overall pattern of secretory proteins had recovered in all cases (Escutia et al. 2006). The conclusion reached is that individual mutations in the different *S. lividans sip* genes, except for *sipY*, do not seem to exert a severe effect on protein secretion, as observed previously (Palacín et al. 2002), due to the compensatory effects of the minor SPases. Thus, the existence of several type I SPases in bacteria reflects the need to ensure the secretion of the necessary extracellular proteins for their survival in their natural environment, affording the bacteria more fitness in changing environmental conditions, an objective that seems to be wisely achieved in the case of soil Gram-positive bacteria, by favouring compensatory effects over substrate specificity.

Transport to the membrane

The synthesis of secretory proteins takes place with a signal peptide at their amino end, which serves as a recognition signal for cytosolic chaperons, such as SecB in *E. coli*. The tight folding of the precursor protein is prevented by these chaperons, which help to direct the pre-secretory protein to the translocation sites at the membrane (Fekkes and Driessen 1999). This mechanism is called post-translational translocation and is mainly used for protein secretion in *E. coli* via the general secretion route (Sec pathway). The genome of the Gram-positive bacterium *Bacillus subtilis* does not have a gene encoding an equivalent SecB protein (Kunst et al. 1997), and the genome sequence of the *S. coelicolor* bacterium (Bentley et al. 2002), which is closely linked to *S. lividans*, as well as the available sequence of the *S. lividans* TK24 genome (<http://www.ncbi.nlm.nih.gov>) also revealed the absence of a *secB* gene, thereby rendering a particular appeal to the research of alternative means of transporting secretory proteins in these bacteria.

The co-translational protein targeting mechanism involving the signal recognition particle (SRP) seems to be conserved from bacteria to mammals (Keenan et al. 2001; Cao and Saier, 2003; Pohlschöder et al. 2004). In mammalian cells the translocation of proteins across the

endoplasmic reticulum membrane occurs via the interaction of the SRP with the ribosome nascent chain complex (RNC), and the entire complex is subsequently targeted to the membrane by association with an SRP-receptor complex (Walter and Johnson 1994). *Escherichia coli* possess a similar SRP mechanism (Wolin 1994) comprising a 4.5 S RNA (scRNA), Ffh, a homologue of the mammalian SRP54 protein and FtsY, with a 300-residue long C-terminal domain resembling that of the mammalian SRP-receptor alpha-subunit, being all the bacterial SRP components essential for cell growth. Major interactions among SRP components are evolutionary conserved (Miller et al. 1994). The Ffh C-terminal M domain is involved in the interaction with the nascent polypeptide chain and the scRNA, representing the minimal functional SRP. The FtsY N-terminal A domain contains the membrane-targeting signal and varies among FtsY homologues, which is probably in line with the different modes for targeting FtsY to the membrane (Powers and Walter 1997; Millman and Andrews 1999; Herscovits et al. 2000). The SRP and the SecA/SecB-based secretion systems belong to two different targeting pathways in *E. coli* that function in a substrate-specific manner (Beck et al. 2000). Integral membrane proteins are targeted to the cytoplasmic membrane by SRP (de Gier et al. 1996; Ulbrandt et al. 1997). However, the postulated role of SRP in the export of *E. coli* SecB-independent secretory proteins has been ruled out (Beha et al. 2003).

A functional interaction has been detected between SecA and Ffh in *B. subtilis* (Bunai et al. 1999). This co-translational mechanism ought to be of greater importance in Gram-positive bacteria, where the SRP may interact with the signal sequence of the newly made protein and FtsY during targeting, as well as with the ribosome, and eventually with the translocon. The signal peptides of the *B. subtilis* secretory proteins seem to be more hydrophobic and longer than those of *E. coli*, and it has been claimed that these signal peptide characteristics may allow the *B. subtilis* SRP system to target both secretory and membrane proteins (Herscovits et al. 2000). Thus, the *B. subtilis* SRP system may be responsible for targeting both membrane and secretory proteins (Oguro et al. 1996; Bunai et al. 1996; Hirose et al. 2000).

The length and hydrophobicity of the signal peptides of *S. lividans* secretory proteins seem to be more similar to those of *B. subtilis* than to those of *E. coli*, therefore it seems reasonable to assume that the same intracellular assignments are conducted by the *B. subtilis* and *S. lividans* SRP systems. The *S. lividans* SRP system consists of a functional signal recognition particle, a ribonucleoprotein, composed of Ffh and an 82 nt long RNA (Palacín et al. 2003) and the small size of this RNA, as in *E. coli*, seems to leave no room for other proteins to adhere to the

complex. The receptor protein, FtsY, also forms part of the *S. lividans* SRP system and its expression takes place throughout cellular growth, similarly to that of scRNA and Ffh. No mutants have been reported in any of the three corresponding genes, which may indicate the possible essential nature of the *S. lividans* SRP components. The *ffh* transcript seems to contain an internal downstream box (Wu and Janssen 1996) to ensure translation of the processed messenger, a feature also shared by the *sip* transcripts (Parro et al. 1999). Co-immunoprecipitation studies confirmed that the *S. lividans* SRP, despite being minimal in composition, is involved in targeting secretory proteins (Palacín et al. 2003).

A comparison of the hydrophobic profiles of FtsY proteins from *E. coli*, *Mycobacterium leprae*, *M. tuberculosis* and *S. coelicolor* (Bibi et al. 2001) suggested the presence of a hydrophobic N-terminal segment that could identify the three *Actinobacteria* FtsY homologs as integral membrane proteins. The use of an *S. lividans* strain lacking the gene coding for the major type I signal peptidase has permitted finding evidence of an in vivo interaction of the FtsY receptor with the SRP, as well as FtsY involvement in targeting secretory proteins to the membrane via the existence of a soluble FtsY, which acts as a functional cytoplasmic receptor for the SRP (Palomino and Mellado 2005). Inactivation of the eukaryotic translocon causes accumulation of membrane-bound SRP-RNC and its receptor, by diminishing the transfer of the signal peptide from the SRP54 to the vacant translocon complexes (Song et al. 2000). Similarly, the SipY mutation may temporarily block the translocon and trigger the accumulation of the SRP pre-protein at the membrane.

Thus, the *S. lividans* SRP also seems to evidence an ability to interact with the translocon by escorting the pre-protein to the translocation complex and possibly not being detached from the membrane until the cleavage of the signal peptide permits the complete release of the secretory protein. The whole process, which takes place efficiently in a sequential manner, is regularly coupled, and SRP as well as FtsY are released from the translocon almost simultaneously for their re-utilisation to target more pre-secretory proteins. An illustration of this model is depicted in Fig. 1. The SRP pathway is not affected by the overproduction of model secretory proteins, confirming that the respective level of SRP components is not a limiting factor for secretory protein overproduction in *S. lividans* (Palacín et al. 2002).

Translocation and membrane protein insertion

Once the secretory proteins have been targeted to the cytoplasmic membrane, translocation is believed to occur

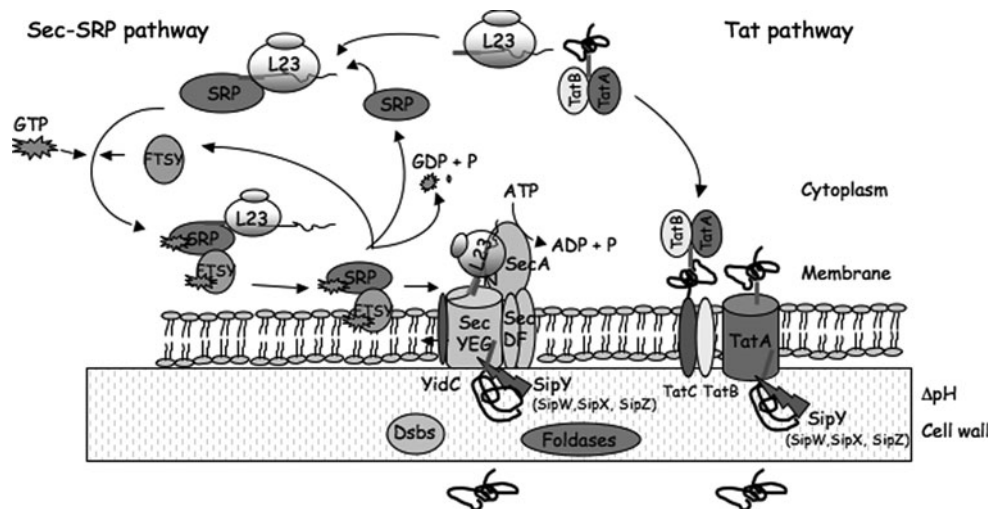


Fig. 1 Secretion pathways in *Streptomyces lividans*. The left section of the figure schematises the major secretory route (Sec pathway), where the nascent polypeptide interacts with the minimal SRP to be targeted to the translocation complex at the membrane with the help of the FtsY receptor in a GTP-dependent process. Once the polypeptide is cleaved by the signal peptidase(s), the SRP components are released and the process is repeated. Improperly folded Sec-dependent mature proteins need the action of foldases or thiol-disulfide isomerases (Dsbs) to acquire their active conformation.

as in *E. coli*, where SecY, SecE and SecG form an heterotrimeric stable complex constituting the channel that conducts the protein to be translocated (Driessen and Nouwen 2008). From the genome sequence analysis it is known that streptomycetes seem to have only one gene for each of these Sec proteins (Bentley et al. 2002), as is also the case for SecA, somehow supporting the assumption of comparable functioning of their *E. coli* counterparts, as *S. lividans* SecA is required in vitro for the secretion of the Sec-dependent α -amylase (Blanco et al. 1998). Two *secG* homologous genes have been identified in *S. coelicolor*, *S. lividans* (SCO1944; Palomino and Mellado 2008) and *B. subtilis* (*yvaL*; Van Wely et al. 1999) chromosomes, and their encoded proteins are found to have a functional homology to that of *E. coli*. Depletion of the *S. lividans secG* does not seem to cause a cold-sensitive phenotype as it happens when the *E. coli* or *B. subtilis* are depleted of their corresponding *secG* genes, and, at standard growth temperature, SecG deficiency seems to have almost a negligible effect in these two bacteria, while this deficiency does confer a more noticeable effect in *S. lividans* where extracellular protein secretion is impaired and the overproduction of a model secretory enzyme is delayed (Palomino and Mellado 2008). In *E. coli* the SecYEG channel associates with the heterotrimeric membrane complex formed by SecD, SecF and YajC proteins, although YajC is not required for the complex function, and the complex itself does not seem to be needed for primary translocation

Protein YidC has been included although its functionality has not been demonstrated in streptomycetes, as yet. The right section of the figure shows how the nascent polypeptide of a Tat-dependent secretory protein is targeted to the membrane. The mature protein is released fully folded to the cell wall after its leader peptide is cleaved by the signal peptidase(s). The figure is adapted from Fig. 4 (Palomino and Mellado 2005) inspired by Fig. 3 (Fekkes and Driessen 1999)

(Driessen and Nouwen 2008). Genes equivalent to these have been found in the *S. coelicolor* genome, yet their functionality has not been determined experimentally. YidC has been identified as an essential membrane protein that assists in the insertion of some membrane proteins in *E. coli* (Driessen and Nouwen 2008), and two genes potentially encoding equivalent proteins have been found in the *S. coelicolor* genome (Bentley et al. 2002). Yet, again, the functionality of their encoded proteins still needs experimental assessment.

The twin-arginine translocation (Tat) pathway

The Tat pathway is an export pathway that has been described in several bacteria and in the thylakoid membranes of plant chloroplasts. Secretory proteins exported by the Tat route are transported across the cytoplasmic membrane in a folded state. This makes the Tat pathway particularly interesting as a potential secretory route to be exploited for the secretion of homologous or heterologous proteins in *Streptomyces* strains.

The Tat pathway consists of three proteins (TatA, TatB and TatC) in *E. coli* and in *Streptomyces* strains (Fig. 1), while *B. subtilis* and archaeobacteria harbour Tat systems with only two Tat proteins (Dilks et al. 2005; Jongbloed et al. 2004; Pop et al. 2002). The N-terminal of the proteins targeted to the Tat route in eubacteria contains a conserved

motif R-R-x- Φ - Φ , where x is usually a polar amino acid and Φ is a hydrophobic amino acid (Berks 1996; Stanley et al. 2000; Widdick et al. 2006). Different algorithms of a predictive nature have been designed to recognise Tat leader peptides, and the use of these algorithms has helped to predict the existence in *S. coelicolor* of a large number of Tat-dependent proteins (Bendtsen et al. 2005; Dilks et al. 2003). From the 145 to 189 Tat-dependent proteins predicted in silico in *S. coelicolor*, a total of 27 have been unequivocally established as Tat-dependent, when membrane proteins from TatC deficient strains were compared to those of the isogenic wild type strain (Widdick et al. 2006). Up to 127 proteins have been predicted to be Tat-dependent in the *S. coelicolor* closely-related strain, *S. lividans* (Schaerlaekens et al. 2004), however, experimentally, the final number has not yet been established. The annotated sequence of the *S. coelicolor* genome includes 819 secretory proteins, which clearly indicates that the 27 assigned Tat-dependent proteins constitute a reduced number when compared with that of the remaining of the secretory proteins, thereby confirming the subsidiary nature of the Tat route for secretion. Moreover, the experimentally identified 27 Tat-dependent proteins in *S. coelicolor* represent a significantly large number of proteins using the Tat pathway for secretion, when compared to the very few found in other bacteria. This substantiates the interest of this secretory route in streptomycetes for the secretion of proteins correctly folded at the bacterial supernatant, although the real usefulness of the Tat system for this purpose still needs to be experimentally assessed. In streptomycetes, the existence and mode of action of proteins with the capacity to intervene in the correct folding of extracellular proteins exported by the Sec route, such as foldases or thiol-disulfide isomerases, equivalent to those described in other eubacteria, still remains to be elucidated.

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